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Independence of circadian entrainment state and responses to melatonin in male Siberian hamsters

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Abstract

Background: Seasonal fluctuations in physiology and behavior depend on the duration of nocturnal melatonin secretion programmed by the circadian system. A melatonin signal of a given duration, however, can elicit different responses depending on whether an animal was previously exposed to longer or shorter photoperiod signals (i.e., its photoperiodic history). This report examined in male Siberian hamsters which of two aspects of photoperiod history – prior melatonin exposure or entrainment state of the circadian system – is critical for generating contingent responses to a common photoperiodic signal.

Results: In Experiment #1, daily melatonin infusions of 5 or 10 h duration stimulated or inhibited gonadal growth, respectively, but had no effect on entrainment of the locomotor activity rhythm to long or short daylengths, thereby demonstrating that melatonin history and entrainment status could be experimentally dissociated. These manipulations were repeated in Experiment #2, and animals were subsequently exposed to a 12 week regimen of naturalistic melatonin signals shown in previous experiments to reveal photoperiodic history effects. Gonadal responses differed as a function of prior melatonin exposure but were unaffected by the circadian entrainment state. Experiment #3 demonstrated that a new photoperiodic history could be imparted during four weeks of exposure to long photoperiods. This effect, moreover, was blocked in animals treated concurrently with constant release melatonin capsules that obscured the endogenous melatonin signal: Following removal of the implants, the gonadal response depended not on the immediately antecedent circadian entrainment state, but on the more remote photoperiodic conditions prior to the melatonin implant.

Conclusions: The interpretation of photoperiodic signals as a function of prior conditions depends specifically on the history of melatonin exposure. The photoperiodic regulation of circadian entrainment state contributes minimally to the interpretation of melatonin signals.

Background

Many mammalian species undergo marked fluctuations in reproductive physiology and behavior on a seasonal basis. These annual variations are driven by programmed changes in the pattern of melatonin secretion from the pineal gland, which is under tight circadian control [1,2]. In long daylengths (LDs) of summer (e.g., 16 h light, 8 h dark daily; 16L:8D), light entrains the circadian pacemaker such that it programs a short duration of elevated nocturnal melatonin secretion. Under short daylengths

(SDs) of winter (e.g., 8L:16D), the circadian system programs secretion of a long melatonin signal. The circadian pacemaker also drives seasonal changes in locomotor activity such that, in nocturnal animals, the active fraction of the daily rest/activity cycle is longer in SD than in LD [1]. Although tightly correlated with melatonin duration, the activity rhythm is mechanistically independent of the pineal melatonin rhythm [1,3].

A central role of melatonin signal duration on reproduction has been established in studies of pinealectomized hamsters given subcutaneous melatonin infusions over several weeks [4–7]. Exposure to signals characteristic of the winter solstice (e.g., a 12 h melatonin signal) prevents gonadal growth or induces gonadal regression, whereas signals reflective of the summer solstice (e.g., a 4 h melatonin signal) support the reproductive phenotype [8]. Closer to the vernal and autumnal equinoxes, however, the absolute duration of photoperiodic signals is far less predictive of the reproductive response. An intermediate length signal (e.g., 12L:12D or 14L:10D) can either stimulate or inhibit the gonadal axis depending on whether hamsters were previously housed in shorter or longer daylengths, respectively [9–11]. Melatonin infusions of intermediate durations likewise can induce opposite reproductive responses among animals with different photoperiodic histories [12].

The present experiments assess the specific dimensions of photoperiodic history that are necessary and sufficient to generate divergent responses to photoperiodic signals. In nearly all studies in this domain, two distinct factors have been confounded – namely, the duration of the prior melatonin exposure and the entrainment status of the circadian system (i.e., whether it is in a LD or SD state). A critical role of melatonin history per se was suggested in the one study that specifically attempted to dissociate these two factors [13]. Male Syrian hamsters in 14L:10D were injected daily with melatonin or saline before lights off to generate two groups with presumably similar entrainment states but with melatonin histories corresponding to ~10L:14D (melatonin-injected) or 14L:10D (saline-injected). After 8 weeks, injections were discontinued and hamsters were moved to 12L:12D to assess whether melatonin injections had imparted a short-day photoperiodic history as would be indicated by a stimulatory response to 12L:12D. Saline-injected hamsters underwent gonadal regression in 12L:12D, but gonadal growth was induced among males previously injected with melatonin. But because melatonin-injected hamsters transferred to 8L:16D also exhibited gonadal growth, the response in 12L:12D may have reflected refractoriness to inhibitory photoperiods rather than reinterpretation of 12L:12D as a stimulatory photoperiod.

The second factor – circadian entrainment status – is largely unexplored in terms of its effects on the establishment of photoperiodic history. Melatonin infusion and injection studies that have incidentally included groups exposed to incongruent daylengths and melatonin signals (e.g., a 10 h melatonin signal during maintenance in 16L:8D, which corresponds naturally to a 5 h melatonin signal) typically employed extreme melatonin durations. Under these conditions, photoperiodic history effects may be absent or masked by responses to abrupt and extreme changes in photoperiod conditions [14]. A role of the circadian system in photoperiodic history was suggested in a second experiment by Karp et al. [13], who manipulated the free-running rhythm of the pacemaker. Various other observations further suggest that circadian systems may be involved in reception of melatonin signals more generally [15–18].

To disentangle the importance of circadian entrainment state versus melatonin exposure on photoperiodic responses, I experimentally dissociated these variables in two separate paradigms. Previous work has demonstrated that relatively brief exposure to 15L:9D is sufficient to render intermediate daylengths (e.g., 13.5L:10.5D) as inhibitory [19,20]. Because in other contexts these latter daylengths support reproductive development, the inhibition following exposure to 15L:9D indicates the acquisition of a long-daylength photoperiodic history. In this report, I test whether entrainment of the circadian system to 15L:9D is sufficient to induce a long-day photoperiodic history, or whether animals must experience the melatonin signal naturally associated with that photoperiod. To accomplish this dissociation, animals were given continuous release melatonin capsules during exposure to 15L:9D in order to obscure the endogenous signal encoding daylength. Hamsters were then probed for their reproductive responses to intermediate photoperiods following removal of the capsules.

Because photoperiod history effects that are important under naturalistic conditions may differ from those seen in common laboratory conditions [14], a separate study was undertaken with more gradual manipulations of melatonin signal duration. Prior work on LD animals established that gonadal regression is induced by exogenous melatonin infusions gradually lengthening from 5 h to 7.5 h over 12 weeks [12]. With such a naturalistic pattern of gradually changing melatonin signals, regression is initiated when melatonin durations are still quite short in absolute terms (6.7 h). Conversely, gonadal growth is stimulated in SD hamsters if the melatonin series is gradually reduced in duration from 10 h to the same intermediate value, and stimulation is first evident when melatonin duration is still long (8.3 h) in absolute terms. Finally, an identical pattern of long, but decreasing

melatonin durations is reproductively inhibitory to LD animals, presumably because the abrupt change from endogenous short signals to the 10 h infusion overrides the effects of the subsequent decreases in duration [12]. The present report assessed whether responses to similar intermediate melatonin durations depended on melatonin history or on circadian entrainment state.

Although endogenous melatonin production is under circadian control, there is compelling evidence for a reciprocal influence as well. In several rodent species, daily timed melatonin infusions or bolus injections entrain activity rhythms of animals maintained under constant dim light or darkness [21,22]. In a light/dark cycle, moreover, daily melatonin injections at ZT8 markedly phase-advanced activity onset of male Siberian hamsters [23]. If circadian entrainment state and melatonin history are to be disentangled experimentally, it needs to be determined that the former is not altered by melatonin infusions of different durations. Thus, Experiment #1 demonstrated that circadian entrainment status, as assayed by the locomotor activity rhythm, was determined by photoperiod and not affected by simultaneous exposure to discrepant melatonin infusions. This procedure was used as a pre-treatment in Experiment #2, which showed that the response to gradually changing, intermediate duration melatonin signals depends on prior melatonin exposure and not on the entraining photoperiod. Finally, Experiment #3 demonstrated that interference with an endogenous melatonin rhythm prevents the imparting of a new photoperiodic history.

Results

Experiment #1

Representative actograms of LD and SD hamsters infused with 5 h or 10 h of melatonin daily are shown in Figure 1A,1B,1C,1D. In 37 of 38 hamsters, activity rhythms were robust enough for clear identification of activity onset, and activity offset was discernible in 36. Phase angle of entrainment (difference between time of lights off and activity onset) was not significantly altered by photoperiod (Fig. 1E; $F = 1.9$, d.f. = 1,33; $p > 0.15$) nor by melatonin duration ($F = 0.0$; d.f. = 1,34, $p > 0.80$). Activity duration (α) was significantly greater in SD than in LD (Fig. 1F; $F = 174$, d.f. = 1,33; $p < 0.001$) but was not significantly different following 10 h versus 5 h melatonin infusions ($F = 0.7$; d.f.= 1,33; $p > 0.30$).

Cessation of melatonin infusion and release into constant darkness revealed no evidence of prior rhythm masking, and activity onsets were continuous with those observed under entrained conditions (Fig. 1A,1B,1C,1D). Neither photoperiod ($F = 2.5$, d.f. = 1,33; $p > 0.10$) nor melatonin infusion duration ($F = 1.2$, d.f. = 1, 33; $p > 0.25$) significantly affected the period of the free-running rhythm (data not shown).

Final paired testis weights differed significantly as a function of melatonin duration (602 ± 83 versus 88 ± 21 mg for 5 h and 10 h infusions, respectively; $F = 34.0$, d.f. = 1,34, $p < 0.001$) but not entrainment condition (360 ± 81 versus 329 ± 91 mg for LD and SD, respectively; $F = 0.1$, d.f. = 1,34, $p > 0.70$). Final body weight exhibited the same dependence on melatonin duration (41.1 ± 1.0 versus 33.8 ± 1.0 g for 5 h and 10 h infusions, respectively; $F = 26.4$, d.f. = 1,34, $p < 0.001$) and independence of entrainment condition (36.7 ± 1.1 versus 38.3 ± 1.5 g for LD and SD, respectively; $F = 1.2$, d.f. = 1,34, $p > 0.25$).

Experiment #2

Figure 2A illustrates the melatonin infusions delivered in Experiment #2 to hamsters maintained in LD or SD. After six weeks of pre-treatment with the same conditions described in Experiment #1, estimated testis volume (ETV) and body weights were reduced in hamsters given 10 versus 5 h melatonin infusions (Week 0 data in Fig. 2B,2C; $F = 38.5$; d.f. = 1, 50; $p < 0.001$ for ETV; $F = 10.1$; d.f. = 1, 50; $p < 0.01$ for body weight). For each of these measures, there was no significant main effect of photoperiod ($F = 0.4$; d.f. = 1, 50; $p > 0.50$; $F = 0.0$; d.f. = 1,50; $p > 0.80$, respectively) nor any interaction of photoperiod with melatonin duration ($F = 0.9$; d.f. = 1,50; $p > 0.70$; $F = 0.9$; d.f. = 1, 50; $p > 0.30$, respectively). Melatonin signals decreasing in duration from 10 h at Week 0 to 7.5 h at Week 12 induced significantly different gonadal growth patterns depending on whether hamsters received 5 h or 10 h melatonin pre-treatment ($F = 23.8$; d.f. = 3, 150; $p < 0.001$). The photoperiod in effect during these infusions had no effect on gonadal growth (Fig. 2B; $F = 1.0$; d.f. = 3, 150; $p > 0.30$) and there was no interaction of melatonin pre-treatment and entrainment condition ($F = 0.3$; d.f. = 3, 150; $p > 0.80$). Body weight trajectories were also influenced by melatonin pre-treatment (Fig. 2C; $F = 4.9$; d.f. = 3, 150; $p < 0.01$) but not by entrainment condition ($F = 1.7$; d.f. = 3, 150; $p > 0.15$). Likewise, there was no significant interaction of these two variables ($F = 0.7$; d.f. = 3; $p > 0.50$).

At no individual time point was there a significant effect of photoperiod on body weight or ETV ($p > 0.10$). Hamsters given the longer melatonin duration during pre-treatment, however, had larger gonads at Week 18 (ETV: $F = 5.3$; d.f. = 1, 50; $p < 0.05$; paired testis weight: $F = 6.2$; d.f. = 1, 50; $p < 0.05$). Body weights of hamsters pre-treated with 10 h versus 5 h melatonin were also lower through Week 6 ($F = 5.6$; d.f. = 1, 50; $p < 0.05$).

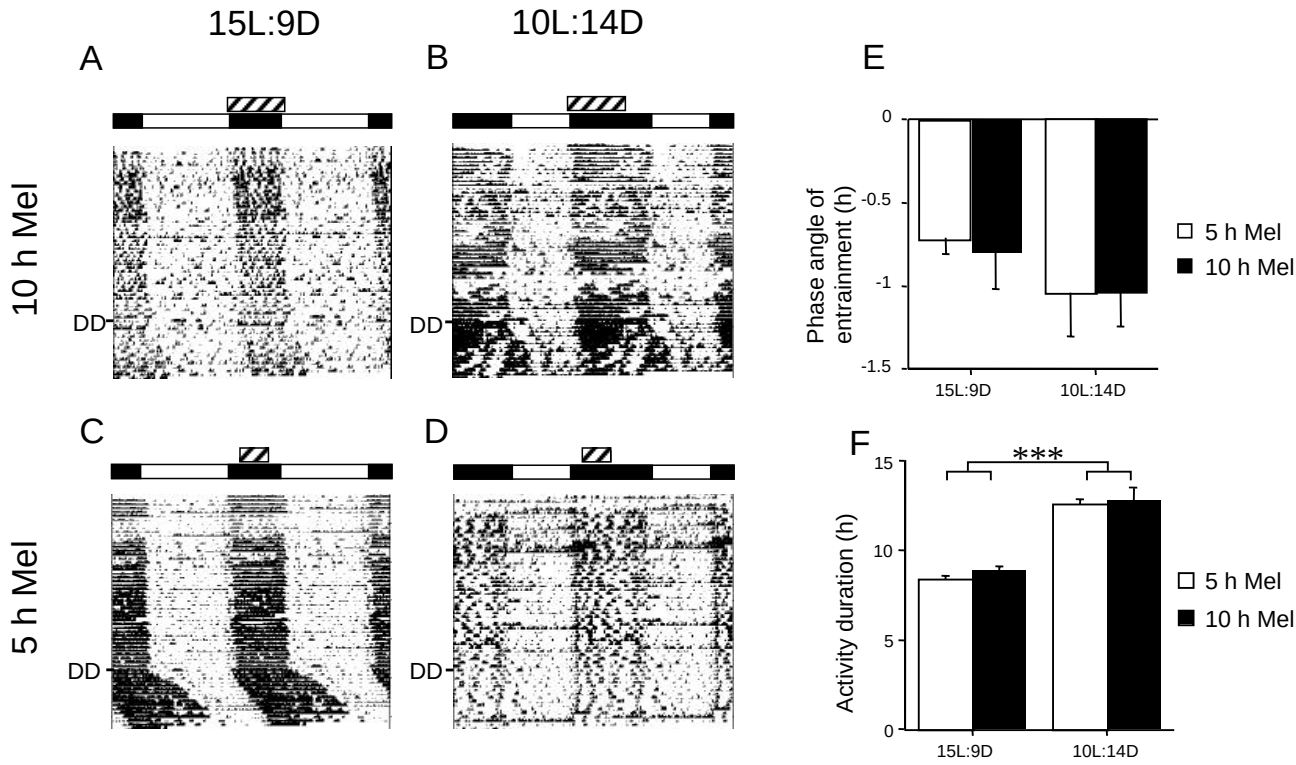

Figure 1
Representative double-plotted actograms (A-D) of hamsters in Experiment #1. Animals were pinealectomized, exposed to long (15L:9D) or short (10L:14D) photoperiods and simultaneously infused daily with melatonin for 5 h or 10 h. Following 6–7 weeks of infusions, catheters were disconnected and hamsters were exposed to constant darkness (DD) as indicated along the left margin of the actogram. The light:dark cycle is indicated with white and black bars, respectively, above each actogram, and the time of daily melatonin infusion is denoted with a crosshatched rectangle. Mean (± sem) phase angle of entrainment (time of lights off minus time of activity onset) (E) and activity duration (F) of hamsters in each group. Asterisks indicate a statistically significant effect of photoperiod (*** $p < 0.001$).

Experiment #3

This experiment assessed whether a photoperiodic history would be imparted if the endogenous melatonin signal was obscured with a constant release melatonin capsule.

Photoperiodic responses before and during melatonin and beeswax implants

At 4 weeks of age, body weight and gonad size reflected the photoperiod that hamsters had been exposed to beginning at Week 2 (Week 4 data in Fig. 3B,3D; $F = 61.5$, d.f. = 46, $p < 0.001$ and $F = 266$, $p < 0.001$, respectively). Following implantation of a constant release melatonin capsule at Week 4, the prior photoperiod continued to predict BW and ETV through Week 8 (Fig. 2B,2D; $F = 26.9$, d.f. = 1,33, $p < 0.001$; $F = 281$, d.f. = 1,33, $p < 0.001$ for body weights and ETV, respectively). In contrast, the photoperiod during the melatonin implant (Weeks 4–8) had no effect on body weight or ETV during this interval ($F = 2.0$, d.f. = 1,33, $p > 0.15$; $F = 0.1$, d.f. = 1,33, $p > 0.70$, respectively).

Transfer of beeswax-implanted hamsters from SD to LD at Week 4, however, acutely increased body weight and gonad size (Fig. 3B,3D). Compared to melatonin-implanted hamsters exposed to identical photoperiods, those given blank capsules over Weeks 4–8 had

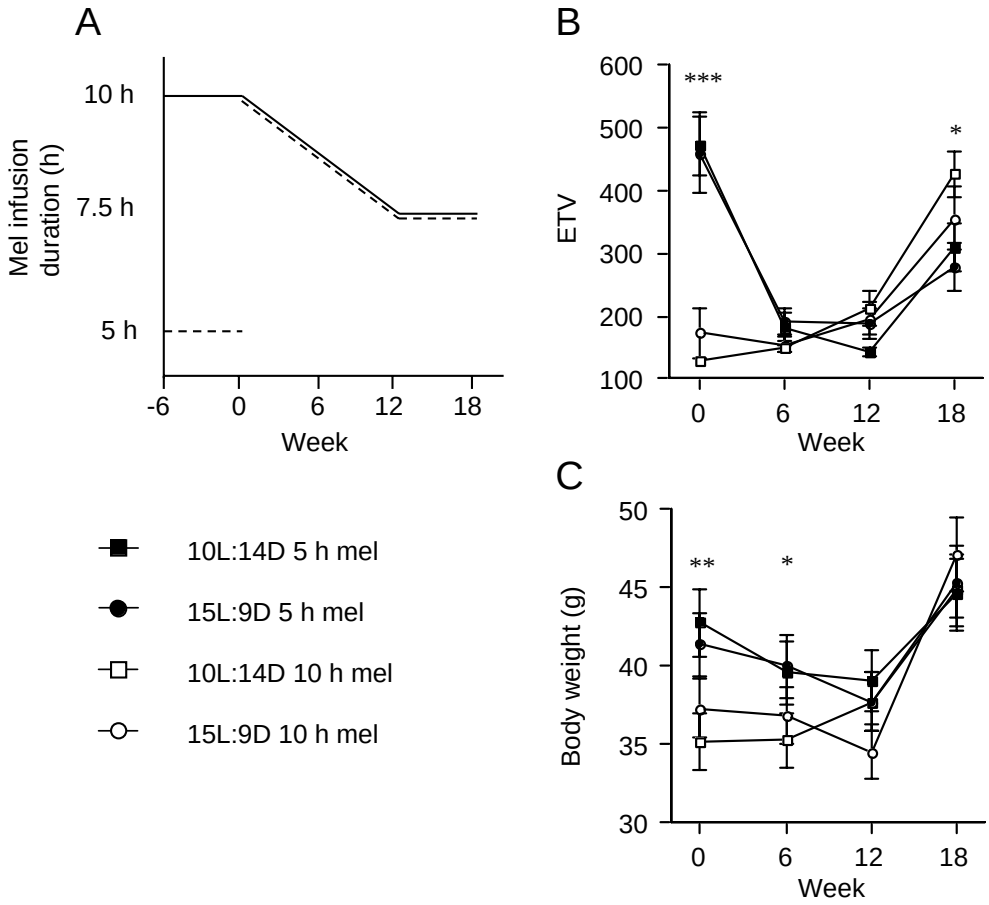

Figure 2
Melatonin infusion schedule and photoperiodic responses of hamsters in Experiment #2. Panel A illustrates the two melatonin infusion protocols (dashed and solid lines, respectively). Both groups received melatonin signals gradually decreasing in duration from 10 h at week 0 to 7.5 h at week 12 followed by 6 additional weeks of 7.5 h signals. The groups differed in the duration of the melatonin during a 6 week pre-treatment (5 h versus 10 h melatonin, solid symbols and open symbols, respectively). These two regimens were administered to animals entrained to either 15L:9D (circles) or 10L:14D (squares) throughout. Mean (± sem) estimated testis volume (B) and body weights (C) in each of the four resulting conditions. Asterisks indicate time points in which there were significant effects of melatonin duration during pre-treatment (*** $p < 0.001$; ** $p < 0.01$; * $p < 0.05$).

significantly higher average and more quickly increasing body weights (main effect of Implant: $F = 13.5$, d.f. = 1,12, $p < 0.01$; Implant * Time interaction: $F = 18.7$, d.f. = 2, 24, $p < 0.001$) and ETVs ($F = 7.2$, d.f.= 1,12, $p < 0.05$, $F = 79.8$, d.f. = 2, 24, $p < 0.001$, respectively). Animals that remained in LD, in contrast, showed no main effect of implant condition on body weight ($F = 0.2$, d.f. = 1,13, $p > 0.65$) or ETV ($F = 2.4$, d.f. = 1,13, $p > 0.10$). Beeswax implants, however, significantly accelerated testicular growth ($F = 7.9$, d.f. = 2,26; $p < 0.01$).

Removal of melatonin implants and test for photoperiodic history in 13L:11D

Following removal of melatonin implants, the gonadal response in 13L:11D depended on the photoperiod during Weeks 2–4 (Fig. 3C,3E). Trough values of ETV dipped lower among hamsters that had been exposed during Weeks 2–4 to LD versus SD (Table 1; $F = 4.2$; d.f. = 1,28; $p = 0.05$). The photoperiod during melatonin treatment, in contrast, had no effect on this measure ($F = 0.1$; d.f. = 1,28; $p > 0.70$). Similarly, the fraction of hamsters with ETV dropping below 300 units in 13L:11D also differed

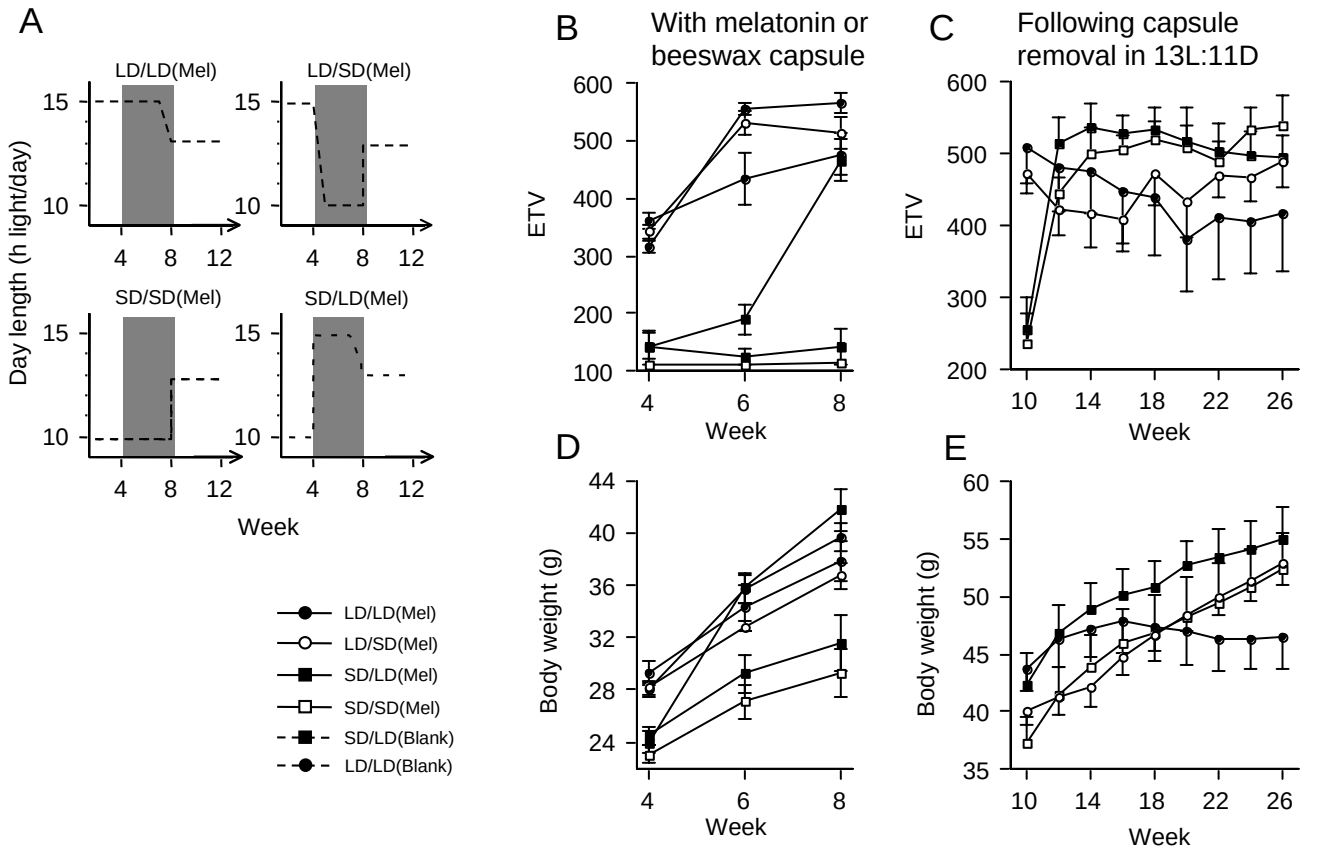

Figure 3
Experimental protocol and photoperiodic responses of hamsters in Experiment #3. Mean (± sem) estimated testis volume (ETV) (B, C) and body weight (D, E) of hamsters during four weeks of exposure to implants of melatonin in beeswax or beeswax alone (B, D) and during 14 weeks of exposure to 13L:11D (C, E). In (A), panels illustrate the daylength to which the circadian system was presumably entrained. The shaded area indicates where melatonin capsules obscured the endogenously generated melatonin signal associated with the ambient daylength. Hamsters exposed to long (LD) or short (SD) daylengths during Weeks 2–4 (15L:9D versus 10L:14D) were maintained in those photoperiods (SD/SD and LD/LD) or transferred to the opposite condition from Weeks 4–7 or Weeks 4–8 (SD/LD and LD/SD; see text for details). All hamsters were in 13L:11D from Week 8 onwards.

Table 1: ETV minimum (mean ± SEM) and fraction of hamsters exhibiting threshold gonadal regression in 13L:11D.

	Week 2–4	Week 4–8	ETV minimum	Fraction regressed
Mel capsule	LD	LD	335 ± 71	4 / 8
	LD	SD	370 ± 48	5 / 10
	SD	LD	456 ± 27	0 / 7
	SD	SD	455 ± 24	0 / 7
			*$p = 0.05$	**$p < 0.01$
Beeswax capsule	LD	LD	386 ± 57	2 / 5
	SD	LD	229 ± 59	3 / 5

*significant main effect of Week 2–4 photoperiod in two-factor ANOVA **significant effect of Week 2–4 photoperiod by Fisher's exact test

depending on the Week 2–4 photoperiod ($p < 0.01$) but not the photoperiod during melatonin capsule exposure ($p > 0.90$).

Photoperiod prior to melatonin capsule implantation affected trajectories of gonad size in 13L:11D (Weeks 10–26; Fig. 3C). Whereas hamsters housed in SD during Weeks 2–4 exhibited rapid gonadal growth to a high plateau value, those weaned into LD showed gradual decreases in ETV from initially high values (Week 2–4 Photoperiod * Time interaction: $F = 21.0$; d.f = 8,216; $p < 0.001$). This effect of Week 2–4 photoperiod held in pairwise comparisons of hamsters kept in LD during Weeks 4–8 (Fig. 3C; SD/LD versus LD/LD; $F = 10.4$; d.f. = 8,114; $p < 0.001$) or in SD (SD/SD versus LD/SD; $F = 11.2$; d.f. = 8,112; $p < 0.001$). The photoperiod during melatonin capsule implantation (Weeks 4–8), however, also exerted a discernible impact on gonadal growth (Week 4–8 Photoperiod * Time interaction: $F = 3.4$; d.f. = 8,216; $p < 0.001$). This effect of the Week 4–8 photoperiod was apparent among those hamsters that had earlier been exposed to LD (LD/LD versus LD/SD; $F = 3.0$; d.f. = 8,128; $p < 0.005$), but not those weaned into SD (SD/LD versus SD/SD; $F = 1.2$; d.f. = 8,88; $p > 0.20$).

Body weight trajectories over Weeks 10–26 likewise were affected by photoperiods during Weeks 2–4 (Fig. 3E; $F = 3.5$; d.f. = 8,216; $p < 0.001$) and Weeks 4–8 ($F = 6.7$; d.f. = 8,216; $p < 0.001$) and these two variables interacted ($F = 3.2$; d.f. = 8, 216; $p < 0.05$). The long-term effect of Week 2–4 photoperiod was restricted to hamsters that been exposed to LD during melatonin implants. Week 2–4 photoperiod had no long-term effect in those animals kept in SD over Weeks 4–8 (Fig. 3E; LD/SD versus SD/SD; $F = 1.0$; d.f. = 8, 112; $p > 0.40$). In contrast, hamsters maintained in LD from weaning through capsule implantation responded to 13L:11D with eventual decreases in body weight compared to those hamsters treated similarly but weaned into SD (Fig. 3E; SD/LD versus LD/LD; $F = 4.7$; d.f. = 8,104; $p < 0.001$). As indicated above, the photoperiod during melatonin implantation also influenced growth trajectories. For hamsters weaned into SD, the photoperiod during melatonin implantation exerted no effect (SD/LD versus SD/SD; $F = 0.2$; d.f. = 8,88; $p > 0.90$). Hamsters weaned into LD and transferred to SD during melatonin capsule exposure exhibited monotonic increases in body weight, whereas those in LD continuously from weaning showed later decreases in body weight in 13L:11D (LD/SD versus LD/LD; $F = 17.5$; d.f. = 8,128; $p < 0.001$).

Discussion

The simultaneous presentation of incongruent photoperiods and exogenous melatonin signals induced a dissociation between two types of photoperiodic variables. On the one hand, the circadian pattern of locomotor activity was affected only by photoperiod and not by the duration of infused melatonin. In contrast, body weight and reproductive responses were largely determined by the pattern of melatonin infusions and not by the entraining photoperiod. In general, the results implicate the duration of the antecedent daily melatonin signal as the critical parameter that imparts photoperiodic history to hamsters.

While timed melatonin exposure entrains circadian rhythms of several mammalian species maintained under constant conditions [21,22], there is a dearth of information about potential entraining effects of melatonin presented simultaneously with a light/dark cycle. In one such study, pharmacological dosages of melatonin (25 μg) injected daily at ZT8 phase-advanced onset of wheel-running activity and lengthened activity duration (α) in Siberian hamsters maintained in 16L:8D [23]. As these entrainment effects were reversed when animals became photorefractory, the authors suggested that they might depend on changes in gonadal steroid concentrations rather than direct entraining actions of melatonin. Arguing against this interpretation, the present use of long and short melatonin infusion durations at a physiological dosage (< 0.006 μg/h) generated large group differences in reproductive status without any differential effect on the entrained pattern of the locomotor activity rhythm. The earlier reported influence on entrainment may relate to the high dosages injected.

The demonstrated independence of melatonin infusion duration and circadian entrainment status allows an analytical separation of the role of these two variables in photoperiodism. Whereas a paramount role for melatonin duration has been generally accepted, a minority view has held that there exists a circadian rhythm in responsiveness to melatonin [17,24,25]. Under short, but not long day lengths, the endogenous melatonin signal is thought to coincide with a circadian rhythm of melatonin sensitivity and thereby to induce gonadal regression. The evidence in support of this view, however, remains equivocal. Based on insights from a prior critique of photoperiodism research [14], Experiment #2 was designed to optimize conditions for detecting any physiological role of circadian entrainment on photoperiodic responsiveness to melatonin. Specifically, I hypothesized that the importance of melatonin duration might have been artifactually accentuated under standard laboratory conditions where animals are exposed to unchanging photoperiodic conditions for several weeks and then to abrupt changes in signal duration. Under more naturalistic conditions where photoperiodic signals change daily and gradually, the pattern of change rather than the absolute duration of photoperiodic signals is more predictive of photoperiodic responses [9].

As indicated above, photoperiod conditions shown in Experiment #1 to differentially entrain the locomotor activity rhythm had no substantial effect on reproductive responses of hamsters monitored in Experiment #2. This was true whether or not animals received abrupt or only gradual changes in melatonin duration (i.e., animals pretreated with 5 h or 10 h melatonin, respectively). Thus, a circadian effect on gonadal responsiveness to melatonin infusions was not revealed using experimental paradigms that more closely simulated ecological conditions than did previous studies.

The minimal role of circadian entrainment history on photoperiodic responsiveness was corroborated in Experiment #3, which employed a different method to dissociate circadian and melatonin histories. By raising systemic concentrations of melatonin chronically, constant release melatonin capsules obscure the rhythmic variation in endogenous signal produced in the pineal gland [26]. As in past studies, animals with such capsules were generally insensitive to concurrent manipulations of photoperiod. It is possible that 4 weeks of chronically elevated melatonin concentrations is itself a potent photoperiodic signal as it is in other species [27], but there is little evidence for this in adult Siberian hamsters [28]. When capsules were removed, the photoperiod time measurement system was functionally restored. As would be expected based on previous reports [19,20,29,30], blank-implanted controls animals previously housed in longer daylengths exhibited the classic response to exposure to intermediate photoperiods – partial gonadal regression (Table 1). Comparable responses were observed in melatonin-implanted animals that had experienced long daylengths prior to the implant (open and closed circles; Fig. 3C). In contrast, animals exposed to short daylengths prior to melatonin implants were universally stimulated by this intermediate photoperiod (open and closed squares; Fig. 3D). Critically, in neither instance did the photoperiod during the melatonin implant affect the degree of gonadal regression or the fraction of animals meeting the threshold for an inhibitory gonadal responses in 13L:11D (Table 1; open versus closed symbols; Fig. 3C). Photoperiodic history effects on gonadal function, therefore, cannot be induced by the immediately prior circadian entrainment status. Instead, the results implicate the earlier pattern of melatonin as the critical parameter imparting photoperiodic history to animals. Finally, 4 weeks of constant release melatonin is insufficient to impart a specific photoperiodic history of its own, but instead allows animals to "remember" the conditions to which they were previously exposed. In this regard the constant presence of melatonin shares properties with the complete absence of melatonin, which in another study, was consistent with a similar photoperiodic memory for at least 6 weeks [19].

Two results suggest a possible a melatonin-independent effect of entrainment status on photoperiodic history. First, among hamsters weaned into 15L:9D, those transferred to 10L:14D during melatonin exposure (open circles, Fig. 3C) manifested an earlier reduction in ETV compared to those kept in 15L:9D (closed circles, Fig. 3C). Second, body weights of the former hamsters increased monotonically whereas the latter animals reached an early plateau (open and closed circles, Fig. 3D). These effects may relate to small differences in melatonin exposure at the time capsules were removed (see Experiment 3 Methods for explanation) or, in the case of body weight, previously described differential regulation of body weight and reproduction [31–33].

Conclusions

In conclusion, whereas melatonin may affect the circadian system in the absence of other zeitgebers, physiological concentrations have no demonstrable effect on entrainment in the presence of a competing light/dark cycle. Exposure to a long or short melatonin signal, however, is sufficient to induce a photoperiodic history, even when it contradicts that encoded by the entrainment status of the circadian pacemaker. If photoperiodic history effects are present in humans, self-administration of melatonin may be sufficient to impart them in the absence of any entraining actions on circadian rhythms.

Methods

All hamsters were derived from a breeding colony founded on stock provided by Dr. Bruce Goldman (University of Connecticut, Storrs) in 1995. Except during melatonin infusions and activity monitoring, hamsters were housed in polypropylene cages (27 × 16 × 13 cm, 1–3 hamsters/cage) on corn cob bedding with food (Mouse Chow #5015, Purina Mills, St. Louis, MO) and water available ad libitum. Light intensity was 100–400 lux at the level of the cage floor. Except where noted hamsters were housed under standard LD (15L:9D; lights on 0500 PST) or SD (10L:14D; lights on 1000 PST) conditions.

Melatonin Infusions

Hamsters for melatonin infusions were pinealectomized under sodium pentobarbital anesthesia (50 mg/kg). After hamsters were placed in a stereotaxic apparatus, a 2 mm hole was trephined above the superior sagittal sinus and the pineal was removed with fine forceps. Several days after recovery, hamsters were catheterized as described previously [34], transferred to circular plastic cages (20 cm diam × 25 cm high), and thereafter infused nightly with melatonin as described below. Melatonin for infusions was prepared by dissolving 5 mg melatonin (Sigma) in 1 ml 70% EtOH and diluting 1:79 with physiological saline. Frozen for future use, aliquots were later thawed and further diluted 1:100 to produce an infusate delivered at a

rate of 0.017 ml/h at a dosage of 10 ng/h melatonin. Infusion pumps (Razel Scientific, Stamford, CT) were reloaded with fresh melatonin solution every 3–4 days during the day so that any small melatonin bolus inadvertently released during this procedure would not lengthen the programmed nighttime signal.

Testis measurements

In Experiments #2 and #3, testis size was monitored noninvasively by measuring the length and width of the left testis under light isoflurane anesthesia. The product of the testis width squared and testis length yields an estimated testis volume (ETV) that is highly correlated with paired testis weight. Correlation coefficients, r, routinely exceed 0.90 over the range of 80–800 mg [35].

Experiment #1

This experiment assessed whether the duration of daily melatonin infusion (5 h versus 10 h) altered the entrainment of locomotor activity rhythms in long and short daylengths (15L:9D and 10L:14D), respectively.

Dams and their male offspring, born over a 10-day interval, were either retained in LD or transferred to SD when pups were 12–15 days old. All subjects were pinealectomized between 21–25 days of age and catheterized 7 days later. Thereafter, locomotor activity rhythms were monitored with passive infrared motion detectors (Coral Plus, Visonic, Bloomfield, CT) mounted ~23 cm above the cage floor. For each photoperiod, melatonin was infused nightly for 5 h (n = 10 and 9 for LD and SD hamsters, respectively; melatonin onset 2200 PST) or 10 h (n = 10 and 9, for LD and SD hamsters, respectively; melatonin onset 1930 PST). After 6–7 weeks, catheters were cut where they exited the skin. At the regular light:dark transition the lights were permanently extinguished to allow assessment of the free-running locomotor activity rhythms over the next 14 days. Animals were then euthanized with pentobarbital, and paired testis weights were collected.

Analysis

To assess phase angle of entrainment, a 24 h histogram was prepared for each animal with the last 13 days of activity in the light:dark cycle. Beginning 2 h before the light offset, activity onset was defined as the first point in the activity profile where values exceeded the overall 24 h mean and were sustained over the next 30 minutes. Ending 2 h after the light onset, activity offset was defined as the last point that exceeded the 24 h mean for 30 minutes. Activity duration was the difference between these two values. For three hamsters, the activity record was of very poor quality during the analysis interval but was clearer 3 weeks prior, and data from this earlier interval were substituted. The freerunning rhythm in constant darkness was calculated by least squares regression through 14 consecutive activity onsets using ClockLab software (Actimetrics, Evanston, IL). Entrainment parameters were assessed with two-factor analysis of variance (ANOVA) with photoperiod (LD vs SD) and melatonin pre-treatment duration (10 h vs 5 h) as independent factors (Statview 5.0; SAS Institute, Cary, NC).

Experiment #2

This experiment assessed whether circadian entrainment state and prior melatonin history could each alter the response to a regimen of gradually decreasing melatonin signals that elicited marked photoperiodic history effects in past studies. Male hamsters were initially treated as described in Experiment #1 to yield four groups of pinealectomized hamsters exposed to LD or SD with simultaneous exposure to long (10 h; n = 9, 15 for LD, SD) or short (5 h; n = 16, 14 for LD, SD, respectively) melatonin infusions. Following six weeks in these conditions, all groups were subsequently exposed to a 12 week pattern of melatonin infusion that shortened gradually in duration from 10 h to 7.5 h (~2 min/day). Hamsters were then given an additional 6 weeks of 7.5 h infusions. Body weight and ETV were determined at six week intervals beginning at the onset of the gradually changing melatonin signals (Week 0).

Analysis

ETV and body weight data were analyzed with between-subjects ANOVA. Group differences in patterns of gonadal and somatic growth were assessed with repeated measures ANOVA. These analyses invariably yield main effects of time. As a change in dependent measures over time is not surprising, these ubiquitous main effects are not reported in the interests of clarity. Reported instead are interactions of time with photoperiod, or time with melatonin duration. These interactions reflect group differences in the pattern of growth.

Experiment #3

This experiment determined the role of entrainment status versus melatonin exposure for the imparting of a long-day photoperiodic history that normally occurs with 4 weeks of exposure to 15L:9D. Specifically, I assessed whether entrainment state was sufficient to impart this history if the endogenous melatonin signal was obscured with a constant release melatonin capsule. Male and female hamsters were paired in LD. At 15 days of age (Week 2), litters and dams remained in LD or were transferred to SD, and males were weaned within 4 days. At 28 days of age (Week 4), animals were given subcutaneous melatonin implants. Hamsters were first anesthetized with isoflurane vapors, and two 8 mm capsules were inserted through a small dorsal midline incision just caudal to the neck. The wound was closed with surgical

staples. Capsules were prepared by adding melatonin powder to heated beeswax (1:24 ratio by weight) and pumping the melted solution into Silastic tubing (1.47 × 1.96 mm ID × OD, Dow Corning, Midland MI).

Immediately following capsule implantation at Week 4, hamsters from LD remained in LD (LD/LD Mel, n = 8) or were transferred to SD (LD/SD Mel; n = 10). Hamsters from SD remained in that photoperiod (SD/SD Mel, n = 9) or were transferred to LD (SD/LD Mel, n = 8). Additional hamsters from LD and from SD were implanted with capsules filled with beeswax alone and maintained in LD (LD/LD Blank, n = 6) or transferred to LD (SD/LD Blank, n = 5, respectively).

Whether the manipulations induced long-day or short-day photoperiodic histories was indicated by the gonadal response in an intermediate photoperiod (13L:11D). Hamsters were transferred from SD to 13L:11D (lights on 0700 PST) at Week 8. Because re-entrainment to shorter photoperiods occurs more slowly than re-entrainment to longer photoperiods [35], hamsters from LD were transferred to 13L:11D after only 3 weeks (Week 7) to allow them to lengthen their endogenous melatonin signal during the final week of exposure to the capsule. The present study did not attempt to verify that all groups were similarly entrained to 13L:11D immediately following removal of capsules. All capsules were removed under isoflurane anesthesia at Week 8.

Analysis

Testis size and body weight were measured every two weeks from 4 to 26 weeks of age. The photoperiods in effect from Weeks 2–4 and from Weeks 4–8 were independent factors in ANOVAs run on body weight and ETV data. Where repeated measures ANOVAs were employed, main effects of time were omitted in the interests of clarity. Group differences in the proportion of hamsters that reduced ETV below 300 units (~40% reduction from LD baseline) in 13L:11D were assessed by Fisher's exact probability test (JMP IN 4.0; SAS Institute, Cary, NC). The results were not sensitive to the specific value of this threshold.

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