The Effects of Spaceflight on the Rat Circadian Timing System

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ABSTRACT

Two fundamental environmental influences that have shaped the evolution of life on Earth are gravity and the cyclic changes occurring over the 24-hour day. Light levels, temperature, and humidity fluctuate over the course of a day, and organisms have adapted to cope with these variations. The primary adaptation has been the evolution of a biological timing system. Previous studies have suggested that this system, named the circadian (*circa* ~ about; *dies* ~ a day) timing system (CTS), may be sensitive to changes in gravity. The NASA Neurolab spaceflight provided a unique opportunity to evaluate the effects of microgravity on the mammalian CTS. Our experiment tested the hypotheses that microgravity would affect the period, phasing, and light sensitivity of the CTS. Twenty-four Fisher 344 rats were exposed to 16 days of microgravity on the Neurolab STS-90 mission, and 24 Fisher 344 rats were also studied on Earth as one-G controls. Rats were equipped with biotelemetry transmitters to record body temperature (T_b) and heart rate (HR) continuously while the rats moved freely. In each group, 18 rats were exposed to a 24-hour light-dark (LD 12:12) cycle, and six rats were exposed to constant dim red-light (LL). The ability of light to induce a neuronal activity marker (*c-fos*) in the circadian pacemaker of the brain, the suprachiasmatic nucleus (SCN), was examined in rats studied on flight days two (FD2) and 14 (FD14), and postflight days two (R+1) and 14 (R+13). The flight rats in LD remained synchronized with the LD cycle. However, their T_b rhythm was markedly phase-delayed relative to the LD cycle. The LD flight rats also had a decreased T_b and a change in the waveform of the T_b rhythm compared to controls. Rats in LL exhibited free-running rhythms of T_b and HR; however, the periods were longer in microgravity. Circadian period returned to preflight values after landing. The internal phase angle between rhythms was different in flight than in one-G. Compared with control rats, the flight rats exhibited no change in HR. Finally, the LD FD2 flight rats demonstrated a reduced sensitivity to light as shown by significantly reduced *c-fos* expression in the SCN in comparison with controls. These findings constitute the first demonstration that microgravity affects the fundamental properties of the mammalian circadian timing system, specifically by influencing the clock's period, and its ability to maintain temporal organization and phase angle of synchronization to an external LD cycle.

INTRODUCTION

Two of the most fundamental environmental influences that have shaped the evolution of organisms on Earth are gravity and the cyclic changes occurring over the 24-hour day. The rotation of the Earth relative to the Sun creates the fluctuation in light and dark, temperature, and humidity over the course of a day. Organisms have adapted in many ways to cope with these cyclic variations in the environment. The primary adaptation has been the evolution of a biological timing system, which anticipates these daily fluctuations. This system has been named the circadian (*circa* ~ about; *dies* ~ a day) timing system (CTS). For example, although daily rhythms in sleep/wake, body temperature, hormonal activity, and performance have been measured in many organisms, they are not simply passive responses to changes in the environment, but rather are generated by a neural pacemaker, the circadian clock, located in the brain (Moore-Ede, 1982). In mammals, this neural pacemaker is called the suprachiasmatic nucleus (SCN). The SCN contains a genetic clock that affects our physiology to create the circadian rhythms of the body. In addition to possessing an endogenous clock, the SCN detects cues from the environment so that internal rhythms can keep time with the external day—a process called entrainment. The primary environmental time cue or zeitgeber (*zeit* ~ time; *geber* ~ giver) detected by mammals is light. All of the circadian rhythms of the body must be coordinated to peak or fall at certain times of the day. Therefore, there is a specific time when body temperature is high to meet the activities of the day, when the stomach is active to anticipate the arrival of food, or when the hormone melatonin is secreted to affect sleep. All of these physiological circadian rhythms must peak at their own specific time for the body and mind to operate efficiently. When these various circadian rhythms are no longer coordinated in time, body temperature may peak at bedtime or food may arrive when the stomach isn't ready. As a consequence, the mind and body do not operate well and difficulties arise in coping with stress. People experience this with jetlag, shift work, and as new parents. It takes time for circadian rhythms to readjust and regain temporal coordination. Until then, individuals will not be at their best. Controlled animal studies in our laboratory have shown the difficulty in coping with a stressor when circadian rhythms are not coordinated (Fuller, 1978).

The gravity of the Earth (one-G) is a constant environmental force that has shaped the anatomy and physiology of virtually every organism. Animal studies have shown that exposure to high gravity levels (hypergravity) reduces body mass, decreases food and water intake, increases metabolism, and decreases activity, body temperature, and performance. A very interesting finding was that animals exposed to twice normal gravity (two-G) exhibited a dramatic change in the amplitude and phase of the circadian rhythms of heart rate, body temperature, and activity, suggesting a disruption in rhythm coordination (Fuller, 1994). We were also interested in whether exposure to two-G might affect the function of the clock responsible for generating circadian rhythms, the SCN. We tested this question using two groups of rats. One group was exposed to two-G for two days, while a control group remained at one-G. At the appropriate time, all of the rats were exposed to light for two hours. Immediately after the light exposure, we used the biological marker *c-fos* to see if the light activated neurons in the SCN. Control rats that remained at one-G had a high number of *c-fos*-activated neurons within the SCN. However, rats at two-G did not have a significant number of *c-fos*-activated neurons in the SCN (Murakami, 1998). This study demonstrated that exposure to two-G could affect the normal function of the pacemaker generating circadian rhythms. Such a disruption in circadian rhythms and SCN function could help to explain the underlying causes of space adaptation syndrome, sleep disturbance, and reduced performance experienced by astronauts in space.

Often, to study the mammalian biological clock, a circadian scientist will house the subjects (animals or humans) in an environment with no time cues. This is referred to as "constant conditions" and can occur either in constant darkness or in constant light. Under constant conditions, the clock is no longer synchronized by environmental time cues, and thus is free to express its own internal period. The period of the clock differs slightly from individual to individual, but it is extremely stable within an individual. By using constant conditions in the Neurolab experiment, we were able to look at the internal or "free-running" period of the clock on the ground and in space. Any changes in the period of the clock during spaceflight could then be interpreted as an effect of gravity on clock function.

The Neurolab mission (STS-90) gave us the unique opportunity to study the effects of the microgravity of space on rat body temperature and heart rate and their respective circadian rhythms. For the first time, we were able to study the effect of microgravity on mammalian circadian rhythms in constant lighting conditions where there were no specific time cues. This allowed us to test the hypothesis that microgravity affects the circadian timing system, including the amplitude and period (i.e., timing) of the circadian pacemaker. In addition, we were able to test the hypothesis that microgravity affects the normal response of the SCN to light by measuring *c-fos*, a marker for neuronal activity. Revealing the physiological, circadian, and neural pacemaker responses to spaceflight may help us understand the process of adaptation to microgravity.

METHODS

Subjects and Housing – Forty-eight adult male Specific Pathogen-Free Fisher 344 rats, weighing 350–370 grams, were used in this experiment. Care of the rats met all National Institutes of Health standards outlined in the *Guide for the Care and Use of Laboratory Animals*, and was approved by both the University of California (UC) Davis and NASA Institutional Animal Care and Use Committees. During preflight and postflight recording periods, the rats were housed individually in standard vivarium cages. During the flight recording period, the flight and ground control groups were housed individually in the NASA Research Animal Holding Facility cages. Food and water were available ad libitum during the entire experimental protocol.

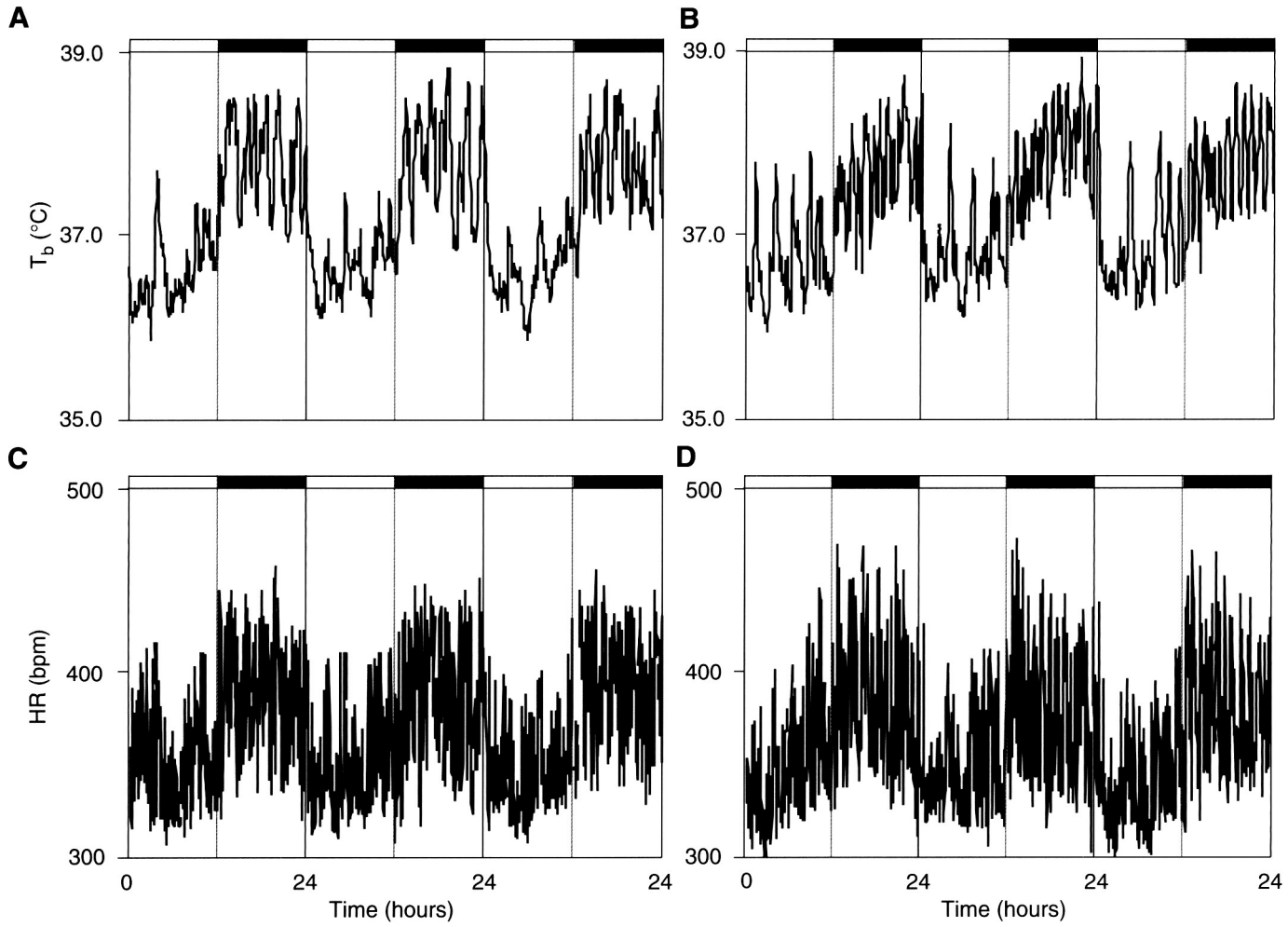

Figure 1. Graphs of three days of body temperature (T_b; °C) and heart rate (HR; beats/minute) data are plotted for a CTL rat (A & C) and an FLT rat (B & D). These data are from rats in a light-dark (LD) cycle during the flight portion of the experiment. The timing of the LD cycles is indicated by the light and dark bars at the top of each graph. Distinct circadian rhythms of T_b and HR are evident in both rats. As is expected in a nocturnal animal, both T_b and HR are higher during the dark, the rat's active period.

Experimental Protocol – Each rat had a biotelemetry unit placed in the abdomen for chronic recording of body temperature (T_b) and heart rate (HR). The rats were divided into two groups: a flight (FLT) group (n=24) and a ground control (CTL) group (n=24). Telemetry data were collected during a two-week preflight and a 16-day flight period. The LL CTL and FLT animals were also measured during a two-week postflight period. In both the FLT and CTL groups, 18 of the rats were housed in a 24-hour light-dark cycle (LD 12:12; 100:0 lux) and six were housed in constant dim red-light (LL; 30 lux). The constant dim red-light permitted monitoring of the rats by the crew during the flight. Brain tissue was collected on four different days during flight: on flight day two (FD2) and flight day 14 (FD14) and postflight on post-recovery day two (R+1) and post-recovery day 14 (R+13). Identical procedures and timelines were used for the ground CTL group. Prior to tissue collection, one-half of the rats in each group were exposed to a light pulse. A light pulse of 100 lux was delivered for 60 minutes at a time of day when light is known to elicit robust *c-fos* expression in the SCN in one-G. All brain tissue was fixed in 4% paraformaldehyde. Sections of the SCN were then made and stained for *c-fos* expression using methods described below.

Biotelemetry – A custom telemetry receiver system (Neurolab Biotelemetry System) was developed by NASA Ames Research Center (Sensors 2000 Program) using commercially available components (Konigsberg Instruments, Inc., Pasadena, CA). This system allowed for heart rate and body temperature to be transmitted via radio waves to a recording system while the rats moved about freely (see technical report by Buckey and Fuller in this publication).

Under anesthesia, a sterilized biotelemetry transmitter was inserted into the abdomen. Electrocardiogram leads and an antenna were positioned under the skin. The rats recovered for two weeks before recording began.

c-fos Immunohistochemistry – The brains were fixed in 4% paraformaldehyde fixative and placed in 30% sucrose solution. Brains from each group were then frozen and sectioned

coronally on a microtome in 50-μm sections. Sections were washed, treated with antibody and dye to reveal the presence of *c-fos*, and mounted on slides for examination under the microscope according to the procedure of Murakami et al., 1998. The results were quantified by counting the number of *c-fos* immunopositive cells in each SCN section.

Circadian and Statistical Analyses – Phase, mean, and amplitude of the T_b and HR rhythms were determined using a least-squares sine/cosine fit. The average daily mean, rhythm amplitude (calculated as the mean to maximum of the best-fit Fourier function) and acrophase (calculated as the time of the maximum of the best-fit Fourier function) of T_b and HR were calculated for each group. The difference, in hours, between the acrophase of T_b and the onset of the light (Lon=hour 0) was used to compare the relative timing of the rhythms in an LD cycle. The relative timing of the acrophases of the two rhythms was used to examine the relationship between the T_b and HR rhythms in LL. Period (τ) was calculated by both Enright's Periodogram method and a linear/nonlinear least-squares cosine fit spectral analysis. Repeated-measures analysis of variance was used to compare preflight (one-G), flight (μG), and postflight (one-G) data. Specific mean comparisons were made using Tukey's HSD post-hoc test. An α of <0.05 was considered statistically significant. All data are summarized as mean±standard error.

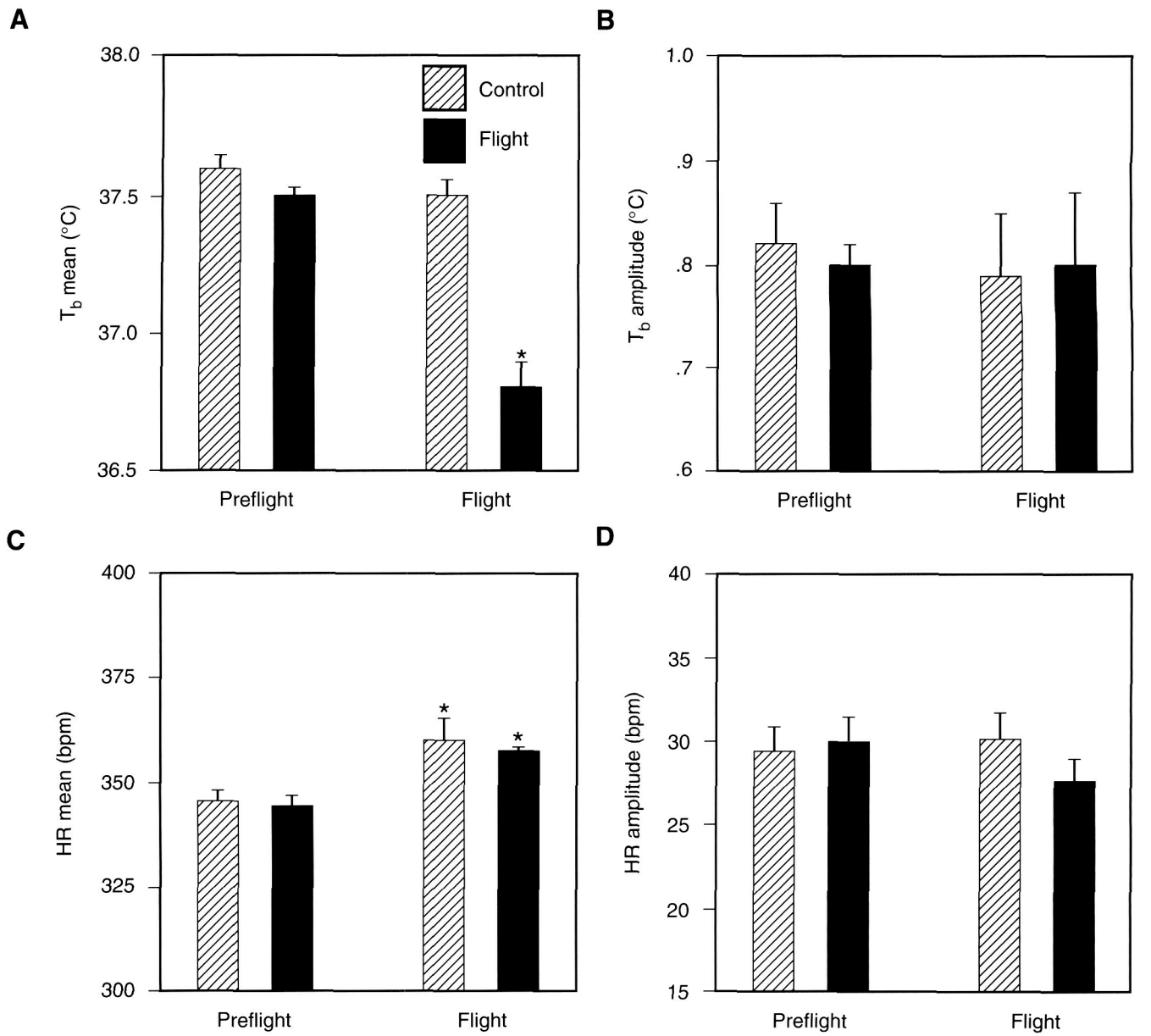

Figure 2. Daily mean (A) body temperature (T_b; °C) and (C) heart rate (HR; beats/minute) are presented as histograms along with average amplitude of the respective rhythms (B & D). These histograms are averages (+ SEM) from all LD CTL and FLT rats for the preflight and flight periods. In the FLT rats, the mean daily T_b was significantly lower during flight than preflight. Mean HR was significantly increased for both CTL and FLT rats during flight compared to preflight. There were no other significant differences between FLT and CTL or between experiment segments. (* = $p < 0.05$)

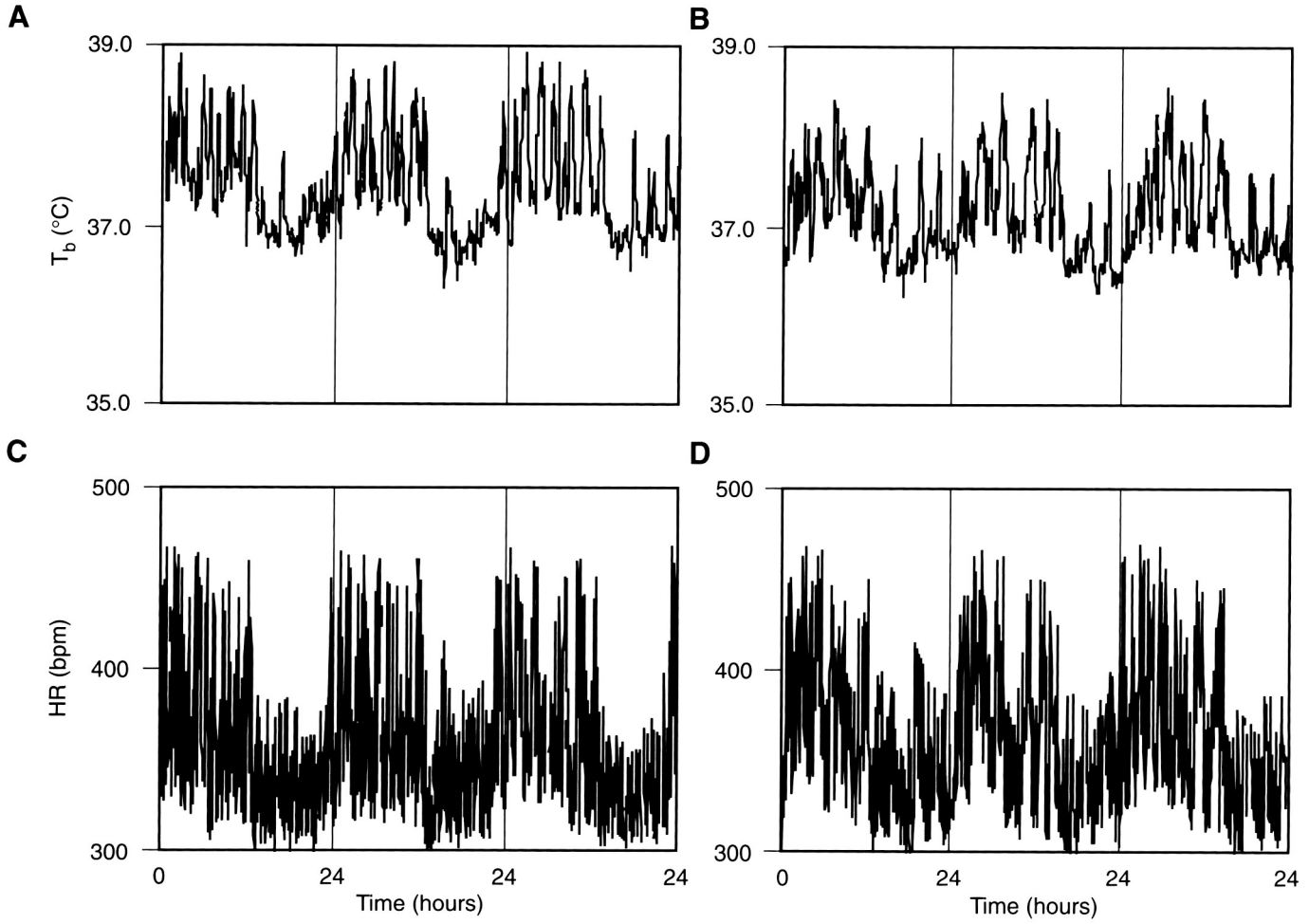

Figure 3. Graphs of three days of body temperature (T_b; °C) and heart rate (HR; beats/minute) are plotted for a CTL rat (A & C) and an FLT rat (B & D). These data are from rats in constant dim red-light (LL) during the flight portion of the experiment. Persistent circadian rhythms are evident in both variables in each rat.

RESULTS

LD Body Temperature (T_b) – Examples of T_b circadian rhythms recorded in LD during the flight period are shown in Figure 1. Data from a CTL rat are shown in Figure 1A and from a FLT rat in Figure 1B. Body temperature is higher during the dark, as expected in a nocturnal animal. Average mean T_b and circadian amplitude are presented as histograms in Figure 2. Mean daily T_b for the LD rats significantly decreased from preflight to flight (Figure 2A). CTL and FLT groups did not differ preflight. No changes were seen between mean daily T_b in CTL from preflight to flight. The mean daily circadian T_b amplitude (Figure 2B) did not differ significantly between preflight and flight or between FLT and CTL groups.

LD Heart Rate (HR) – HR data recorded from the same rats at the same time as the T_b data shown in Figures 1A and 1B are presented in Figures 1C (CTL) and 1D (FLT). HR is highest when the rats are awake and active, during the dark. This corresponds to the time of the highest T_b. The average mean and circadian amplitude are presented in Figures 2C and 2D. Mean daily HR of both CTL and FLT rats was significantly higher during flight. Since the response was similar in both groups, it is likely due to the changes in the cage environment between preflight and flight. The mean daily amplitude of the HR circadian rhythm (Figure 2D) was not significantly different between preflight and flight or between the FLT and CTL groups.

LL Body Temperature (T_b) – Circadian rhythms of T_b and HR recorded under LL are presented in Figure 3. Data from a CTL rat are shown in Figure 3A and from a FLT rat in Figure 3B. Clear, persistent T_b circadian rhythms are evident in LL. The daily mean and circadian amplitude are graphed in Figure 4. As in the LD FLT rats, mean T_b was lower during flight; however, mean T_b did not differ significantly among preflight, flight, and postflight periods (Figure 4A). In contrast to the LD FLT rats, in LL the average T_b rhythm amplitude was significantly higher in microgravity compared to pre- and postflight (Figure 4B). In CTL rats, the mean circadian T_b rhythm did not change over time. Mean amplitude of the circadian T_b rhythm also did not differ between FLT and CTL in preflight or postflight measurements (Figure 4B).

LL Heart Rate (HR) – As with T_b, HR circadian rhythms persisted in LL in both CTL (Figure 3C) and FLT (Figure 3D) rats. As seen in LD, mean daily HR in both FLT and CTL groups exhibited a significant increase from preflight to flight

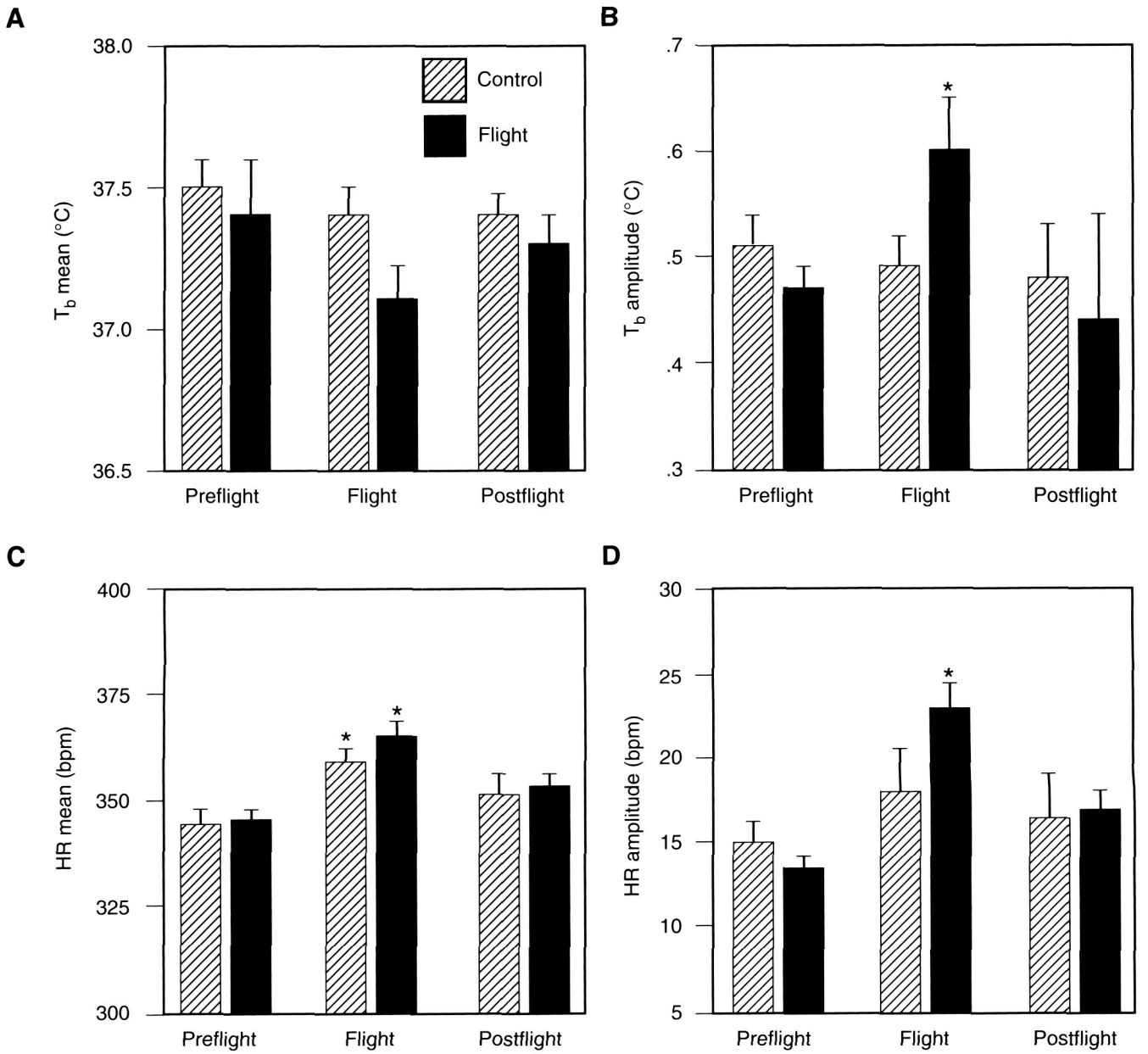

Figure 4. Daily mean (A) body temperature (T_b; °C) and (C) heart rate (HR; beats/minute) are presented as histograms along with the amplitudes of the respective rhythms (B & D). These data represent averages (+ SEM) from all LL CTL and FLT rats for the preflight, flight, and postflight periods. As was seen in the LD FLT group, there was a reduction in the average daily mean Tb during flight in the LL FLT animals, but it did not achieve significance. There was a significant increase in the amplitude of the T_b rhythm in the LL FLT rats during flight compared to pre- and postflight. As was seen in the LD rats, the LL CTL and FLT rats demonstrated a significant increase in mean HR during flight compared to pre- and postflight. There were no other significant differences between FLT and CTL groups or between experiment segments. (*=$p<0.05$)

(Figure 4C). The mean amplitude of the HR rhythm also increased significantly preflight to flight in the FLT rats (Figure 4D). The mean amplitude of the HR rhythm did not differ significantly between preflight and postflight. Daily mean HR and average amplitude of FLT rats were not significantly different from CTL during pre- and postflight (Figure 4D).

LD T_b-HR Phase – There was a significant phase delay in the timing of the T_b rhythm during flight in FLT rats compared to CTL rats during the flight period (Figure 5A) and compared to preflight. The HR rhythm of the FLT group appeared to have a less-stable phase relationship with the LD cycle as compared to the CTL group; however, the difference was not significant.

LL T_b-HR Phase – In LL, the relative timing of the T_b and HR rhythms was altered during spaceflight compared to preflight and postflight as well as to CTL measurements (Figure 5B). As was seen in the LD FLT rats, there was a significant delay in the relative timing of the T_b rhythm, resulting in a

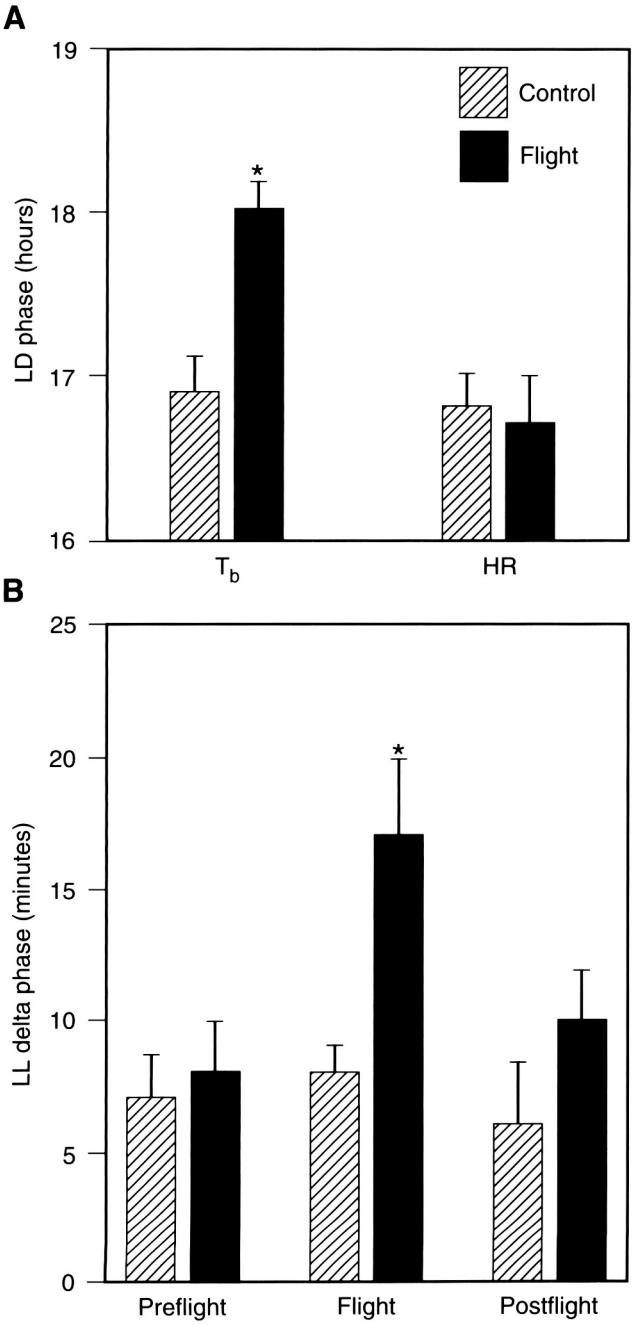

Figure 5. The relative timing of the body temperature (T_b) and heart rate (HR) rhythms during flight are presented as histograms. (A) shows the average (+SEM) time of acrophase (hours after light onset) of the T_b and HR rhythms for the LD CTL and LD FLT groups. The T_b rhythm was significantly delayed in the FLT group compared to the CTL group. However, there was no difference in the timing of the HR rhythm. Data from the LL CTL and LL FLT groups for the preflight, flight, and postflight segments are presented in (B). Since there was no LD cycle to use as a reference point, data are expressed as the average difference (+SEM) in time in minutes between the acrophase of the two rhythms. During flight, there was a significant delay in the relative timing of the T_b rhythm with respect to the HR rhythm in the FLT group compared to pre- and postflight and compared to the CTL group. (*=$p<0.05$)

longer time lag between the acrophases of the two rhythms. Preflight the relative timing of the two rhythms in the FLT and CTL LL groups was similar to that of the preflight FLT and CTL LD groups.

LL Circadian Period – Recording data under LL allowed us to calculate the period of the T_b and HR rhythms. The FLT LL group had a significant increase in the period (τ) of both the T_b (Figure 6A) and the HR (Figure 6B) rhythms in microgravity as compared to preflight and postflight measurements. In contrast, the LL CTL group did not show any significant changes in τ over time. Preflight and postflight τ was similar for both FLT and CTL groups.

LD SCN c-fos – To test the effects of microgravity on the sensitivity of the circadian pacemaker to light, we used a one-hour light pulse to stimulate production of *c-fos* (a marker for neuronal activity) expression in SCN neurons. We compared the number of *c-fos*-labeled neurons in the SCN of rats exposed to light with rats that were not exposed to a light pulse. FLT and CTL animals were exposed to a one-hour light pulse on FD2, FD14, R+1, and R+13. Since the production of *c-fos* in response to light differs over the day, all light pulses were given at the same time beginning at two hours after lights-out. Figure 7 shows a photomicrograph of the SCN of two CTL LD animals. The rat labeled Dark (Figure 7A) did not receive a light pulse, while the rat labeled Light (Figure 7B) did. The *c-fos* produced in response to the light pulse is visible in the darkly stained cells (Figure 7B; arrows). No such staining is visible in the Dark rat. This normal pattern of *c-fos* expression in light-pulsed rats was seen in all CTL rats. However, when compared with the CTL rats exposed to light, the FD2 FLT rats exposed to light had significantly lower levels of *c-fos* expression. A similar depression of *c-fos* expression, although not significant, was also observed in the R+1 FLT rats exposed to light. The FD14, R+1, and R+13 FLT rats exposed to light demonstrated a pattern of response that was closer to the response of the CTL rats. Although fewer *c-fos* immunopositive cells appeared in the FLT group compared to the CTL group, the difference was never significant.

DISCUSSION

This study demonstrated that rats exposed to spaceflight exhibit significant changes in circadian timing. The flight rats maintained in a normal LD cycle had T_b rhythms that were consistently delayed throughout the flight. In contrast, the HR rhythm maintained a relatively normal phase relative to the LD cycle during flight. As a consequence, the T_b and HR rhythms had an altered and less stable phase relationship during flight. This response has also been documented in monkeys (Fuller, 1996) and humans (Gundel, 1993). This loss of internal coordination of circadian rhythms is consistent with the condition of jetlag in humans. The present results in rats suggest that astronauts living in the space environment for extended periods of time, such as on the space station, could experience desynchronized circadian rhythms. Therefore, the

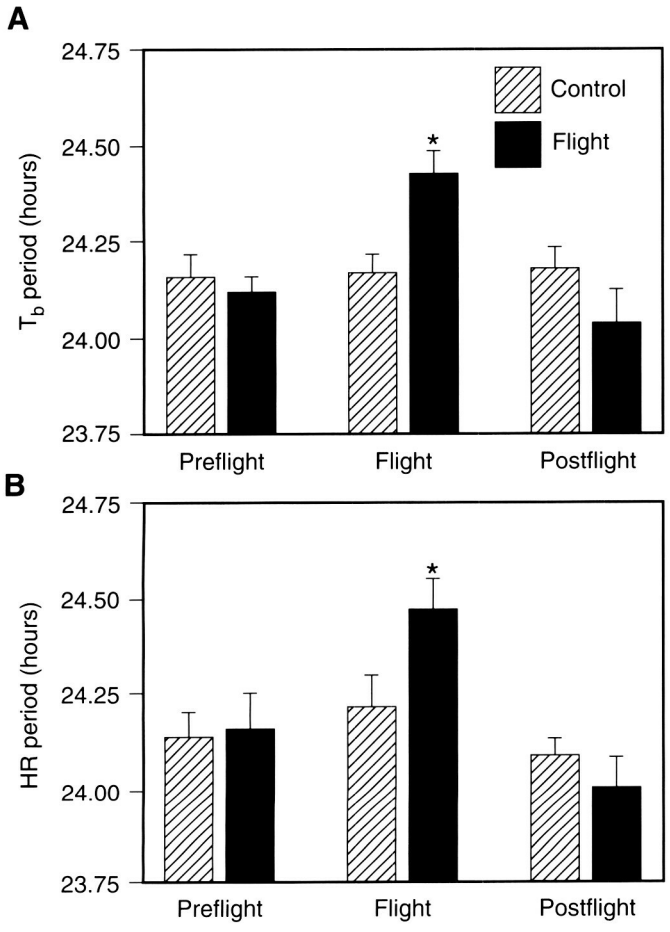

Figure 6. Average period (hours + SEM) of the (A) body temperature (T_b) and (B) heart rate (HR) rhythms of the FLT LL and CTL LL rats are presented as histograms for the preflight, flight, and postflight segments of the experiment. There was a significant increase in the period of both rhythms in the FLT group during flight compared to the preflight and postflight as well as compared to the CTL group. (*=p<0.05)

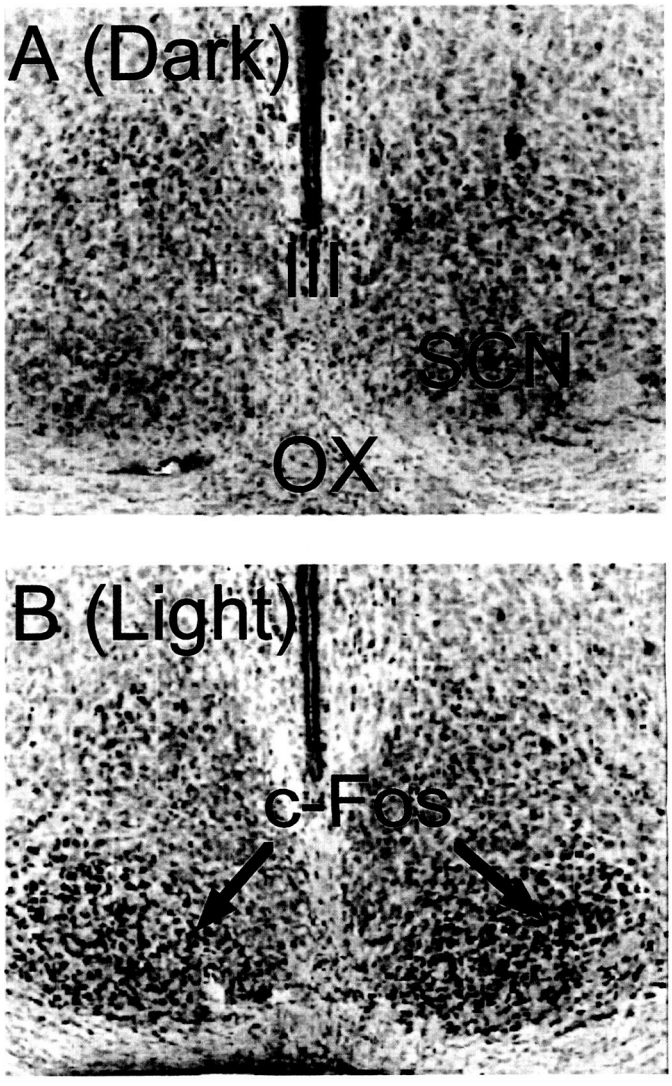

Figure 7. Samples of SCN from two CTL LD rats treated to reveal the presence of *c-fos*. A light pulse was presented to the rat in (B) at a time of day it would be expected to elicit *c-fos* expression within the SCN. The rat in (A) did not receive a light pulse, and thus acted as a control. As expected, without a light pulse (A), there was no expression of *c-fos*. In contrast, the *c-fos* expressed in response to the light pulse (B) is evident as the darkly stained cells (Arrows). (III = third ventricle; OX = optic chiasm)

astronauts may feel as if they are jet-lagged and have performance deficits even though they are maintained in a normal LD cycle.

This study also demonstrated that there was a significant decrease in the induction of *c-fos* in SCN neurons by a light pulse during the early part of the flight. This could be caused by a decrease in the sensitivity of either the eye or the SCN to light, or to a shift in the phase of the pacemaker such that light exposure occurred during a less-sensitive period. It will be important to understand which of these alternatives occurs during spaceflight since each can have a direct effect on how well light entrains circadian rhythms of humans in microgravity conditions. When humans do not receive adequate light levels to entrain circadian rhythms, T_b rhythms can become out of phase with the other rhythms. Therefore, the evidence for reduced responsiveness of the SCN to light in the FLT rats provided by lower *c-fos* induction could explain the gradual change in phase of the T_b rhythm relative to both HR rhythms and the LD cycle. In humans, a syndrome that has been linked to possible circadian dysfunction is seasonal affective disorder or winter depression. An effective clinical treatment for seasonal affective disorder has been to expose patients to light of sufficient intensity. This light treatment not only causes remission of depression, but it also shifts the patient's circadian rhythms.

It will be important to understand if there is also a critical threshold of the light level required on the space station to minimize the risk of circadian dysfunction.

The flight rats maintained in LL exhibited a significant increase in the period of their circadian rhythms. It is highly likely that the period of the pacemaker (SCN) was affected by spaceflight since both T_b and HR circadian rhythms exhibited an identical change in period. In addition, the timing of the T_b rhythm became delayed in flight compared to the HR rhythm. Both of these responses (i.e., period and phase differences) returned to preflight values after the flight. It is not clear how spaceflight can induce such changes in the circadian pacemaker. Changes in the intensity of ambient light levels during LL can significantly alter the period of circadian rhythms (Moore-Ede, 1982). The reduced *c-fos* response in SCN neurons following a light pulse might suggest that a change in the light input to the pacemaker was responsible for the change in period. However, reduced photosensitivity of the pacemaker, analogous to a decrease in ambient light intensity, would be expected to shorten the period of the rhythms as shown in previous observations of intensity-period relationships.

The vestibular system's response to changes in gravity has been shown to cause a number of autonomic effects. It is possible that the vestibular system affects the SCN either directly or indirectly through autonomic nuclei. New animal models (knockouts, transgenic) have recently been developed to explore physiological changes related to vestibular system function. Our recent studies, using several new genetic mouse models, suggest that the vestibular system may mediate the effect of gravitational changes on circadian rhythms (Murakami, 1998; Fuller, 2000). Further studies employing these new genetic mouse models will increase our understanding of the neural mechanisms mediating the physiological responses and process of adaptation to space.

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