PART III

4.1.- OBJECTIUS

4.1.- OBJECTIUS

L'objectiu principal de la segona part d'aquest treball ha estat l'estudi del desenvolupament i efecte de les condicions ambientals en els ritmes circadiaris de rates transgèniques hipertenses de la soca TGR(mREN2)27. Aquesta part està formada per dos experiments amb els següents objectius:

1.- Estudiar el desenvolupament dels ritmes circadiaris d'activitat motora, freqüència cardíaca i pressió sanguínia de les rates transgèniques hipertenses de la soca TGR(mREN2)27. Les rates TGR(mREN2)27 pertanyen a una soca que es caracteritza per tenir un gen addicional de renina de ratolí, que produeix una hiperactivitat del sistema renina-angiotensina. Una altra característica important i curiosa d'aquestes rates és que en l'edat adulta l'acrofase del seu ritme de pressió arterial està endarrerida 12 hores respecte de la dels ritmes de freqüència cardíaca i d'activitat motora. S'ha demostrat que la pressió sanguínia de rates TGR de 5 setmanes de vida és semblant a la de rates control Sprague-Dawley, tant en els seus nivells com en el seu ritme circadiari. Poc temps després, però, la pressió comença a pujar, alhora que l'acrofase del ritme s'endarrereix progressivament. El nostre objectiu ha estat el d'estudiar en profunditat el desenvolupament dels ritmes circadiaris d'activitat motora, freqüència cardíaca i pressió sanguínia de rates joves TGR des del punt de vista cronobiològic.

2.- Estudi de l'efecte de cicles de llum-foscor de període inferior a les 24 hores en rates TGR joves i adultes. S'ha observat que cicles llum-foscor de període inferior a les 24 hores indueixen una dissociació del ritme circadiari d'activitat motora en rates Wistar i en ratolins Swiss. Aquests animals (tant joves com adults) manifesten un ritme d'activitat motora amb dos components: un d'encarrilat a la llum (i per tant amb el mateix període que el cicle extern de llum-foscor) i un component no encarrilat a la llum (amb un període al voltant de les 24 hores). Donat el peculiar desenvolupament dels ritmes circadiaris de les rates TGR i la possibilitat que donen els aparells de telemetria de mesurar els ritmes circadiaris d'activitat motora, freqüència cardíaca i pressió sanguínia, el nostres objectius han estat: 1) observar si els cicles curts de llum-foscor són capaços de produir una dissociació dels ritmes d'activitat motora, freqüència cardíaca i pressió sanguínia de rates TGR i 2) observar quin és l'efecte de cicles curts de llum-foscor en rates TGR joves durant el desenvolupament del seus ritmes circadiaris i comparar-lo amb l'efecte sobre rates TGR adultes amb ritmes circadiaris madurs.

4.1.- OBJECTIVES

The aim of the second part of this work has been the study of the development and effect of the environmental conditions on the circadian rhythms of young transgenic hypertensive TGR(mREN2)27 rats. This part is composed by two experiments with the following objectives:

1.- Study of the development of the circadian rhythms of motor activity, heart rate and blood pressure of transgenic hypertensive TGR(mREN2)27 rats. TGR(mREN2)27 rats are a hypertensive strain characterised by the presence of an additional mouse renin gene, which leads to overactivity of the renin-angiotensin system. Another important and curious characteristic they have, is that in the adulthood, the acrophase of their blood pressure rhythm is delayed about 12 hours with respect of that of heart rate and motor activity. It has been demonstrated that blood pressure of five-week-old TGR rats is similar to that of control Sprague-Dawley rats both in levels and in circadian rhythmicity, and that the increase in blood pressure starts thereafter, and goes in parallel with the delay of the circadian acrophase. Our aim has been to study more in depth the development of the circadian rhythms of motor activity, heart rate and blood pressure of young TGR rats from the chronobiological point of view.

2.- *Study of the effect of light-dark cycles of a period inferior to 24 hours on young and adult TGR rats. Light-dark cycles of a period of less than 24 hours induce the dissociation of the circadian rhythm of motor activity in Wistar rats and in Swiss mice. These animals (both young and adult) manifest a motor activity rhythm with two components: a light-entrained component (with the same period as the light-dark cycle), and a non-light-entrained component (with a period of around 24 hours). Given the peculiar development of the circadian rhythms of TGR rats, and the possibility that telemetry has given to measure the motor activity, heart rate and blood pressure rhythms, our aim has been to: 1) observe whether short light-dark cycles are capable of inducing a dissociation of the rhythms of motor activity, heart rate and blood pressure of TGR rats; and 2) observe what is the effect of short light-dark cycles on young TGR rats during the development of their circadian rhythms, and to compare it with adult TGR rats with mature circadian rhythms.*

4.2.- EXPERIMENT 9

CIRCADIAN PACEMAKER FUNCTION AND ENTRAINMENT DURING MATURATION OF TRANSGENIC HYPERTENSIVE TGR(mREN2)27 RATS AND SPRAGUE-DAWLEY RATS

Chronobiology International, acceptat, 2001.

Resum

Objectiu: La soca de rates TGR és una soca modificada genèticament que es caracteritza perquè les rates desenvolupen hipertensió entre la cinquena i la desena setmana de vida. Curiosament, s'ha observat que rates TGR adultes sotmeses a cicles llum-foscor de 24h de període mostren un ritme circadiari de pressió sanguínia invers als d'activitat motora i freqüència cardíaca, de manera que els valors més alts de pressió se situen durant la fase de repòs, en comptes de situar-se a la fase activa. L'objectiu del present experiment ha estat el d'analitzar detingudament el desenvolupament dels ritmes d'activitat motora, freqüència cardíaca i pressió arterial en rates joves TGR i comparar-lo amb el de rates control, sota cicles llum-foscor de 24 hores de període.

Material i mètodes: Cinc rates mascle transgèniques heterozigotes de la soca TGR(mRen2)27 i cinc rates mascle control de la soca Sprague-Dawley van arribar al laboratori amb 4 setmanes d'edat. Cadascuna d'elles es va col·locar en una gàbia individual i sota cicles llum-foscor de 24 hores de període. Al cap de tres dies se'ls va implantar un aparell de telemetria que mesurava l'activitat motora, la freqüència cardíaca, la pressió arterial sistòlica i la pressió arterial diastòlica. Immediatament després de l'operació es van començar a enregistrar les variables citades anteriorment durant 40 dies.

Resultats: Els resultats confirmen que la pressió sanguínia augmenta a partir de la cinquena setmana de vida, arribant a un nivell assimptòtic a l'onzena setmana. Paral·lelament a aquest increment en els nivells de pressió, un endarreriment en l'inici de la fase alfa d'aquest ritme respecte dels ritmes d'activitat motora i freqüència cardíaca i també, un descens dels valors de freqüència cardíaca. En general, els nivells de freqüència cardíaca i d'activitat motora, així com la potència de tots els ritmes circadiaris examinats, eren superiors en les rates TGR que en les rates Sprague-Dawley.

Conclusions: La modificació genètica present en les rates TGR no només produeix hipertensió, sinó que a més podria estar involucrada en la generació del patró circadiari alterat de pressió sanguínia. A més, els nostres resultats semblen indicar que aquesta modificació genètica també podria estar interferint o modificant el funcionament del sistema circadiari de les rates TGR, a través de l'alteració de les vies d'entrada cap al rellotge circadiari i/o del mateix rellotge.

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CIRCADIAN PACEMAKER FUNCTION AND ENTRAINMENT DURING MATURATION OF TRANSGENIC HYPERTENSIVE TGR(mREN2)27 AND SPRAGUE-DAWLEY RATS

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ABSTRACT

TGR(mREN2)27 (TGR) transgenic rats develop hypertension due to the mouse mRen-2 gene inserted in their genome. At 5 weeks of age, the blood pressure of TGR rats starts rising, until a maximum is reached at 10 weeks of age. Adult TGR rats show peak values of blood pressure (BP) during the light phase, while heart rate (HR) and motor activity (MA) peak at night. In the present experiment, we evaluated the evolution of circadian rhythms in motor activity, heart rate, and blood pressure of TGR and Sprague-Dawley (SD) rats under 12h light-dark cycles (LD 12:12). Results confirmed that the blood pressure of TGR rats starts to increase at 5 weeks of age, reaching a plateau by the 11th week. Parallel to the increase in blood pressure levels, there was a decrease in the period length of the blood pressure rhythm, a delay in the onset of the alpha phase of the blood pressure rhythm with respect to that of motor activity and heart rate, and a decrease in heart rate levels. In all of the variables studied, the alpha phase of SD rats always started before darkness, whereas that of TGR rats started after lights off. In general, heart rate and motor activity levels of TGR rats were higher than those of SD rats. The amplitude of the circadian rhythms studied was

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greater in TGR rats than in SD rats. The present results suggest that the different evolution of circadian rhythms in TGR and SD rats might be due to differences in the functioning of the entrainment pathway or the circadian clock itself, which can be detected in young rats and that are probably caused by the expression of the mouse transgene. (*Chronobiology International, 18(4), 627–640, 2001*)

Key Words: Blood pressure; Circadian rhythm; Heart rate; Hypertension; Motor activity; Telemetry; Transgenic (mREN2)27 rat

INTRODUCTION

The TGR (mREN2)27 (TGR) transgenic rat strain is used as a model of genetic hypertension. These rats were generated (1) by random integration of the mouse mRen-2 gene into the genome of Sprague-Dawley (SD) rats. Homozygous TGR rats develop severe hypertension and show a high mortality rate if not treated with angiotensin-converting enzyme inhibitors. In heterozygous TGR rats, blood pressure (BP) starts to rise at 5 weeks of age, reaching a plateau between 10 and 11 weeks of age (2). The BP levels reached by heterozygous TGR rats are lower than those reached by homozygous TGR rats (3). The hypertensive animals also develop a progressive left ventricular hypertrophy (4), arterial medial thickening, and nephrosclerosis (5).

It is thought that the high BP levels in TGR rats are due to the expression of the mouse Ren-2 gene in the rat's adrenal gland (5–7). Moreover, it has been found that plasma active renin, plasma prorenin, adrenal renin, and BP increase in parallel with aging, whereas kidney renin decreases (2). Therefore, the increase in renin levels with age is thought to be related to the increase in BP.

An important characteristic of adult TGR rats is that they show an inverse circadian BP profile, with peak values occurring during the resting phase of the animals, whereas heart rate (HR) and motor activity (MA) rhythms are undisturbed (8). The development of the inverse circadian BP profile appears to take place at the same time as the development of hypertension (9).

The present study analyzed in depth the development of the motor activity, heart rate, and blood pressure of young TGR rats compared to those of SD control rats under 12h light-dark cycles (LD 12:12).

MATERIALS AND METHODS

Five heterozygous male transgenic rats [TGR(mREN2)27-MolGene] and five Sprague-Dawley controls (Mol: SPRD Han) 4 weeks of age were obtained from M+B A/S (Ry, Denmark). They were then housed individually in plastic cages (380 × 220 × 150 mm) for 3 days with free access to food (Haltungsfutter-Ratten/Mäuse, Altromin, Lage, Germany) and water, in controlled environmental

conditions, at an ambient temperature of 23°C ± 1°C. The rats were kept under LD 12:12 at a light intensity of 100 lux during the light phase throughout the experiment.

Immediately after implantation of pressure transmitters (previously described in Ref. 8), MA, HR, systolic blood pressure (SBP), and diastolic blood pressure (DBP) were monitored telemetrically, with measurements taken every 5 minutes using the DataQuest® system (Data Sciences, St. Paul, MN). Data were obtained for 40 days. The experiment was performed in adherence to the *Guide for the Care and Use of Laboratory Animals*, published by the National Institutes of Health, and was approved by German federal regulations (RP Karlsruhe, Az. 35-9185.81/1/99).

To study the development of the four variables monitored, the sampled data were divided into four stages of 10 days each: stage 1 (days 1–10), stage 2 (days 11–20), stage 3 (days 21–30), and stage 4 (days 31–40). The circadian rhythms of MA, HR, SBP, and DBP for each of the stages were analyzed by means of a Lomb and Scargle periodogram (10,11). In addition, a Fourier series was fitted to the data, from which the amplitude and the power content (PC) of the first harmonic (period = 24h) were obtained.

To study the inversion of the blood pressure profile, the entrainment characteristics of each group of rats were studied through the phase angle or psi, which is the phase relation between the zeitgeber and the studied rhythm. To calculate psi, and for each rat's variable at each stage, the time (in minutes) between lights off and the onset of activity (when the variable moved above the median) was measured from the mean waveform plotted at modulo 24h. 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.

Analysis of variance (ANOVA) was used for statistical analysis. Graphs and calculations were made using the integrated package for chronobiology analysis El Temps (copyright A. Díez-Noguera, University of Barcelona, Spain, 1999). For the representation of the double plots, and for all of the variables studied, the daily (24h) mean was calculated, and it was then subtracted from each individual value. Negative values were considered as zero. Data are shown as mean plus or minus standard deviation unless otherwise indicated.

RESULTS

As seen with the double plots (Fig. 1), there were marked differences between the two strains of rats in the development of the circadian rhythms of the distinct variables studied.

First, the 24h mean values of BP of TGR rats were significantly higher in SD rats than in TGR rats in the first stage ($P < .001$, Figs. 2a and 2b). However, BP values of both SD and TGR rats increased with time ($P < .001$), but in TGR rats, this increase was more pronounced: The total increase in SBP throughout

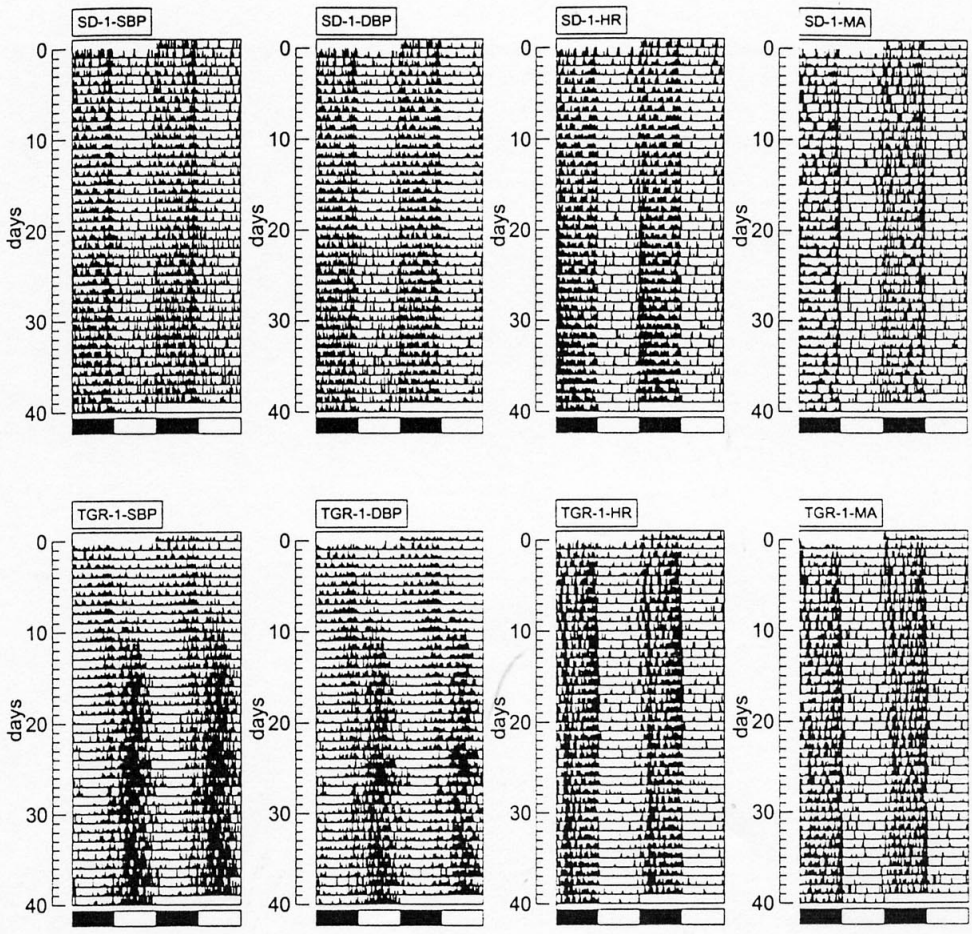

Figure 1. Double plots at modulo 24h of a representative animal from each group. The upper graphs correspond to an SD rat and the lower to a TGR rat. From left to right, the graphs represent the systolic blood pressure (SBP), the diastolic blood pressure (DBP), the heart rate (HR), and the motor activity (MA) rhythms. The vertical axis indicates the days of the experiment, starting after the implantation of the telemetric devices in rats 4.5 weeks old. The lower bar indicates the LD cycle: The black part represents 12h darkness, and the white part indicates 12h light.

the stages was 12.67 ± 7.04 mmHg in the SD rats and 82.53 ± 5.52 mmHg in the TGR rats; similarly, the total increase in DBP was 9.46 ± 3.23 mmHg in the SD rats and 53.85 ± 4.59 mmHg in the TGR rats. On the other hand, the mean daily values of HR of TGR rats were higher than those of SD rats in the first stage ($P < .001$), and although these values tended to decrease with age in both strains, TGR rats continued to have higher 24h mean values of HR ($P < .05$, Fig. 2c). The 24h mean MA also increased with time, but only in the first stage, after which it reached a plateau (Fig. 2d); the TGR rats showed higher values than SD rats throughout the stages ($P < .001$).

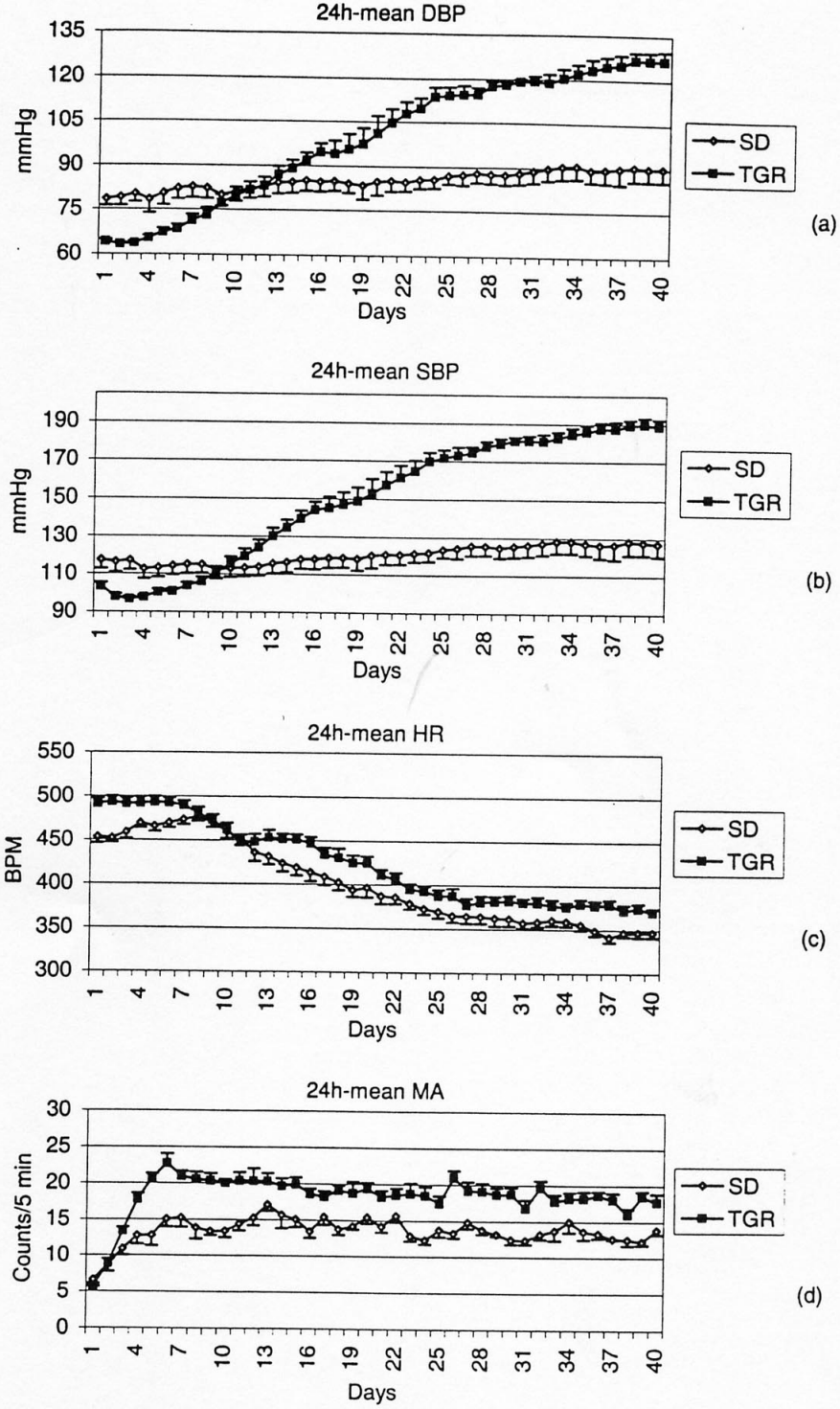

Figure 2. Progress in time of the 24h mean of each of the variables studied throughout the experiment and for both SD and TGR rats: (a) diastolic blood pressure (DBP); (b) systolic blood pressure (SBP); (c) heart rate (HR); and (d) motor activity (MA). Day 1 on the horizontal axis corresponds to rats 4.5 weeks old. BPM, beats per minute. Means ± SE of 5 rats per strain.

Second, SD rats, from the first day of the experiment, were entrained to the LD cycle with a constant period of 23.97h ± 0.51h for all the stages and for all the variables studied (MA, HR, DBP, and SBP) (Fig. 3). In contrast, for the TGR rats, there were differences depending on the variable studied: HR and MA rhythms were entrained to the external LD cycle from the first stage, maintaining a period of 24.01h ± 0.12h throughout the stages (Figs. 3c and 3d), whereas DBP and SBP had a period of 24.80h ± 0.19h in the first stage, which decreased gradually until it reached a value of 24.06h ± 0.14h in the third stage and was maintained thereafter (Figs. 3a and 3b).

As we found in the present study that the amplitude and the power content of the first harmonic are parallel, only the results obtained for the power content are shown. First, we found statistically significant differences among the variables studied ($P < .001$): HR had the highest values, followed by MA, and finally by SBP and DBP. We also observed that amplitude values tended to increase with development ($P < .01$), continuously in the case of the HR, to the end of the monitoring period. In the case of the other variables, they reached a plateau between the second and third stages (Fig. 4). For all the variables, TGR rats, although they started with similar values of amplitude in the first stage (at least in SBP and DBP), showed a greater increase than SD rats. Consequently, TGR rats had higher amplitude values by the end of the monitoring period than SD rats in all of the variables studied.

There were also differences in psi between the two strains of rats: In SD rats, psi was maintained constant throughout the stages and for all the variables studied, with a value of −0.61h ± 0.40h; that is, the onset of the alpha phase always occurred about half an hour before the lights went off (Fig. 5). In TGR rats, however, psi of SBP and DBP increased with age, starting from 5.51h ± 0.90h in the first stage, and reached a value of 11.27h ± 2.18h in the third stage, which was maintained in the subsequent stages (Figs. 5a and 5b). Psi of HR remained constant throughout the stages, with a value of 0.13h ± 0.94h (Fig. 5c); finally, psi of MA increased gradually from a value of −0.36h ± 0.49h in the first stage to a value of 2.47h ± 0.49h in the fourth stage (Fig. 5d). It is worth noting that, by the end of the monitoring period and independent of the value of psi, the onset of the alpha phase in the TGR rats was always delayed with respect to the onset of darkness.

DISCUSSION

With the results of the present experiment, we confirmed previous observations in that the development of hypertension in young TGR rats is parallel with the reversal of their BP profile, and that these changes start at around 5 weeks of age. The new and most important finding of the present experiment is the observation that the BP rhythm of 5-week-old TGR rats submitted to a 24h LD cycle has a period of around 25h, which subsequently decreases until it entrains

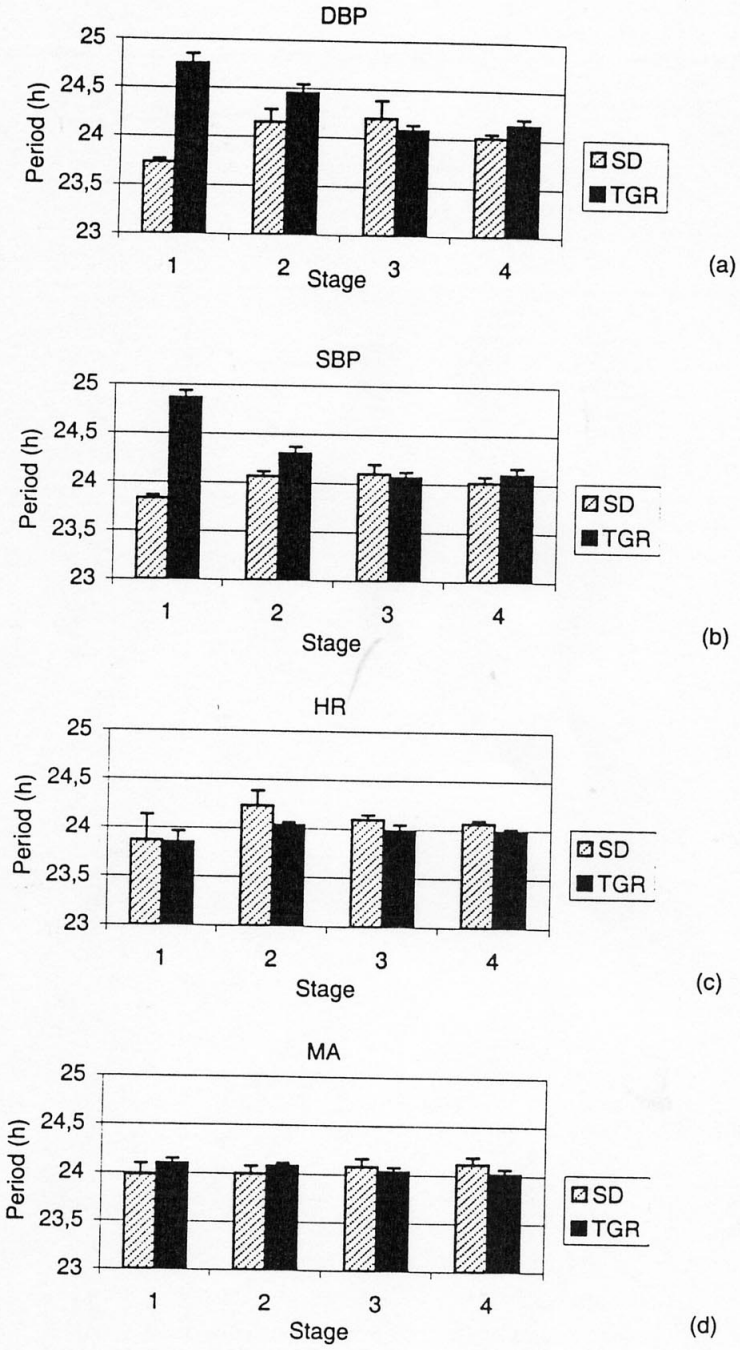

Figure 3. Progress in time of the period of the circadian rhythm of each of the variables studied throughout the experiment and for both SD and TGR rats: (a) diastolic blood pressure (DBP); (b) systolic blood pressure (SBP); (c) heart rate (HR); and (d) motor activity (MA). BPM, beats per minute. Means ± SE of 5 rats per strain.

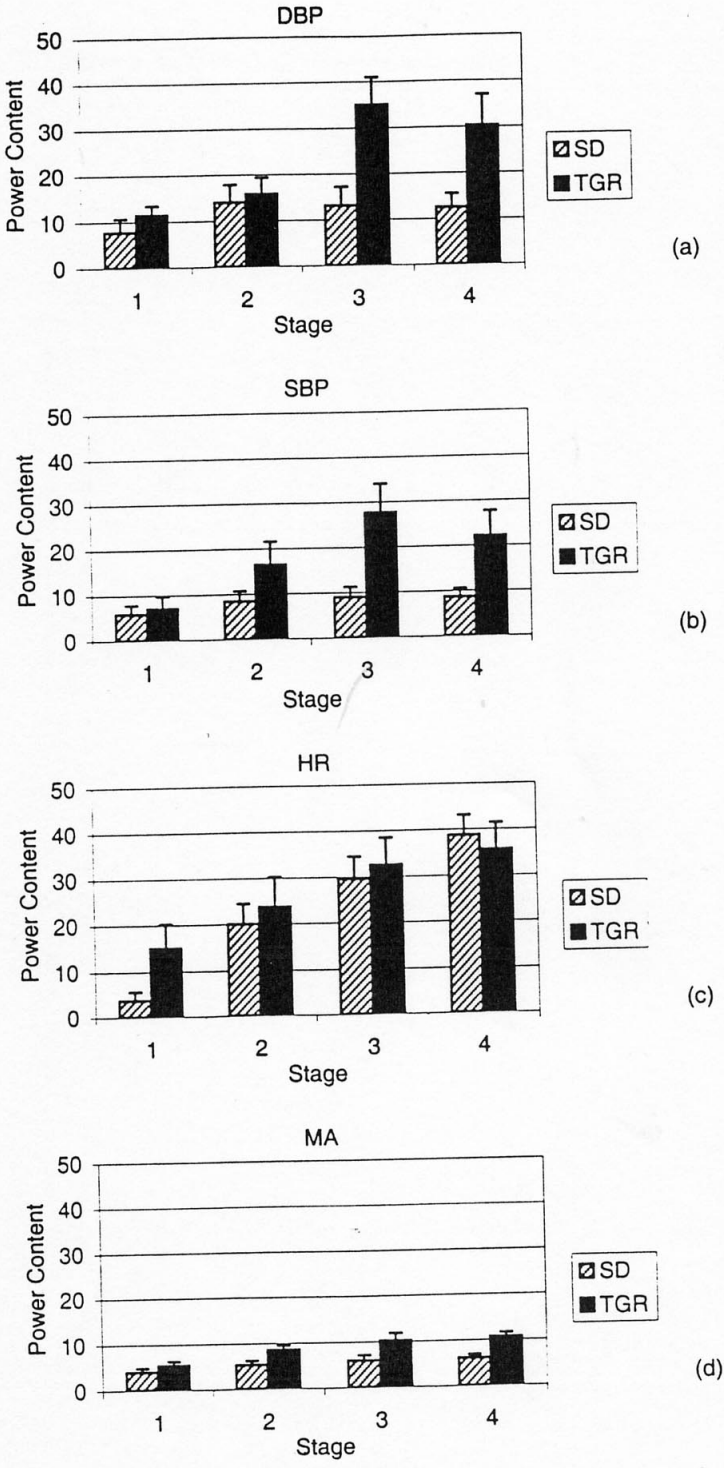

Figure 4. Progress in time of the power content of the first harmonic of the circadian rhythm of each of the variables studied throughout the experiment and for both SD and TGR rats: (a) diastolic blood pressure (DBP); (b) systolic blood pressure (SBP); (c) heart rate (HR); and (d) motor activity (MA). BPM, beats per minute. Means ± SE of 5 rats per strain.

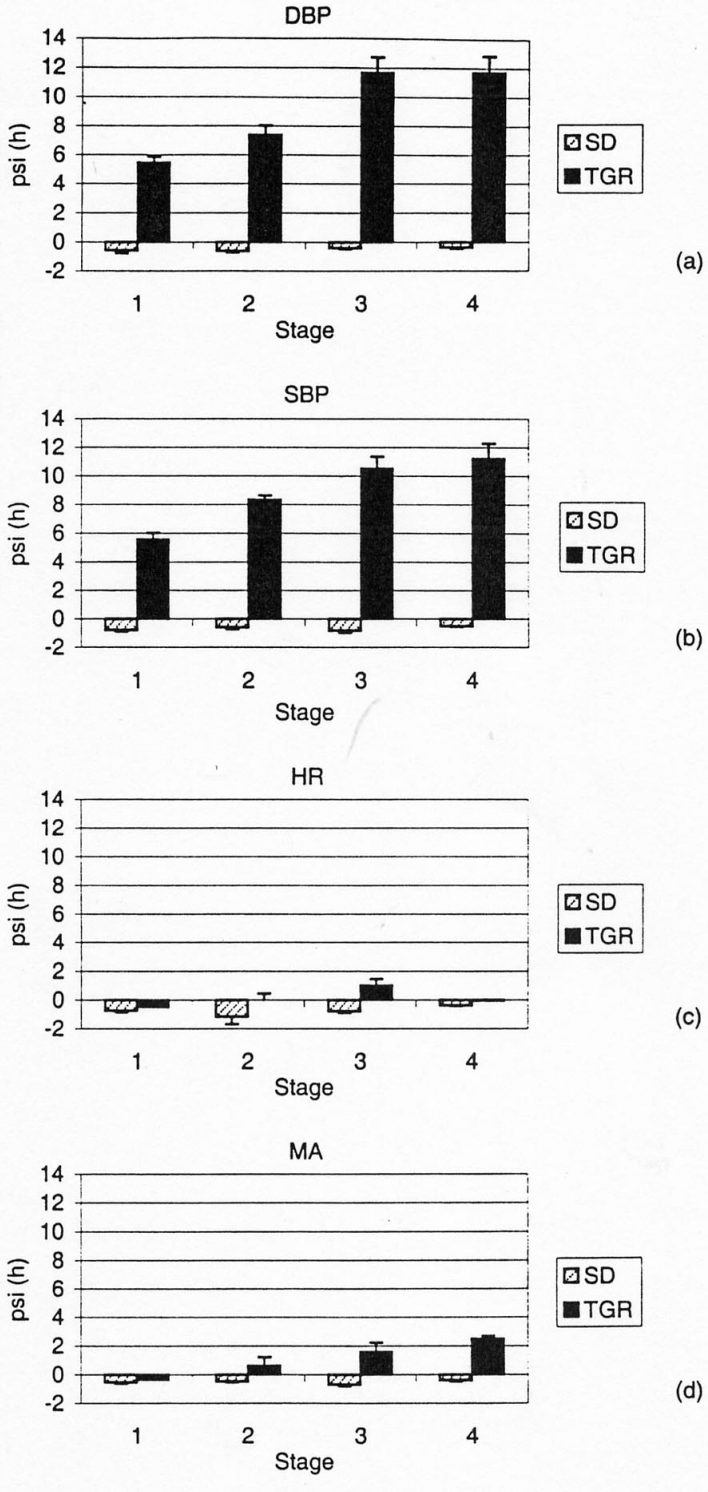

Figure 5. Progress in time of psi of each of the variables studied throughout the experiment and for both SD and TGR rats: (a) diastolic blood pressure (DBP); (b) systolic blood pressure (SBP); (c) heart rate (HR); and (d) motor activity (MA). Means ± SE of 5 rats per strain.

to the external LD cycle 3 weeks later. Probably, this change of period is related to the previously observed change of the phase of the rhythm, but the reason why the phase of the BP rhythm is finally shifted by about 11h with respect to the phase of MA and HR is still not clear. The finding that the MA, HR, and BP rhythms develop in a distinct way, in terms of period and psi, appears to indicate that each of these variables is controlled differently. If we consider that the circadian system is organized into several functional populations of oscillators (12–15), it could be suggested that at least three different populations of oscillators exist, which would independently drive MA, HR, and BP.

It is believed that the rise in BP during maturation of TGR rats is mainly due to the overproduction of renin in the adrenal due to the expression of the Ren-2 mouse gene in this gland (2). It has also been shown that urinary excretion of deoxycorticosterone, corticosterone, and aldosterone was significantly elevated in young TGR rats during the developmental phase of hypertension, but not in adult animals (3). Therefore, it appears that the adrenal expression of the transgene not only stimulates renin synthesis, but also stimulates corticosteroid production and increases its sensitivity to adrenocorticotropic hormone. This hypothesis is supported by the observation that plasma corticosterone is markedly higher in TGR rats compared to SD rats during the light phase (16). The high corticosterone levels could result in an alteration of the BP regulation and could also affect the regulation of the activity of the hypothalamo-pituitary-adrenal (HPA) axis, which could in turn modify the regulation of the circadian system. Indeed, HPA axis disturbances have been directly related to behavioral pathologies (17–21), and both the HPA axis and the circadian system are required in adaptive processes (22).

The disturbed rhythm of BP is not caused by renal and vascular damage secondary to the hypertension as the increase in BP and the reversal of the circadian BP profile develop in parallel (9). Moreover, the suprachiasmatic nuclei (SCN) of the hypothalamus, site of the master biological clock in mammals, appear to be responsible for the generation of the BP rhythm, as SCN-lesioned TGR rats lose all their circadian rhythms, including that of BP (23). The additional mouse renin gene has also been found to be expressed in the hypothalamus of TGR rats (24), and although the rise in BP in TGR rats is thought to be due to the expression of the transgene in the adrenal gland, we think that the expression of the mouse gene in the hypothalamus, and perhaps also in the SCN, could contribute to alter the generation of the BP rhythm, possibly via the adrenal cortex. It has been recently demonstrated that the SCN utilizes the autonomic nervous system pathway to communicate with the adrenal cortex (25), and several studies indicate that corticosterone secretion in the adrenal cortex, including its circadian variation, is influenced by the SCN (26–32).

An alteration of the circadian system due to the expression of the renin gene was also observed in an earlier study, in which the response to a light pulse was found to differ between TGR and SD rats, both in the phase shift induced in the rhythms of HR, MA, and BP and in the induction of c-fos expression in

the SCN (33). The different responses to light could be caused by two factors: on the one hand, a different light perception or transmission to the SCN and, on the other hand, a different functioning of the SCN. Hypertension is known to produce changes in the capillary network of the retina of rats, such as increased tortuosity, generalized narrowing of the vessels, and arteriovenous crossing effects (34). It has also been reported that long-term hypertension causes degeneration of the outer retinal layer due to obstructive changes in the choroidal vasculature (35). Although TGR rats could have an altered retina due to hypertension or to the expression of the mRen-2 gene at this level, it has been observed that retinal degeneration in some rodents does not imply changes in light response (36,37).

These findings suggest that the different responses to a light pulse observed between TGR and control SD rats (33) might not only be explained by retinal damage, but also by an alteration of the circadian system. In such a case, not only the regulation of the circadian rhythm of BP would be altered in TGR rats, but also the rest of the rhythms would be. In the present experiment, when comparing SD and TGR rats, we have indeed observed significant differences in the psi and power content of the rhythms of MA and HR, variables that are closely related to the inherent characteristics of the clock (38). Consequently, the mouse Ren-2 gene carried by TGR rats not only appears to induce hypertension, but also its expression might also be involved in the altered circadian BP rhythm generation; in addition, our results indicate that the mouse gene could interfere with or modify the normal functioning of the circadian system of TGR rats, by altering the input pathway to the pacemaker, to the circadian clock itself, or to both at the same time.

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4.3.- EXPERIMENT 10

EFFECT OF SHORT LIGHT-DARK CYCLES ON YOUNG AND ADULT TGR(mREN2)27 RATS

Chronobiology International, acceptat, 2001.

Resum

Objectiu: Diversos experiments demostren que en sotmetre rates a cicles LD de període curt (inferior a les 24 hores) es produeix una dissociació del seu ritme d'activitat motora en dos components de diferent període. En aquest estudi s'ha sotmès a diversos individus de la soca de rates hipertenses TGR(mREN2)27 (TGR) a cicles LD de 22 hores de període per tal de: 1) observar si hi ha una dissociació en els ritmes de pressió sanguínia, freqüència cardíaca i activitat motora en aquesta soca de rates; 2) estudiar com els cicles LD de 22 hores influencien el desenvolupament dels ritmes circadiaris de rates TGR joves i 3) comparar l'efecte de cicles LD de 22 hores en els ritmes circadiaris de rates TGR joves i adultes.

Material i mètodes: Deu rates mascle transgèniques heterozigotes de la soca TGR(mRen2)27 van arribar al laboratori amb 3 setmanes d'edat. Cadascuna d'elles es va col·locar en una gàbia individual i sota cicles LD de 24 hores de període. Al cap d'una setmana se'ls va implantar un aparell de telemetria que mesurava l'activitat motora (MA), la freqüència cardíaca (HR), la pressió arterial sistòlica (SBP) i la pressió arterial diastòlica (DBP). Quan els animals tenien 5 setmanes d'edat (dia 1 de l'experiment), 5 rates es van sotmetre a cicles LD de 22h de període (grup G22) i les altres 5 es van mantenir sota cicles LD de 24h de període (grup G24). Quan els animals tenien 11 setmanes d'edat (dia 45 de l'experiment) el grup G24 es va sotmetre a cicles LD de 22h de període.

Resultats: Independentment de l'edat, quan les rates TGR se sotmeten a cicles LD curts manifesten una dissociació dels seus ritmes de MA, HR, SBP i DBP. Aquests ritmes presenten dos components: un component encarrilat al cicle LD extern (i per tant amb un període de 22 hores) i un component no encarrilat pel cicle extern (amb un període d'entre 24 i 25 hores). També hem observat que les rates del grup G22 tenen, amb l'edat, un augment de BP més gran que les rates del grup G24, manifestant una major mortalitat deguda a hipertensió maligna. Per últim, sembla que les rates TGR joves tenen més dificultats a encarrilar a cicles LD curts que les adultes i també, que les rates joves tenen una mortalitat més elevada per hipertensió maligna que les adultes quan estan sotmeses a cicles de període 22 hores.

Conclusions: Els cicles LD de 22 hores de període aplicats a rates TGR indueixen una dissociació dels ritmes circadiaris de MA, HR, SBP i DBP en dos components. Aquests cicles sembla que tinguin un major efecte estressant en les rates TGR hipertenses, que no pas en rates Wistar normotenses, especialment en les rates TGR joves durant el desenvolupament de la hipertensió. Aquest fet, juntament amb l'observació que les característiques de la dissociació dels ritmes difereixen entre aquestes dues soques de rates, suggereix que potser el seu sistema circadiari també és diferent.

CHRONOBIOLOGY INTERNATIONAL, 18(4), 641–656 (2001)

EFFECT OF SHORT LIGHT-DARK CYCLES ON YOUNG AND ADULT TGR(mREN2)27 RATS

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ABSTRACT

Animals placed under short light-dark (LD) cycles show a dissociation of their circadian rhythms. However, this effect has only been studied in Wistar rats and with the motor activity (MA) rhythm. Thus, in the present experiment, we studied in TGR(mREN2)27 (TGR) rats, a strain of hypertensive rats, the effect of a short LD cycle on the circadian rhythms of MA, heart rate (HR), and blood pressure (BP). Our aim was (1) to investigate whether the exposure of TGR rats to a short LD cycle induced a dissociation of their circadian rhythms, (2) to study the effect of short LD cycles on the development of the circadian rhythms of TGR rats, and (3) to compare the effect of short LD cycles on young and adult TGR rats. One group of TGR rats was maintained under LD cycles of 22h periods (group G22). The progress in time of their rhythms was compared to that of TGR rats of the same age that had been kept under LD cycles of 24h periods (group G24). For the third point, the rhythms of a group of 5-week-old TGR rats kept under LD 22h cycles (young rats) were compared to those of a group of 11-week-old TGR rats (adult rats). Results showed that there is a dissociation of the circadian rhythms of all the variables monitored in TGR rats maintained under

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LD 22h cycles, independent of age. We have also found that group G22 showed a higher increase in BP with age and a higher mortality due to malignant hypertension compared to group G24. Finally, it seems that it is harder for young rats to entrain to short LD cycles than for adult rats, and young rats have a higher mortality due to malignant hypertension than adult rats. In conclusion, we demonstrated that short LD cycles produce a dissociation in the HR, BP, and MA circadian rhythms. The results of this experiment, compared to those previously obtained in Wistar rats, suggest that the light perception, the responses of the circadian system to light, or both are altered in the TGR rats. (*Chronobiology International, 18(4), 641–656, 2001*)

Key Words: Blood pressure; Development; Dissociation; Heart rate; Motor activity; TGR rats

INTRODUCTION

Although for some years it has been demonstrated that the suprachiasmatic nuclei (SCN) of the hypothalamus constitute the main circadian clock in mammals (1), it is still not clear how the clock is organized to generate the circadian rhythms. Nevertheless, some clues seem to indicate strongly that the SCN are formed of multiple oscillators: first, the finding that individual neurons of the SCN can express independently phased circadian rhythms (2), and second, the observation that the circadian rhythms of many vertebrates can dissociate, or "split," into two distinct components (3).

Splitting occurs in various vertebrate species, including lizards, birds, and mammals, following chronic exposure to constant light (4–11). Remarkably, few studies have described the occurrence of a dissociation of the rhythm in animals submitted to light-dark (LD) cycles. This was produced in the motor activity rhythm of hamsters by nonphotic stimulations (12) or in the motor activity rhythm and feeding behavior of Wistar rats submitted to short LD cycles (13–16).

Transgenic TGR(mRen-2)27 (TGR) rats, created in 1990 by Mullins by transfection of the mouse mRen-2 renin gene into the genome of Sprague-Dawley rats (17), are a very interesting strain of rats from the chronobiological point of view. In heterozygous male TGR rats, blood pressure (BP) starts to increase at 5 weeks of age, and a maximal value of approximately 240 mmHg is reached at 10 weeks of age (18,19). In parallel with this rise in BP, a continuous delay in the onset of the circadian acrophase is observed, leading to a complete reversal of the rhythm in BP at an age of 8 weeks, with peak values occurring during the resting phase of the animals, whereas 24h profiles of motor activity and heart rate peak during the activity phase (19,20). It also has been observed that the response of TGR rats to a light stimulus is lower compared to that of Sprague-Dawley controls (21), indicating that the circadian system of TGR rats might be somehow altered.

Apart from measuring the responses of an animal to a given zeitgeber,

another way to study the functioning of the circadian system is by exposure of this animal to LD cycles of varying lengths. Hence, in the present experiment, we submitted TGR rats to a LD cycle of a period of 22h (which has been shown to produce dissociation of the motor activity and feeding rhythms of Wistar rats) and monitored the circadian rhythms of BP, heart rate (HR), and motor activity (MA). Our aims were (1) to observe whether there is a dissociation of the BP, HR, and MA rhythms in TGR rats exposed to a short LD cycle, (2) to study how a short LD cycle influences the development of the circadian rhythms in young TGR rats, and (3) to compare the effect of a short LD cycle on young and on adult TGR rats.

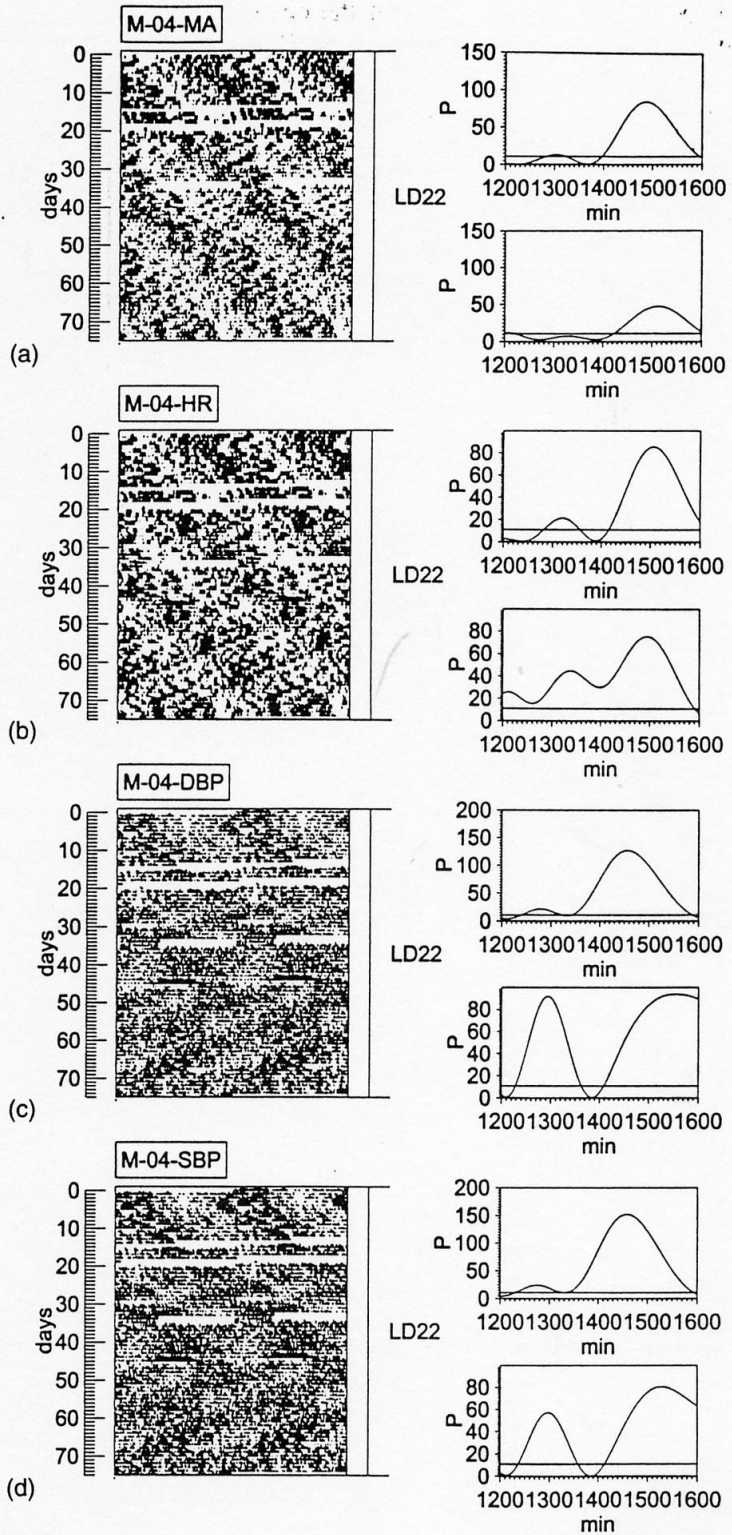
MATERIALS AND METHODS

Ten male heterozygous transgenic rats (strain TGR(mREN2)27-MolGene) 3 weeks of age were purchased from M+B A/S (Ry, Denmark). They were then housed individually in plastic cages ($380 \times 220 \times 150$ mm) under a 12h light-dark cycle (LD 12:12), with a light intensity of around 100 lux during the light phase. After adjustment to the animal facility for 1 week, the rats were equipped with pressure transmitters as described elsewhere (20) and MA, HR, systolic blood pressure (SBP), and diastolic blood pressure (DBP) were monitored. Measurements were taken every 5 minutes using the DataQuest® system (Data-Sciences, St. Paul, MN).

After the implantation of the transmitters, the animals were left to recover from the operation for 1 week. When the animals were 5 weeks old (day 1 of the experiment), 5 rats were submitted to LD cycles of a 22h period (LD 11:11, group G22), and the other five rats were submitted to LD cycles of a 24h period (LD 12:12, group G24) to study the effect of the lighting conditions on the development of the BP profile. At 11 weeks of age (day 45 of the experiment), group G24 was transferred to LD cycles of a 22h period to study the effect of a short LD cycle on adult TGR rats.

Temperature was maintained constant throughout the experiment (23°C ± 1°C), and the rats had free access to water and food (standard diet for rodents, Altromin, Lage, Germany). The experiment was performed in adherence to the *Guide for the Care and Use of Laboratory Animals*, published by the National Institutes of Health, and was approved by German federal regulations (RP Karlsruhe, Az. 35-9185.81/1/99).

To study the effect of LD 22h cycles on the development of the BP profile, data were taken from days 1 to 22 of the experiment because 3 of the 5 rats of the G22 group died of malignant hypertension between days 23 and 25 of the experiment. To compare the effects of LD 22h cycles on young and on adult rats, data from days 1 to 10 (5-week-old rats) and from days 45 to 54 of the experiment (11-week-old rats) were used. In all cases, circadian rhythms of MA, HR, SBP, and DBP were analyzed by means of a Lomb and Scargle periodogram


M-04-MA
days
LD22
P
min
(a)
M-04-HR
(b)
M-04-DBP
(c)
M-04-SBP
(d)

(22,23). A Fourier series was fitted to the data, and the amplitude and power content (PC) of the first harmonic of the circadian rhythm were thus obtained. Data were analyzed using the integrated package for chronobiology El Temps (copyright A. Díez-Noguera, University of Barcelona, Spain, 1999). For the representation of the double plots and for all of the variables studied, the daily (24h) mean was calculated and was then subtracted from each individual value. Negative values were considered as zero. Analyses of variance (ANOVA) and *t* test were used for statistical analysis. Data are shown as mean plus or minus the standard deviation unless otherwise indicated.

RESULTS

Dissociation

The double plots show that TGR rats manifest a dissociation of the circadian rhythms of SBP, DBP, HR, and MA when exposed to LD 22h cycles (Figs. 1 and 2). The dissociation is observed both in young rats during the development of their BP profile and in adult rats with mature circadian rhythms. In all the variables measured, the circadian rhythm showed two components: a light-entrained component (entrained to the external LD 22h cycle and thus with a period of around 22h) and a non-light-entrained component (which seems to be free running, with a period longer than 24.5h). These two rhythmic components were also reflected in two statistically significant peaks in the periodogram.

Effect of 22-Hour Light-Dark Cycles on the Development of the Circadian Rhythms

The 24h means showed that the gradual rise in BP was more pronounced in group G22 than in group G24 ($P < .05$), despite the fact that, on day 1 of the experiment, there were no statistically significant differences between the groups (Fig. 3). The 24h mean HR of G22 rats did not decrease as much as that of G24

Figure 1. Double plots at modulo 24h of a representative animal from group G22. This group of rats was maintained under light-dark cycles of a 22h period throughout the experiment, as indicated by the axis on the right of the graph. Also on the right of the graph, the periodograms of young (days 1 to 10 of the experiment) and adult rats (days 45 to 54 of the experiment) are shown. Figures 2a, 2b, 2c, and 2d represent, respectively, the motor activity (MA), the heart rate (HR), the diastolic blood pressure (DBP), and the systolic blood pressure (SBP) rhythms. The vertical axis on the left of the graph indicates the days of the experiment (day 0 corresponds to 5-week-old animals); blank spaces indicate data missing due to technical problems. The vertical axis of the periodogram represents the power of the peak; the horizontal axis represents the period, in minutes, of the peak; and the horizontal line indicates the level of significance.

rats; therefore, HR values tended to be higher in the G22 group, predominantly in the second half of the observation period ($P < .05$). There were no statistically significant differences between the 24h means in MA between the two groups. Also, by day 25 of the experiment, none of the rats of the G24 group had died, whereas 3 of 5 of the rats of the G22 group had died of malignant hypertension (means of the 3 rats on the day of death: DBP 196.43 ± 22.17 mmHg and SBP 262.08 ± 23.36 mmHg).

The double plots showed that the animals of the G22 group manifested a dissociation of the circadian rhythm in all of the variables studied, with the presence of a light-entrained component (LEC-G22) and a non-light-entrained component (NLEC-G22) (Fig. 2). On the other hand, rats of the G24 group were completely entrained to the LD 24h cycle, showing a circadian rhythm with only one entrained component (LEC-G24) (Fig. 3).

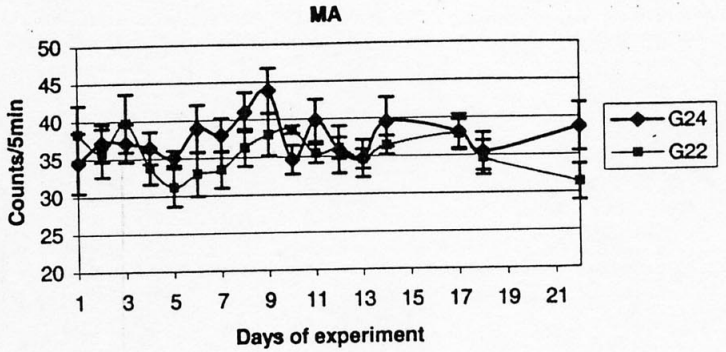
There were no statistically significant differences in the periods of the different variables monitored either in the LEC-G22 or in the NLEC-G22. The average period of the LEC-G22 was 21.97h ± 0.47h, and the average period of the NLEC-G22 was 25.07h ± 0.19h. The period of the LEC-G24 was 24.13h ± 0.10h for BP and MA and 23.98h ± 0.07h for HR (Fig. 4a).

The amplitude and PC of the first harmonic of the NLEC-G22 were higher than those of the LEC-G22 for all of the variables measured ($P < .01$). The amplitude and PC of the LEC-G22 were always lower than those of the LEC-G24 ($P < .001$). Finally, the amplitude and PC of the LEC-G24 were higher than those of the NLEC-G22 ($P < .05$), but only statistically significant for HR and MA (Figs. 4b and 4c).

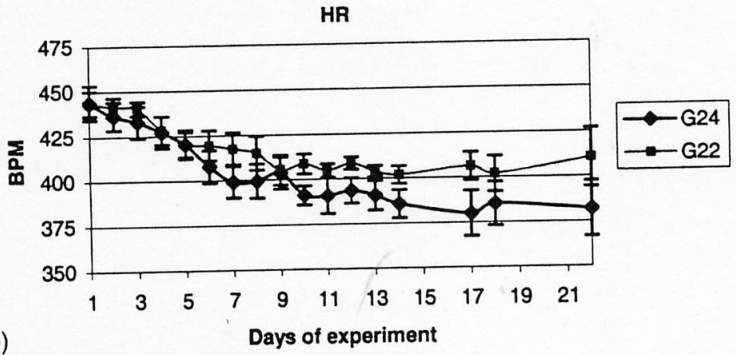
Effect of 22-Hour Light-Dark Cycles in Young and in Adult Rats

As seen with the double plots, the circadian rhythms of both young and adult TGR rats dissociated into two components under LD 22h cycles (Figs. 2

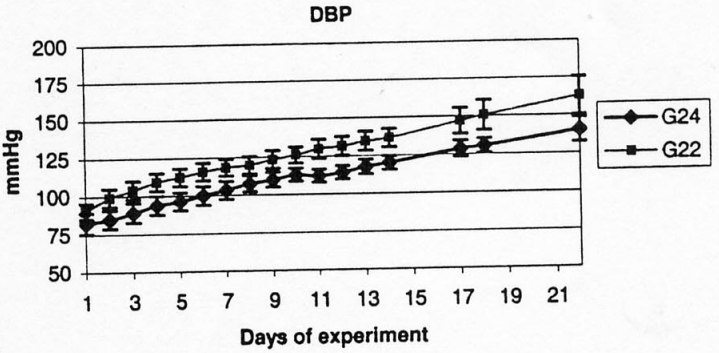
Figure 2. Double plots at modulo 24h of a representative animal from group G24. This group of rats was maintained under light-dark cycles of a 24h period for 45 days. The light-dark schedule is shown in the upper bar: The white part indicates 12h of light, and the black part indicates 12h of darkness. On day 46 of the experiment, the rats were transferred to a LD cycle of a 22h period by shortening the light phase to 11h. The axis on the right of the graph indicates the lighting regime throughout the experiment. Also on the right of the graph, the periodograms of young (days 1 to 10 of the experiment) and adult rats (days 45 to 54 of the experiment) are shown. Figures 3a, 3b, 3c, and 3d represent, respectively, the motor activity (MA), the heart rate (HR), the diastolic blood pressure (DBP), and the systolic blood pressure (SBP) rhythms. The vertical axis of the graph indicates the days of the experiment (day 0 corresponds to 5-week-old animals); blank spaces indicate data missing due to technical problems. The vertical axis of the periodogram represents the power of the peak; the horizontal axis represents the period, in minutes, of the peak; and the horizontal line indicates the level of significance.


MA
Counts/5min
50
45
40
35
30
25
20
1 3 5 7 9 11 13 15 17 19 21
Days of experiment
G24
G22

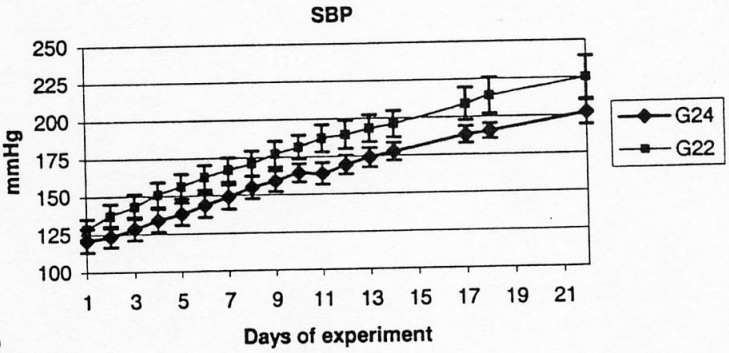
(a)


HR
BPM
475
450
425
400
375
350
1 3 5 7 9 11 13 15 17 19 21
Days of experiment
G24
G22

(b)


DBP
mmHg
200
175
150
125
100
75
50
1 3 5 7 9 11 13 15 17 19 21
Days of experiment
G24
G22

(c)


SBP
mmHg
250
225
200
175
150
125
100
1 3 5 7 9 11 13 15 17 19 21
Days of experiment
G24
G22

(d)

and 3). After 25 days in LD 22h cycles, 3 of 5 of the young rats had died, whereas only 1 of 5 of the adult rats had died.

Within the young animals, and for all the variables measured, the amplitude and PC of the first harmonic of the NLEC was always higher than those of the LEC ($P < .05$). On the other hand, no significant differences in the amplitude and the PC between the LEC and the NLEC were found within the adult animals for any of the variables measured.

No statistically significant differences were found in the period of the LEC, either between the variables monitored or between young and adult rats, with an average period of $21.82h \pm 0.48h$ (Fig. 5a). In contrast, there were significant differences in the period of the NLEC between young and adult rats: In the BP rhythm, young rats tended to have a longer period than adult rats, although this difference was only statistically significant for DBP ($P < .05$, Fig. 5d).

The LEC of the rhythm of the cardiovascular variables of adult rats tended to have higher amplitude and PC than those of young rats, although this difference was only statistically significant for HR ($P < .05$, Figs. 5b and 5c), whereas the amplitude of the LEC of the rhythm of MA was higher in young than in adult rats ($P < .05$, Fig. 5b). In the case of the NLEC, the amplitude and PC of the rhythm of BP in young rats tended to be higher than that of adult rats, although only the difference of PC of the SBP rhythm was statistically significant ($P < .05$, Figs. 5e and 5f). There were no statistically significant differences in the amplitude and PC of the rhythm of HR between adult and young rats; finally, the amplitude of the rhythm of MA was higher in young than in adult rats ($P < .01$, Fig. 5f).

DISCUSSION

Here, for the first time, we present evidence that short LD cycles not only produce a dissociation of the circadian rhythm of MA, but also a dissociation of the circadian rhythms of BP and HR of young and adult TGR rats. In each of these rhythms, two circadian components of different periods appear: a component entrained to the external LD cycle and a nonentrained component. On the basis of the dissociation of a rhythm and of other observed phenomena such as splitting and the maturation of the circadian system (24), we can consider the circadian system as being formed by a population of autonomous circadian oscillators. In this multioscillatory model (25–28), each oscillator would have a dif-

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Figure 3. Progress in time of the 24h mean of each of the variables studied during days 1 to 22 of the experiment. Data from days 15, 16, and 19 to 21 are missing due to technical problems. Means ± SE of 5 rats per group. BPM, beats per minute; DBP, diastolic blood pressure; HR, heart rate; MA, motor activity; SBP, systolic blood pressure.

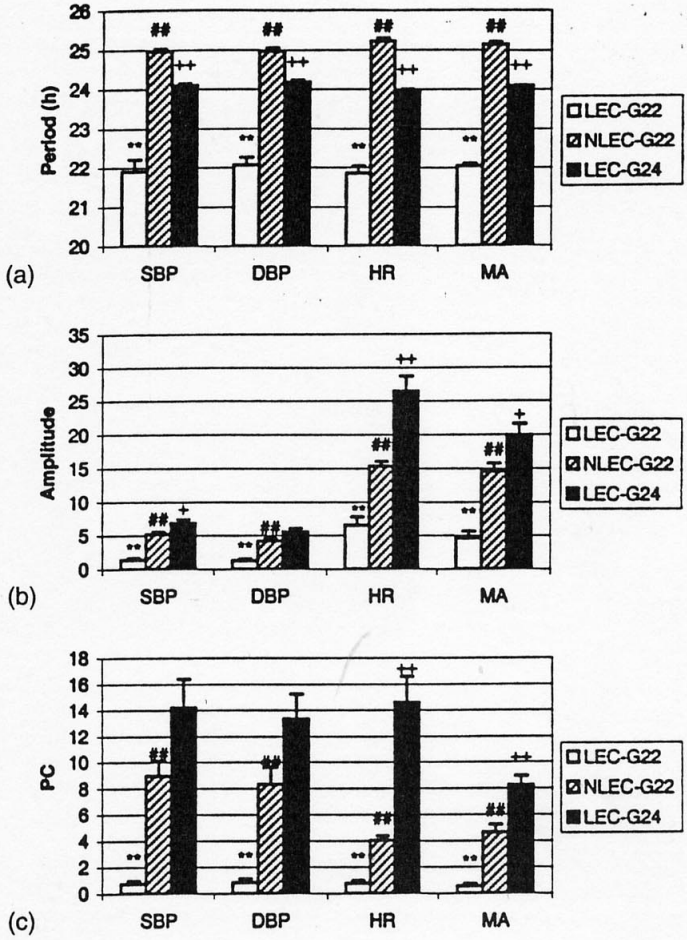

Figure 4. (a) Period, (b) amplitude, and (c) power content of the first harmonic for each of the rhythms of the G22 and the G24 groups corresponding to days 1 to 22 of the experiment (see text for more details). Means ± SE of 5 rats per strain. $^{**}P < .005$ (LEC-G22 compared to LEC-G24); $\#\#P < .005$ (LEC-G22 compared to NLEC-G22); $+P < .05$ (NLEC-G22 compared to LEC-G24); $++P < .005$ (NLEC-G22 compared to LEC-G24).

ferent frequency within the circadian range; therefore, not all the oscillators would necessarily be entrained by a specific zeitgeber. Accordingly, when the period of the external cycle is similar to the endogenous period of the system, the oscillators will run in close phase and will generate a single rhythm. This is the case in group G24: As the period of the external LD cycle (24h) resembles the endogenous period (ca. 24.5h), only the LEC is observed. On the other hand, when the period of the external cycle deviates from the endogenous period, there might be only one group of oscillators able to entrain to the external cycle. This

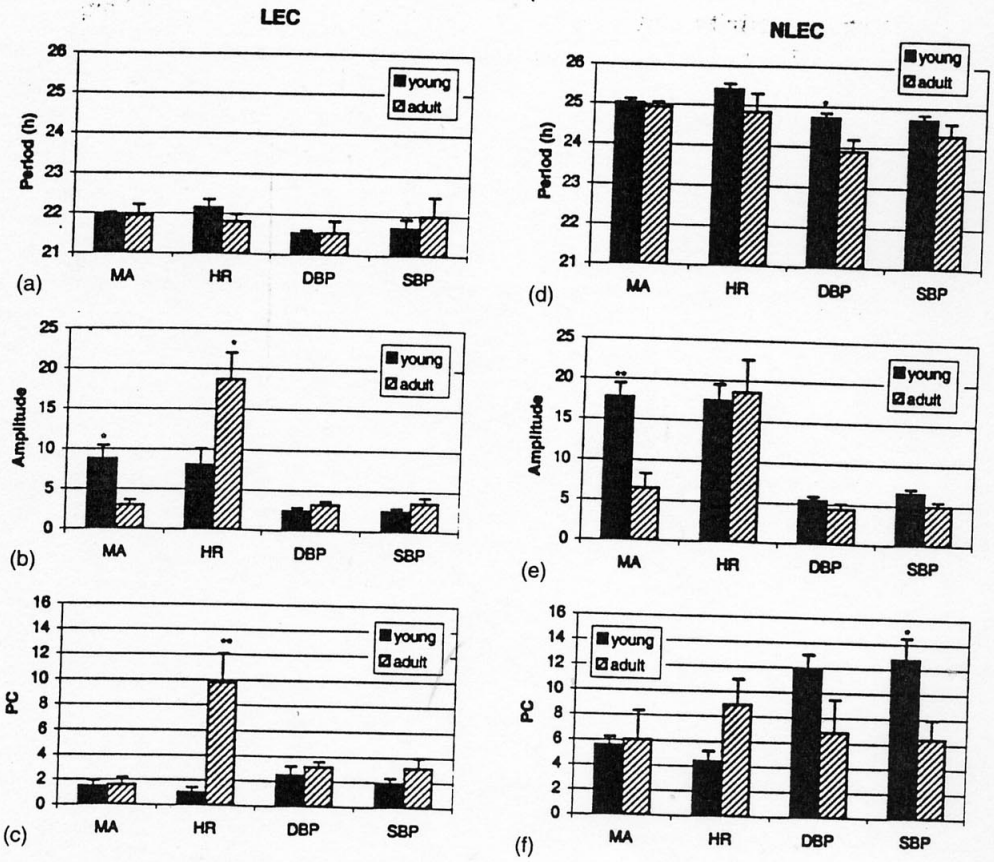

Figure 5. Period, amplitude, and power content of the first harmonic of the light-entrained component (LEC) (left, top to bottom, respectively) and the non-light-entrained component (NLEC) (right, top to bottom, respectively) for each of the variables measured, corresponding to days 1 to 10 of the experiment (young rats) or to days 45 to 54 of the experiment (adult rats). See text for more details. Means ± SE of 5 rats per strain in young rats and means ± SE of 4 rats per strain in adult rats. $*P < .05$; $**P < .01$.

is the case in G22 animals, in which the LEC would be generated by a small number of entrained oscillators in addition to the masking effect of light, and the NLEC would be generated by the oscillators that have not been able to entrain.

Although the nature of the oscillators is unknown, some observations point to the neurons as being the principle candidates, as cultured individual neurons of the SCN are able to generate spontaneous discharges (28–31) and can express independently phased circadian firing rhythms (2). The synaptic communication and other complex interactions are able to synchronize the circadian rhythms in individual SCN neurons (32) and therefore to generate the rhythm of the circadian pacemaker (33). Moreover, Zlomanczuk et al. (34) found a correlation be-

tween electrical activity of the SCN and wheel-running behavior of hamsters, demonstrating that the splitting observed in the overt rhythm of wheel-running activity also occurred in the SCN firing profile.

In TGR rats, higher BP at a younger age appears to be important in determining the risk of onset of malignant phase hypertension (35). In the present experiment, the higher 24h mean BP values of G22 rats compared to those of G24 rats would explain the high number of G22 rats that developed malignant hypertension and a higher mortality due to this disease compared to the G24 group. The high BP and HR values developed by G22 rats could be due to a stress effect produced by the abnormal short LD cycle. In fact, restraint stress causes tachycardia and pressor responses in rats (36), probably via the activation of the hypothalamic-pituitary-adrenal axis, which would cause an increase in plasma corticosterone concentration (37). In addition, it has been found that spontaneously hypertensive rats have a higher reactivity to stress in BP than Wistar-Kyoto rats (36,38); interestingly, no animal died in the experiments with Wistar rats exposed to short LD cycles (13,14,39), suggesting that the mechanism used by transgenic hypertensive rats to cope with stress is impaired, resulting in an additional risk factor in hypertension. Hence, in G22 rats, the stress of being submitted to short LD cycles has been added to the stress created by hypertension, leading to increased cardiovascular complications and death. Moreover, short LD cycles seem to have a greater stress effect on young than on adult TGR rats (3 of 5 of the young rats vs. only 1 of 5 of the adult rats died within 25 days), probably because in the case of young rats, the exposure to LD 22h cycles coincided with the development of hypertension and, simultaneously, with their abnormal BP profile (19).

Here, we have found that the period of the NLEC of the BP rhythm of the young rats was significantly different from that of the HR and MA rhythms, whereas in the adult rats, no such difference was observed. Previous experiments showed that the period length of the BP rhythm was approximately 25h and differed from that of HR and MA even when TGR rats were submitted to LD 24h cycles (Canal-Corretger et al. unpublished data), and together with a difference in period length, a difference in the acrophase of the BP rhythm was observed (19). However, this difference between period lengths is only observed in young TGR rats at the time when the BP rises (Canal-Corretger et al. unpublished data), and thus it is probably related to the development of the BP circadian rhythm. The hypertension of TGR rats is believed to be produced by the expression of the mouse Ren-2 gene in the adrenal gland of the rat (40–42), and it has been observed that the rise in plasma active renin, plasma prorenin, and adrenal renin parallels that in BP (43). However, it is still not clear which mechanisms induce the changes in period and in the circadian phase in these rats, although the renin-angiotensin system and the adrenal steroids seem to play an important role (44).

In young TGR rats, we have observed that the amplitude of the first harmonic of both LEC and NLEC of the MA rhythm is higher than in adult rats.

This could be related to the decrease in the total amount of MA that TGR rats experience with aging (19). But, it is worth paying attention to the finding that, in young rats submitted to LD 22h cycles, the NLEC was more important (had higher amplitude and power content) than the LEC for all the variables studied, whereas in adult rats, both components were equally important. This is in contrast with the results found for Wistar rats submitted to LD 22h cycles, for which the LEC component was more important (it explained a higher percentage of overall variance) than the NLEC independent of age (13,14). Therefore, it appears that either light perception or the functioning of the pacemaker differs in the two strains of rats.

Although one reason for this difference could be the effect of hypertension on the pacemaker, as changes in SCN morphology and function have been associated with the development of this illness both in rats (45,46) and in humans (47,48), we believe that the circadian system of TGR rats is altered due to the expression of the mouse Ren-2 gene. Zhao et al. (49) found that the additional mouse gene is also expressed in the hypothalamus of TGR rats. In support of our hypothesis are the observations that a single light pulse at CT14 (circadian time 14) does not produce any significant phase shift of HR and BP rhythms or induce c-fos expression in the SCN of TGR rats in contrast to control Sprague-Dawley rats (21). Taken together, these data support the notion that, in TGR rats, the perception of light, the transformation of the light signal to the pacemaker, and/or the response of the circadian system to light is/are impaired.

In conclusion, here we have demonstrated that short LD cycles produce a dissociation of the circadian rhythms of MA, HR, and BP of TGR rats into two components. The short LD cycle seems to have a greater stress effect in TGR rats than in Wistar rats, especially in the young TGR rats during the development of hypertension. This finding, together with the observation that the characteristics of the dissociation observed in TGR rats differ from those of Wistar rats, suggests that their circadian systems also differ, possibly because of the transgene expressed in TGR rats.

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4.4.- DISCUSSIÓ

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Les rates TGR(mRen2)27 (TGR) són una soca hipertensa creada per inserció a l'atzar del gen mRen2 de ratolí dins el genoma de rates Sprague-Dawley (SD) (Mullins et al. 1990). Les rates TGR homozigotes pel gen mRen2 pateixen una hipertensió fulminant que els provocarà la mort si no són degudament tractades. Així doncs en aquests estudis es van emprar rates TGR heterozigotes, ja que poden viure llarg temps sense tractament i per tant, es poden estudiar els seus ritmes circadiaris sense cap tipus d'interferència externa. Una característica de la soca TGR és que les rates presenten una disfunció renal, glomeruloesclerosi, hipertròfia del ventricle esquerre i engruiximent de la capa medial de les artèries, que van progressant amb l'edat (Bachmann et al. 1992, Springate et al. 1994).

Pel que fa a l'origen de la hipertensió en les rates TGR, es creu que aquesta és deguda a l'expressió del gen que se li ha insertat de ratolí, ja que això provocaria una hiperactivació del sistema renina-angiotensina (Lemmer et al. 2000b). Aquesta hipòtesi es veu reforçada per dues observacions: en primer lloc, el transgen està "hiper-expressat" en teixits extra renals i especialment en el còrtex adrenal (Bader et al. 1992, Yamaguchi et al. 1992, Peters et al. 1993, Rubattu et al. 1994), mentre que l'expressió renal de la renina endògena està suprimida i a més, la renina activa circulant és majoritàriament d'origen adrenal (Véniant et al. 1995, Peters et al. 1996); en segon lloc, s'ha observat que l'administració tant d'inhibidors de l'enzim convertidor d'angiotensina (ECA), com d'antagonistes del receptor AT1 d'angiotensina aconsegueix disminuir els valors de pressió arterial (PA) de rates TGR fins a nivells normals (Bader et al. 1992, Hirth-Dietrich et al. 1994, Lemmer et al. 1994, Moriguchi et al. 1994, Böhm et al. 1995, Schneko et al. 1995).

Les rates TGR s'usen com a model animal de la hipertensió secundària en humans i a part d'això, també són molt interessants des del punt de vista cronobiològic, ja que s'ha observat que els animals adults d'aquesta soca manifesten un ritme de PA en fase inversa amb els ritmes d'activitat motora (AM) i de freqüència cardíaca (FC). Concretament, mentre que els nivells més elevats d'AM i de FC es troben durant la nit (fase d'activitat), els valors màxims de PA ocorren durant el dia (fase de repòs) (Lemmer et al. 1993). En rates TGR joves d'unes 4 setmanes de vida, però, tant els valors com el patró circadiari de PA són normals i no és fins a la cinquena setmana de vida que, paral·lelament a un increment dels nivells de PA també s'observa un canvi progressiu de fase del ritme de PA que culminarà, entre la desena i l'onzena setmanes d'edat amb el patró descrit anteriorment (Witte i Lemmer 1999).

En l'Experiment 9 hem observat interessantment que en rates de 5 setmanes d'edat, simultàniament a un increment de PA, també hi ha una incapacitat per encarrilar al cicle extern de llum-foscor (LD 12:12h), possiblement deguda a l'endarreriment gradual de l'hora d'inici de

la fase alfa del ritme de PA. Aquest procés acaba al voltant de la desena setmana de vida, en la qual les rates pateixen hipertensió i tot i que el seu ritme de PA està completament encarrilat al cicle extern de llum-foscor, presenta un desfasament d'unes 12 hores respecte del dels ritmes d'AM i FC. Curiosament, però, entre rates TGR i rates control SD, no només hi ha diferències en l'evolució del ritme de PA, sinó que també n'hi ha en l'evolució dels ritmes d'AM i FC: mentre que a les 5 setmanes d'edat les rates SD ja manifesten uns ritmes circadiaris ben encarrilats amb el cicle LD extern, amb un període, relació de fases i una potència del ritme estables, les rates TGR encara modifiquen aquestes variables fins a la desena setmana de vida, abans no les estabilitzen. Així veiem que l'evolució dels ritmes circadiaris de les rates TGR i SD difereix. Tanmateix, les diferències entre aquestes dues soques de rates no són només observables en rates joves durant la maduració dels ritmes, sinó que també es poden veure en rates adultes, per exemple, en la relació de fases entre els ritmes circadiaris i el cicle extern: en rates SD la fase alfa dels ritmes estudiats s'inicia sempre abans que s'apaguin els llums, mentre que en rates TGR la fase alfa comença després de l'inici de foscor. A més a més recentment s'ha observat que tant l'expressió de mRNA de c-fos induïda per la llum en el NSQ, com els efectes d'un pols de llum en la fase dels ritmes circadiaris també són diferents entre la soca hipertensa i la control (Lemmer et al. 2000a). Així doncs, totes aquestes dades en conjunt semblarien indicar que el funcionament del sistema circadiari d'aquestes dues soques de rates no és idèntic.

La raó d'aquesta divergència la podríem buscar, en primer lloc, a nivell del sistema receptor de la informació lluminosa externa. En efecte, s'ha demostrat que la hipertensió causa danys en la xarxa capil·lar de la retina de rates (Bhutto and Amemiya 1997) i que a llarg termini produeix una degeneració de la capa externa de la retina (Sakaguchi 1997), lloc on se situen els cons i bastons, principals fotoreceptors de la retina. Tot i això, estudis en ratolins transgènics amb degeneració retinal demostren que aquests continuen responent a la llum igual que ho fan els ratolins controls amb retines intactes (Foster et al. 1993, Lucas et al. 1999). Per consegüent, tot i les possibles diferències que hi pugui haver a nivell retinal entre rates hipertenses TGR i rates SD normotenses, caldria buscar també en d'altres nivells l'origen de les diferències entre aquestes dues soques de rates. Per una banda, el fet que els ritmes circadiaris de PA i FC de les rates TGR no canviïn de fase, ni s'expressi c-fos després d'un pols de llum (Lemmer et al. 2000a) fa pensar que la transmissió de la informació lluminosa cap al rellotge biològic podria estar alterada en aquesta soca de rates. Per altra banda, el fet que, a diferència de les rates control, les rates TGR no presentin una expressió rítmica de mRNA de c-fos en el NSQ sota cicles LD, ni una variació espontània dels nivells d'aquest gen en DD (Lemmer et al. 2000a); i que tant l'evolució dels seus ritmes circadiaris sota cicles LD, com la relació de fases que finalment estableixen aquests ritmes amb el cicle LD extern també difereixin, indueix a pensar que potser el mateix rellotge biològic estigui funcionant diferentment en ambdues soques de rates. El mateix gen addicional de renina, doncs, a part de produir el desenvolupament d'una

hipertensió severa, també podria estar afectant els mecanismes pels quals el rellotge regula els ritmes circadiaris. De fet, s'ha trobat que el transgen s'expressa a l'hipotàlem de rates (Zhao et al. 1993) i per tant, potser també ho faci en el NSQ.

En l'Experiment 10 i per primera vegada, s'ha demostrat que cicles LD de període 22h produeixen una dissociació dels ritmes circadiaris d'AM, FC i PA de rates TGR, tant joves com adultes, en dos components, un d'encarrilat al cicle extern (LEC) i un altre de no encarrilat a la llum (NLEC). S'ha demostrat amb anterioritat respecte del LEC que, tot i que en part pugui ser producte d'un emmascarament produït per la llum, també és producte d'un mecanisme encarrilador. Aquesta última afirmació és corroborada per les següents troballes: 1) la relació de fases entre el LEC i el cicle extern canvia segons el període del cicle LD, 2) si després de sotmetre les rates a cicles LD curts es passen a DD, l'inici del ritme en curs lliure estarà en fase amb la del LEC previ i 3) els postefectes observats en rates un cop s'han passat a DD depenen del període del cicle LD previ i per tant, depenen del període del LEC (Vilaplana et al. 1997, Campuzano et al. 1998, Campuzano et al. 1999).

Les causes de la dissociació del ritme es poden explicar fàcilment assumint un model multioscil·latori del sistema circadiari (Díez-Noguera 1994, Rosenwasser i Adler 1986, Miller 1998, Honma et al. 1998). Segons aquest model, el sistema circadiari estaria format per un conjunt d'oscil·ladors autònoms acoblats entre sí que generarien els ritmes circadiaris. De tota manera, cada oscil·lador tindria una freqüència diferent dins el rang circadiari, de manera que no tots els oscil·ladors haurien de ser necessàriament encarrilats per un determinat *zeitgeber*. Això podria explicar perquè quan un animal se sotmet a un cicle extern de període similar al període endogen (per exemple de 24h), es manifesta només un sol component; mentre que quan un animal està sotmès a un cicle extern de període allunyat de l'endogen, potser només un petit grup d'oscil·ladors hi podrà encarrilar (i per tant generarà el LEC) i la resta d'oscil·ladors que no han encarrilat generaran el NLEC (Vilaplana et al. 1997).

La teoria que múltiples oscil·ladors formen el sistema circadiari es veu reforçada amb els resultats de l'Experiment 10, els quals mostren que les característiques dels dos components manifestats per les rates TGR sotmeses a cicles LD de 22h de període no són iguals en els diversos ritmes estudiats (AM, FC i PA). Aquests resultats, juntament amb els de l'Experiment 9, en el qual s'ha observat que, dins el grup de rates transgèniques, la relació de fases o psi és diferent segons el ritme estudiat, suggereixen que cadascun d'aquests ritmes està controlat per un grup d'oscil·ladors diferents. Per altra banda, també s'ha observat que després d'un pols de llum a CT15, en les rates TGR s'observa un endarreriment de la fase del ritme d'activitat motora, mentre que no es produeix cap canvi de fase significatiu en els ritmes de FC i PA (Lemmer et al. 2000a) i en canvi en rates SD es produeixen canvis de fase en tots aquests ritmes. Assumint

que cadascun d'aquests ritmes està controlat per un grup d'oscil·ladors diferents, podrem explicar perquè les característiques d'aquests ritmes són diferents.

La naturalesa d'aquests oscil·ladors és encara desconeguda de moment, però tot fa pensar que podrien ser neurones, ja que neurones individuals de NSQ en cultiu són capaces de generar ritmes circadiaris espontanis de freqüència de descàrrega (Honma et al. 1998, Bos i Mirmiran 1990, Liu et al. 1997, Herzog et al. 1998) i poden expressar ritmes de descàrrega amb fases independents (Welsh et al. 1995). A més, diversos tipus d'interaccions entre neurones individuals del NSQ poden sincronitzar els seus ritmes circadiaris (Shirakawa et al. 2000) i per tant generar el ritme circadiari del rellotge biològic (Mirmiran et al. 1995). En relació amb la manifestació d'un ritme de dos components, s'ha demostrat en hàmsters que l'*splitting* observat en el ritme circadiari d'AM també apareix en el ritme d'activitat elèctrica del NSQ (Zlomanczuk et al. 1991), cosa que suggereix que en la dissociació observada en el ritme d'AM de rates potser també ocorre el mateix.

Les rates de la soca TGR-Han (les usades en els nostres experiments) tenen una baixa incidència d'hipertensió maligna, concretament aquesta és del 18% en mascles (Whitworth et al. 1994) i en canvi, en l'Experiment 10 ha estat d'un 40%. Aquesta dada sembla suggerir que el fet de sotmetre les rates TGR a cicles LD de període 22h, capaços d'induir la dissociació dels seus ritmes circadiaris, els provoca una gran situació d'estrès, capaç fins i tot d'augmentar la mortalitat per hipertensió maligna. Aquesta situació d'estrès, a més, sembla que es veu agreujada quan coincideix amb el desenvolupament dels ritmes circadiaris, ja que: 1) la incidència d'hipertensió maligna en rates joves en el nostre experiment ha estat del 60%, mentre que en les rates adultes ha estat del 20%; 2) l'augment de PA en les rates joves sotmeses a cicles LD de 22h ha estat més pronunciat que en les rates de la mateixa edat sotmeses a cicles LD de 24h i 3) en rates joves sotmeses a cicles LD de 22h, la manifestació del NLEC és molt més marcada que la del LEC.

Cal esmentar també que hem trobat diferències en la manifestació del LEC i NLEC en rates TGR, comparat amb estudis anteriors realitzats amb rates normotenses Wistar. Aquests últims estudis, al contrari que els resultats de l'Experiment 10, indicaven que no hi havia diferències en quant a la manifestació dels dos components entre rates joves i adultes i també s'havia observat que el LEC sempre era molt més marcat que el NLEC (Vilaplana et al. 1997, Campuzano et al. 1998). Les diferències en quant a manifestació del ritme entre rates joves i adultes que hem trobat, podrien estar relacionades amb tots els processos que acompanyen el desenvolupament i maduració dels ritmes circadiaris en rates TGR, ja explicats amb anterioritat. Ara bé, pel que fa a la importància relativa entre el LEC i el NLEC, la hipòtesi -anteriorment proposada- que la transmissió de la informació lluminosa pot estar alterada en les rates TGR, pot ser també vàlida aquí. En efecte, s'ha demostrat recentment que la manifestació de dos components com a conseqüència de sotmetre una rata a cicles LD de període inferior a les 24h

depèn de la intensitat de llum (Cambras et al. 2000). Concretament, s'ha observat que sota intensitats de llum baixes s'afavoreix la dissociació del ritme d'AM en dos components. Per tant, si la transmissió de la informació lluminosa estigués alterada en les rates TGR de manera que fossin menys sensibles a la llum, això podria explicar l'elevada importància que té el NLEC respecte del LEC en aquesta soca de rates i també, perquè no augmenta l'expressió de mRNA de c-fos en el NSQ després d'un pols de llum (Lemmer et al. 2000a). De tota manera el fet que, després d'un pols de llum, es produeixi un canvi de fase en les rates TGR (almenys en el ritme d'AM) similar al produït en rates SD mostra que aquestes rates sí que són sensibles a la llum.

4.4.- DISCUSSION

TGR(mRen2)27 (TGR) rats are a hypertensive strain created by random transfection of the mRen2 gene of mouse into the genome of Sprague-Dawley (SD) rats (Mullins et al. 1990). Homozygous TGR rats for the mRen2 gene develop fulminant hypertension, which is lethal unless they are treated pharmacologically. Hence, we used heterozygous TGR rats, as they can live longer without treatment, and so their circadian rhythms can be studied without any external interference. These rats are characterised by glomerulosclerosis, progressive left ventricular hypertrophy and arterial medial thickening, which occur with increasing age (Bachmann et al. 1992, Springate et al. 1994).

It has been suggested that hypertension in TGR rats might be produced by the expression of the mouse renin gene, which would induce hyperactivation of the renin-angiotensin system (Lemmer et al. 2000b). This hypothesis is supported by two observations: on the one hand, the transgene is overexpressed in extrarenal tissues, especially in the adrenal cortex (Bader et al. 1992, Yamaguchi et al. 1992, Peters et al. 1993, Rubattu et al. 1994), whereas the expression of endogenous renal renin is suppressed, and the circulating active renin is chiefly of adrenal origin (Véniant et al. 1995, Peters et al. 1996); on the other hand, the administration of angiotensin-converting enzyme inhibitors and angiotensin AT1 receptor antagonists decreases the blood pressure (BP) of TGR rats to normal levels (Bader et al. 1992, Hirth-Dietrich et al. 1994, Lemmer et al. 1994, Moriguchi et al. 1994, Böhm et al. 1995, Schneko et al. 1995).

TGR rats are used as animal models of secondary hypertension in humans and they are also useful for chronobiological studies because adult rats show an inverse BP rhythm corresponding to the motor activity (MA) and heart rate (HR) rhythms. Specifically, MA and HR are maximal at night (activity phase), whereas BP peaks during the day (resting phase) (Lemmer et al. 1993). However, 4-week-old TGR rats have normal values and normal circadian rhythm of BP, but at around 5 weeks of age, in parallel with an increase of BP, there is a progressive phase shift, which will end with the above-mentioned inverse BP rhythm between the 10^{th} and the 11^{th} week (Witte and Lemmer 1999).

In Experiment 9 we show that at 5 weeks of age, BP starts to increase and the animal cannot entrain to the LD cycle (LD 12:12h), probably owing to the progressive delay of the alpha phase of the BP rhythm. This process ends around the 10^{th} week of age, when rats suffer from hypertension and show a circadian rhythm of BP fully entrained to the external LD cycle, but delayed around 12 hours with respect to the MA and HR rhythms. However, TGR and SD rats differ not only in the evolution of their BP rhythm, but also in the evolution of the MA and

HR rhythms: 5-week-old SD rats show fully entrained circadian rhythms, with stabilised period, phase relation and power content, in contrast to TGR rats, which modify these variables until the 10th week of age. These differences are also observed in adult rats, e.g. in the phase relation between the circadian rhythms and the external LD cycle: in SD rats, the alpha phase always starts before lights off, whereas in TGR rats the alpha phase starts after lights off. Moreover, both the c-fos mRNA expression produced by light in the suprachiasmatic nucleus (SCN) and the phase shifts induced by a light pulse on the circadian rhythms also differ between the hypertensive and the control rats (Lemmer et al. 2000a). These data suggest that the functioning of the circadian system of the two strains of rats is not identical.

The cause of these dissimilarities can be sought, in the first place, at the light reception level. Hypertension alters the capillary network of the retina of rats (Bhutto and Amemiya 1997) and long-term hypertension causes degeneration of the outer retinal layer (Sakaguchi 1997), where the rods and cones, major photoreceptors of the retina, can be found. However, several studies on transgenic mice have revealed that the response to light of degenerated retina is indistinguishable from that of intact retina (Foster et al. 1993, Lucas et al. 1999). Therefore, even if TGR and SD rats differ at the retinal level, we should examine other levels. On the one hand, the circadian rhythms of BP and HR were not phase-shifted, nor was the c-fos mRNA inducted after a light pulse in TGR rats (Lemmer et al. 2000a), suggesting that the transmission of photic information to the circadian pacemaker is altered. On the other hand, TGR rats do not show rhythmic expression in c-fos mRNA in the SCN under LD cycles or a spontaneous variation in c-fos mRNA in DD (Lemmer et al. 2000a). Furthermore, the development of the circadian rhythms under LD cycles, and the phase relation between these rhythms and the LD cycle also differ between the two strains, which suggests that their circadian pacemakers function in a different way. Thus, the mouse renin gene, in addition to producing severe hypertension, may also affect the mechanisms by which the clock regulates the circadian rhythms. Indeed, it is expressed in the hypothalamus of rats (Zhao et al. 1993) and probably in the SCN.

Experiment 10 shows, for the first time, that LD cycles of a 22h-period dissociate the circadian rhythms of MA, HR and BP in young and adult TGR rats into two components, a light-entrained component (LEC) and a non-light-entrained component (NLEC). It has been previously demonstrated that the former, although it might be in part the result of a masking effect of the light-dark cycle, it is also the result of an entrainment mechanism. This is corroborated by the following findings: 1) the phase relation between the LEC and the LD cycle varies with the period of this cycle, 2) if the rats are transferred to DD after short LD cycles, the onset of the free-running rhythm will be in phase with that of the previous LEC, and 3) the after-effects observed after transference to DD depend on the period of the previous LD cycle, and

hence on the period of the LEC (Vilaplana et al. 1997, Campuzano et al. 1998, Campuzano et al. 1999).

This dissociation can be easily explained on the basis of the multioscillatory model of the circadian system (Díez-Noguera 1994, Rosenwasser and Adler 1986, Miller 1998, Honma et al. 1998). According to this model, the circadian system is composed by a group of autonomous oscillators coupled to each other that generate the circadian rhythms. Each oscillator has a specific frequency in the circadian range, so that not all have to be necessarily entrained by a specific Zeitgeber. *This may explain why an animal placed under an LD cycle of a period similar to its tau (for example 24 hours) shows only one component, whereas when it is placed under an LD cycle of a period far from its tau, only a small group of oscillators entrain (and thus generate the LEC) while the non-entrained oscillators generate the NLEC (Vilaplana et al. 1997).*

The multioscillatory nature of the circadian system is supported by the results of Experiment 10, which show that the characteristics of the two components expressed by the TGR rats placed under 22h-period LD cycles vary according to the rhythm studied (MA, HR or BP). These results, together with those of Experiment 9, which showed that within transgenic rats, the relation of phases or psi depended on the rhythm studied, suggest that each of these rhythms is controlled by a specific set of oscillators. Moreover, after a light pulse at CT15, there is a phase delay in the MA rhythm, and no significant phase shift in the rhythms of HR or BP in TGR rats, whereas there are phase delays in the MA, HR and BP rhythms of SD rats (Lemmer et al. 2000a). Therefore, the fact that the characteristics of these rhythms are not always identical can be explained on the assumption that each is controlled by a different group of oscillators.

The nature of oscillators is unknown, but neurones are the principal candidates, because cultured individual neurones of the SCN generate spontaneous circadian rhythms of discharges (Honma et al. 1998, Bos and Mirmiran 1990, Liu et al. 1997, Herzog et al. 1998) and they can also express independently-phased circadian firing rhythms (Welsh et al. 1995). Furthermore, several types of interactions in individual SCN neurones synchronise their circadian rhythms (Shirakawa et al. 2000), and therefore generate the rhythm of the circadian pacemaker (Mirmiran et al. 1995). For a rhythm with two components, it has been shown in hamsters that the splitting in the circadian rhythm of MA also occurs in the rhythm of electrical activity of the SCN (Zlomanczuk et al. 1991), which may also be the case for the dissociation of the MA rhythm that we observed.

The TGR-Han strain of rats (used in our experiments) shows low incidence of malignant hypertension: 18% in males (Whitworth et al. 1994). However, in Experiment 10 we found an incidence of 40%. Thus, 22h-period LD cycles, which induce the dissociation of the circadian rhythms of TGR rats, may be highly stressful and increase mortality by malignant hypertension.

Moreover, this stressful situation may be worsened if it coincides with the development of the circadian rhythms because: 1) the incidence of malignant hypertension in young rats in our experiment was of 60%, whereas that of adult rats was of 20%; 2) the increase in BP observed in young rats placed under 22h-period LD cycles was higher than that of rats of the same age but placed under 24h-period LD cycles; and 3) in young rats placed under 22h-period LD cycles the manifestation of the NLEC was stronger than that of the LEC.

We would also like to highlight that the manifestation of the LEC and the NLEC in TGR rats differed from that reported elsewhere for normotensive Wistar rats. In these rats there were no differences in the manifestation of the two components between young and adult rats and the LEC was always more marked than the NLEC (Vilaplana et al. 1997, Campuzano et al. 1998). The differences between young and adult rats found in Experiment 10 may be associated with the previously explained processes that take place during the development and maturation of the circadian rhythms in TGR rats. Regarding the relative importance of the LEC and the NLEC, the hypothesis of an alteration of the photic information transduction pathway in TGR rats may also be valid here. Indeed the manifestation of the two components in the MA of a rat placed under LD cycles of a period inferior to 24 hours depends on light intensity (Cambras et al. 2000). Low intensity promotes the dissociation of the rhythm of MA into two components. Therefore, if the transmission of photic information was altered in TGR rats in such a way that these were less sensitive to light, this might explain the higher importance of the NLEC when compared with the LEC, and also why the expression of c-fos mRNA in the SCN does not increase after a light pulse (Lemmer et al. 2000a). Nevertheless, the fact that after a light pulse there is a phase shift (at least in the MA rhythm) similar to that produced in SD rats indicates that TGR rats are sensitive to light.

CONCLUSIONS

5.- CONCLUSIONS

La conclusió general d'aquest treball és que les condicions ambientals d'il·luminació en què un animal ha crescut afectaran la manifestació dels seus ritmes circadiaris. Concretament, en cadascuna de les parts del treball hem obtingut les següents conclusions:

Part I.- Efecte de les condicions ambientals d'il·luminació durant les primeres setmanes de vida, en el desenvolupament del sistema circadiari.

1) Les condicions d'il·luminació en què una rata ha estat criada tenen un efecte més important en el desenvolupament del seu ritme circadiari que el ritme de la mare.

2) En la rata i en el ratolí, les condicions d'il·luminació en què han estat sotmesos durant l'alletament són crítiques pel desenvolupament del seu ritme circadiari d'activitat motora.

3) La llum constant durant l'alletament té un efecte protector front l'arritmicitat induïda per la llum constant en l'adult. Aquest efecte protector es manté al llarg de la vida de la rata.

4) La manifestació del ritme d'activitat motora de la rata adulta depèn del nombre de dies en què aquesta va estar sotmesa a llum constant durant l'alletament, requerint-se 12 dies o més de llum constant durant l'alletament per a què aquesta manifesti un ritme circadiari sota condicions de llum constant.

5) La manifestació del ritme d'activitat motora de la rata adulta depèn del dia concret en què aquesta va estar sotmesa a llum constant durant l'alletament, trobant-se un període crític al voltant del dia 16 després del naixement.

6) Les condicions d'il·luminació en què una rata es troba durant l'alletament tenen efecte sobre:
    - la potència del ritme circadiari d'activitat motora sota condicions de foscor constant, de llum constant i de cicles de llum-foscor;

- la fase d'encarrilament del ritme d'activitat motora sota cicles de llum-foscor de 22, 23 i 27 hores de període;
- la resposta fàsica a un pols de llum.

7) Aquestes diferències en la potència no depenen de la capacitat visual de la rata.

8) En la rata, una única injecció subcutània de melatonina a CT 10 o a CT 12 no té cap efecte evident sobre el ritme circadiari d'activitat motora.

9) En la rata, els estímuls no fòtics afecten els ritmes circadiaris de forma més feble que els estímuls lluminosos.

Part II.- Desenvolupament i efecte de les condicions ambientals en els ritmes circadiaris de rates transgèniques hipertenses de la soca TGR(mREN2)27.

1) Entre la cinquena i la desena setmanes de vida les rates TGR(mREN2)27 (TGR) presenten un augment dels nivells de pressió arterial, un endarreriment en l'inici de la fase alfa del ritme de pressió arterial respecte la dels ritmes d'activitat motora i freqüència cardíaca, una disminució dels nivells de freqüència cardíaca i un augment de l'activitat motora diària.

2) Les rates TGR i les Sprague-Dawley (SD) presenten diferències en la fase d'encarrilament a un cicle de llum-foscor de 24 hores.

3) Les rates TGR sotmeses a cicles llum-foscor de 22 hores de període presenten una dissociació dels seus ritmes d'activitat motora, freqüència cardíaca i pressió arterial en dos components, un d'encarrilat al cicle extern (i per tant amb un període de 22 hores) i un altre de no encarrilat (amb un període proper a les 24.5 hores); independentment de l'edat.

4) Les rates hipertenses TGR joves sotmeses a cicles LD de 22 hores presenten una major incidència de casos d'hipertensió maligna que les rates TGR adultes.

5.- CONCLUSIONS

The general conclusion of the present study is that the light environment in which an animal is raised will affect the manifestation of its circadian rhythms. Specifically, in each part of the study the following conclusions have been obtained:

Part I.- Effect of the lighting conditions during the first postnatal weeks on the development of the circadian system.

1) The lighting conditions in which a rat has been reared have a more important effect on the development of its circadian rhythm than the rhythmicity of the dam.

2) In the rat and the mouse, the lighting conditions in which they were placed during lactation are critical for the development of their circadian rhythm of motor activity.

3) Constant light during lactation has a protective effect against the arhythmicity induced by constant light in the adult. The protective effect is maintained throughout the life span of the rat.

4) The manifestation of the motor activity rhythm in the adult rat depends on the number of days in which it was kept under constant light during lactation. Twelve or more days of constant light during lactation are necessary to manifest a circadian rhythm under constant light.

5) The manifestation of the motor activity rhythm in the adult rat depends on the specific day in which it was kept under constant light during lactation. There is a critical period around postnatal day 16.

6) The lighting conditions in which a rat is kept during lactation affect:

- *the power of the circadian rhythm of motor activity under constant darkness, constant light and under light-dark cycles;*
- *the phase of entrainment of the motor activity rhythm under 22h-, 23h- and 27h-period light-dark cycles;*
- *the phasic response to a light pulse.*

7) The differences in the power of the rhythm do not depend on the visual capacity of the rat.

8) In the rat, a single subcutaneous injection of melatonin at CT10 or CT12 do not have an evident effect on the circadian rhythm of motor activity.

9) In the rat, the non-photic stimuli have a weaker effect on the circadian rhythms than the photic stimuli.

Part II.- Development and effect of the lighting conditions on the circadian rhythms of hypertensive TGR(mREN2)27 rats.

1) Between the 5th and the 10th postnatal weeks, TGR(mREN2)27 (TGR) rats show an increase in the levels of blood pressure, a delay in the onset of the alpha phase of the blood pressure rhythm with respect to the motor activity and heart rate rhythms, a decrease in the levels of heart rate and an increase in the daily motor activity levels.

2) TGR and Sprague-Dawley (SD) rats show differences in the phase of entrainment to a 24h-period light-dark cycle.

3) TGR rats kept under 22h-period light-dark cycles show a dissociation of the motor activity, heart rate and blood pressure rhythms into two components: one that is entrained to the external light-dark cycle (and thus has a period of 22 hours) and the other that is not entrained (with a period close to 24.5 hours); independently of the age.

4) Hypertensive young TGR rats kept under 22h-period light-dark cycles show a greater incidence of malignant hypertension than adult TGR rats.

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ANNEX

CONSTANT BRIGHT LIGHT (LL) DURING LACTATION IN RATS PREVENTS ARHYTHMICITY DUE TO LL

***Physiology & Behavior 63*: 875-882, 1998.**

Resum

Objectiu: En experiments previs vam observar que les rates que havien nascut i crescut en llum constant, en l'edat adulta eren capaces de manifestar, a diferència de les rates nascudes en d'altres condicions, un ritme circadiari d'activitat motora sota condicions de llum constant. L'objectiu del present experiment és el d'investigar si la manifestació d'aquest ritme circadiari en llum constant és transitòria i per tant, és només característica de les primeres setmanes de vida, o bé si roman al llarg de la vida de l'animal.

Material i mètodes: Sis rates Wistar femella van arribar al nostre laboratori el dia 16 de gestació. El dia del naixement, les cries es van barrejar, de manera que cada mare alimentava 5 o 6 mascles i 5 o 6 femelles de diverses ventrades. Tres lots es van transferir a foscor constant (grup DD) i els altres tres es van sotmetre a il·luminació constant (grup LL). L'alletament va durar 22 dies, després dels quals les cries es van separar de les mares. El dia del deslletament doncs, es van agafar 4 mascles i 4 femelles de cadascun dels grups i es van posar en gàbies individuals amb actímetre d'infraroig, per tal d'enregistrar-ne la seva activitat motora fins al final de l'experiment. Totes les rates es van sotmetre, en una primera etapa, a condicions d'il·luminació constant, amb accés a l'aigua i al menjar *ad libitum*. En etapes successives es van anar alternant condicions de foscor constant, de llum constant i cicles de llum-foscor de 24 hores de període. En cadascuna de les etapes s'estudiaven les característiques del ritme, per tal de veure si hi havia influència de les condicions de llum viscudes durant l'alletament en la manifestació del ritme d'activitat motora de les rates. La durada total del període registrat en aquest experiment va ser d'un any.

Resultats: No hi ha diferències pel que fa a la capacitat d'encarrilament d'ambdós grups de rates a cicles llum-foscor, ni tampoc en la manifestació del ritme en condicions de foscor constant. La majoria de les rates del grup LL són capaces de mantenir un ritme circadiari en condicions de llum constant al llarg de tot l'experiment, encara que hi ha diferències en la manifestació del ritme entre mascles i femelles: tot i que la majoria de mascles del grup LL s'han tornat arrítmics al final de l'experiment, la majoria de les femelles continuen manifestant un ritme clarament circadiari.

Conclusions: La presència de llum constant durant l'alletament té un efecte protector respecte l'arritmicitat induïda per la llum constant en l'adult, que es manté al llarg de la vida de l'animal. Aquest fet només és clarament observable en les femelles.

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Constant Bright Light (LL) during Lactation in Rats Prevents Arhythmicity Due to LL

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CAMBRAS, T., J. VILAPLANA, A. TORRES, M. M. CANAL, N. CASAMITJANA, A. CAMPUZANO AND A. DÍEZ-NOGUERA. *Constant bright light (LL) during lactation in rats prevents arhythmicity due to LL.* PHYSIOL BEHAV **63**(5) 875–882, 1998.—Light has a strong effect on the circadian system. Light–dark (LD) cycles are the main zeitgebers for practically all organisms, and the exposure of animals to constant bright light (LL) alters the manifestation of circadian rhythms. In rats, exposure to LL in adulthood produces an arhythmic pattern in their motor activity, with a large number of ultradian components. In previous experiments, we found that rats born and kept under LL during lactation develop, after weaning, a circadian rhythm which is maintained for at least a couple of months. Here, we examined motor activity rhythms under LL of two groups of rats which differed in the lighting conditions under which they were kept during lactation: 1) rats kept under LL during lactation (LL-rats), which manifested a circadian rhythm after weaning, and 2) rats kept under constant darkness (DD-rats), which were arhythmic after weaning. We investigated whether the presence of rhythmicity under LL in LL-rats is a transitory effect or whether it persists throughout most of the life of the rat. Moreover, we examined motor activity rhythms of both groups of rats under different lighting conditions to find out other possible differences in the manifestation of their circadian rhythms. Results showed that there are no differences in the capacity of entrainment of both groups of rats to LD cycles or in the rhythm that rats show under DD. Most of the LL-rats maintained their circadian rhythms for the duration of the experiment (1 year), although we found differences in the rhythms manifested between males and females. We found that most of the LL-males became arhythmic; consequently, at the end of the experiment, there were no differences in the number of males showing circadian rhythm in the LL- and DD-groups. Most of the females in the LL-group showed a clear circadian rhythm under LL during the entire experiment. Thus, LL during lactation has a protective effect against the disruptive effect of LL on the circadian rhythm, although it is only clearly manifested in females. © 1998 Elsevier Science Inc.

Constant light Development Circadian rhythm

UNDER constant lighting conditions the circadian system is free running, and for most organisms, an animal's endogenous rhythm, with period τ, is manifested in its behavior and physiology. τ depends on the lighting conditions: in nocturnal animals, it lengthens when light intensity increases. The exposure of animals to LL, which has a strong effect in the retina (20), is also one of the conditions that reflects the effect of light on the circadian system. Splitting (1) and the presence of a large number of ultradian components are the best known consequences of the exposure of an animal to LL. When adult rats are subjected to LL, they lose their circadian rhythms after several days or weeks and manifest an arhythmic pattern (5) with a large number of ultradian components with an unstable phase relationship (10).

In previous experiments, we found that when rats were born and maintained under LL with the same light intensity that produces arhythmicity in adult rats (300 lux), they showed an ultradian pattern in their motor activity for the first 10 days after weaning, but later developed and maintained a circadian rhythm (3). This could be produced by either alterations in the circadian system or a loss of sensitivity in the retina. This circadian rhythm has a longer period than that observed in rats under DD, and the stability of the waveform depends on the light intensity (22). The manifestation of this rhythm depends on the lighting conditions during lactation but does not appear to depend on the rhythm of the dam (2). Here, we investigate whether the presence of the rhythm under LL of rats maintained under LL during lactation remains throughout most of the life span of the animal or whether, on the contrary, the manifestation of the rhythm under LL is a transitory effect, only observed during the first weeks after weaning. Also, we wanted to explore the different functioning of the circadian system of these rats when they are submitted to different light patterns in their adulthood (LD, DD, or LL).

MATERIALS AND METHODS

Experimental Design

Six pregnant female Wistar rats (Charles River, St. Aubin les Elbeuf, France) were supplied to the laboratory on Day 16 of gestation. The rats were housed individually in transparent Makrolom™ cages (50 × 25 × 12 cm) under a light–dark cycle (LD

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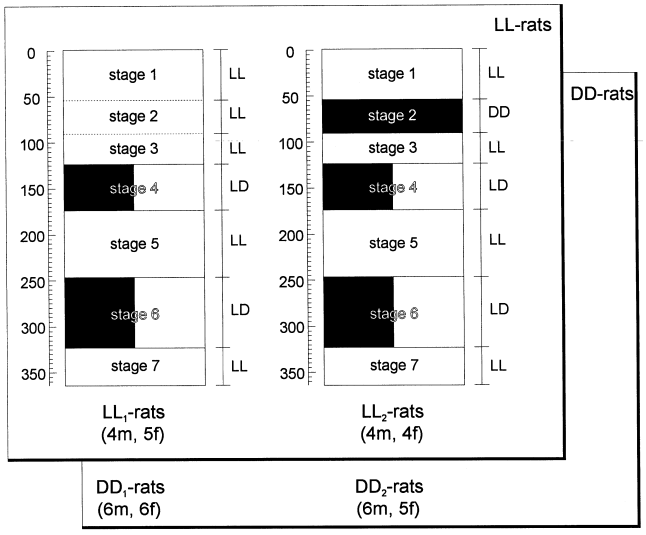

FIG. 1. Scheme of the lighting conditions and groups of rats used in the experiment.

12 h:12 h, light intensity = 270–300 lux). Rats were maintained under these conditions until delivery (5 days later). When they were 1 day old, the litters were cross-fostered in such a way that each litter contained about the same number of males and females (between 5 and 6 of each sex) and each dam fed at least one pup of each original offspring. The day after delivery, three of the new litters were transferred to DD (0.5 lux of dim red light) and the other three to LL (270–300 lux). Animals remained under these conditions until they were 22 days old, when they were weaned and isolated in individual cages (25 × 25 × 12 cm) with water and food available ad libitum. From this day on, motor activity was recorded. The number of rats used for the experiment was 8 males and 9 females, born and maintained under LL during lactation (LL-rats), and 12 males and 11 females, born and maintained under DD during lactation (DD-rats). Lighting conditions were the same for both groups of rats and adhered to the following schedule (see Fig. 1):

Stage 1 (Days 1–55): LL; 55 days

Stage 2 (Days 56–91): Half of the animals of each group were transferred to DD (groups LL_2 and DD_2) and the other half remained under LL (groups LL_1 and DD_1); 36 days.

Stage 3 (Days 92–125): LL; 34 days

Stage 4 (Days 126–175): LD (12 h:12 h); 50 days

Stage 5 (Days 176–248): LL; 73 days

Stage 6 (Days 249–324): LD (12 h:12 h); 76 days

Stage 7 (Days 325–360): LL; 36 days

In all cases, light was 300 lux of cool white light, and darkness was 0.5 lux of dim red light.

Later, a second experiment was carried out to test the capacity of young LL- and DD-rats to synchronize to LD cycles of low intensity (L: 5–8 lux). To do so, two new groups of young rats obtained, selected, and maintained in a manner similar to that in the previous experiment were used. LL-rats (5 males and 3 females) were kept during lactation under LL, and DD-rats (4 males and 4 females) were kept under DD. From the day of weaning (Day 1 of the experiment) the lighting conditions were the same for both groups of rats:

Stage 1 (Days 1–38): LL (300 lux); 38 days

Stage 2 (Days 39–69): DD; 31 days

Stage 3 (Days 70–99): LD (light = 5–8 lux); 30 days

Stage 4 (Days 100–124): DD; 25 days

In all cases, approximately every 10 days, cages were cleaned and the rats were weighed. The motor activity of each rat was recorded by an optical detection system of two crossed perpendicular infrared beams, situated on a plane 3 cm above the floor of the cage. Data were automatically recorded every 15 min since the day of weaning until the end of the experiment and saved on floppy disks for further analysis.

Mathematical and Statistical Analysis

The period of the motor activity rhythm for each of the different stages of each experiment was determined by the periodogram of Sokolove and Bushell (18), with data previously smoothed by a moving average corresponding to 4-h recording bins (16 data points). Because the stages had different numbers of days and we wanted to be able to compare the results, we carried out the analysis with data corresponding to blocks of 25 consecutive days each. In the case of long stages (such as Stage 5), a periodogram analysis was carried out only in two blocks of 25-day data each. The percentage of variance (PVAR) explained by the highest peak obtained in the periodogram was used as an indicator of the importance of the circadian rhythm in the motor activity pattern. Moreover, each data set was divided into data subsets of length equal to the mean τ, and after that, a Fourier analysis was applied to each subset. The power content of the first harmonic of the spectra (circadian harmonic) can also be considered as an indicator of the importance of the circadian system, and it was plotted versus time (days of the experiment) as an indicator of the evolution of the circadian rhythm throughout the experiment.

ANOVA was performed by means of two linear models using the PVAR as the dependent variable. In the first model, we used as independent variables the stage of the experiment (Stage 1, Stage 2 under DD, Stage 2 under LL, Stage 3 after LL, and Stage 3 after DD) and the interactions of these variables with sex and lighting conditions during lactation (20 variables total). In the second model, we did not differentiate between those rats that were under DD in Stage 2; thus, this stage was not included in the analysis. In this second model, the independent variables were the stage of the experiment (Stage 1, Stage 3, Stage 4, Stage 5, and Stage 6) and the interactions with sex and lighting conditions during lactation (20 variables total). In both models, contrast hypotheses were carried out.

The exact Fisher probability test was applied to compare the number of animals of each group that manifested a circadian rhythm in the different stages. In this case, the presence of the rhythm was a dichotomous variable and was considered present if the highest peak of the periodogram was statistically significant ($p < 0.05$ after applying Bonferroni's correction).

The presence of cataracts in the rats was evaluated by visual inspection occasionally during the cage changes and at the end of the experiment, considering the degree and the extent of the lens opacity.

RESULTS

The double-plotted actograms (Figs. 2 and 3) show, generally, that LL-rats exhibited a circadian rhythm under LL but that DD-rats did not. Under DD, all the rats showed a circadian rhythm with τ shorter than that observed in rhythmic rats under LL. The capacity to detect the light changes was also confirmed by the capacity of all the rats to synchronize to LD cycles. Because the record of the motor activity was very long and there were several groups of animals and lighting conditions, we present the analysis of the different parts of the experiment separately, in order to make the results easy to follow. Still, statistical analysis was carried out

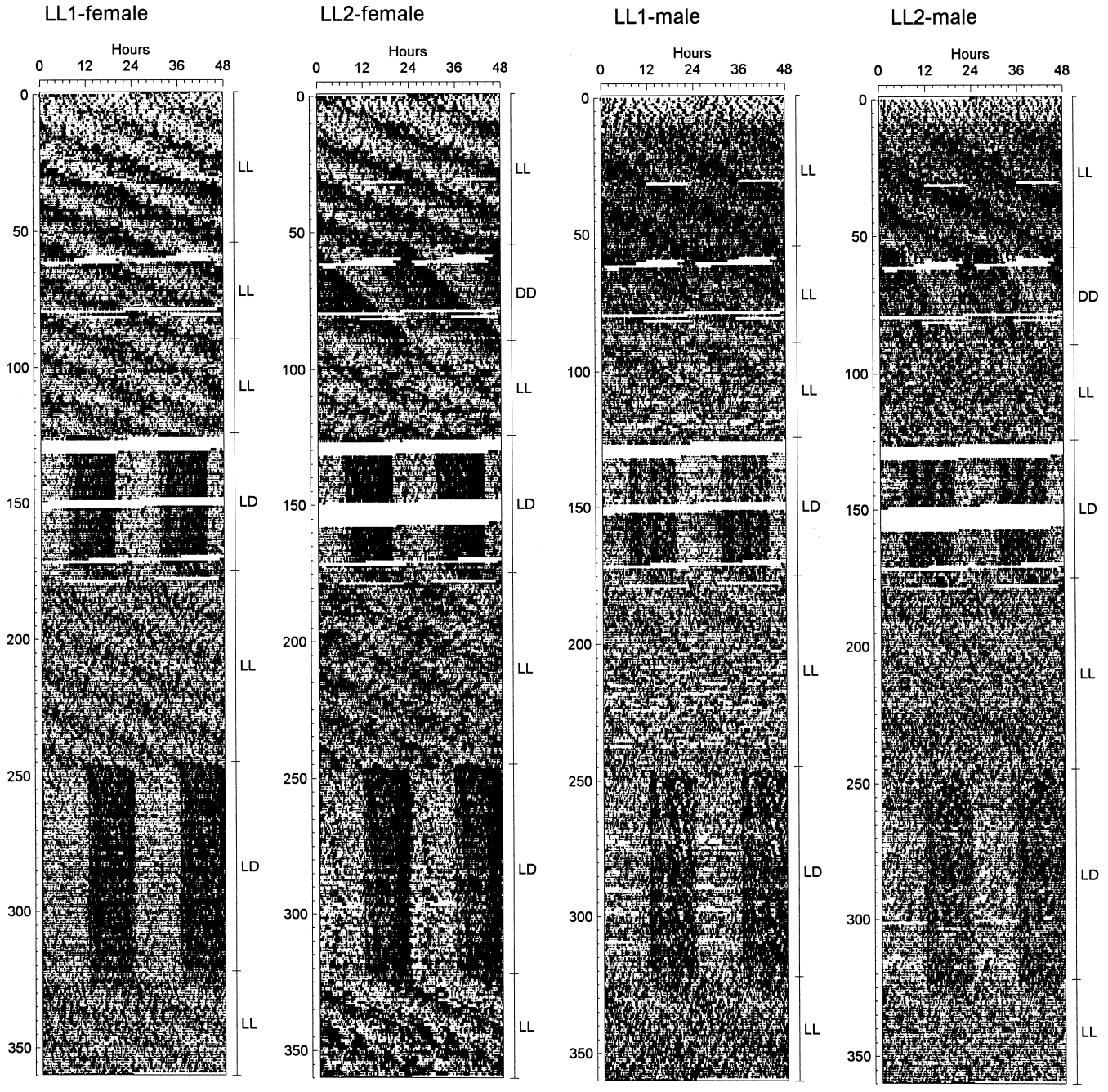

FIG. 2. Double-plotted actograms of representative rats belonging to the LL-group. For each graph, the left axis indicates the days of the experiment and the first day corresponds to the first postweaning day. The right axis indicates the illumination condition of each stage.

with all the data simultaneously according to the linear models explained above. Here, we only report the statistically significant results ($p < 0.05$).

Stages 1, 2, and 3 (Days 1–125)

Here, we group the results of these three stages because half of the animals were under LL during these three stages and the other half were under DD during the second stage (groups LL_2 and DD_2). From the analysis of these stages we can carefully assess the following aspects:

Circadian rhythm under LL over 125 days. To study the evolution of the circadian rhythm, only those animals that were continuously under LL were used (groups LL_1 and DD_1). The evolution of the power content of the circadian harmonic throughout these days was calculated and used as an indicator of the evolution of the magnitude of the circadian rhythm. This variable increased until Days 40–50 and later decreased (see Fig. 4).

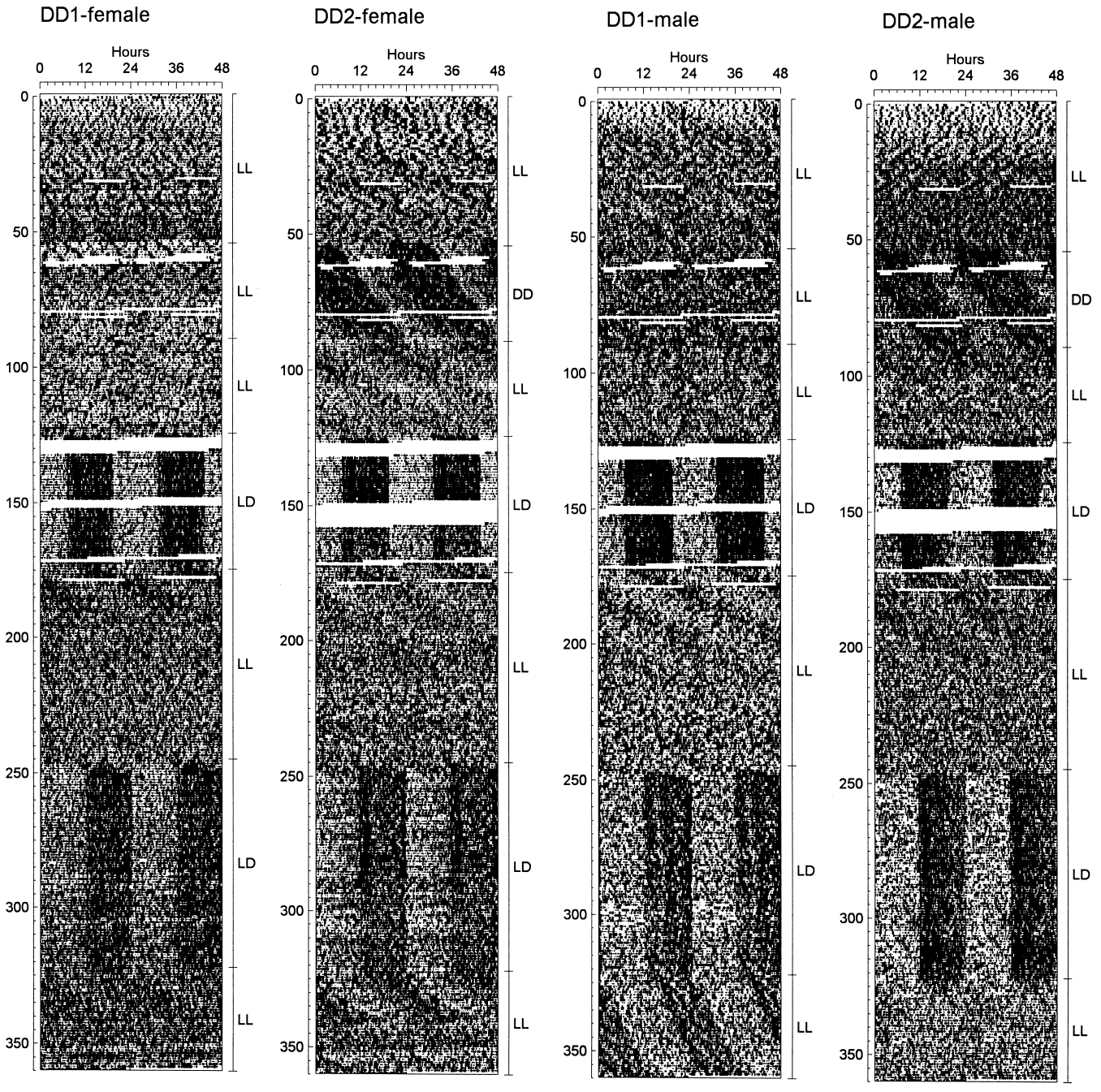

FIG. 3. Double-plotted actograms of representative rats belonging to the DD-group. For each graph, the left axis indicates the days of the experiment and the first day corresponds to the first postweaning day. The right axis indicates the illumination condition of each stage.

During this stage, the PVAR was higher in LL-rats than in DD-rats, with females having higher values than males.

Circadian rhythm under DD (Stage 2). During this stage, LL_2- and DD_2-rats showed similar values of τ and PVAR. Only sex was a significant factor ($p < 0.05$) determining the value of PVAR, which was higher in females than in males. Thus, the manifestation of the endogenous rhythm under DD does not seem to be dependent on the lighting conditions during lactation.

Circadian rhythm under LL (Stage 3). In this stage, we found no differences between LL_1- and LL_2-rats or between DD_1- and DD_2-rats. This indicates that DD does not affect the manifestation of the rhythm under LL. LL-rats show statistically significantly higher values of PVAR than DD-rats. In all cases, females had higher values than males. In the case of both female and male LL-rats, the PVAR in this stage is significantly lower than those found in Stage 1. The same results were found considering LL_1 and LL_2 separately. These results show that in LL-rats the PVAR decreases through time independently from DD in Stage 2. In

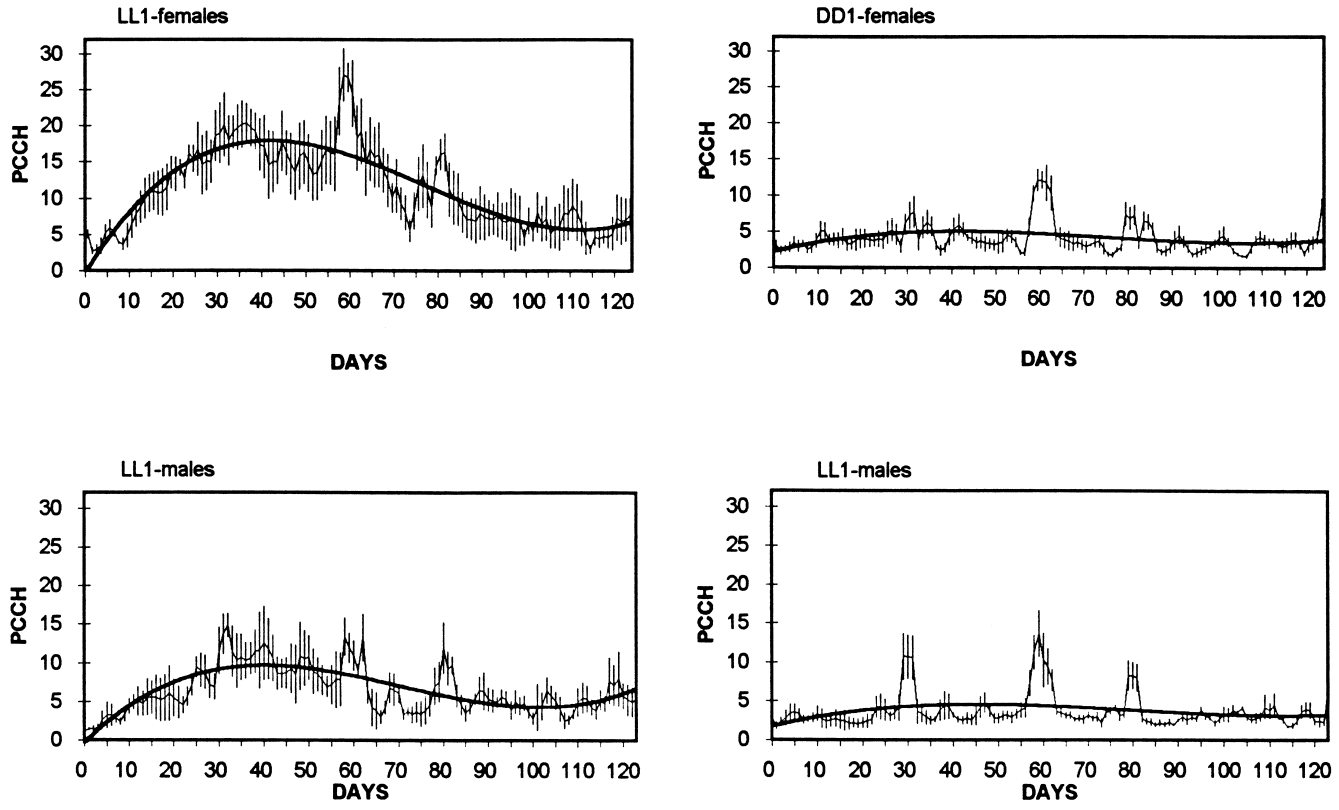

FIG. 4. Evolution under LL of the power content of the first harmonic obtained by Fourier analysis. The values correspond to the mean value (and SE) of the animals of each group. To visualize the tendency, data have been adjusted to a polynomial function (third degree).

DD-rats, the PVAR was always low, with no differences between Stages 1 and 3.

As in these stages we found no differences between LL_1 and LL_2 or between DD_1 and DD_2 and as it was more important for the purpose of this study to differentiate between the conditions during lactation, the following stages were analyzed with the second linear model in which we did not differentiate between those rats that were kept under DD in Stage 2.

Stage 4 (Days 126–175): LD

All rats, when submitted to LD cycles, were able to synchronize, and a 24-h rhythm is significantly present in the periodogram. The factor of sex was significant in this stage: females had higher values of the PVAR than males in both the LL- and DD-groups. Moreover, there were no differences between LL- and DD-females, but DD-males showed higher PVAR values than LL-males.

Stage 5 (Days 176–248): LL

Because this stage was longer than the others, here we have considered two substages: Stage 5.1 (Days 176–201) and Stage 5.2 (Days 220–245). In general, the PVAR is higher in Stage 5.1 than in Stage 5.2, but the ANOVAs reveal that only DD-females show higher values in Stage 5.1 than in Stage 5.2. We interpret this as indicating that in DD-females a circadian rhythm appeared after LD exposure, but its amplitude decreased progressively under LL. In the other rats, the rhythm was maintained or was not present during this entire stage. Here, as the PVAR is in all cases close to the limit of significance, even in those animals in which the rhythm is significant, we found no differences between LL- and DD-rats or between males and females.

Stage 6 (Days 249-324): LD

Again, all the rats were able to synchronize to 24 h. Females showed again higher PVAR than males. LL-males, especially LL_2-males, showed a lower PVAR than the other groups. Perhaps one explanation for the low values of PVAR in males could be that they showed lower values in motor activity than females, and this is reflected in the waveform of the rhythm. However, we found no explanation for the lower values of PVAR of LL-males compared with that of DD-males, because they show no differences in motor activity or body weight.

Stage 7 (Days 325–360): LL

In this stage, we found no differences between the groups. In each group, some of the rats showed a circadian rhythm, which disappeared after a few weeks, but other rats showed a clear circadian rhythm throughout this stage. However, in three rats (one DD_1-male and two DD_1-females) a circadian rhythm was clearly manifested (see Fig. 2, rat DD_1-male) with a τ of 24 h 30 min, which was shorter than that manifested under LL (about 25 h 50 min) and close to that of the rats under DD. These three rats developed cataracts in both eyes, which means that they may have perceived a lower light intensity than before; this is reflected in the τ shortening. This is the reason why the results of this stage have not been included in the global analysis.

In our opinion, the most important result of this experiment is

the following: Considering the number of rats in both the LL- and DD-groups that show a significant circadian rhythm in all of the stages (excluding Stage 7), we found that 10 of 17 LL-rats and 5 of 23 DD-rats showed a circadian rhythm, and these ratios were statistically significantly different from each other (Fisher exact probability test: $p < 0.019$). Considering both sexes separately, we found 3 of 8 LL-males and 2 of 12 DD-males that showed a significant circadian rhythm in all light conditions, although these distributions were not statistically significantly different (Fisher: $p < 0.29$). However, 7 of 9 LL-females and 3 of 12 DD-females showed a significant circadian rhythm in all of the stages (Fisher: $p < 0.034$). This shows that in most of the LL-rats, especially females, the manifestation of the circadian rhythm under LL is not a transitory effect.

The results of the second experiment showed that LL-rats had a clear circadian rhythm under LL (mean τ : 25 h 47 min, SE: 8.5 min), whereas DD-rats did not. Under DD, there were no differences in the rhythms manifested between the two groups (τ: 24 h 40 min, SE: 7.5 min) or in their abilities to synchronize to LD cycles of low light intensity. Thus, as in this experiment we only wanted to test if the animals were able to synchronize to LD cycles with low light intensity (5–8 lux) and the results were affirmative, without differences between LL- and DD-rats, we have not included the graphs in this paper, and we only took the results for discussion.

DISCUSSION

In this experiment, we found that LL during lactation prevents the arhythmicity caused by LL in adulthood; LL-rats were able to develop and maintain a circadian rhythm, whereas DD-rats were arhythmic. This protection was clearly seen during the first months after weaning, when the external conditions did not change from LL. In most of the LL-rats, especially females, this protection seemed to be present during at least the first year of life, even after the lighting conditions changed to DD or to LD. There was no difference between the rhythm manifestation of LL-rats and DD-rats under DD. Nor was there a difference between these groups in their capacity to synchronize to LD cycles with high and low light intensities.

Although LL-rats show a circadian rhythm, the amplitude (power content) of this rhythm changes during the first 4 months under continuous LL. The power content of the circadian rhythm increases from the first day after weaning until Day 30 and later this value decreases from about Day 70 on, although it remains higher than in the case of DD rats, which always showed a low, mostly not significant, value in this variable. This suggests that LL inhibits the manifestation of the circadian rhythm only after the rhythm has been established. Considering a multioscillatory system (4), we can suggest a different effect of LL in both groups of rats: In DD-rats the circadian system is developed during lactation, and thus, when after weaning they are transferred to LL, light may inhibit the neuronal communication (coupling between the oscillators) in the already established circadian system; thus, the circadian rhythm is not manifested. Although we can also think that DD may be an abnormal condition for the development of the circadian system, we do not consider its effect, because rats kept under DD during lactation behave like adult rats in their rhythmic behavior: they do not show a circadian rhythm in their motor activity under LL. In the case of LL-rats, LL may cause a delay in the development of the communication between the elements of the circadian system, which will not be achieved until several weeks after weaning. This delay permits an adaptation to the external lighting conditions and consequently, the circadian rhythm can be expressed. This implies that the adaptation to the environmental conditions (i.e., LL) is established during the first period of life and this generates the persistence of the rhythmicity under LL, which, at least in females, remains for the first year of life. However, the manifestation of the circadian rhythm in LL-rats decreases once the maximum has been reached. This might indicate that perhaps the sensitivity to LL increases after some time under LL, but this is in contradiction with the damage produced in the retina by LL.

One could consider that the protection of LL and the different manifestations of motor activity after weaning between the two groups of rats are a consequence of a retinal alteration due to LL during lactation. Neonatal treatment with monosodium glutamate (MSG), which produces retinal damage and alterations in the intergeniculate leaflet, causes protection toward LL (6): Saline-treated rats were arhythmic under LL, and MSG rats show a period of about 24 h in this condition. However, in the mentioned experiment, the mean period of MSG rats under LD is 24.45 h (24.15 h in saline-treated rats), making suspect the complete ability of these rats to entrain. In our case, the rats that manifested a rhythm under LL followed Aschoff's rules; their period under LL, with a mean value of 25.8 h, was longer than that under DD (24.45 h). Under LD the period was always 24 h. Thus, the different manifestation of the rhythmicity under LL between the two groups of rats cannot be interpreted considering only retinal damage. However, even when rats in this study showed retinal damage, the retina was still functional and sent information about lighting conditions to the suprachiasmatic nuclei (SCN); hence, rats synchronized to both LD cycles (with high and low light intensities). In fact, it has already been demonstrated that rats are able to synchronize to LD cycles of dim red light after exposure to LL (17).

The extent of retinal damage appears to vary with age, prior lighting history, and genetic and dietary background [for a review, see (20)]. For instance, rats reared in the dark adapt to the environment by increasing the amount of rhodopsin in the retina, and these rats are more susceptible to light-induced retinal damage than age-matched rats reared in weak cyclic light (15). Thus, this suggests that the group more prone to retinal damage may be the DD-rats. The exposure to LL (1290 $\mu W/cm^2$) for 12 weeks is highly effective in completely or nearly completely destroying photoreceptors in the albino rat (16). Thus, taking into account the large span of time that animals were under LL, we believe that retinal damage should not be different from one group to another and should not be the cause of the different rhythm manifestation in the two groups of rats. It must also be considered that the three rats that manifested cataracts belong to group DD_1 (born under DD and for the first 125 days after weaning, under LL without interruption), which may indicate a special sensitivity of this group to constant light. When, at the end of the experiment, the visual organ is altered (cataracts), the rhythm is clearly manifested in a different way: the τ of the rhythm is more similar to that of rats kept under DD. However, the information that the pacemaker receives about light is still enough to entrain the rat to LD cycles. We cannot know whether the presence of cataracts is due to the age, the lighting conditions, or both; in any case, it causes the rats to be less sensitive to LL, although the τ does not seem to change proportionally to cataract formation. This fact indicates that the rhythm manifested by LL-rats under LL should not be due to a lack of light perception, because τ is much longer than under DD, but it may be due to the adaptation of the circadian pacemaker to LL. Thus, we can suppose that although the retina could be damaged because of LL-conditions, there are still some cells able to send light information to the SCN. Actually, the circadian system may be adapted to the environmental conditions through photoreceptors different from those that mediate the light perception of the visual system (7). The retina shows a certain plasticity (13); in damaged retina due to LL exposure in which nearly all rods and a substantial

proportion of cones degenerated, there is sprouting of processes to incorporate retinal elements into the entrainment pathway which serves to maintain entrainment. Thus, as the retina is still functional, we may consider that the differences due to the different rhythm manifestation should be found in the structure of the pacemaker.

Alterations of the circadian system by LL have been studied by Steinhorst et al. (19), who examined the neurohistochemical structure of the SCN correlated with the manifestation of the circadian rhythmicity in rats under LD and in rhythmic and arhythmic rats under LL (150 lux). They showed correlation between the area stained with neurophysin (vasopressin (VP) precursor in the SCN) and the manifestation of rhythmicity and suggested that the morphology of the circadian system is determined by the manifestation of the circadian rhythm but not by the external lighting conditions. We could, thus, assume that during the first months after weaning, rats that were under LL during lactation may have the same structure of the circadian system as those rats maintained under LD or DD. However, after weaning and under LL, DD-rats may show a different structure in the circadian system similar to that of adult rats under LL. These differences were clear during the first 2 months after weaning, but less after the change in the lighting conditions. It has also been found that VP rhythm is present in rats under LL for the first 5–12 postnatal days, and also with a period of about 25.3–25.7 h (11), which indicates that arhythmicity by light is not produced at such an early age. Furthermore, in other experiments we found that when young rats are born under abnormal LD cycles with a 4-h period (21), the circadian rhythm is present, although masked by this ultradian rhythm. Nevertheless, when adult rats are maintained under the same ultradian LD cycles, they show a complete dissociation of the rhythm (23). All this indicates that young animals have the ability to adapt to the external conditions in such a way as to ensure that the circadian system may always generate a circadian rhythm.

Although the presence of the circadian rhythm under LL seems to be a transitory effect in males because they soon become arhythmic, this is not the case for females, in which the rhythm under LL persists even when the animals are transferred to other lighting conditions. We showed that the number of females that show a rhythmic behavior in LL is higher in LL-rats than in DD-rats (in males this difference is not significant). This suggests that our initial hypothesis—that the effect of LL during a determined stage of the circadian development may affect the manifestation of the rhythm for the whole life of the rat—may be valid, especially in females. This also suggests that the circadian pacemaker and also light perception may be different in males and females. Sex differences in rhythm manifestation (24) and in the circadian pacemaker itself (8,9) have been found. The fact that mainly females manifest a circadian rhythm could also be related to the effects of motor activity on the circadian pacemaker. In our experiment, females showed higher motor activity than males. This may be due to the smaller size of females compared with males, which could favor more movement in the cage. However, some of the females under LL show permanent estrous (12), which increases motor activity. Whatever the reason, the feedback loop of motor activity (14) may simply be stronger in females than in males and consequently, the circadian rhythm may be clearly manifested. In conclusion, this experiment indicates that, in most of the LL-rats, especially most of the females, the effects of lighting conditions during lactation may not be a transitory effect and that they could affect the manifestation of circadian rhythms under LL for a lifetime. Because the function of the retina does not seem to cause the different rhythm manifestation between the two groups of rats, these differences may then be in the structure of the circadian system itself. Thus, this experiment opens up the possibility that lighting conditions during early ages of development could affect the manifestation of circadian rhythmicity and even, perhaps, the morphology of the SCN.

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"... we must not look upon science as a "body of knowledge", but rather as a system of hypotheses; that is to say, as a system of guesses or anticipations which in principle cannot be justified, but with which we work as long as they stand up to tests..."

Karl R. Popper

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