Daily Exposure to a Nonphotic Stimulus Can Alter Photoperiodic Response to Short Days in Hamsters (43732)

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Abstract. **The ability of mammals to measure seasonal changes in daylength depends upon a circadian clock and the phase-relationship between this clock and the light:dark cycle. Recently, a number of pharmacological and nonpharmacological stimuli have been shown to have pronounced effects on the phase of the circadian clock of rodents. The objective of the present study was to determine if a drug-induced change in the phase-relationship between a measurable circadian rhythm (i.e., wheel running behavior) and the light:dark cycle would alter the effects of the light cycle on the neuroendocrine-gonadal axis. Adult male hamsters with regressed testes due to exposure to an inhibitory 10:14-hr light:dark cycle were daily injected with vehicle or the short-acting benzodiazepine, triazolam, while remaining on short days, while a control group of hamsters was transferred to a photostimulatory 14:10-hr light:dark cycle. Two other groups of hamsters with regressed testes were blinded and daily injected with vehicle or triazolam. The injections were timed to occur about 4 hr before activity onset because previous studies had demonstrated that injections of triazolam at this time can lead to a phase advance in the activity rhythm. The circadian rhythm of wheel running behavior was measured in all the animals maintained on the 10:14-hr light:dark cycle in order to monitor circadian phase. While no testicular growth was observed after 25 days of vehicle injections, growth was observed in the triazolam-treated animals that was comparable to that observed in control animals transferred to long days. Testicular growth in triazolam-treated animals was associated with an earlier onset of locomotor activity, when compared with the vehicle-treated animals. Importantly, triazolam had no effect on the testicular size of blind animals. These results indicate that daily injections of triazolam can stimulate neuroendocrine-gonadal activity by altering the phase-relationship between the cycle and the circadian clock involved in photoperiodic time measurement, and that agents which can affect the clock may be useful in altering seasonal cycles.** [P.S.E.B.M. 1994, Vol 206]

There is now substantial evidence indicating that photoperiodic time measurement (i.e., the measurement of the seasonal change in daylength) in both birds and mammals depends upon the phase-relationship between the light:dark cycle and an internal circadian or 24-hr clock (1–4). The results from a number of different experiments indicate that if light is present at certain times of the circadian cycle, the day will be interpreted as being a long one, regardless of the absolute amount of light to which the animal may be exposed, and the reproductive response of the animal will be the same as if the animal were exposed to normal long days (1–4).

The importance of the circadian clock for measuring the seasonal change in daylength raises the possibility that manipulation of the internal clock by experimental interventions could lead to a change in the reproductive response of an animal to a given daylength. For example, a drug-induced change in the phase-relationship between the circadian system and

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the light:dark cycle could result in a misreading of how long the day actually is, since different phase points of the circadian clock would be coincident with times of light and dark than under drug-free conditions. Theoretically, there are at least two possible ways in which a drug could alter the phase-relationship between the circadian system and the light:dark cycle (5). First, the acute administration of a drug might induce a direct phase-shift in the circadian clock governing rhythmicity; such a phase-shift might alter the entrainment pattern to the entraining light cycle. Second, the chronic administration of a drug might lead to a change in period of the underlying clock; such a change in period would lead indirectly to a change in the phase-relationship between the circadian clock and the light:dark cycle since this phase-relationship is dependent on the period of the endogenous circadian clock (6, 7).

To our knowledge, the only substance that has been successfully used to alter the photoperiodic response by inducing a change in the phase-relationship between the light:dark cycle and the internal circadian clock is deuterium oxide (D_2O). Eskes and Zucker demonstrated that while testicular regression normally occurs in hamster exposed to an 10:14-hr light:dark cycle (i.e., 10 hr of light per 24 hr), providing D_2O (7.5%) in the drinking water prevented testicular regression, presumably due to the known effects of D_2O on lengthening the free-running period of the circadian clock (8). Indeed, in hamsters maintained on an 10:14-hr light:dark cycle and provided with D_2O, activity onset was delayed by about 4 hr when compared with control animals. This change in the phase-relationship between activity onset and the light:dark cycle resulted in lights-on occurring about 8 hr after the onset of activity, a time when light is known to be stimulatory to neuroendocrine-gonadal activity in this species (3).

Recent experiments indicate that treatment with the short-acting benzodiazepine, triazolam, can induce both phase-dependent shifts in entrained rhythms as well as induce changes in the period of the underlying circadian clock (9–12). These results indicate that triazolam can have both direct and indirect effects in altering the phase-relationship between the light:dark cycle and circadian rhythms, and raise the possibility that such changes in phase could lead to an altered photoperiodic reproductive response (13, 14). In the present study we sought to test the hypothesis that a triazolam-induced change in the phase-relationship of the activity rhythm to the light:dark cycle would lead to an altered response of the neuroendocrine-gonadal axis to the inhibitory effects of short days.

Materials and Methods

Male golden hamsters (Mesocricetus auratus LAK:LVG-[SYR]), raised in our breeding colony from animals purchased from Charles River Lakeview (Newfield, NJ), were kept six per cage in a light-controlled room until the age of 7 weeks. The animals were maintained under a 14:10-hr light:dark cycle (lights-on 07:00–19:00 hr) with light intensity averaging 600 lux at cage floor level (United Detector Technology lightmeter).

At the age of 7 weeks, 47 hamsters were transferred to a dim 10:14-hr light:dark cycle (light intensity ≈10–30 lux at cage floor level). After 9 weeks of this particular lighting regimen, all but seven hamsters of this cohort were moved to individual cages equipped with a running wheel to allow for the continuous recording of locomotor activity (15), while the remaining seven animals were kept group-housed. One week later, the 42 animals still alive were assigned to five different groups. Control hamsters kept group-housed from the beginning of the study were returned to a 14:10-hr dim light:dark cycle and left undisturbed for 25 days ($C_{14L:10D}$, $n = 7$). Two groups of animals were maintained on the original 10:14-hr light:dark cycle and received daily injections of either vehicle ($V_{10L:14D}$, $n = 10$) or triazolam ($T_{10L:14D}$, $n = 10$) for 25 days. Two additional groups of hamsters were bilaterally enucleated under deep pentobarbital anesthesia (80–100 mg/kg) and subjected to a treatment consisting of daily injections of the vehicle dimethylsulfoxide (V_{bl}, $n = 7$) or triazolam (T_{bl}, $n = 8$) for 25 days in a row. Animals were blinded rather than exposed to DD for these last two groups to insure that they were not accidentally exposed to any light. After 25 days of these experimental conditions, living animals of all groups were transferred to constant darkness (DD) at the usual time of lights-off, and remained under DD conditions for 6 days before termination of the experiment. Three hamsters died during the course of treatment. Throughout the entire study, hamsters were provided with food and water *ad libitum*.

The volume of each injection of vehicle (DMSO) or triazolam (Halcion; Upjohn Company, Kalamazoo, MI) given to the animals was 0.1 ml, and the daily dose of triazolam was 0.1 mg. The first vehicle or triazolam injection was timed to occur ≈4 hr before the expected onset of locomotor activity on this day, and further injections were given each day at the same clock time. The timing of treatment with triazolam was critical for the design of these studies since previous studies have demonstrated that the effects of triazolam on phase of the activity rhythm under both entrained and free-running conditions are dependent on the circadian time of drug treatment. Thus, depending on the time of treatment, injections of triazolam can phase delay, phase advance, or have no effect on the rhythm of activity (13).

Under light ketamine anesthesia, right testis width was repeatedly assessed in all hamsters on five differ-

ent occasions: first, on the day of transfer from the 14:10-hr to the 10:14-hr light:dark cycle, then, after 6, 9, and 10 weeks under the 10:14-hr light:dark cycle, and finally, before transfer of the animals to DD. At the end of the experiment animals were sacrificed by decapitation, and both testes were removed and weighed.

No attempt was made to quantify the phase relationship between the onset of activity and the light: dark cycle curing the time of the injections due to the possible masking effects of treatment on activity onset. Instead, an eye-fitted straight line was drawn through the onset of locomotor activity of each individual animal for the 6-day interval after release into DD, and was extrapolated backwards to establish the onset of activity on the last day of drug injection under the light:dark cycle. Gonadal measurements (i.e., testis widths and pair testes weights) and estimated phase relationships between the activity rhythm and the light-dark cycle were compared between different groups of animals by using an unpaired Student's *t* test for independent samples. In comparing gonadal measurements within groups of hamsters, paired Student's *t* tests (modified by Bonferonni) were used.

Results

Just prior to their transfer from the 14:10-hr to the 10:14-hr light:dark cycle, all hamsters had large testes (mean testis width = 11.1 ± 0.1 mm, n = 47). As expected from the results of previous studies (3, 16), exposure to short days induced testicular regression in all hamsters: after 10 weeks of exposure to short days, the mean testis width in the 42 living animals averaged 6.1 ± 0.1 mm, and there was no significant difference in testis size between the five groups of animals.

Reexposure to 14:10-hr light:dark cycles induced partial testicular recrudescence in $C_{14L:10D}$ animals; mean testis width averaged 8.0 ± 0.7 mm after 25 days of 14:10-hr light:dark cycles, and testes weight averaged 1.19 ± 0.29 g at the end of experiment (Fig. 1 and 2). Vehicle injections had no stimulatory effect on gonadal function of hamsters kept under the 10:14-hr light:dark cycles: testis width in $V_{10L:14D}$ animals averaged 6.5 ± 0.1 mm on the day of the last injection, and paired testes weight 0.27 ± 0.03 g at the end of experiment. In contrast, triazolam injections had a clear stimulatory effect on the gonadal axis of hamsters maintained under the 10:14-hr light:dark cycle: mean testis width averaged 8.4 ± 0.3 mm in $T_{10L:14D}$ animals at the end of drug treatment, a value significantly larger ($P < 0.001$) compared with testis width in $V_{10L:14D}$ animals, and compared with $T_{10L:14D}$ animals before drug treatment ($P < 0.001$). Similarly, at the end of the experiment, mean testes weight was significantly larger in $T_{10L:14D}$ animals (0.97 ± 0.16 g, n = 10) compared with $V_{10L:14D}$ animals ($P < 0.002$). Importantly, both testis widths at the end of drug treatment and pair testes weight at the end of experiment were similar in $T_{10L:14D}$ and in $C_{14L:10D}$ animals ($P > 0.3$) (Fig. 1 and 2).

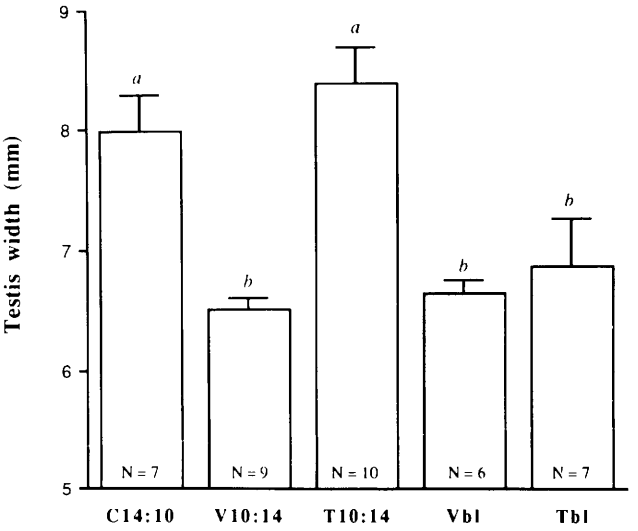

Figure 1. Mean (±SEM) right testis width (mm) in the five groups of hamsters. After 25 days of exposure to long days, control hamsters exhibited partial testicular recrudescence ($C_{14L:10D}$ group). There was no significant increase of testicular growth in sighted hamsters kept on short days and treated with vehicle ($V_{10L:14D}$ group). In contrast, testicular growth was observed in hamsters kept on short days and treated with triazolam ($T_{10L:14D}$ group) and was comparable to testicular growth observed in $C_{14L:10D}$ animals. No significant increase in testis width was observed in blind hamsters treated with vehicle (V_{bl} group) or triazolam (T_{bl} group). Numbers at the bottom of each column designate the number of animals per group at the time measurements were taken. *a*, The groups of animals that were not significantly different from each other; *b*, the groups of animals that were significantly different from *a*. ($P < 0.001$). Note: all hamsters showed testis regression after 10 weeks of exposure to short days (mean testis width = 6.1 ± 0.1 mm).

Repeated injections of vehicle or triazolam did not induce significant changes on gonadal size of blind hamsters: testis widths at the end of drug treatment were similar in V_{bl} (6.6 ± 0.2 mm, n = 6) and T_{bl} (6.9 ± 0.4 mm, n = 7) animals ($P > 0.6$). Those values were not significantly different from testis width values before drug treatment ($P > 0.1$ in both groups) (Fig. 1). Similarly, mean pair testes weights were similar in V_{bl} (0.35 ± 0.04 g) and T_{bl} (0.48 ± 0.1 g) animals at the end of the experiment ($P > 0.2$) (Fig. 2).

Daily injections of vehicle given to $V_{14L:10D}$ hamsters had no apparent effect on the phase-relationship between the onset of activity and the time of lights-off, and the mean onset of locomotor activity on the last day of drug treatment was calculated to occur 105 ± 11 min after lights-off (Fig. 3). Activity onset occurred near the time of the injection in $T_{10L:14D}$ hamsters, and the onset of activity on the last day of drug treatment was calculated to occur 4 ± 42 min after the time of

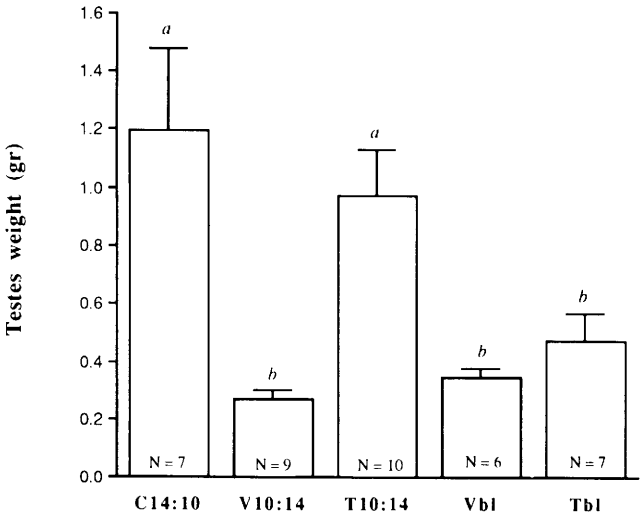

Figure 2. Mean (±SEM) paired testes weight (g) in the five groups of hamsters at the end of the experiment. The testes of control hamsters in the $C_{14L:10D}$ group and those of hamsters in the $T_{10L:14D}$ group exhibited a similar partial recrudescence. Testes weights in these two groups of animals were significantly larger than in hamsters in the $V_{10L:14D}$ group and in the V_{bl} group or the T_{bl} group. See Figure 1 legend for further details.

lights-off, a value significantly earlier than that observed in the $V_{14L:10D}$ animals ($P < 0.05$) (Fig. 3). The rhythm of wheel running behavior was not entrained by the daily injections of vehicle in any of the V_{bl} hamsters, and the onset of locomotor activity on the last day of vehicle treatment varied between 360 and 540 min after lights-off in this group of animals (Fig. 4). In contrast, activity onset occurred near the time of the injection in T_{bl} hamsters, and the onset of locomotor activity on the last day of drug treatment was calculated to be between 3 and 4 hr before lights-off, indicating that the animals were entrained by the daily injections of triazolam (Fig. 4).

Discussion

These results indicate that treatment with triazolam can induce testicular recrudescence in hamsters maintained on an inhibitory daylength. The absence of any stimulatory effect of triazolam on testicular size in blind hamsters indicates that this drug does not have any direct stimulatory effect on the neuroendocrine-gonadal axis. Instead, the stimulatory effects of triazolam on testicular size appear to be due to the effects of the drug on the circadian clock involved in measuring the length of the day. Daily injections of triazolam induced a change in the phase-relationship between the light:dark cycle and the circadian clock which resulted in a light cycle that is normally inhibitory to gonadal function now being stimulatory to neuroendocrine-gonadal activity. A phase advance and/or a shortening of the period of the circadian clock induced by triazolam altered the phase-relationship of the activity rhythm to the light:dark cycle in such a manner that the onset of activity occurred within a few minutes of lights-off. The photosensitive phase (i.e., the circadian time when the presence of light triggers a long-day response) in the hamster begins about 0.5 hr prior to the onset of activity and ends about 11 hr after activity onset (3). Thus, the new phase-relationship between the light:dark cycle and the circadian clock which was induced by triazolam apparently resulted in light now being coincident with the photosensitive phase of the circadian clock.

These results represent the second demonstration that a drug-induced change in the way hamsters en-

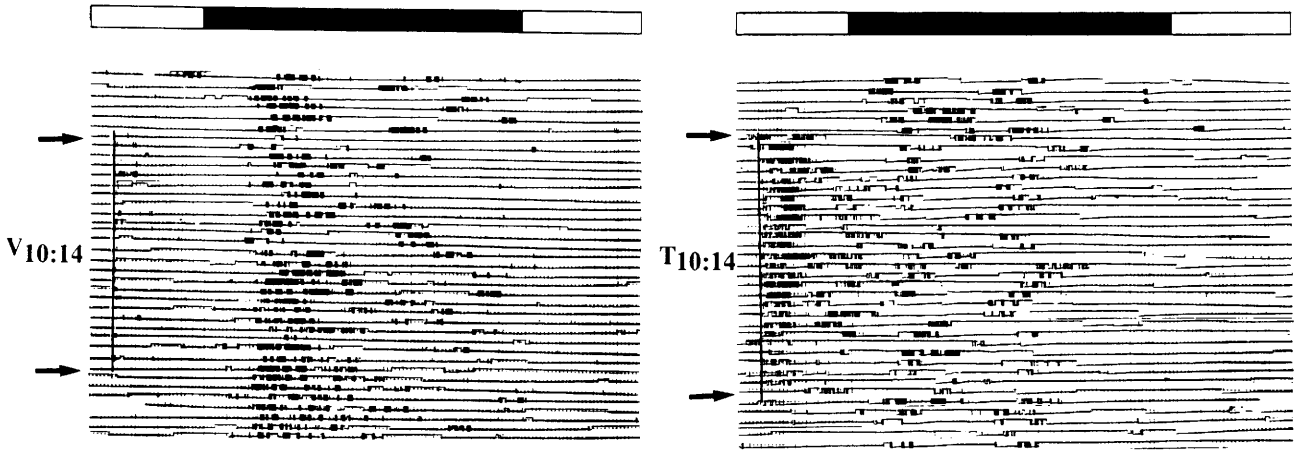

Figure 3. Continuous wheel running activity records from two representative sighted hamsters injected with vehicle or triazolam. The two animals were exposed to a similar 10:14-hr light:dark cycle for the first 32 days of this record, and were then transferred to DD for the last 6 days of the experiment. Starting on day 7 of these records, these animals were subjected to daily injections of vehicle (V, left panel) or 0.1 mg triazolam (T, right panel) every 24 hr for 25 days in a row. Successive days are plotted from top to bottom. Arrows on the left of each record designate the first and last day of injections with vehicle or triazolam, and the second arrow designates the day of transfer to DD. The vertical black bars within the records designate the exact time of the injections of vehicle or triazolam.

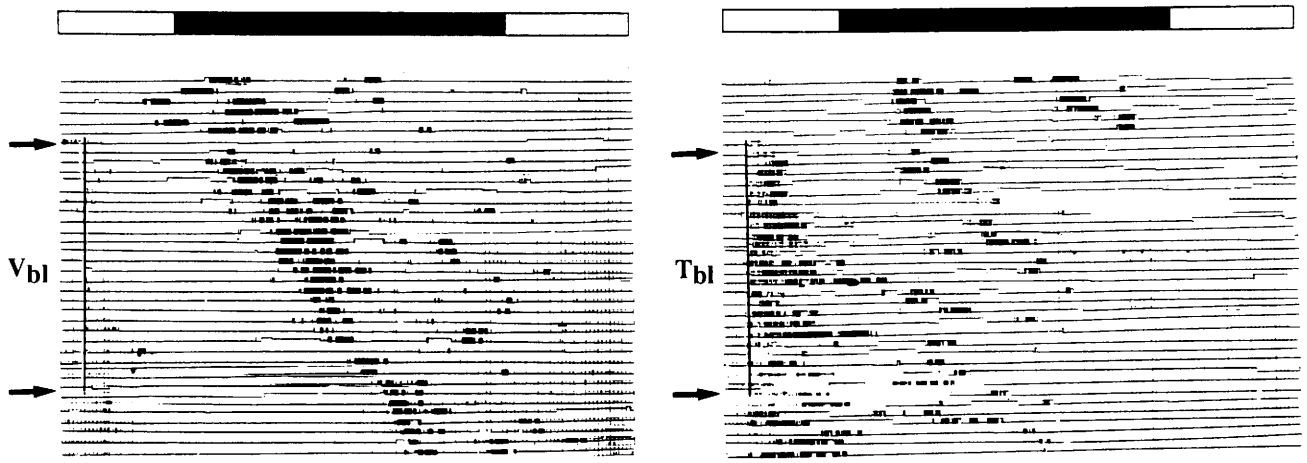

Figure 4. Continuous wheel running activity records from two representative blind hamsters injected with vehicle or triazolam. The two animals were transferred to DD for the last 6 days of the experiment. See Figure 3 for further details.

train to a light:dark cycle can alter the response of the reproductive system to an inhibitory daylength. Using a different experimental protocol, Eskes and Zucker demonstrated that testicular regression which normally occurs during exposure to a 10:14-hr light:dark cycle could be prevented by chronic treatment with D_2O in the drinking water (8). Treatment with D_2O delayed the onset of activity by about 4 hr, thus resulting in coincidence of lights-on with a phase of the activity rhythm about 8 hr after activity onset. As noted above, the photosensitive phase in hamsters appears to span a region of the circadian cycle from near activity onset until about 11 hr later. Taken together with the present findings it appears that a drug-induced advance or delay in the circadian clock under entrained conditions can alter the photoperiodic response to the inhibitory effects of short days, and that such changes in phase can prevent as well as reverse the inhibitory effects of short days.

Triazolam acts by potentiating the action of the inhibitory neurotransmitter gamma-aminobutyric acid (GABA) (17). Although GABA is found in the suprachiasmatic nuclei (SCN), the location of the central pacemaker in mammals (18), the phase shifting effects of triazolam are not thought to be due to a direct effect on the SCN, but rather are due to the effects of the drug on the overall activity state of the animal. Triazolam induces an acute increase in locomotor activity in the hamster, and if this acute increase in activity is prevented, phase shifts in the circadian clock are not observed (12). Other agents which induce an acute increase in activity, such as the availability of a novel running wheel for a short period of time, or exposure to a 3–6 hr pulse of darkness on a background of constant light, also induce phase-shifts in the circadian clock (12, 19, 20), and the phase response curves which have been generated for different agents which induce activity have similar shapes and response regions (21).

The phase shifting effects of activity-inducing stimuli on the circadian clock might be mediated by the lateral geniculate nuclei (LGN) and/or by raphe inputs to the SCN. The LGN pathway involves axonal projections from retinal ganglion cells to the intergeniculate (IGL) leaflet of the LGN, and projections from the IGL to the SCN (18). The demonstration that lesions of the LGN can block the phase shifting effects of triazolam is compatible with an effect of benzodiazepines on the circadian clock via an effect on an LGN input to the SCN (22). On the other hand, the SCN also receive intense serotoninergic projections from the median and dorsal raphe (23, 24), and microinjections of 5-HT 1A agonists in the lateral ventricle induce an increase in locomotor activity in rats and phase shifts that are similar to phase shifts induced by triazolam (25). Since physical activity significantly elevates brain serotonin levels (26), it is possible that the phase shifting effects of activity-inducing stimuli are mediated through serotoninergic pathways.

If the effects of triazolam on entrainment to the inhibitory 10:14-hr light:dark cycle and its subsequent effects on the photoperiodic response are due to the effects of the drug on the activity state of the animal, then it is anticipated that other agents which induce phase shifts in the circadian clock by inducing an acute increase in activity would also be able to alter the response of the reproductive system to a given photoperiod. Theoretically, it should be possible to alter the photoperiodic response via both pharmacological and/or nonpharmacological perturbations which influence circadian phase.

The physiological mechanisms by which the circadian clock ultimately influences hypothalamic-pituitary-gonadal activity involves the pineal hormone, melatonin (27–29). In hamsters as well as other species, it has been established that it is the circadian control of the duration of the nocturnal release of melatonin which determines whether an animal will inter-

pret the day as being long or short. Thus, in hamsters, during exposure to short days (e.g., 10:14-hr light: dark) melatonin levels are maintained at a high level for a sufficiently long enough period of time such that the long duration melatonin signal is inhibitory to neuroendocrine-gonadal activity. Although circulating melatonin levels were not measured in the present study, the working hypothesis is that the effects of triazolam on the phase relationship between the circadian clock and the light:dark cycle resulted in an alteration in the duration of nocturnal melatonin production, such that the pattern was not similar to that observed in hamsters transferred on long days. New melatonin assays that allow for the detection of circulating melatonin levels in rodents (Maywood *et al.*, personal communication) should now make it possible to correlate circulating melatonin levels in individual animals in which the photoperiodic response has been altered by changing the phase-relationship between the circadian clock and the light:dark cycle.

While the results of the present study demonstrate once again the importance of the circadian clock in photoperiodic time measurement, they also raise the possibility that it might be possible to take a pharmacological approach to alter seasonal rhythms for practical purposes. Changes in the light:dark cycle have been shown to influence the breeding season, reproductive hormone levels and milk production in various farm animals including sheep (30), pigs (31), cattle (32), and horses (33). However, attempts to alter the seasonal reproductive response of agricultural animals by manipulating the length of the day face many formidable obstacles that involve the rigid control of the light:dark cycle in an agricultural setting (5). An alternative approach to manipulating the light:dark cycle would be to use pharmacological agents that would fool the animal into responding as if it were exposed to long days when in fact the days were still short. Similarly, light has been used to treat depression in patients with seasonal affective disorder (SAD) by exposing humans to bright light during the early morning or late evening hours: a therapeutical intervention which may not always be practical (34). Being able to alter the phase of circadian rhythms, particularly those involved in conveying information about the seasonal change in daylength, might be useful in treating abnormalities associated with disorders of seasonal cycles.

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