THE EFFECTS OF HYPOXIA AND HYPERCAPNIA
ON
HAMSTER ACTIVITY RHYTHMS

by

Tim M. Jarsky

A thesis submitted in conformity with the requirements for the degree of Master of Science, Graduate Department of Zoology, in the University of Toronto

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Golden hamsters (*Mesocricetus auratus*) were exposed to 3 h of constant hypoxia (O$_2$ concentration = 5%), episodic hypoxia (O$_2$ concentration = 17-5%), or constant hypercapnia (CO$_2$ concentration = 15%) at CT6, CT11 and/or CT15 to determine if a phase shift in activity rhythms is induced by a respiratory stimulus. Hamsters were kept in a 14:10 LD cycle before the pulse and at the onset of a respiratory stimulus they were released into constant darkness (DD). A separate experiment measuring metabolism (oxygen consumption; VO$_2$) and inspiratory ventilation (V$_t$) using open flow respirometry and whole-body plethysmography, respectively, demonstrated that all chemical stimuli were powerful respiratory stimuli. Constant hypoxia caused 0.2 ± 0.06 h, 0.3 ± 0.08 h, 0.4 ± 0.1 h phase delays in hamster activity rhythms at CT6, CT11 and CT15, respectively, when the first day post pulse was used to calculate phase shifts. However, when the constant hypoxia experiment was repeated at CT6 and a post-pulse regression line was used to calculate the phase shift no phase change was induced. Constant hypoxia given on 3 consecutive days at CT6 caused a 0.68 ± 0.18 h phase delay using both calculation methods. Episodic hypoxia caused a 0.16 ± 0.06 h phase delay at CT15. Hypercapnia did not alter activity rhythms. Apparent phase delays obtained using the first day post pulse for phase shift calculation were strongly correlated with reductions in activity levels on the day of the pulse. These results suggest that the phase delays in the circadian rhythm of activity induced by the different kinds of hypoxia are mediated by acute decreases in activity levels. It is concluded that respiratory stimuli have little or no direct influence on circadian rhythms in activity in hamsters. Episodic hypoxia did not induce long term facilitation (LTF) or phase advances as hypothesized. Absence of LTF may represent an adaptation to a burrowing lifestyle.
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This study is one in a series designed to determine if and how the circadian system interacts with respiration. The purpose of this study was to determine whether a respiratory stimulus can phase shift the circadian pacemaker, the suprachiasmatic nucleus (SCN), in hamsters. It is known that respiration is controlled by a system with multiple levels involving simple stimulus response reactions (feedback control) and more complex central command of rhythm generation and sensory information integration (feedforward control). However, the possibility that respiratory control could also involve modulation by the circadian timing system has rarely been considered.

The rationale for this study is based on work in rats, humans and ducks which exhibit a time-of-day dependent sensitivity to a respiratory stimulus (Peever and Stephenson 1997, Raschke and Moller 1989, Woodin and Stephenson 1998). This finding suggests that the respiratory system may experience modulation by the circadian timing system. If respiration is under circadian control it raises the possibility that a respiratory stimulus could affect the clock as it has been shown that manipulation of many clock controlled variables affect clock function (Boulos and Rusak 1982, Mrosovsky 1988, Reebs and Mrosovsky 1989, Mrosovsky and Salmon 1989). Thus, the following experimental objectives were developed:

1. To determine if continuous hypoxia (5%), episodic hypoxia (5-17%), and hypercapnia (15%) can phase shift the activity rhythms in the golden hamster.

2. To characterize the respiratory responses to hypoxia (5%), episodic hypoxia (5-17%), and hypercapnia (15%) in the hamster.

The focus of this introduction is to review the relevant literature on mammalian circadian rhythms and respiratory control. The evidence which indicates that respiration is likely to be subject to circadian control, the rationale for hypothesizing that respiratory outputs might feedback on the circadian pacemaker, and the practical implications of such feedback are also
experimental animal and respiratory stimuli. Where possible, anatomical, physiological, and behavioral data are integrated in an attempt to illuminate possible mechanisms of respiratory feedback on the SCN.

REVIEW OF CIRCADIAN RHYTHMS

Circadian rhythms are physiological processes and/or behavioral systems that oscillate under constant environmental conditions with a period close to, but rarely exactly, 24 hours. The timing of such behaviors or physiological processes is controlled by an internal clock (endogenous pacemaker). The endogenous pacemaker has two principal properties: it is normally synchronized (entrained) to the light-dark cycle but, in the absence of temporal cues, it 'free runs' with a period approximating 24 hours (Moore 1995). Endogenous control of physiological and behavioral systems confers the ability to anticipate environmental changes allowing an organism to avoid dangers and exploit the advantages of events such as the day-night cycle and the seasons of the year (Foster 1993). Therefore, a major action of the environment is to entrain the internal system to a period of precisely 24 hours, so that it and the expressed rhythms that it controls will be in an adaptive temporal relationship with the day-night cycle (Menaker et al. 1978).

Three basic elements are necessary in order for an organism to predict dawn and dusk. The organism must have an internal timer, producing a stable self-sustained oscillation. The internal time signal must be entrained by an environmental time signal (Zeitgeber). Finally, the temporal information provided by the clock must eventually be transduced into a signal that can drive the appropriate rhythms in physiology and behavior in a precise manner.

In fact, precision is one of the most striking features of biological clocks. The accuracy is evident in rodents which have an inclination for exercise on running wheels. The time at which rodents begin and continue exercise (activity onset) can have a standard error which can sometimes be about a minute, from day to day, under constant conditions. In other words, the
Photic Influences on Circadian Clocks

The primary strategy used to understand the influence of light on the clock has been the administration of light pulses to animals, usually nocturnal animals, kept in constant darkness while monitoring wheel running activity. The predicted time of activity onset on the day of the pulse is subtracted from the time of activity onset on the day following the pulse to give the immediate phase change. The size of the phase change depends upon the time of day at which the light pulse is presented. This is illustrated by a phase response curve (PRC; Fig. 1.1). Photic stimulation produces no change of phase if the pulse is given in the hamster’s subjective day. However, light pulses evoke phase delays when presented in early evening, and phase advances when given late in the subjective night (Summer and McCormack 1984). The magnitude of light-induced phase shifts also depends on intensity, wavelength, and duration of light exposure (Takahashi et al. 1984, Nelson and Takahashi 1991). The effect of both phase advances and phase delays in response to light is to keep activity confined to the dark portion of the light-dark cycle in nocturnal animals.

The anatomical route by which light modifies the circadian pacemaker in mammals starts at the eye. All vertebrates, including mammals, appear to have specialized non-visual (i.e. not involved in spatio-temporal imaging) photoreceptors. In mammals these photoreceptors are located in the retina (Foster 1993). This specialized population of photic receptors mediates photic entrainment of the SCN (Moore 1995). The SCN is the site of the master oscillator or clock and its function is the generation of circadian rhythms (Rusak and Zucker 1979, Miller 1993). The SCN is localized in a region of the hypothalamus, just dorsal to the optic chiasma,
FIGURE 1.1 Phase response curve for novelty induced wheel running (non-photic) and light pulses (photic). Modified from Mrosovsky et al. 1992 and Takahashi et al. 1984. Shaded regions indicate times at which respiratory stimuli were given in the present study. By convention in nocturnal animals CT12 is the time at which activity onset occurs.
neurons and associated glia (Miller 1993). The SCN and the retinal cells are connected via the retinohypothalamic tract (RHT) (Moore and Lenn 1972, Eichler and Moore 1974). The RHT has several components, however, the largest projection is to the SCN (Johnson et al. 1989). In addition to the RHT, an indirect projection to the SCN originating in retinorecipient cells of the intergeniculate leaflet (IGL) of the lateral geniculate nucleus - the geniculohypothalamic tract (GHT) - is also involved in entraining circadian rhythms to the light-dark cycle (Pickard et al. 1987).

The cellular and molecular mechanisms by which light causes phase shifts of the SCN pacemaker are not known. However, recently, clear photic regulation of immediate-early gene expression in the SCN has been identified and has been correlated with the effects of illumination of the retina. It has been demonstrated that light pulses that induce phase shifts also induce Fos protein expression in the SCN and IGL regions (Kornhauser et al. 1990, Rea 1989, Rusak et al. 1990). Photic induction of Fos in the SCN is gated by the circadian clock. C-fos is a proto-oncogene, a member of a family of transcription factors (Kornhauser et al. 1993) and it appears to function in cellular signal transduction by coupling transient stimuli to gene regulation in the nucleus (Muller et al. 1984, Greenberg and Ziff 1984). The discovery of c-fos within the SCN enables Fos expression to be used as a marker for light cells within the SCN.

In addition to pulses of light, pulses of darkness can phase shift the activity rhythms of hamsters kept in constant light. Hamsters maintained under constant illumination and exposed to 2 or 6 hour pulses of darkness undergo phase changes, both transient and long lasting (Boulos and Rusak 1982). These results were interpreted by the authors to indicate that the PRC for dark pulses is the mirror image of the PRC for light pulses. However, Reebs et al. (1989) demonstrated that running activity or a correlated variable mediates the phase-advancing effects of dark pulses on hamster circadian rhythms. It was demonstrated that dark pulses induce activity and when activity is blocked during a dark pulse, phase shifts no longer occur or are minimized (Reebs et al. 1989, Van Reeth and Turek 1989). Therefore dark pulses may affect the
Therefore dark pulses may be considered as non-photic rather than photic stimuli, as far as the clock is concerned.

**Non-photic Influences on circadian clocks.**

In addition to environmental stimuli such as light, behavioral experiences (non-photic stimuli) are capable of altering the phase of circadian rhythms. The possibility that clock-controlled variables might have feedback effects has been considered by a number of investigators, as it seems likely that something controlled by the clock could also affect the clock. Sexual arousal (Honrado and Mrosovsky 1989), hoarding opportunities (Rusak et al. 1988), novel running wheels (Reebs and Mrosovsky 1989a), triazolam, a benzodiazepine (Turek 1988), dark pulses, social interactions between males and cage changes (Mrosovsky 1988) are examples of non-photic phase-shifting stimuli. A goal of most early research on non-photic stimuli was to demonstrate the existence of feedback and assess its strength (Mrosovsky 1996). The most effective of the above mentioned non-photic stimuli is novelty-induced wheel running. In the golden hamster three hours of wheel running in the subjective day produces up to 3-hour phase advances, while smaller phase delays (30 min.) occur if pulses are given in the subjective night (Fig. 1.1) (Mrosovsky et al. 1992). The shape of the novelty-induced wheel running PRC is similar, but not necessarily identical, to those of other non-photic stimuli for which PRC have been plotted.

Activity is a common feature of many non-photic stimuli studied. Triazolam, for example, initiates prolonged bouts of activity. However, when activity is blocked following injection, phase shifts are greatly reduced (Mrosovsky et al. 1989). For most of these procedures it has been demonstrated that phase shifts or entrainment usually do not occur if running is absent or prevented (Mistleberger et al. 1996). Therefore, it is likely that the phase-resetting
signal is a neural or physiological correlate of induced activity. However, it is not known whether it is activity per se, or some associated variable that is critical in producing the phase shift.

Many non-photic stimuli have some or all of the following components in common:
1. Activity must last a long time (Gannon and Rea 1995). The maximum phase advance for novelty-induced wheel running often requires as much as three hours of running. This contrasts with the light PRC where large phase changes can be obtained with relatively short light pulses of only a few minutes (Mrosovsky 1996).
2. Novelty. The largest phase changes are obtained when the stimulus is used for the first time. However, novelty may just be a way of evoking arousal or activity (Mrosovsky 1996).
3. Motivation. Mistlberger (1991) suggested that 'the motivational significance of a scheduled behavioral arousal' and the 'motivational context within which physical activity occurs' may be important.
4. Reward. A common interpretation is that non-photic stimuli are rewarding. However, the necessity of reward for non-photic shifting is by no means solidly established (Mrosovsky 1996).

None of the experiments done to date clearly demonstrate the role(s) of any of these variables in phase-resetting. However, there are circumstances where induced activity has not caused phase shifts (Sayeski et al. 1990, Edgar 1991 Janik and Mrosovsky 1993, Mrosovsky and Biello 1994, De Vries and Meijer, unpublished, cited in Mrosovsky 1995, Mistlberger 1996). These experiments, and particularly the experiment where hamsters which did not run in a novel wheel ("sluggards") but would run in the cold and did not phase-reset (Janik and Mrosovsky 1993, Mrosovsky and Biello 1994), have led to the hypothesis that it is not activity but the motivational context which determines phase-resetting. The question of relative contributions of activity time, novelty, motivation and reward remains unresolved.
The central neural pathways which mediate non-photic phase shifts are not known. However, stimuli that induce locomotor activity produce changes in the phase of free-running activity rhythms, with a PRC that is similar to that produced by stimulation of the geniculohypothalamic tract (GHT) (Moore 1994). In addition, phase shifts to non-photic stimuli are eliminated by lesions of the intergeniculate leaflet (IGL) (Johnson et al. 1989, Janik and Mrosovsky 1994, Wickland and Turek 1994). The GHT may be a pathway through which activity or a correlated variable provides input to the SCN to modulate pacemaker function (Wickland and Turek 1994).

The IGL receives serotonergic input from the dorsal raphe nucleus (Meyer-Bernstein and Morin 1996). The dorsal raphe is rich in benzodiazepine receptors (Michels et al. 1990), therefore, the projection from the dorsal raphe to the IGL may have a role in mediating non-photic phase shifts (Meyer-Bernstein and Morin 1996). Neuropeptide Y (NPY) is a major neurotransmitter of the GHT (Albers and Ferris 1984). The SCN also receives serotonergic inputs from raphe nuclei (Morin 1994). The serotonergic system apparently provides inhibitory modulation of the circadian system's response to light (Duncan et al. 1988, Meijer et al. 1988, Morin and Blanchard 1991) but the role of serotonin in non-photic phase shifting is not as clear.

Pharmacological manipulations of serotonergic neurotransmission, using agonists, affects the free-running pacemaker both in vitro (Prosser et al. 1990, Lovenberg et al. 1993) and in vivo (Edgar et al. 1993). Specifically, quipazine, a non-specific serotonin agonist, applied to rat SCN slices produced a PRC that was more similar to that evoked by non-photic stimuli than that produced by light pulses (Prosser et al. 1990). Also, serotonergic antagonists impair arousal-induced phase shifts of the circadian system in the Syrian hamster (Sumova et al. 1996). However, in hamsters quipazine did not phase shift activity rhythms but 8-OH-DPAT, a 5HT1A and 5HT7 receptor agonist, did (Bobrzynska et al. 1996a).

The firing rate of serotonergic cells in the dorsal raphe is directly correlated with the degree of arousal of the animal (Jacobs and Azmitia 1992). Serotonergic terminals are found
projection to the SCN does not appear to be involved in non-photic phase shifting as phase shifts persist after 95% serotonin depletion in the hamster SCN (Bobrzynska et al. 1996b). Despite the fact that serotonin depletion in the SCN does not influence arousal induced phase shifts, it still remains possible that endogenous serotonergic pathways are mediators of non-photic entrainment of the circadian system. One possibility is the serotonergic projection from the dorsal raphe to the IGL (Meyer-Bernstein and Morin 1996). Serotonergic mediation does not exclude the possibility that other afferent systems also contribute to entrainment by arousal (Sumova et al. 1996).

Neuropeptide Y transmission from the IGL to the SCN appears to be necessary for at least one type of non-photic phase shifting (novelty-induced wheel running) in one species (hamsters) (Biello et al. 1994). Injections of NPY into the SCN produce phase shifts (Albers and Ferris 1984, Huhman and Albers 1994, Biello and Mrosovsky 1993). These NPY induced phase shifts are still obtained when hamsters are prevented from being very active (Biello et al. 1994). NPY antiserum administration to the SCN blocks the phase-advancing effect of novelty-induced wheel running (Biello et al. 1994). Thus, phase-shifts induced by wheel running depend on the NPY projection from the IGL to SCN in hamsters. It remains to be determined how important NPY and serotonin are in other species or in clock-resetting by other manipulations.

Light pulses that induce phase shifts also induce Fos protein expression in SCN and IGL regions. However, the role of c-fos in non-photic phase shifting is not clear. Non-photic manipulations induce Fos expression in the SCN (Amir and Stewart 1996). However, there is no difference in Fos levels in the SCN across time of day. This indicates that the sensitivity of SCN cells to non-photic manipulations is not circadian in nature. In contrast to the SCN, the IGL Fos expression in response to non-photic treatment is phase-dependent. These results suggest that cells in SCN and IGL respond to several types of non-photic manipulations (Amir and Stewart 1996).
A third manipulation exists which has a PRC different from the photic and non-photic PRC. If hamsters are prevented from being active during their active period it causes phase delays as large as 45 min. However, restraint at other times of the day does not induce phase changes (Van Reeth et al. 1991). This result suggest that reductions in activity can cause phase delays.

REVIEW OF RESPIRATION

Respiratory Neuroanatomy.

The brain mechanisms controlling breathing in intact mammals are subject to blood and brain gas and pH values and behavioral state (e.g. emotive, cognitive, or movement related). In its simplest form, neural respiratory control is an oscillatory process that starts with the alternating activation of inspiratory and expiratory neurons in the brain stem (Eldridge and Millhorn 1986). The anatomical location of the respiratory control centers have been fairly well elucidated (Fig. 1.2). The purpose of this section is to familiarize the reader with the primary respiratory brain centers.

The phrenic motor neurons driving the diaphragm, and the intercostal motoneurons driving the intercostal muscles are located in the spinal cord and receive their rhythm mainly from the medullary respiratory neurons. The isolated medulla oblongata is able to generate a respiratory rhythm in the absence of peripheral feedback (Feldman and Smith 1995). In the medulla, respiratory neurons are found in three primary locations, the dorsal respiratory group (DRG), the ventral respiratory group (VRG), and the motor nuclei of some cranial nerves. The mechanism by which the basic rhythm is generated is still under intense investigation. In vitro studies have identified putative respiratory pacemaker cells in the pre-Botzinger complex (Smith et al. 1991) but these have not been found in intact adult preparations. Neuronal network models of central rhythm generation are often proposed (e.g. Cohen 1979, Duffin 1991) but no single model has yet received convincing empirical validation.
location of the main groups of respiratory neurons. NTS nucleus tractus solitarius, DRG dorsal respiratory group, VRG ventral respiratory group. Modified from Mateika and Duffin (1995).
numerous inputs from both central and peripheral neural sources (Feldman and Smith 1995). These sources are concerned with the homeostatic functions of respiration, especially in relation to regulation of the partial pressures of $O_2$ and $CO_2$, body temperature, and the acid-base balance of the body fluids. Other important inputs include mechanoreceptors which serve to modulate the breath to breath activity of respiratory muscles, and the influence of arousal state which can alter numerous properties of the control system.

Sensory Input.

The partial pressures of oxygen ($P_{aO_2}$) and carbon dioxide ($P_{aCO_2}$) in arterial blood are sensed by the peripheral chemoreceptors located in the carotid and aortic bodies (Fidone and Gonzales 1986). The former are positioned along the two common carotid arteries and the latter are located along the aortic arch. They relay information to the NTS in the medulla via the glossopharyngeal and/or vagus nerves. The peripheral chemoreceptors are primarily asphyxia sensors as the sum of the response to hypoxia and hypercapnia administered separately is less than the response to the same levels of hypoxia and hypercapnia given together (asphyxia) (Duffin 1990).

When all peripheral chemoreceptors are denervated an increase in ventilation in response to inhaled $CO_2$ is still observed (Bruce and Cherniack 1987). This indicates that there are receptors in the brain which are excited by changes in $CO_2$, called central chemoreceptors. The central chemoreceptors are thought to be located along the ventrolateral surface of the medulla in a region called the ventrolateral medullary shell (VMS) (Loeschcke et al. 1970, Mitchell et al. 1963, Schlaefke 1981). The VMS is exposed to cerebrospinal fluid (Bruce and Cherniack 1987). The central chemoreceptors probably monitor changes in $[H^+]$ in the interstitial fluid of the brain rather than the direct measurement of $CO_2$ levels (Cherniack and Longobardo, 1995). The changes in the partial pressure of $CO_2$ at the central chemoreceptors lag behind those of arterial blood (Duffin 1990).
A. Ventilation

Hypoxia is any reduction in the oxygen partial pressure or content of blood. Upon exposure to hypoxia, for example by inhalation of air low in $O_2$, lung ventilation increases abruptly then it declines. The latter part of this biphasic response is referred to as the hypoxic ventilatory decline (HVD) (Fig. 1.3). The response is dependent on the severity and duration of the hypoxia and is species and body size dependent (Mortola and Gautier 1995). Differences in HVD among species are evident in animals which live either permanently (fossorial) or intermittently (semifossorial) in burrows. In burrows the gas composition can be substantially asphyxic (Kuhnen et al. 1987). Fossorial animals such as hamsters tend to have a diminished response to hypoxia and a blunting of the ventilatory response to hypercapnia. These characteristics are believed to be genetic traits for which the expression does not depend on environmental conditions. This conclusion is based on the fact that adult hamsters which have never been exposed to burrows have the same respiratory response as those raised in burrows (Mortola 1991).

The acute response to hypoxia is dramatically modified depending on the severity and duration of hypoxic exposure. For example, more prolonged exposure to hypoxia (hours to several days) produces a secondary increase in breathing, a phenomenon that has been termed ventilatory acclimatization to hypoxia (Fig. 1.3) (Bisgard and Neubauer 1995). Metabolic rate, temperature and the hypocapnia that accompanies hyperventilation, are only a few of the many variables known to change during hypoxia and influence respiratory drive (Mortola and Gautier 1995).

The mechanisms underlying this complex respiratory response to hypoxia are not fully understood. However, it is widely accepted that peripheral chemoreceptors are almost exclusively responsible for the observed initial increase of ventilation, whereas the subsequent ventilatory attenuation is of central origin and not the result of an alteration in hypoxic peripheral chemoreceptor discharge (Millhorn et al. 1984, Neubauer et al. 1985,
responses to sustained moderate or severe hypoxia. The figure is based on long term, intermediate, and short term experiments on different species at ambient temperatures below thermoneutrality. \( \dot{V}_e \) expired minute ventilation, \( \dot{V}_{O_2} \) oxygen uptake (metabolism). Dotted red line indicates where the metabolic response is variable.
Biphasic Response

- Arousal
- Depression
- Acclimatization

\[ \dot{V}_E \]
\[ \dot{V}_{O_2} \]

Time

0 min hrs days
chemoreceptors with regard to ventilatory stimulation during hypoxia (Bisgard and Neubauer 1995).

When the peripheral chemoreceptors are removed, hypoxia tends to decrease ventilation (Cherniack et al. 1970, Orr et al. 1975). This occurs because hypoxic blood stimulates an elevation of cerebral blood flow which in turn washes CO₂ out of the central chemoreceptor tissues. This withdraws the CO₂ ventilatory stimulus from the central chemoreceptors, thus reducing ventilatory drive. Estimates of the central chemoreceptor sensitivity to CO₂ can be, on average, halved because of the cerebral blood flow effect (Duffin 1990).

Several nonspecific neuronal responses to hypoxia are known that could be responsible for hypoxic ventilatory depression. They can be classified into presynaptic and postsynaptic events. Presynaptic reductions in neurotransmitter release, reuptake, or synthesis reduce the net excitatory input required to reach the threshold for neuronal activation. Postsynaptic events result in an initial transient intracellular alkalization that is rapidly followed by an intracellular acidosis, a small rise in intracellular [Ca²⁺], an increase in potassium conductance, membrane hyperpolarization, and a reduction in cell excitability (Bisgard and Neubauer 1995). In addition, reductions in O₂ availability promote metabolic production of adenosine and lactic acid, which act as neuromodulators to reduce excitability of CNS neurons (Neubauer et al. 1990, Yan et al. 1995).

Not all areas of the brain are equally susceptible to the depressant action of hypoxia (Chapman et al. 1979, Tenney and St. John 1980). At certain levels of hypoxia, areas of the brain that stimulate ventilation may be more depressed than areas that decrease ventilation. Thus the net ventilatory response to exposures to hypoxia is determined by the time constants of the stimulating effects of hypoxia on the peripheral chemoreceptors and the depressant effects on inhibitory and excitatory regions of the brain (Cherniack and Longobardo, 1995). Therefore, it is hypothesized that the biphasic hypoxic response is the result of those effectors which have short time constants and powerful stimulatory effects acting early to cause the increase in
effects, resulting in a subsequent reduction in ventilation (Mortola and Gautier 1995). The central depression affects regions in or above the upper pons rostral to the midbrain/pontine junction in the rabbit (Martin-Body and Johnston 1988), and may reside in the red nucleus (Waites et al. 1996).

B. Long-term Facilitation

An additional affect of hypoxia occurs when hypoxic bouts are given in short succession (episodic hypoxia). Episodic hypoxia may produce prolonged augmentation of breathing which is maintained for an hour or more following the termination of the stimulus (Millhorn et al. 1980a, Cao et al. 1992, Powell and Aaron 1993, Bach et al. 1992, Turner and Mitchell 1997). A long-lasting increase on ventilatory drive (long-term facilitation) was evoked in awake dogs by short exposure to repetitive isocapnic hypoxia (Cao et al. 1992). In the anesthetized and ventilated cat, repeated carotid sinus nerve stimulation induced a progressive increase of baseline normoxic phrenic nerve activity (Milhorn et al. 1980a, b). The hypoxic bouts were of short duration and therefore the ventilatory response was characteristic of the increase in ventilation which occurs before the onset of hypoxic ventilatory decline during more prolonged stimuli. Since the increase in ventilation is due to peripheral chemoreceptor output it is possible that the sustained high level of ventilation is due at least in part to altered peripheral chemoreceptor output following stimulation.

The mechanism by which repetitive hypoxic exposure induces an increase in ventilatory drive is unknown. It is possible that either carotid body input or central nervous system hypoxia may mediate the long-lasting respiratory stimulation after cessation of hypoxic stimulation (Cao et al. 1992). However, there is evidence that central serotonergic neurons in the raphe obscurus nucleus of the brain stem could mediate the long-lasting stimulation of respiratory drive. When a serotonin receptor antagonist was microinjected into the raphe nuclei of the cat long-term facilitation was not evoked (Millhorn et al. 1980b, Bach and Mitchell 1996). Also,
response in goats (Henderson et al. 1996). There are many other potential mechanisms. It is possible that brief but repetitive exposures to hypoxia may raise metabolic rate as a result of sympathetic nervous system activation (Cao et al. 1992). This in turn would influence normoxic ventilation. Unfortunately steady state O₂ consumption or CO₂ production recordings have not yet been obtained in studies of long-term facilitation.

C. Arousal State

Studies in sleeping humans and other mammals have shown that increases in both hypoxia and hypercapnia can cause arousal from sleep (Bowes et al. 1981, Gothe et al. 1981, Neubauer et al. 1981). Unless changes in ventilation are great, the ventilatory movements do not produce awakening by themselves (Neubauer et al. 1981). In addition, peripheral chemoreceptor stimulation was shown to evoke a cardiorespiratory response similar to that evoked by electrical stimulation of the defense arousal areas of the brain, leading to the suggestion that carotid chemoreceptor stimulation may activate the brainstem defence arousal system (Marshall 1987). It has been demonstrated in cats and rats anaesthetized with Althesin (alphaxalone-alphadalone) that carotid chemoreceptor stimulation is one of the many different types of stimuli that serve as excitatory inputs to the brainstem defense areas (Hilton and Marshall 1982, Marshall 1984). Stimulation of the defense reaction by carotid body chemoreceptor stimulation was previously overlooked because the anesthetics used in respiration experiments blocked the response.

The defense reaction is characterized by hyperventilation, tachycardia, renal and mesenteric vasoconstriction, skeletal muscle vasodilatation, pupillary dilation, retraction of the nictitating membrane, and piloerection (Marshall 1987). This set of responses is termed the visceral alerting response (VAR). The VAR is superimposed on the primary hypoxic response of bradycardia and vasoconstriction. In the conscious animal the response of carotid body chemoreceptor stimulation is dependent on the strength of the stimulus and activity already
accompanying fear and rage reaction can best be understood as adaptations which prepare an organism to cope with an emergency and specifically to perform the extreme muscular exertion of flight or attack. Stimulation of the defense arousal areas is an appropriate reaction to hypoxia as escape from the hypoxic environment is another form of respiratory compensation.

D. Hypometabolism and Thermogenesis

A reduction in $\dot{V}O_2$ occurs with the HVD during hypoxia. The hypoxic hypometabolism has been observed in cats (Gautier et al. 1989), rats (Gautier et al. 1992), hamsters (Mortola 1991), and many other rodents (Frappell et al. 1992). The reduction in $\dot{V}O_2$ during hypoxia is inversely dependent on body size (Frappell et al. 1992). However, the reduction in $\dot{V}O_2$ during moderate hypoxia is not a universal occurrence. The $\dot{V}E/\dot{V}O_2$ ratio (metabolism-specific expiratory minute ventilation) is relatively similar among species, and in hypoxia it increases approximately by the same magnitude (Mortola et al. 1989, Frappell et al. 1992). During hypoxia the ambient temperature ($T_{amb}$) can cause changes in $\dot{V}E$ and $\dot{V}O_2$, however, the $\dot{V}E/\dot{V}O_2$ ratio remains constant. For example, hypoxia given at thermoneutrality (25°C) in rats almost doubles $\dot{V}E$ and has a minimal hypometabolic effect (Gautier et al. 1992, Saiki et al. 1994). In the cold (5°C) metabolism decreases by approximately 30% and ventilation increases by only 15% (Fig. 1.3). In both cases the same hypoxic stimulus caused the same hyperventilation i.e. same $\dot{V}E/\dot{V}O_2$ ratio, but with different combinations of $\dot{V}E$ and $\dot{V}O_2$. Cold hypoxic exposure also reduces the threshold for nonshivering thermogenesis (Kuhnen et al. 1987) indicating a lowering in the set point for body temperature ($T_b$). From this information it has been hypothesized that the hypometabolic effect is due to a reduction in thermogenesis.

A reduction in thermogenesis can not be the only explanation for the observed hypoxic hypometabolism as in some species, and particularly in newborns, hypometabolism can manifest even at thermoneutrality. A possible explanation is that $O_2$ exerts its effect on metabolic rate via direct effects on cellular function. A drop in $O_2$ supply can interfere with a
phosphorylation (Mortola and Gautier 1995). Despite the fact that changes in Po$_2$ and Tamb influence many variables, including physiochemical relations determining the acid-base status of blood and brain tissue, the respiratory output seems to perfectly track the metabolic response.

RESPONSES TO HYPERCAPNIA

Hypercapnia is an increase in the CO$_2$ partial pressure of blood. Hypercapnia stimulates lung ventilation (Chapin 1954, Mortola 1991, Saiki and Mortola 1996). In the human, the central chemoreceptors mediate a linear increase in ventilation as arterial CO$_2$ increases above a threshold of about 40 mmHg (Duffin 1990). The magnitude and relative contribution of the carotid body to the CO$_2$ response increases with hypoxia (Cherniack and Longobardo, 1995). In hamsters, it has been demonstrated that there is a biphasic response of ventilation to hypercapnia which peaks at an inhaled CO$_2$ concentration of approximately 21% (Chapin 1954). Hypercapnia has three primary effects which determine the final respiratory output. Hypercapnia hyperpolarizes respiratory neurons and thereby decreases their excitability (Wyke 1963). It causes metabolic depression due to acidemia (Stupfel 1974). Finally, hypercapnia stimulates catecholamine release which induces thermogenesis (Tenney 1956, Mortola and Gautier 1995).

CIRCADIAN RHYTHMS AND RESPIRATION

Hypoxia and hypercapnia, through the respiratory system, have a wide range of effects on other physiological systems. These physiological systems, such as the sleep-wake cycle, metabolism, thermoregulation, also affect respiration. All of these effectors of respiration have in common a strong interaction with the circadian system. In addition, exercise (activity) is a behavioral state that has profound effects on respiration and is also under control of the circadian system. The purpose of this section is to give a brief review of the effect that the sleep wake cycle, metabolism, thermoregulation, and exercise have on respiration. All these systems
separation into sections is artificial, purely for the purpose of clarity of presentation.

A. Sleep

In the waking state, automatic and behavioral influences interact to establish the level and pattern of breathing (Cherniack and Longobardo, 1995). However, during sleep different states and stages associated with characteristic electroencephalograph (EEG), behavioral, and physiological changes can affect the control of breathing. Sleep begins with the slow-wave or quiet stage. This stage causes significant changes in respiration. Minute ventilation decreases because of reductions in tidal volume and breathing frequency (Cherniack and Longobardo, 1995). Breathing in slow-wave sleep is thought to be governed almost exclusively by automatic mechanisms. Absence of environmental stimulation seems to account for the depression of ventilation observed in slow-wave sleep (Orem 1995, Philipson and Bowes 1986).

In humans, REM sleep replaces slow-wave sleep at intervals of 60-90 min. The REM sleep has been divided into tonic and phasic periods (Heller 1987). The primary change in ventilation in REM sleep is an increased variability from breath to breath. The source of this variability is the specific changes in the brain and brainstem that occur in relationship to the phasic events of this sleep state (Pack 1995).

In general, sleep is usually associated with decreased ventilation, increased resting levels of $P_{aCO_2}$, reduced $P_{aO_2}$ (Birchfield et al. 1958, Gothe et al. 1981) and causes a reduced metabolic rate (Brebbia and Altshuler 1965). Studies in humans and diurnal animals show that metabolic rate falls gradually during sleep, reaches a minimum late in the night, then rises before awakening (Fraser et al. 1989). The observed fall in organismal metabolic rate during sleep may be caused by a general reduction in tissue metabolic rate (Shapiro et al. 1984), or a state dependent change in a specific mechanism such as muscle activity (Kleitman 1963), or thermoregulatory set-point (Fraser et al. 1989). An alternative view is that the changes during sleep are a consequence of circadian variations in metabolic activity (White et al. 1985) or body
mainly due to the change in arousal state (Fraser et al. 1989).

B. Metabolism

Circadian rhythms of oxygen consumption ($\dot{V}O_2$) have been described in white-footed mice and pocket mice (Chew et al. 1965, Kayser and Heusner 1967). The daily increase in $\dot{V}O_2$ often precedes the daily onset of activity and the onset of food intake (Aschoff and Pohl 1970). Ventilation levels change accordingly to meet the changing metabolic demands. The rhythm in $\dot{V}O_2$ and the rhythm in body temperature vary with time of day in a similar fashion. It was shown in birds as well as in man that when body temperature increased by 0.5 °C $\dot{V}O_2$ increased by 20-40% during the resting stage of the activity rest cycle (Aschoff and Pohl 1970). However, during the active phase $\dot{V}O_2$ increased by 10-25% in response to a similar change in body temperature. These results are compatible with the idea that the rhythm of $\dot{V}O_2$ and the rhythm of body temperature each are controlled by a separate circadian oscillator (Aschoff and Pohl 1970).

C. Thermogenesis

Body temperature varies throughout the day according to its own rhythm. The respiratory system is a major heat loss site, so changes in ventilatory pattern can alter heat loss from the lung. The daily changes in body temperature are a strong influence on the final ventilatory level. The underlying mechanisms responsible for the temperature cycle are unknown. It has been shown that the rhythm in body temperature is not solely due to changes in metabolic rate (Aschoff 1970, Heller and Glotzbach 1977). In the pigeon, central nervous system mechanisms controlling arousal states and circadian rhythmicity have separate and additive influences on temperature regulation (Heller et al. 1983).
Exercise causes an increase in ventilation that is proportional to metabolic rate. \( \dot{V}_E \) increases not only to satisfy the higher metabolic demands but also to increase the respiratory evaporative heat loss (Mortola and Gautier 1995). Ventilation is increased during exercise by signals arising from thermal receptors, from proprioceptors in the limbs, and from the CNS. The intensity of such signals is not necessarily proportional to metabolism. \( P_{\text{aCO}_2} \) is held virtually constant during exercise despite large increases in CO\(_2\) production. This could be explained as an effect on breathing of a signal that is directly coupled to metabolic rate. Because such a metabolic signal would not be reduced by increasing ventilation, breathing during exercise might be under substantial feed-forward rather than feedback control (Mateika and Duffin 1995, Cherniack and Longobardo 1995).

**Daily rhythms in respiration by masking**

The above mechanisms all directly influence respiration. Consequently, the daily rhythms in these physiological systems could give rise to a daily rhythm in respiration (i.e. masking). The existence of a underlying rhythm in respiration could only be demonstrated by experiments which control for these confounding variables.

**CAN RESPIRATORY STIMULI PHASE-SHIFT THE SCN?**

The following evidence indicates that respiration is a potential candidate for circadian control:

1. Biological clocks coordinate daily changes in thermoregulation, metabolism, sleep-wake cycle, and activity. These clock-controlled physiological systems and activity have strong interactions with each other and with respiration.

2. Circadian rhythms have been demonstrated in different cardiovascular parameters (heart rate, blood pressure) (Gautherie 1973, Halberg 1969, Lucente et al. 1987, Mainardi et al. 1987). Since respiration and cardiovascular systems serve the same primary function and since neural
circadian control of respiration.

3. It has been determined that maternal pinealectomy alters the daily pattern of fetal breathing in sheep (McMillen et al. 1990). Melatonin is rhythmically released by the pineal gland during the dark hours and crosses the placenta into fetal circulation providing the fetus with information about time of day.

4. There is preliminary evidence suggesting that ventilatory chemoreflexes exhibit day-night changes in rats (Peever and Stephenson 1997), humans (Raschke and Moller 1989), and ducks (Woodin and Stephenson 1998).

The above discussion considers whether respiration is affected by the circadian timing system. It is important to emphasize that the above findings are suggestive, but not conclusive, and experiments have not yet been completed that control for the confounding effects of correlated rhythms (sleep state, metabolism, activity etc.).

Another unanswered question concerns whether respiration can affect the clock. Phase changes have been obtained by manipulation of many clock controlled variables (e.g. Aschoff 1965, Eastman 1982, Turek and Gwinner 1982, Rusak et al. 1989, Mrovsovsky 1988). Furthermore, since humans, rats and ducks exhibit a time of day dependent change in apparent chemoreceptor sensitivity which suggests the respiratory system may be modulated by the circadian system, it is possible that respiration could be involved in a feedforward-feedback loop with the circadian system. The possibility that respiration could affect the clock is not just of theoretical interest, it also has practical implications, since it raises the possibility that an experiment on one day may affect the results of the same test at the same time on the next day. This is not an unreasonable hypothesis as a common characteristic of respiratory experiments is daily repetition of the protocol and day to day variability in the data.

The experiment reported in this thesis tests the feedback portion of the loop. It was hypothesized that respiratory stimuli can reset the SCN, as indicated by a phase change in an overt circadian rhythm.
RATIONALE FOR THE FEEDBACK HYPOTHESIS

A. Direct Feedback

A study on humans demonstrated that exposure to altitude and hypoxia simultaneously caused a transient phase shift in several physiological parameters (e.g. peak expiratory air flow, grip strength, oral temperature) (Ashkenazi et al. 1982). However, the effects of the altitude associated reduction in barometric pressure and hypoxia were not separated and therefore the effect of each stimulus is not distinguishable. Nonetheless, at least one neural pathway exists between the respiratory system and the SCN making feedback a possibility. The NTS is the recipient of carotid body chemoreceptor output. The NTS has an efferent to the dorsal raphe nucleus which primarily releases epinephrine (Hokfelt et al. 1974, Aghajanian and Wang 1977). The dorsal raphe then have a serotonergic projection to the IGL (Vertes 1991, Waterhouse et al. 1993, Meyer-Bernstein and Morin 1996). It may be through this neural connection that phase shifts were obtained when humans were exposed to altitude and hypoxia.

An alternative means by which a respiratory stimulus could affect the SCN is that hypoxia could cause a general reduction in neuronal excitability thereby altering clock function. It is also feasible for hypercapnia to have a similar effect. Metabolic effects are to be expected in non-fossorial animals exposed to CO₂ at concentrations higher than 10%, since an acute fall of pH impairs the utilization of glucose and fat by an inhibition of the activity of pH-dependent enzymes.

In contrast, episodic hypoxia strongly stimulates the defense arousal areas, and may cause general arousal. A common characteristic of non-photic stimuli is that they induce arousal. In fact, entrainment has been obtained by rattling the bars of bird cages (Reebs et al. 1989), or even dropping cages (Enright 1975), both are situations which would stimulate the defense reaction. Therefore, it is plausible that episodic hypoxia might produce a PRC similar to that of other non-photic stimuli. Since hypercapnia also stimulates carotid body chemoreceptors, it may also stimulate the defense reaction.
The sleep-wake cycle, thermoregulation, and metabolism are all influenced by respiratory stimuli (Mortola and Gautier 1995). Both hypoxia and hypercapnia can disrupt sleep patterns (Neubauer et al. 1981, Marshall 1987). As well, hypoxia can cause a reduction in body temperature and metabolic rate (Kuhnen et al. 1987, Mortola 1991). Hypoxia and hypercapnia may also affect the level of activity due to the stress placed on the cardiovascular system (Aaron et al. 1992). This effect may be particularly apparent if the stimulus falls within the daily activity period. A change in activity may in turn reset the SCN (Reebs et al. 1989). If the behavioral entrainment system is non-specific, as has been suggested (Mrosovsky et al. 1989), it would be expected that a variety of behavior altering stimuli like hypoxia and hypercapnia would be capable of producing phase shifts. It is evident that the respiratory stimuli have a wide range of effects on physiological systems and behaviors and the respiratory stimuli can potentially affect the SCN via these systems and not directly.

Obviously, there are many alternative mechanisms by which the respiratory system could affect the SCN and if feedback occurs further experiments will be required to determine the mechanism. The primary purpose of the present study is to test for the existence of such feedback in the respiratory and circadian system of the hamster.

EXPERIMENTAL ANIMAL SELECTION

The golden hamster (Mesocricetus auratus) was chosen for this experiment because they have high cycle to cycle circadian timing accuracy so that small phase shifts can be identified. Hamsters do, however, present a disadvantage due to their respiratory adaptations to a fossorial lifestyle. The relative insensitivity of their respiratory system to hypoxia and hypercapnia requires that the respiratory stimuli be much more severe than would be used for non-fossorial rodents.
There are two primary methods by which PRCs are obtained. The Aschoff Type I (AI) (Aschoff 1965), the most commonly used of the two methods, has the animals free running in constant conditions (DD or LL) and a pulse (eg. light, wheel running, respiratory stimulus) is then given at the desired circadian time and the phase change is measured. However, a practical disadvantage of this technique is that it is difficult to give pulses at the same CT to many animals as activity onsets are spread out over the day because individual animals have different free running periods.

The Aschoff Type II test (AII) (Aschoff 1965) was the test used in this study because it enables pulses to be given to many animals at the same CT. Animals are at similar CTs because prior to administration of the pulse they are entrained to a LD cycle (Moore-Ede 1982). At the onset of the pulse animals enter constant conditions. Allowance must be made for the effects on the circadian system caused by the release from LD to constant conditions (DD or LL). The transition from LD to DD commonly causes phase advances of about 30 min. (Mrosovsky and Salmon 1990, Mrosovsky 1991, Janik and Mrosovsky 1993). Thus, a disadvantage of this technique is that two variables are manipulated simultaneously. This problem is overcome by comparing the resultant phase change with that from control studies in which the animal was released from the same LD cycle into constant conditions but with no pulse given. Phase changes are interpreted relative to the control. For example, if a pulse attenuates the phase advance observed in controls, it is inferred that it caused a phase delay.

**RESPIRATORY STIMULI SELECTION**

In choosing the respiratory stimuli three parameters need to be taken into account: type, level, and duration. Three hours was chosen as the standard duration of all the respiratory stimuli because non-photic stimuli tend to be long-lasting (Mrosovsky 1996). Also, in hamsters the largest phase changes for novelty-induced wheel running are obtained after about three hours of continuous running (Reebs et al. 1989). Continuous hypoxia, episodic hypoxia, and
discussed earlier.

Pulse levels of $O_2$ and $CO_2$ were based on hamster burrow concentrations. For the changes in $O_2$ and $CO_2$ levels to be considered respiratory stimuli it was assumed that they must be greater than what a hamster would normally encounter in the burrow. Hamster burrow concentrations of $CO_2$ can be as high as 10.8% (Kuhnen et al. 1987). Ventilation in the hamster has been shown to increase steadily from 0% to 21% $CO_2$ (Chapin 1954, Walker et al. 1985, Mortola 1991). Based on this information and the fact that the $CO_2$ analyzer had a upper working limit of 15.9%, 15% was the chosen level of hypercapnia. Burrow $O_2$ concentration has been measured at 10% (Kuhnen et al. 1987). Therefore, 5% $O_2$ was deemed to be a sufficient stimulus. Although $O_2$ and $CO_2$ pulse levels appear significantly more extreme than burrow levels, this is not the case, because in the burrow the conditions are asphyxic. Asphyxia is a much stronger respiratory stimulus than $O_2$ and $CO_2$ administered separately.

*Respiratory Stimuli Response Characterization*

The respiratory responses to each of the stimuli were recorded. This was done to confirm that the pulse was in fact stimulating the respiratory system. Furthermore, knowledge of the respiratory response might help in the interpretation of the activity rhythms data.
Materials and Methods

Effect of respiratory stimuli on hamster activity rhythms

Animals and Housing

Eighteen male virus-free golden hamsters (*Mesocricetus auratus*) aged 42 days were obtained from Harlan Sprague-Dawley (Indianapolis, IN). They were housed singly in plastic cages (45 X 25 X 20 cm) with 17.5 cm diameter running wheels. The running surface of the wheels was covered with 4 mm plastic mesh. Temperature during experiments was 20 ± 2°C. Purina 5001 pellets and water were available ad libitum. Routine maintenance, when necessary, was performed quietly. Bi-weekly cage changes entailed providing the animals with a clean cage containing wood chips (N.E. Products Corp. Hardwood laboratory bedding), food and water.

Nine hamster cages were housed in each of two sealed animal chambers (Fig. 2.1). The chambers were impermeable to light. Two 2.44 m cold white fluorescent bulbs provided approximately 140 lux within the chamber during lights-on. Light levels were measured with a Gossen, Lunasix 3 light meter. Light function within the chamber was monitored by a photocell which, when activated by light, caused a pen deflection on an Esterline-Angus event recorder.

The animal chambers were ventilated continuously at a rate sufficient to maintain CO₂ concentration less than 0.5%. 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. However, the high gas flow rates required to rapidly raise and lower oxygen or carbon dioxide concentrations were observed to cause small (12 mmH₂O) increases in pressure.
activity rhythms.
Animal Chamber

Circulation Fan

Not Shown Gas Sample outlet

Fluorescent Light

Photocell

Gas Inlet

Ventilation Duct
Data recording instruments were kept outside of the experimental room to avoid disturbing the animals when collecting data. Wheel revolutions were detected by micro switches hard wired to an Esterline-Angus event recorder (Model 620X).

Data Analysis

Part 1. Aschoff Type II (AII) Experimental Design.

The AII experimental design was used for all non-photic manipulations because it enabled pulse administration to occur at the same circadian time for all animals. Hamsters were kept in LD 14:10 for 3 days or until they were entrained (Aschoff 1965). The hamsters were then simultaneously transferred into darkness and given a pulse on day 4. They remained in darkness from day 5 until day 7. Phase shifts were calculated by subtracting activity onset on the day before the pulse (day 3) from activity onset of the day following the pulse (day 5). Activity onset was defined as: 1. A continuous 5 min. bout of activity followed by at least one additional 5 min. bout within the subsequent 30 min. (Lees et al. 1983). 2. Onset had to follow at least 6 hr. without activity which met onset criteria (Mrosovsky 1988).

Part 2. Modified AII Experimental Design

The respiratory stimuli for which apparent phase shifts were observed were repeated at CT6 using a modified AII procedure (Bobrzynska et al. 1996a) to check for the occurrence of transients. A regression line was calculated for 6 consecutive activity onsets in DD, starting on the third day following the pulse. The predicted activity onset on the day following treatment was estimated by backward extrapolation of the post-pulse regression line. It was assumed that the onset of activity on the day of the experimental treatment was the same as it was on the day before the pulse. The phase shift was defined as the difference between the onset on the last pre-pulse day and the predicted onset on the day following the pulse (Bobrzynska et al. 1996a). The modified AII result was then compared with the unmodified AII phase shift calculation.
Total daily wheel-running time was quantified to determine the effect of the respiratory stimulus on activity. One bin of activity was defined as a 10 min section of continuous pen deflections. The effect of a pulse or sham pulse was calculated by subtracting the activity on the day of the pulse from that on the day before the pulse. Changes in activity were expressed either as a percent or in number of 10 min. bins. The activity change of the tests was then subtracted from the activity change of the control to quantify the effect of the respiratory stimuli on activity. Activity change for consecutive days of hypoxia was calculated by subtracting the three days immediately before the pulse from the three days during the pulse.

The total difference in activity between the test and the control (test activity change - control activity change) were also compared to the phase shift to determine if a significant correlation existed.

Part 4. Statistical Analysis of Actogram Data

The effects of the various pulses were assessed by comparing test data to the appropriate controls. Significance was tested using a two-tailed, paired student t-test. Correlations were tested using the Pearson Product - Moment Correlation coefficient (r). Differences are considered significant at the 95% confidence level (P < 0.05).

Non-photic Stimuli

Novelty-Induced Wheel Running

Purpose: To confirm that the experimental animals would respond in the expected way to an established standard test.

Methods: A procedure similar to that applied by Reebs and Mrosovsky (1989) was used. Hamsters (21 weeks old) were placed in running-wheels similar to the ones in their home cages, but encased in Plexiglas, for three hours at CT6. Lights were then turned-off. Wheel revolutions were counted by electronic counters. An infrared night scope was used to remove the hamsters
shifts were measured with the unmodified AII procedure described above.

Cage Changing

Purpose: The AII procedure has been shown to be a valid method to study circadian rhythms (Mrosovsky 1996). In addition, it has been shown to be in agreement with AI (Aschoff 1965) for several stimuli which produce relatively large phase shifts (i.e. novelty-induced wheel running, triazolam, NPY). However, I remained unsure as to whether the AII method had the resolution to yield significant small phase shifts with only 9 hamsters because the size of the phase shift associated with the transfer from a LD cycle to constant darkness is quite variable. It ranges from almost nothing (Reebs and Mrosovsky 1989, Biello and Mrosovsky 1993), to about half an hour (Mrosovsky and Salmon 1990). Cage changes have been show to cause small phase shifts (i.e. 25 min.) (Mrosovsky et al. 1989). A cage change experiment was carried out to determine if a sample size of 9 is sufficient to identify significant small phase shifts.

Methods: The cage change was performed at CT6. Eight hamsters (16 weeks old) were transferred to cages previously occupied by other hamsters, and one hamster received a fresh cage. Following the cage change the lights were switched off. The control experiment consisted of lights out at CT6 on a later date in the same group of hamsters at age 18 weeks.

Respiratory Stimuli

Three hour long pulses of sustained hypoxia (inhaled O₂ concentration = 5% ), episodic hypoxia (inhaled O₂ concentration alternated between 17% and 5%), or sustained hypercapnia (inhaled CO₂ concentration = 15% ) were given at CT6, and CT11 and/or CT15 (Fig 1.1). A respiratory pulse was obtained by blowing the appropriate gas into the chamber until the desired concentration levels were obtained. Immediately prior to the respiratory stimuli, in addition to the transfer to constant darkness, normal chamber air ventilation was shut-off. Following the pulse ventilation was turned back on. During all respiratory stimuli O₂ and CO₂ were monitored by
were hard wired to a computer (MacClassic) for recording.

Control experiments consisted of substituting compressed air for the gas (i.e. nitrogen, oxygen and CO₂) used in a particular test pulse. Flow rates into the chamber were matched to those used in each test pulse. As in the tests, chamber air ventilation and lights were shut-off at the onset of the pulse. Following the sham pulse, chamber ventilation was reinstated. Control phase-shifts were calculated using the AII procedure described above. Control experiments were done for each respiratory manipulation.

Visual Observation of Hamster Behaviour During Sustained Hypoxia and Hypercapnia

- Purpose: Visual observations were performed in order to ensure that the respiratory stimuli did not induce behavioural changes that would indicate that the animals were in discomfort.

- Methods: Three hamsters were placed in a sealed Plexiglas chamber and exposed to hypoxia and hypercapnia at CT6 for a minimum of 30 min., or until what appeared to be a steady state. Observations were manually recorded.

Hypoxia

- Purpose: To determine if a 3 h. exposure to 5% O₂ can cause a phase shift in the circadian wheel running rhythm.

- Methods: Hypoxia was obtained by blowing compressed nitrogen into the sealed animal chamber until oxygen levels fell to 5%. Hypoxia was then sustained with a low flow of nitrogen. Following 3 hours of 5% O₂ the nitrogen was shut off. Nitrogen flow at the onset of the pulse was monitored by an uncalibrated custom built, high capacity, flow indicator. For the remainder of the pulse, nitrogen flow (approx. 2.5 L/min) was monitored by a calibrated flow meter (Model N014-96, Cole-Parmer Instrument Co.). Hamsters were 23-27 weeks old during these tests.
Additive Effect of Hypoxia on Phase Shifts.

Purpose: To determine whether exposure to 3 hr. of 5% O₂ on three consecutive days at CT6 would cause a phase change in hamster activity rhythms. This experiment was intended to determine whether a hypoxic stimulus given on consecutive days at the same time would have a cumulative effect. A further experiment was conducted to determine if the effect of constant hypoxia on activity was maintained in a 14:10 LD cycle.

Methods: Hypoxic bouts were given on three consecutive days for three hours at CT6. Hamsters (aged 20 weeks) entered DD at the onset of the first hypoxic pulse or remained in 14:10 LD throughout the experiment.

Episodic Hypoxia

Purpose: To determine whether a series of short exposures to hypoxia can cause phase shifts in hamster wheel running rhythms.

Methods: Episodic hypoxia was induced by alternately ventilating the chamber with nitrogen and oxygen. Oxygen levels oscillated between 5% for 3 min. and 17% for 3 min. for a total duration of three hours. The timing of O₂ and N₂ flow was automated (Fig 2.2). In each case gas flow was controlled by a solenoid valve (models 01-10 and V52LB2026, M&M International and Honeywell Inc.). The electronic circuit controlling each solenoid valve consisted of a timer-controlled AC supply in combination with a feedback system that served as a limit switch to ensure that oxygen concentration did not oscillate past user-defined limits (Fig. 2.2 A). Each feedback system consisted of an oxygen-dependent voltage from an oxygen analyzer which sampled from the animal chamber.

Episodic hypoxia was initiated by activating the timer which turned on the set-point comparator which controlled the solenoid valve in the nitrogen supply line. This comparator was activated by oxygen levels above 5%. If the input to the comparator was above 5% it sent power to
episodic hypoxia. Red line represents the currently active feedback loop. Black line represents the currently inactive feedback loop. As drawn, the diagram represents the state of the system at the beginning of a hypoxic pulse (i.e. initiation of N₂ flow).

B. A graph of the O₂ oscillations during episodic hypoxia. Solid red lines indicate a device is active. Solid black lines indicate a device is inactive. The beginning of a hypoxic pulse is represented by the points marked with an asterisk (*).
within the chamber fell to 5% the set-point comparator would cause the solenoid valve to close
stopping N\textsubscript{2} flow and preventing oxygen from dropping any further. The nitrogen set-point
comparator was then deactivated by a timer, thus preventing the N\textsubscript{2} solenoid from opening again in
the same cycle. At the same time the oxygen set-point comparator was activated by its timer. The
set-point comparator which controlled O\textsubscript{2} flow was set so that oxygen levels below 17% would
open the solenoid valve controlling oxygen flow to the chamber. This would result in oxygen
levels rising in the chamber until they reached 17%. These feedback loops were alternately
activated by the timers in a 3 minutes on, 3 minutes off sequence, resulting in a 6 min. duty cycle.
For each three minute section gas levels were changing for approximately two minutes and held
constant for the remaining minute. Hamsters were 30-34 weeks old during these tests.

Hypercapnia

Purpose: To determine whether hypercapnia can phase change hamster activity rhythms.

Methods: CO\textsubscript{2} was maintained at 15% for 3 hours by blowing compressed CO\textsubscript{2} into the
chamber at approximately 0.2 L/min. Hamsters were 35-37 weeks old during these tests.

Respiratory Responses to Hypoxic and Hypercapnic Pulses
These experiments were designed to study the metabolic and ventilatory responses to hypoxic and
hypercapnic stimuli at CT6.

Apparatus Design
A 6-channel open-circuit whole-body plethysmograph (Bartlett and Tenney 1970) was used to measure lung ventilation and metabolic rate (Fig 2.3). Whole-body plethysmography
operates on the principle that during inspiration air is warmed and humidified and, in a fixed
volume chamber, this causes an increase in pressure (Chapin 1954). Pressure changes were
monitored between a reference and an animal chamber using a differential pressure transducer
B. Schematic diagram of a single channel of the six channel open-circuit plethysmograph. Thick solid lines indicate primary animal chamber flow. Thin solid lines represent the air flow for gas analysis and pressure transducer ports. Dotted arrows indicate the direction of gas flow.
The differential pressure transducer was hard wired to a digital data acquisition system (MacLab/8, Analog Digital Instruments Ltd.) for recording of the respiratory wave form. Seven 850 ml sealed Plexiglas cylinders served as six animal chambers and one reference chamber. The chambers were submerged in a water bath maintained at a constant temperature of 22 -23°C.

Air-flow through the chambers was generated by cylinders of premixed compressed gas. The gases passed through a regulator and a series of valves which maintained flow in each chamber at 1.5 litres min.\(^{-1}\). Gas was dried prior to flow measurements and humidified before entering the animal chambers. During ventilation measurements flow into and out of the chamber were sequentially shut-off. At all other times gas exited each chamber via a common outlet pipe which terminated in a jar which housed a digital hygrometer (Model 57550-21, Canadawide Scientific Co.). Between the chamber and the common outlet pipe was a one-way valve which allowed outlet gas samples to be taken from each chamber. Outlet gas concentrations were measured using oxygen and carbon dioxide analyzers (Model S-3A/1 and Model 3D-3A, Ametek Corp.). Immediately prior to entering the analyzers the outlet gas passed through a desiccating column of Drierite (WH. Hammond Drierite Co.).

Calculation of Ventilatory Parameters

Inspired ventilation \((\dot{V}_I) = f \cdot V_T\) where, \(f\) is the number of breaths in a minute \((f = 60/\text{T}_{\text{TOT}})\), and \(V_T\) is the tidal volume (ml, BTPS). \(V_T\) was calculated using the equations developed by Jacky (1980):

\[
V_T = \left[\frac{(P_m/P_{\text{cal}}) \cdot V_{\text{cal}} \cdot G_A}{[1-(T_I/T_{\text{TOT}}) \cdot (1-G_A/G_N)]}\right]
\]

\(P_m\) = respiratory pressure deflection
\(P_{\text{cal}}\) = the pressure deflection of a known volume gas
\(V_{\text{cal}}\) = volume of the gas injected into the chamber
\(T_I\) = average time for one inspiration
\(T_{\text{TOT}}\) = average time for one complete respiratory cycle
Gₐ is a dimensionless proportionality constant relating the expansion of the warmed and humidified inhaled gas to the total volume of inhaled gas (Jacky 1980):

\[
G_a = \frac{[T_b (P_b - P_{C_{H_2O}})] / [T_b (P_b - P_{C_{H_2O}}) - T_c (P_b - P_{A_{H_2O}})]}{T_b = \text{body temperature (°K)}} \]
\[
T_c = \text{animal chamber temperature (°K)} \]
\[
P_b = \text{barometric pressure in the chamber (mmHg)} \]
\[
P_{A_{H_2O}} = \text{water vapour pressure of the gas in the alveoli (mmHg)} \]
\[
P_{C_{H_2O}} = \text{water vapour pressure of the gas in the chamber (mmHg)} \]

Gₙ is a dimensionless proportionality constant relating the contraction of the exhaled gas as it cools to Tₙ to the volume of exhaled gas (Jacky 1980):

\[
G_n = \frac{T_b (P_b - P_{N_{H_2O}}) / [T_b (P_b - P_{N_{H_2O}}) - T_n (P_b - P_{A_{H_2O}})]}{P_{N_{H_2O}} = \text{water vapour pressure of saturated gas at the nares (mmHg)}} \]
\[
T_n = \text{nasal temperature (°K)} \]

Tₙ used to calculate Vᵣ were estimated from separate experiments (see Appendix 1).

**Calculation of Metabolic Rate**

Metabolic rate or oxygen consumption (\(\dot{V}o_2\), ml min⁻¹ STPD) was measured by open flow respirometry (Withers 1977):

\[
\dot{V}o_2 = \frac{[\dot{V}_i (FIO_2 - FEo_2)] / [1-(1-RE) FEo_2]}{\dot{V}_i = \text{dry gas flow out of the animal chamber (ml min⁻¹ STPD)}} \]
\[
FIO_2 = \text{fractional concentration of oxygen entering the chamber} \]
\[
FEo_2 = \text{fractional concentration of oxygen leaving the chamber} \]
RE is the respiratory exchange ratio where:

\[
RE = \frac{(F_{ECO_2} - F_{ICO_2})}{(F_{IO_2} - F_{EO_2})}
\]

**Experimental Protocol**

The oxygen and carbon dioxide analyzers were calibrated against gas samples of known \(O_2\) and \(CO_2\) concentrations before each experiment. The differential pressure transducer was calibrated in the prepulse period while the animals were in the chambers by injecting 1 ml of air several times and recording the pressure signal (Fig 2.4).

The hamsters were weighed and then placed into the animal chambers about 35 min. before CT6. Several ventilation and metabolic rate measurements were taken in the pre-pulse period. To simulate the transfer to DD which occurred in the circadian experiments, the animal chambers were covered with an opaque lid. To reduce the transduction of light into the chambers via apparatus tubing room light during the experiment was provided by a dim red light. The circadian photoreceptors have a very low quantum sensitivity to wavelengths greater than 600 nm (Takahashi et al. 1984).

Six hamsters (aged 36-38 weeks) were exposed to air, constant hypoxia, episodic hypoxia, and hypercapnia in four separate experiments. Each pulse started at CT6. After four minutes the system reached equilibrium and three consecutive measurements of metabolic rate and one ventilation measurement were taken. Then, at each subsequent 20 min. interval a ventilation and a metabolic rate measurement were taken. Air was reintroduced to the system following the last measurement at 3h. Measurements were taken at 4, 20, 40, and 60 min. of the recovery period. The protocol was modified during the episodic pulse. The first pulse measurement was taken at 20 min instead of 4 min. The recovery period was also extended from one to two hours to see if long-term facilitation occurred. Since the equilibration time of the plethysmograph was 4 min. the changes in \(O_2\) levels
circuit plethysmography. CO₂ and O₂ represent the percentage of carbon dioxide or oxygen in the animal chamber. Vent'n represents the ventilatory trace measured in volts. Dotted vertical line indicates where a 1 ml calibration injection was given.
measurements could not be taken until equilibration was reached resulting in the extension of a duty cycle until measurements were completed. After the experiments the hamsters were removed from the animal chamber and returned to their home cages.

**Statistical Analysis of Respiratory Data**

All statistics were computed using Data Desk software (Data Description, Inc.). Significance was tested using a repeated measures ANOVA. If a significant difference was obtained a post-hoc Least Significant Difference Test was used for multiple comparisons. Differences are considered significant at the 95% confidence level ($P < 0.05$).
Chapter 3

Results

Non-photic Stimuli

Three hours of novelty-induced wheel-running at CT6 produced a mean phase advance of 3.2 ± 0.12 h in hamsters which ran more than 4000 revolutions ($n = 8$) (Fig. 3.1). A single hamster ran 1627 revolutions and did not phase shift. The mean number of wheel revolutions was 6776 ± 956 ($n = 9$). The maximum number of revolutions obtained over the three hour pulse was 10 186.

The mean cage change phase advance at CT6 (0.88 ± 0.13 h) was significantly larger than the control response (0.47 ± 0.06 h) (Fig 3.2).

Turning the lights-off produced a significant phase advance (0.52 ± 0.04 h) in circadian rhythms when administered at CT6 (Fig. 3.3). The phase advances obtained in both animal chambers (labelled Box 1 and Box 2 in Fig. 3.3) were not significantly different from each other.

Respiratory Control Experiment

A three hour pulse of air at CT6 caused no significant change in the respiratory parameters measured (Fig. 3.4). Furthermore, the prepulse measurements obtained in the constant hypoxia (5%), episodic hypoxia (5-17%), hypercapnia (15%) experiments, were not significantly different from the air pulse values. Consequently, the prepulse measurements from each experiment were used to measure the effect of each respiratory stimulus, not the air pulse. This was done to minimize the effect of possible differences between days.
Figure 3.6 Actogram illustrating phase shift after novelty-induced wheel-running. Horizontal arrows indicate the period of novel wheel running. DD started immediately after all the animals were placed in their novel wheels. Vertical arrow indicates the time at which lights turned-off during LD 14:10. Time 24:00 to 08:00 is not shown, no wheel running took place during this period. The hamster in this example ran 6086 wheel revolutions during the 3 h period in the novel wheel.
Cage Change

Phase Change (h)

n = 6
Lights out CT6

Phase Change (h)

<table>
<thead>
<tr>
<th>Box 1</th>
<th>Box 2</th>
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<tbody>
<tr>
<td>n = 9</td>
<td>n = 9</td>
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</table>
frequency. B. $V_T$, tidal volume. C. $V_T/T_I$, tidal volume divided by the mean inspiratory time (mean inspiratory airflow). D. $\dot{V}_I$, inspiratory minute ventilation. E. $\dot{V}O_2$, rate of oxygen consumption. F. $\dot{V}_I/\dot{V}O_2$, metabolism specific inspiratory minute ventilation. Where error bars are not visible they are within the point.
Air

(A) 

$\dot{f}$ (cycles min$^{-1}$)

(B) 

$V_T$ (ml)

(C) 

$\frac{V_T}{V_I}$ (ml s$^{-1}$)

Time (min)
Air

\[ \dot{V}_I \]  
(ml min\(^{-1}\))

\[ \dot{V}_{O_2} \]  
(ml min\(^{-1}\))

\[ \dot{V}_I / \dot{V}_{O_2} \]

Time (min)
1. Constant Hypoxia

Visual observations indicated that hamsters were initially aroused from sleep by 5% O₂. After 3 - 4 min. the hamsters curled-up in a corner of their cage and appeared to fall asleep. Sleep was occasionally interrupted by periods of feeding and grooming. Hamsters started to shiver immediately following the return to air. Shivering was visible for several min..

1.a. Respiratory responses

Three hours of constant hypoxia at CT6 stimulated the hamster respiratory system (Fig. 3.5). \( f \) increased to approximately double the prepulse values (51.8 ± 5.6 breaths min \(^{-1}\)) within 4 min. after the onset of hypoxia and remained significantly elevated above resting for a further 120 min. of hypoxia. Four min. after the return to air, \( f \) was elevated to 235 ± 32 breaths min \(^{-1}\), more than four times the prepulse value and approximately twice the hypoxic values. \( f \) returned to prepulse levels 40 min. after the return to air (Fig 3.5 A).

The response of \( V_T \) to hypoxia was biphasic (Fig 3.5 B). \( V_T \) was significantly elevated above resting levels (1.82 ± 0.11 ml) 20 min. after the onset of hypoxia (2.49 ± 0.22) and subsequently fell to prepulse values. \( V_T \) remained depressed for the remainder of the hypoxic pulse. Four min. after the return to air \( V_T \) was significantly elevated above prepulse levels. \( V_T \) had returned to prepulse values after 20 min. in air.

The response of \( V_T/T_I \) was biphasic, peaking at 20 min. (13.0 ± 0.9 ml s \(^{-1}\)) (Fig 3.5 C). Four min. after the return to air \( V_T/T_I \) was elevated to 24.6 ± 4.1 ml s \(^{-1}\). Twenty min. after the return to air \( V_T/T_I \) returned to prepulse values (4.5 ± 0.2 ml s \(^{-1}\)).

The \( V_I \) response to hypoxia was biphasic (Fig 3.5 D). \( V_I \) returned to prepulse values (89 ± 7 ml min \(^{-1}\)) following the peak at 20 min. (244.3 ± 23.3), at 60, 80, 120, 160, and 180 min. of the hypoxic pulse (144.3 ± 20.3, 147.0 ± 34.7, 135.3 ± 15.4, 121.7 ± 18.1, 141.6 ± 14.1, respectively). \( V_I \) was elevated to 569 ± 88 ml min \(^{-1}\), over six times prepulse values, 4 min. after the return to air. \( V_I \) returned to prepulse values 40 min. after the return to air.
180 min. (*) significant change from prepulse values. (†) significant decrease within the hypoxic pulse from the peak value at 20 min. (Ψ') measurement is significantly different from all other values.
min-l) until 60 min. (1.89 ± 0.17 ml min⁻¹) after the onset of hypoxia. \( \dot{V}_O_2 \) was depressed below prepulse \( \dot{V}_O_2 \) for the remainder of the pulse. Four min. after the return to air metabolic rate was elevated to 10.9 ± 0.4 ml min⁻¹. \( \dot{V}_O_2 \) returned to resting levels 40 min. after the return to air (Fig 3.5 E).

The response of \( \dot{V}_1/\dot{V}_O_2 \) was variable (Fig. 3.5 F). \( \dot{V}_1/\dot{V}_O_2 \) appeared to be elevated to three to four times resting values at the onset of hypoxia, however, it only became statistically significantly elevated above prepulse values (25.6 ± 2.1) at 100, 140, and 180 min (125.8 ± 26.3, 106.4 ± 27.8, 100.2 ± 14.9, respectively) after the onset of hypoxia. \( \dot{V}_1/\dot{V}_O_2 \) returned to prepulse values four min. after the return to air.

1.6 Wheel running rhythms

According to the AII phase shift calculation method hypoxia caused a reduced phase advance at CT6, CT11, and CT15 relative to the control (Fig. 3.6 and 3.7). The difference in phase advance between the test and the control was 0.2 ± 0.06 h, 0.3 ± 0.08 h, 0.4 ± 0.1 h at CT6, CT11, CT15 respectively.

Since constant hypoxia caused a significant phase delay relative to the control, the test was repeated at CT6 to determine if the phase shift obtained was permanent. The effect of the pulse on the phase of activity was calculated using the AII and modified AII experimental design (Fig. 3.8 and 3.9). Use of the first day post-pulse to calculate the phase shift resulted in a phase change (0.3 ± 0.1) significantly reduced from the control pulse (0.73 ± 0.14 h) but not statistically different from the earlier test at CT6 (0.51 ± 0.13). The post pulse regression line predicted a first-day post-pulse that was statistically different from the actual first-day post-pulse. When the predicted first day post pulse was used to calculate the phase shift (0.83 ± 0.17) it was not statistically different from the sham pulse (0.91 ± 0.06 h).
Hypoxia caused a significant reduction in activity at CT6, CT11, and CT15 relative to the control (-16.5 ± 2.47, -17.3 ± 3.51, -16.0 ± 3.11 # of 10 min. bins, respectively) (Fig 3.10). The reduction in activity was similar at all CTs. The sham pulses did not cause significant changes in activity. The control pulses caused small increases which were not statistically different from zero (Fig. 3.10).

2. Consecutive Days of Hypoxic Pulses

2.a. Wheel running rhythms

The results of 3 consecutive days in which 3 h long pulses of constant hypoxia were administered are illustrated in Fig 3.11. Consecutive hypoxia caused a reduced phase advance relative to that of the sham pulse (net phase delay = 0.68 ± 0.18 h) (All experimental design) (Fig 3.12). However, when the hamsters remained in LD during the pulse regime activity onset returned to prepulse values by the first day following the final pulse. On the days in which pulses occurred activity onsets were variable but consistently delayed.

2. b. Activity

Consecutive days of hypoxia significantly reduced activity levels by 24 ± 7 10 min. bins relative to the control over all three days.

3. Episodic Hypoxia

3.a. Respiratory responses

Three hours of episodic hypoxia at CT6 induced a significant respiratory response (Fig. 3.13). Episodic hypoxia did not induce long-term facilitation of ventilation following termination of the stimulus. Respiratory frequency did not significantly increase above prepulse values (70 ± 11.4 breaths min⁻¹) during episodes of 5% O₂. However, during episodes of 17% O₂ f (122.8±
hatching indicates a control experiment. (*) significant difference from control.
Constant Hypoxia (5% O₂)

![Bar chart showing Phase Change (h) for CT6, CT11, and CT15 under constant hypoxia conditions.

- CT6: n = 9
- CT11: n = 9
- CT15: n = 8

The data indicates a significant phase change under hypoxic conditions, with CT11 showing the most pronounced effect.

---

67
CT15. Test and sham pulse time is demarcated by the horizontal arrows. Vertical arrows indicate when the lights were turned off in the 14:10 LD cycle before the pulse. Animals were transferred to DD at the onset of each pulse.
methods. Cross hatching indicates control experiment. (*) significant difference from the control.
Constant Hypoxia (5% O₂)

1st day post pulse vs. regression analysis

![Bar graph showing phase change (h) with error bars for n=9 in 1st day post pulse and regression for CT6.](image)

- 1st day post pulse
- CT6
- Regression
- n=9
Horizontal arrows demarcate when the pulse was given. Vertical arrows indicate when the lights went out during LD 14:10. Hamsters entered DD at the onset of hypoxia.
control experiment. (*) significant difference from the control.
Constant Hypoxia (5% O₂)

Activity Change

CT6  CT11  CT15

n = 9  n = 9  n = 9

% Activity Change

*
CT6 in DD at the onset of the first pulse, and LD for the duration of the experiment. Cross hatching indicates control experiment. (*) significant difference from the control.
Consecutive Days of Hypoxia

Phase Change (h)

1.50
1.25
1.00
0.75
0.50
0.25
0.00

LD
DD
DD

1st day post pulse
regression

*
constant hypoxia at CT6. Vertical arrows indicate when the lights went out during LD 14:10. Hamsters entered DD at the onset of the first pulse.
prepulse levels four min. after the return to air (Fig 3.13 A).

\( V_T \) did not change significantly from prepulse values (1.71 ± 0.21 ml) during hypoxic episodes or after 4 min. into the post pulse recovery period. During episodes of 17% O\(_2\) \( V_T \) was reduced (0.81 ± 0.06 ml) to about 47% of prepulse values (Fig 3.13 B).

\( V_T/T_1 \) was elevated relative to prepulse levels (4.9 ± 0.24 ml s\(^{-1}\)) during all hypoxic episodes. The response of \( V_T/T_1 \) during hypoxic episodes was biphasic, \( V_T/T_1 \) peaked at 20 min. (12.1 ± 0.9 ml s\(^{-1}\)) and subsequently declined to its minimum hypoxic value at 180 min. (8.57 ± 0.456 ml s\(^{-1}\)). \( V_T/T_1 \) remained depressed during hypoxic episodes relative to the 20 min. peak value for the remainder of the stimulus. \( V_T/T_1 \) returned to prepulse values during each 17% O\(_2\) episode and throughout the recovery period (Fig 3.13 C).

\( \dot{V}_1 \) values did not change significantly from prepulse values (96.4 ± 6.4 ml min\(^{-1}\)) during all 17% O\(_2\) episodes. \( \dot{V}_1 \) responded biphasically during the hypoxic episodes. \( \dot{V}_1 \) peaked at twenty min. (229.7 ± 12.4 ml min\(^{-1}\)), and declined until the last measurement at 180 min. (161 ± 29.7 ml min\(^{-1}\)) (Fig 3.13 D).

\( \dot{V}O_2 \) in air remained at prepulse levels (3.5 ± 0.3 ml min\(^{-1}\)) during all the 17% O\(_2\) episodes. However, after 100 min. \( \dot{V}O_2 \) was significantly depressed below prepulse values during 5% O\(_2\) episodes (mean \( \dot{V}O_2 = 1.7 ± 0.2 \) ml min\(^{-1}\)) (Fig 3.13 E).

\( \dot{V}_1/\dot{V}O_2 \) was elevated to approximately 2.2 times prepulse levels during 5% O\(_2\) episodes, and \( \dot{V}_1/\dot{V}O_2 \) returned to prepulse levels during the 17% O\(_2\) episodes and the recovery period (Fig 3.13 F).

3.8b. Wheel running rhythms

Episodic hypoxia did not produce a significant phase change relative to the control experiments at CT6 and CT11. At CT15 episodic hypoxia caused a phase advance (0.14 ± 0.06 h) that was significantly reduced relative to the control experiment (0.31 ± 0.07 h) (Fig 3.14 and 3.15).
Figure 5.4c Respiratory response to episodic hypoxia. Episodic hypoxia was initiated at time 0 and terminated at 180 min. (*) significant change relative to prepulse values. (Ψ) measurement that is significantly different from all other measurements.
Episodic Hypoxia

A.

\[ f \] (cycles min\(^{-1}\))

B.

\[ V_T \] (ml)

C.

\[ V_T/T_I \] (ml s\(^{-1}\))

Time (min)
experiments. (*) significant difference from control.
Episodic Hypoxia

Phase Change (h)

<table>
<thead>
<tr>
<th>Condition</th>
<th>n = 9</th>
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<tbody>
<tr>
<td>CT6</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>CT11</td>
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<td></td>
</tr>
<tr>
<td>CT15</td>
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</table>
Horizontal arrows demarcate when the pulse was given. Vertical arrows indicate when the lights went out during LD 14:10. Hamsters entered DD at the onset of episodic hypoxia.
Episodic hypoxia caused a significant reduction in activity at CT6, CT11, and CT15 (-7.22 ± 1.75, -11.3 ± 2.6, -15.3 ± 4.0 # of 10 min. bins, respectively) relative to the respective controls (Fig 3.16).

4. Hypercapnia

Behavioral responses of the hamsters to 15% CO₂ were similar to those during 5% O₂. However, no shivering was observed to occur following the return to air.

4.a. Respiratory responses

Three hours of hypercapnia (15% CO₂) at CT6 induced a significant respiratory response (Fig. 3.17). Respiratory frequency was only significantly elevated above prepulse values (53.5 ± 3.9 breaths min⁻¹) at 10 min. and 160 min (99.4 ± 27.3 breaths min⁻¹, 84.3 ± 11 breaths min⁻¹ respectively) after the onset of hypercapnia (Fig. 3.17 A).

\( V_T \) was elevated significantly relative to prepulse values (2.68 ± 0.26 ml) by 20 min (4.3 ± 0.16 ml) (Fig. 3.17 B). \( V_T \) remained at approximately double prepulse values for the duration of the pulse. \( V_T \) declined to prepulse values by the fourth min. of the recovery period.

\( V_T/T_i \) rose significantly in comparison to prepulse values (5.5 ± 0.46 ml s⁻¹) by the tenth min. of hypercapnia (10.97 ± 0.51 ml s⁻¹) (Fig. 3.17 C). \( V_T/T_i \) remained elevated for the duration of the pulse. \( V_T/T_i \) returned to prepulse values within 4 min. after termination of the pulse.

\( \dot{V}_i \) was significantly elevated (295.2 ± 16.9 ml min⁻¹) relative to prepulse values (133.4 ± 12 ml min⁻¹) for the entire pulse (Fig. 3.17 D). All recovery measurements were statistically equal to the prepulse values.

\( \dot{V}_O_2 \) was significantly lower than prepulse values (4.06 ± 1.25 ml min⁻¹) at 7, 10, 160, and 180 min. (2.24 ± 0.14 ml min⁻¹, 2.31 ± 0.19 ml min⁻¹, 2.03 ± 0.32 ml min⁻¹, 1.96 ± 0.27 ml min⁻¹, respectively) of the hypercapnic pulse. \( \dot{V}_O_2 \) returned to prepulse values by the first air measurement following the pulse (4 min.) (Fig. 3.17 E).
control experiment. (*) significant difference from the control.
concluded at 180 min. (*) significant change from prepulse values.
measurement (133.5 ± 19.6, t = 4min). $\dot{V}_1/\dot{V}O_2$ remained elevated for the duration of the hypercapnic pulse. $\dot{V}_1/\dot{V}O_2$ returned to prepulse values by the first measurement taken in the recovery period (t = 184 min.) (Fig. 3.17 F).

4.b. Wheel running rhythms

Three hours of constant hypercapnia at CT6 and CT15 did not cause a significant phase change relative to the controls (Fig. 3.18 and 3.19).

4.c. Activity

Activity was significantly reduced relative to the control experiments at CT6 and CT15 (-8.5 ± 1.8, -11.8 ± 2.6 number of 10 min. bins) (Fig 3.20). Activity was not affected by the sham pulses.

5. Activity vs. Phase Change.

Changes in activity were not significantly correlated ($r = 0.011$) with phase changes (difference between test and control experiments) when individual data points were used (Fig 3.21 A). However, when two outliers are removed the correlation coefficient $r$ is raised to 0.262 ($P < 0.05$). When the mean phase changes were plotted against mean activity changes between the pulse and control, reductions in activity were significantly correlated with phase delays ($r = 0.92; P < 0.001$) (Fig 3.21 B). The differences between the two tests indicates that individual variability is the reason no significant correlation was obtained when all individual data points were used. The calculated $r^2$ value was 84% indicating that 16% of the variance in the group data cannot be explained by changes in activity.
control experiments.
Horizontal arrows demarcate when the pulse was given. Vertical arrows indicate when the lights went out during LD 14:10. Hamsters entered DD at the onset of hypercapnia.
Figure 3.2: Mean activity change ± SEM for hypercapnia. Cross-hatching indicates control experiment. (*) significant difference from the control.
Hypercapnia

Activity Change

% Activity Change

CT6

CT15

n = 9

n = 9

n = 9

n = 9

*
B. Activity vs. phase change group data for all respiratory stimuli. $O_2CT6 / O_2CT11$

/ $O_2CT15$, group data for constant hypoxia. $EO_2CT6 / EO_2CT11 / EO_2CT15$, group data for

episodic hypoxia. $CO_2CT6 / CO_2CT15$, group data for hypercapnia. $3O_2CT6$, group data for

consecutive days of hypoxia.
Discussion

This study has shown that hypoxia can cause small phase delays in hamster activity rhythms. It is unclear whether phase shifts were transient or long lasting. The phase delays may have been mediated through reductions in activity levels. The phase delay caused by hypoxic pulses presented on three consecutive days did not occur when the hamsters remained in a regular LD cycle.

Standard Non Photic and Respiratory Stimuli

The main purpose of this study was to determine whether chemical stimuli that are known to affect lung ventilation will also influence the phase of the circadian pacemaker, as measured by the timing of the onset of wheel-running activity. Preliminary experiments were conducted to confirm (a) that the chosen gas concentrations elicited a respiratory response in the hamster, and (b) that the hamster wheel running rhythms would respond to standard non-photic stimuli in the predicted way under the present experimental conditions.

The whole body-plethysmography experiments confirmed that 3 h pulses of constant hypoxia (O$_2$ concentration = 5%), episodic hypoxia (O$_2$ concentration = 17 - 5%) and constant hypercapnia (CO$_2$ concentration = 15%) significantly affected lung ventilation and metabolic rate.

Novelty-induced wheel running and cage changes were chosen as standard non-photic stimuli that are known to cause large and small phase advances, respectively, when presented at CT6 (Mrosovsky 1988, Reebs et al. 1989). The mean phase shift in response to 3 h of novelty induced wheel running was 3.2 h, which is similar to results previously obtained in hamsters (Reebs et al. 1989, Janik and Mrosovsky 1993). Furthermore, a cage change at CT6 produced a statistically significant mean phase advance of 25 min., again similar to the phase advances of about half an hour observed by Mrosovsky (1988). Novelty-induced wheel-running and the cage
produce data similar to those obtained in other studies.

**Constant Hypoxia**

The $\dot{V}_l$ response elicited by hypoxia was biphasic, similar to those previously reported in the literature (see Mortola and Gautier 1995 for review). Changes in $\dot{V}_l$ over the 3 h pulse were almost entirely caused by changes in $V_T$. Respiratory frequency was elevated by hypoxia until the last two measurements ($t = 140, 160 \text{ min.}$) taken in $5\% O_2$, whereas $V_T$ responded in a biphasic manner with a statistically significant decline from the peak value by the first measurement ($t = 40 \text{ min.}$) after the peak. The increase in $\dot{V}_l$ at the onset of hypoxia is attributed to peripheral chemoreceptor stimulation (Nishimura et al. 1987), whereas the attenuation of $\dot{V}_l$ is caused by washout of $CO_2$ from the central chemoreceptors (Neubauer et al. 1985) and a reduced central drive for respiration (Millhorn et al. 1984, Neubauer et al. 1985, Martin-Body and Johnston 1987). In rabbits the central depressant effects originate in regions rostral to the midbrain/pontine junction (Martin-Body and Johnston 1988), and possibly in the red nucleus (Waites et al. 1996).

The hypoxic hypometabolism observed in the present experiment has previously been observed in cats (Gautier et al. 1989), rats (Gautier and Bonora 1992), hamsters (Mortola 1991), and many other rodents (Frappell et al. 1992). The reduction in metabolic rate is attributed to a inhibition of thermogenesis and direct effects on cellular function (Mortola and Gautier 1995). The temperature data and the shivering following the hypoxic pulse indicate that thermogenesis was inhibited in the present experiment.

Hypoxia typically causes a sustained increase in the $\dot{V}_l/\dot{V}O_2$ ratio at the onset of hypoxia (Mortola et al. 1989, Frappell et al. 1992). The $\dot{V}_l/\dot{V}O_2$ ratio remains relatively constant despite the fact that $\dot{V}O_2$ decrease over the hypoxic period. In the present experiment 3 h of constant hypoxia (5%) induced an increase in the $\dot{V}_l/\dot{V}O_2$ ratio at the onset. However, the $\dot{V}_l/\dot{V}O_2$ ratio was variable throughout the pulse. Changes in the $\dot{V}_l/\dot{V}O_2$ ratio were mainly caused by changes in $\dot{V}_l$. 

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in metabolic rate following the return to air. The increase in VO₂ coincided with the behavioral observation of shivering. The shivering likely occurred to compensate for the reduction in T_b which took place during hypoxia (Mortola and Dotta 1992, Saiki et al. 1994). The increase in VO₂ following the return to air was likely the result of a combination of shivering and non-shivering thermogenesis (brown fat metabolism) (Girardier and Stock 1983).

Constant hypoxia administered at CT6, CT11 and CT15 caused a significant 12-18 min. reduction in the instantaneous phase advance relative to the control when the first day post-pulse was used to calculate the instantaneous phase shift. It is assumed that the reduced phase advances obtained in the Aschoff type II test (hamsters entered DD at the onset of the pulse) would have been phase delays in an Aschoff type I test (where hamsters are in DD for the duration of the experiment) because the phase advance caused by the transition from LD to DD does not occur in the Type I test (Moore-Ede et al. 1982). However, when the first day post-pulse activity onset was determined by extrapolation of the post-pulse regression line, the phase shift obtained was not statistically different from the control at CT6. This result would indicate that the reduced phase advance obtained on the first-day post-pulse at CT6 was a temporary response (i.e. transient). Based on the present data, I tentatively conclude that a single 3 h pulse of constant hypoxia (5%) did not cause a significant long-lasting phase shift in the activity rhythms of hamsters despite the fact that it was a powerful respiratory stimulus. Further experiments, using the AI procedure, are needed to clarify this contradictory result.

Consecutive Days of Hypoxia

Calculation of the phase change caused by 3 h of constant hypoxia administered on three consecutive days using the post-pulse regression line and the first-day post-pulse resulted in a phase advance that was 41 ± 10 min. less than the control advance. Consequently, consecutive days of hypoxia caused a long lasting phase delay in the activity rhythms of the hamster. This
Humans, rats and ducks exhibit a time of day dependent change in apparent chemoreceptor sensitivity which suggests that the respiratory system may be modulated by the circadian system (Raschke and Moller 1989, Peever and Stephenson 1997, Woodin and Stephenson 1998). Furthermore, constant hypoxia can phase delay hamster activity rhythms. If respiration is under circadian control and manipulation of respiration does phase shift circadian rhythms in respiration, as the aforementioned results suggest, then it is possible that a respiratory stimulus on one day can alter the response to the same stimulus on the next day at the same time. This type of feedforward-feedback interaction would be of interest to investigators who use respiratory stimuli to determine the mechanisms involved in respiratory control. Such investigators would benefit from a tool to control for respiratory stimulus induced phase shifts.

Maintaining hamsters in a 14:10 LD cycle during consecutive days of hypoxia eliminated the phase change obtained when animals free-ran in DD following the first pulse. This result would suggest that, with hypoxia in hamsters at least, a maintained LD cycle can prevent a respiratory stimulus from phase shifting activity rhythms. Light is the dominant entrainer of circadian rhythms. However, it has previously been shown that a non-photic stimulus (novelty induced wheel running) can reduce the phase advancing effects of a weak light pulse (10 lux) (Ralph and Mrosovsky 1992). In addition, preliminary experiments have demonstrated that stronger light pulses (100 lux) eliminated the blunting effect that the non-photic stimulus had on the light pulse (Mrosovsky 1993). It appears that possible effects that non-photic stimuli may have on light pulses may be light intensity dependent. Since light levels were approximately 140 lux in the present experiment it is not surprising that the small phase shifts induced by hypoxia were eliminated by light. To further ensure that the experimental subject is at the same circadian time, investigators should allow several days between experiments as this would give the subjects time to reentrain to the prevailing LD cycle.
It has previously been reported that an acute exposure to low barometric pressure can cause phase changes in human circadian rhythms (Ashkenazi et al. 1982). A 30 min. exposure to a simulated altitude of 7620m in combination with a 2-3 min. pulse of hypoxia “early in the day” resulted in transient phase delays of up to three hours in certain physiological and cognitive performance parameters (oral temperature, grip strength, peak expiratory air flow and cognitive ability tests) in human subjects. At 7620 meters the decrease in barometric pressure from 1 atm. to 0.35 atm. causes the partial pressure of oxygen to fall to approximately 57 mmHg. This is equivalent to breathing a gas mixture containing approximately 7.5% O₂ at sea level. The experimental subjects remained in LD following the pulse and all parameters reentrained by three days post pulse. Ashkenazi et al. (1982) concluded that altitude and/or hypoxia would have caused a long lasting phase change had the experimental subject been isolated from all time cues following the pulse. However, there are other ways to interpret this result. Altitude and/or hypoxia may have altered the phase angle of entrainment to the LD cycle implying a change in the period of the SCN output. Altitude and/or hypoxia may have had a direct effect on the measured variables and not on the clock which controls them (i.e. masking).

Due to the various possible interpretations of the Ashkenazi et al. study (1982) it is difficult to make comparisons with the present study. Difficulties in comparing the two studies are further compounded by differences in duration and intensity of hypoxia, species differences, and the combination of hypoxia and altitude. It is possible that the change in pressure was at least partially responsible for the phase shift in human subjects as it has been demonstrated in pocket mice that circadian rhythms in Tₜ are entrained by large cyclic pressure changes (Hayden and Lindberg 1969). The fact that phase delays were obtained in both experiments lends weight to the hypothesis that hypoxia caused the phase shift in the Ashkenazi et al. study (1982), although the results of the present experiment do not support the role of a single pulse of hypoxia as a powerful inducer of phase shifts.
Episodic Hypoxia

The respiratory response to episodic hypoxia observed in hamsters differed in two respects from those observed previously in rats, cats, goats and dogs (Millhorn et al. 1980ab, Bach et al. 1992, Cao et al. 1992, Powell and Aaron 1993, Turner and Mitchell 1997). Despite the fact that each hypoxic episode was followed by an air episode, the hypoxic episodes appeared to have had a cumulative effect, resulting in a biphasic response similar to that seen during constant hypoxia. Mechanisms mediating the ventilatory decline may be the same mechanisms that are believed to be involved in the $\dot{V}_l$ attenuation to constant hypoxia (HVD). These include washout of CO$_2$ from central chemoreceptors due to increased cerebral blood flow stimulated by hypoxic blood (Duffin 1990), or pre- and post-synaptic events causing altered neuronal threshold for excitability within the respiratory control network (Bisgard and Neubauer 1995). The neurotransmitter GABA may be a key mediator of the HVD because GABA antagonists can reverse respiratory depression (Melton et al. 1990). Finally, reductions in oxygen availability promote the metabolic production of adenosine and lactic acid which act as neuromodulators to reduce excitability of CNS neurons (Neubauer et al. 1990, Yan et al. 1995). Changes in some or all of these parameters may cause the progressive hypoxic ventilatory decline seen during episodic hypoxia.

Long-term facilitation (LTF) is a long-lasting increase of ventilation after cessation of a series of brief hypoxic challenges (Cao et al. 1992). LTF was not induced by episodic hypoxia in hamsters as no increase in $\dot{V}_l$ over the prepulse values was observed during the two-hour recovery period. Previous experiments which have evoked LTF in awake dogs and goats have used a 80 and 93% arterial blood saturation (Cao et al. 1992, Turner and Mitchell 1997). Duty cycles were in the range of 2-5 min. of hypoxia followed by 2-5 min. normoxia lasting for 40 to 90 min. (Bach et al. 1992, Turner and Mitchell 1997). Most experiments which induce LTF have maintained arterial isocapnia during episodic hypoxia (Millhorn et al. 1980ab, Bach et al. 1993, Turner and Mitchell 1997) but not all (Powell and Aaron 1993). It appears that the regimen used
differences in the episodic hypoxia protocol were responsible for the lack of LTF. However, further experiments, with various gas concentrations (8% O₂, 8% O₂ and 3% CO₂, 5% O₂ and 2% CO₂) have failed to elicit LTF in the hamster (Stephenson, unpublished data).

One speculative explanation for the absence of LTF in hamsters is that they may have evolved defense arousal system which is not activated by hypoxia. During hypoxia, stimulation of the defense arousal system results in the release of catecholamines in the cat and rat (Hilton and Marshall 1982, Marshall 1984). Recruitment of the defense arousal system as part of the general hypoxic response is interpreted as a beneficial adaptation which allows an animal to prepare itself to escape from a hypoxic environment (Marshall 1987). One of the mechanisms postulated to be responsible for LTF is catecholamine release which results in an increase in metabolic rate (Cao et al. 1992). Repeated bouts of hypoxia, it is hypothesized, may result in a build-up of catecholamines leading to a sustained elevated level of metabolism and \( \dot{V}_1 \) following the pulse. Since hamsters frequently encounter asphyxic conditions in the burrow (Kuhnen et al. 1987), they may have adapted a respiratory response that does not involve recruitment of the defense arousal system, and the subsequent release of catecholamines, as there is no need for escape from the burrow.

It was hypothesized that LTF may cause phase shifts in hamster activity rhythms because the release of serotonin in the central nervous system is necessary for the inducement of LTF in cats (Millhorn et al. 1980b) and serotonin may be involved in non-photic phase shifting as there is a serotonergic projection from the dorsal raphe to intergeniculate leaflet (IGL) (Meyer-Bernstein and Morin 1996). Phase shifts to non-photic stimuli are eliminated by lesions of the IGL (Johnson et al. 1989, Janik and Mrosovsky 1994, Wickland and Turek 1994). Since, LTF was not induced by episodic hypoxia this hypothesis remains untested.

Episodic hypoxia did not cause a phase shift in hamster running activity at CT6 and CT11. However, a statistically significant 8 ± 3.3 min. reduced phase advance relative to the control was obtained at CT15. Since the respiratory response to episodic hypoxia was similar to
that of constant hypoxia, it is possible that the same mechanism was responsible for the delayed activity onset on the first day post pulse.

Episodic hypoxia occurs commonly in patients with sleep apnea where frequent apneic episodes result in recurrent bouts of oxyhemoglobin desaturation in sleep. The findings of episodic hypoxia tests would suggest that, in hamsters at least, circadian rhythms are not disrupted by cyclic reductions in the oxygen content of blood. The clinical relevance of this finding is limited because the atypical respiratory response of the hamster suggests that the phase response could also be atypical.

**Hypercapnia**

The ventilatory response to hypercapnia was consistent with responses previously reported in the literature (Chapin 1954, Walker et al. 1985, Maskrey 1990). The effect of hypercapnia on metabolism in the present study is interesting because the effects of CO$_2$ on metabolism have not been studied exhaustively. The results presently available on adult mammals appear to be contradictory, since hypercapnia has been said to increase, have no effect, or decrease metabolic rate (Stupfel 1974, Jennings and Laupacis 1982, Kaminski et al. 1985, Gautier et al. 1993). However, comparison of data between published studies, and the present unpublished data, suggest that the metabolic response to hypercapnia may be concentration dependent. For example, in adult and newborn rats 2-5% CO$_2$ had no effect on metabolism (Saiki and Mortola 1996), whereas 19.5-32.5% CO$_2$ caused a significant decrease in metabolic rate (Stupfel 1974). In the present study, 15% CO$_2$ reduced metabolism to about two-thirds of resting levels. In animals exposed to CO$_2$ concentrations higher than 10%, metabolic effects are to be expected, since an acute fall of pH impairs the utilization of glucose and fat due to an inhibition of the activity of pH-dependent enzymes (Stupfel 1974, Kuhnen et al. 1987). The fall in pH is dependent on the buffering capacity of the blood which tends to be higher in fossorial animals (Boggs et al. 1984). At lower levels of CO$_2$ sympathetic reactions to the chemoreceptor stimulation may prevail (Saiki and Mortola 1996).
significant phase change in hamster activity rhythms. However, the hypercapnic pulse at CT6 was a powerful respiratory stimulus.

**Mechanism of Phase Shifts**

Although it was hypothesized that the respiratory stimuli would be arousing, the respiratory data and behavioural observations indicate that the stimuli were in fact depressing. Consequently, the possibility that an arousing respiratory stimulus can cause a phase shift in hamster activity rhythms remains untested. Nonetheless hypoxia did induce phase delays. There are several mechanisms which could explain the effect that hypoxia had on circadian wheel running rhythms:

1. Phase delays might occur if hypoxia slowed or temporarily stopped the pacemaker. Hypoxia can cause reductions in neuronal excitability (Bisgard and Neubauer 1995). However, whether or not hypoxia-induced reductions in neuronal excitability have any effect on the SCN is unknown. The SCN may in fact be able to compensate for changes in neuronal excitability as it does compensate for changes in temperature (Rawson 1960, Richter 1975, Gibbs 1983). The circadian system is considered to be temperature-compensated because temperature changes do not affect circadian rhythms to the degree that they affect biochemical reactions (Moore-Ede et al. 1982).

2. A variety of stimuli that induce changes in locomotor activity also induce phase-dependent shifts in the circadian rhythm of locomotor activity in hamsters (Boulos and Rusak 1982, Mrosovsky 1988, Reebs and Mrosovsky 1989, Mrosovsky and Salmon 1990). Therefore, the role that changes in activity, caused by constant hypoxia and episodic hypoxia, may have had on the circadian rhythm of locomotor activity should be considered.

Reductions in activity were significantly correlated with instantaneous phase delays calculated using the first-day post-pulse (Fig. 3.21). Reduction in activity might have the same effect as immobilization experiments where preventing a hamster from running during the highly
Hypoxia may be an indirect way of immobilizing hamsters. However, if reductions in activity induce phase delays why did hypercapnia, which caused significant reductions in activity, fail to induce phase delays?

Hypercapnia may not have caused phase delays because there may be a minimum threshold for the reduction in activity. Activity thresholds have been demonstrated in novelty induced wheel running experiments which typically require that a hamster run more than 4000 revolutions, in a 17 cm diameter wheel, before a phase shift occurs (Janik and Mrosovsky 1993). Perhaps the reduction in activity caused by hypercapnia was not large enough to cause a phase change in hamster activity rhythms.

The correlation between activity reduction and phase delays is complicated by the fact that the first day post pulse and regression analyses gave conflicting results for constant hypoxia at CT6 (i.e. phase delay vs. no phase delay, respectively). It is possible that the regression analysis did not yield a significant delay because the sample size was too small, although, there is no trend in the data which would indicate this. It is also possible that the reduction in activity caused by hypoxia is close to the threshold for activity reduction induced phase delays. Further experiments are required to determine the role of activity reductions in hypoxia induced phase delays.

3. Constant hypoxia caused a reduction in $T_b$ and metabolic rate, and subsequent shivering in the recovery period. It has been demonstrated in rats and hamsters that periods of hypothermia induce phase delays at several circadian times (Rawson 1960, Richter 1974, Gibbs 1983). Therefore, changes in body temperature may be responsible for the phase delays caused by hypoxia. The phase shifts caused by the reductions in $T_b$ have been interpreted as the result of $Q_{10}$ effects combined with unknown clock mechanisms which partially compensate for the temperature changes. $Q_{10}$ is a measure of the temperature-sensitivity of rates of reaction, and the rate of most biochemical processes changes two- or threefold with each 10°C change in temperature (that is, $Q_{10} = 2-3$) (Moore-Ede et al. 1982). Based on the conclusions of the
induced hypothermia may be responsible for the phase delays.

However, the $T_b$ manipulation experiments (Rawson 1960, Richter 1974, Gibbs 1983) did not control for confounding behavioral correlates. Examination of the representative actograms in the Rawson (1960) study reveals that hypothermic pulses visibly reduced activity levels even when they occurred outside of the period when hamsters were normally active. For example, mice experienced a reduction in $T_b$ to an average of 31.2 °C (min. of approx. 28°C) over 3 h, which caused a 35 min. phase delay and what appears to be a 40 - 60% reduction in activity (Rawson 1960). It may be that the phase delays obtained for reductions in $T_b$ in mice were partially the result of reduced activity levels as immobilization of hamsters during their active period also causes phase delays (Van Reeth et al. 1991). It appears that the mechanisms postulated to be mediating hypothermia-induced phase changes need to be re-examined to account for changes in activity levels.

The fact that activity changes may play a role in the phase delays caused in the temperature manipulation studies does not mean that changes in $T_b$ do not have an effect on biological time-keeping. However, if a small reduction in temperature over a short period of time causes a large reduction in activity, as in the present experiment, it seems likely that the changes in activity would have a stronger effect on the circadian activity rhythms of hamsters than reductions in $T_b$ would. It would be interesting to know whether changes in activity correlated with the phase changes in the temperature experiments, as they did in the present study.

4. The evidence for reductions in activity mediating the changes in activity rhythms by hypoxia is correlative and therefore not conclusive. Thus, it is still possible that the respiratory stimulus is directly responsible for the phase delays. However, it is unlikely that changes in a specific respiratory parameter is directly responsible for the phase delays. All the respiratory stimuli used in the present experiment resulted in changes in respiration. All the respiratory stimuli caused increases in $f$, $V_T$, $\dot{V_l}$, $\dot{V_l}/\dot{V}_O_2$, and $V_T/T_1$ and a reduction in $\dot{V}_O_2$, yet phase shifts were only induced by hypoxic stimuli. No correlation was detected between any of the
similar respiratory effects does not imply that they were mediated by the same respiratory mechanism and therefore it is still possible that the hypoxic stimuli were directly responsible for the observed phase delays.

*Respiratory Stimuli and Activity*

The mechanism by which hypoxia and episodic hypoxia induced large reductions in activity levels is unknown. Reductions in activity caused by pulses which occurred during the active period can conceivably be explained by experiments which demonstrated that $\dot{V}O_2\text{max}$ (maximum rate of oxygen consumption) is reduced at high altitude (Tucker et al. 1984, Cymerman et al. 1989). It is possible that severe hypoxia could reduce $\dot{V}O_2\text{max}$ to a level where wheel running is no longer aerobically sustainable.

The mechanisms mediating the reduction in activity caused by hypoxic pulses that occurred outside of the active period (i.e. CT6) are more difficult to understand because hamsters would often run immediately following a pulse that occurred during the active period or even between hypoxic episodes. Perhaps running following the pulse which occurred during the active period (CT11 and CT15) is for a different reason than the running following a pulse which occurred outside the active period (CT6). Shivering following a hypoxic pulse and temperature data indicate that $T_b$ was reduced during hypoxic pulses. Perhaps the hamsters were running to rewarm following the pulse. It has previously been demonstrated that reduced ambient temperatures cause hamsters to run (Mrosovsky and Biello 1994, Mistlberger et al. 1996).

The attenuation in activity following a pulse at CT6 could be caused by long lasting changes in blood lactate flux. Exposure of mammals to hypoxia results in immediate hyperventilation which in turn, leads to respiratory alkalosis because $CO_2$ is blown off. The alkalosis is compensated principally by reduced net acid excretion via the kidneys (Gonzalez and Clancy 1986). If the kidneys were to maintain a low level of acid excretion following the pulse it is possible that the lactate levels normally produced during activity bouts could not be removed.
reduced kidney lactate excretion would contribute to muscle fatigue and may reduce the overall activity levels. However, a reduction in the rate of acid excretion has only been demonstrated after several weeks at high altitude (Gonzalez and Clancy 1986, Brooks et al. 1991). Furthermore, the time constant of changes in kidney excretion rates in hypoxia and on the return to normoxia are unmeasured. Other possible mechanisms include a reduction in energy substrates such as glycogen or long lasting changes in neurochemicals.

**Future Experiments**

The fact that three powerful respiratory stimuli did not directly influence the activity pacemaker in hamsters raises several questions:

1. Do hamsters exhibit a time-of-day sensitivity to a respiratory stimulus?

   Daily changes in respiratory chemosensitivity have already been demonstrated in humans, rats and ducks (Raschke and Moller 1989, Peever and Stephenson 1997, Woodin and Stephenson 1998). It would also be expected that hamsters would have a daily rhythm in chemosensitivity.

2. a. Can LTF be induced in fossorial animals? 2. b. Do respiratory stimuli cause phase shifts in non-fossorial animals?

   One of the major reasons for maintaining that hypoxia and hypercapnia could cause phase shifts in hamster activity rhythms is that these respiratory stimuli have been shown to be arousing by stimulation of the defense reaction in several non-fossorial species (see Marshall 1987 for review). Arousal is a common feature in many non-photic stimuli (Mrosovsky 1996). However, the behavioral and respiratory data obtained in the present study suggests that hypoxia and hypercapnia were not arousing in the hamster. The possibility that adaptations to a burrowing lifestyle have eliminated the defense reaction from the hypoxic and hypercapnic respiratory response might explain the absence of both LTF and phase shifts in activity rhythms.
mammals and (b) if respiratory stimuli cause phase shifts in non-fossorial mammals.

3. What is the role of exercise in hypoxia induced phase delays?

Reductions in activity levels are the most likely cause of the observed phase delays. Therefore, it would be interesting to see if periods of immobilization similar to those induced by hypoxia would cause similar phase delays. Matching phase delays would lend weight to the hypothesis that hypoxia induced phase shifts are activity mediated. Developing a curve for different levels of immobilization versus the phase shift obtained would also determine if an activity threshold exists for the phase shifts caused by reductions in activity.

Critique of the Method

Despite the fact that the apparatus was capable of producing data similar to that of other studies using non-photic stimuli, the lack of a permanent phase shift by a single pulse of hypoxia at CT6 suggests that the sample size may have been too small. Repetition of the experiment would elucidate the effect of hypoxia on hamster activity rhythms.

Whole-body plethysmography demonstrated that constant hypoxia, episodic hypoxia and hypercapnia evoked a respiratory response. However, $T_b$ and $T_N$ used to calculate $V_T$ were estimated from separate experiments. Since a 1°C error in $T_b$ would cause a 5% error in $V_T$ the results of the statistical tests could have been affected.

In order to obtain an accurate measurement of $T_b$ implantable radio transmitters should have been used. However, transmitters were unavailable. The remaining option was the use of thermocouples to obtain rectal temperature. Obtaining temperature measurements in this way may cause hamsters discomfort due to the total restraint required and therefore placed limits on the measurements that could be obtained. Hamsters have been immobilized for three hours in other work (Van Reeth et al. 1990) and therefore this was taken to be the upper limit of restraint time.
made:

1. The relationship between $T_b$ and $T_N$ in air was assumed to be the same during hypoxia. Over the small range of $T_b$ observed in air there was no correlation between $T_b$ and $T_N$, supporting the assumption that the difference between $T_b$ and $T_N$ is constant.

2. $T_b$ was assumed to fall at a constant rate throughout the period of hypoxia. This assumption is supported by the fact that the reduction on $T_b$ during hypoxia is linear over 2 h period of observation. However, it is possible that the assumption caused a underestimation of $T_b$ during the final hour of constant hypoxia.

3. $T_b$ was assumed to be unchanged during episodic hypoxia. Assuming that each four minute hypoxic period within the episodic pulse caused the same reduction in $T_b$ as the initial four minutes of constant hypoxia, there would be a 0.1°C reduction in $T_b$.

Consequently, any changes in $T_b$ during episodic hypoxia would cause 0.5% change in $V_T$ calculations. However, since episodic hypoxia evoked a hypometabolism it is likely that temperature did fall further than 0.1°C. Overestimating temperature would exaggerate the difference in $V_T$ and $V_I$ between pre-pulse and pulse.

Despite the fact that these assumptions probably caused small errors in $V_T$ it is unlikely that they would have resulted in misinterpretation of the respiratory responses.

**Conclusion**

The results of this study would suggest that respiratory stimuli do not directly phase shift the SCN. Since the small phase delays obtained correlate with reductions in activity it is quite possible that alterations in behavioral states were responsible for the phase shifts obtained. This study appears to be another example of the importance of activity and/or its correlate in phase shifting of the SCN in hamsters.
The possibility that respiratory stimuli might modify the performance of the circadian pacemaker has not previously been considered by most investigators. Although the results of the present experiment indicate that respiratory stimuli do not cause large phase shifts, it is important not to overgeneralize from these results. The possibility exists that adaptations to a fossorial lifestyle may have prevented the respiratory stimuli from inducing phase shifts. Further investigations are necessary to determine possible interactions between respiration and the circadian system.


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The correlation of nasal temperature with body temperature

Pressure changes associated with ventilation are dependent upon changes in temperature of the inspired air. During inhalation the air is heated to $T_b$. Air is subsequently cooled rapidly to $T_N$ during exhalation (Epstein and Epstein 1978, Jacky 1980). Consequently, $VT$ calculations rely on a measure of $T_b$ and $T_N$. In order for the respiratory experiment to demonstrate that the respiratory stimuli are in fact stimulating the respiratory system a normoxic-pulse comparison was all that was necessary. As long as $T_b$ remains constant, any error associated with inaccurate temperature data will be systematic with negligible effects on the comparisons.

Hypercapnia is known not to affect $T_b$ at a $T_c$ of about 20°C (Stupfel et al. 1974, Saiki and Mortola 1996, Peever and Stephenson 1997). However, $T_b$ has been shown to fall during continuous hypoxia (Frappe et al. 1992, Mortola and Dotta 1992). To estimate for the reduction in $T_b$, an experiment was conducted in which measured $T_b$ was measured over a two hour hypoxic pulse and a one hour recovery. It was assumed that $T_b$ remained constant throughout the episodic hypoxia pulse.

Methods:

Five hamsters were used. All experiments were performed between CT6 and CT9. The hamsters were restrained in a Plexiglas cylinder which was just small enough to prevent the hamsters from turning around but allowed some head and leg movement. $T_b$ was measured by placing a thermocouple probe (Bat-10, Physiotemp) approximately 4 cm into the rectum. $T_N$ was obtained by placing a similar thermocouple in the expiratory airstream immediately outside the nostril. The thermocouples were hard wired via a universal amplifier (model G-4123-01, Gould Electronics) to a computer (MacLab/8, Analog Digital Instruments Ltd.) for recording. Once the rectal thermocouple was in place the animals were left for ten minutes. $T_b$ was recorded over the entire experimental period while $T_N$ was obtained for at least two uninterrupted fifteen second intervals.
restrainer were placed in the plethysmograph where they were exposed to 5% O₂ for two hours and then air for one hour. The third hour of T_b was obtained by extrapolation of a regression line based on the first two hours of hypoxia. The animals were not exposed to three hours of hypoxia in the restrainer in order to reduce their discomfort.

Results:

The mean T_b was 37.1 +/- 0.2 °C (n = 5) in animals breathing air. The mean T_N was 31.4 +/- 0.5 °C (n = 5). There was no correlation between T_b and T_N over the small range of T_b used in the experiment (P < 0.05). The relationship between T_b and T_N in hypoxia and hypercapnia was assumed to be the same as it was in air.

The effect of episodic hypoxia on T_b is unknown. The equation of the regression line of T_b during hypoxia is (Fig A1.1):

\[ T_b = -0.034(\text{Time}) + 37.7 \]
represent T_N in air. Solid circles represent T_b during 5% constant hypoxia.