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ORIGINAL ARTICLE

Melatonin administration modifies circadian motor activity under constant light depending on the lighting conditions during suckling

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Early lighting conditions have been described to produce long-term effects on circadian behavior, which may also influence the response to agents acting on the circadian system. It has been suggested that melatonin (MEL) may act on the circadian pacemaker and as a scavenger of reactive oxygen and nitrogen species. Here, we studied the oxidative and behavioral changes caused by prolonged exposure to constant light (LL) in groups of rats that differed in MEL administration and in lighting conditions during suckling. The rats were exposed to either a light–dark cycle (LD) or LL. At 40 days old, rats were treated for 2 weeks with a daily subcutaneous injection of MEL (10 mg/kg body weight) or a vehicle at activity onset. Blood samples were taken before and after treatment, to determine catalase (CAT) activity and nitrite level in plasma. As expected, LL-reared rats showed a more stable motor activity circadian rhythm than LD rats. MEL treatment produced more reactivity in LD- than in LL rats, and was also able to alter the phase of the rhythm in LD rats. There were no significant differences in nitrite levels or CAT activity between the groups, although both variables increased with time. Finally, we also tested depressive signs by means of sucrose consumption, and anhedonia was found in LD males treated with MEL. The results suggest that the lighting conditions in early infancy are important for the long-term functionality of the circadian system, including rhythm manifestation, responses to MEL and mood alterations.

Keywords: Anhedonia test, circadian rhythm, depression, early lighting, melatonin, oxidative stress

INTRODUCTION

Circadian rhythms have their origin in adaptation to the cyclic environment generated by the earth's rotation and are manifested in most physiological or behavioral variables. The principal mammalian pacemaker is located in the suprachiasmatic nuclei (SCN) of the hypothalamus, which exhibits daily cycles of neuronal activity and gene and protein expression (Moore et al., 2002). Moreover, many other subordinate clocks are present in other organs and tissues (Albrecht, 2012).

Light is the environmental factor that has the main influence on circadian patterns, inducing responses such as: entraining the SCN to the 24-h environment, rhythm phase shifts or melatonin (MEL) suppression. These classical responses of the circadian system are mainly due to intrinsically photosensitive retinal ganglion cells (ipRGC) that contain the photopigment melanopsin (Schmidt et al., 2011a, b) although classical photoreceptors, rods and cones, are also involved in these responses (Lucas et al., 2012). ipRGC directly project to the ventrolateral area of the SCN, but also to many other areas in the brain that control other functions including mood (Schmoll et al., 2011).

Information on the light–dark (LD) environment reaches the pineal gland, which acts as an interface between the circadian pacemaker and the light information system. The hormone MEL is mainly secreted by the pineal gland, although other extrapineal sources such as the intestine retina or other sides of the brain have also been described (Acuña-Castroviejo et al., 2014; Jimenez-Jorge et al., 2007). MEL possesses chronobiotic properties, which influence circadian rhythms. MEL can induce the clock phase to advance when it is administered in the late evening in humans or 2 h before darkness in rodents, and it has also been described as a coupling agent among the components of the circadian system (Agez et al., 2009; Hardeland et al., 2011 for review). MEL has many other functions, since it may also induce immunomodulatory responses, neuroprotection, oncostatic effects and it is also a strong

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scavenger with antioxidant properties (Carpentieri et al., 2012; Hardeland et al., 2003; Reiter et al., 2000).

Constant light (LL) is an abnormal situation that has dramatic effects on circadian rhythm manifestation. The problem with LL is the presence of light in what is supposed to be the dark stage. This produces many disturbances in the circadian system, mainly manifested by rhythmic alterations in animals' behavior. In rats, LL lengthens the period of the activity circadian rhythm and finally induces arhythmicity, which may occur under bright light, >100 lx, (Chiesa et al., 2010; Eastman & Rechtschaffen, 1983; Honma et al., 1996) or in dim light, <1 lx, (Cambras & Díez-Noguera, 2012). This effect may be due to an uncoupling effect of the oscillators within the circadian pacemaker (Chiesa et al., 2010; Ohta et al., 2005). Moreover, light at night suppresses melanopsin expression in the adult retina (Hannibal et al., 2005).

After a long term under LL, MEL levels are still detectable with no differences between day and night (Aguzzi et al., 2006; Tapia-Osorio et al., 2013) or with peaks of secretion at random, that are not associated with the manifestation of the motor activity (Aguzzi et al., 2006). Moreover, light at night has been described to induce oxidative stress (Hardeland et al., 2003) and to lead to alterations in metabolism (Coomans et al., 2013), weight gain (Borninger et al., 2014) and a depressive state (Fonken & Nelson, 2013; Tapia-Osorio et al., 2013).

However, the effect of LL on animal behavior is not the same if animals have been submitted to LL during early stages. Early light experiences can have lasting effects into adulthood. The developmental processes that take place in the brain during the perinatal period are very susceptible to external agents, which may modify the behavior of individuals or the responses to stressors in adulthood (Aubrecht et al., 2014; Mirescu et al., 2004). The circadian system is not an exception and its development is influenced by external stimuli. Rat SCN is not totally mature on the day of birth and most connections, to and from the SCN, develop postnatally (Brooks & Canal, 2013). The first 3 weeks after birth (the suckling period) appear to be critical for the maturation of the circadian system and for the establishment of synchronization of the pup's circadian rhythms to the environment (Brooks & Canal, 2013). Thus, it is not surprising that exposure to light at this age has an effect on the development of the circadian system.

Previous studies have shown that light experienced during the first postnatal weeks has long-lasting effects on animal's behavior. Rats reared under LL, a condition that induces arrhythmicity in adult animals, will show a clear circadian rhythm in their behavior when exposed to LL later in life. This rhythm is only dependent on the environmental light experience and not on the mother's rhythmicity (Cambras et al., 1997, 1998). Due to this response, postnatal LL exposure provides a unique situation in which intact animals of the same species and genetic background will manifest two different rhythmic patterns under the same lighting conditions in adulthood: rats reared during the first 3 weeks under LD will show arrhythmicity, while those reared under LL will be rhythmic. Whether the presence of the circadian rhythm under LL is adaptive or maladaptive remains unknown. Nonetheless, this abnormal situation is a good model to examine the effect of early light conditions on the development of the circadian system, due to the characteristic and easily visible effect that it produces on rat behavior.

Therefore, since LL may affect the expression of the circadian rhythm, oxidative stress and mood, it is a good condition to prove the effects of the early lighting environment on the functioning of the circadian system. Thus, in this experiment, we wanted to test whether lighting conditions during suckling that induce different rhythmic manifestations in rats could also affect responses mediated by the MEL system. Thus, we tested the capacity of MEL to synchronize the rhythm under bright light as well as its possible antioxidative effect and antidepressant role as a function of the lighting conditions in the early postnatal life.

To do so, we studied the effects of MEL administration on motor activity rhythm under prolonged exposure to LL in two groups of rats, of both sexes, which differed in lighting conditions during suckling: either a LD cycle or LL.

MATERIALS AND METHODS

Animals

Male and female Wistar rats were used for the experiment. Ten pregnant females arrived at our lab from Charles River, France, when they were at the 17th day of gestation. They were housed with free access to food and water under a 24-h LD cycle (L: 12 h 500 lx, D: 12 h 0.2 lx of dim red light). On the day of delivery, the pups were distributed at random between the females. From the day of birth (postnatal day 0, P0) to the weaning day (P22), 2 l were kept under constant bright light (LL, 500 lx) and 3 l under 24 h LD cycles (LD: 500 lx during light and 0.2 lx of dim red light during darkness). The rest of the animals were used for other experiments. All the experimental procedures complied with the institutional guidelines for the care and use of laboratory animals established by the Ethical Committee for Animal Experimentation at the University of Barcelona and in accordance with the ethical standards of the Chronobiology International journal (Portaluppi et al., 2010).

Animal grouping and treatment

On the day of weaning, four experimental groups were made and named according to the lighting conditions during suckling (LL or LD) and the later MEL treatment (MEL, or vehicle, VEH). Therefore, the groups were as follows: LD-VEH (seven females and four males);

LD-MEL (seven females and four males); LL-VEH (seven females and three males) and LL-MEL (six females and five males). Moreover, four males and three females of the same age were always maintained under LD cycles and were used as the control group for biochemical analysis.

On the day of weaning, rats were moved to individual cages and all of them were submitted to 24 h LD cycles with ''light at night'' (lL cycles: 12 h dim white light, 2 lx (l) and 12 h bright light, 500 lx (L). These lighting conditions allowed the maintenance of temporal external references, that would help to deduce the phase of the rhythm to later apply MEL at the onset of the activity.

On day 18 after weaning, rats were transferred to constant bright light (LL) and remained under this condition for the rest of the experiment which lasted 80 days. In Figure 1, the experimental procedure in time can be observed. Animals always had access to food and water *ad libitum*. Two days later, the rats, which were now at day 20 after weaning – the age when the circadian rhythm phase is considered to be stabilized (Albert et al., 2013) – were treated for 2 weeks with a subcutaneous injection of MEL at a dose of 10 mg/kg body weight (2 ml/kg of a solution of 5 mg MEL/ml) or the VEH (10% of ethanol in saline), at a time corresponding to the onset of activity (onset of dim light) in the previous lL cycle. Blood samples were taken, from the saphenous vein, before (between CT3 and CT6) and after treatment, to determine catalases (CAT) activity and nitrite level. Since after treatment, rats of LD group had a less-defined circadian pattern and some of them were arrhythmic, sampling time was chosen considering that most of LL rats (all rhythmic) were under CT3–CT7. Afterward CT was calculated for each rat and results were examined accordingly.

Between days 73 and 78, anhedonia was tested by means of sucrose consumption (D'aquila et al., 1997; Forbes et al., 1996).

The motor activity of each animal was continuously recorded by means of activity meters placed outside the cage that used two perpendicular crossed infrared beams situated 6 cm above the floor of the cage. Each beam interruption represented an activity count that was registered and stored in 15-min bins for further analysis. The light was recorded at the same time as the activity in a separate channel connected to a photocell pulse generator.

Oxidative stress

Blood samples were drawn in EDTA tubes to separate erythrocytes (for the CAT activity) and plasma (for the nitrite levels) and centrifuged at 5200 *g* for 15 min at 4 °C. The erythrocytes were subsequently washed twice with two volumes of 0.9% sodium chloride solution to remove plasma remnants. Following this, the erythrocytes were hemolyzed with a phosphate-EDTA buffer (10–1 mM, pH 6.25) diluted 1/20.

Catalase activity

Catalase activity (EC 1.11.1.6) was determined in hemolyzed erythrocytes according to Aebi (1984). CAT activity was assayed in 50 mM potassium phosphate buffer pH 7.4 and 0.3 M H_2O_2 at 240 nm in

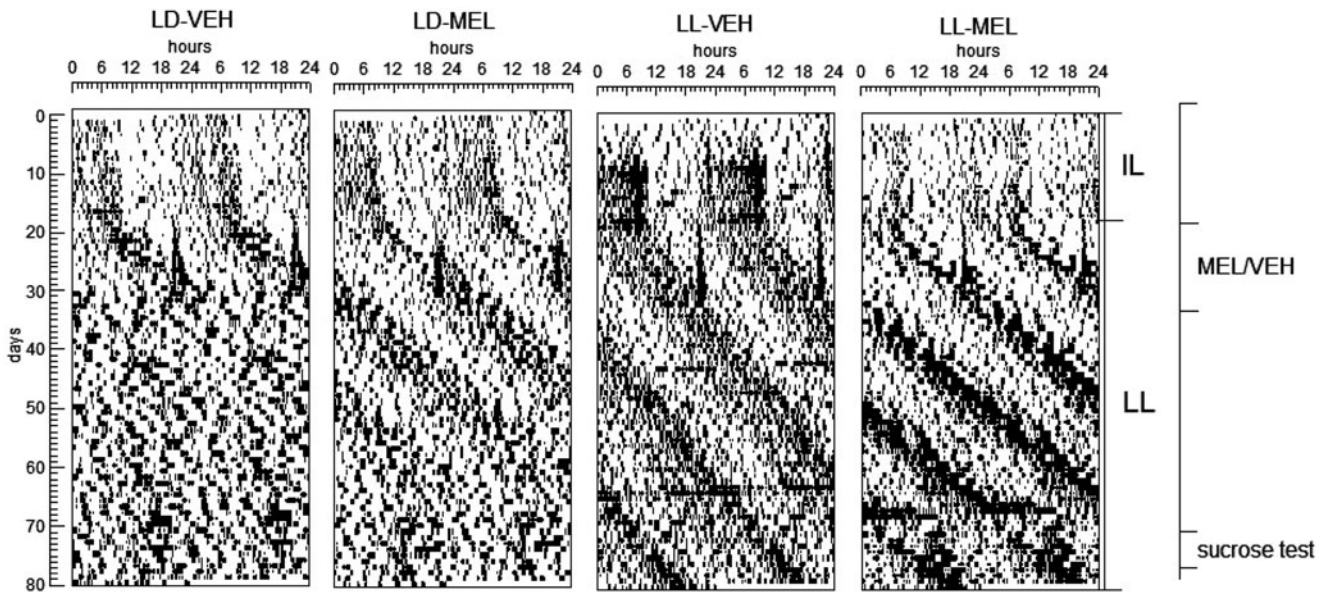

FIGURE 1. Actograms of a representative rat of each group. The first day of the actograms correspond to the day of weaning (P22). Groups named LL were reared under LL during suckling and those named LD were reared under 12-h light:12-h dark cycles. Bars on right indicate the procedure. The outside bar: from days 18 to 33, rats received daily administration of melatonin (MEL) or vehicle (VEH); between 72 and 76 days, the sucrose consumption was tested (including the previous days for adaptation to the solution). The inside bar on the right indicates lighting conditions in each experimental stage: lL, dim–bright light cycles (LD cycles of 12 h of 2 lx and 12 h 500 lx of white light) and LL, constant bright light of 500 lx.

a spectrophotometer. The H_2O_2 decomposition rate was directly proportional to enzyme activity. To compare the amount of CAT from different samples, we calculate the units of CAT per gram of hemoglobin (Hb). The Hb content was measured in hemolyzed erythrocytes with the HemoCue Hb 201 (Angelholm, Sweden).

Nitrite levels

The production of NO was measured indirectly via its stable metabolite nitrite using Griess reagent, as per the manufacturer's instructions (Sigma-Aldrich, Madrid, Spain, 0.4 g Griess reagent with 10 ml nitrite-free MQ water). In brief, for the determination of plasma nitrite, 5 µl of 30% $ZnSO_4$ solution was added to 50 µl of plasma and centrifuged at 12 000 *g* for 10 min at 4 °C in order to remove protein (Ghasemi et al., 2007). Both the supernatant sample and the Griess reagent were then added equally to the 96-well plate. The 96-well plates were allowed to develop for 10 min at room temperature, and the absorbance was measured using a plate reader at 540 nm. The nitrite concentration was calculated via comparison to a standard reference of $NaNO_2$ solutions at concentrations of 5, 25 and 50 mM.

The amount of nitrite was referenced to the amount of protein in the sample in order to make comparisons between groups. Protein concentrations were determined using the Bradford test (1976) and the Bio-Rad protein reagent.

For both CAT activities and nitrites levels, the results were expressed considering the mean data of control animals as 1 and the values of the samples proportional to it.

Sucrose preference test

This test was used as an indicator of anhedonia which may reflect some form of affective disorders, including depression (Aebi, 1984; D'Aquila et al., 1997). The test was carried out in the animals' home cages. For the task, rats were presented with two bottles of water: one contained plain drinking water, and the second a 1% sucrose solution. Prior to testing, rats had only the sucrose solution for 24 h. Then, for a further 24 h, they were habituated to the presence of two drinking bottles (one containing sucrose solution and the other water). On the day of the test (after 2 days with only water consumption), rats were presented again with the two bottles, and could choose which bottle to drink from 24 h. Water and sucrose solution were measured every 8 h and the positions of the two bottles were switched to reduce any confounding effect produced by a side bias. Sucrose preference was calculated as the percentage of the volume of sucrose intake over the total volume of fluid intake during the 24 h.

Data analysis

Circadian rhythmicity was determined using data from: (1) days 1 to 18, under bright and dim light cycles, (2) days 18 to 33, corresponding to the first MEL administration, (3) days 34 to 54 and (4) days 54 to 74, to analyze the rhythm after treatment. A chi-squared periodogram was calculated to determine the main circadian periodicity. The scanning range was from 20 to 27 h in steps of 5 min, and the threshold for statistical significance was $p = 0.05$, with the Bonferroni correction for multiple estimations within the periodogram. The software used for the calculations implements the method of Sokolove & Bushell (1978). This analysis provided the value of the significant free running period in the data series, and the percentage of variance explained by this rhythm (PV). PV can be used as an indirect measure of the daily phase stability and as an indicator of the significance of the rhythm, since greater PV indicate repetitive and stable patterns on a daily basis.

A sequential periodogram based on 10-day data was also calculated in steps of 24 h throughout the experiment, to determine the tau changes and the evolution of the PV over time. In this way, the period of each day corresponded to that of the rhythm of the following 10-day block.

The mean motor activity of rats every 15 min was also calculated. Moreover, to assess the increase in activity due to the reaction to the treatment, motor activity was calculated in blocks of 2 h each and referred to as a percentage of the total activity for 24 h: block 1: 2.5–0.5 h prior to treatment; block 2: 0.5 h before administration to 1.5 h after administration (to ensure that all the reactivity is included in the interval); block 3: 1.5–3.5 h after administration.

To test the phase control of MEL on the activity rhythm, we calculated the relationship between the phase of the free running rhythm after treatment and the time of injection. To do this, we first calculated for each rat the period of its free-running rhythm. Then, we adjusted the data corresponding to the 15 days after treatment to a sinusoidal curve with this period. Then, the grouping of the acrophases of the individual rhythms of animals from each group was tested by means of Rayleigh's *Z*-test. The time 0 corresponded to the time of the injection.

The analysis of the motor activity temporal series was carried out using the integrated package for chronobiology ''El Temps'' v.251 A. Díez-Noguera, Universitat de Barcelona, 2011. The statistical analysis was performed with the PASW 18.0 package. A general linear model was applied to the different variables, considering as factors: sex, light patterns during lactation, treatment (MEL or VEH) and the interactions among them. When sex was not significant, a new analysis was carried out considering the other factors. Then, depending on the results, *post hoc* comparisons were carried out using Bonferroni's correction. Numerical results are expressed as mean ± SEM.

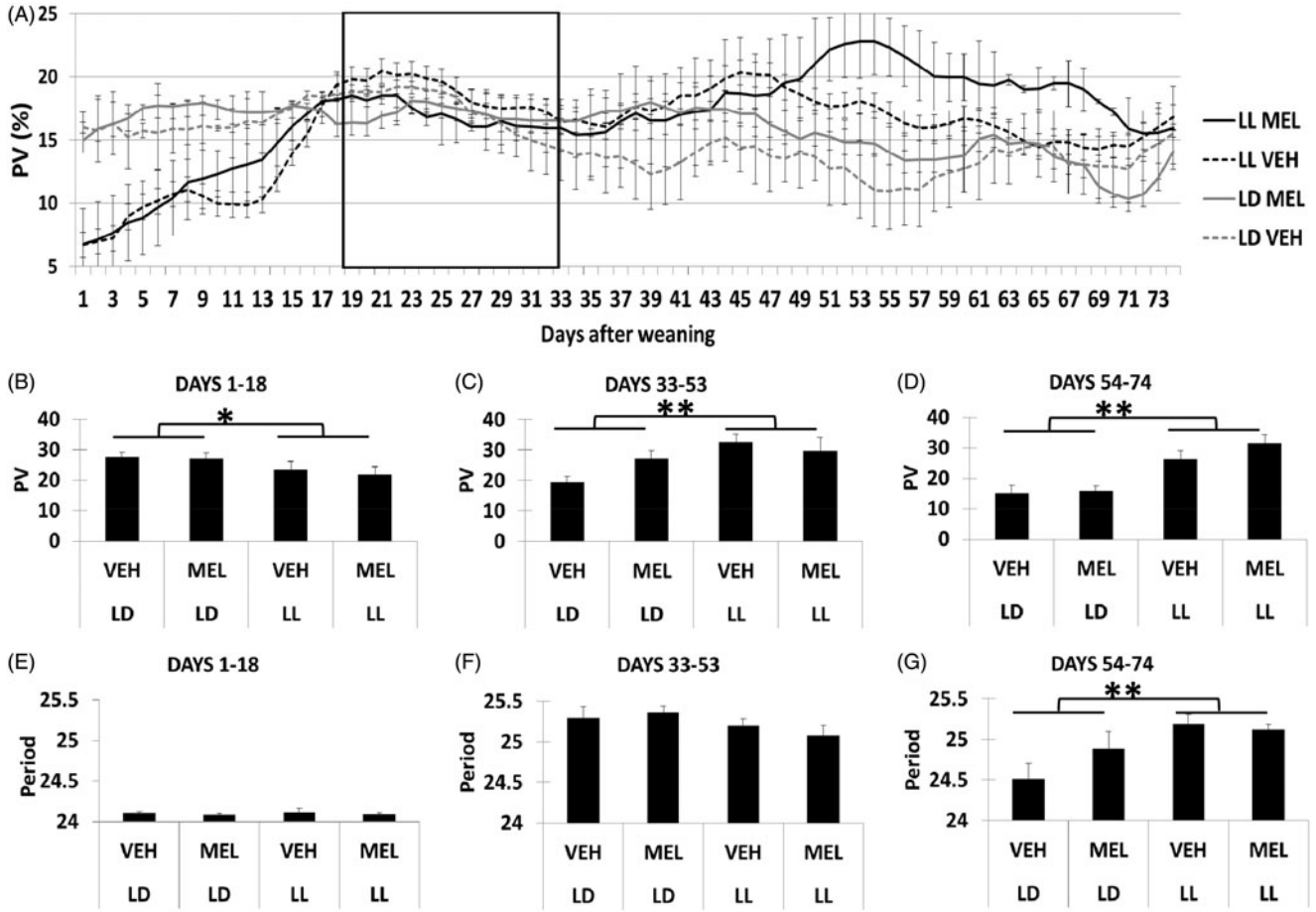

FIGURE 2. Percentage of variance explained by the circadian rhythm (PV) throughout the experiment. (A) Evolution of the PV for each of the experimental groups. The rectangle indicates the time that the animals received the daily administration of MEL or VEH. The other graphs represent the mean values of the periodogram analysis, carried out with individual data from the days indicated in the graphs: (B–D) mean PV and (E–G) mean period, in hours. In all the cases, data are presented as mean ± SEM. Asterisks indicate statistically significant differences: $^{*}p<0.05$, $^{**}p<0.001$.

RESULTS

Body weight

An ANOVA of body weight at day 78, considering sex, lighting conditions during suckling, and treatment as independent factors, indicated that sex was a statistically significant factor ($F_{1,36}$: 628.97, $p<0.01$), as was treatment ($F_{1,36}$: 4.281; $p<0.05$). Animals treated with MEL had a lower body weight than those treated with the VEH. The mean body weight for females was: 198.3 g (SEM: 2.99) for control and 191.7 g (SEM 3.9) for MEL treated rats; and for males, 348.8 g (SEM: 12.7) in the case of control and 326.11 (SEM: 6.8) for MEL treated. However, when an ANOVA was carried out for each sex separately, neither the treatment nor the lighting conditions were found to be statistically significant.

Motor activity rhythm

As a general view (Figure 1), rats had different circadian patterns along the experiment. All the LL rats showed a free running circadian rhythm during the entire experiment. However, LD rats showed a more disrupted pattern of motor activity, which became arrhythmic nearly 50 days after weaning. In both cases, the masking effect due to reactivity to the treatment injection can be observed as an increase in motor activity, although it is more noticeable in LD rats.

To examine the rhythmic expression of motor activity throughout the experiment, we calculated a periodogram with 10-day data sets in steps of one daytime. As shown in Figure 2(A), the percentage of variance (PV) evolved with time according to the lighting conditions during weaning, but during the time that the VEH or MEL administration took place no differences were found. Later, evolution was different among the groups. The LL-MEL group was the one with the strongest manifestation of the circadian rhythm and LD-VEH the one with the lower PV values.

To compare the rhythm between the groups, we calculated three periodograms for each animal's data, in blocks of ~20 days each, corresponding to days 1–18 (under lL) and after the MEL/VEH administration (days 33–53 and 54–74).

An ANOVA was carried out for the PV of each one of the blocks, first considering sex as an independent variable. However, since the differences were not

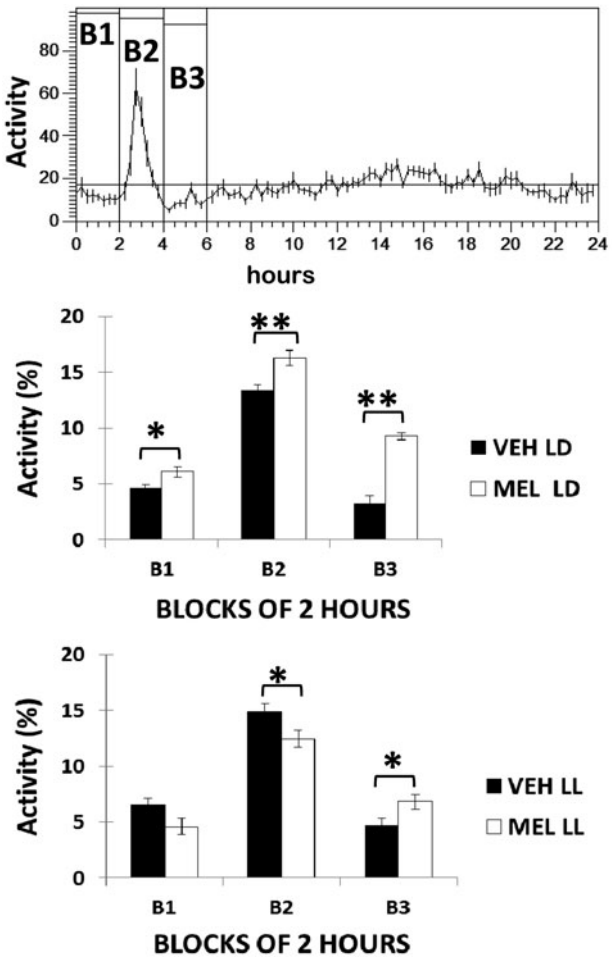

FIGURE 3. Activity response to melatonin (MEL) or vehicle (VEH) administration. Upper graph: example of a 24-h rhythm profile obtained from data during the 15 days of the administration, to indicate the reactivity induced by the injection, and the three 2-h blocks whose activity was analyzed for each animal and expressed as a percentage of the mean activity during 24 h. Lower graphs: mean percentage of activity for each one of the three blocks: middle graph, LD rats; and lower graph, LL rats. Black bars correspond to rats treated with the vehicle and white bars with melatonin. In all the cases, data are presented as mean ± SEM. Asterisks indicate statistically significant differences: $^{*}p<0.05$, $^{**}p<0.001$.

statistically significant, we carried out new analyses without this factor. For block 1 (days 1–18), all the rats were synchronized to the 24-h light cycle, and thus, this was the mean period of the rhythm in all the groups (Figure 2E). The PV (Figure 2B) was higher in LD than LL groups (ANOVA, $F_{1,40}$: 4.341, $p<0.05$). However, as can be observed in Figure 2(A), the PV of LL groups increased during the first 18 days to reach the same level as LD groups at the onset of the administration.

The first 20 days after MEL/VEH administration (days 33–53), lighting conditions during weaning was a significant factor that influenced the overt rhythm. LL rats had higher PV (Figure 2C) than LD groups (ANOVA, for PV: $F_{1,40}$: 6.634, $p<0.05$), although the values of the period were not significantly different (Figure 2F). No differences due to treatment were detected.

During days 54–74, the differences in rhythm due to the lighting conditions were more significant for PV ($F_{1,40}$: 26.48, $p<0.01$) (Figure 2D), and at this stage the period of LL rats was longer than that of LD rats ($F_{1,37}$: 7.857, $p<0.05$) (Figure 2G). No effect of treatment was detected.

When treatment was given, all the rats exhibited an increment in activity. The reactivity to the administration was analyzed by carrying out a mean profile in bases of 24 h, and then calculating the motor activity in blocks of 2 h. The activity in each block of 2 h was referred to as a percentage of the total motor activity in 24 h (Figure 3). When expressed as a percentage, there were no sex differences in the activity levels and this factor was not considered.

An ANOVA for each of these blocks indicated that there was a statistically significant interaction between treatment and lighting conditions during suckling. Thus, we carried out a second ANOVA separately for the LL and LD groups. In the LD groups, MEL significantly increased activity in the three blocks (block 1: $F_{1,21}$: 7.136, $p<0.05$; block 2: $F_{1,21}$: 13.69, $p<0.01$ and block 3: $F_{1,21}$: 50.06, $p<0.01$). However, this did not occur in the LL group. In LL rats, no differences were found between MEL or VEH administration in block 1, but in block 3 the MEL rats had higher activity than VEH ($F_{1,19}$: 5.288, $p<0.05$). However, in block 2, rats treated with MEL had lower activity than those treated with VEH, ($F_{1,19}$: 4.876, $p<0.05$).

To examine the phase control of rhythm by the MEL injection, Rayleigh's Z-test was used to calculate the grouping of the acrophases of the free running rhythm after treatment, in relation to the time of administration (Figure 4). All the acrophases were significantly grouped, but those corresponding to the LD-MEL group were phase-shifted by ~4.6 h, compared to all the other groups: Watson–Williams test, LD-MEL versus LD-VEH ($F_{1,19}$: 47.25, $p<0.001$), versus LL-MEL ($F_{1,19}$: 11.24, $p<0.01$) and versus LL-VEH ($F_{1,19}$: 12.97, $p<0.01$).

Oxidative stress

Catalase activity and nitrites were compared before and after the injection of MEL and VEH from blood samples. As a control, we used a group of LD rats that had always been under LD cycles and had the same age as the experimental groups. In these rats, no differences between the two samples were found in CAT activity or nitrites and thus the mean value was referred to as 1, to which all the other values were referred.

In the case of samples taken after treatment, the circadian time corresponding to the blood sampling for each animal was calculated. No significant differences were found according CT (calculated, first fitting a sinusoidal curve, and then by an ANOVA between four groups of CT) Thus, we considered all the values together.

There were no significant differences in CAT activity and nitrite levels among the groups due to the lighting

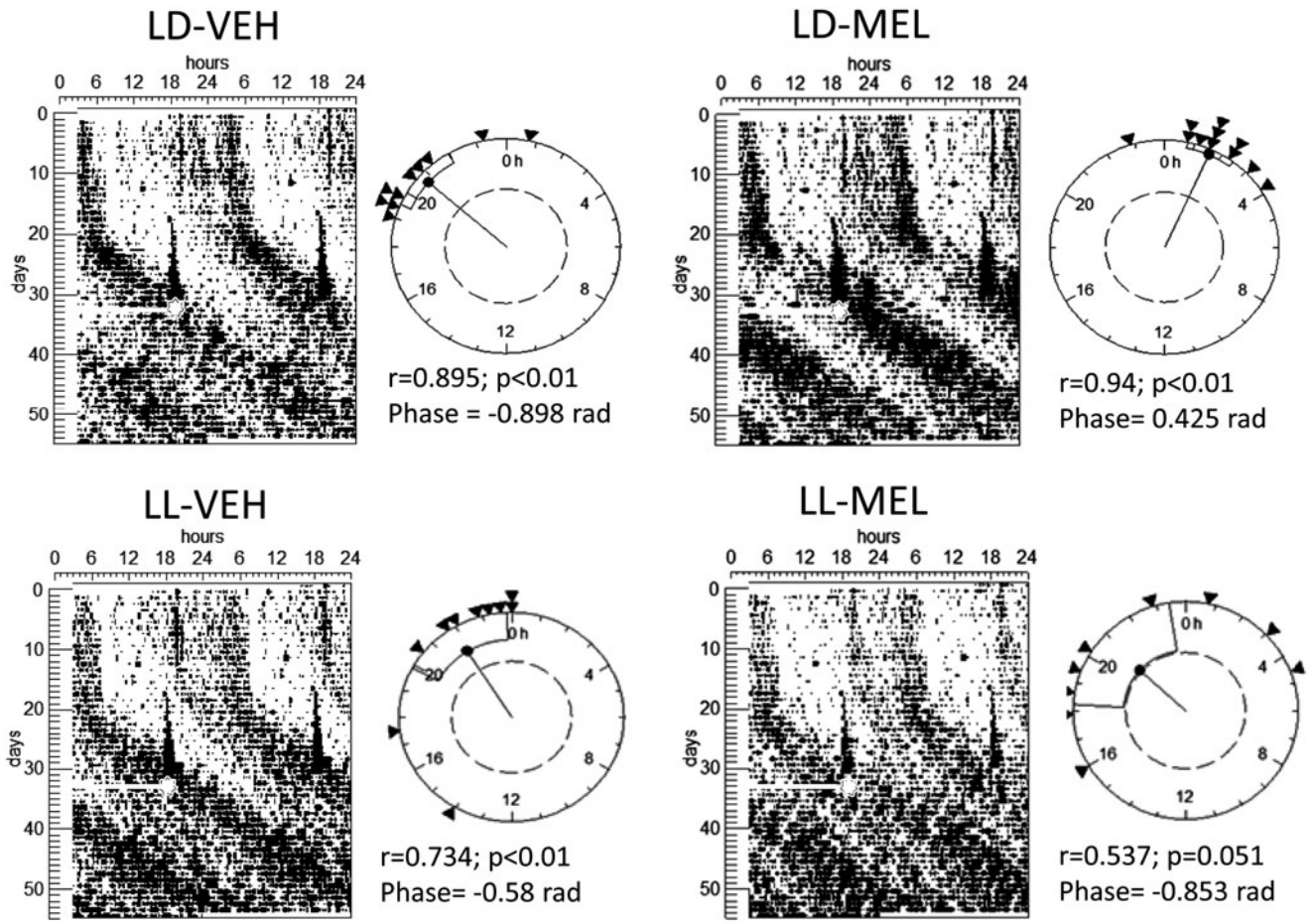

FIGURE 4. Phase control of the rhythm due to melatonin or vehicle administration for each group of rats. Double-plotted actograms represent the mean activity of all the rats in each group during the first 50 days of the experiment, in order to emphasize the common traits. White bar in the middle left of the actogram indicates the last day of injections and the white asterisks show the time of administration, which is considered the reference time of the phase of the rhythm. Of note: the LD-MEL group has a more visible rhythm due to synchronization among the rats and higher reactivity to the injections. Circular graphs indicate the result of Rayleigh's *Z*-test. Outer cycles indicate 24 h, and 0 time corresponds to the time of the injection. The inner circle indicates the threshold for statistical significance of grouping at $p = 0.05$. The vector (r) indicates the phase of the group (mean value [black point at the end] and the confidence interval). Notice that LD-MEL is the group whose phase is different from the rest.

conditions or the MEL treatment (Figure 5). However, in all the cases, the levels after treatment were higher than in the control group: for CAT activity (t_{46}: 2.136, $p<0.05$) and for nitrites (t_{16}: 2.72, $p<0.05$). This suggests that the increase may have been due to the time the animals had been under LL. The increase in CAT activity after treatment was significant in LD groups (ANOVA, $F_{1,35}$: 5.37, $p<0.05$) and the increase in nitrite levels after treatment was significant in both groups (ANOVA, $F_{1,33}$: 3.99, $p<0.05$ for LD groups, and $F_{1,33}$: 4.81, $p<0.05$ for LL groups).

Anhedonia test

In most of the groups, the percentage of sucrose consumed by the rats stood at ~80% (Figure 6). An ANOVA revealed a higher preference of sucrose in females than in males ($F_{1,39}$: 16.6, $p<0.05$). However, interactions by ''sex and group'', ''sex and treatment'' and ''sex and light'' were also significant. Thus, new ANOVA were carried out separately for each group. No differences among groups were found in females, although in the case of males, only the LD-MEL group had a sucrose preference low enough to suggest anhedonia compared to LD-VEH (ANOVA, $F_{1,6}$: 25, $p<0.01$).

Levels of MEL under LL in LD or LL-reared rats (Supplementary Material)

In a previous experiment (data not published before and shown here as Supplementary Material), we tested the plasma MEL levels of rats born and suckled in LL or LD conditions. We found that plasma MEL levels showed marked variations among individuals in the peak levels and in the circadian time that the maximum level was found (Figure S1 in Supplementary Material). No differences between LD or LL rats could be observed.

DISCUSSION

In this experiment, lighting conditions during suckling modified the circadian behavior of rats as well as the

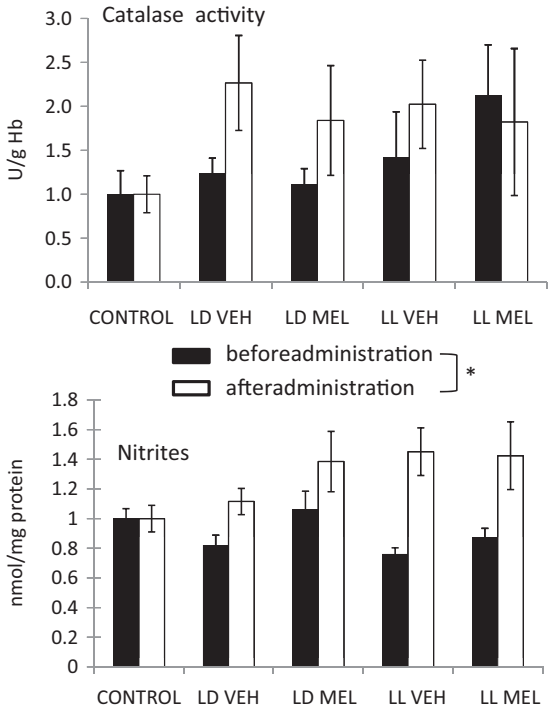

FIGURE 5. Oxidative variables: catalase activity and nitrite concentration for each one of the experimental groups, before (black bars) and after (white bars) the daily injection of melatonin (MEL) or vehicle (VEH). Control group corresponds to rats of the same age, always maintained under 24-h LD cycles. In all the cases, data are presented as mean ± SEM. Statistically significant differences ($^*p < 0.05$) were found between values before and after treatment for both variables, considering all the groups together.

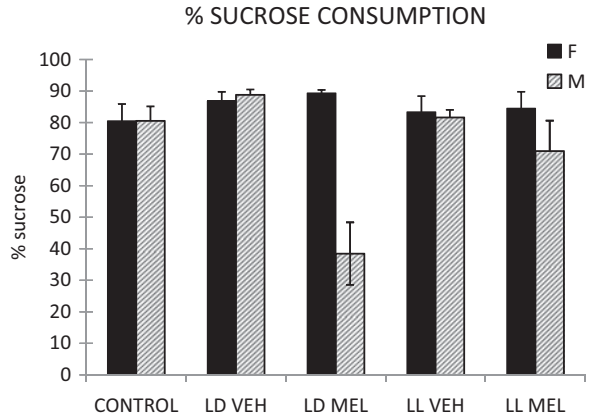

FIGURE 6. Sucrose consumption expressed as a percentage of the total liquid consumption in 24 h (mean ± SEM) for each group of rats according to the sex (F: females, M: males). Anhedonia was only found in males of the LD-MEL group, which differed from the rest of the groups ($p < 0.05$).

effect of MEL on their circadian rhythm. MEL had more influence on the circadian rhythm of animals reared under LD. However, there was no impact on the oxidative system, which could be explained by the strong effects of LL.

As expected (Cambras et al., 1998, 2014; Canal-Corretger et al., 2000), in this experiment the motor activity of LL rats followed a rhythmic pattern while LD rats became arrhythmic. However, unlike in other experiments, both groups of rats were first submitted to 24 h cycles of bright–dim light and then received daily MEL for 2 weeks. Thus, under these circumstances, the characteristic motor activity pattern of both groups was not visible until day 50 after weaning approximately, when LD rats started to become arrhythmic. Thus, it is noticeable that the LL during weaning has a long-lasting effect on the circadian system. However, although all the LD rats will become arrhythmic, LD-VEH loses first the circadian rhythmicity than LD-MEL, as seen in Figure 2.

The bright–dim light cycle induced a 24-h activity pattern in all the rats, which was necessary to test MEL effects at a determined circadian time. Under this situation, animals that were under LD, which later became arrhythmic, were those that showed a more stable circadian rhythm. This stability could be due to the cyclic environment during the suckling period, due to the previous LD cycle, or to the rhythmic dam.

The effects of MEL were slightly visible in the body weight: rats treated with MEL were lighter than the respective counterparts. This effect on body weight has already been reported and it is related to an increase in motor activity in the dark phase (Terrón et al., 2013). In our case, since all the rats were under LL, a situation that increases body mass (Miler et al., 2014; Vinogradova & Anisimov, 2013), MEL might have prevented the body weight increase due to LL.

MEL may increase motor activity, alertness and body temperature in nocturnal animals (Bilu & Kronfeld-Schor, 2013). Here, all the rats showed an increase in motor activity due to reactivity to the injection, but this increase was higher in the LD group treated with MEL. Moreover, LD rats treated with MEL showed a phase difference of the circadian rhythm, with respect to the other groups. We cannot deny that this phase shift could also be due to the masking effect caused by higher motor activity induced by the injection. However, in any case, both responses suggest higher susceptibility of the LD group to the external MEL. Differences in the pharmacological effects of MEL according to early lighting conditions could be due to the coupling of the circadian system. Light may uncouple the oscillators that form the circadian pacemaker (Chiesa et al., 2010; Ohta et al., 2005) and thus, arrhythmic LD-reared rats might have a circadian system that is weakly coupled. Since a weak circadian system may be more sensitive to external stimuli (Cambras et al., 2011), this could also apply to the effects of MEL.

The response of the circadian system to this hormone could also be explained by a variation in sensitivity of the MEL receptors in the SCN, due to LL exposure in early life. Both LD- and LL-reared rats maintained detectable, similar plasma MEL levels (Supplementary Material), although peak levels, and the circadian time at which they occurred, are rather variable. Thus, in both rats (rhythmic and arrhythmic), MEL rhythms are

desynchronized from the activity patterns, as has already been described in rats under LL (Aguzzi et al., 2006; Fukuhara et al., 2005). Since, MEL plasma levels were similar among the groups, we hypothesize that MEL receptors of LD rats could be more sensitive than those of the LL group, producing stronger responses to MEL administration. MEL receptors have been detected at fetal day 18, but they are regulated postnatally (Zitouni et al., 1996). Consequently, LL at this age might modify the sensitivity or the expression of MEL receptors in the SCN.

LL exposure may also induce anhedonia (Tapia-Osorio et al., 2013), which fits with the consideration that LL is a depressive agent (Mendez et al., 2012). In our experiment, this effect was only found in LD reared male rats treated with MEL, which showed a decrease in sucrose consumption. LD rats were those with a more disrupted circadian rhythm, which may induce humor alterations, as may occur in human circadian misalignment (Salgado-Delgado et al., 2011). However, anhedonia was not found in LL rats, which indicates that LL during suckling may protect against LL effects in adults (Martynhak et al., 2011).

It is noticeable that rats only received MEL for 15 days and the anhedonia test was carried out 2 months later. However, we must take into account the age of the rats when MEL was administered, since stimuli applied at an adolescent age could produce long-lasting effects on the circadian manifestation (Albert et al., 2013). Other reports found enduring effects of MEL interacting with life stressors. It has been demonstrated that early life adversities influence diurnal primates' circadian system in terms of responsiveness to MEL (Rawashdeh & Dubocovich, 2014). Moreover, hamsters submitted to prenatal restraint and treated postnatally with MEL showed depressive-like behavior. This supports the hypothesis that MEL and exposure to stressors early in life interact to alter adult affective disorders (Aubrecht et al., 2014). Hence, faced with an anomalous situation, such as continuous light, the MEL response may differ according to the early lighting conditions.

Sex differences were found in the link between corticosterone levels and MEL. MEL had more antidepressive effects in females than in males (Hill et al., 2003). We might hypothesize that LD groups would have higher corticosterone levels (Tapia-Osorio et al., 2013) than LL groups and that the interaction between high corticosterone and MEL could be responsible for the anhedonic behavior of the LD-MEL males. Differential responses to pharmacological drugs such as MEL may indicate modifications in brain neurochemistry targeted by the environment (Kraemer & Clarke, 1990; Rawashdeh & Dubocovich, 2014), which suggests that during ontogeny, neural wiring and chemistry are influenced by environmental factors. The different response in the anhedonia test of LD males might indicate different pharmacological effects of MEL according to the sex of the animal and early environmental lighting. The use of animals of both sexes should always be encouraged in pharmacological and physiological studies.

Circadian responses and mood behavior can be affected by light, which may be due to variations in the retinal pigment melanopsin (Roecklein et al., 2013). The morphology or function of melanopsin cells could be modified by light conditions during early life.

Studies in retinas from mouse pups suggest that the number of melanopsin-immunoreactive cells around birth is not dependent on the ambient lighting conditions, since the number of immunostained melanopsin cells did not differ between LL, DD or LD born rats at P1 (González-Menéndez et al., 2010, 2011). However, melanopsin system might be affected by ambient light conditions during postnatal development. In this sense, LL has been described to suppress melanopsin to almost undetectable levels after 5 days (Hannibal et al., 2005). In a previous study, we found out that rats under LL, born and reared under LD, DD or LL, showed a similar degree of retinal degeneration, due to prolonged LL exposure. However, melanopsin-expressing RGCs was detected in all of them, although the levels of immunostaining were lower than in control rats always maintained under LD conditions (Cambras et al., 2014). Perhaps their functioning may have been modified by light, which would explain the different responses of the circadian system found in this experiment. However, we cannot exclude that perhaps other retinal photoreceptors such as the visual rod cells can be completely degenerated after such a long-lighting experience, and this could contribute to a different response to light. We must take into account that MEL was not found to affect the oxidative stress induced by, and there were no differences due to early lighting conditions, which may be attributed to the independence of the oxidative system to the melanopsin cells. The levels of both oxidative variables increased over time, which could be attributed to a strong and accumulative effect of continuous light on oxidative variables, or to the stress caused by the daily injections, that masks the effect of MEL as antioxidant agent (Carpentieri et al., 2014; Reiter et al., 2000; Vishwas et al., 2013).

In summary, this experiment suggests that lighting conditions in early infancy may modify the further functioning of the circadian system and related structures, and influence rhythmic behavior, responses to MEL, and the anhedonic response.

DECLARATION OF INTEREST

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Supplementary material available online.
Supplementary Figure S1.