

Circadian clock resetting by arousal in Syrian hamsters: the role of stress and activity

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Mistlberger, R. E., M. C. Antle, I. C. Webb, M. Jones, J. Weinberg, and M. S. Pollock. Circadian clock resetting by arousal in Syrian hamsters: the role of stress and activity. *Am J Physiol Regul Integr Comp Physiol* 285: R917–R925, 2003. First published July 3, 2003; 10.1152/ajpregu.00222.2003.—Circadian rhythms in the Syrian hamster can be markedly phase shifted by 3 h of wheel running or arousal stimulation during their usual daily rest period (“subjective day”). Continuous wheel running is predictive but not necessary for phase shifts of this “nonphotic” type; hamsters aroused by gentle handling without running can also show maximal shifts. By contrast, physical restraint, a standard stress procedure and thus presumably arousing, is ineffective. To resolve this apparent paradox, phase-shifting effects of 3-h sessions of restraint or other stress procedures were assessed. In a preliminary study, phase shifts to arousal by gentle handling were significantly potentiated by the cortisol synthesis inhibitor metyrapone, suggesting that stress-related cortisol release may inhibit phase shifts to arousal. Next, it was confirmed that restraint in the subjective day does not induce phase shifts, but behavioral observations revealed that it also does not sustain arousal. Restraint combined with noxious compressed air blasts did sustain arousal and induced a significant cortisol response compared with arousal by gentle handling but did not induce shifts. Restraint combined with continuous horizontal rotation was also ineffective, as was EEG-validated arousal via confinement to a pedestal over water. However, 3 h of resident-intruder interactions (an intense psychosocial stress) or exposure to an open field (a mild stress) did induce large shifts that were positively correlated with indexes of forward locomotion. The results indicate that large phase shifts associated with arousal in the usual sleep period are neither induced nor prevented by stress per se, but are dependent on the expression of at least low levels of locomotor activity. Sustained arousal alone is not sufficient.

circadian rhythms; nonphotic zeitgeber; wheel running; sleep deprivation; immobilization stress; psychosocial stress; cortisol; electroencephalogram

IN 1988 IT WAS FIRST REPORTED that brief interactions between socially isolated Syrian hamsters in the middle of their usual rest period (for nocturnal animals, the “subjective day”) can reliably induce small (~40 min) phase advance shifts of their free running circadian activity rhythms (31). Small advance shifts could also be induced by nonsocial disturbances, such as changing the animals’ cage or litter (31), whereas the same stimulation in the latter half of the active period (the “subjective night”) could induce small phase delays. Much larger phase advance shifts, in the range of 2–4 h, could be induced by confining hamsters to a novel wheel for 3 h (36). Animals that ran continuously, accumulating 5,000 or more wheel revolutions, with rare exceptions shifted, whereas those that did not run, with rare exceptions, did not shift. Phase shifts induced by other stimuli, including triazolam injections and exposure to a 6-h dark interval in otherwise constant light, were soon shown also to depend on the expression of wheel running activity; shifts were blocked by physical restraint (41, 43) and strongly attenuated by confinement to a small nest box (34, 35). These and other studies demonstrated that activity or arousal during the usual sleep period can be a potent nonphotic zeitgeber (entraining stimulus), capable of shifting or entraining free running rhythms or altering the phase of entrainment to a light-dark (LD) cycle (for additional references, see 29, 33).

Although intense or continuous locomotor activity was implicated as a critical factor for clock resetting in response to arousal procedures, the results did not rule out the possibility that hamsters need only be kept awake for large phase shifts to occur. A role for arousal independent of continuous running was clearly established using the sleep-deprivation procedure of gentle handling (i.e., occasional light air puffs or touch when animals assumed a sleep posture). This procedure fully mimicked the large phase-advance shifting effects of continuous novelty-induced wheel running, despite minimal locomotion and intervention (3, 27). In a subset of hamsters in one study, no relation was detected between phase shifting and general cage activity (3). In aggregate, these results indicate that arousal, or sleep loss, is sufficient to reset the circadian pacemaker in Syrian hamsters and that “exercise,” i.e., intense or continuous running, is not necessary.

A remaining puzzle in clarifying the behavioral state correlates of nonphotic clock resetting are reports that physical restraint in the middle of the usual rest period

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does not induce phase shifts in hamsters. Restraint is a well known stress stimulus and would thus be expected to arouse sleeping hamsters. If arousal without exercise is sufficient to induce phase shifts, then restraint should also be an effective zeitgeber. Yet, in the three available studies, restraint during the rest period induced no shifts and prevented shifts to triazolam injections or dark pulses (39, 41, 43). Three explanatory hypotheses will be considered. 1) The restraint procedure used in these hamster studies may not have produced a sustained arousal. 2) The stress of restraint may inhibit nonphotic phase shifting. 3) Some minimal level of locomotion may be necessary for arousal-induced phase shifts. There is no means by which to refute the first hypothesis, as behavioral or physiological measures of arousal or stress were not reported in the hamster restraint studies (39, 41, 43). There is no direct evidence to refute the second hypothesis; however, evidence consistent with the hypothesis includes observations that injections of cortisol or the glucocorticoid agonist dexamethasone do not entrain or phase shift free running rhythms in Syrian hamsters (2, 16) and that acute stressors do not induce phase shifts in another species, the rat (23, 24; but note that large phase shifts in response to activity or arousal have not yet been demonstrated in rats). Finally, there is no evidence to refute the third hypothesis. Whereas the sleep-deprivation procedure of gentle handling minimizes locomotion, the hamsters in these studies were not prevented from moving (3, 27). The reported lack of correlation between general cage activity levels and phase shifts to this procedure does not rule out the possibility that some minimal level of locomotion is necessary for clock resetting.

We report here the results of several experiments designed to assess the contributions of stress and locomotion to nonphotic phase shifting in Syrian hamsters. We first examined whether phase shifts to sleep deprivation and restraint could be enhanced by pharmacological blockade of cortisol synthesis. We then examined whether the conventional restraint procedure sustains arousal and whether increasing the stress load during restraint might induce phase shifts. We measured cortisol (Cort) to determine if sleep deprivation by gentle handling, confinement to a novel running wheel, and stress-loaded restraint are in fact differentially stressful. Finally, we compared the phase-shifting effects of three additional stress procedures in which locomotion was either prevented [confinement to a pedestal over water (37)] or permitted [resident-intruder interaction and confinement to a novel open field (17)]. The results support *hypothesis 1* (the conventional restraint procedure does not sustain arousal), refute *hypothesis 2* (stress does not preclude the induction of large "nonphotic" phase shifts), and are consistent with *hypothesis 3* (some minimal level of forward locomotion is necessary for large phase shifts).

METHODS

Animals and housing. Young adult male Syrian golden hamsters (80–140 g; LVG:Lak) were used in all experiments

(Charles River, Montreal, PQ, Canada). The animals recovered from shipping for 10–14 days in a group colony room before transfer to individual polypropylene cages (45 × 25 × 20 cm) equipped with wire mesh floors and running wheels (17.5-cm diameter). Wheel running cages were housed in ventilated isolation cabinets with controlled lighting (LD 14:10, ~30 lx:0 lx). Food and water were available ad libitum. Wheel running activity was detected by microswitches monitored continuously by computer. Activity data were summed and stored to disk at 10-min intervals and periodically transferred to a Macintosh computer for analysis.

Phase shift measurements. In all but one experiment, the Aschoff type II method was used for measuring phase shifts (see Ref. 32). Hamsters were maintained in LD until the day of the experimental procedure. Lights were turned off when the procedure was initiated and were kept off for 3–5 days. A computer algorithm was used to identify the onset of the main period of daily wheel running, designated circadian time (CT12), by convention (each complete circadian cycle is comprised of 24 equal "circadian hours"). The average time of activity onset for 4–5 days before the day of the experimental manipulation was compared with the time of activity onset on *day 2* of constant dark (DD) after the manipulation. A within-subject design was used in most experiments, so that phase shifts to experimental procedures were expressed relative to phase shifts to the appropriate control procedures (typically lights off at the same time, with no additional manipulation; see Fig. 1, A and B). After DD, the LD cycle was reinstated for at least 5 days before additional testing.

Phase shifts to sleep deprivation by gentle handling: effects of Cort synthesis inhibitor. Hamsters ($n = 9$) were subjected to sleep deprivation by gentle handling, as described previously (3, 27, 38). Briefly, cage lights were dimmed to ~1 lx (red incandescent; DDred) at zeitgeber time 6 (ZT6, i.e., 6 h before the usual time of lights off, designated ZT12 by convention). Cage tops were removed and the hamsters were observed continuously for 3 h. Food and water were available, but running wheels were locked. If the hamsters attempted to adopt a sleep posture, they were stimulated by tapping on the cage, a light air puff, or light touch. At the end of the procedure, the wheels were unlocked and the lights were turned off for at least 3 days. All hamsters received one 3-h sleep deprivation counterbalanced for order with one control test (DD at ZT6, no behavioral intervention). These data were reported previously (3) and are included here to illustrate typical phase shift responses to sleep deprivation and permit within-animal comparisons with the drug condition. To test whether phase shifts induced by sleep deprivation are influenced by a cortisol response to the procedure, the hamsters were subjected to two additional 3-h sleep deprivations, preceded at ZT23 and ZT4.5 by injections of the corticosteroid synthesis (11β -hydroxylase) inhibitor metyrapone (50 mg/kg, Sigma-Aldrich) or vehicle (40% polyethyleneglycol in saline). The hamsters were reentrained to LD 14:10 for at least 5 days before each test. Tests in four hamsters indicated that metyrapone alone did not induce phase shifts.

Phase shift effects of restraint and stress-loaded restraint. Hamsters ($n = 12$) entrained to LD 14:10 were subjected to a DD control test and three physical restraint tests. Restraint was accomplished by placing hamsters into polyvinylchloride tubes (15-cm long, 4-cm internal diameter) inside a wire cage (0.5-cm² mesh) that blocked both ends but allowed air and waste to pass. The animals could move horizontally ~6 cm, but could not turn around. Hamsters were restrained from ZT6 to ZT9 in DDred, after which they were returned to their

home cages and left undisturbed in DD for 3 days. Hamsters were reentrained to LD for at least 5 days between tests.

During the first restraint test, behavioral state was scored every minute as active wake (eyes open, overt attempts to move), quiet wake (eyes open, motionless), and behavioral sleep (eyes closed and motionless). Behavioral observations were made using an infrared viewer.

During the second and third restraint tests, counterbalanced for order, the hamsters were stimulated with compressed air to interrupt behavioral sleep. During one test, a light puff of air was delivered to the face at 1-min intervals using an aerosol dusting can (30 psi; Memorex Duster). During the other test, a high-intensity air "blast" was applied at interstimulus intervals of ~30 s using compressed air tanks providing 110 psi through 3/8-in. tubing. Sound pressure level of the compressed air was measured at 107 dB (Quest Technologies, model 2900 dB meter). This procedure is designated "stress-loaded" restraint. Due to the near continuous intervention, no attempt was made to score behavioral state.

A second group of hamsters ($n = 11$) was subjected to the 3-h restraint procedure combined with continuous vestibular stimulation, produced by horizontal rotation on a cart at ~120°/s. Behavioral state was noted but not recorded as the procedure appeared to produce sustained quiet or active wake.

Cort responses to sleep-deprivation procedures. To determine whether the sleep-deprivation procedures of gentle handling, stress-loaded restraint, and confinement to a novel running wheel (a standard procedure for inducing large phase shifts) are differentially stressful, the effect of these procedures on Cort release was measured. Naive hamsters ($n = 96$) were housed in running wheel cages and entrained to LD 14:10 for at least 10 days. On the test day, lighting was reduced to DDred at ZT6, and the hamsters were subjected to either the control procedure (undisturbed in home cage), sleep deprivation by gentle handling, stress-loaded restraint, or confinement to a 33-cm diameter novel running wheel. Twenty-four hamsters were assigned to each group, and eight hamsters from each group were killed at ZT6.5, ZT7.5, and ZT9 (i.e., at 30, 90, and 180 min after the onset of the procedures). For blood collection, the animals were transferred singly to another room and decapitated (the order of death was counterbalanced across groups). Blood was collected into chilled Eppendorf tubes containing EDTA, centrifuged at 3,600 rpm for 10 min, and stored at -70°C. Total Cort (bound plus free) was measured by radioimmunoassay in plasma extracted in 95% ethanol. Tracer [³H]Cort (cat# 07-121026), unlabeled Cort for standards (cat# 07-121040), and anti-Cort (cat# 07-121015) were obtained from ICN (Costa Mesa, CA). Dextran-coated charcoal was used to absorb and precipitate free steroids after incubation. Samples were counted in Scintisafe Econo2 (Fisher Scientific, Nepean, ON, Canada). Intra- and interassay coefficients of variation were <4.3% and 5.6%, respectively.

Phase shift effects of sleep deprivation by pedestal over water. Hamsters ($n = 12$) were housed in wheel running cages and entrained to LD 14:10 for at least 10 days. Each hamster received three 6-h sessions on a 4.3-cm diameter pedestal set 1.5 cm above 13-cm water (23°C) in a Plexiglas cage (20 × 30 × 55 cm). The cage was enclosed by a standard stainless steel lid providing food and water ad libitum. Plastic barriers were placed in the water around the pedestal, so that if hamsters chose to swim, they could not reach the sides of the cage. These preliminary tests established that the procedure prevented sleep and that the hamsters could stay out of the water. After a few swims in the preliminary tests,

all hamsters did stay out of the water. The hamsters then received, at 7-day intervals, a control test (DD for 3 days beginning at ZT6) counterbalanced for order with a 3-h (ZT6–9) sleep deprivation on the pedestal.

EEG recordings were conducted on four additional hamsters to confirm that the pedestal technique prevents sleep. Electrodes ($n = 4$ stainless steel jeweler screws for differential recording of midline EEG and lateral EEG and $n = 2$ subcutaneous wire electrodes for recording EMG from the nuchal muscle) were implanted using standard stereotaxic procedures and pentobarbital sodium (50 mg/kg) anesthesia. The pins from all six electrodes were connected to a plastic headcap bonded to the skull with dental acrylic.

After 5–7 days of recovery from surgery, the hamsters were habituated to the recording cable for 3–7 days in an electrically shielded recording chamber. The cable was left connected for 24 h before the baseline recording day. On the day after baseline, the hamsters were placed on the pedestal over water from ZT6 to ZT9, with lights lowered to DDred. EEG and electromyogram (EMG) signals were amplified (Grass model 79D, Grass Instruments), digitized (sampling rate of 250 Hz), and stored on computer using AcqKnowledge software (Biopac, Goleta, CA). Behavioral states were determined in 10-s epochs on the basis of four electrographic measures: unfiltered lateral EEG, lateral EEG filtered for delta waves (1–3 Hz), midline EEG filtered for bandwidths including theta (5–15 Hz), and EMG (high-pass filtered at 50 Hz). Each epoch was classified as whichever state was predominant, according to criteria validated previously (Ref. 4, see also 38). Wakefulness (W) was identified by low-amplitude lateral EEG, absence of delta waves, only occasional regularity in midline theta EEG, and high-amplitude EMG. This electrophysiological state corresponded to behavioral alertness with eyes open. Slow-wave sleep (SWS) was identified by high-amplitude lateral EEG, presence of delta waves, spikiness in midline theta EEG, and low-amplitude EMG. Rapid eye movement sleep (REMS) was identified by low-amplitude lateral EEG, absence of delta waves, sinusoidal midline theta rhythms, and muscle atonia. Also recognized were transitional sleep (TS; high-amplitude lateral EEG, absence of delta waves, spikiness in midline theta EEG, and low-amplitude EMG) and low-amplitude sleep (LS; low-amplitude cortical EEG, absence of delta waves, spikiness in midline theta EEG, and low-amplitude EMG).

Phase shift effects of social stress: resident-intruder paradigm. Hamsters ($n = 12$) were housed in running wheel cages and entrained to LD 14:10. The six lightest hamsters (123 ± 19 g) were designated as "intruders", and the six heaviest (145 ± 5 g) as "residents." At ZT6 on the test day, a 3-h resident-intruder test was conducted in DDred. Each intruder was rotated between the cages of two resident hamsters at 15-min intervals, after an initial 30-min interval. Thus each intruder interacted with two different residents and each resident with two different intruders. Sessions were videotaped using an infrared camcorder to score behavior. Running wheels were locked during the first test and unlocked during the second test. If any animal was observed to adopt a sleep posture, a light air or touch stimulus was applied. Phase shifts were quantified relative to a control DD test.

Videotapes were scored in 1-min intervals by trained observers. Behavioral variables quantified included fight, chase, flee, walk, defensive subordinate posture, offensive attack posture, nesting, number of minutes during which each subject spent some time wheel running (wheel unlocked condition), and number of experimenter interventions to prevent a sleep posture. These variables were used to determine

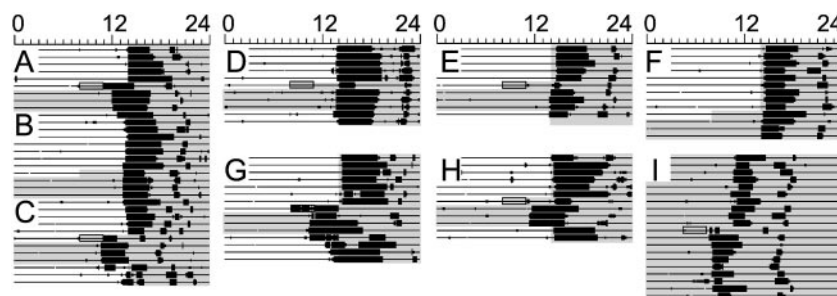


Fig. 1. Wheel running of representative hamsters subjected to control and arousal procedures. On each chart, each line represents 24 h of recording, plotted in 10-min bins from *left to right*. Consecutive days are aligned vertically. Heavy bars on the lines indicate time bins when wheel counts were registered. Lights off [light/dark (LD) 14:10 or constant darkness (DD)] is denoted by the shading. Rectangles indicate 3-h arousal procedures conducted in DD under red incandescent (DDred). *A*: large phase advance (120 min) induced by the sleep deprivation procedure of gentle handling beginning at zeitgeber time (ZT)6 (6 h before the usual time of lights out). *B*: control procedure of lights out at ZT6 (5 min phase advance). *C*: large phase advance (190 min) by sleep deprivation in the same hamster after treatment with the cortisol synthesis inhibitor metyrapone. *D*: stress-loaded restraint procedure (20 min advance). *E* and *F*: sleep deprivation by confinement to a pedestal over water (50 min advance) and control procedure for same hamster (30 min advance). *G*: large phase advance (240 min) in a subordinate hamster after 3 h of psychosocial stress. *H*: large phase advance (180 min) in a dominant hamster after 3-h of social interaction. *I*: large phase advance (120 min) in a hamster after 3 h in a novel open field.

the dominance status of the hamsters, and activity and intervention measures were correlated with the size of phase shifts.

Phase shift effects of open field stress. Hamsters ($n = 12$) used in the resident-intruder test were reentrained to LD for 3 wk and then maintained in DDred. During the third week in DDred, each subject was transferred at CT6 to a 1-m square open field apparatus for 3 h under the same dim lighting. The open field was made of 60-cm-high white plastic walls and floor. A 250-ml, 6-cm diameter glass beaker, with horizontal stripes of white tape, was placed in the center of the floor. An overhead infrared camera coupled to a micro-computer by an image analyzer (Chromotrack, San Diego Instruments, San Diego, CA) was used to track movements. Data were sampled every 0.055 s. The minimum displacement necessary for detection of movement was set at 4 cm. For analysis purposes, the open field was divided on the computer tracker file into an outer ring (0–15 cm from the wall), a middle ring (15–30 cm from the wall), an inner ring (30–50 cm from the wall), an object zone (15 cm in diameter centered around the glass beaker), and four corner areas (each 15 × 15 cm). Before each trial, the open field and glass beaker in it were cleaned with a solution of 70% alcohol. The trial began with the subject being placed in the open field facing the wall. The tracker recording began simultaneously with the release of the animal via a remote start switch. The trial duration was 180 min, with data summaries automatically taken each minute. Light taps on the floor or walls of the open field were used to prevent sleep postures as necessary. At the end of the trial the subject was promptly removed and returned to its home running wheel cage.

Phase shifts were measured by fitting separate regression lines to activity onsets for 10 days preceding and after the open field test. The following measures were recorded: the time spent in each of the five regions of the open field (the 3 rings, the object area, and the corner areas); the number of entries into each of the same five regions; the total distance traveled; and the number of crossings of 20 × 20-cm imaginary divisions of the open field.

Data analysis. The phase shift, cortisol, and behavioral measures were evaluated by ANOVA with Student-Newman-Keuls post hoc analyses or paired *t*-tests with Bonferroni corrections. Pearson product-moment correlation coefficients

were calculated to examine relationships between phase shifts and behavioral variables. Means in the text are reported as $\pm$ SE.

RESULTS

Phase shifts to 3-h sleep deprivation are enhanced by metyrapone. There was a significant main effect of treatment on phase shifts ($F_{2,16} = 30.9$, $P < 0.0001$; Figs. 1, A–C, and 2). Mean phase shifts induced by 3 h of sleep deprivation were significantly larger in the metyrapone condition (149 ± 12 min) compared with the vehicle condition (107 ± 16 min), and both were significantly larger than the mean shifts in the control condition (11 ± 7 min).

Restraint procedures are differentially arousing but do not induce phase shifts. All hamsters restrained for 3 h from ZT6 to ZT9, by the conventional method (i.e.,

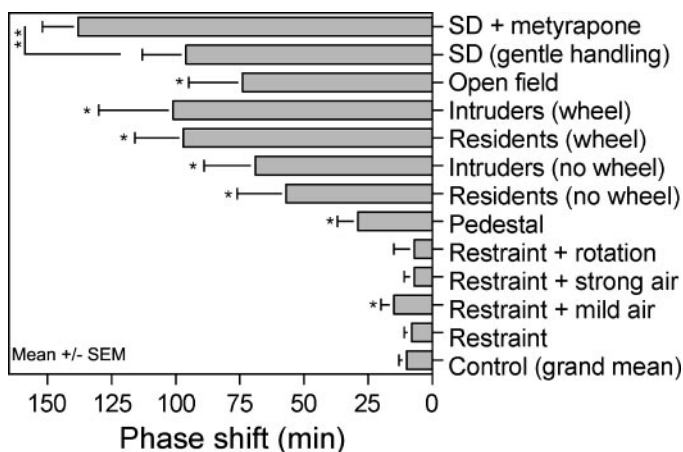


Fig. 2. Group mean ($\pm$ SE) phase advance shifts in response to arousal procedures, reported here as difference scores relative to the within-subject DD control procedures. Bar representing "control condition" is a grand mean of all control tests. All statistical comparisons were made within subject. SD, sleep deprivation by gentle handling. *Significantly different from within-subject control condition at $P < 0.05$.

without air stimulation or other interventions), exhibited behavioral sleep within the first hour. Mean sleep latency was 39 ± 8 min and mean sleep accumulation was 120 ± 8 min. Quiet wake occupied 9 ± 2 min and active wake 52 ± 28 min. Restraint combined with low-intensity air stimulation appeared to reduce behavioral sleep, whereas high-intensity compressed air applied at ~ 30 -s intervals prevented behavioral signs of sleep.

Despite differential effects on behavioral state, phase shifts to these procedures were uniformly small (e.g., Fig. 1D), and treatment means were 20 min or less. Although there was a significant main effect of treatment ($F_{2,22} = 5.43$, $P < 0.05$), only restraint combined with low-intensity air stimulation differed significantly from the control condition ($P < 0.05$), and the magnitude of the difference was only 9 min. There were no differences among the three restraint procedures (Fig. 2). In the conventional restraint condition (no air stimulation) there was no relationship between the size of the phase shift and the amount of time in either wake ($r = 0.09$, $P > 0.1$) or behavioral sleep ($r = -0.16$, $P > 0.1$).

Hamsters subjected to vestibular stimulation by horizontal rotation during restraint exhibited continuous behavioral arousal (eyes open), but no significant phase shift compared with the control procedure (26 ± 10 vs. 19 ± 5 min, in the restraint and control procedures, respectively; $P > 0.1$).

Sleep-deprivation procedures are differentially stressful: Cort measurements. There was a significant main effect of circadian time ($F_{2,81} = 6.96$, $P = 0.002$) and treatment ($F_{3,81} = 24.62$, $P < 0.001$, Fig. 3) on plasma Cort. Pairwise comparisons revealed that at the 30-min sampling point, Cort levels did not differ across procedures. At the 90-min sampling point, only the restraint procedure differed significantly from the undisturbed control condition. At the 180-min sampling point, both gentle handling and restraint were associated with significant elevations of Cort. However, in the gentle handling group, only three of eight hamsters exhibited higher Cort levels at 180 min. Hamsters confined to a running wheel for 3-h did not exhibit significant Cort elevations at any time point by comparison with the control group.

Sleep deprivation by the pedestal over water method does not induce large phase shifts. During baseline EEG recording sessions from ZT6 to ZT9, hamsters averaged $24.8 \pm 0.8\%$ W, $42 \pm 1.7\%$ SWS, $17.4 \pm 0.3\%$ REMS, $3.6 \pm 0.6\%$ LS, and $2.2 \pm 0.4\%$ TS. During the 3-h session on the pedestal over water, the hamsters exhibited continuous waking, i.e., eyes open with low-amplitude desynchronized lateral and midline EEG and continuous high-amplitude EMG, reflecting a tonically high muscle tone required to remain upright on the pedestal (Fig. 4).

Hamsters used for phase shift studies also exhibited continuous behavioral alertness with eyes open while on the pedestal. The hamsters did not fall into the water. A small but significant group mean phase advance shift was observed (59 ± 11 min, compared with

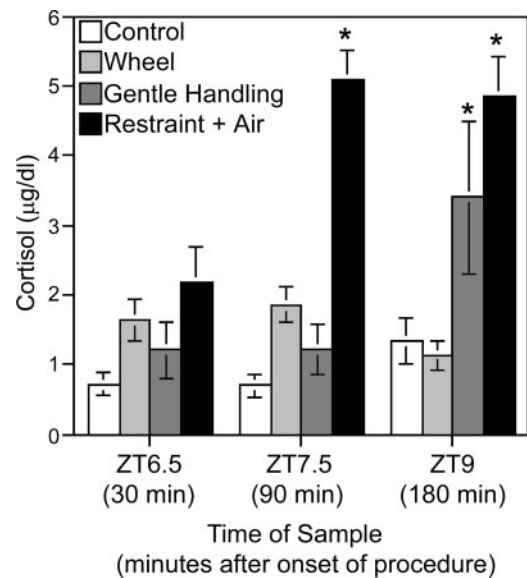


Fig. 3. Plasma cortisol responses to control and arousal procedures at 3 sampling points. Each bar represents the mean ($\pm$ SE) of 7 or 8 hamsters. *Significant ($P < 0.05$) difference from control condition for that sampling point. Control, lights off with no other intervention; Wheel, confinement to a novel running wheel; Gentle handling, arousal enforced by air puffs or gentle touch to prevent sleep postures; Restraint + air, arousal enforced by confinement to a restraint tube and nearly continuous blasts of compressed air (stress-loaded restraint).

28 ± 4 min in the control condition; paired $t = 3.63$, $P < 0.05$; Figs. 1E and 2). However, only one shift exceeded 47 min by comparison with the control procedure, and 9 of 12 shifts were less than 30 min.

Resident-intruder interactions induce large phase shifts related to measures of activity. Dominance status in the hamster dyads was consistent with the relative size of the protagonists and the site of the encounter. Of the 24 total tests, involving 12 hamsters tested twice each, once with the wheel locked and once with the wheel open, the larger resident hamster was dominant in 21 cases, exhibiting chasing, offensive attack postures and no fleeing. One resident hamster exhibited neither chasing nor fleeing on either trial, another exhibited fleeing and no chasing on one trial, and one intruder hamster exhibited both chasing and fleeing on one trial. The remaining intruder hamsters exhibited only fleeing and defensive/submissive postures.

There was no relation between phase shifts and resident status. Compared with the DD control condition, residents shifted 62 ± 18 and 98 ± 34 min on the first and second test, respectively, whereas intruders shifted 72 ± 28 and 82 ± 43 min, respectively (all significantly different from control, $P < 0.05$; Fig. 2). Phase advance shifts >140 min occurred in one resident and two intruders in the first test, and in two residents and three intruders in the second test, for a total of eight large shifts in 24 total tests, involving three of six residents and four of six intruders (e.g., Fig. 1, F and G). Resorting the groups to account for the social status outliers produced virtually the same numerical outcomes. Phase shifts on the two tests were

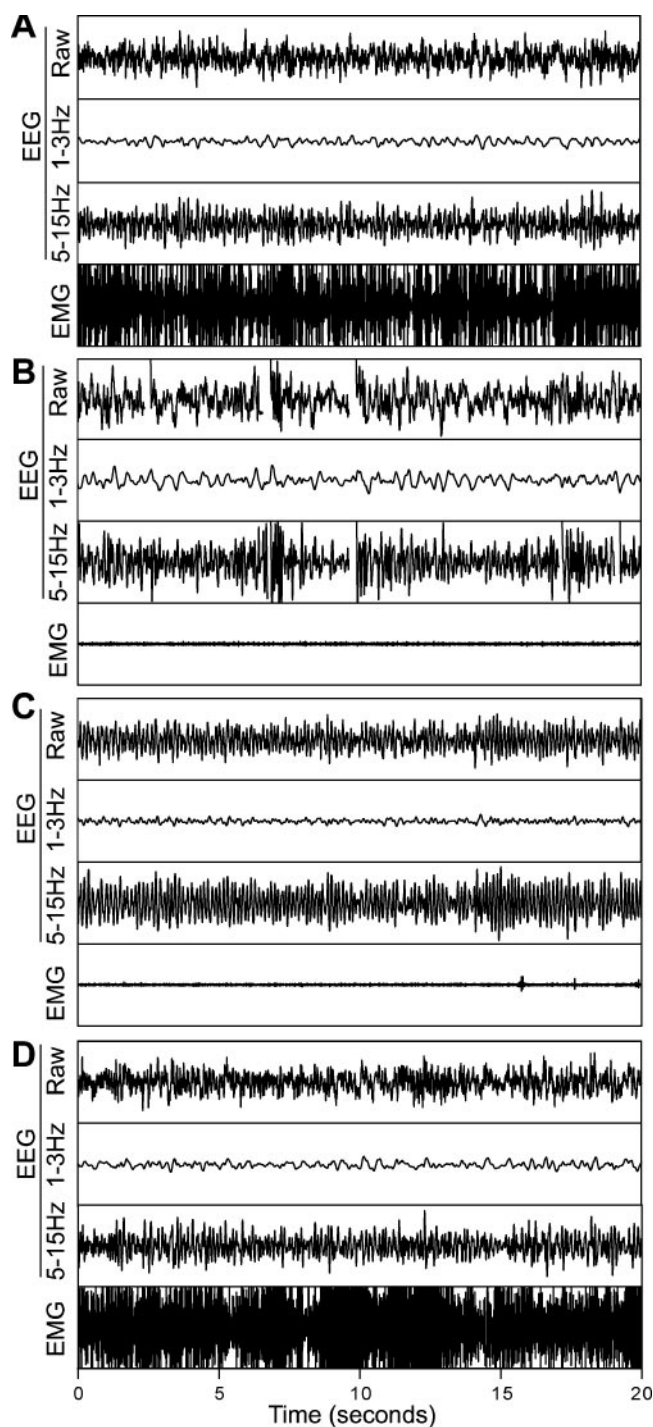


Fig. 4. Samples of polygraphically defined sleep and wake states (A: waking; B: slow-wave sleep; C: rapid eye movement sleep) in a hamster during baseline recording (A-C) and while confined to a pedestal over water (D; sleep deprivation). EMG, electromyogram.

positively correlated across hamsters ($r = 0.79$, $P < 0.005$).

There was a strong positive relation between phase shifts and an aggregate measure of activity created by combining the number of episodes of chasing and fleeing (note that 10 of 12 hamsters exhibited either one or the other). This combined activity score correlated

+0.59 ($P < 0.05$) with phase shift magnitude in *test 1*, and +0.48 in *test 2* ($P < .05$). In *test 2*, the number of minutes during which running was observed (revolutions not recorded) was not significantly correlated with phase shifts ($r = 0.31$, $P > 0.1$).

Phase shifts were inversely related to the number of interventions required to prevent sleep postures in both *test 1* ($r = -0.61$, $P < 0.05$) and *test 2* ($r = -0.56$, $P < 0.05$). More interventions were used on residents than on intruders (12.7 vs. 8.8 and 8.3 vs. 4.7 during *tests 1* and *2*, respectively), but this was only a statistical trend ($P > 0.1$). There was a substantial group difference in the number of minutes of wheel running observed, with intruders averaging 44 ± 11 min and residents only 9 ± 10 min (independent $t = 2.58$, $P < 0.05$).

Open field exposure induces large shifts related to measures of forward locomotion. Hamsters placed into the open field at CT6 remained close to the walls during the first minute, explored the inner ring of the field within the first 2 min, and investigated the center field object within the first 3 min. All hamsters remained spontaneously active during the first 30 min, but needed at least a few interventions to interrupt sleep postures after that. Phase shifts to this open field test ranged from -23 to 177 min and averaged 74 ± 21 min. Total activity in the open field was not significantly related to the phase shifts ($r = -0.28$). However, the total activity score is an integrated measure of activity related to forward locomotion, grooming, object manipulation, scratching at the corners, and other significant movements. Therefore, activity in the corners and in the outer and inner rings of the open field was quantified separately, along with the number of area crossings. Both total time and activity level in the corners were inversely related to phase shifting ($r = -0.79$ and -0.77 , respectively, $P < 0.001$). Notably, most attempts to sleep occurred in the corners. By contrast, total time and activity in the center of the open field were positively correlated with shifting ($r = +0.76$ and 0.71 , respectively, $P < 0.001$). The total number of crossings between 20-cm-square areas within the open field (the most direct measure of forward locomotion) correlated 0.67 with phase shifts ($P < 0.005$).

To further explore the apparent relation between locomotion and shifting, the hamsters were divided into shifters (>80 min, $n = 6$, mean = 133 ± 16 min) and nonshifters (<15 min, $n = 5$, mean = 4 ± 5 min). Shifters exhibited significantly more time and activity in the inner ring and significantly less in the outer ring and the corners ($P < 0.002$ for each comparison). Shifters exhibited 2.85-fold more line crossings than nonshifters over the entire 3 h ($P < 0.005$), and 4.41-fold more during the last hour of the test ($P < 0.001$). Although the groups did not differ in the general activity score, the shifters showed progressively more time in the inner ring of the open field while the nonshifters showed progressively more time in the corners (the "sleep attempt" zone). The two groups did not differ in either measure during the first 30 min,

suggesting no group differences in "anxiety" (operationalized by thigmotaxic behavior) during initial exposure to the open field.

DISCUSSION

The results of this study permit several substantive conclusions concerning the role of stress and locomotor activity in circadian clock resetting by arousal in hamsters. First, it is clear that stress does not mediate phase shifts to arousal procedures, because the stress-loaded restraint procedure failed to significantly alter circadian phase despite sustained behavioral arousal and a strong cortisol response. This result is consistent with evidence that neither cortisol nor dexamethasone entrain or phase shift free running rhythms in hamsters (2, 16) and that psychosocial stress during the mid-rest period does not phase shift circadian rhythms in another species, the rat (23, 24; but note that there are no reports yet that rats exhibit phase shifts to arousal in the mid-rest period). Previous studies using physical restraint during the mid-rest period also reported no phase shifts in hamsters, but the degree to which the conventional restraint procedure is stressful throughout a 3-h test is uncertain given our observations that all hamsters immobilized without additional intervention exhibited behavioral sleep within, on average, 37 min. The one discordant empirical finding is that intraperitoneal injections of saline late in the rest period can induce small phase shifts (~60 min) in hamsters (12, 22). These shifts were positively correlated with a cortisol response but not with acute induction of wheel running. However, this procedure was not effective in the mid-rest phase, and it is unknown whether the shifts were dependent on cortisol release, locomotor activity other than running, or some other factor.

A second substantive conclusion is that stress does not prevent large phase shifts to arousal in the mid-rest period. We were led to consider this novel hypothesis by the prior evidence that restraint, a presumably arousing procedure, does not produce shifts, and by our anecdotal observations that a few hamsters that appeared very stressed by the sleep deprivation procedure of gentle handling (based on defensive body postures and vocalizations) did not exhibit phase shifts (M. C. Antle, unpublished observations). Tantalizing support for this hypothesis was provided by our first experiment, showing that a cortisol synthesis inhibitor metyrapone, at doses that block the corticosterone response to restraint in rats (8), may potentiate phase shifts to sleep deprivation by gentle handling. However, our subsequent studies appear to refute the hypothesis; large shifts were evident in socially subordinate hamsters transferred between the cages of two dominant hamsters for 3 h (a strong stressor; 17) and in hamsters confined to a novel open field for 3 h (a milder stressor). Why metyrapone was effective in the first experiment remains unclear, but it is worth noting that the mean phase shift in that condition was not

greater than that exhibited by other groups subjected to sleep deprivation without metyrapone (see 3, 27).

A third substantive conclusion is that some minimal level of locomotion is necessary to induce phase shifts by arousal in the mid-rest period. This conclusion may have already been assumed based on the results of prior studies showing that restraint or confinement to a small nest box can block or attenuate phase shifts to activity-inducing stimuli (e.g., triazolam, dark pulses) and that hamsters that fail to run in novel wheels with rare exceptions fail to shift. An obvious conclusion from those results is that activity is necessary for shifts to occur, but such a conclusion oversteps the data. Our behavioral observations revealed that restrained hamsters fall asleep, and we have previously noted that hamsters that fail to run when confined to a novel wheel also fail to stay awake. If hamsters are not kept awake, then the absence of phase shifts to restraint or confinement could be due to the absence of running or the lack of arousal. Thus these studies do not permit a conclusion that locomotor activity is necessary for phase shifting. Indeed, the results of our two previous sleep deprivation studies demonstrated that continuous running activity is not necessary for phase shifting and that very large shifts can be induced despite low levels of activity (3, 27). Nonetheless, hamsters in those studies were able to move about in their cages. The results of the experiments reported here constitute the clearest evidence so far that the expression of locomotor activity is indeed necessary for phase shifts to behavioral arousal. Restrained hamsters kept aroused for 3 h did not phase shift. Hamsters confined to a small platform stayed awake for 3 h but showed only small or no phase shifts relative to the control procedure. Some hamsters subjected to psychosocial stress showed very large phase shifts, and across hamsters, shift magnitude was related to a composite activity measure (chasing and fleeing) and unrelated to social status in the dyads. Some hamsters confined to a novel open field also exhibited very large phase shifts and, again, shifting was related to indexes of forward locomotion (e.g., number of area crossings). Activity does not need to be either continuous or intense, but some minimal level appears to be necessary.

This conclusion suggests an explanation for individual differences in phase shift responses to sleep deprivation by gentle handling procedures. Some hamsters fail to shift, and this has been related to the number of interventions required to prevent behavioral sleep (3). Nonshifters appear sleepier and make more attempts to sleep. We also noted that some hamsters that appeared very stressed required few interventions yet still failed to shift. In both types of hamsters, phase shifts may be small or absent because total locomotor activity over the 3 h is low, in the one case because of sleepiness and in the other because of prolonged freezing responses. In a pilot study, we failed to see phase shifting in response to continuous rewarding lateral hypothalamic stimulation applied to hamsters in the mid-rest period; these hamsters were awake but did not have to produce an operant response to obtain

stimulation, and moved very little during the procedure (M. S. Pollock, M. C. Antle, and R. Mistlberger, unpublished observations). Thus even strongly rewarding stimuli may fail to induce phase shifts in the absence of locomotion or operant responses that require motor output.

These results also have implications for elucidating the neural pathways by which nonphotic stimuli induce phase shifts. There is convergent evidence that the thalamic intergeniculate leaflet (IGL) is a gateway through which arousing stimuli shift the master circadian pacemaker located in the suprachiasmatic nucleus (SCN). The IGL contains neuropeptide Y (NPY)-releasing neurons that project to the SCN (30) and that express FOS protein in hamsters that are aroused for 3 h in the mid-rest period, either by confinement to a novel running wheel (14, 26) or by gentle handling (3). Ablation of the IGL blocks circadian phase or period changes induced by wheel running or saline injections in hamsters, rats, or mice (15, 18, 20, 21, 44). Microinjections of NPY into the SCN area induce phase shifts that mimic those induced by arousal (1), and phase shifts induced by running are blocked by microinjections of NPY-antibody (5). However, several issues remain to be resolved. The aspects of behavioral arousal coded for by IGL efferents to the SCN are unknown. Our behavioral analyses suggest that this pathway may not be activated (e.g., as assessed by FOS induction) by arousal procedures associated with little or no movement and no phase shifts. If NPY efferents are activated by such procedures, this would suggest that NPY release is necessary but not sufficient for clock resetting to arousal, and that one or more other pathways must participate. In mice, serotonin efferents also appear to be necessary (10, 20), although the evidence for this in hamsters is equivocal at best (reviewed in Ref. 29). Also remaining to be identified are the pathways that transmit information about behavioral state to the IGL. There are several candidates from among the monoamine and neuropeptide systems that regulate behavioral state (25, 42). Again, our behavioral data suggest that critical pathways may be preferentially activated during arousal accompanied by locomotion. Whether such pathways encode motor output or somatosensory or proprioceptive feedback from movement is an open question. The lack of phase shifts in restrained hamsters subjected to rotational stimulation, at a rate known to disrupt spatial memory in rodents (e.g., Ref. 11), suggests that vestibular correlates of movement are not a sufficient stimulus.

Phase advance shifts induced in hamsters by the procedures of gentle handling or confinement to a novel wheel can be detected within 1 h of the end of the procedure, using the circadian rhythm of light-induced c-Fos expression in the SCN as a cellular level phase marker (3; see also 22). In the current study, confinement to a novel wheel was not associated with a significant elevation of Cort, and sleep deprivation by gentle handling was associated with elevated Cort only in three of eight hamsters sampled at ZT9. If by this time the circadian clock were already phase advanced

2–3 h by these procedures, as inferred from the FOS data, then a significant elevation of Cort might be expected by virtue of the new circadian phase (i.e., basal Cort exhibits a significant elevation as the pacemaker approaches the beginning of the subjective night). The absence of elevated Cort, particularly in the wheel confinement group, suggests a lag in resetting of the Cort rhythm, by comparison with the circadian rhythm of light-induced FOS that is intrinsic to SCN neurons.

Manipulations of behavioral state can have potent effects on the circadian system of Syrian hamsters. There are, however, significant species differences; whereas other rodents, including nocturnal rats and mice and the diurnal European ground squirrel, can also be entrained by a daily exercise schedule, these animals do not exhibit large or reliable phase shifts in response to arousal during the mid-subjective day (9, 13, 19, 28). The Syrian hamster may be an outlier species in the responsiveness of its circadian clock to behavioral manipulations in the subjective day, but only a handful of species have been examined so far. Several studies of humans have produced evidence consistent with a phase shifting effect of exercise in the subjective night or late in the subjective day (6, 7, 40). The design of these studies was informed by the early evidence that continuous wheel running was highly predictive of phase shifting in hamsters confined for 3 h to a novel wheel. However, our sleep deprivation studies demonstrate that continuous running is not required to induce maximal phase advance shifts (3, 27). The results of the present study extend this analysis by showing that the expression of locomotor activity is necessary, although even low levels appear sufficient. Perhaps casual walking, standing, or other low-intensity activities may prove to be sufficient in humans as well.

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REFERENCES

1. **Albers H and Ferris C.** Neuropeptide Y: role in light-dark cycle entrainment of hamster circadian rhythms. *Neurosci Lett* 50: 163–168, 1984.
2. **Albers HE, Yogev L, Todd RB, and Goldman BD.** Adrenal corticoids in hamsters: role in circadian timing. *Am J Physiol Regul Integr Comp Physiol* 248: R434–R438, 1985.
3. **Antle MC and Mistlberger RE.** Circadian clock resetting by sleep deprivation in the Syrian hamster. *J Neurosci* 20: 9326–9332, 2000.
4. **Bergmann BM, Winter JB, Rosenberg RS, and Rechtschaffen A.** NREM sleep with low-voltage EEG in the rat. *Sleep* 10: 1–11, 1987.
5. **Biello SM, Janik D, and Mrosovsky N.** Neuropeptide Y and behaviorally induced phase shifts. *Neuroscience* 62: 273–279, 1994.

6. Buxton OM, Frank SA, L'Hermite-Baleriaux M, Leproult R, Turek FW, and Van Cauter E. Roles of intensity and duration of nocturnal exercise in causing phase delays of human circadian rhythms. *Am J Physiol Endocrinol Metab* 273: E536–E542, 1997.
7. Buxton OM, Lee CW, L'Hermite-Baleriaux M, Turek FW, and Van Cauter E. Exercise elicits phase shifts and acute alterations of melatonin that vary with circadian phase. *Am J Physiol Regul Integr Comp Physiol* 284: R714–R724, 2003.
8. Calvo N, Martijena ID, Molina VA, and Volosin M. Metyrapone pretreatment prevents the behavioral and neurochemical sequelae induced by stress. *Brain Res* 800: 227–235, 1998.
9. Edgar DM and Dement WC. Regularly scheduled voluntary exercise synchronizes the mouse circadian clock. *Am J Physiol Regul Integr Comp Physiol* 261: R928–R933, 1991.
10. Edgar DM, Reid MS, and Dement WC. Serotonergic afferents mediate activity-dependent entrainment of the mouse circadian clock. *Am J Physiol Regul Integr Comp Physiol* 273: R265–R269, 1997.
11. Gibson BM, Shettleworth SJ, and McDonald RJ. Finding a goal on dry land and in the water: differential effects of disorientation on spatial learning. *Behav Brain Res* 123: 103–111, 2001.
12. Hastings MH, Mead SM, Vindlacheruvu RR, Ebling FJ, Maywood ES, and Grosse J. Nonphotic phase shifting of the circadian activity rhythm of Syrian hamsters: the relative potency of arousal and melatonin. *Brain Res* 591: 20–26, 1992.
13. Hut RA, Mrosovsky N, and Daan S. Nonphotic entrainment in a diurnal mammal, the European ground squirrel (*Spermophilus citellus*). *J Biol Rhythms* 14: 409–419, 1999.
14. Janik D, Mikkelsen JD, and Mrosovsky N. Cellular colocalization of Fos and neuropeptide Y in the intergeniculate leaflet after nonphotic phase-shifting events. *Brain Res* 698: 137–145, 1995.
15. Janik D and Mrosovsky N. Intergeniculate leaflet lesions and behaviorally-induced shifts of circadian rhythms. *Brain Res* 651: 174–182, 1994.
16. Janik D, Nest KJ, and Janiga MA. Exogenous corticosteroid and shifts of circadian rhythms in hamsters. *Chronobiol Int* 18: 203–213, 2001.
17. Koolhaas JM, De Boer SF, De Rutter AJ, Meerlo P, and Sgoifo A. Social stress in rats and mice. *Acta Physiol Scand Suppl* 640: 69–72, 1997.
18. Kuroda H, Fukushima M, Nakai M, Katayama T, and Murakami N. Daily wheel running activity modifies the period of free-running rhythm in rats via intergeniculate leaflet. *Physiol Behav* 61: 633–637, 1997.
19. Marchant EG and Mistlberger RE. Morphine phase-shifts circadian rhythms in mice: role of behavioural activation. *Neuroreport* 7: 209–212, 1995.
20. Marchant EG, Watson NV, and Mistlberger RE. Both neuropeptide Y and serotonin are necessary for entrainment of circadian rhythms in mice by daily treadmill running schedules. *J Neurosci* 17: 7974–7987, 1997.
21. Maywood ES, Smith E, Hall SJ, and Hastings MH. A thalamic contribution to arousal-induced, non-photic entrainment of the circadian clock of the Syrian hamster. *Eur J Neurosci* 9: 1739–1747, 1997.
22. Mead S, Ebling FJ, Maywood ES, Humby T, Herbert J, and Hastings MH. A nonphotic stimulus causes instantaneous phase advances of the light-entrainable circadian oscillator of the Syrian hamster but does not induce the expression of c-fos in the suprachiasmatic nuclei. *J Neurosci* 12: 2516–2522, 1992.
23. Meerlo P and Daan S. Aggressive and sexual social stimuli do not phase shift the circadian temperature rhythm in rats. *Chronobiol Int* 15: 231–240, 1998.
24. Meerlo P, van den Hoofdakker RH, Koolhaas JM, and Daan S. Stress-induced changes in circadian rhythms of body temperature and activity in rats are not caused by pacemaker changes. *J Biol Rhythms* 12: 80–92, 1997.
25. Meyer-Bernstein EL and Morin LP. Differential serotonergic innervation of the suprachiasmatic nucleus and the intergeniculate leaflet and its role in circadian rhythm modulation. *J Neurosci* 16: 2097–2111, 1996.
26. Mikkelsen JD, Vrang N, and Mrosovsky N. Expression of Fos in the circadian system following nonphotic stimulation. *Brain Res Bull* 47: 367–376, 1998.
27. Mistlberger R, Belcourt J, and Antle M. Circadian clock resetting by sleep deprivation without exercise: dark pulses revisited. *J Biol Rhythms* 17: 227–237, 2002.
28. Mistlberger RE. Effects of daily schedules of forced activity on free-running rhythms in the rat. *J Biol Rhythms* 6: 71–80, 1991.
29. Mistlberger RE, Antle MC, Glass JD, and Miller JD. Behavioral and serotonergic regulation of circadian rhythms. *Biol Rhythm Res* 31: 240–283, 2000.
30. Moore RY and Card JP. Intergeniculate leaflet: an anatomically and functionally distinct subdivision of the lateral geniculate complex. *J Comp Neurol* 344: 403–430, 1994.
31. Mrosovsky N. Phase response curves for social entrainment. *J Comp Physiol [A]* 162: 35–46, 1988.
32. Mrosovsky N. Methods of measuring phase shifts: why I continue to use an Aschoff type II procedure despite the skepticism of referees. *Chronobiol Int* 13: 387–392, 1996.
33. Mrosovsky N. Locomotor activity and non-photic influences on circadian clocks. *Biol Rev Camb Philos Soc* 71: 343–372, 1996.
34. Mrosovsky N and Salmon P. Triazolam and phase-shifting acceleration re-evaluated. *Chronobiol Int* 7: 35–41, 1990.
35. Rees SG, Lavery RJ, and Mrosovsky N. Running activity mediates the phase-advancing effects of dark pulses on hamster circadian rhythms. *J Comp Physiol [A]* 165: 811–818, 1989.
36. Rees SG and Mrosovsky N. Effects of induced wheel running on the circadian activity rhythms of Syrian hamsters: entrainment and phase response curve. *J Biol Rhythms* 4: 39–48, 1989.
37. Suchecki D, Lobo L, Hipolide D, and Tufik S. Increased ACTH and corticosterone secretion induced by different methods of paradoxical sleep deprivation. *J Sleep Res* 7: 276–281, 1998.
38. Tobler I and Jaggi K. Sleep and EEG spectra in the Syrian hamster (*Mesocricetus auratus*) under baseline conditions and following sleep deprivation. *J Comp Physiol [A]* 161: 449–459, 1987.
39. Van Reeth O, Hinch D, Tecco JM, and Turek FW. The effects of short periods of immobilization on the hamster circadian clock. *Brain Res* 545: 208–214, 1991.
40. Van Reeth O, Sturis J, Byrne MM, Blackman JD, L'Hermite-Baleriaux M, Leproult R, Oliner C, Refetoff S, Turek FW, and Van Cauter E. Nocturnal exercise phase delays circadian rhythms of melatonin and thyrotropin secretion in normal men. *Am J Physiol Endocrinol Metab* 266: E964–E974, 1994.
41. Van Reeth O and Turek FW. Stimulated activity mediates phase shifts in the hamster circadian clock induced by dark pulses or benzodiazepines. *Nature* 339: 49–51, 1989.
42. Vrang N, Mrosovsky N, and Mikkelsen JD. Afferent projections to the hamster intergeniculate leaflet demonstrated by retrograde and anterograde tracing. *Brain Res Bull* 59: 267–288, 2003.
43. Wickland C and Turek FW. Phase-shifting effect of triazolam on the hamster's circadian rhythm of activity is not mediated by a change in body temperature. *Brain Res* 560: 12–16, 1991.
44. Wickland C and Turek FW. Lesions of the thalamic intergeniculate leaflet block activity-induced phase shifts in the circadian activity rhythm of the golden hamster. *Brain Res* 660: 293–300, 1994.