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Refractoriness to a Static Melatonin Signal Develops in the Pituitary Gland for the Control of Prolactin Secretion in the Ram

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ABSTRACT

Hypothalamo-pituitary disconnected (HPD) and control Soay rams were treated chronically for 48 wk with s.c., continuous-release implants of melatonin while under long days (16L:8D). The implants produced continuously elevated blood concentrations of melatonin 2–3 times higher than the normal nocturnal maximum. The long-term treatment induced a biphasic effect on prolactin secretion in both the HPD and control rams, with a marked decrease in the blood prolactin concentrations for 10 wk followed by a gradual increase. The introduction of a second melatonin implant after 20 wk failed to affect prolactin secretion. The treatment with melatonin also caused a dynamic effect on FSH secretion, but this occurred in the control rams only. Blood concentrations of FSH in the HPD rams were very low throughout, but there were minor changes in testicular diameter that were correlated with variations in prolactin. Overall, the results support the conclusion that 1) melatonin acts primarily in the pituitary gland to affect prolactin secretion, and partial refractoriness develops at this level for control of prolactin; and 2) melatonin acts most probably in the hypothalamus to affect gonadotropin secretion, and refractoriness develops at the level of the neural tissue regulating GnRH release for control of gonadotropins.

INTRODUCTION

In mammals, the pineal hormone melatonin transduces the effects of photoperiod to coordinate cyclical changes in reproduction, growth, molting, and other characteristics and to synchronize these to the seasons of the year [1–3]. Melatonin is secreted only at night, and the duration of secretion varies with night length providing a durational-variable endocrine signal that is thought to act in the brain and pituitary gland via specific membrane-bound receptors to transduce the complex neuroendocrine effects. The target sites and the mechanisms of action of melatonin, however, remain largely unresolved [4].

In sheep, exposure to a change from long to short days results in a sequence of neuroendocrine responses similar to those observed in autumn under natural conditions. These include an increase in the secretion of LH and FSH, the functional activation of the reproductive axis in both rams and ewes [5–7], and a decrease in the secretion of prolactin and the associated development of the denser winter pelage [8, 9]. These effects occur with a latency of several weeks. In the long term, exposure to short days results in the development of photorefractoriness with termination of the sexually active phase and the reversal of the initial responses. These chronic effects occur with a latency of several months [10–12]. If the exposure to constant short days is very protracted, circannual rhythms in the seasonal parameters may be expressed with a loss of the normal temporal association between the various endocrine rhythms (e.g., gonadotropin and prolactin secretion [13]). Since both the acute and the chronic endocrine effects of short days can be induced in pinealectomized ewes by the appropriate daily infusion of melatonin [14, 15] it is clear that the effects are transduced by the melatonin signal. Furthermore, a similar sequence of responses can be induced in both rams and ewes housed under long days by the administration of a constant-release implant of melatonin that permanently increases the circulating concentrations of melatonin above normal nocturnal values [16, 17]. Thus a continuous melatonin signal can mimic the biological effects of an intermittent, daily, long-duration melatonin signal (short day) under the appropriate conditions.

Recent studies involving the placement of micro-implants of melatonin in different sites in the brain and pituitary gland, in sheep maintained under long days, have provided evidence that melatonin acts locally in the mediobasal hypothalamus (MBH), and/or in the adjacent pars tuberalis (PT) of the pituitary gland, to mediate the multiple effects of photoperiod [18–20]. The ventromedial region of the MBH appears to be an important site for the regulation of GnRH-induced LH secretion with melatonin possibly acting through dopaminergic neurones [21, 22], while the PT, which expresses the highest concentrations of melatonin receptors [23, 24], may mediate the effects of melatonin on prolactin secretion [25, 26]. Definitive evidence that melatonin acts at least in part on the pituitary gland, independently of the hypothalamus, to regulate prolactin secretion, has been provided by studies in hypothalamo-pituitary disconnected (HPD) rams [27, 28].

With evidence for differential regulation of gonadotropin and prolactin secretion by melatonin, the aim of the current study was to investigate whether both systems (brain vs. pituitary gland) show time-dependent changes, equivalent to photoinduction and photorefractoriness, in response to a constant melatonin signal. This was studied in HPD and intact control rams housed under long days and treated with s.c., continuous-release implants of melatonin for 48 wk. The first prediction was that HPD rams would show a long-term cycle of suppression and reactivation of prolactin secretion, with corresponding changes in the pelage, similar to that expressed by control rams. This result would indicate that time-dependent changes occur in the responsiveness to melatonin in the prolactin-regulatory site in the pituitary gland, independent of the hypothalamus. The second prediction was that the HPD rams would show no long-term cycle of activation and inactivation of gonadotropin secretion and testicular activity due to the lack of input from the hypothalamus, but that this cycle would be clearly expressed by control rams. This result would confirm the dependence on hypothalamic GnRH for the synthesis and release of gonadotropins and would indicate that time-de-

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pendent changes may occur in the responsiveness to melatonin in the gonadotropin-regulatory site in the MBH.

MATERIALS AND METHODS

Animals and Routine Measurements

Adult rams of the Soay breed of feral sheep, which show pronounced photoperiodically regulated cycles in testicular activity, coat growth, and other seasonal characteristics, were used in the study [6, 29]. The animals were adult (5–6 yr old at start of experiment) and had a mean body weight of 48.6 kg. They were housed permanently in light-controlled rooms and routinely exposed to alternating 16-wk periods of long days (16L:8D) and short days (8L:16D) to entrain the long-term endocrine cycles. Light intensity was approximately 160 lux at the animals' eye level. The time of lights-on was constant (0800 h), and adjustments in photoperiod were achieved by abruptly changing the time of lights-out by 8 h. The animals received a maintenance standard diet of grass nuts, with hay and water ad libitum. Temperature in the animal rooms was regulated with a variable airflow system and heating to maintain the ambient temperature between 10 and 20°C. The overall mean temperature during the 4 phases of the experiment (see below) were 18.2, 15.7, 11.9, and 11.0°C.

To record the long-term endocrine and morphological changes, blood samples were collected from the jugular vein by venipuncture twice weekly from each ram during the light phase (between 1000 and 1200 h). The samples were heparinized, and the plasma was separated by centrifugation within 30 min and stored at −20°C until the concentrations of FSH, prolactin, and melatonin were measured by RIA. Every 2 wk the diameter of the testes was measured, and the intensity of the sexual skin coloration in the inguinal region was visually scored as an index of androgen secretion [30]. The pattern of wool growth and molting on the scrotum was also recorded to correlate with the changes in prolactin secretion [19].

Experimental Manipulations

Two groups of age-matched, adult Soay rams were maintained in adjacent rooms under the artificial lighting conditions. Group 1 consisted of long-term HPD rams (HPD group, n = 7) in which the HPD operation had been performed by the method of Clarke, Cummins, and de Kretser [27, 31] approximately 3 yr earlier. Group 2 consisted of intact rams (control group, n = 7) that either received the sham-HPD operation at the same time as the surgery in the HPD group (n = 4) or received no surgery (n = 3). All the HPD rams showed the expected clinical signs of permanent hypothalamo-pituitary disconnection including polyuria, gonadal regression, and increasing obesity [27, 32]. No hormonal replacement therapy was given to the HPD rams, and they remained in good health.

Following an identical photoperiodic history, both groups of animals were exposed to a change from short to long days and then maintained on long days throughout the experiment, which lasted 80 wk. After 18 wk, all rams were treated with an s.c. implant of melatonin to maintain the circulating concentrations of melatonin 2–3 times higher than the normal nocturnal concentrations observed in untreated rams [16]. Such treatment had been shown to induce a long-term sequence of testicular changes (activation, regression, and reactivation) similar to that induced by continuous exposure to short days [16]. The melatonin implants consisted of a sealed envelope (surface area 32 cm^2) of 0.127-mm Silastic sheeting (Bibby Sterilin, Aberbargoed, Mid Glamorgan, UK) containing 850 mg melatonin (Genzyme, Haverhill, Suffolk, UK). The implants were incubated overnight in a large volume of 0.9% saline and washed before implantation to prevent an initial high release of melatonin. Each animal received a single implant at the start (experimental [exp.] Week 18), placed beneath the skin over the rib cage using local anesthetic, and a second implant after 20 wk (exp. Week 38) to further increase the circulating concentrations of melatonin. All implants were removed after 48 wk (exp. Week 66), and the observations continued for a further 14 wk while the animals remained under long days (80 wk total; Fig. 1).

On two occasions, once before the implantation of melatonin (exp. Week 12) and once after the introduction of the first melatonin implant (exp. Week 22, Fig. 1) sequential blood samples were collected from all rams every hour for 24 h to monitor the diurnal rhythm in the blood concentrations of melatonin. To facilitate the repeated sampling, a polythene cannula was inserted into the jugular vein on the day before each study, and blood samples were collected via a 3-way tap using heparinized saline to maintain patency of the indwelling cannula. The heparinized blood samples were collected onto ice, and the plasma was separated within 30 min and stored at −20°C until the melatonin concentrations were measured by RIA.

RIAs

The concentrations of FSH and prolactin were measured in weekly blood samples collected from the rams using routine RIAs validated for sheep plasma for FSH [33] and for prolactin [34]. The FSH assay had a lower limit of detection (10% decrease in binding relative to Bo) of 0.2 μg NIDDK-FSH-RP2/L plasma, and intra- and interassay coefficients of variation (CV) of 9.2% and 10.2%, respectively, based on low-, medium-, and high-quality control samples measured in 10 assays. The corresponding values for the prolactin assay were 0.5 μg/L NIH-PRL-S13, 6.5%, and 9.8%, respectively. The concentrations of melatonin in both the hourly and weekly plasma samples were measured using a direct RIA [35] as previously validated for sheep plasma [36]. The assay was performed using the antibody (Stockgrand, Guildford, Surrey, UK) at a working dilution of 1:3000, tritiated melatonin (Amersham, Amersham plc, Buckinghamshire, UK), and melatonin as standard (Genzyme). The sensitivity of the assay was 40–60 pM, and the mean intra- and interassay CVs were 6.8% and 9.2%, respectively. All sequential samples from one animal for either the hourly or weekly series were included in the same assay, and normally samples from HPD and control rams were paired and included in the same assay.

Statistical Analysis

The half-weekly profiles of prolactin and melatonin during the period of treatment with the melatonin implants were examined for significant changes with respect to time (weeks of experiment) by ANOVA with repeated measures. For this the data for the first 40 wk of treatment with melatonin were divided into 4 consecutive 10-wk periods (20 sample points per animal) to assess significance of the time-dependent changes at different phases of the study. The periods were as follows: first 10 wk with one implant—exp. Weeks 19–28; second 10 wk with one implant—exp. Weeks 29–38; first 10 wk with two implants—exp. Weeks

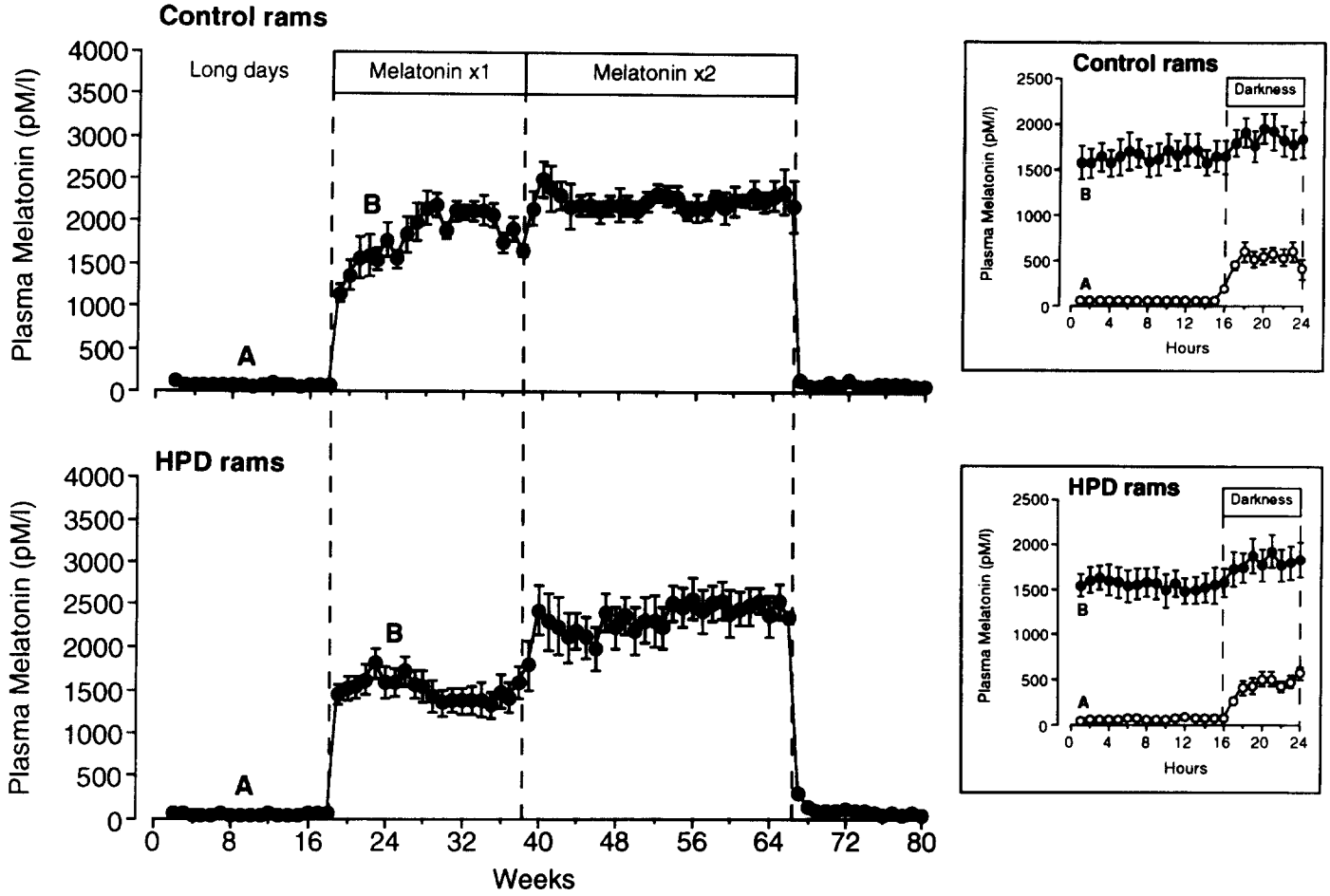

FIG. 1. Weekly changes in the blood plasma concentrations of melatonin in the light phase in control and HPD Soay rams (left upper and lower panel, respectively) treated with one s.c. Silastic implant containing melatonin at Week 18, and a second implant at Week 38, during exposure to long days (16L:8D) for a total of 80 wk; the implants were removed at Week 66. Panels on the right show the hourly changes in blood plasma concentrations of melatonin throughout 24 h for the two groups, before (time A) and after (time B) the onset of the treatment with melatonin. All values are mean ± SEM (n = 7).

39–48; second 10 wk with two implants—exp. Weeks 39–48. These periods were preselected based on the latency of the various phases of the responses to melatonin [16]. The mean hormonal concentrations for all four periods were compared between HPD and control groups by Student's *t*-test. The linear correlations between prolactin concentrations and time for each 10-wk period were calculated using Cricket Graph software (Cricket Graph Inc., Philadelphia, PA). The times of maximum and minimum testis diameter, maximum and minimum plasma FSH concentrations, and minimum plasma prolactin concentrations were assessed for each animal using a 3-point moving average for each parameter, and the values were expressed in weeks relative to the start of the period of implantation with melatonin (exp. Week 18) as a group mean ± SEM. These values were compared between HPD and control groups by Student's *t*-test.

RESULTS

Melatonin Concentrations in Peripheral Blood

The weekly changes in the daytime blood plasma concentrations of melatonin in the control and HPD rams throughout the 80-wk experiment are summarized in Figure 1. Hourly profiles for 24 h before and after the onset of treatment with melatonin are also illustrated. The introduction of the first melatonin implant at Week 18 produced an immediate increase in blood plasma concentrations of melatonin in both control and HPD rams. The mean concentrations were 2- to 3-fold higher than the maximum concentrations observed at night before implantation (see natural 24-h profile due to endogenous melatonin secretion, Fig. 1) and were maintained throughout 24 h. The mean concentrations increased further after 20 wk following the introduction of the second melatonin implant. There were no significant differences in mean concentrations of melatonin between control and HPD rams during the different periods of implantation, except during period 2, when the animals had one melatonin implant (exp. Weeks 29–38, Fig. 1). At this time the circulating concentrations of melatonin were significantly higher in the control compared with the HPD rams (mean ± SEM: 2002.8 ± 54.5 vs. 1402.6 ± 22.2 pM, control vs. HPD rams, respectively; $p < 0.001$).

Prolactin Secretion and the Pelage Cycle

The long-term effects of the experimental manipulations on prolactin secretion and the timing of the pelage molt in the rams are summarized in Figures 2 and 3. In both control and HPD rams, the blood plasma concentrations of prolactin were increased at the start of the experiment due to exposure to long days. Implantation with melatonin was

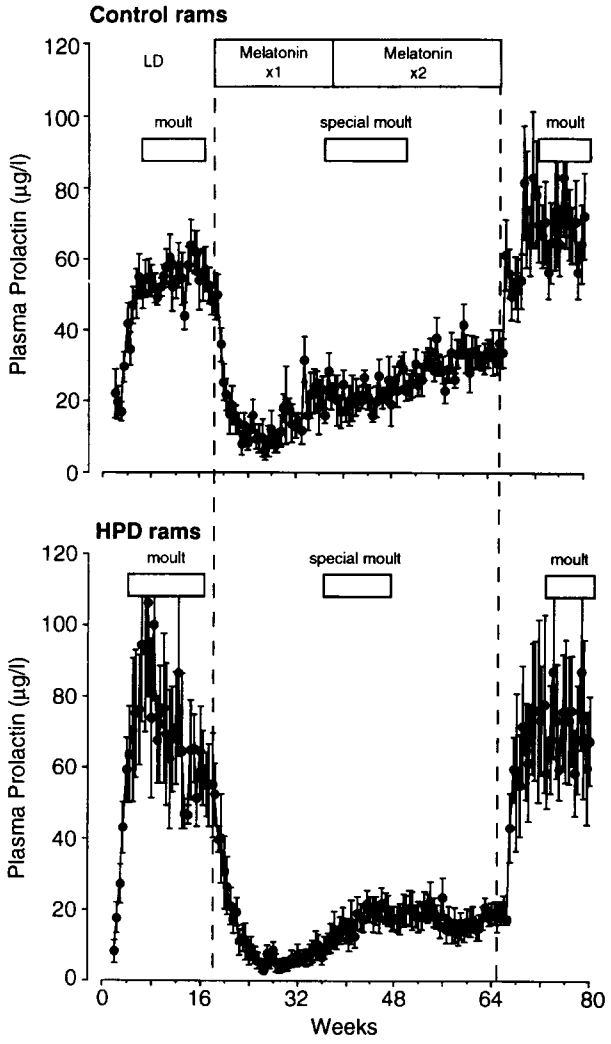

FIG. 2. Weekly changes in the blood plasma concentrations of prolactin in control (upper) and HPD Soay (lower) rams treated with s.c. Silastic implants containing melatonin for 48 wk during exposure to long days (see Fig. 1). The open horizontal bar represents the duration of the pelage molts on the scrotum in each group. The values are mean ± SEM (n = 7).

associated with a marked decrease in the concentrations of prolactin in both groups, beginning within 1 wk and extending throughout the 48 wk of treatment. Maximum suppression, however, occurred at 8–12 wk from the introduction of the first melatonin implant, and there was a partial recovery in the concentrations of prolactin during the remainder of the treatment in both control and HPD rams. The statistical analysis revealed a significant ($p < 0.001$) time-dependent decrease in prolactin concentration during the first 10 wk with one melatonin implant in the control and HPD animals (exp. Weeks 19–28, negative slope), and a significant ($p < 0.001$) time-dependent increase in prolactin concentration during the second 10 wk with one melatonin implant in both groups (exp. Weeks 29–38, positive slope). These changes were characterized by fitting a linear regression to the data for the two periods (Fig. 3). There were also significant ($p < 0.05$) time-dependent increases in prolactin concentrations during the treatment with two melatonin implants (control rams: exp. Weeks 49–58, positive slope; HPD rams: exp. Weeks 39–48, positive slope), although there was considerable variation between animals. The removal of the melatonin implants after 48 wk was followed by a rapid increase in the plasma concentrations of prolactin in both control and HPD rams, and the concentrations returned to the pretreatment values characteristic of long days within 1 wk (Fig. 2).

Comparisons between the plasma concentrations of prolactin in the control and HPD rams revealed that the concentrations were significantly ($p < 0.05$) increased in the HPD rams compared with the controls during the pretreatment period, with much greater variation between animals. During the melatonin treatment the concentrations of prolactin were significantly ($p < 0.05$) lower in the HPD rams compared with the controls (second period with one implant, exp. Weeks 29–38, and second period with two implants, exp. Weeks 49–58). There was no significant difference in the mean concentrations of prolactin between the two groups during the posttreatment period (Fig. 2).

The changes in the growth and molting of the pelage in the control and HPD rams were closely associated with the long-term changes in the patterns of prolactin secretion (Fig. 2). Molting (normal spring molt) and the reinitiation of wool growth occurred following the major increase in

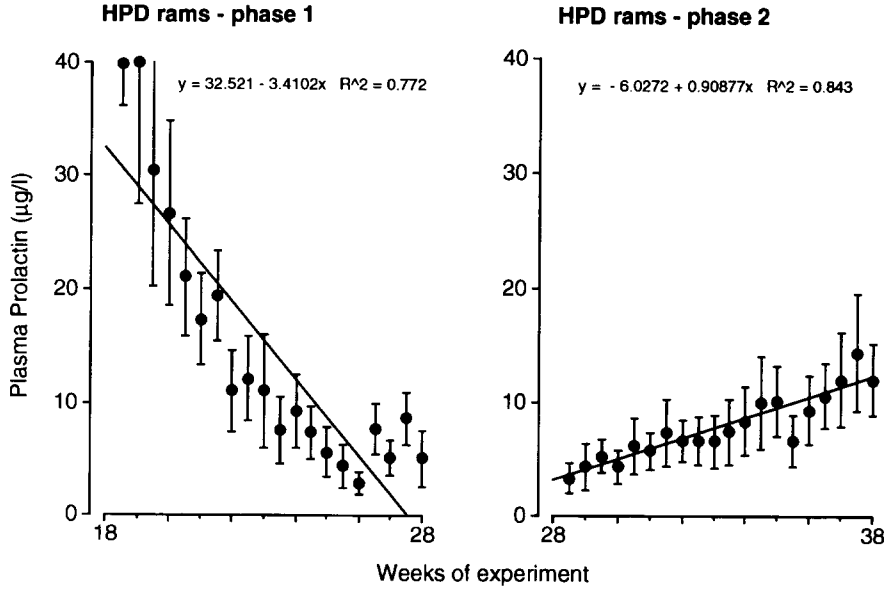

FIG. 3. Linear regression fitted to the mean blood plasma prolactin concentrations in the HPD rams for the first (left) and second (right) 10 wk during which the animals were treated with a single s.c. Silastic implant containing melatonin (see Fig. 1).

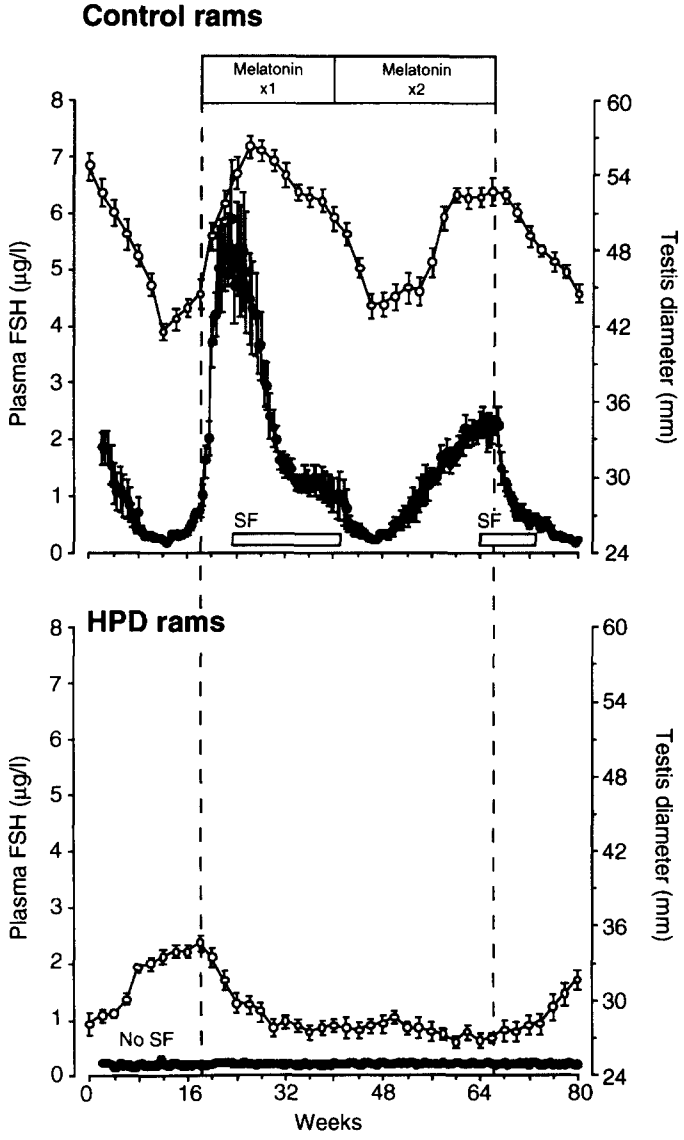

FIG. 4. Weekly changes in the blood plasma concentrations of FSH (solid circles) and diameter of the testes (open circles) in control (upper panel) and HPD Soay rams (lower panel) treated with Silastic implants containing melatonin for 48 wk during exposure to long days (see Fig. 1). The open horizontal bar represents the duration of the period of the sexual skin flush in the inguinal region (SF, androgen response) in each group. The values are mean ± SEM (n = 7).

plasma prolactin concentrations at the start of the experiment (associated with the switch from short to long days), and toward the end of the experiment (associated with the removal of the melatonin implants, Fig. 2), in both groups. During the initial phase of the treatment with melatonin, a long winter pelage developed in both groups (exp. Weeks 22–38). This was gradually molted midway through the period of implantation with melatonin, in association with gradual increase in the plasma concentrations of prolactin (exp. Weeks 36–50). This molt was unlike the normal spring molt in that there was a delay between the casting of the old wool fibers and the regrowth of new fibers, and the new coat included fibers similar to those of the winter pelage (molt from a winter to a winter coat termed "special molt"; Fig. 2).

FSH Secretion and Testicular Activity

The effects of the experimental manipulations on FSH secretion and testicular activity in the rams are illustrated in Figure 4. In the control rams the blood plasma concentrations of FSH decreased at the start of the experiment due to transfer to long days and were beginning to increase again at the time of implantation with melatonin (exp. Week 18, Fig. 4). The melatonin treatment was followed by a rapid increase in concentrations of FSH that reached a maximum after 3–8 wk, followed by a progressive decline. The introduction of the second melatonin implant at 20 wk was associated with a further decrease to a minimum at 30 wk into the treatment (exp. Week 48); the period of low gonadotropin secretion was notably synchronized in all animals. The concentrations of FSH then increased again until the end (exp. Week 48, Fig. 4). The removal of the melatonin implants after 48 wk resulted in a rapid decline in the concentrations of FSH. The statistical analysis of the FSH profile in the control rams revealed significant ($p < 0.001$) time-dependent changes during all 4 phases of treatment with melatonin, with increases during the first 10-wk period with one implant (exp. Weeks 19–28) and during the second 10-wk period with two implants (exp. Weeks 49–58), and decreases during the second 10-wk period with one implant (exp. Weeks 29–38) and during the first 10-wk period with two implants (exp. Weeks 39–48). In the control rams there were changes in the diameter of the testes that were closely associated with the changes in the plasma concentrations of FSH. Testicular growth occurred during the initial increase in FSH concentrations early into the treatment with melatonin. This was followed by full testicular involution and then regrowth related to the decrease and increase in FSH concentrations during the treatment with melatonin. The androgen-dependent sexual skin coloration reappeared biphasically in association with the cycle in testicular activity (Fig. 4).

In the HPD rams the plasma concentrations of FSH were very low throughout the study, and there was no detectable change associated with treatment with melatonin (Fig. 4). The testes in the HPD rams were markedly smaller than in the control rams even during the phase of testicular involution in these animals. In the HPD rams there was an increase in testicular diameter at the start of the experiment and a long-term decrease following implantation with melatonin. These changes were reversed after removal of the melatonin implants (Fig. 4). There was no development of the androgen-dependent sexual skin coloration in the HPD rams at any stage.

DISCUSSION

The first conclusions that can be drawn from this study are that melatonin acts primarily in the pituitary gland to inhibit prolactin secretion and that the pituitary gland becomes partially refractory to this effect with prolonged exposure to a static/continuous melatonin stimulus. This is based on the way the treatment with a continuous-release implant of melatonin caused long-term changes in prolactin secretion that were similar in both the HPD and control rams. These changes are unlikely to be the result of environmental influences, since the animals remained indoors in their home pens and received a standardized diet, the ambient temperature was regulated within the range 10–20°C, and the photoperiod was constant 16L:8D. The HPD operation totally removes any hypothalamic regulation of prolactin secretion, as evidenced by the absence of prolactin responses to stress-provocation tests and the treatment with centrally active compounds including serotonin [27] and the D2 receptor antagonist, sulpiride [28]; thus it is unlikely

that the long-term changes in prolactin secretion are caused by centrally mediated responses. The results provide strong support for the conclusion that melatonin acts directly in the pituitary gland to affect the function of the lactotrophs. The partial recovery in prolactin secretion shown by these animals after 10 wk of treatment with exogenous melatonin indicates a progressive loss of responsiveness to melatonin at the level of the pituitary gland under chronic stimulation. At this time the introduction of a second melatonin implant, which further boosted the circulating concentrations of melatonin, failed to suppress blood prolactin concentrations consistent with a change in tissue responsiveness. Moreover, the timing and magnitude of the long-term prolactin response to melatonin were remarkably similar to those in the control rams, providing evidence that a functional hypothalamus is not required to mediate the principal effects of melatonin on prolactin secretion. The use of continuous-release implants of melatonin is a pharmacological approach to investigate the action of melatonin since the target tissues are exposed to a continuous high-level stimulus. A very similar long-term pattern in the blood prolactin concentrations occurs, however, in rams exposed to prolonged short days in which case the intermittent, long-duration melatonin signal induces the effect [10]. This supports the view that the suppression of prolactin secretion followed by a partial recovery (partial refractoriness) is a normal physiological response to a short-day melatonin signal and that this is due to the action of melatonin in the pituitary gland.

The mechanism by which the melatonin signal affects prolactin secretion within the pituitary is not fully resolved. High-affinity melatonin receptors are expressed in the PT and zona tuberalis, but not by lactotrophs [37]. The most plausible hypothesis, therefore, is that melatonin acts on cells in the PT/zona tuberalis and that these relay the effect to the lactotrophs by way of a paracrine factor. The inhibitory effect of melatonin on forskolin-induced cAMP in PT cells in primary culture has been investigated in detail, and most recently it has been shown that PT cells produce an unidentified peptide or peptides (called tuberalin) that stimulate gene expression in lactotrophs and other pituitary cells and promote prolactin secretion [38, 39]. Accordingly, a long-duration melatonin signal from short days, or a continuous melatonin signal from a melatonin implant, may suppress prolactin secretion by inhibiting the release of tuberalin from the PT. The refractory response could be due to the reduced responsiveness of the melatonin target cells in the PT and to the partial recovery of tuberalin secretion acting to restimulate the lactotrophs. There have been no studies in sheep to investigate the cellular mechanisms associated with refractoriness of the pituitary gland. In the Siberian hamster, the binding of [^{125}I]melatonin in PT cells and the cAMP-inhibitory response to melatonin are not decreased in short-day-refractory animals [40], and a similar lack of relationship between melatonin binding and photorefractoriness has been observed during the annual cycle in the male mink [41].

The second conclusion is that, in contrast to the control of prolactin, melatonin is totally dependent on an intact hypothalamo-pituitary system to affect gonadotropin secretion. This is based on the very clear differences in the pattern of FSH secretion in the HPD and control rams. In the HPD group, the blood concentrations of FSH were very low throughout the study and there were no changes related to the treatment with the constant-release implants of melatonin. In the control group, however, the treatment with melatonin caused very marked changes in the blood concentrations of FSH with activation, involution, and reactivation of the reproductive axis during prolonged treatment. The long-term pattern in testicular activity was very similar to that observed previously in intact Soay rams exposed to a prolonged period of short days [16]. The absence of a gonadotropin response in the HPD rams is consistent with the obligatory requirement for GnRH to stimulate the synthesis and secretion of FSH and LH from the gonadotrophs [31]. Moreover, the marked changes in gonadotropin secretion in the control rams can be assumed to reflect changes in the release of GnRH from the hypothalamus, since these dictate the reproductive responses to photoperiod and melatonin in the intact animal [6, 42]. Experimental studies in which both rams and ewes have been treated chronically with micro-implants of melatonin, placed in different areas of the brain and pituitary gland, have provided evidence that melatonin acts in the MBH and not in the pituitary gland to affect GnRH-induced gonadotropin secretion [18, 19, 26]. The local administration of melatonin in the PT affects prolactin but not gonadotropin secretion [26]; thus it is unlikely that the PT is involved in mediating effects on the gonadotropin/gonadal axis. This is consistent with the current results, since the PT remains in association with the rest of the pituitary gland in the HPD ram, and these animals show prolactin but not gonadotropin responses to melatonin. The mechanism by which a long-duration or a continuous melatonin signal acts in the MBH to activate gonadotropin secretion, and later to inactivate the system (refractory response), is still largely unresolved. One notable observation was that the introduction of the second melatonin implant at 20 wk, when gonadotropin secretion was in decline, was associated with a further decrease in the blood concentrations of FSH, with hormonal profiles of all animals remarkably synchronized. This is consistent with the concept that the refractory state is positively induced, perhaps by the activation of inhibitory pathways, rather than being a passive response to long-term exposure to a constant melatonin signal.

The final conclusion that arises from these results is that melatonin acts through both prolactin and gonadotropin secretion to influence the testicular and pelage cycle in the ram. The potential effect of prolactin in the testis in the absence of gonadotropins is indicated by the changes in testicular size in the HPD rams. In these animals the testes were permanently more regressed than in the control animals as expected due to the very low circulating concentrations of FSH, but the testes showed a low-amplitude cycle that was correlated with the changes in prolactin secretion (increased under long days and decreased during the treatment with the melatonin implants, a pattern opposite to that seen in the controls). This association has been described previously in HPD rams [32] and is thought to result from the action of prolactin on Leydig cells promoting steroidogenesis, and/or from the direct action of prolactin on germ cells to increase the efficiency of spermatogenesis. The sexual skin coloration was permanently absent in the HPD rams; thus there was no evidence of peripheral androgen secretion in response to prolactin in these animals. In the control rams there was a close correlation between the changes in testicular size and the blood concentrations of FSH, which is consistent with the predominant role of gonadotropins in stimulating both steroidogenesis and spermatogenesis in the ram [43]. Since prolactin secretion is increased in the sexually inactive phase in intact rams, the presumption is that prolactin normally acts to maintain the

functional integrity of the testes when gonadotropin secretion is reduced in the sexually inactive phase (priming role) and this permits the rapid reactivation in response to gonadotropins once stimulated by short days (or treatment with a melatonin implant as in the current study). Prolactin is also known to act on the skin to induce the reactivation of the hair follicles and molting of the old pelage [44, 45], and this effect was evident in both the HPD and control rams. A normal molt occurred following the major increase in blood prolactin concentrations induced by long days (pretreatment and posttreatment), while an abnormal molt occurred after the minor increase in prolactin during the treatment with melatonin. At this time a winter-type rather than a summer-type pelage developed after molting, which indicates that the pelage type is dictated by the endocrine status at the time of hair-follicle reactivation [45–47]. In addition, the pelage fibers in the HPD rams resembled those of a castrated ram and were notably finer than those in the control rams, presumably due to the absence of androgens, which act in the hair follicle in conjunction with prolactin to affect the characteristics of the hair fiber [48, 49].

In summary, this study provides firm support for the "dual site hypothesis" [50]. This proposes that melatonin acts primarily in the pituitary gland to regulate prolactin secretion and in the hypothalamus to regulate gonadotropin secretion. This is the first evidence that prolonged exposure to melatonin causes partial refractoriness at the level of the pituitary gland, and it is probable that the mechanisms involved in the refractory responses are different for the regulation of prolactin and gonadotropins. The differential regulation of these two systems provides an explanation of how the prolactin-pelage and gonadotropin-gonadal axis of an animal can be dissociated by experimental manipulations [51–55], how the gonadal and pelage axes can have a different critical day length [56], and how species or breeds can differ in timing to the gonadal cycles (long-vs. short-day breeders) but have similar summer/winter pelage cycles [57, 58].

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