Effects of aging on the circadian rhythm of wheel-running activity in C57BL/6 mice

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Valentinuzzi, Veronica S., Kathryn Scarbrough, Joseph S. Takahashi, and Fred W. Turek. Effects of aging on the circadian rhythm of wheel-running activity in C57BL/6 mice. Am. J. Physiol. 273 (Regulatory Integrative Comp. Physiol. 42): R1957–R1964, 1997.—The effects of age on the circadian clock system have been extensively studied, mainly in two rodent species, the laboratory rat and the golden hamster. However, less information is available on how aging alters circadian rhythmicity in a commonly studied rodent animal model, the mouse. Therefore, in the present study we compared the rhythm of wheel-running activity in adult (6–9 mo) and old (19–22 mo) C57BL/6 mice maintained under different lighting conditions for a period of 4 mo. During this period, mice were subjected to phase advances and phase delays of the light-dark (LD) cycle and eventually to constant darkness (DD). In LD (12 h light, 12 h dark), old mice exhibited delayed activity onset relative to light offset and an increase in the variability of activity onset compared with adult mice. After a 4-h phase advance of the LD cycle, old mice took significantly longer to reentrain their activity rhythm when compared with adult animals. Old mice also demonstrated a decline in the number of wheel revolutions per day and a tendency toward a decrease in the length of the active phase. An increase in fragmentation of activity across the 24-h day was obvious in aging animals, with bouts of activity being shorter and longer rest periods intervening between them. No age difference was detected in the maximum intensity of wheel-running activity. In DD, the free-running period was significantly longer in old mice compared with adults. In view of the rapidly expanding importance of the laboratory mouse for molecular and genetic studies of the mammalian nervous system, the present results provide a basis at the phenotypic level to begin to apply genetic methods to the analysis of circadian rhythms and aging in mammals.

activity rhythms; Mus musculus; light-dark cycles; free-running period

PROPER PHASE RELATIONSHIPS among numerous physiological and behavioral 24-h rhythms, as well as between these rhythms and daily environmental cycles, are crucial for the optimal health of the organism and its adaptation to the environment (1, 13, 18). Age-related changes in the circadian timing system can profoundly influence these relationships. Rodents are common models for the study of both the circadian system and its aging. Age-related changes in circadian rhythms have been well documented in several species, particularly the laboratory rat and the Syrian (golden) hamster. Typical changes include shortening of the circadian period (10, 14, 28, 30), alteration in the phase angle of entrainment to the light-dark (LD) cycle (10, 20, 31), fragmentation of the activity rhythm (7, 11, 20), decreased precision in onset of daily activity (12, 20, 31), altered rates of reentrainment following a shift in the LD cycle (33), and alterations in the response to the phase-shifting effects of light (19, 32) and nonphotic stimuli (23).

Although there is considerable information on aging of the circadian system in rats and hamsters, less is known about the aging of the circadian clock in the mouse. The only age-related changes reported in mice have been in the activity-to-rest ratio, amplitude of activity, and free-running period. As shown in other species, old mice show a decrease in the activity-to-rest ratio (3, 24) and in the amplitude of wheel-running activity (17, 24, 29). The effect of aging on the free-running period has not been consistent, perhaps due to differences in lighting conditions during or before measurement or to the use of different strains of mice or the age of the animals at testing. Welsh et al. (29) observed a longer period for sleep-wake cycles in older C57BL/6 and C57BL/10 mice. Whereas Teena and Wax (24) observed no significant age-related change in the period of the C57BL/6 strain, Pittendrigh and Daan (15) detected a shorter period in the same strain and in 26-mo-old DBA mice, but only after several months in constant darkness (DD). In a study analyzing aftereffects of different day lengths, Davis and Menaker (3) observed a shortening of period in 10-mo-old C57BL/6 mice compared with 2.5-mo-old animals. However this difference was apparent only after several weeks in DD. Possidente et al. (17) detected longer periods in 7-mo-old C57BL/6 mice compared with 1.5-mo-old mice after entrainment to different photoperiods. In the present study, we examined the rhythm of locomotor activity in old (19–22 mo) and adult (6–9 mo) C57BL/6Nia mice in LD and DD conditions. We quantitated several aspects of this overt rhythm, including phase angle of entrainment to the LD cycle, variability in activity onset, rate of reentrainment to an LD cycle, total wheel revolutions performed per day (amplitude), peak intensity of wheel-running activity, the number of bouts of activity per cycle, the duration and size of these bouts, the length of the active phase, and the free-running period in DD.

MATERIALS AND METHODS

Animals and experimental treatment. Twelve male C57BL/6 mice (Mus musculus) were purchased from the National Institute on Aging at 4 (n = 6) and 16.5 mo (n = 6) of age. On
arrival, mice were housed in individual cages (15 × 32 cm) equipped with running wheels (11 cm in diameter). Husbandry activities were performed every 2 wk; this never coincided with experimental manipulations. Cages were placed in a light-proof chamber, and animals were maintained in a cycle of 12-h light (300 lx) and 12-h darkness (0 lx), in constant ambient temperature (21 ± 1°C) with food and water available ad libitum. At 6 and 19 mo of age, the animals were submitted to a 4-h phase advance of the LD cycle and 2 wk later to a 4-h phase delay. The mice were then maintained on this 12:12-h LD cycle (LD 12:12) for 3 mo. Finally, at 9 and 22 mo of age the mice were placed in DD and remained in this condition for 45 days. Wheel-running activity was continuously recorded with an on-line data acquisition system (Stanford Software Systems, Stanford, CA).

Data analysis. Determination of daily activity onset was required to calculate the phase angle of entrainment, to determine precision of activity onset, to establish rate of reentrainment, and for calculation of length of the active phase. To determine onset of locomotor activity, data for each 24-h cycle were divided into 5-min bins. Onset was defined as the first bin where activity was greater than or equal to 10% of peak activity and followed by three out of the next six bins having at least 10% peak activity. Additionally, determination of daily offset of wheel-running activity was also required for the calculation of length of the active period. Offset was defined as the last bin where activity was ≥10% of peak activity preceded by three of six bins having at least 10% of peak activity. Application of these criteria defined onsets and offsets for each circadian cycle (obtained by using an in-house data analysis program), which was in good agreement with visual inspection of the actograms. Alpha (the time in hours between activity onset and offset) was determined between cycles 20 and 30 in DD.

The phase angle of entrainment to the LD cycle was defined as the number of minutes that activity onset preceded (+) or followed (−) the onset of darkness. This phase was determined beginning 10 days after the last shift of the LD cycle, over a 10-day interval, for each animal. Precision of activity onset was defined as the daily deviation from each animal’s mean phase of entrainment. Specifically, we took the standard deviation of the phase of activity onset calculated previously for each animal. A grand mean of variability in activity onset was obtained for the groups of adult and old mice.

The number of days until stable reentrainment of the activity rhythm following a shift of the LD cycle was determined. Entrainment to the new LD cycle was considered complete when activity onset was within 20 min of the baseline phase for that animal during the 10-day period before the 4-h phase advance of the lights.

The free-running period was evaluated by means of a χ² periodogram (Chronobiology Kit, Stanford Software System), based on an interval of 10 days, beginning 20 days after the animals were released in DD. The analysis was performed with a 5-min step size and a range of period from 22 to 26 h. In addition, the total daily wheel-running activity for each animal was obtained through the Activity Count Program of the Chronobiology Kit for an interval of 10 days during LD and DD conditions. The maximum revolutions per minute (rpm) was defined as the maximum running-wheel revolutions per minute observed in a given cycle. This number was averaged over a 10-day interval for each animal.

Division of activity into bouts was performed as described previously by our laboratory (11). Briefly, we determined that an interval of rest of 20 min or longer separated intrabout intervals from interbout intervals using a log survivor function of all rest intervals over the course of several cycles from several different mice. To eliminate low-amplitude noise artifacts in the records, an additional requirement of wheel-running intensity of at least 6 rpm was introduced in the bout detection algorithm. These criteria were applied to several cycles from each animal, 20 days after release in DD, and the number of bouts of activity per circadian cycle was compared between the two age groups. The duration in minutes of each bout and the number of wheel revolutions performed per bout (bout size) were also analyzed.

The results are reported as means ± SE. Unpaired Student’s t-tests were used to make statistical comparisons between age groups.

RESULTS

Parameters measured in LD conditions. A statistically significant difference (t = −3.87, P < 0.01) between age groups was observed in the phase of entrainment to the LD 12:12 cycle (Fig. 1, A and B). Wheel-running behavior in adult mice began a few minutes after the onset of darkness each day. The mean phase angle of entrainment in the adult group was 11.4 ± 4.2 min. In contrast, activity onset in aged animals always occurred substantially later in the evening, with wheel-running in this group beginning an average of 52.8 ± 9.6 min after lights went out. In addition, precision of activity onset was significantly different (t = −2.49, P < 0.05) in the two age groups (Fig. 1C). The time of daily activity onset under entrained conditions varied only by 9 ± 1 min within the group of adult mice. In contrast, old mice showed irregular activity onsets, which varied by 33 ± 7 min from day to day. The precision in adult animals and the lack of precision in the old group was reflected in the individual records (Fig. 1A). Day-to-day variability in activity onset ranged from 6 to 13 min in individual adult mice. The most precise old mouse exhibited a day-to-day variability in the time of activity onset of 24 min, and the least precise older mouse varied by an average of 1 h 11 min per day.

An age-related difference in the time to reentrain after a 4-h phase advance of the LD cycle was observed. Adult mice resynchronized to the new light schedule on the following day, whereas old mice took an average of 8.7 ± 0.73 days to attain their characteristic phase angle of entrainment (Fig. 2, A and B). This difference between adult and old mice was statistically significant (t = 9.59, P < 0.001). In marked contrast, reentrainment to a 4-h delay of the LD cycle occurred within the first cycle for both age groups (data not shown).

A statistically significant difference between age groups was observed in total wheel-running activity (t = −4.04, P < 0.01). When averaged over an interval of 10 days, the total number of revolutions performed per cycle was 34,300 ± 3,500 for adult animals compared with 18,150 ± 1,800 revolutions/day in old animals (Fig. 3). The range was 20,800–42,600 revolutions/day for adult mice, compared with 12,600–25,500 revolutions/day for old animals. The highest intensity wheel-running activity in revolutions per minute did not differ significantly between age groups. Adult ani-
Parameters measured in DD conditions. Old mice used in this experiment had an average free-running period of $23.96 \pm 0.05$ h, whereas the adult group averaged $23.53 \pm 0.09$ h as assessed by $x^2$ periodograms on days 20-30 in DD (Fig. 4, A and B). This difference in circadian period was statistically significant ($t = 4.16$, $P < 0.005$); thus aging is associated with a longer circadian period in C57BL/6 mice. For this same 10-day interval in DD, the length of the active period was determined. Although no statistically significant age-related difference was observed for this parameter of the locomotor rhythm, old animals tended to show a shorter active period. The time elapsed between activity onset and offset averaged $10.8 \pm 0.78$ h in old animals and $12.8 \pm 1.35$ h in adult mice. Total wheel revolutions performed per cycle was also measured in DD, where each age group performed approximately the same number of wheel revolutions per circadian cycle as found under LD conditions (data not shown); thus the age-related change in the amplitude of the locomotor rhythm persists under constant conditions.

The fragmented appearance of the actograms generated from wheel-running behavior of old mice compared with those derived from adult mice was quantified in our analysis of the number of bouts of wheel-running activity per cycle. Figure 5A shows that old mice exhibit more bouts ($4.95 \pm 0.51$) of wheel-running activity per cycle than adult animals ($3.7 \pm 0.64$). This difference was statistically significant ($t = 3.00$, $P < 0.05$). Bout duration and bout size also manifest an effect of age. The duration of activity bouts (Fig. 5B) was lower in old animals ($98.0 \pm 17.0$ min) than in adult animals ($158 \pm 18$ min). This difference was statistically significant ($t = -2.38$, $P < 0.05$). Bout size (Fig. 5C) was lower in old mice ($3,054 \pm 1,039$ revolutions/bout) compared with adult mice ($7,403 \pm 1,676$ revolutions/bout). This parameter showed a high level of variability, the range being 2,631-11,778 revolutions.
in adult animals and 645–6,620 revolutions in old animals. Because of this, the t-test revealed a $P = 0.052 \ (t = -2.21)$. The variability in bout size among adult mice results from the fact that the active period almost uniformly begins with a large consolidated bout of wheel running, which persists for several hours. The records of old mice are also characterized by one or two large bouts and a number of small bouts of activity each cycle.

**DISCUSSION**

The present results indicate that the aging process significantly affects the circadian system in C57BL/6 mice. Several parameters of the circadian rhythm of locomotor activity in mice show clear and defined differences between old and young animals. Aging not only alters the free-running period but also the pattern of entrainment to an LD cycle, the time it takes for the circadian rhythm of locomotor activity to reentrain to a new LD cycle, and other characteristics such as precision of activity onset, fragmentation of locomotor activity, and total wheel revolutions performed.

One of the most striking age-related changes observed in these experiments is the increase in the free-running period in DD. This result supports previous work indicating lengthening of the activity period with age in house mice. The 0.43-h mean difference observed between adult and old mice here is comparable to the difference reported by Possidente and co-workers (17). However, these investigators used young and adult animals that were aged 1 and 7.5 mo, respectively. In the present study, the mice were 9 and 22 mo of age at the time we measured the free-running period. A change in period appears to be a characteristic change in the aging circadian system, and this is convincing evidence that the clock itself is altered during senescence (10, 28, 30). Indeed, other investigators have provided direct evidence of physiological and biochemical age-related changes in the biological clock localized in the suprachiasmatic nucleus of the hypothalamus of mammals (1, 18, 23).

We detected a significant delay in the time of activity onset in old mice compared with adults when the mice were maintained on a LD 12:12 cycle. The delayed phase angle of entrainment in older mice is consistent with the observed lengthening of the free-running period. According to the entrainment theory (13), an increase in period would result in a change in the phase relationship between the entraining LD cycle and the activity rhythm such that less light falls in the delay region of the phase-response curve (PRC) to light during steady-state entrainment. In other words, in aged mice the endogenous period gets closer to the entraining agent (zeitgeber) period so that the daily delay required for appropriate entrainment is reduced. This causes older mice to delay activity onset until later...
in the evening. Additionally, Daan and Pittendrigh (2) provided evidence of a direct relationship between the free-running period and the response to resetting stimuli (i.e., fast pacemakers tend to be more delayed or less advanced by light than slow pacemakers). Thus we could probably expect a change in the PRC of old animals. In this case, an increase in period would be correlated with a smaller delay zone compared with young animals, a change that would favor entrainment in old animals. The measurement of a PRC to light in old mice would test this hypothesis. Changes in the phase-shifting effects of light pulses have been observed in aging Syrian hamsters (19, 32).

Fig. 4. A: representative activity records of young (top) and old (bottom) mice entrained to an LD 12:12 cycle and subsequently placed in constant darkness (DD) on day 48. Successive days are plotted from top to bottom, and x-axis represents a 48-h period of activity. Black bar on top indicates dark phase of the LD cycle. B: mean ± SE of free-running period in DD of old and young mice expressed in h. Circadian period is significantly longer in old animals (*P < 0.01).

Fig. 5. A: mean ± SE number of bouts of wheel-running activity per cycle for old and young animals in DD conditions. Old animals show a significantly higher number of bouts per cycle (*P < 0.05). B: mean ± SE of bout duration in min for old and young animals in DD conditions. Bout duration in old animals was significantly shorter (*P < 0.05). C: mean ± SE of bout size expressed in number of revolutions per bout for old and young animals in DD conditions. Old animals show smaller bout size (*P = 0.052).
Day-to-day variability in activity onset increases significantly in old animals. Data like these may be taken as an indication of how tightly a circadian rhythm is coupled to the zeitgeber. The decrease in the stability of the phase control in old animals suggests a decrease in the coupling strength between the LD cycle and the pacemaker (9, 18). Indeed, the threshold of light required to shift the pacemaker is increased in old hamsters (32), which would be expected to decrease the efficacy of photic entrainment in older animals. Whether similar age-related changes in the threshold for photic entrainment of mice also occur remains an open question.

Aging was characterized by a dramatic decrease in the capability to reentrain to a phase shift in the LD cycle. Old mice required 8 days to reentrain to a 4-h phase advance, whereas adult animals adjusted to the new LD cycle in 1–2 days. An almost immediate 4-h phase advance in the adult animals may seem at variance with the small advance region evident in the PRC to light of this species (2, 21). However, a PRC generated in response to 15-min light pulses may not be quantitatively useful in interpreting how an animal will respond when the entire light-dark cycle is advanced 4 h. Indeed, longer light pulses have been shown to produce bigger advances in the circadian system of mice (Ref. 26; M. H. Vitaterna, personal communication). In addition, in the present experiment, the new LD cycle may be inducing the 4-h phase shift by two different mechanisms. First, light is present during the whole advance region of the PRC after the 4-h advance to the new LD cycle. In addition, these mice would not experience any of the phase-delaying effects of the previous LD cycle. The second mechanism that may contribute to a rapid phase advance is the possibility that darkness occurring in the middle of what would have been the light phase provides an activity-inducing stimulus. Activity at this phase of the cycle has been shown to advance the circadian system of mice (4, 8).

The effect of age observed in the rate of reentrainment could be related, in part, to activity itself. The activity induced by darkness may be lower in old animals, and consequently the feedback on the clock would be expected to be lower. Aging is known to alter the feedback effects of the activity/rest cycle in Syrian hamsters (23, 25). Indeed, we observed decreased locomotor activity in old mice throughout the cycle, as discussed below. In this sense, not only may old mice respond with less activity to the dark but this activity is less efficient in its feedback on the clock.

Rate of phase shift may also be taken as a measure of strength of coupling to external zeitgebers. When the coupling between the LD cycle and the pacemaker is strong, resynchronization to a new LD cycle is fast, which is what we observe in adult mice. On the other hand, the decrease in the rate of reentrainment in old animals could be indicative of a decreased coupling strength. Hamsters also show age-related changes in the ability to reentrain. Old hamsters reentrain more rapidly than young ones after an advance shift of the LD cycle and reentrain more slowly than young hamsters after a delay shift of the LD cycle (31). The age-related changes in the ability to reentrain of these two species are consistent with the age-related change in the free-running periods observed. Mice show a lengthening of their period, which means they need to phase advance more to reentrain to an advancing LD cycle. On the other hand, hamsters show a decrease in their free-running period, leading to a faster reentrainment to a phase advance of the LD cycle and to a slower reentrainment to a phase delay of the LD cycle. Reentrainment to a 4-h phase delay, in the present study, was immediate for both groups of mice. The lack of observable transients in this case probably results from the inhibitory effect (i.e., masking) that light is known to have on the wheel-running activity of mice.

Aging mice in this study show a significant increase in the number of discrete bouts of wheel-running activity per cycle. Previous studies from our laboratory have demonstrated that the activity pattern of aged hamsters is also fragmented into a greater number of discrete bouts (7, 11, 20). In some models of the circadian system, bouts of activity are controlled by individual circadian oscillators that are themselves part of a coupled oscillator system (3, 16, 22). The fragmentation of the activity period exhibited by old mice in this study suggests that, as in hamsters (20), the process of aging may also influence the phase or strength of coupling among individual oscillators.

The increased bouts of activity in senescent mice were characterized by being of shorter duration and fewer total wheel revolutions. This correlates well with the significant decrease in overall wheel-running activity we observed and has been noticed previously by other investigators (17, 24, 29). We do not believe this decreased level of activity results from the old animals being less healthy than the adult animals because we found no significant difference in the maximum rate of wheel-running activity achieved by the old and adult mice. Changes in the temporal distribution of intense wheel-running behavior rhythm can have major feedback effects on the period of the circadian clock (4). The increased period observed in aging animals could be a manifestation of their lower level of activity. The lack of precision in activity onset could also be a consequence of the same effect. Changes in these parameters, activity level and precision of activity onset, seem to occur in parallel. For example, young hamsters decrease total activity and lose precision in their activity onsets when transferred from an LD 14:10 to an LD 6:18 cycle (5). It would be interesting to perform a longitudinal study to determine whether these two measures also age at the same rate.

Although a wide variety of age-related alterations in circadian rhythmicity has been observed (1, 18, 23), little is known about the functional significance of these changes. In view of the central role played by the circadian clock system in the regulation of diverse physiological and behavioral variables, age-related changes can be expected to have an important impact.
on the health of the organism. At the same time, the relationship between circadian rhythms and external time cues probably does much to maximize the survival of each species in a environment where food supplies and predator activity are themselves cyclic (9). A loss of this temporal organization during aging may mean increased exposure to predators and/or a diminished efficiency in obtaining nutrients, ultimately threatening survival.

Perspectives

Aging is associated with numerous changes in the expression of both physiological and behavioral 24-h rhythms in mammals. Changes that occur in rodent animal model species within the first 2 yr of life are similar to those observed in humans after many decades. Thus there is a great deal of interest in understanding the genetic and physiological basis for age-related changes in rhythmicity in animal models of aging and in the use of such models for testing the effectiveness of various countermeasures for preventing and/or attenuating the effect of age on the circadian clock system. Although the mouse is the mammalian model of choice for elucidating the genetic basis of behavioral and physiological processes, only a few previous studies have explored how advanced age impacts on the murine circadian clock system.

Recently, we reported the isolation of a single gene mutation in the mouse (Clock) that changes two central properties of circadian rhythms: the intrinsic period and the persistence of rhythmicity in DD (27). The isolation of the Clock mutant mouse opens up a new set of questions that can be addressed with respect to interactions between the circadian system and aging. Preliminary observations suggest that young heterozygous Clock mutant mice display many of the changes in circadian properties seen with aging in wild-type mice (unpublished observations). Thus the clock mutation and the aging process may be acting on the circadian system in a similar manner. The recent cloning (6) of this Clock gene and the characterization of age-related changes in the circadian rhythm of locomotor activity presented in this study open the possibility of eventually using the tools of molecular genetics to unravel the physiological basis of age-related changes in the circadian clock system of mammals.

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