Failure of melatonin to influence endogenous opioid effects on LH secretion in the anoestrous ewe*

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Summary. In May mature seasonally anoestrous ewes were implanted with melatonin which advanced the onset of cycles by about 1 month. The LH response to an opioid antagonist, WIN-3, was determined 5, 15, 25 and 60 days after melatonin implantation, by intravenous administration of WIN-3 (12.5 mg/dose) 4 times at 15-min intervals during both the 1st and the 5th hour of an 8-h treatment period. There was no effect of WIN-3 at 5, 15 and 25 days after melatonin implantation. At 60 days LH concentration and pulse frequency were significantly increased ($P < 0.05$ and < 0.01 respectively) in response to WIN-3 treatment, but only in those animals which had begun reproductive cycles, an effect known to be mediated by the presence of progesterone. We were therefore unable to find evidence to support the hypothesis that the influence of melatonin in advancing the breeding season may be via an opioidergic pathway.

Keywords: opioids; LH; melatonin; ewes

Introduction

The major environmental factor controlling the onset and duration of seasonal breeding in ewes is photoperiod. Short days stimulate, and long days inhibit breeding activity (Yeates, 1947; Hafez, 1952), and the pineal hormone melatonin drives the reproductive response to daylength in this species (Bittman *et al.*, 1983). The duration of the nocturnal rise in plasma melatonin concentrations is dependent on the length of the dark period and its longer duration in short days induces normal reproductive function (Kennaway *et al.*, 1977; Rollag *et al.*, 1978). Exogenous melatonin has been applied to anoestrous ewes such that the resultant pattern of circulating melatonin mimics the endogenous pattern of secretion under short days and advances the onset of ovarian activity in these animals (Kennaway *et al.*, 1982a; Nett & Niswender, 1982; Arendt *et al.*, 1983). Nevertheless, the precise neuroendocrine mechanism by which melatonin functions is not known. Evidence accumulated over the past few years has implicated an inhibitory role for endogenous opioids in the control of LH secretion in sheep (Trout & Malven, 1984; Ebling & Lincoln, 1985; Schanbacher, 1985; Brooks *et al.*, 1986). Furthermore, the efficacy of opioid inhibition appears to fluctuate with changes in the photoperiod-induced breeding activity in this species. For instance, our previous studies showed that the opioid antagonist naloxone elevated LH concentrations during the breeding season (short days), but not during long day-induced seasonal anoestrus (Brooks *et al.*, 1986). Also, Ebling & Lincoln (1985) reported that a seasonal change in the endogenous opioid control of LH secretion occurs in Soay rams in parallel to the gonadal cycle, the LH response to naloxone and morphine being minimal in the non-breeding season at the time of testicular regression.

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Studies in long-day breeding hamsters have provided evidence to suggest that photoperiod can modulate hypothalamic opioid activity; short photoperiod greatly reduces the LH response to naloxone (Swann & Turek, 1983; Eskes *et al.*, 1984) and significantly increases hypothalamic β -endorphin content (Kumar *et al.*, 1984). One way by which photoperiod could influence LH responses to opioids in seasonally breeding mammals is via melatonin which could establish an opioid-mediated LH control mechanism. The present study was, therefore, carried out to test this hypothesis by administering an opioid antagonist and measuring the LH response at intervals after implanting ewes with melatonin during seasonal anoestrus. The opioid antagonist WIN 44441-3 (WIN-3, Sterling Winthrop; see Ward *et al.*, 1983) was used. Extensive studies involving this compound in sheep have previously been described (Yang *et al.*, 1988).

Materials and Methods

Animals. Sixteen seasonally anoestrous Suffolk-cross mature ewes (mean body weight $\pm$ s.e.m., 70.3 ± 1.5 kg), all of which had given birth to lambs in the spring, were used. They were kept outside on a University farm pasture except for intensive blood sampling periods, during which they were brought indoors and housed under conditions of natural daylight and temperature and fed a diet of ewe concentrates and hay. Water was available at all times.

Experimental design. After confirmation of anoestrous status by laparoscopy, animals were randomly allocated to 4 equal groups: Group 1, ethanol-treated controls (ethanol was used to dissolve WIN-3); Group 2, treated with WIN-3; Group 3, fitted with low-release melatonin implants, and treated with WIN-3; Group 4, fitted with high-release melatonin implants and treated with WIN-3. A single Silastic melatonin implant was inserted subcutaneously in the axilla region of each animal on 25 May. After 24 days, the implants in Group-3 sheep were replaced, and all implants then remained *in situ* until the end of the experiment. Implants, diameters 0.29 and 0.4 cm, length 3 and 4 cm for low- and high-release respectively and containing 70 mg melatonin in a silicone base, were provided by Dr J. C. Bouffault and Synkron Co., Virginia, USA. They were designed to maintain plasma melatonin concentrations around 100–200 pg/ml for at least 6 weeks (J. C. Bouffault, personal communication).

To evaluate the degree of opioid involvement in LH suppression after short- and long-term exposure to exogenous melatonin, WIN-3 was administered to ewes in Groups 2, 3 and 4 at 5, 15, 25 and 60 days after insertion of the implants.

Treatment and collection of blood samples. A jugular vein cannula was inserted into each animal on the day before the start of each WIN-3 treatment and accompanying intensive blood sampling. Eight injections of ethanol (0.75 ml/injection) were given at 15-min intervals to all ewes during the 1st and 5th hour of an 8-h control period starting at 08:00 h. This was followed by 8 injections of WIN-3 (Groups 2, 3 and 4; 12.5 mg in 0.75 ml ethanol/injection) or ethanol alone (Group 1; 0.75 ml/injection), starting at the same time on the following day. Blood samples (3 ml) for LH determinations were collected from all the animals at 15-min intervals throughout the injection period and for a further 8 h starting at 08:00 h on the day after. Each injection was given immediately after the withdrawal of a blood sample. The WIN-3 injection regimen was designed to minimize any behavioural responses which could derive from continuous long-term exposure to the drug.

Blood samples (5 ml) for progesterone measurements were taken twice weekly by jugular venepuncture from the time of melatonin implantation to determine the time of onset of ovarian cyclicity. In addition, hourly blood samples (10 ml) were collected during the 2nd and 3rd challenge with WIN-3 (i.e. at 25 days and 60 days after the implantation) and those taken at 17:00 h subsequently used for the determination of plasma melatonin concentrations. All blood samples were centrifuged immediately and the plasma was collected and stored at -20°C until assayed.

Hormone assays. Plasma LH concentrations were determined by the double-antibody radioimmunoassay method of McLeod *et al.* (1982). NIH-LH-S24 was used as standard and all results were expressed in terms of this preparation. The sensitivity of the assays averaged 0.13 ng/ml. Inter- and intra-assay coefficients of variation (CV) were 11.2% and 13.3%, respectively. Plasma progesterone was measured by the radioimmunoassay method of Haresign *et al.* (1975). The sensitivity of the assay was 0.1 ng/ml and the inter- and intra-assay CVs were <10%. Melatonin assay was carried out at the University of Surrey as described by English *et al.* (1986). Assay sensitivity was 5 pg/ml and the intra-assay CV was <10%.

Statistical analysis. LH pulses were identified by the three criteria described by Goodman & Karsch (1980). A pulse was identified if: (1) the peak associated with the pulse occurred within two samples of the previous nadir; (2) the amplitude of the pulse exceeded the sensitivity of the assay; (3) the LH level at the peak exceeded the 95% confidence limits of the LH concentration at both the preceding and subsequent nadirs. The amplitude of each pulse was defined as the LH concentration at the peak minus that at the preceding nadir. Differences in plasma mean LH concentration and pulse amplitude were evaluated by a split-plot analysis of variance and frequencies of LH pulses by analysis of deviance (Nelder & Wedderburn, 1972). Analysis of deviance was also applied to determine whether the time of onset of ovarian cyclicity was different between melatonin-implanted and control ewes.

Results

Effects of melatonin implantation on plasma concentrations of melatonin and LH and the onset of oestrous cyclicity

As shown in Fig. 1, melatonin implants resulted in a rise in circulating melatonin concentrations, being 198 ± 33 and 91 ± 13 pg/ml for ewes implanted with high- and low-release implants compared to 10 ± 0.5 pg/ml for non-implanted controls at 25 days after implantation and 65 ± 7.0 , 55 ± 14 and 12 ± 1.3 pg/ml at 60 days after implantation respectively.

Fig. 1. Plasma melatonin concentrations (at Day 25 after melatonin implantation) and the time of onset of cyclic activity in each ewe.

Plasma LH concentrations during the control periods were not different in melatonin-implanted and non-implanted ewes at any of the times studied (see Table 1).

The time of the onset of ovarian cyclicity was assessed from progesterone data (values > 1 ng/ml for at least 7 days followed by a fall and a 2nd rise a few days later were considered to reflect normal ovarian activity). Although there was overlap, ovarian cyclicity occurred significantly earlier ($P < 0.05$) in melatonin-treated sheep, being 46, 60, 70 and 88 days in Group 4 and 55, 66 and 74 days in Group 3 compared with 70, 88, 88 and 116 days in Group 1 and 56, 81, 95 and 118 days in Group 2 (mean $\pm$ s.e.m. 65.6 ± 5.2 and 89.0 ± 7.5 days after implantation; Fig. 1). There was no correlation between the date of onset of ovarian cyclicity and plasma melatonin concentrations (see Fig. 1).

LH responses to WIN-3

Plasma LH concentrations, pulse amplitude and frequency for all treatment groups on all treatment occasions are summarized in Table 1. At 5, 15 and 25 days, there were no significant effects of WIN-3 upon any of the LH parameters studied in sheep in Groups 3 and 4 and those in Groups 1

Table 1. Mean LH concentration, pulse amplitude and pulse frequency for ewes in all groups during control (C), treatment with ethanol or WIN-3 (T) and post-treatment (P) periods

Group	Time (days)*	No.	Mean LH (ng/ml)			Pulse amplitude (ng/ml)			Pulse frequency (no./8 h)		
			C	T	P	C	T	P	C	T	P
1 (ethanol)	15	4	0.44	0.24	0.25	1.28	0.67	0.49	2.0	1.0	0.8
	25	4	0.50	0.63	0.60	1.44	2.99	1.61	1.5	1.8	1.8
	60	4	0.58	0.64	0.54	2.07	3.08	1.85	1.8	1.5	1.5
2 (Win-3)	5	4	0.56	0.32	0.44	3.46	0.48	2.15	1.5	1.5	0.8
	15	4	0.58	0.26	0.55	2.17	0.63	1.45	1.5	0.8	1.8
	25	4	0.77	0.73	0.82	1.63	0.96	1.87	2.5	1.8	2.5
3 (low-release melatonin + Win-3)	60	3†	0.45	0.41	0.46	0.67	0.25	0.35	1.7	1.7	2.0
	5	4	0.51	0.42	0.58	1.85	0.89	1.96	1.5	1.5	2.0
	15	4	0.42	0.28	0.36	0.94	0.30	1.48	1.0	0.8	1.3
4 (high-release melatonin + Win-3)	25	4	0.70	0.46	0.51	1.79	0.58	1.55	1.3	1.8	1.5
	5	4	0.40	0.29	0.58	1.21	0.28	2.48	1.5	1.3	2.0
	15	4	0.55	0.57	0.51	1.99	0.81	2.06	2.0	1.8	1.8
3 + 4 (melatonin + Win-3, cyclic)	25	4	0.56	0.44	0.45	1.29	0.78	1.03	2.3	1.8	1.8
	60	3§	0.53 ^a	0.91 ^b	0.60 ^a	0.55	0.78	0.38	2.3 ^a	5.3 ^c	2.3 ^a
3 + 4 (melatonin + Win-3, not cyclic)	60	4	0.42	0.51	0.59	0.48	0.49	0.85	1.5	2.5	3.3

*Days after implantation of the melatonin treated groups.
†Standard error of the difference between means incorporating all three periods (C, T and P).
‡One sheep had a preovulatory-type LH surge during sampling period P and the results are not included in the statistical analysis. Mean LH concentrations for this sheep for periods C, T and P were 0.33, 0.29 and 11.2 ng/ml respectively. There was no apparent effect, therefore, of WIN-3 treatment.
§One sheep died 35 days after implantation with melatonin.
a vs b, $P < 0.05$; a vs c, $P < 0.01$.

and 2. At 60 days, WIN-3 treatment induced a significant ($P < 0.05$) elevation in mean LH concentrations from 0.53 to 0.91 ng/ml as a result of an enhancement ($P < 0.05$) in the pulse frequency, only in 3 melatonin implanted animals (2 in Group 4 and 1 in Group 3) which had begun oestrous cyclicity. In contrast, WIN-3 did not affect LH secretion in the other melatonin-implanted animals or in the non-implanted controls (see Table 1).

Discussion

Subcutaneous implantation of melatonin into seasonally anoestrous ewes resulted in an elevation of day-time circulating melatonin to values similar to those observed in non-implanted animals during the night (Kennaway *et al.*, 1982b). The resultant increased melatonin concentration may therefore be responsible for the earlier onset of oestrous cyclicity seen in the melatonin-implanted animals. This finding is consistent with previous studies in which either melatonin implantation (English *et al.*, 1986) or melatonin feeding (Kennaway *et al.*, 1982a; Arendt *et al.*, 1983; Stellflug *et al.*, 1988) were demonstrated to advance the onset of breeding activity by periods of from 2 to 10 weeks. The lack of apparent correlation between circulating melatonin concentrations and the time of onset of oestrous cyclicity in the present study (see Fig. 1) accords with the view that, above a certain threshold the duration rather than the concentration of melatonin secretion is critical to its effects on the seasonal cycle (Bittman & Karsch, 1984). The present demonstration that implantation of melatonin into ewes did not affect their tonic LH secretion is in line with previous findings that pinealectomy did not alter circulating LH concentrations in the ewe (Roche *et al.*, 1970). The fact that WIN-3 failed to elevate LH concentration in melatonin-implanted or non-implanted control sheep at 5, 15 and 25 days after the melatonin implantation, and that, at 60 days, WIN-3 significantly enhanced LH secretion only in 3 melatonin-implanted ewes which had exhibited ovarian cyclicity indicates that melatonin *per se*, at least at a level which was sufficient to advance the breeding season, is unlikely to induce reproductive cyclicity by initially switching LH control to an opioid-mediated system. Since the earlier onset of the oestrous cycle seen in melatonin-treated animals is presumably the consequence of melatonin administration and the plasma melatonin concentrations in implanted animals were equivalent to those occurring naturally during a short photoperiod, the possibility that the induced melatonin concentrations may have been insufficient to trigger an endocrine response seems remote. It is recognized that the design of the experiment, in which cyclicity was determined by plasma progesterone measurement in retrospect, precluded a detailed examination of WIN-3 challenge immediately before the onset of ovarian cyclicity. However, the one sheep which came into this category showed no response to WIN-3 as close as 24 h before an ovulatory surge of LH (see footnote, Table 1).

It has been shown that pinealectomy disrupts a diurnal [met]enkephalin rhythm in the rat diencephalon (Kumar *et al.*, 1982). Pinealectomy in mice has also been demonstrated to abolish the day-night rhythm of morphine analgesia (Lakin *et al.*, 1981). In addition, short photoperiod causes an elevation in hypothalamic [met]enkephalin content and serum β -endorphin-like immunoreactivity levels in the hamster while pinealectomy abolishes this elevation (Kumar *et al.*, 1984). All these data support the idea that short-photoperiod-induced pineal activity may activate the hypothalamic opioid system in other species. Also, several groups of investigators have demonstrated that short-day exposure eliminates the LH response to naloxone in hamsters (Swann & Turek, 1983; Eskes *et al.*, 1984; Roberts *et al.*, 1985a, b). In an attempt to determine whether the changes in sensitivity to naloxone were determined directly by changes in photoperiod or were a consequence of reduced concentrations of gonadal steroids, Roberts *et al.* (1985a) found that the chronic maintenance of high serum testosterone levels failed to restore the LH response to naloxone in castrated male hamsters exposed to an inhibitory photoperiod. In contrast, pinealectomy maintained the LH response to naloxone in intact animals subjected to short days. Therefore, Roberts *et al.* (1985a) concluded that photoperiod-induced changes in the ability of naloxone to

elicit an LH release are mediated via the pineal gland but are independent of changes in circulating steroid concentrations. Implants of melatonin in rams maintained under long days induced testicular reactivation and during the early stages there were increased LH responses to naloxone treatment (Lincoln, 1988).

The observation reported here that melatonin does not modulate the opioid/LH axis in ewes is therefore at variance with findings in other species and there appears also to be a difference between male and female sheep.

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