Effects of hypoxia and hypercapnia on circadian rhythms in the golden hamster (Mesocricetus auratus)

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Jarsky, Tim M. and Richard Stephenson. Effects of hypoxia and hypercapnia on circadian rhythms in the golden hamster (Mesocricetus auratus). J Appl Physiol 89: 2130–2138, 2000.—This study was designed to determine whether respiratory stimuli can influence the mammalian circadian timing system. Three-hour pulses of hypoxia (inspired O2 concentration = 8%) or hypercapnia (inspired CO2 concentration = 11%) were presented for 7 days at midsubjective day (circadian time 6–9) under constant darkness. Hypoxic and hypercapnic pulses caused cumulative phase delays of 46.4 ± 6.9 and 25.9 ± 12.3 min, respectively. Distance run per day was significantly reduced on hypoxic and hypercapnic pulse days, compared with nonpulsed days. Phase shifts were correlated with the reduction in daily running activity (multiple \( r^2 = 0.521, P = 0.036 \)), metabolic depression (multiple \( r^2 = 0.772, P < 0.001 \)), and reduction in body temperature (multiple \( r^2 = 0.539, P = 0.027 \)), but not lung ventilation (multiple \( r^2 = 0.306, P = 0.414 \)) during pulses. We conclude that hypoxia and hypercapnia can influence the phase and quantity of activity in free-running hamsters.

Body temperature; metabolism; nonphotic stimuli; respiration

RECENT WORK IN HUMANS, RATS, and ducks has demonstrated a time-of-day-dependent change in respiratory responsiveness to hypoxic and hypercapnic stimuli (24, 29, 33, 38), suggesting that the respiratory system is modulated by the circadian timing system.

It is well established that manipulation of many clock-controlled variables will affect clock function (21), which raises the possibility that there may be a reciprocal interaction between the circadian timing and the respiratory control systems. An effect of respiratory stimuli on circadian timing mechanisms could have important implications, for example, by contributing to sleep disruption in disorders, such as sleep apnea syndromes and nocturnal asthma, in which hypoxia and hypercapnia occur (23), as well as for the design of long-term respiratory studies in which daily repetitions of stimuli are used.

Several studies report data that suggest that chemical respiratory stimuli could have long-lasting effects on the circadian clock. A study on simulated high altitude (7,620 m) in a hypobaric chamber, during which the oxygen masks of three human subjects were removed for 2–3 min, concluded that acute exposure to severe hypoxia caused a transient phase shift in several physiological variables (peak expiratory air flow, grip strength, and oral temperature) (2). However, the subjects were maintained in a light-dark (LD) cycle after the test, thus complicating interpretation of apparent phase shifts.

Another study examined the potential role of elevated CO2 concentration ([CO2]) in circadian rhythm disturbances (32). Four male subjects were studied in an environmental chamber before and during 24 days of elevated inspired [CO2] (~2%). Activity (wrist actigraphy), rectal temperature, and urine analyses (cortisol, catecholamines, and 6-hydroxymelatonin sulphonate) were reported. Small effects on circadian temperature rhythm amplitudes were observed, but confounding factors, including LD cycle and competing experimental objectives, rendered the data inconclusive.

Recently, two groups (20, 31) concluded that prolonged exposure (7 days and 63 h, respectively) to hypoxia or hypercapnia can reduce the amplitude of circadian temperature rhythms in rats. One of these studies (31) kept the animals on a 12:12-h LD cycle, preventing an analysis of the effects of hypoxia on circadian phase and period. In the other study (20), poststimulus period and phase were found to be unaffected under free running conditions. However, a prolonged hypoxic stimulus could conceivably have had opposing effects on phase at different times of day that resulted in no net phase change. Analyses of phase and period during the stimulus were prevented by the severe attenuation of the rhythm amplitude (20).

To reexamine this question, we studied wheel running behavior in golden hamsters (Mesocricetus auratus), because this has been shown to be a reliable and precise marker of circadian phase in this species. Hamsters were held in an environment free of time cues and were exposed to scheduled pulses of hypoxia or hypercapnia to test the hypothesis that respiratory stimuli can induce

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long-lasting changes in the phase, period, and quantity of wheel running activity.

MATERIALS AND METHODS

Animals and Housing

Eight male, virus-free golden hamsters (Mesocricetus auratus) were obtained from Charles River Laboratories (Montreal, PQ) after the experimental protocols were approved by the University of Toronto Animal Care Committee. All animals participated in experiments 1 and 2 and were ~40–45 days old on arrival at the laboratory. They were housed singly in plastic cages (45 × 25 × 20 cm) equipped with 17.5-cm-diameter running wheels. The running surface of the wheels was covered with plastic mesh (4 mm). Purina 5001 pellets and water were available ad libitum.

Eight hamster cages were placed in a single sealed animal chamber. Cage walls were opaque, thus preventing visual contact between experimental subjects. The chamber was impermeable to external light and was equipped with timer-controlled fluorescent lights that provided ~140 lux light intensity at the level of the cage floor. Four fans ensured full mixing of the air in the chamber and cages, and, except during pulses, the animal chamber was ventilated continuously with air at a rate sufficient to maintain [CO2] < 0.5%. Although ammonia was not measured, the high ventilation rate and mixing fans were assumed to prevent accumulation of this gas as they did for CO2. The air or test gas was distributed evenly within the chamber via a multiport ventilation duct and four mixing fans. Gas exhaust ports were designed to minimize pressure fluctuations when gas flow rates were adjusted and to prevent light from entering the chamber. Temperature within the closed chamber was 20–22°C.

Respiratory stimuli (pulses) were presented simultaneously to all hamsters by manipulation of the oxygen concentration ([O2] = 8% for hypoxia) or [CO2] (11% for hypercapnia) of the air within the animal chamber. Immediately before the pulse, normal chamber air ventilation was terminated. Hypoxia was induced by briefly blowing pure N2 into the chamber, and hypercapnia was induced by briefly blowing pure CO2 into the chamber. No attempt was made to maintain isooxic conditions, and elevation of [CO2] during hypercapnic pulses produced a mild reduction in [O2] (to ~17%). Gases were supplied from pressurized gas cylinders, and control of [O2] or [CO2] was automated. Gas flow to the chamber (air, N2 or CO2) was controlled by a series of solenoid valves. Power supply to the solenoid valves was controlled by a timer that was set to a cycle of 3 h on, 21 h off. A paramagnetic O2 analyzer (model 572, Servomex) and an infrared CO2 analyzer (model CD-3A, Ametek) were used to monitor chamber [O2] and [CO2], respectively. The analog voltage outputs of the analyzers were connected to a custom-built electronic comparator that closed the solenoid valve supplying N2 or CO2 when gas concentrations exceeded the user-defined limits (i.e., <8% for [O2] and >11% for [CO2] in the present study). A small leak prevented further depletion of O2 or accumulation of CO2 due to animal metabolism, and the solenoids were briefly triggered approximately every 20 min to maintain gas concentrations at their target levels. At the end of the 3-h pulse, airflow was reinstated. “Sham” pulses using compressed air instead of N2 or CO2, were used as a control for factors such as noise, humidity, or pressure changes (10).

Experiment 1: Effect of Scheduled Respiratory Stimuli on Wheel Running Behavior

A modified Aschoff type I experimental design (1) was used. The onset of wheel running activity was used as a circadian phase marker and was arbitrarily defined as circadian time (CT) 12 during constant darkness (DD). Activity onset was defined as a continuous 5-min bout of wheel running, followed by at least one additional 5-min bout within the subsequent 30 min (26). Wheel revolutions were detected by micro switches and were recorded on a Maclab/8 (AD Instruments) data acquisition system.

Hamsters were kept in a LD cycle (14-h light, 10-h darkness) to align their circadian rhythms. Cages were changed, and then the hamsters were released into DD for at least 7 days or until a stable free-running period was apparent. The hamsters (now aged 250–255 days) were then exposed to daily 3-h pulses of hypoxia ([O2] = 8%) for 7 days, starting at ~CT6. The respiratory stimuli were given at CT6 because established nonphotic zeitgebers are known to be effective at this time (21). CT6 was estimated for each hamster on the first pulse day by extrapolation of the free running onset times of the previous 5 days; the pulse was initiated at the average CT6 for the group and then at the same clock time each day thereafter. After the last pulse day, hamsters were allowed free run in DD for a minimum of 7 additional days. Cages were then changed, and the animals were returned to LD for at least 7 days. This procedure was repeated in the same animals for pulses of hypercapnia ([CO2] = 11%, [O2] = 17%) at age 285–290 days and for sham (compressed air) pulses at age 337–342 days.

In preliminary control studies, two different groups of eight male hamsters were used. In one group, the phases of wheel running onset were separated by entraining the animals to individual 14:10-h LD cycles in which lights off occurred at different times of day. The animals were then placed in the animal chamber in DD, and the sham test procedure was conducted as described above. The first sham pulse began at the following CTs: 0, 1, 5, 7, 8, 12, 18. In another group of hamsters, the free running period (T) was >24 h [i.e., pulse period (T) < τ]. In another group of hamsters, τ was <24 h when sham experiments were conducted (i.e., T > τ).

Experiment 2: Physiological Effects of Hypoxic and Hypercapnic Pulses at CT6

A whole body plethysmograph was used to measure lung ventilation and metabolic rate. Six 850-ml sealed Plexiglas cylinders served as animal chambers (n = 4) and reference chambers (n = 2). One reference chamber was a nonventilated thermobarometer for use during lung ventilation measurements. The other reference chamber served as a source of inlet gas for respiratory gas exchange measurements. All chambers were submerged in water at room temperature (22–23°C). The chambers were ventilated in parallel using premixed compressed gases (air: [O2] = 21%, [CO2] = 0%; hypoxia: [O2] = 8%, [CO2] = 0%; hypercapnia: [O2] = 17%, [CO2] = 11%). Gas was dried (using a column of Drierite before flow measurements were taken (1.5 l/min) and then humidified before it entered the animal chambers. A small fan was used in each chamber to ensure complete mixing of the gas.

Respiratory gas exchange (metabolic rate) was estimated by open-flow respirometry (37). The time constant of the system was 35 s; thus gas concentrations were at steady state by 3 min after a change in flow. [O2] and [CO2] of inlet gas and the outlet gases of each of the four animal chambers were measured sequentially for ~5 s each. [O2] and [CO2] were
measured using O<sub>2</sub> and CO<sub>2</sub> analyzers (models S-3A/1 and 3D-3A, Ametek). The gas passed through a desiccating column (Drierite) immediately before entering the analyzers.

Gas exchange measurements were immediately followed by lung ventilation measurements. This involved closing the inlet and outlet of an animal chamber and recording the pressure deflections induced by lung ventilation within the closed chamber (3, 4). A differential pressure transducer (model DP45–14, Validyne Engineering) was used to measure the difference in pressure between the thermobarometer reference chamber and an animal chamber. Pressure deflections were calibrated in each animal chamber by injections of 1 ml of air with the animals in place. Animal chamber temperatures were assumed to equal that measured in the reference chamber, as confirmed in preliminary studies. Relative humidity in each animal chamber was assumed to equal that measured in the combined outflow gas.

Body temperature (T<sub>b</sub>) of each animal was monitored throughout the experiment by calibrated radiotransmitters (model TA10TA-P20, Data Sciences International) that were implanted into the peritoneal cavity while the hamster was under halothane anaesthesia. The animals were given 2 wk to recover from the surgery before experiments began. The hamsters (now aged 379–384 days) were weighed and then placed into the animal chambers under dim red light ~1 h before CT6. A lid shielded the animal chamber, and a small hole was used for visual observation of the animals under dim red light. The hamsters were exposed to 3-h pulses of air, hypoxia, or hypercapnia on 3 separate days, and at least 2 days separated experiments for each animal.

Gas exchange and ventilatory measurements were taken three times before the pulse. Pulses were initiated at CT6, and, after 5 min, gas exchange and ventilation were measured. Measurements were repeated every 20 min thereafter for 3 h. Air was reintroduced to the system after the last pulse measurement was taken at 3 h, and measurements were then taken at 5, 20, 40, and 60 min of the recovery period. After the experiments, the hamsters were removed from the animal chamber and returned to their home cages.

Data Analysis

T calculation. Although the respiratory stimuli began at the same clock time each day, the time to final pulse concentration was variable due to changes in inlet gas flow rates. Pulse onset was arbitrarily defined as the time that the chamber gas concentration reached 50% of the final pulse concentration. T was calculated using the slope of a least-squares linear regression fitted through the seven sequential daily pulse onset times.

τ calculation. Activity period (τ) was calculated as the slope of a least-squares linear regression fitted through the wheel running onset times. “Prepulse” τ was calculated using the activity onset times on the 5 days immediately preceding the first pulse day. “Pulsed” τ was based on data from the last 5 of the 7 days on which respiratory pulses were delivered. “Postpulse” τ was calculated for activity onsets on the third through seventh days after the last pulse day. Pulsed τ was compared with T and the prepulse τ to evaluate whether entrainment had occurred.

Phase shift calculation. Both pre- and postpulse regression lines were extrapolated to the first pulse day. The phase shift was defined as the difference between the onset times predicted by the two regression lines for the first pulse day.

Activity level calculation. Activity level was defined as total wheel revolutions per day. Mean prepulse activity levels were calculated using the 5 days immediately before the first pulse day. Mean pulsed activity levels (total wheel revolutions per day) were based on data from the last 5 of the 7 pulse days. Prepulse and pulsed activity levels were compared to determine whether the respiratory stimuli affected the quantity of wheel running.

Calculation of ventilatory parameters. Inspired ventilation (V<sub>I</sub>) = f × VT, where f is the number of breaths per min, and VT is the tidal volume (ml, STPD). VT was calculated using the equations developed by Drorbaugh and Fenn (4)

\[
VT = \frac{(P_w/P_{cal})V_{cal}[(T_b(P_B - P_{H_2O}))]}{[T_b(P_B - P_{H_2O}) - T_b(P_B - P_{H_2O})]}
\]

where P<sub>n</sub> is the respiratory pressure deflection; P<sub>cal</sub> is the pressure deflection of a known volume of gas; V<sub>cal</sub> is the volume of gas injected into the chamber; T<sub>b</sub> is body temperature (°K); T<sub>c</sub> is animal chamber temperature (°K); P<sub>B</sub> is barometric pressure in the chamber (mmHg); P<sub>H_2O</sub> is saturated water vapor pressure of the gas in the alveoli (mmHg); and P<sub>C_H_2O</sub> is water vapor pressure of the gas in the chamber (mmHg).

Calculation of metabolic rate. Metabolic rate was estimated as the rate of oxygen consumption (V<sub>O_2</sub>, ml/min, STPD), as measured by open flow respirometry (37)

\[
V_{O_2} = \frac{[V(F_{IO_2} - F_{EO_2})]/[1 - (1 - RE)F_{EO}]}{(1 - (1 - RE)F_{EO})]
\]

where V is dry gas flow entering the animal chamber (ml/min, STPD); F<sub>IO_2</sub> is the fractional concentration of oxygen entering the chamber; and F<sub>EO</sub> is the fractional concentration of oxygen leaving the chamber.

RE is the respiratory exchange ratio and was calculated as follows

\[
RE = \frac{(F_{ECO_2} - F_{ICO_2})/(F_{ICO_2} - F_{EO_2})}{(1 - (1 - RE)F_{EO})]
\]

where F<sub>ECO_2</sub> is the fractional concentration of carbon dioxide leaving the chamber, and F<sub>ICO_2</sub> is the fractional concentration of carbon dioxide entering the chamber.

Statistical analysis. All statistics were computed using SYSTAT software (SPSS). Significance was tested using a general linear model, with sample time and hamster as factors. A general linear model was used because it accounts for the variability between individuals and allows for missing data points. When a significant difference was obtained, a Tukey’s post hoc test was used for multiple pairwise comparisons. Differences are considered significant at the 95% confidence level (P < 0.05).

Because the same animals were used in experiments 1 and 2, changes in activity and physiological parameters caused by the respiratory stimuli were analyzed for correlations with the phase changes obtained. Changes in physiological parameters were calculated by subtracting the mean prepulse value from the mean pulsed value for each animal. Activity changes were calculated as described in Activity level calculation. Correlations were tested using a general linear model, with the hamster acting as the categorical variable.
RESULTS

Experiment 1: Effect of Scheduled Respiratory Stimuli on Phase, Period, and Quantity of Wheel Running

Controls. Daily wheel revolutions and the phase and period of onset of wheel running were unaffected by 3-h pulses of air beginning at CT6 on 7 consecutive days (Fig. 1). This was also confirmed in two additional groups of hamsters: when the first pulse began at a variety of CTs (0, 1, 5, 7, 8, 10, 12, and 18) and when T > τ and T < τ (data not shown). Consequently, the prepulse days from each experiment were used to measure the effect of each respiratory stimulus to minimize the effect of possible differences (e.g., age-related) between experiments. In addition, a separate group of hamsters was exposed to a novel wheel for 3 h, beginning at CT6, which induced phase advances of 3.2 ± 0.1 h. This positive control (12) demonstrated that large phase shifts are possible under the present laboratory conditions.

Hypoxia. Seven consecutive days of 3-h hypoxic pulses (\[O_2\] = 8%) beginning at CT6 caused a cumulative phase delay of 46.4 ± 6.9 min (Figs. 1 and 2). Hamsters exhibited a prepulse τ of 23.88 ± 0.01 h. Postpulse τ (23.86 ± 0.02 h) was not significantly different from prepulse τ. In the last five hypoxic pulse days, τ (23.93 ± 0.02 h) was not significantly different from T (23.96 ± 0.00 h). Daily wheel revolutions on days with hypoxic pulses (6,395 ± 901 wheel revolutions, 3.71 ± 0.53 km) were significantly reduced from the prepulse values (9,180 ± 1,378 wheel revolutions, 5.32 ± 0.80 km).

Hypercapnia. Seven consecutive days of 3-h hypercapnic pulses at CT6 caused a cumulative but nonsignificant (P = 0.143) phase delay of 25.9 ± 12.3 min (Figs. 1 and 2). Postpulse τ (23.87 ± 0.02 h) was not significantly different from prepulse τ (23.89 ± 0.01 h). In the last five hypercapnic pulse days, τ (23.94 ± 0.02 h) was not significantly different from T (23.95 ± 0.00 h). Daily wheel revolutions on days with hypercapnic pulses (6,504 ± 1,127 wheel revolutions, 3.77 ± 0.67 km) were significantly lower than the prepulse values (8,255 ± 1,703 wheel revolutions, 4.79 ± 0.99 km).

Fig. 1. Cumulative phase shifts (mean ± SE; n = 8) after 7 consecutive days of 3-h pulses [delivered at circadian time (CT)6–CT9] of hypoxia (\[O_2\] concentration = 8%), hypercapnia (\[CO_2\] concentration = 11%), and control tests in which air was substituted for test gas. *Significantly different from control.

Fig. 2. Actograms of representative animals illustrating phase change of wheel running activity due to hypoxic (A) and hypercapnic (B) pulses. Animals were free running in constant darkness. Each line of the actogram represents a 24-h day, and consecutive days are plotted beneath each other. The ordinate for each day is plotted as a histogram of distance run (m) per 2 min, vertical scale 0–150 m. Horizontal, double-headed arrows indicate the time of hypoxic or hypercapnic pulses. Dashed regression lines illustrate the wheel running rhythm periods over 5 days before (prepulse) and after (postpulse) the respiratory pulses. The cumulative phase delay induced by the pulses was calculated by extrapolation of the regressions to the first pulse day.

Experiment 2: Physiological Effects of Hypoxic and Hypercapnic Pulses at CT6

Controls. Figure 3 shows that a 3-h pulse of air (sham pulse) at CT6 had no significant effect on lung ventilation, \(\dot{V}_{\text{O}_2}\), or \(T_b\). Furthermore, for the group as a whole, the sham pulse values were not significantly different from those measured during the prepulse interval in the hypoxia and hypercapnia experiments. Consequently, to minimize the effect of possible differences between days in individual animals, the prepulse measurements from each experiment were used to measure the effect of each respiratory stimulus.

\(T_b\) declined slightly, but not significantly, during the first 65 min after the animals were introduced into the animal chamber and remained relatively constant (35.9 ± 0.1°C) for the remainder of the control experiment.
inspired ventilation (V′I, BTPS). and concluded at 240 min.

A hypoxia (98.4 % O2 concentration = 8%) began at CT6. Pulses were initiated at 60 min and concluded at 240 min. A: body temperature (Tb). B: minute inspired ventilation (V′I). C: rate of oxygen consumption (V′O2). D: metabolism-specific ventilation (V′I/V′O2). Values are means ± SE; n = 8. *Significantly different from mean prepulse value (P < 0.05).

**Hypoxia.** Visual observations indicated that hamsters were initially aroused from sleep by introduction of 8% O2 into the plethysmograph. After 3–4 min, the hamsters curled up and appeared to resume sleeping. Sleep was occasionally interrupted by periods of feeding and grooming. It is assumed that similar behavioral events occurred during pulses in experiment 1, but no attempt was made to account for changes in behavioral state in the data analyses. Hamsters started to shiver immediately after the return to air. Shivering was visible for several minutes.

Hypoxia caused an increase in V′I within 5 min after the start of the pulse (Fig. 3). V′I averaged over the entire pulse was 158.3 ± 3.4 ml/min, which was statistically significantly greater than prepulse values (98.4 ± 7.3 ml/min). V′I further increased, to 322.6 ± 31.7 ml/min, over three times prepulse values, at 5 min after the return to air. V′I returned to prepulse values 40 min after the return to air.

V′O2 was significantly lower than prepulse values (3.9 ± 0.2 ml/min) at 5, 20, and 40 min (2.2 ± 0.1, 2.5 ± 0.2, and 2.5 ± 0.1 ml/min, respectively) after the onset of the hypoxic pulse. V′O2 steadily increased thereafter but remained below prepulse values throughout the pulse. Five minutes after the return to air, V′O2 was elevated to 10.0 ± 0.5 ml/min, which was significantly above prepulse levels. V′O2 returned to prepulse levels by 20 min after the return to air.

Metabolism-specific ventilation (V′I/V′O2) was significantly above prepulse values (27.2 ± 2.7) for the entire pulse (60.3 ± 2.2) for all but one measurement (i.e., at time = 200 min in Fig. 3, 52.2 ± 3.2) and returned to prepulse values within 5 min after the return to air.

Tb was significantly lower than the prepulse value (36.2 ± 0.1°C) after 5 min of hypoxia (33.8 ± 0.7°C) and remained depressed for the duration of the pulse. Tb reached a nadir of 31.7 ± 0.3°C at 235 min (Fig. 3). Tb began to rise by the first measurement in air (5 min) but required 1 h to return to prepulse levels.

**Hypercapnia.** Behavioral responses of the hamsters to 11% [CO2] were similar to those during hypoxia, with the exception that shivering was not observed during posthypercapnic recovery in air.

V′I was significantly elevated relative to prepulse values (279.6 ± 32.0 vs. 139.6 ± 3.3 ml/min, respectively) for the entire hypercapnic pulse (Fig. 3). All postpulse measurements were statistically similar to the prepulse values.

V′O2 was slightly, but not significantly, lower than prepulse values (3.8 ± 0.1 ml/min) by 5 min (2.4 ± 0.1 ml/min) after the onset of hypercapnia. V′O2 then returned to prepulse levels by 40 min after the onset of hypercapnia and remained at that level for the duration of the pulse. V′O2 was variable during the recovery period but did not deviate significantly from the prepulse values.

The V′I/V′O2 response to hypercapnia was biphasic. V′I/V′O2 was significantly raised to 3.5 times the prepulse values after 5 min of hypercapnia (38.3 ± 0.1 vs. 133.5 ± 19.6, respectively), then fell to 64.5 ± 5.5 one hour after the pulse onset, and remained there for the duration of the hypercapnic pulse. V′I/V′O2 returned to prepulse values by 5 min into the recovery period.

Hypercapnia caused a nonsignificant reduction in Tb for the duration of the pulse (Fig. 3). Tb returned to prepulse values (35.3 ± 0.07°C) by 5 min into the recovery period.

**Correlation Analysis**

The phase changes caused by the respiratory stimuli in experiment 1 were significantly correlated with the reductions in daily wheel running activity (Fig. 4) and also correlated with the responses of V′O2 and Tb to hypoxic and hypercapnic pulses in experiment 2. However, the ventilatory responses (V′I) to hypoxia and hypercapnia in experiment 2 were not correlated with phase shifts observed in experiment 1.

**DISCUSSION**

The results of this study support the hypothesis that chemical respiratory stimuli can influence the output of the circadian pacemaker, as measured by the timing and intensity of wheel running activity in the golden hamster.
Respiratory stimuli influence circadian rhythms

This study has shown that respiratory stimuli beginning at CT6 can cause permanent phase delays in the circadian activity rhythm. In the present study, the difference in τ and T was intentionally small, as preliminary studies had shown that phase responses to hypoxia and hypercapnia were small (12). Indeed, this was the rationale for subjecting the animals to several consecutive daily stimuli, instead of the more conventional single pulse. For the hypoxic stimuli, period difference (T − τ) averaged 4.8 min, requiring a cumulative phase delay of 33.6 min for full entrainment over 7 days. For hypercapnic stimuli, T − τ averaged 3.6 min, requiring a cumulative phase delay of 25.2 min for full entrainment over 7 days. The observed permanent phase delay induced by hypercapnia averaged 25.9 min, thus supporting the suggestion that the animals were entrained. However, the observed permanent phase delay induced by hypoxia averaged 46.4 min, which exceeds that required for entrainment and suggests a progressive increase in the phase angle between hypoxia onset and wheel running onset. This could imply that either insufficient time was allowed to achieve stable entrainment or, alternatively, that hypoxia does not entrain the circadian clock but, instead, has a nonspecific effect on phase. The latter suggestion is supported by preliminary data in which small phase advances were observed after 3-h hypoxic pulses initiated at CT11 and CT15 (12). Additional studies in a different group of animals have also shown that phase advances are not observed at any CT, and hamsters did not entrain to hypoxia when T < τ (A. Y. Dunn and R. Stephenson, unpublished data).

Period of Wheel Running Rhythm

All eight animals exhibited a τ < 24 h in DD. Seven consecutive daily pulses of hypoxia or hypercapnia resulted in a significant lengthening of the apparent wheel running period. In both cases, scheduled pulses of respiratory stimulant appeared to entrain the circadian rhythm in wheel running (Fig. 2), although although it is recognized that a 7-day test is too short to make firm conclusions regarding entrainment. Further research is needed to confirm that hamsters will exhibit stable entrainment to scheduled hypoxic or hypercapnic pulses. In the week after the last pulse day, the animals resumed their free run with a period that was not significantly different from that during the prepulse days. The absence of significant after effects on τ suggests that the intrinsic τ was unchanged by scheduled respiratory stimuli. Similar pre- and poststimulus τ was also reported for rats exposed to a week of continuous exposure to hypoxia or hypercapnia (20). Thus the effects of the scheduled pulses on wheel running onset times were probably mediated by phase delays rather than a lengthening of τ.

Phase of Wheel Running Rhythm

Wheel running behavior occurred predominantly in the subjective night (Fig. 2), confirming previous observations in the golden hamster (26). Under control conditions, the hamsters ran an average of 4.41 ± 1.05 km per subjective night in DD, 71% of which was concentrated in the first 4 h of the night. A 3-h pulse of hypoxia or hypercapnia during the subjective day (CT6–CT9) did not stimulate wheel running during the pulse (an example is shown in Fig. 2) but, instead, had a delayed effect on the distance run in the subsequent night. It is unknown whether this effect was specific to wheel running or whether total activity was suppressed on the nights after pulses. It is possible that wheel running was replaced by other active behaviors.

Saiki and Mortola (29) have shown that acute hypoxia can abolish the day-to-night differences in lung ventilation, metabolic rate, and T_b in 6-day-old rat pups. Furthermore, prolonged (7 days) hypoxia and hypercapnia reduced the amplitudes of circadian rhythms in T_b, V_{O2}, and general body movements in adult rats (20). Salloum et al. (31) also observed a suppression of T_b rhythm amplitude in rats exposed to 63 h of continuous hypoxia. The present study differs from these studies in that our hamsters were normoxic and normocapnic for >3 h before activity onset during each of the pulse days, but wheel running activity was still reduced. It is of interest to note that, in a preliminary study, our laboratory found that hamsters began running on the wheel ~2–4 h after the termination of a 3-h hypoxic pulse initiated at CT11 (12). This suggests (but does not prove) that the daily reduction in wheel running activity may be a masking effect of the stimulus on activity, rather than an affect on the amplitude of the output of the circadian pacemaker. Further research is needed to resolve this question.

Quantity of Wheel Running

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All eight animals exhibited a τ < 24 h in DD. Seven consecutive daily pulses of hypoxia or hypercapnia resulted in a significant lengthening of the apparent wheel running period. In both cases, scheduled pulses of respiratory stimulant appeared to entrain the circadian rhythm in wheel running (Fig. 2), although although it is recognized that a 7-day test is too short to make firm conclusions regarding entrainment. Further research is needed to confirm that hamsters will exhibit stable entrainment to scheduled hypoxic or hypercapnic pulses. In the week after the last pulse day, the animals resumed their free run with a period that was not significantly different from that during the prepulse days. The absence of significant after effects on τ suggests that the intrinsic τ was unchanged by scheduled respiratory stimuli. Similar pre- and poststimulus τ was also reported for rats exposed to a week of continuous exposure to hypoxia or hypercapnia (20). Thus the effects of the scheduled pulses on wheel running onset times were probably mediated by phase delays rather than a lengthening of τ.

Phase of Wheel Running Rhythm

This study has shown that respiratory stimuli beginning at CT6 can cause permanent phase delays in the circadian activity rhythm. In the present study, the difference in τ and T was intentionally small, as preliminary studies had shown that phase responses to hypoxia and hypercapnia were small (12). Indeed, this was the rationale for subjecting the animals to several consecutive daily stimuli, instead of the more conventional single pulse. For the hypoxic stimuli, period difference (T − τ) averaged 4.8 min, requiring a cumulative phase delay of 33.6 min for full entrainment over 7 days. For hypercapnic stimuli, T − τ averaged 3.6 min, requiring a cumulative phase delay of 25.2 min for full entrainment over 7 days. The observed permanent phase delay induced by hypercapnia averaged 25.9 min, thus supporting the suggestion that the animals were entrained. However, the observed permanent phase delay induced by hypoxia averaged 46.4 min, which exceeds that required for entrainment and suggests a progressive increase in the phase angle between hypoxia onset and wheel running onset. This could imply that either insufficient time was allowed to achieve stable entrainment or, alternatively, that hypoxia does not entrain the circadian clock but, instead, has a nonspecific effect on phase. The latter suggestion is supported by preliminary data in which small phase delays of similar magnitude were observed after 3-h hypoxic pulses initiated at CT11 and CT15 (12). Additional studies in a different group of animals have also shown that phase advances are not observed at any CT, and hamsters did not entrain to hypoxia when T < τ (A. Y. Dunn and R. Stephenson, unpublished data).
Nonphotic stimuli presented at CT6 usually evoke phase advances in the hamster wheel running rhythm (21), whereas we have found that respiratory stimuli presented at this time cause phase delays. The majority of nonphotic stimuli that have been shown to affect long-term changes in circadian phase, including access to a novel wheel, social interactions, and bolus injections of benzodiazepines (26, 35), have been shown to induce an acute increase in locomotor activity during the stimulus. It is unclear whether activity per se, or a correlated variable such as arousal, novelty, motivation, or reward, is critical in producing the phase shift (21). Significantly, Van Reeth et al. (34) found that enforced immobility induced phase delays in hamsters when imposed in subjective night but not in subjective day. In the present study, both hypoxia and hypercapnia had delayed effects on activity, reducing wheel running intensity during subjective night. This presents a possible mechanism for the hypoxia- and hypercapnia-induced phase delays, a suggestion that is supported by the significant correlation between the magnitude of the phase shift and the depression of wheel running (Fig. 4).

Physiological Responses to Pulses and Relationships to Circadian Phase Shifts.

Experiment 2 was conducted for two reasons: to confirm that the [O₂] and [CO₂] (8 and 11%, respectively) used in experiment 1 will significantly stimulate the hamster respiratory system and to correlate the measured physiological responses with the phase shifts observed in experiment 1.

Tb. Several studies have found that, whereas the circadian system is temperature compensated, temperature pulses can nevertheless induce phase shifts and ambient temperature cycles can entrain circadian rhythms both in vivo and in vitro (5, 28). In the present study, hypoxia caused a significant reduction in Tb, and hypercapnia induced a transient, but nonsignificant, reduction in Tb. Acute profound hypothermia has been shown to induce phase shifts in hamsters and other rodents (8, 9, 25, 27), suggesting that acute Tb changes may have mediated the phase responses to respiratory gas pulses. However, the studies cited above used intense environmental cooling to induce hypothermia and are therefore not directly comparable to the present study. Furthermore, these earlier studies did not control for confounding behavioral correlates. For example, examination of the published representative actograms (9, 25, 27) reveals that hypothermic pulses usually reduced subsequent nocturnal activity levels, even when hypothermia occurred during subjective day. Thus phase delays generated by cold-induced hypothermia (9, 25, 27) and hypoxia or hypercapnia (present study) could both be an indirect effect of reduced levels of activity (34).

V̇O₂. The hypoxic hypometabolism observed in the present experiment has previously been observed in numerous species (6). The reduction in V̇O₂ during acute hypoxia has been attributed to an inhibition of thermogenesis (19). In the present study, the decrease in V̇O₂ during hypoxia and the threefold increase in V̇O₂ coincidental with visible shivering after the return to air are consistent with this hypothesis.

The effect of hypercapnia on metabolism in adult mammals is unclear because hypercapnia has been reported to increase (14, 15), have no effect (13, 30), or to decrease (7, 17, 22) metabolic rate. In the present study, 11% [CO₂] reduced V̇O₂ to a minimum of ~75% of prepulse levels, but this effect was nonsignificant and transient, lasting <1 h (Fig. 3). Duration of exposure, [CO₂], ambient temperatures, and species all varied between studies, but none of these could be consistently linked to differences in results.

The acute effects of the hypoxic and hypercapnic pulses on V̇O₂ and Tb in experiment 2 were significantly correlated with the phase responses to similar stimuli in experiment 1 (Fig. 4). V̇O₂ and Tb correlated, so it is not possible to distinguish their relative potential as mediators of the phase delay. One possibility is that the combination of low temperature and oxygen lack caused an acute slowing of the circadian clock. However, this is not supported by the recent report that a week of hypoxia, accompanied by low Tb for at least the first 24 h, did not result in a phase delay on the resumption of nornoxia in rats (20).

Vt. Hypoxia has been reported to evoke a sustained increase in Vt/V̇O₂ in hamsters and other rodents (6, 18, 19), and this was confirmed in the present experiment. The ventilatory response to hypercapnia was also consistent with responses previously reported for the hamster (3, 11, 18, 36). The increase in Vt found in the present study was primarily caused by changes in Vt. Respiratory frequency remained relatively constant in hypoxia and hypercapnia. The ventilatory responses to pulses were not correlated with phase shifts.

In summary, hypoxia and hypercapnia induced ventilatory stimulation, together with behavioral, metabolic, and thermoregulatory depression in hamsters. The results of the correlation analysis exclude elevated lung ventilation as a mediator of the effects of 3-h hypoxic and hypercapnic pulses at CT6 on the circadian timing system. However, a potential role for metabolic and/or thermoregulatory mechanisms in mediating the observed changes in wheel running rhythms requires further study.

Perspectives

This study has demonstrated that hypoxic and hypercapnic stimuli can have a statistically significant effect on circadian phase and activity levels. Gregarious rodents living in burrow systems may benefit from a nonphotic zeitgeber to coordinate the activity of the group at times when exposure to sunlight is infrequent. Respiratory gases could be postulated to serve as such a zeitgeber, because oscillations of CO₂ and O₂ in the burrow system will be maximal when activity is synchronized. However, the present study does not support this scenario for the hamster. The levels of hypoxia and hypercapnia used in this study were se-
lected to be in the extreme range of values recorded for captive hamsters living in a burrow system (16). Despite their severity, the phase-shifting effects of these stimuli were small (<5 min/day) and would, therefore, be predicted to have a very weak entraining effect under more moderate, natural conditions. Furthermore, hypoxia and hypercapnia were shown to induce only phase delays and would, therefore, be ineffective as a zeitgeber for an animal whose intrinsic circadian period is longer than the period of the oscillating gas concentrations.

A similar argument can be made regarding the potential role of hypoxia and hypercapnia in disruption of circadian organization of sleep-wake cycles in sleep-disordered breathing syndromes. Any effect of respiratory gas fluctuations on circadian timing is likely to be inconsequential, unless the human circadian timing system is considerably more sensitive to these stimuli than that of the hamster.

Finally, because respiratory chemoreflex responsiveness varies with time of day (22, 33) and manipulation of respiratory gases causes circadian phase delays, a respiratory stimulus on one day might alter the response to the same stimulus (at the same clock time) on the next day. This “feedback” effect would be of significance to investigators who use respiratory stimuli on successive days in chronic animal models of respiratory gas fluctuations on circadian timing is likely to be of significance to investigators who use respiratory stimuli on successive days in chronic animal models of respiratory gas fluctuations on circadian timing is likely to be inconsequential, unless the human circadian timing system is considerably more sensitive to these stimuli than that of the hamster.

In conclusion, this study has confirmed that hypoxia and hypercapnia can influence the phase of the circadian pacemaker of the hamster and thus raises interesting questions about the mechanisms involved. However, the effect was modest and is unlikely to represent a confounding factor in experimental design or in the etiology of respiratory-related sleep disorders.

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