The Effect of Old Age on the Free-Running Period of Circadian Rhythms in Rat

Wil Witting, Majid Mirmiran, Nico P. A. Bos, and Dick F. Swaab

Netherlands Institute for Brain Research, Amsterdam, The Netherlands

Summary: The free-running period is regarded to be an exclusive feature of the endogenous circadian clock. Changes during aging in the free-running period may therefore reflect age-related changes in the internal organization of this clock. However, the literature on alterations in the free-running period in aging is not unequivocal. In the present study, with various confounding factors kept to a minimum, it was found that the free-running periods for active wakefulness, body temperature, and drinking behavior were significantly shorter (by 12–17 min) in old than in young rats. In addition, it was found that the day-to-day stability of the different sleep states was reduced in old rats, whereas that of the drinking rhythm was enhanced. Transient cycles were not observed, nor were there any age-related differences in daily totals of the various sleep-wake states. The amplitudes of the circadian rhythms of active wakefulness, quiet sleep, and temperature were reduced, whereas those of paradoxical sleep and quiet wakefulness remained unchanged. **Key Words:** Free-running period—Circadian rhythm—Circadian clock—Suprachiasmatic nucleus—Aging.

Deterioration of circadian rhythmicity has long been recognized as an inevitable fate affecting all aging animals, including man (1–4). Of all behavioral parameters studied, the free-running period (FRP) is the only one representing an exclusive feature of the endogenous circadian clock. Age-related changes in the FRP may therefore disclose changes in the internal organization of this clock (5–7), which is believed to be the suprachiasmatic nucleus (SCN; 8). However, closer examination of the literature on age effects with respect to the FRP showed that the findings are far from unequivocal. Although some investigators have reported shortening, others have found lengthening or no changes at all in the FRP during aging (Table 1).

When reviewing the literature it becomes apparent that several studies were not designed to investigate aging effects on the FRP in the first place. Consequently, many studies may have been liable to confounding factors such as differences in locomotor activity levels (37–43) or lighting conditions (44). Moreover, in many

Received February 10, 1993; accepted with revisions August 31, 1993.

Address correspondence and reprint requests to Dr. W. Witting, Department of Cardiology, Thorax-center, University Hospital Groningen, P.O. Box 30001, 9700 RB Groningen, The Netherlands.

TABLE 1. An overview of the articles that studied free-running rhythms and are cited with respect to aging

Article	Animal	Measured variable(s)	Free-running period	Age (yr)	Activity	Amplitude	Period analysis
Richter, 1922 (9)	Rat	MA		+ (10)	↓	↓	
Eskin, 1971 (10)	Sparrow	MA	0				EF
	Mouse	RWA	0		↓		EF
Pittendrigh and Daan, 1974 (12)	Hamster	RWA	↓	- (13)	↓		EF
	Deermouse						
	P.m.	RWA	↓	- (14)	↓		EF
	P.I.	RWA	↓	+ (14)	↓		EF
Wax, 1975 (15)	Mouse	RWA	0	+ (16)	↓	↓	EF
Wax and Goodnick, 1975 (17)	Mouse	BP	↑	+ (16)	↓	↓	EF
Pittendrigh and Daan, 1976 (18)	Mouse	RWA	0	+ (16)	↓		EF
Wax, 1977 (19)	Mouse	RWA	↑/0	+ (16)	↓	↓	EF
Wenzel et al., 1977 (20)	Mouse	MA	0	+ (16)	↓		FTD
Kenagy, 1978 (21)	Ground squirrel	RWA	0		0	0	EF
	Kangaroo rat	RWA	0		0	0	EF
	Human	T	0	- (23)			EF
Wever, 1979 (22)	Rat	MA/T	0	- (10)		↓	CF
Mosko et al., 1980 (24)	Cockroach	RWA	0	+ (25)			EF
Page and Block, 1980 (25)	Hamster	RWA	↑	- (13)	↓	↓	EF
Davis and Menaker, 1980 (26)	Hamster	RWA	↑	- (13)	↓	↓	EF
Davis and Menaker, 1981 (27)	Human	T/MA/SW	↓/0	- (23)	↓	↓	PLM
Weitzman et al., 1982 (28)	Human	T	0	- (23)			EF
Wever, 1984 (29)	Mouse	RWA	↑/0	+ (16)	↓	↓	PLM
Welsh et al., 1986 (30)	Rat	SW	↓/0	+ (32)		0	CF
Van Gool et al., 1987 (31)	Hamster	RWA	0	+ (13)			EF
Morin, 1988 (33)	Human	T/SW	0	+ (23)	↓	↓	PLM
Monk and Moline, 1988 (34)	Rat	FI	0	+ (10)	0		EF
Rietveld et al., 1988 (35)	Rat	SW/T/D	↓/0	+ (10)		↓	PLM
Witting et al., 1994 (present study)	Rat			+ (10)	0	↓	PLM

In only a few studies were both the animals old according to the definition of Comfort (36) and a significant shortening of the free-running period was found. Where several age-groups were studied, the reported effects concern the oldest group. Age refers to whether (+) or not (-) the definition for old animals was met; the numbers in the age column are references to the cited literature on average life-span or 50% point of mortality.

↑, lengthening of the free-running period or an increase in amplitude or activity with age; ↓, shortening or reduction; 0, no age-related change; P.m., *P. maniculatus*; P.I., *P. leucopus*; RWA, running wheel activity; T, temperature; MA, movement activity; SW, sleep-wake; FI, food intake; D, drinking; BP, bar pressing; EF, eye-fit with or without regression; CF, cosine-fit; PLM, periodogramlike method; FTD, Fourier transform + decomposition.

articles even the oldest group appeared not to be very old according to Comfort (36), who defined "aged" as the age beyond the 50% point of mortality. Sometimes the reported differences among ages did not concern the oldest groups. Therefore, we felt the need to reexamine the effect of old age on the FRPs for the different states comprising the sleep-wake rhythm, as well as the circadian rhythms of temperature and drinking behavior.

MATERIAL AND METHODS

Animals

Nine young (7–9 months of age) and seven old (31–33 months of age) male Brown Norway rats (TNO, Rijswijk, The Netherlands) were anesthetized with Hypnorm (10 mg fluanison + 2 mg fentanyl/ml; 0.05–0.10 ml/kg) to allow implantation of electroencephalographic (EEG) and electromyographic (EMG) electrodes for chronic sleep-wake recordings. Two stainless steel screw electrodes were placed ~2 mm anterior to λ and bregma, respectively, and about 2 mm left from the sagittal suture for recording of EEG. Two double-twisted nichrome wire-electrodes were placed into the dorsal neck muscles for EMG recording. A thermistor (Siemens KTY 12) measured temperature on the skull. Lick detectors were used to monitor drinking activity. The time between surgery and the present experiment was 2½ months. Just before the free-running experiment, the rats were kept under 12-h light:dark conditions for at least 10 days. After surgery and throughout the experiment, the rats were housed separately in sound-proof plexiglass cages (390 × 272 × 495 mm; temperature 20–22°C) and had free access to food and water. The animals were kept in a free-running condition for 2 weeks, during which time a dim red light (0.1 lux) was on continuously.

Data Acquisition

A computerized on-line sleep-wake classification system determined the behavioral states in four animals simultaneously with a sampling rate of 0.1 Hz (45). In addition, for each animal the system stored one analog and one digital channel, which were used for monitoring temperature and drinking behavior, respectively.

Statistics

The data were pooled per 150 s and filtered using a low-pass infinite impulse response filter with moderately sharp cut-off at a period of 6 h (46). This procedure permitted the subsequent periodogram analysis (47) to be performed with relatively high resolution. During the past decade, periodogram analysis has been the method of choice in many circadian experiments (see Table 1). However, as Enright (48) recently discussed, the method does not cope well with phase jumps or shifts. In this article we avoided this problem by analyzing many short epochs instead of one long epoch. Pittendrigh and Daan (18) stated, when using the eye-fit method, that "the rhythm's precision is detectable in relatively short-term recordings of a week or so . . ." Our experience with the periodogram method combined with digital filtering agrees with this observation. Therefore, the periodogram analysis was moved through the 14 days of free-running data with a 6-day window in steps of 1 day, with a

FIG. 1. FRPs ($\pm$ SEM) of young (light bars) and old rats (dark bars). The FRPs of active wakefulness (AW), drinking behavior (D), and temperature (T) were significantly shorter in old as compared with young rats (** $p < 0.01$; * $p < 0.05$). No changes were found for quiet sleep (QS), paradoxical sleep (PS), or quiet wakefulness (QW). In the latter variable a circadian rhythm was sometimes just detectable (see Fig. 2), which may account for the somewhat deviant values for QW.

resolution of 150 s. The median values of the FRPs thus obtained were used for statistical comparison by means of Student's *t* test (two-tailed). The amplitudes of the rhythms and the total amount of time spent in the various behavioral states were calculated for the same 6-day epochs. Amplitudes were calculated using a procedure that searched for the maximum difference between two halves of the circadian cycle.

Amplitudes and daily totals of the sleep-wake states were corrected for differences in FRP and also compared by means of the *t* test. The presence of transients [i.e., "cycles of rapidly changing duration intervening between two steady states, each characterized by a nearly stable τ " (18)] was determined by comparing the FRPs of the first and last obtained 6-day windows, using the paired *t* test. Age-related changes in the day-to-day stability of the rhythms were assessed by means of analysis of variance, comparing the within-subjects variance over the peak values for all 6-day windows in each age group (49). For all tests, $p < 0.05$ was considered statistically significant.

RESULTS

All sleep-wake recordings were usable except for one of the old rats. Temperature was successfully recorded in five young and seven old rats. Drinking behavior data were obtained from all rats.

Period Length

The FRPs of active wakefulness, temperature, and drinking behavior were 17.4, 12, and 13.8 min shorter, respectively, in the old rats as compared with the young rats (Fig. 1).

Amplitude

Age-related changes in the amplitude were also observed (Fig. 2). For active wakefulness, quiet sleep, and temperature, the amplitude was lower in the old than in the

FIG. 2. Amplitudes ($\pm$ SEM) of the free-running rhythms of young (light bars) and old rats (dark bars). The amplitudes of active wakefulness, quiet sleep, and temperature were significantly lower in old as compared with young rats. No changes were found for paradoxical sleep or quiet wakefulness. For explanation of abbreviations see Fig. 1 legend.

young group. The amplitude of the drinking rhythm was not assessed because of the large variation among the animals, which was probably due to differences in the sensitivity of the lick detectors.

Total Amounts

No differences were observed in the total time spent in any of the behavioral states (Fig. 3).

Transients

None of the variables showed significant differences between the FRPs of the first and last 6-day windows, either for the age groups separately or when combined (all p

FIG. 3. Daily totals ($\pm$ SEM) of the four sleep-wake states of young (light bars) and old rats (dark bars). None of these variables showed a significant age-related change. For explanation of abbreviations see Fig. 1 legend.

values > 0.05 ; data not shown), thus suggesting that transients were absent from our data set.

Day-to-Day Stability

The day-to-day stability of free-running rhythms changed during aging, but not in the same way for all variables. Whereas all sleep-wake states were significantly less stable in the old rats [active wakefulness, $F(42,61) = 3.28$, $p < 0.001$; quiet sleep, $F(42,61) = 37.84$, $p < 0.001$; paradoxical sleep, $F(42,61) = 23.34$, $p < 0.001$; quiet wakefulness, $F(42,61) = 3.34$, $p < 0.001$], the drinking rhythm was significantly more stable in the aged group ($F(61,49) = 5.23$, $p < 0.001$). Stability of circadian temperature rhythm was not significantly affected by age.

DISCUSSION

The main purpose of our study was to examine the effect of old age on the length of the FRP, while minimizing possible confounding factors. Thus, the experiment was performed in constant darkness and no running wheel was present. Furthermore, no differences among the groups were observed in the mean daily amounts of sleep and wakefulness, and the old group of animals was truly old according to the criterion of Comfort (36). Finally, transients could not be detected statistically. In this way, three of six examined FRPs were significantly shorter in the old group. Still, it should be considered that some other putative confounding factors, such as the effects of long-lasting solitary confinement, cannot as yet be evaluated. For example, the weight gain of our rats during the experiments was higher than expected on the basis of the weight curves obtained from the same animals before the experiment, when the rats were still housed in groups. Furthermore, no statement can be made at this time about any qualitative differences, such as the intensity of activity during active wakefulness or the depth of sleep between young and old animals.

Although our results concerning the effect of old age on the FRP are in agreement with those of several other studies, the data appear to conflict with certain other articles on this subject (Table 1). Therefore, possible factors that may have contributed to the discrepancies in the literature, which have been cited with respect to age-related changes in the FRPs, will be discussed.

Age

The finding in many articles that the FRP changes during aging does not imply that these changes were specific for very old animals. In fact, changes in FRP have been reported throughout the whole life cycle. For example, the aging effect observed by Davis and Menaker (26) should be regarded as a result of development, because their reported change occurred during the first 18 weeks of life. In one longitudinal study (18), the greatest shortening of the FRP was found in young rats, suggesting changes during puberty or adulthood rather than in old age. When "aged" is defined as the age of animals beyond the 50% point of mortality (16,36), almost half of all published studies fail to fulfill this criterion (Table 1). In one study (33) various groups of

animals were classified as old, whereas in fact only the oldest group met the accepted criterion.

Light

A broad range of light intensities has been used during the various experiments, ranging from constant light and/or darkness to self-selected light-dark schedules. Because light is one of the main modulators of circadian rhythms, it is conceivable that some of the reported changes in FRP were not primarily due to endogenous changes in the pacemaker itself, but rather to an age-dependent response to ambient light intensities. The effect of the intensity of constant light on circadian rhythms is known as Aschoff's rule (44): in nocturnal animals the FRP of the rhythm increases with increasing light intensity. Although Wax (17,19) did not use constant light but self-selected light-dark schedules, this resulted in a longer exposure to light in the old groups in comparison with the young ones. Therefore, this increased exposure could have led to longer FRPs in the older groups. As was suggested by Morin (33), other studies (12,15,27) also may have been susceptible to "unusual lighting conditions" that "prevailed during or prior to the measurement of the circadian period."

Activity Level

Similar to the effect of light, experimental conditions that allow animals to express more movement activity render a shorter FRP (37,39,41-43). Several investigators have noticed distinct differences between young and old animals in the influence of environmental stimuli on the amount of movement/activity. Richter (9) reported that young rats expressed increased activity when placed in a running wheel, whereas old rats did not. This is compatible with the finding of Pittendrigh and Daan (12). These investigators observed that both period length and α (i.e., active period, as determined by running wheel) decreased with age instead of changing in opposite direction, which led them to suggest the existence of two distinct processes. Finally, Welsh (30) also remarked that "some of the differences [in FRP] between young and old animals might be explained by a tendency of intense locomotor activity to prolong episodes of wakefulness." Because the presence of a running wheel may have induced differences in FRP between young and old animals, it is important to note that running wheels were not used in our present experiment, nor were any differences observed in the total time spent in the four sleep-wake states (Fig. 3).

Transients

Transients are defined as "cycles of rapidly changing duration intervening between two steady-states, each characterized by a nearly stable τ . The two steady-states are typically separated by no more than 10 transients—sometimes by only one" (18). Some authors use the term "after-effects" instead. True after-effects, defined as "long-lasting, slowly decaying, changes in pacemaker period incurred by prior experience" (18) will not be considered in this article. Clear transients are usually observed only during large changes in circadian period length. Van Gool et al. (31) suggested that their results might have been biased by such transients, because the FRP was

assessed shortly after transition from the 12-h light:dark cycle into constant darkness. However, after entrainment to a 24-h LD cycle, only one study (12) found such an effect, whereas three (15,21), including the present study, did not. Finally, age-related differences in transients have never been reported.

Other Parameters

The deterioration of circadian rhythms is not only expressed by changes in the FRP. In fact, the extent of the changes in other parameters may be several times greater than those observed in the FRP. Day-to-day stability in the circadian rhythmicity of sleep-wake states was, for instance, greatly decreased in old animals. Comparable age-related findings have been reported on the rhythms of running wheel (33), temperature (29), and food intake (29). The increased day-to-day stability of the FRPs of drinking behavior in old rats may be related to the fact that the mean daily water consumption of the old group was considerably higher than that of the young group (32.8 ± 8.2 vs. 22.3 ± 2.7 ml/day, two-sided *t* test, $p < 0.05$), a phenomenon that has been ascribed to an impaired ability to concentrate urine and a diminished tubular responsiveness to vasopressin in kidney of aged rats (50,51). The amplitude changes are also consistent with most of the data reported in the literature (Table 1). The suggestion made in a previous study (31) that the age-related amplitude reduction found under a 24-h light/dark cycle might primarily reflect difficulties in entrainment to a 24-h cycle is not supported by our present data, where, under free-running conditions, three of five measured amplitudes were clearly reduced.

The Circadian Clock

Age-related functional changes may reflect changes in the internal organization of the endogenous clock, in which the SCN plays a major role. Indeed, morphological changes in this structure have been reported with age, both in humans and in rats (5-7). It is worth mentioning in this respect that in patients with Alzheimer's disease, both the SCN (7) and the circadian rest-activity rhythms (52) are severely affected. Experimentally induced partial lesions of the SCN in rodents also result in both a shortening of the FRPs as well as in changes in other parameters such as reduction of amplitudes. However, although partial lesioning may thus serve as a model for functional deterioration during aging, the extent of the experimental lesions has to be large (53) as compared with the morphological destruction found during aging. Therefore, even after 20 years, Eskin's remark (11) that "it is often not clear what measurements of FRP, widely reported in literature, mean in terms of the state of the underlying circadian system" still holds.

Aging has often been reported to have a perturbing influence on circadian rhythmicity. This article emphasizes that the question of the FRP changes in old animals is in fact a highly complex one, because of the large number of confounding variables. Articles concerning circadian rhythms in old age frequently cite results based on findings in young or adult laboratory animals, or data that are likely to have been biased in one or more ways. Nevertheless, the present data show that even in a situation where confounding factors are kept to a minimum, a small but significant effect of old age on the period length of free-running rhythms can be detected.

Acknowledgment: We thank M.A. Corner, R. Nooy, J. Overdijk, H. Stoffels, and D. van der Werf for their valuable contributions. Old animals were funded by the 'NWO-bank voor oude proefdieren.' This study was supported by STIPT, project 'Ritmemeter,' MTR 89026.

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