0013-7227/05/$15.00/0
Printed in U.S.A.

Endocrinology 146(9):3782–3790

doi: 10.1210/en.2005-0132

Photorefractoriness in Mammals: Dissociating a Seasonal Timer from the Circadian-Based Photoperiod Response

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In seasonal animals, prolonged exposure to constant photoperiod induces photorefractoriness, causing spontaneous reversion in physiology to that of the previous photoperiodic state. This study tested the hypothesis that the onset of photorefractoriness is correlated with a change in circadian expression of clock genes in the suprachiasmatic nucleus (circadian pacemaker) and the pars tuberalis (PT, a melatonin target tissue). Soay sheep were exposed to summer photoperiod (16-h light) for either 6 or 30 wk to produce a photostimulated and photorefractory physiology, and seasonal changes were tracked by measuring the long-term prolactin cycles. Animals were killed at 4-h intervals throughout 24 h. Contrary to the hypothesis, the 24-h rhythmic expression of clock genes (*Rev-erbα, Per1, Per2, Bmal1, Cry1*) in the suprachiasmatic nucleus and PT reflected the ambient photoperiod/melatonin signal and not the changing physiology. Contrastingly, the PT expression of α-glycoprotein hormone subunit (*αGSU*) and *βTSH* declined in photorefractory animals toward a short day-like endocrinology. We conclude that the generation of long-term endocrine cycles depends on the interaction between a circadian-based, melatonin-dependent timer that drives the initial photoperiodic response and a non-circadian-based timer that drives circannual rhythmicity in long-lived species. Under constant photoperiod the two timers can dissociate, leading to the apparent refractory state. (*Endocrinology* 146: 3782–3790, 2005)

MANY MAMMALS USE the predictability of the annual cycle in day length to synchronize long-term rhythms in physiology and behavior to the seasonal environment. This is achieved through a specialized sensory-neuroendocrine mechanism wherein retinal photoreception is transduced into a nycthemeral pattern of melatonin secretion (1). Light regulates the melatonin rhythm in two ways: first by synchronizing a circadian pacemaker in the suprachiasmatic nucleus (SCN) that controls the activity of the pineal gland and second by acute inhibition of melatonin production. The duration of melatonin secretion then provides an endocrine index of night length and thus day length.

Melatonin target tissues interpret the changes in the nightly melatonin signal to time seasonal physiology to the annual photoperiodic cycle. Long duration melatonin signals promote a winter physiology and short duration signals promote a summer physiology. This has been elegantly demonstrated in pinealectomized hamsters and sheep in which melatonin is replaced via programable infusions pumps, and infusion duration varied to activate the two types of seasonal physiology (2). The effects of photoperiod/melatonin on diverse aspects of seasonal physiology (*e.g.* reproduction, body weight, pelage molt) are believed to be mediated by melatonin receptors expressed in a range of different neural, pituitary, and possibly peripheral target cells (3).

An important feature of the seasonal timing mechanism is its capacity to monitor photoperiodic history. This is particularly evident at the spring and autumn equinoxes when seasonal mammals have a different physiology, despite the photoperiod and the associated melatonin signal, being the same at the two times of year. Analysis of this phenomenon has led to the characterization of a process known as photorefractoriness, whereby animals held on constant photoperiod undergo spontaneous reversion in physiology to a state associated with the opposite photoperiod (4–7). In seasonal rodents, exposure to prolonged short photoperiod (SP) causes gonadal regression and the development of a winter phenotype over an interval of about 12 wk, and this is followed by a progressive and regulated recovery back to a summer physiology within a further period of 12–20 wk, with no change in the prevailing photoperiod. This SP refractory response permits animals in the natural winter environment to reactivate physiology in anticipation of spring, without the requirement for the stimulus of long photoperiod (LP) (4).

In longer-lived species (*e.g.* ground squirrels, mustelids, sheep, and deer), exposure to a fixed LP can also cause refractoriness and reversion to a winter-like physiology, and when the photoperiod is held constant for a sufficiently long period, these animals express alternating transitions in seasonal physiology every 10–12 months as an endogenous circannual rhythm (8–13). Because the dynamics of the physiological changes during the development of photorefractoriness and during the onset of circannual rhythms are similar and can be revealed by exposure to constant photoperiod, it

First Published Online May 26, 2005

Abbreviations: CV, Coefficient of variation; DIG, digoxigenin; *αGSU*, α-glycoprotein hormone subunit; LP, long photoperiod; PM, postmortem; PT, pars tuberalis; SCN, suprachiasmatic nucleus; SP, short photoperiod; VEGF, vascular endothelial growth factor; ZT, Zeitgeber time.

***Endocrinology* is published monthly by The Endocrine Society (http://www.endo-society.org), the foremost professional society serving the endocrine community.**

is likely that they share common underlying control mechanisms (3, 14). These phenomena indicate that the photoperiod/melatonin signaling mechanism represents a means of initiating or synchronizing intrinsic long-term processes to the external seasonal environment, rather than merely a means of directly driving physiological transitions.

The anatomical level at which photorefractoriness develops and the mechanisms involved are important and unresolved issues in the study of seasonal time keeping. Refractoriness does not appear to develop at the level of melatonin synthesis by the pineal gland because several studies demonstrate that the melatonin signal continues to faithfully reflect photoperiod in photorefractory animals (15–17). Moreover, treatment with a standardized daily infusion of melatonin induces refractoriness in sheep after several months (18), as does treatment with constant-release implants of melatonin (19, 20). Studies on tissue responsiveness to melatonin have focused on the pituitary pars tuberalis (PT) because of its high expression of type one melatonin receptors (MT_1) and because it can be studied *in vitro* as well as *in vivo* (21, 22). The principal second messenger response to melatonin, the inhibition of cAMP synthesis, does not change in the PT of SP-refractory Siberian hamsters (23), and such animals continue to express SP patterns of cAMP-regulated gene expression (17). Contrastingly, prolactin-associated responses in the PT do change during SP refractoriness. This includes the reactivation of α-glycoprotein subunit (*αGSU*) gene expression (24) and an increase in prolactin releasing activity (17). Hence, it appears that photorefractoriness develops in melatonin-responsive tissues, unrelated to a change in the primary melatonin response.

It has been proposed that photoperiodic information processing in the PT depends on the modulation of circadian clock gene expression by melatonin. Melatonin directly stimulates the expression of *Cry1* in the PT of sheep and rodents, and this results in a peak of *Cry1* expression in the PT shortly after dark onset (25, 26) (our unpublished observations). Additionally, melatonin withdrawal at dawn, coupled with the adenylate cyclase sensitizing effects of prolonged melatonin exposure during the night (27, 28), induces a cAMP stimulated peak of *Per1* expression in the morning. These actions of melatonin thus provide a decoding mechanism whereby photoperiod may dictate PER/CRY protein complex formation to control PT transcriptional output (29). Furthermore, photorefractoriness may develop due to a progressive change in the circadian pattern of PT clock gene expression occurring independently of the melatonin signal (3). In one study in SP-refractory hamsters, the morning peak in *Per1* expression in the PT remained suppressed as in SP-responsive animals, which appears to contradict this hypothesis (17); however, gene expression in peripheral tissues reverted spontaneously to a high amplitude, LP-like rhythmic pattern (30).

Based on these considerations, we analyzed mRNA for multiple clock genes (*Rev-erbα*, *Per1*, *Per2*, *Bmal1,* and *Cry1*) in the PT, as well as in the SCN, of LP and LP-refractory Soay sheep. The clock genes were selected to represent the positive and negative feedback elements of the circadian clock (31) and because *Cry1* expression in the PT has been shown to be activated by melatonin at the onset of darkness (25). The prediction was that if photorefactoriness results from a dissociation between the melatonin signal and clock genes rhythms, the amplitude and/or relative phasing of clock gene expression in the PT would differ significantly between LP and LP-refractory animals. We measured the long-term changes in prolactin secretion as an endocrine response regulated by the PT (16, 20, 21, 25). We also measured the expression *of αGSU, βTSH*, vascular endothelial growth factor (*VEGF*), and *VGF* as markers of PT function (24, 32–34). The prediction was that RNA abundance for one, or more, of these PT genes would decline in LP-refractory sheep in parallel with the spontaneous decline in prolactin secretion associated with photorefractoriness.

Materials and Methods

Animals and routine measurements

Experiments were conducted in accordance with the U.K. Animals (Scientific Procedures) Act of 1986. The Soay sheep were obtained from specialist breeders in Scotland (35). The animals were housed in light-sealed rooms in single-sex groups and fed a standardized diet of grass pellets (500 g/animal, Vitagrass, Cumbria, UK) given daily 1 h into the light phase. There was free access to hay and water. White florescent strip lights provided approximately 160 lux at the animals' eye level during the light phase. Dim red light (<5 lux) was provided during the dark phase. Daily locomotor activity was recorded continuously on a group basis using infrared sensors coupled to a Mini Mitter VitalView system (Sunriver, OR) (35).

The long-term changes in physiology were followed by measuring the changes in circulating concentrations of prolactin in blood samples collected from the jugular vein twice weekly from representative animals (12/group; six female and six male). Samples were placed in heparinized tubes and the blood plasma separated by centrifugation within 30 min and stored at −20 C until used for the hormone assay. In the males, changes in testis size and pelage molt were routinely monitored (35).

Experimental design

Forty-eight pubertal Soay sheep, 8 months old at the start of the study, were brought indoors in midwinter (December) and preconditioned to short days (8-h light, 16-h darkness). Zeitgeber time (ZT) 0 was taken as time of lights on throughout; light changes were achieved by altering the time of lights-out by 8 h. After 8 wk, all animals were switched to long days (16-h light, 8-h darkness) to commence the experiment. One treatment group (n = 24, 18 females, six males) was exposed to long days for 12 wk, followed by short days for 12 wk, and finally returned to long days for 6 wk to produce photoresponsive animals in which prolactin secretion was predicted to be increasing due to the long-day photoperiod (LP group). The second treatment group (n = 24; 18 females, six males) was studied in parallel and remained on long days for the full 30 wk of the experiment. This was predicted to produce photorefractory animals in which prolactin secretion was spontaneously decreasing due to the prolonged long photoperiod treatment (LP-R group).

One week before the end of the experiment, when all animals were under long days, blood samples were collected hourly for 24 h from 12 animals/group (same animals as used for the routine sampling) to measure the daily melatonin rhythm. For the frequent sampling, the animals were fitted with a cannula placed in the jugular vein on the day before study, kept patent with heparinized 0.9% saline, and blood samples were collected from a three-way tap to avoid disturbance. Blood samples (3 ml) were separated and the plasma stored as for the routine samples.

At the end of the study, batches of animals (three females and one male) were killed by an injection of pentobarbitone at 4-h intervals (n = 4/time point) over 24 h, starting at ZT 11. The hypothalamus and upper pituitary gland tissue was removed as a single block from the skull within 10 min of death, snap frozen in isopentane and dry ice at −30 C, and stored at −80 C until analyzed for mRNA. Material from the complete group was frozen within 40 min of the ZT target time, and the

photoperiod in the two treatments was staggered by 1 h to facilitate the sampling.

Additional material

Frozen PT tissues from Soay sheep exposed to SP and LP for 6 wk (29) were also used for comparison with the current LP and LP-R groups. These tissues had been collected from similar aged animals at the same six clock times across 24 h (n = 4/time point) and were stored at −80 C.

In situ hybridization and scanning densitometry

Coronal cryosections (20 μm) through the SCN and PT were prepared in advance, thaw mounted onto glass slides in sequential order, and stored at −80 C. Expression of the clock genes *Rev-erbα, Per1, Per2, Bmal1*, and *Cry1* in the PT and SCN was analyzed by radioactive *in situ* hybridization using homologous RNA probes for *Per1*, *Per2*, *Bmal1*, and *Cry1*, as described previously (29). The *Rev-erbα* probe was based on the murine sequence (36) and was generously supplied by Professor Ueli Schibler, University of Geneva, Switzerland. Sections were hybridized overnight at 60 C with 5×10^5 cpm of probe per slide and the next day subjected to RNase A digestion and stringency washes in sodium citrate buffer to remove nonspecific probe hybridization. Slides were then dehydrated in graded ethanol solutions and exposed to autoradiographic film (Biomax; Kodak, Sigma UK, Poole, Dorset, UK) initially for 5 d. Exposure duration was then optimized for each gene by repeat film exposures, depending on labeling intensity in the SCN and PT regions; these ranged from 2 (cry1) to 10 (VGF) d. Films were scanned on an 1640XL transmittance scanner (Epson UK, Hemel Hempstead, Hertfordshire, UK), with OD standards and background subtracted; calibrated OD measurements of gene expression in the SCN and PT were performed using NIH-Image. All analyses were performed blind to treatment identity.

The expression of a range of genes associated with physiology in the PT were also studied by *in situ* hybridization on the residual cryosections of PT; these were *αGSU* and *βTSH* (32), *VEGF* (33), and *VGF* (34). The *αGSU* and *βTSH* probes were based on the rat sequences, the *VEGF* probe on the caprine sequence and the *VGF* probe on the Siberian hamster sequence. These probes were generously provided by Chin and colleagues (37), Klosen *et al.* (38), Redmer *et al.* (39), and Barrett *et al.* (34), respectively. To provide better resolution of PT histology, *αGSU* and *βTSH* expression was visualized by digoxigenin (DIG)-based *in situ* hybridization as described previously (40). This was performed in animals from the LP and LP-R groups only at ZT 3 due to the limited supply of tissue.

Endocrine assays

The concentration of prolactin in the weekly blood plasma samples was measured by a standardized RIA validated for ovine plasma (41). The assay had a lower limit of detection of 0.5 ng/ml plasma and intra- and interassay coefficients of variation (CVs) of 7.0 and 9.5%, respectively, based on five assays. Melatonin concentrations in the hourly blood samples were measured by RIA (42) using a commercial antibody (PF-1288; SPI-BIO, Paris, France). The sensitivity of the assay was 5.0 pg/ml plasma and the assay CVs were less than 15%.

Statistics

Quantified gene expression and plasma hormone concentrations were analyzed for effects of treatment (LP, compared with LP-R group) and time (week of study or time of day) by two-way ANOVA, with *post hoc* comparisons using Bonferroni's test. For the 24-h melatonin profiles, the period of melatonin secretion was calculated for each animal defined by the number of consecutive hourly samples with melatonin concentrations greater than the basal values by intraassay CV × 2. The duration and peak concentrations of melatonin for the LP and LP-R groups and other single point parameters were compared by Student's *t* test. Locomotor activity patterns were measured on a group basis only and representative, double-plotted actograms covering a total 24 wk (wk 6–30 of the experiment) are presented using the Mini Mitter software (VitalView) for LP and LP-R groups (see Fig. 2).

Results

Generation of a photorefractory physiological state

Prolactin secretion, pelage molt, and gonadal activity. The two lighting regimens induced the predicted long-term changes in prolactin secretion in the sheep (Fig. 1A). The initial exposure to LP caused a progressive (approximately 20-fold) increase in plasma prolactin concentrations over the first 10 wk. There was an associated pelage molt in all animals. In the LP group, the switch to SP suppressed prolactin concentrations until wk 24, when the second exposure to LP reactivated the prolactin axis. At the termination of the study, prolactin concentrations in LP animals were rapidly increasing and all animals were reinitiating a second pelage molt. In the LP-R group, the prolonged exposure to long days produced activation, followed by a progressive decline in prolactin concentrations from wk 10 until the end of the study, although concentrations always remained above SP-suppressed values (Fig 1A). There was no synchronous second pelage molt in the LP-R group. The statistical analysis demonstrated a significant ($P < 0.001$, two-way ANOVA) group × time interaction; plasma prolactin concentrations were significantly different between the LP and LP-R groups from 14 to 30 wk of the study, except for the time of crossover at wk 28 (Fig. 1A).

In the male animals at the end of the study, testis size was decreasing in the LP group (consistent with long-day-induced inhibition of the reproductive axis) and increasing in the LP-R group (consistent with the development photorefractoriness for the reproductive axis). This was reflected by differences in testis weight between groups at wk 30 (testis weight: 86.9 ± 4.3 and 126.1 ± 9.5 g for the LP and LP-R groups, respectively; $P < 0.001$, Student's *t* test). Overall, these data demonstrate that the LP and LP-R groups were in clearly distinct photostimulated and photorefractory states at wk 30 when tissues were collected at postmortem (PM, Fig. 1A).

Endocrine expression in the PT. DIG-labeled *in situ* hybridization revealed the presence of *αGSU* and *βTSH* mRNA throughout the ovine PT (Fig. 1B). In the case of *αGSU*, a scattered minority (less than 5%) of all labeled cells showed particularly intense expression and were variably present in all tissue sections analyzed (Fig. 1B, *top panel*). There was no corresponding subgroup of cells seen in sections stained for *βTSH* (Fig. 1B, *bottom panel*). The overall density of the hybridization signal for *βTSH* was consistently increased in the LP compared with the LP-R animals (Fig. 1B, *lower panel*).

Radioactive *in situ* hybridization analysis of PT *αGSU* mRNA levels in tissues from the previous study in LP and SP acclimatized sheep demonstrated that the expression of *αGSU* is photoperiod dependent, with levels under SP being approximately half of those seen under LP ($P < 0.001$, two-way ANOVA) (Fig. 1C, *top panel*). In the present study animals, *αGSU* expression was found to be time dependent in the LP-R but not the LP animals ($P < 0.001$ for treatment × time interaction). In the LP-R group, expression levels in the midlight phase were indistinguishable from those in LP animals, but expression declined from this point forward to the following morning. This produced a reduction in mean levels

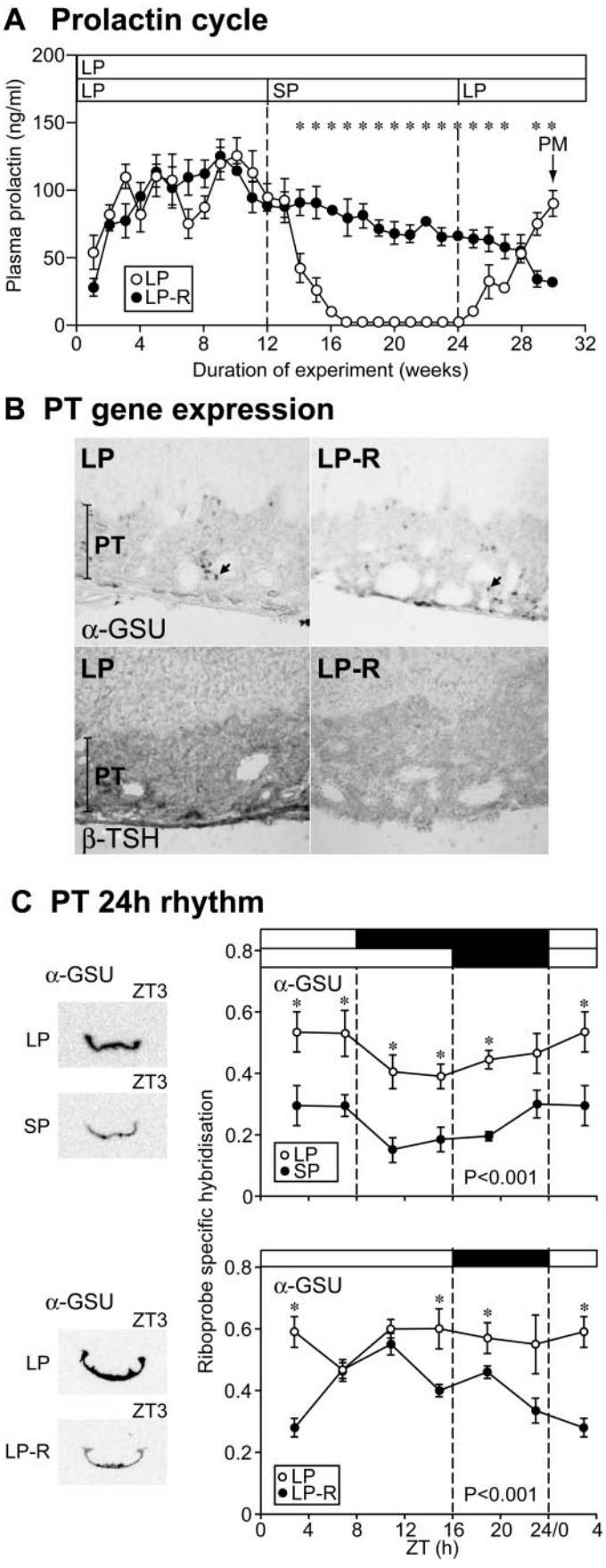

FIG. 1. A, Weekly changes in blood plasma concentrations of prolactin in LP and LP-refractory (LP-R) Soay sheep, throughout the 30-wk experiment. Values are mean ± SEM, n = 12/group. PM denotes the time of collection of tissues for gene analyses. *Asterisks* indicate times of significant difference between groups ($P < 0.05$, two-way ANOVA with *post hoc* test). B, DIG-labeled *in situ* hybridization images of *αGSU* subunit and *βTSH* gene expression in representative sections of PT from LP and LP-R Soay sheep. Sparse, densely expressing *αGSU* cells are indicated (*arrows*). C, Twenty-four-hour profiles of *αGSU* expression in PT in LP and SP Soay sheep [tissues from previous study (29), *upper panel*] and LP and LP-R Soay sheep [current animals, *lower panel*], measured by radioactive *in situ* hybridization. Values are mean ± SEM, n = 4/group. *Horizontal bars* show time of daylight (*open*) and darkness (*closed*). *Asterisks* indicate times of significant difference between groups ($P < 0.05$, two-way ANOVA with *post hoc* test), and the significance level for the overall time × group interaction is indicated.

of *αGSU* expression of about 40% in LP-R animals, compared with LP controls ($P < 0.001$, effect of treatment); values remained above those in the SP treatment (LP-R, compared with SP, $P < 0.001$). The decline in the *βTSH* expression in LP-R animals measured at ZT3 closely paralleled the decline in *αGSU*, but this was not analyzed through 24-h due to lack of tissue.

Autoradiographic images of PT sections from both SP and LP-R animals showed the maintained presence of isolated hot spots of intense *αGSU* expression in animals that were developing a SP-like endocrine physiology. These spots correspond to the minor population of intensely labeling cells described above and probably represent invasive gonadotropes seen at the interface with the pars distalis (38). VEGF and VGF mRNA was also measured in the PT, but the level of expression was independent of time or treatment (data not shown).

Overall, the *αGSU* and *βTSH* data demonstrate that the endocrine cells of the PT had changed toward a SP-like phenotype in LP-R animals.

Circadian rhythmicity in the photorefractory state

Circadian locomotor activity. The daily locomotor activity patterns of the animals were recorded continuously (Fig. 2A). Activity was diurnal and crepuscular with a peak after lights-on associated with feeding time and a minor peak anticipating lights-off. The period of activity was more restricted in the LP group during exposure to short days, and there was a period of readjustment lasting about a week after the abrupt light changes. This was seen as a phase advance in the onset of activity after the switch between long and short days (Fig. 2A, wk 12). At the end of the study, locomotor activity patterns were similar in the LP and LP-R groups, despite the differences in seasonal physiology.

Melatonin secretion. The corresponding 24-h melatonin profiles were determined in the week before the end of the study (Fig. 2B). Plasma melatonin concentrations were consistently increased during the 8-h dark phase in all animals with no differences in any of the melatonin parameters between the LP and LP-R groups. The duration of increased melatonin concentrations was 8.33 ± 0.13 and 8.50 ± 0.14 h (mean ± SEM), and the mean nocturnal melatonin concentration was

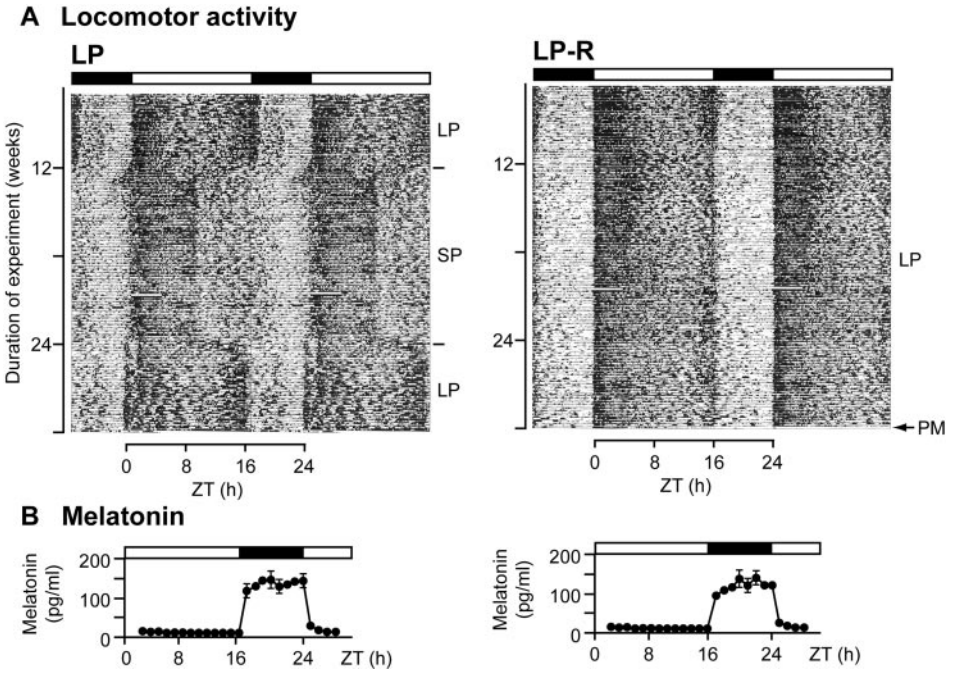

FIG. 2. A, Representative double-plotted locomotor actograms for 6–30 wk of the experiment for LP and LP-refractory (LP-R) Soay sheep. The photoperiod treatment is shown to the right for each actogram. PM denotes time of collection of tissues for gene analyses. B, Twenty-four-hour profiles in blood plasma concentrations of melatonin in the final week of the study for the two groups. Values are mean ± SEM, n = 10/group, expressed relative to ZT (ZT 0 = time of lights on), and aligned to the actograms. *Horizontal bars* show time of daylight (*open*) and darkness (*closed*).

123.7 ± 16.5 and 137.5 ± 17.6 pg/ml plasma for the LP and LP-R groups, respectively (NS, Student's *t* test).

Clock gene expression in the SCN. The status of the core circadian pacemaker in the SCN was assayed by analysis of circadian clock gene expression (Fig 3, A and B). All five genes assayed (*Rev-erbα*, *Per1*, *Per2*, *Bmal*, and *Cry1*) were rhythmically expressed and showed peak mRNA values at times of day consistent with our previous work (29). For four of the five genes, there was no significant effect of treatment on the temporal pattern of expression. For the remaining gene, *Bmal1*, the profile appeared to be slightly flattened in LP-R animals ($P < 0.05$ for treatment × time interaction), although cycle mean levels of expression were not significantly different. Hence, the transition to the LP-R state had negligible impact on the core circadian clockwork (Fig. 3) or the light/SCN control of melatonin secretion (Fig. 2).

Clock gene expression in the PT. All the clock genes measured in the SCN were also rhythmically expressed in the PT but with a different, tissue-specific 24-h pattern (Fig. 4, A and B). Peak mRNA values for *Rev-erbα* and *Per1* occurred in the early light phase at ZT 3; and *Per2* expression peaked slightly later (ZT7). *Bmal1* expression was highest late in the day (peak ZT 11–15), and maximum *Cry1* expression occurred as expected in the early dark phase (ZT 19). The mRNA profiles for all genes were strikingly similar in the two treatment groups ($P > 0.05$ for treatment and treatment × time interaction in all cases) and indistinguishable from those we have previously described in LP-acclimated animals (25, 29).

Discussion

These results provide strong evidence to refute the hypothesis that a change in clock gene expression in the PT regulates photorefractoriness. Prolactin secretion declined in the sheep exposed to prolonged LP as seen previously (11, 16, 35), demonstrating the development of a photorefractory state. There was also a clear decline in *αGSU* mRNA levels in the PT associated with the spontaneous decrease in prolactin secretion, which is consistent with a functional role of the PT in regulating prolactin release (17, 20, 21). The 24-h clock gene rhythms, however, faithfully tracked the melatonin signal encoding the photoperiod and not the changing physiological state that occurred over the period of 30 wk under constant photoperiod.

Whereas both SP and LP photorefractoriness are well characterized at the whole-organism level, studies of the underlying control mechanisms have been limited to SP-R in hamsters (17, 24, 43). In sheep, the prolactin and gonadotropin axes undergo both SP-R and LP-R responses indicating that the mechanisms may be different in long-lived species. The LP-R response in prolactin secretion is very clearly expressed in hypothalamo-pituitary disconnected sheep, supporting the view that refractoriness develops within the pituitary gland, probably within the PT in which MT_1 melatonin receptors are expressed at notably high levels (16, 20). We therefore mapped the development of LP-R in the Soay sheep model in intact animals and collected brain and pituitary tissues at the transition time when both mean prolactin levels and the trajectory of change in prolactin secretion diverged dramatically from that seen in LP control animals. We examined the RNA expression of four gene-coding hormones or growth factors expressed in the PT (*αGSU*, βTSH, VEGF, and VGF); of these, *αGSU*, was expressed throughout the PT and declined toward a SP-like state in the photorefractory animals. Due to tissue limitations, we did not quantify *βTSH* expression, but our DIG-based *in situ* results suggest that the decline in *αGSU* expression occurs in *βTSH*-expressing cells and that this gene also undergoes an SP-like decline in expression in LP-R animals. We found no change in *VEGF* or *VGF* expression, suggesting that these genes are not photo-

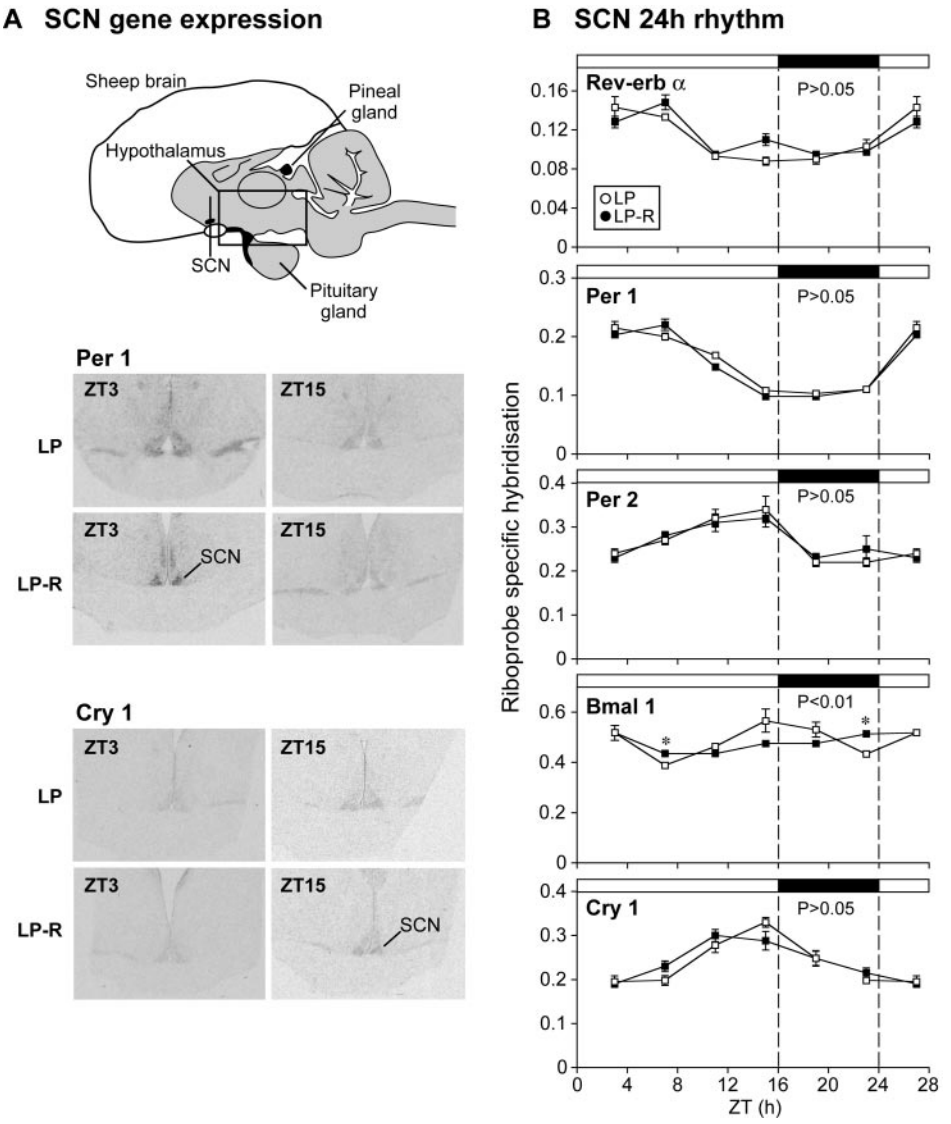

FIG. 3. A, *Above*, Diagram of a sheep brain showing location of coronal sectioning of the SCN; *below*, representative *in situ* hybridization images of *Per1* and *Cry1* gene expression in SCN in LP and LP-refractory (LP-R) Soay sheep at ZT 3 and ZT 15. B, Twenty-four-hour profiles for *Rev-erbα*, *Per1*, *Per2*, *Bmal1*, and *Cry1* gene expression in SCN in LP and LP-R Soay sheep, measured by radioactive *in situ* hybridization. Values are mean ± SEM, n = 4/group. *Horizontal bars* show time of daylight (*open*) and darkness (*closed*). *Asterisks* indicate times of significant difference between groups ($P < 0.05$, two-way ANOVA with *post hoc* test), and the significance level for the overall time × group interaction is indicated.

periodically modulated in the ovine PT. Previous studies in hamsters demonstrate that PT *αGSU* mRNA values increase in SP-R hamsters as prolactin secretion rises, and this precedes changes in the expression of gonadotropin subunits in the pars distalis (24). Moreover, PT explants from hamsters show increased production of prolactin releasing activity with the development of SP refractoriness (17). Collectively these results support the concept that refractoriness occurs somewhere between the melatonin target cell and the PT paracrine output.

In mammals, photoperiod time measurement is circadian based due to regulation of the melatonin signal by the SCN (1, 44). We measured the expression of five clock genes in the sheep SCN but found no significant effect of the LP-R state on the expression of four of them. For a fifth, *Bmal1*, there was a slight amplitude reduction in the 24-h rhythm but no change in mean levels. Given that nuclear BMAL1 protein, unlike PER1 (45), is not believed to undergo significant circadian variation in the SCN (46), we consider this observation in sheep to be of minor importance. Furthermore, the fact that both the 24-h patterns of melatonin secretion and the locomotor actogram profiles were similar in LP and LP-R animals confirms the stability of the circadian system under prolonged LP. Hence, it is most likely that the cellular events leading to the LP-R state in sheep occur downstream of melatonin signal production and independent of core circadian timing within the SCN.

Within the PT, melatonin acts through compound effects on adenylate cyclase, involving both acute suppression and time-dependent sensitization (22), which is believed to account for the morning peak in *Per1* expression (28). In the recent study of the SP-R state in hamsters, photorefractoriness was not associated with a change in the expression of *Per1* or of another cAMP-regulated gene, inducible cAMP early repressor (17). This result agrees with the earlier demonstration that adenylate cyclase activity in the PT remains melatonin sensitive in PT explants from SP-R hamsters (23). In the current study, entry into the LP-R state also failed to influence patterns of *Per1* expression in the PT, consistent with the view that alterations in melatonin-dependent reg-

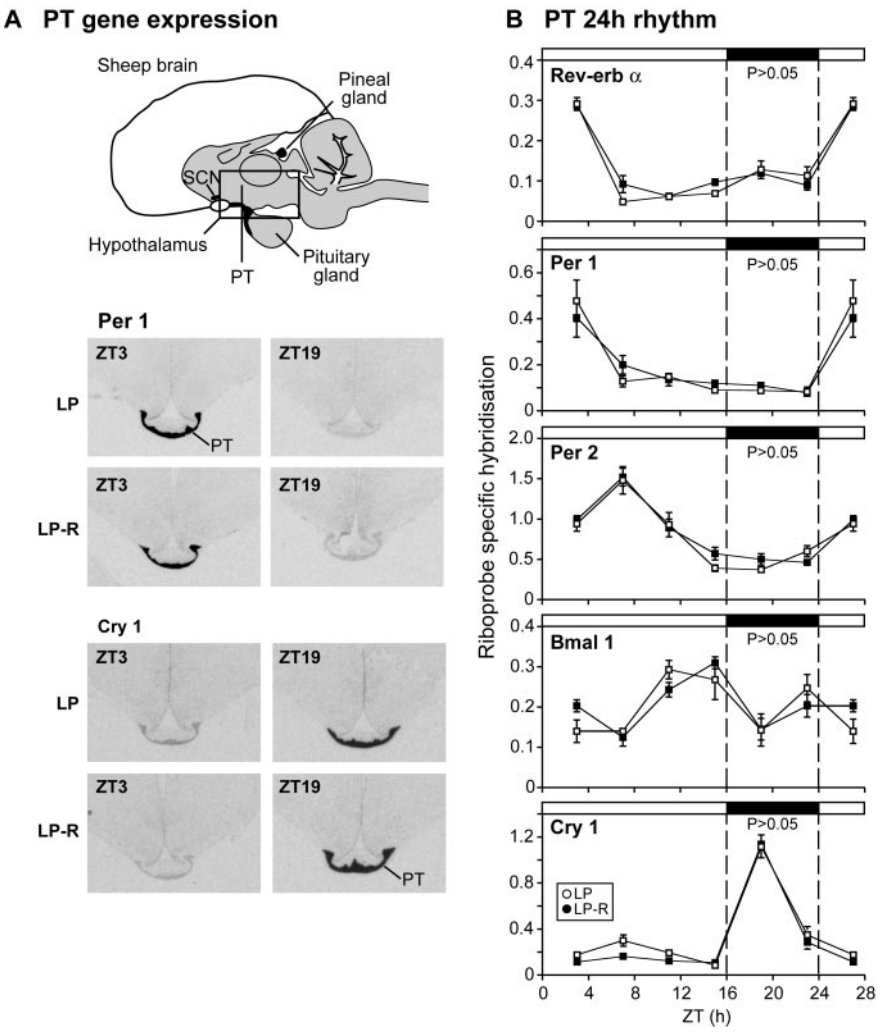

FIG. 4. A, *Above*, Diagram of a sheep brain showing location of the coronal sectioning of the PT; *below*, representative *in situ* hybridization images of *Per1* and *Cry1* gene expression in PT in LP and LP-refractory (LP-R) Soay sheep, at ZT 3 and ZT 15. B, Twenty-four-hour profiles for *Rev-erbα*, *Per1*, *Per2, Bmal1*, and *Cry1* gene expression in PT in LP and LP-R Soay sheep, measured by radioactive *in situ* hybridization. Values are mean ± SEM, n = 4/group. *Horizontal bars* show time of daylight (*open*) and darkness (*closed*). The significance level of the overall time × group interaction is indicated.

ulation of cAMP response element-containing genes does not induce the development of the LP-R state.

Whereas *Per1* is one core member of the complex of clock genes that control circadian physiology, other members of this complex are controlled through different combinations of transcriptional elements, including E-boxes, and retinoid-related orphan receptor response elements (RORES) (31). Recent data suggest that *Cry1* is directly regulated by melatonin (25, 26), but it is probable that other clock genes are not directly controlled by melatonin in the PT. We therefore examined the expression patterns of a range of clock genes (*Rev-erbα*, *Per2*, *Cry1*, and *Bmal1*) in the sheep PT. The results demonstrate a remarkably tight maintenance of circadian rhythmicity from the photostimulated to the photorefractory condition. Thus, neither the melatonin signal induction of *Cry1* at the onset of darkness nor the relative phasing of clock gene rhythms across the 24 h change in the PT during long-term transitions in seasonal physiology.

The term photorefractoriness implies loss of responsiveness to photoperiod, but it is clear this is not the result of insensitivity of the target tissues to melatonin. PT tissue still expresses melatonin-binding sites, binds iodomelatonin, and shows cAMP-mediated responses (23), and photorefractory animals rapidly respond to a change in the duration of the melatonin signal with altered prolactin secretion (16). The data presented here demonstrate that PT cells continue to monitor the duration of the melatonin signal, and hence prevailing photoperiod, at the level of clock gene transcription. This observation indicates that the role of melatonin effects on clock gene rhythms in seasonal timing is limited to the photoperiodic input pathway: other, as-yet-unidentified processes, must be responsible for long-term changes in physiology leading to the expression of photorefractoriness.

Hence, our data are consistent with published models for circannual rhythmicity that invoke dual timing processes (3, 12): a circadian based photoperiod timer and a circannual timer. We speculate for prolactin secretion that both these processes operate within the pituitary gland. The former process is proposed to depend on a clock gene-based readout of the melatonin signal within the MT_1-expressing cells of the PT, whereas the latter is clock gene independent.

The noncircadian basis to the circannual timer is supported by studies demonstrating that the circannual transitions between summer and winter physiology persist after optic nerve section (ferret) (10), SCN lesion (ground squirrel) (48, 49) (sheep) (50; and Scott, C. J., personal communication), pinealectomy, or superior cervical ganglionectomy (ferret) (10) (sheep) (11, 51), surgical procedures that block the pho-

toperiodic response. Furthermore, entrainment of SCN-intact animals to non-24-h light-dark cycles that change the circadian period has no effect on the timing of such transitions and the circannual cycle (ground squirrel) (52), as also observed in birds (53). Finally, the two-timer concept fits nicely with data from the *tau*-mutant hamster, in which the circadian clock runs with a short period of about 20 h; this mutation perturbs the initial photoperiodic response (circadian based) but not the rate at which photorefractoriness subsequently develops (54) (noncircadian based).

In conclusion, the present study demonstrates that photorefractoriness in the prolactin axis is not associated with changes in the circadian expression of clock genes in the PT, as based on results for five core clock genes. Output-associated gene expression (αGSU and βTSH) is dissociated from clock gene expression in this melatonin-responsive tissue in photorefractory animals. We suggest that the photorefractory state for the prolactin axis is unrelated to changes in the photoperiod/melatonin/clock gene transduction pathway but depends on a distinct molecular timing mechanism regulated within the pituitary gland.

Acknowledgments

The authors thank Norah Anderson, Marjorie Thompson, Ian Swanston, and Irene Cooper for expert technical help with the experiment.

Received February 2, 2005. Accepted May 18, 2005.

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This work was supported by the Medical Research Council (to G.A.L. and H.A.) and the Biological Sciences Research Council (to D.G.H., Grant S/17353).

References

1. **Goldman BD** 2001 Mammalian photoperiodic system: formal properties and neuroendocrine mechanisms of photoperiodic time measurement. J Biol Rhythms 16:283–301
2. **Bartness TJ, Powers JB, Hastings MH, Bittman EL, Goldman BD** 1993 The timed infusion paradigm for melatonin delivery: what has it taught us about the melatonin signal, its reception, and the photoperiodic control of seasonal responses. J Pineal Res 15:161–190
3. **Lincoln GA, Anderson H, Loudon A** 2003 Clock genes in calendar cells as the basis of annual timekeeping in mammals—a unifying hypothesis. J Endocrinol 179:1–13
4. **Reiter RJ** 1972 Evidence for refractoriness of the pituitary-gonadal axis to the pineal gland in golden hamsters and its possible implications in annual reproductive rhythms. Anat Rec 173:365–372
5. **Robinson JE, Karsch FJ** 1987 Photoperiodic history and the changing melatonin pattern can determine the neuroendocrine response of the ewe to daylength. J Reprod Fertil 80:159–165
6. **Hastings MH, Walker AP, Powers JB** 1989 Differential effects of photoperiodic history on the responses of gonadotrophin and prolactin in intermediate daylengths in the male Syrian hamster. J Biol Rhythms 4:335–350
7. **Freeman DA, Zucker I** 2001 Refractoriness to melatonin occurs independently at multiple brain sites in Siberian hamster. .Proc Natl Acad Sci USA 98:6447–6452
8. **Pengelly ET, Amundson SJ** 1974 Circannual rhythmicity in hibernating animals. In: Pengelly ET, ed. Circannual clocks: annual biological rhythms. New York: Academic Press; 96–160
9. **Goss RJ** 1977 Photoperiodic control of antler cycles in deer. iv. Effects of constant light:dark ratios on circannual rhythms. J Exp Zool 201:379–383
10. **Herbert J, Stracey PM, Thorpe DH** 1978 Recurrent breeding season in pinealectomised or optic-nerve-sectioned ferrets. J Endocrinol 78:389–397
11. **Karsch KJ, Robinson JE, Woodfill CJ, Brown MB** 1989 Circannual cycles in luteinizing hormone and prolactin secretion in ewes during prolonged exposure to a fixed photoperiod: evidence for an endogenous reproductive rhythm. Biol Reprod 41:1046–1068
12. **Zucker I** 2001 Circannual rhythms: mammals. In: Takahashi J, ed. Circadian clocks: handbook of behavioral neurobiology. Vol 12. New York: Academic Press; 509–528
13. **Martinet L, Mondain-Monval M, Monnerie R** 1992 Endogenous circannual rhythms and refractoriness of testis activity, moult and prolactin concentrations in mink (*Mustela vison*). J Reprod Fertil 95:325–338
14. **Prendergast BJ, Mosinger Jr B, Kolattukudy PE, Nelson RJ** 2002 Hypothalamic gene expression in reproductively photoresponsive and photorefractory Siberian hamsters. Proc Natl Acad Sci USA 99:16291–16296
15. **Malpaux B, Robinson JE, Brown MB, Karsch FJ** 1987 Reproductive refractoriness of the ewe to inductive photoperiod is not caused by inappropriate secretion of melatonin. Biol Reprod 36:1333–1341
16. **Lincoln GA, Clarke IJ** 2000 Role of the pituitary gland in the development of photorefractoriness and generation of long-term changes in prolactin secretion in rams. Biol Reprod 62:432–438
17. **Johnston JD, Cagampang FRA, Stirland JA, Carr AJF, White MRH, Davis JRE, Loudon ASI** 2003 Evidence for an endogenous *Per* 1 and *ICER*-independent seasonal timer in the hamster pituitary gland. FASEB J 17:810–815
18. **Wayne NL, Malpaux B, Karsch KJ** 1988 How does melatonin code for daylength in the ewe: duration of nocturnal melatonin release or coincidence of melatonin with a light-entrained sensitive period. Biol Reprod 39:66–75
19. **Lincoln GA, Ebling FJP** 1985 Effect of constant-release implants of melatonin on seasonal cycles in reproduction, prolactin secretion and moulting in rams. J Reprod Fertil 73:241–253
20. **Lincoln GA, Clarke IJ** 1997 Refractoriness to a static melatonin signal develops in the pituitary gland for the control of prolactin secretion in the ram. Biol Reprod 57:460–467
21. **Morgan PJ** 2000 The pars tuberalis: the missing link in the photoperiodic regulation of prolactin secretion. J Neuroendocrinol 12:287–295
22. **Hazlerigg DG, Morgan PJ, Messager S** 2001 Decoding photoperiodic time and melatonin in mammals: what can we learn from the pars tuberalis? J Biol Rhythms 16:326–335
23. **Weaver DR, Provencio I, Carlson LL, Reppert SM** 1991 Melatonin receptors and signal transduction in photorefractory Siberian hamsters (*Phodopus sungorus*). Endocrinology 128:1086–1092
24. **Bockers TM, Bockmann J, Salem A, Niklowitz P, Lerchl A, Huppertz M, Wittkowski W, Kreutz MR** 1997 Initial expression of the common α-chain in hypophyseal pars tuberalis-specific cells in spontaneous recrudescent hamsters. Endocrinology 138:4101–4108
25. **Hazlerigg DG, Andersson H, Johnston JD, Lincoln GA** 2004 Molecular characterization of the long-day response in the Soay sheep, a seasonal mammal. Curr Biol 14:334–339
26. **Dardente H, Menet JS, Poirel VJ, Streicher D, Gauer F, Vivien-Roels B, Klosen P, Pevet P, Masson-Pevet M** 2003 Melatonin induces Cry1 expression in the pars tuberalis of the rat. Brain Res Mol Brain Res 114:101–106
27. **Hazlerigg DG, Gonzalez-Brito A, Lawson W, Hastings MH, Morgan PJ** 1993 Prolonged exposure to melatonin leads to time-dependent sensitization of adenylate cyclase and down-regulates melatonin receptors in pars tuberalis cells from ovine pituitary. Endocrinology 132:285–292
28. **von Gall C, Garabette ML, Kell CA, Frenzel S, Dehghani F, Schumm-Draeger PM, Weaver DR, Korf HW, Hastings MH, Stehle JH** 2002 Rhythmic gene expression in pituitary depends on heterologous sensitization by the neurohormone melatonin. Nat Neurosci 5:234–238
29. **Lincoln G, Messager S, Andersson H, Hazlerigg D** 2002 Temporal expression of seven clock genes in the suprachiasmatic nucleus and the pars tuberalis of the sheep: evidence for an internal coincidence timer. Proc Natl Acad Sci USA 99:13890–13895
30. **Carr AJ, Johnston JD, Semikhodskii AG, Nolan T, Cagampang FR, Stirland JA, Loudon AS** 2003 Photoperiod differentially regulates circadian oscillators in central and peripheral tissues of the Syrian hamster. Curr Biol 13:1543–1548
31. **Reppert SM, Weaver DR** 2002 Coordination of circadian timing in mammals. Nature 418:935–941
32. **Bockmann J, Bockers TM, Vennemann B, Niklowitz P, Muller J, Wittkowski W, Sabel BA, Kreutz MR** 1997 Short-photoperiod down-regulation of thyrotropin-α and -β in hamster pars tuberalis-specific cells. Endocrinology 137:1804–1813
33. **Jabbour HN, Boddy SC, Lincoln GA** 1997 Pattern of expression of VEGF and its receptor flt-1 in the ovine pituitary gland: expression is independent of hypothalamic control. Mol Cell Endocrinol 134:91–100
34. **Barrett P, Ross AW, Balik A, Littlewood PA, Mercer JG, Moar KM, Sallmen T, Kaslin J, Panula P, Schuhler S, Ebling FJ, Ubeaud C, Morgan PJ** 2005 Photoperiodic regulation of histamine H3 receptor and VGF mRNA in the arcuate nucleus of the Siberian hamster. Endocrinology 146:1930–1939
35. **Lincoln GA** 2002 Neuroendocrine regulation of seasonal gonadotrophin and prolactin rhythms: lessons from the Soay ram model. Reproduction Suppl 19:131–147
36. **Preitner N, Damiola F, Lopez-Molina L, Zakany J, Duboule D, Albrecht U, Schibler U** 2002 The orphan nuclear receptor REV-ERBα controls circadian transcription within the positive limb of the mammalian circadian oscillator. Cell 110:251–260
37. **Godine JE, Chin WW, Habener JF** 1982 α Subunit of rat pituitary glycoprotein hormones. J Biol Chem 257:8368–8371
38. **Klosen P, Bienvenu C, Demarteau O, Dardente H, Guerrero H, Pevet P,**

Masson-Pevet M 2002 The mt1 melatonin receptor and ROR β receptor are co-localized in specific TSH-immunoreactive cells in the pars tuberalis of the rat pituitary. J Histochem Cytochem 50:1647–1657
39. **Redmer DA, Dai Y, Li J, Charnock Jones DS, Smith SK, Reynolds LP, Moor RM** 1996 Characterization and expression of vascular endothelial growth factor (VEGF) in the ovine corpus luteum. J Reprod Fertil 108:157–165
40. **Morgan PJ, Webster CA, Mercer JG, Ross AW, Hazlerigg DG, MacLean A, Barrett P** 1996 The ovine pars tuberalis secretes a factor(s) that regulates gene expression in both lactotropic and nonlactotropic pituitary cells. Endocrinology 137:4018–4026
41. **McNeilly AS, Andrews P** 1974 Purification and characterization of caprine prolactin. J Endocrinol 60:359–367
42. **Fraser S, Cowan P, Franclin M, Franey C, Arendt J** 1983 Direct radioimmunoassay for melatonin in plasma. Clin Chem 29:396–397
43. **Bittman EL** 1978 Hamster refractoriness: the role of insensitivity of pineal target tissue. Science 202:648–650
44. **Schwartz WJ, de la Inglasia HO, Zlonanczuk P, Illnerova H** 2001 Encoding Le Quattro Staioni within the mammalian brain: photoperiodic orchestration through the suprachiasmatic nucleus. J Biol Rhythms 16:302–311
45. **Field DM, Maywood ES, O'Brien JA, Weaver DR, Reppert SM, Hastings MH** 2000 Analysis of clock proteins in mouse SCN demonstrates phylogenetic divergence of the circadian clockwork and resetting mechanism. Neuron 25: 437–447
46. **von Gall C, Norton E, Lee C, Weaver DR** 2003 Light does not degrade the constitutively expressed BMAL1 protein in the mouse suprachiasmatic nucleus. Eur J Neurosci 18:125–133
47. **Lincoln GA, Andersson H, Hazlerigg D** 2003 Clock genes and the long-term regulation of prolactin secretion: evidence for a photoperiod/circannual timer in the pars tuberalis. J Neuroendocrinol 15:390–397
48. **Dark J, Pickard GE, Zucker I** 1985 Persistence of circannual rhythms in ground squirrels with lesions of the suprachiasmatic nuclei. Brain Res 332:201–207
49. **Lee TM, Zucker I** 1991 Suprachiasmatic nucleus and the photic entrainment of circannual rhythms in ground squirrels J Biol Rhythms 6:315–330
50. **Scott CJ, Jansen HT, Kao CC, Kuehl DE, Jackson GL** 1995 Disruption of reproductive rhythms and patterns of melatonin and prolactin secretion following bilateral lesions of the suprachiasmatic nucleus in the ewe. J Neuroendocrinol 7:429–443
51. **Lincoln GA, Libre EA, Merriam GR** 1989 Long-term reproductive cycles in rams after pinealectomy and superior cervical ganglionectomy. J Reprod Fertil 85:687–704
52. **Carmichael MS, Zucker I** 1986 Circannual rhythms in ground squirrels: a test of the frequency demultiplication hypothesis. J Biol Rhythms 1:277–284
53. **Gwinner E** 1986 Circannual rhythms: endogenous annual clocks in the organization of seasonal processes. In: Farner DS, ed. Zoophysiology. Berlin: Springer Verlag; 54–178
54. **Loudon ASI, Ihara N, Menaker M** 1998 Effect of a circadian mutation on seasonality in Syrian hamsters (*Mesocricetus autarus*). Proc R Soc Lond 265:517–521

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