The Royal Society

Effects of a circadian mutation on seasonality in Syrian hamsters (*Mesocricetus auratus*)

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In Syrian hamsters, exposure to short photoperiods or constant darkness induces a decrease in gonadotrophin secretion and gonadal regression. After 10–12 weeks, animals undergo spontaneous gonadal reactivation, gonadotrophin concentrations rise, and in males, testes size increases and spermatogenesis resumes. The *tau* mutation shortens the period of circadian wheel-running activity by 4 h in the homozygote. Here, we examine the impact of this mutation on the reproductive response to photoperiod change. Seventeen adult *tau* mutant and nine adult wild-type males were housed in complete darkness for 25 weeks and testes size determined at weekly intervals. Gonadal regression and subsequent recrudescence occurred in both groups of animals. Regression occurred more rapidly in *tau* mutants, with a nadir significantly earlier than wild-types but after a similar number of circadian cycles. Rates of testicular recrudescence were similar in both groups. Our data suggest that an acceleration of the circadian period increases the rate of reproductive inhibition in animals exposed to inhibitory photoperiods. Once initiated, the rate of spontaneous reactivation may be independent of the circadian axis.

Keywords: circadian; photorefractory; *tau* mutant; hamster; seasonal

1. INTRODUCTION

Daylength is a key environmental variable regulating reproduction in seasonally breeding mammals. In seasonal Syrian hamsters, artificial manipulations of photoperiod cause changes in gonadal function. Long photoperiods result in an increase in pituitary gonadotrophin secretion and activation of the gonads (Elliott 1976; Stetson & Tate-Ostroff 1981; Hastings *et al.* 1989; Vitaterna & Turek 1993). In contrast, short photoperiods or continuous darkness (DD) initially causes the suppression of gonadotrophins and prolactin, and gonadal atrophy (Gaston & Menaker 1967; Berndtson & Desjardins 1974; Steger *et al.* 1982; Vitaterna & Turek 1993; Stirland *et al.* 1996*a*). However, after approximately 10–12 weeks, photo-inhibited animals undergo spontaneous recrudescence of the neuroendocrine axis and gonadal regrowth occurs, even during photoperiods which initially caused reproductive suppression (Stetson *et al.* 1977; Turek & Campbell 1979; Reiter 1980). This process of 'short-day photorefractoriness' is a universal phenomenon in seasonally breeding mammals.

The circadian system plays a central role in mediating neuroendocrine responses to photoperiod. Paired circadian oscillators located in the suprachiasmatic nuclei (SCN) drive the secretion of a circadian melatonin signal from the pineal gland. When maintained in DD, animals such as Syrian hamsters exhibit circadian rhythms of melatonin secretion, with peak concentrations occurring at the same relative time as circadian activity bouts, as measured by wheel-running activity (Maywood *et al.* 1993). Studies largely undertaken in Syrian hamsters demonstrate that melatonin signal duration is a key component in photoperiodic time measurement (see review, in Bartness *et al.* 1993). Long-term exposure (8 weeks) of pinealectomized animals to repeated daily infusions of long-duration signals ($>8\,\mathrm{h\,d^{-1}}$) induces testicular regression and suppression of prolactin secretion, mimicking responses observed in intact animals exposed to short daylengths. Because the generation of the nocturnal melatonin signal is entrained by the circadian system (Illnerova 1991), and is proportional to the length of the night (i.e. Syrian hamster; Maywood *et al.* 1993), it is clear that scotoperiod duration is paramount in mediating these physiological changes.

The *tau* mutation is a single semi-dominant autosomal mutation, accelerating the period of the circadian activity cycle from 24 h in the wild-type to 20 h in the homozygous mutant (Ralph & Menaker 1988). When *tau* mutants are maintained on 24-h light:dark (L:D) cycles, they exhibit abnormal patterns of entrainment of the circadian activity cycle with an advance of the normal nocturnal activity bouts into the photophase (J. A. Stirland & A. S. I. Loudon, unpublished observations). During such cycles, *tau* mutants are unable to undergo gonadal regression in response to short photoperiods, even as short as 1 h of light per 24 h (1L:23D; Menaker & Refinetti 1993). For wild-type hamsters, the equivalent photoperiod at which gonadal regression occurs is approximately 12L:12D or less (Elliott 1976; Hastings *et al.* 1989; Vitaterna & Turek 1993; Stirland *et al.* 1996*a*). In contrast, *tau* mutants kept on 20-h L:D cycles exhibit a normal photoperiodic response, undergoing full testicular regression at photoperiods of 10L:10D or less (Stirland *et al.* 1996*a*).

Here, we describe a study in which we examined the impact of the *tau* mutation on rates of reproductive

Received 12 August 1997 *Accepted* 5 September 1997

response to inhibitory photoperiods. Because earlier studies indicate that no single photoperiod can be defined which is equivalent for both genotypes, experiments were undertaken by exposing animals to DD. These animals were not provided with access to running wheels, as this is known to attenuate the reproductive response to inhibitory photoperiods. Our results clearly demonstrate that reproductive inhibition occurs at a faster rate in *tau* mutants and by the same ratio as the increased acceleration in circadian period. In contrast, rates of testicular regrowth occurred at similar rates in both genotypes, suggesting an absence of circadian input after the onset of the photorefractory response.

2. MATERIALS AND METHODS

(a) *Animals*

All animals used in these experiments were from Charles River (Lake View Gorge strain). Wild-types were purchased from the supplier (Charles River, Wilmington, MA, USA), while *tau* mutants were bred at the University of Virginia from stock established from the original *tau* mutant individual. The colony origin is described in Loudon *et al.* (1994). Nine wild-type and 17 homozygous *tau* mutant males aged 66–69 d were individually caged in DD for 30 weeks. Prior to exposure to DD, animals were housed under a 14L:10D cycle—a photoperiod which is known to stimulate reproduction in both normal period and *tau* mutant males (Menaker & Refinetti 1993). At the start of the experiment, all males had enlarged testes. All handling, measurements and cage changes were made using infrared viewing equipment, such that animals were maintained throughout in conditions of complete darkness. Animals were not provided with access to running wheels at any stage in the study.

(b) *Measurements*

(i) *Changes in testis index*

Every 7 d, animals were lightly sedated with a gaseous anaesthetic (medoxyfluoroacetate), and the width of the right testis measured using vernier callipers. Animals were weighed at the start of the study, week 0, and at weeks 12 and 25.

(ii) *Statistical analysis*

Data for changes in testicular dimension were analysed by cubic regression analysis. Comparisons between different groups were initially performed on the entire data set by analysis of covariance (ANCOVA) on the linear and quadratic components of cubic regressions (SuperAnova, Abacus, Cherwell Scientific, Oxford, UK). Analysis was confined to the linear and quadratic components in order that comparisons could be made between the initial slopes of decline and subsequent increase, although the full data set contained a significant cubic component. Subsequent comparisons of the slopes of decline or increase were undertaken by linear regression of log-transformation of a subset of the data. For the declining portion, this was taken as weeks 0–10, and for the increasing portion, weeks 12–25. Differences in the mean minimal testis indices were compared for the two groups by the *t*-statistic.

3. RESULTS

At the start of the experiment, wild-types weighed significantly more than *tau* mutants (149.9 ± 6 g ($n=9$) and 109.3 ± 1 g ($n=17$), $p<0.01$). These differences persisted through to the end of the experiment (168.4 ± 6.3 g versus 130.5 ± 2.6 g, $p<0.01$). Similar body-weight differences have been reported in other studies (Stirland *et al.* 1996*a*). There were significant differences in mean testicular widths at the start of the experiment with *tau* mutants having smaller testes (12.3 ± 0.2 mm and 11.05 ± 0.11 mm; $p<0.05$). When corrected for body weight (mm g^{-1}), there were no significant differences between groups. In order to normalize for these differences, changes in testicular width were calculated as a testis index (Vitaterna & Turek 1993), whereby the mean testis width at week 0 was divided by testis width at each subsequent measurement, for each animal (i.e. width relative to week 0).

Changes in mean testis index over the course of the experiment are shown in figure 1*a*. Continuous darkness induced gonadal regression and subsequent gonadal recrudescence in both genotypes. Analysis of covariance of the linear component of the cubic regression revealed that testicular regression occurred more rapidly in *tau* mutants than wild-types ($b=0.023$, s.e.m. 0.007, $p=0.01$). Analysis of the quadratic component of the cubic regression revealed that this component was significantly higher for *tau* than wild-type animals ($c=-0.0010$, s.e.m. 0.0003, $p<0.01$), indicating that time of onset of testicular regrowth occurred earlier in *tau* mutants. The rate of decline of testicular index was 0.100 and 0.077 for *tau* and wild-types, respectively. This was substantiated by comparisons of the mean minimal testis index, which occurred significantly earlier in *tau* mutants, at 11.26 weeks (± 0.36, $p<0.05$), than in wild-types, at 12.66 weeks (± 0.41). A summary of the regression coefficients for the quadratic model used is shown in table 1. The testis index data were log-transformed and rates of decline calculated for weeks 0–10. The slope of decline was significantly greater for *tau* mutants than wild-types ($p<0.01$). There were no significant differences between genotypes in rates of testicular regrowth (weeks 11–20), supporting the conclusions above. In order to account for differences in circadian genotype, changes in testis index were recalculated with respect to the differences in circadian period (a circadian cycle was considered here as 24 h for wild-types and 20 h for *tau* mutants). The data in figure 1*a* are thus replotted in figure 1*b* on the basis of circadian weeks. Following this transformation, there was no significant difference in the rate of decline ($b=0.007$, s.e.m. 0.007, $p \geqslant 0.1$), or subsequent recrudescence ($c=0.00007$, s.e.m. 0.00025, $p \geqslant 0.1$), in testis index. There was also no significant difference in the estimated mean nadir of testis index for both groups (12.56 ± 0.20 and 12.66 ± 0.41 weeks for *tau* and wild-type, respectively). In contrast to the onset of the study, there were significant differences in the testicular indices of the two genotypes from weeks 21–25 ($p<0.01$), with the *tau* mutants exhibiting a significant 'overshoot' in gonadal recovery.

4. DISCUSSION

Our results demonstrate that a mutation which accelerates circadian rhythms also alters long-term rates of change of reproductive function. It is clear that *tau* not only increases the rate of decline of testis size in response to inhibitory photoperiods, but also significantly advances

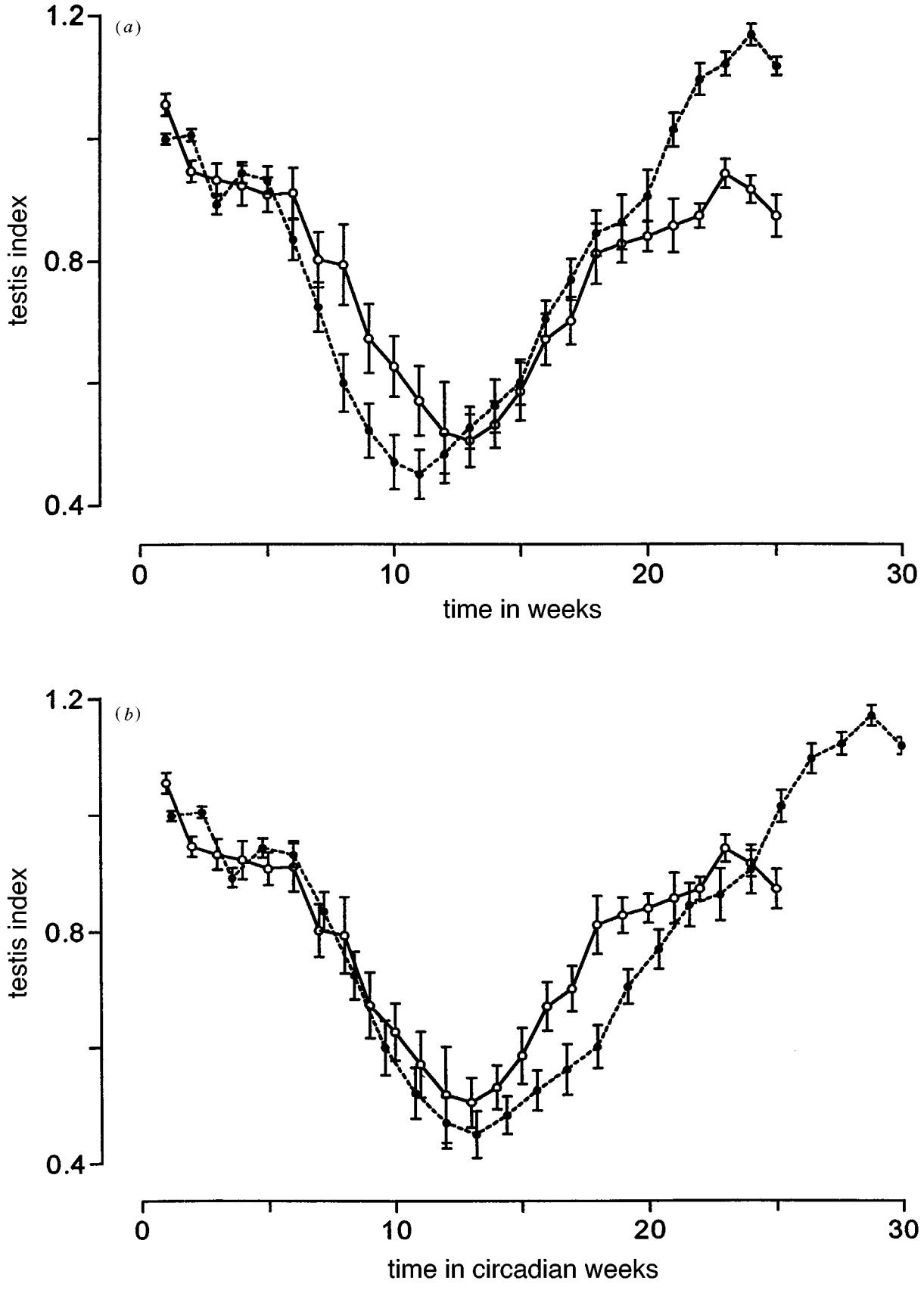

Figure 1. Changes in testis size, as a testis index expressed relative to width in week 0, for *tau* (closed circles) and wild-type (open circles) male hamsters. Data are plotted on a time base relative to weeks (*a*), or corrected for differences in circadian time as circadian weeks (*b*).

Table 1. *Summary of the regression coefficients for the quadratic model used*

	regression coefficient ±s.e.		*p*-value	
	tau	wild-type	*tau*	wild-type
intercept	1.25 (±0.29)	1.15 (±0.031)	0.001	0.001
linear component	−0.123 (±0.006)	−0.077 (±0.005)	0.001	0.001
quadratic component	0.005 (±0.0004)	0.003 (±0.00002)	0.001	0.001

the time of onset of photorefractoriness, as defined by the point at which subsequent testicular recrudescence develops. Comparisons with respect to circadian time indicate that these differences may be attributed to the effect of the *tau* mutation. Here, changes in testicular size are virtually identical for the two genotypes. A number of studies of Syrian hamsters have shown that reduction in gonadotrophin secretion following exposure to such photoperiods is gradual rather than abrupt, with declines in luteinizing hormone (LH) and follicle stimulating hormone (FSH) occurring over 7–11 weeks (Berndston & Desjardins 1974; Turek *et al*. 1975; Steger *et al*. 1982). Thus, our data suggest that the circadian timer may be involved in controlling rates of neuroendocrine response to inhibitory photoperiods.

A plausible explanation is that the differences we have observed are attributable either to an altered pattern of melatonin secretion, or altered responsiveness to the melatonin signal. Our earlier studies of wild-types demonstrate that the duration of melatonin secretion varies in proportion to the length of the dark phase, and that this rhythm is endogenously driven in animals maintained in constant darkness (Maywood *et al*. 1993). Here, circadian activity cycles and melatonin secretion are closely coupled. We have no detailed information on circadian changes in plasma concentrations of melatonin in the *tau* mutant, but our preliminary data indicate that melatonin is also endogenously secreted and coincident with the phase of the nocturnal activity (i.e. every 20 h; R. J. Lucas, J. A. Stirland and A. S. I. Loudon, unpublished results). We have previously shown that pinealectomized *tau* mutants are sensitive to inhibitory (8–10 h) melatonin signals imposed at this frequency (Stirland *et al*. 1995, 1996*b*). One possibility is that an increased frequency of melatonin signals generated by the circadian timer modulates the rate at which gonadotrophin hormone support is withdrawn, following exposure to inhibitory photoperiods. In wild-type animals, experimental manipulations of melatonin signal frequency have been undertaken, in which pinealectomized males were exposed to inhibitory (10 h melatonin) signals for an 8-week period, at frequencies of 20 h or 24 h (Maywood *et al.* 1990). At higher signal frequencies of 20 h, a more potent inhibitory stimulus is observed, with a greater degree of testicular regression and lowered gonadotrophin levels after an 8-week infusion period than when the same signal is presented at 24-h frequencies. Thus, exposure of wild-types to abnormal 20-h melatonin signal frequencies may mimic the effects on the reproductive axis which we have observed in *tau* mutants. Our studies of melatonin-infused pinealectomized animals also suggest that the *tau* mutation has altered the mechanisms responsible for measurement of the melatonin signal. In contrast to wild-type hamsters, *tau* mutants fail to undergo testicular regression when exposed to long-duration melatonin signals at 24-h frequencies (Stirland *et al.* 1996*b*). Such signals are inhibitory in wild-types (Maywood *et al.* 1990). Recent studies of wild-type Syrian hamsters by Powers *et al*. (1997) provide further support for the finding that latency to gonadal regression is more flexible than the time-course of recrudescence. In this study, the shorter the photoperiod to which animals were exposed, the more rapid the rate of regression, but the subsequent rate of recrudescence was unaffected by the duration of inhibitory photoperiod used. This may be attributable to differences in the duration of the melatonin signal generated on these photoperiods, since pinealectomized wild-types exposed to inhibitory long-duration melatonin signals ($12\,h\,d^{-1}$) regressed more rapidly than animals exposed to 8.5-h signals, but in both cases the rate of recrudescence was similar. Thus, the melatonin signal frequency and signal duration interact to alter the rate of gonadal regression but not the subsequent refractory response.

It remains possible that the effects we observed may also be attributable to differences in endogenous gonadotrophin support, due either to the effects of the prior photoperiod history or to pleiotropic effects of the *tau* mutation. Clearly, the two groups experienced different photoperiod histories as both were maintained on 14L:10D cycles prior to the experiment. In addition, our earlier studies of ovariectomized *tau* mutant hamsters demonstrated that *tau* mutants exhibit a reduction in the frequency of LH pulses compared to wild-types, when maintained in conditions of constant bright illumination (Loudon *et al*. 1994). One possibility is that the more rapid regression we observed in the intact males might be attributable to the fact that gonadotrophic support was closer to the margin required for full gonadal maintenance than it was for wild-types, notwithstanding the fact that testicular size relative to body weight was not significantly different in the two genotypes used in the study. Further, it is difficult to explain the more rapid onset of testicular regrowth (and subsequent 'overshoot'; figure 1*a*) in *tau* mutants in terms of the pleiotropic effects of *tau* in reducing gonadotrophin support. We have no explanation for this 'overshoot'. We confined the analysis presented above to comparisons of rates of change as it was not possible to determine whether testicular regrowth had reached a plateau in either genotype by week 25.

We were intrigued that the onset of gonadal recrudescence in the two genotypes commenced after a similar number of circadian cycles, but at different absolute times. This implies that the onset of the refractory neuroendocrine process may depend upon the passage of a fixed number of circadian cycles (approximately 80),

representing an accumulated response to the preceding pattern of exposure to melatonin. In its simplest form, this might suggest that hamsters simply 'count cycles' during the regression phase. Long-term (>10 weeks) studies of response to artificial melatonin signals in pinealectomized hamsters have not been undertaken for technical reasons. However, using seasonally breeding sheep, Karsch and colleagues (Karsch *et al.* 1986) have shown that both long- and short-day photorefractoriness (as measured by changing LH concentrations) can be mimicked by the imposition of continuous long- or short-duration melatonin signals, in ovariectomized, oestradiol-implanted, pinealectomized ewes. In these experiments, long-term exposure to fixed duration melatonin signals resulted in a reversal of gonadotrophin response as the animals became refractory to the melatonin signal.

In conclusion, our study shows that the rate of photoperiodic inhibition is accelerated in *tau* mutants, but that once initiated, reactivation is not associated with circadian genotype. Our data suggest that this component of the seasonal response may not require input from the circadian clock.

We thank Denise Toliver for assistance with the management and care of the animals, Jason Yustein for assistance with the experimental measurements, Dr Andre Gilburn for statistical advice and our colleagues at the NSF Center for Biological Timing for their interest and their comments. The work was supported by an NIH grant (HD 13162) to M.M. and a Wellcome Research Travel Grant to A.S.I.L.

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