Siberian hamsters that fail to reentrain to the photocycle have suppressed melatonin levels

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Ruby, Norman F., Margarita L. Dubocovich, and H. Craig Heller. Siberian hamsters that fail to reentrain to the photocycle have suppressed melatonin levels. Am. J. Physiol. Regulatory Integrative Comp. Physiol. 278: R757–R762, 2000.—Siberian hamsters readily reentrain to a 3-h phase delay of the photocycle (16 h light/day) but fail to reentrain to a 5-h phase delay. This study tested whether melatonin production was suppressed in animals that failed to reentrain. Melatonin was measured on the day before, day of, or several days after each phase shift. Melatonin levels measured 4 h after dark onset were ~83 µg/ml on the day before each phase delay and undetectable (<6 µg/ml) during the light phase on the day of the phase shift. Activity onsets regained their prior phase relationship to the photocycle 4 (3 h) or 5 (5 h) days after the phase shift; on that day, melatonin levels were measured 4 h after dark onset. Melatonin levels were unaffected by the 3-h phase delay (>57.6 µg/ml) but were undetectable after a 5-h phase delay (<8 µg/ml). Thus melatonin remained suppressed only after the phase delay to which hamsters also fail to reentrain. This relationship suggests that the propensity for reentrainment may be influenced by changes in melatonin production following a phase shift of the photocycle.

THE CIRCADIAN SYSTEM of Siberian hamsters (Phodopus sungorus) housed in constant conditions is similar to that of other nocturnal rodents. Their activity rhythms free run in constant darkness with periods close to 24 h and with periods of ~26 h when they are exposed to constant light; light pulses produce phase advances in early subjective night and phase delays in late subjective night (11, 16). In contrast, their response to phase shifts of the light-dark (LD) cycle differ from other rodents in that they fail to reentrain to 5-h phase delays or advances of the photocycle (16 h light/day) even though they readily reentrain to phase shifts of 1 or 3 h (18, 20). Over 80% of hamsters that fail to reentrain free run through the LD cycle for several months with circadian periods generally between 25.0 and 25.5 h that are rarely modulated by the LD cycle. A small minority of animals become arrhythmic within a few days after a 5-h phase shift (20). Because these hamsters are easily desynchronized from the photocycle or are made arrhythmic, they are useful for investigations of physiological mechanisms of photic entrainment.

Melatonin may be involved in these reentrainment phenomena. Synthesis of the pineal gland hormone melatonin is highly photosensitive and inhibited by dim light (13). In mammals, the production of melatonin is stimulated by sympathetic innervation of the pineal gland that is primarily controlled by the hypothalamic SCN that is innervated by retinal ganglion cells (8, 12). Either nighttime light pulses or a phase shift of the LD cycle can, therefore, potentially both phase shift the circadian pacemaker in the SCN and suppress melatonin synthesis. The magnitude of the suppression and the time required for melatonin to recover varies among rodent species. For example, melatonin levels in rats can return to normal 2 h after a light pulse, whereas they remain suppressed for several hours in Siberian hamsters (4, 7, 9). Furthermore, plasma melatonin remains suppressed in these hamsters on the night after the light pulse (9). In rats, the recovery time for melatonin secretion is also related to phase-shift magnitude. The rate-limiting enzyme for melatonin synthesis, N-acetyltransferase, remains completely suppressed for 3–4 days after an 8-h phase advance, but returns on the first night after a 5-h phase advance in rats (6). If decreases in melatonin production diminish the likelihood of reentrainment in Siberian hamsters, this effect should be reversed by the administration of exogenous melatonin. We previously found that the number of hamsters that reentrained to a 5-h phase delay of the photocycle was substantially increased when they were injected daily with melatonin (19). On termination of the treatment, the animals that otherwise would have free run reentrained to the photocycle.

The effects of exogenous melatonin on reentrainment and the possibility that a phase shift of the photocycle may have produced long-term suppression of melatonin prompted an investigation of the effects of a 5-h phase delay of the photocycle on endogenous melatonin levels; therefore, we compared endogenous melatonin levels in hamsters that reentrained to those that failed to reentrain to a phase shift of the photocycle. In previous studies, we found that only 9% of the animals reentrained to a 5-h phase delay, but that 100% of the hamsters reentrained to a 3-h phase delay of the photocycle (18). The differential effects of 3- and 5-h phase delays may be used to evaluate the relationship...
between endogenous melatonin levels and reentrainment. We hypothesized that plasma melatonin levels would return to normal levels after a 3-h, but not after a 5-h, phase delay of the photocycle.

METHODS

Housing conditions. Siberian hamsters (Phodopus sungorus) from the breeding colony were maintained from birth three to four per cage in a 16:8-h LD cycle (lights on at 0200 PST) and an ambient temperature (T_a) of 22°C. Housing conditions and illumination in the hamster colony and experiment rooms were as previously described (20); light intensity was 10–45 lx on the cage floors depending on the location of the cage in the room. Animals were provided cotton batting for nesting material; food (Purina chow #5015) and tap water were available ad libitum.

Activity. Activity was measured by PIR motion detectors mounted on each cage lid as described (18). Each detector is mounted directly above the tip of the water bottle sipper tube so that each time an animal drinks, it breaks the plane of detection and a unit of activity is recorded. Activity levels primarily reflect drinking behavior but also include locomotor activity that occurs directly under the sipper tube. The temporal resolution for detecting successive bouts of activity is 1–2 s between bouts. Activity bouts were summed in 10-min intervals and stored on computer.

Melatonin RIA. At the time of blood withdrawal, hamsters were anesthetized with methoxyflurane vapors (Metofane) until the eye-blink reflex was absent. A heparinized capillary tube (ID 1.1 mm) was used to withdraw 0.35–0.50 ml blood from the retroorbital plexus. Blood was collected in vials containing 30 µl of heparin (1,000 U/ml) that were kept on ice until all samples had been collected and then centrifuged for 5 min (∼1,400 g). Serum from individual samples was then removed by pipette and frozen (−20°C) until assayed for melatonin. Animals were overdosed by injection with pentobarbital sodium (20 mg). Melatonin levels were determined by using the Buhlmann (Alpco, Windham, NH) melatonin RIA kit following extraction of plasma melatonin by reverse-phase column chromatography. This RIA was validated for melatonin measurements in hamster blood (22). All samples were run in the same assay.

Data analysis. The time of activity onset was defined as the first 10-min interval in a circadian cycle when the number of activity bouts increased above the daily mean activity level and was sustained at or above that level for ≥30 min. The times of activity onset are defined relative to dark onset and are negative when dark onset precedes activity onset. Activity onset is the first 10-min interval when the number of activity bouts remains below the daily mean for ≥30 min. Alpha is the time interval between activity onset and offset. ANOVA or t-tests were used to evaluate differences among groups; repeated measures were used where appropriate. Pearson's correlation coefficients were used to test whether melatonin levels and times of activity onset were correlated. The times of activity offset on the last day of each experiment were estimated by a linear regression of the prior 3 days of data. All group values are expressed as the means ± SE.

Experimental protocol. Activity rhythms were monitored in adult (3–4 mo of age) male hamsters for 14 days, and then each animal was randomly assigned to one of three groups. In the first group, blood samples were obtained 4 h after activity onset on the day before the phase shift (day 1; designated Pre-PS). The remaining animals were then exposed to a 5-h phase delay of the LD cycle that was achieved via a lengthening of the light phase; animals remained on the 16:8-h LD photocycle for the remainder of the study. Blood samples were obtained from the second group on the day of the phase shift 30 min before the new time of dark onset (day 0; designated PS). A third group of animals was sampled after the phase shift (day 5; designated Post-PS) on the first day that the mean time of activity onset of this group attained the same phase relationship to the LD cycle that it had before the phase shift (samples obtained 4 h after dark onset).

Three additional groups of animals were treated similarly, except that they were exposed to a 3-h phase delay. Blood samples were obtained from the Post-PS animals in this experiment 4 days after the phase shift because that was the first day that the mean time of activity onset occurred after dark onset. Schematics of this protocol are presented for both experiments (Fig. 1).

Blood samples were obtained 4 h after activity onset (i.e., middark phase) in Pre-PS and Post-PS groups because at that circadian phase, plasma levels of melatonin are at their peak values in Siberian hamsters entrained to a 16:8-h LD cycle (4, 5, 9, 10, 21) and are >50% of their peak values in hamsters entrained to various T-cycles (1). The advantage of this protocol was that it allowed us to obtain blood samples when activity rhythms in both Pre-PS and Post-PS groups had the same phase relationship to the LD cycle. Therefore blood samples were obtained before and after the phase shift at the same zeitgeber times and at the same circadian phase of the animal’s activity rhythms in both experiments.

Fig. 1. Schematic representation of experimental design for 5-h (A) and 3-h (B) phase delays. Blood samples were taken day before (Pre-PS), day of (PS), or after phase shift (Post-PS) on day 5 (A) or day 4 (B) at times indicated (\(\triangledown\)). Means ± SE times of activity onset for each group (\(\bullet\)); error bars are smaller than symbols. Rectangular bars indicate light-dark (LD) cycle phases before and after phase shift.
RESULTS

Experiment 1: 5-h phase delay. Times of activity onset and alphas did not differ among Pre-PS (n = 7), PS (n = 8), and Post-PS (n = 8) hamsters on the day before the phase shift (P > 0.05; Fig. 2, A and C). The phase relationship between activity onset and dark onset in the Post-PS group on days −1 and 5 did not change significantly (P > 0.05; Fig. 2A). The time of activity onset, activity offset, and alpha were plotted for the Post-PS group beginning 3 days before the phase shift (Fig. 3A). Activity onsets were phase delayed by 141.1 ± 12.1 min on the day after the phase shift; the intervals between successive activity onsets increased gradually over the next 5 days and reached a maximum of 53.9 ± 15.0 min between days 4 and 5 (Fig. 3A). Alpha significantly shortened after the phase shift by as much as 98 min on day 2 (P < 0.05), then gradually increased (Fig. 3A).

Plasma melatonin levels were significantly lower in both the PS (P < 0.001) and Post-PS (P < 0.001) groups compared with the Pre-PS group (Fig. 4A). There were no differences in mean melatonin levels between the PS and Post-PS groups (P > 0.05). Melatonin levels did not correlate with times of activity onset established on the days that blood samples were obtained for the Pre-PS (r = 0.15, P > 0.05) or the Post-PS (r = −0.06, P > 0.05; Fig. 4B) group.

Experiment 2: 3-h phase delay. Times of activity onset and alphas did not differ among Pre-PS (n = 9), PS (n = 6), and Post-PS (n = 9) groups on the day before the phase shift (P > 0.05; Fig. 2, B and C). The phase relationship between activity onset and dark onset in the Post-PS group on days −1 and 4 did not differ significantly (P > 0.05; Fig. 2B). Time of activity onset, activity offset, and alpha were plotted for the Post-PS group beginning 3 days before the phase shift. Mean activity onset gradually delayed by ~45 min each day (Fig. 3). Alpha did not change significantly after the phase shift (P > 0.05; Fig. 3B).

Plasma melatonin levels in the Post-PS group were not significantly different from those in the Pre-PS group (P > 0.05; Fig. 4C). Melatonin levels were, however, significantly lower in the PS group compared with the Pre-PS one (P < 0.001; Fig. 4). No significant correlation was found between melatonin levels and the times of activity onset that were established on the days that blood samples were obtained for the Pre-PS (r = 0.22, P > 0.05) or Post-PS (r = −0.10, P > 0.05; Fig. 4D) groups.

Comparisons between 5-h and 3-h phaseshifts. Means ± SE times of activity onset and alphas of Pre-PS, PS, and Post-PS groups did not differ between experiments before or after the phase shifts (P > 0.05; Fig. 2). The range in times of activity onsets for the Post-PS groups was, however, nearly four times greater after the 5-h phase delay than it was after the 3-h one (150 vs. 40 min, respectively; Fig. 4, B and D). Melatonin levels did not differ significantly between experiments among the Pre-PS and PS groups (P > 0.05), but were significantly different between the two Post-PS groups (P < 0.001; Fig. 4, A and C).

DISCUSSION

Melatonin was suppressed in hamsters exposed to a 5-h phase delay of the photocycle but not in those exposed to a 3-h phase delay. After the phase shifts, activity onsets drifted gradually toward the new time of dark onset, and melatonin was sampled in both Post-PS groups on the first day when their mean times of activity onset were the same as they had been before the phase shift. By maintaining this temporal arrangement, we were able to measure melatonin at the same zeitgeber times and at the same circadian phase of the...
animal's activity rhythms in both experiments. This timing was essential because 1) nightly secretion of melatonin begins close to the time of activity onset and ends close to the time of activity offset in this species (15); 2) light suppresses melatonin production (4, 9); and 3) Siberian hamsters exposed to a 5-h phase delay free run indefinitely with tau close to 25.0 h despite the presence of the LD cycle (20). On the basis of the mean times of activity onset and the estimated times of activity offset for both Post-PS groups, blood samples were obtained at the circadian phase when plasma melatonin is normally at its maximum nightly level.

Fig. 3. Times of activity onset (●) and offset (○) and alpha for each Post-PS group after a 5-h (A) or 3-h (B) phase delay; error bars are smaller than some symbols. Projected time of activity offsets (dotted open circle) and time blood samples were obtained (▼) are indicated. Rectangular bars indicate LD cycles before and after phase shifts. Alpha for each day is shown to right of each activity graph. *P < 0.05 compared with alpha on day -1.

Fig. 4. Means ± SE plasma melatonin levels for 5-h (A) and 3-h (C) phase delays for each treatment group. Individual melatonin levels plotted against each animal’s time of activity onset on day that blood samples were obtained for 5-h (B) and 3-h (D) phase shifts. Melatonin levels were not correlated with times of activity onset in either Pre-PS (○) or Post-PS (●) groups for either shift (P > 0.05). LD cycles indicated by rectangular bars (B, D); 0 = dark onset. *P < 0.001 compared with Pre-PS group.
Melatonin levels may remain chronically suppressed in animals that fail to reentrain to a 5-h phase delay of the photocycle. In prior studies, we found that animals that failed to reentrain also free ran with tau close to 25.0 h and had mean alphas of 7.25 h (18). The implication of these values for animals free running in the presence of a 16:8-h LD cycle is that they are exposed to light during some portion of their active phases on 24 of every 25 days of their free run (18). Because the inhibitory effects of light can persist on the night after light exposure (9), melatonin production is probably disrupted or suppressed by light exposure on those 24 days. In the present study, we found that melatonin levels were suppressed on the 1 day of 25 that the animal's active phases were completely shielded from light exposure. Therefore it is likely that melatonin is either secreted in an irregular pattern, or it is chronically suppressed in animals that fail to reentrain to the LD cycle.

The times of activity onset in the Post-PS group exposed to a 5-h phase delay were more variable than in those hamsters exposed to a 3-h one. One implication of this finding is that melatonin was measured over a wider range of circadian times after the phase shift in the 5-h experiment than it was for the 3-h one. Because the onset of the nightly rise of melatonin correlates with time of activity onset (2), this presents a potential problem. Blood samples taken from animals with very delayed (i.e., negative) activity onsets may have been obtained before melatonin achieved its nightly peak plasma levels. Conversely, melatonin may have been suppressed in animals with advanced (i.e., positive) activity onsets because they were exposed to light early in subjective night. Neither of these possibilities can account, however, for the differences in mean melatonin levels between Pre-PS and Post-PS groups in the 5-h experiment because the times of activity onset in all Pre-PS and most Post-PS hamsters occurred within 60 min after dark onset. Therefore, the uniformly low plasma melatonin levels found in Post-PS animals in the 5-h experiment are most likely due to the effects of the phase shift on melatonin production and not to individual differences in the circadian times at which blood samples were obtained.

Our experimental protocol assumed that the hamsters responded to the 5-h and 3-h phase shifts as other hamsters have done in prior experiments where 9 and 100% of animals, respectively, reentrained. We could not verify that the same proportion of animals in this study would have reentrained because animals were killed after blood was withdrawn; however, the failure to reentrain to the same 5-h phase delay used here has been replicated in three separate groups of hamsters (19, 20). It is also unlikely that useful information would have been gained from allowing hamsters to recover because damage to the retroorbital plexus could alter photic input to the pacemaker and diminish the likelihood of reentrainment, thus biasing the results. Furthermore, even when multiple reentrainment signals were present, no more than 14% of the animals reentrained to the 5-h phase delay used in the present study (19).

The response of the circadian pacemaker to 3-h or 5-h phase delays of the photocycle were different for each Post-PS group. On the day after the 5-h phase shift, times of activity onset were phase delayed by over 2 h; daily delays in activity onset then gradually increased from 20 to over 50 min. Alpha was compressed by over 90 min for 2 days after the phase shift and then gradually began to decompress. In contrast, alpha and daily delays (−45 min) in activity onsets remained stable after the 3-h phase delay. The progression of activity onsets after the 3-h phase shift, however, may be due to the interaction of nonparametric and parametric effects of that phase shift on the pacemaker. The large phase shift on the first day after the 5-h phase delay cannot be accounted for entirely by predictions based on the photic phase response curve (PRC) for this species because a 5-h phase shift delayed activity by three times more than the 3-h phase shift. It is unlikely that a 2-h difference in light exposure could produce a threefold increase in phase shift magnitude in a species with a very low amplitude type 1 photic PRC (11, 16). This difference may be explained, however, by the additive effect of a phase shift and an increase in tau. Siberian hamsters free run with tau very close to 24.0 h when released into constant darkness but free run with tau closer to 25.0 h after a 5-h phase delay (11, 16, 20). This increase in tau may be initiated on the day of the phase shift. A 5-h phase delay of the photocycle may simultaneously phase shift activity rhythms and initiate an increase in tau. Tau may continue to increase on subsequent days as nonparametric effects diminish.

Perspectives

The correlation between low melatonin levels in the middle of the night and failure to reentrain after a 5-h phase delay of the LD cycle suggests that there may be a functional relationship between intact entrainment mechanisms and nightly elevations in melatonin. Only the 5-h phase shift both prevented reentrainment (18) and suppressed endogenous melatonin levels, whereas melatonin levels were normal and all animals reentrained to a 3-h phase shift (16). If this correlation represents a functional relationship, then administration of exogenous melatonin should prevent the effects of a 5-h phase delay. In a prior study, hamsters exposed to a 5-h phase delay were injected daily with melatonin at dark onset beginning on the day of the phase shift (19). All of those animals appeared to entrain to the injection regimen, and, after treatment was terminated, 56% of the hamsters maintained photic entrainment to the end of the study, whereas only 9% spontane-
ously reentrained to a 5-h phase shift (19, 20). Thus the failure to reentrain to a 5-h phase shift is greatly attenuated by melatonin replacement. In sharp contrast to these findings are data obtained from other species; pinealectomized rats and golden hamsters both reentrain to phase shifts of the LD cycle (3, 17). Although these data suggest that Siberian hamsters would respond similarly, no studies have exposed pinealectomized hamsters to phase shifts of an LD cycle with a fixed photoperiod. Given the unique reentrainment phenomena described for this species, generalizations from other rodents should be made cautiously. Nevertheless, we cannot rule out the hypothesis that suppression of melatonin may be a consequence, and not a cause, of the failure to reentrain.

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