Active immunization against melatonin in Ile-de-France ewes and photoperiodic control of prolactin secretion and ovulatory activity

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An experiment was conducted to determine whether active immunization against melatonin could modify the perception of abrupt photoperiodic changes in ewes. Two groups each containing six intact Ile-de-France ewes were submitted to alternate periods of short days for 2.5 months and long days for 2.5 days for about 70 weeks. Three series of active immunizations against a melatonin conjugate were carried out during the first of the three long-day periods. Control ewes were actively immunized at the same time against human serum albumin. Blood samples were taken once a week throughout the experiment to measure antibody titre and affinity, and prolactin and progesterone concentrations. Sera of all treated ewes demonstrated higher antibody titres than those of control ewes. Antisera were highly specific, as evidenced by the absence of displacement of iodinated melatonin in the presence of ten melatonin metabolites. Significant effects of photoperiod and of the interaction between treatment and photoperiod on prolactin concentration were detected. Prolactin concentrations in plasma of the control ewes were high during long days and low during short days. However, in the treated ewes, with the exception of the first period of long days, prolactin concentrations were not influenced by photoperiodic changes. Ovulatory activity of control ewes, as demonstrated by progesterone measurements, was stimulated by short days and inhibited by long days. In contrast, ovulatory activity of treated ewes, after a response identical to that of control ewes after the first photoperiodic shift from long to short days, showed a complete desynchronization of ovulatory activity relative to photoperiodic changes. Immunization against melatonin can therefore modify the influence of artificial photoperiodic changes on prolactin secretion and ovulatory activity in ewes.

Introduction

Photoperiod is the environmental cue used by temperate breeds of sheep to regulate their reproductive seasonality (Ortavant *et al.*, 1985). Pinealectomy abolishes the reproductive responses of ewes to photoperiodic changes (Bittman *et al.*, 1983a). Melatonin, an indoleamine, synthesized and secreted by the pineal gland into the general circulation, mediates the photoperiodic drive of reproductive activity (Karsch *et al.*, 1984). In pinealectomized ewes, long periods of infusion of melatonin (16 h day^{-1}) mimic short daylength and stimulate reproductive activity; in contrast, short periods of infusion (8 h day^{-1}) mimic long daylength and inhibit reproductive activity (Bittman *et al.*, 1983b, 1985). Discrete photoperiodic signals, provided by melatonin infusions to pinealectomized ewes, can synchronize the circannual rhythm of LH neuroendocrine

activity (Woodfill *et al.*, 1991). Consequently, if adequate replacement therapies, using melatonin alone in pinealectomized ewes, can completely restore the effects of photoperiodic changes observed in intact ewes, it is possible that under normal conditions, melatonin is the major hormone responsible for the reproductive response of ewes to changes in daylength.

However, the few experiments involving the immunization of sheep against melatonin under natural conditions gave conflicting results in comparison with pinealectomized, melatonin-treated animals. Under such conditions of immunization, ewes (Arendt *et al.*, 1981) and rams (Lincoln and Almeida, 1981) did not show clear-cut changes in their reproductive seasonality.

It is, therefore, of interest to determine whether, in intact ewes, immunoneutralization of melatonin modifies the prolactin response to artificial rather than natural daylength changes, and the ovulatory response to artificial changes in photoperiod. The present experiment was therefore conducted to test

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Fig. 1. Experimental design. Ile-de-France ewes were submitted to alternate periods of 2.5 months of short days (SD, hatched areas) and 2.5 months of long days (LD, white areas), and received three series of immunizations (Imm 1, 2 and 3), each one consisting of four intradermal injections (ID) followed by one intravenous injection (IV). Characterization of antibody was carried out in blood samples obtained twice a month and 5–7 days after IV (**).

the working hypothesis that active immunization against melatonin modifies the effects of abrupt photoperiodic changes on prolactin secretion and ovulatory activity.

A preliminary report of the work was presented at the Sixth Colloquium of the European Pineal Society in Copenhagen in July 1993.

Materials and Methods

Two groups, each containing six intact adult Ile-de-France ewes (*Ovis aries* L.), were submitted to alternating periods of 2.5 months of short days (8 h light:16 h dark) and long days (16 h light:8 h dark). Photoperiodic treatment began on 23 January 1991 and ended on 30 June 1992 (Fig. 1). The two groups of ewes were separated and maintained in two rooms in a light-proof building where ambient temperature changes were identical from one room to another and followed an attenuated seasonal trend linked to climatological conditions. Daylength was regulated by electronic timers that operated fluorescent bulbs providing approximately 300 lux at the level of the animals' eyes. Ewes were fed daily with hay, straw and corn. They had free access to water and mineral licks. All manipulations of ewes were performed in accordance with UK legal requirements.

Three series of active immunizations against a melatonin conjugate or against human serum albumin were carried out at about the times of transition from short days to long days. Melatonin (Aldrich Chimie, Ilkirch) was coupled to HSA (Aldrich Chimie) by glutaraldehyde (Aldrich Chimie) as described by Tillet *et al.* (1986). Each series of immunizations comprised four intradermal injections of the immunogen at weekly intervals, followed by one i.v. injection of the immunogen a month later. The first intradermal injection was given with complete Freund's adjuvant and the second and third injection with incomplete Freund's adjuvant. The i.v.

injection was given without adjuvant. This procedure, which uses a repetition of the primary immunization instead of a single booster immunization, has already been used in sheep with satisfactory results (Tillet *et al.*, 1986). Blood samples for characterization of antibody were collected 5–7 days after the i.v. injection. Antisera were characterized by radioimmunoassay using ^{125}I -labelled melatonin (specific activity $2 \times 10^{-3} \text{ Ci mmol}^{-1}$; Amersham, Paris). Antibody titre was defined as the antisera dilution that binds 50% of the tracer (in all assays 100 μl samples were incubated with 15 300 d.p.m. ^{125}I -labelled melatonin).

Specificity of antisera was characterized by using the displacement of ^{125}I -labelled melatonin in the presence of ten melatonin metabolites, tested in the sera of two representative ewes taken after the third immunization period.

Blood samples were taken once a week throughout the experiment, and always at the same hour relative to lights on. They were obtained by venepuncture of the jugular vein; plasma and serum were separated and stored at -20°C until assayed. Samples taken twice a month were used to measure antibody titre in the serum; samples taken once a week were used for assays of prolactin and progesterone concentrations in the plasma.

Prolactin was assayed in duplicate 10–50 μl aliquots of plasma using the radioimmunoassay method of Kann (1971). Sensitivity was 8 ng ml^{-1} of plasma of NIDDK-o-prolactin-19 for 50 μl . Intra-assay coefficient of variation for two plasma pools averaged 11%. All samples were measured in the same assay.

Progesterone was assayed in duplicate by the method described by Saumande *et al.* (1985), slightly modified as follows: immunoprecipitation was accelerated by adding polyethylene glycol and the second antibody at the same time. All tubes were measured in the same assay. Sensitivity was 0.1 ng ml^{-1} and the intra-assay coefficients of variation for 1.8 ng ml^{-1} and 5.6 ng ml^{-1} plasma were 4.1% and 14.7%,

Fig. 2. Melatonin binding (percentage binding of ^{125}I -labelled melatonin at a dilution 1/10 000) determined in the serum of blood samples taken twice a month from six Ile-de-France ewes that had received three series of immunizations (Imm 1, 2 and 3) against a melatonin conjugate coupled to human serum albumin.

respectively. A plasma progesterone concentration higher than 1 ng ml^{-1} in the blood sample taken once a week was considered to indicate the presence of an active corpus luteum and was used to estimate the date of ovulation, which was arbitrarily defined as 3 days before the first increase in plasma progesterone concentration above 1 ng ml^{-1} (Thimonier, 1978).

Statistical analysis

Statistical analysis was performed by repeated measures analysis of variance, after a logarithmic transformation of prolactin data and testing the effects of immunization treatment, photoperiod and time. In such a model, significant interaction between treatment and photoperiod indicated that prolactin concentrations varied with photoperiod in a different manner between groups. It was followed by Duncan's new multiple range test for differences between treatment groups. All statistical analyses were carried out by means of SuperANOVA program (Abacus Concepts, Berkeley, CA).

Results

Titres and specificity of the antisera

Antibody titres of sera of all ewes immunized against melatonin (MI) were higher than 1/5000 at the third immunization, in contrast to ewes immunized against human serum albumin (HSA). Four MI ewes had a titre between 1/16 000 and 1/32 000 and one ewe a titre higher than 1/32 000 at the time of the second immunization.

Variations in the titres, which were monitored throughout the experiment from the samples taken twice a month, at a dilution of 1/10 000 (Fig. 2), confirmed the preceding results and showed that in two ewes (Nos 9 and 11) melatonin

binding decreased almost to zero between the immunization periods; in ewes 7, 8 and 12 melatonin binding was intermediate between the immunization periods, and ewe 10 had an antisera titre which remained high between the immunization periods.

Antisera were highly specific, as evidenced by the absence of displacement of ^{125}I -labelled melatonin in the presence of ten melatonin-related compounds (serotonin, *N*-acetylserotonin, tryptamine, 5-methoxytryptamine, 6-hydroxymelatonin, tryptophol, 5-methoxytryptophol, tryptophan, 5-hydroxytryptophan and 5-hydroxyindol acetic acid) tested in the sera of two representative ewes (Nos 10 and 12) taken after the third immunization period.

Prolactin

Significant effects of photoperiod and of the interaction between treatment and photoperiod on prolactin concentration were detected ($P = 0.0016$ and $P = 0.0362$, respectively). The effect of treatment was not significant.

Prolactin concentrations in the plasma of the HSA ewes were high during long days and low during short days (Figs 3 and 4). In contrast, in the MI ewes, with the exception of the first period of long days (when antibody titres were not yet high), prolactin concentrations were not influenced by photoperiodic changes (HSA ewes: short days: 35, 59 and 49 versus long days: 158, 143 and 103 ng ml^{-1} , compared with MI ewes: short days: 62, 67 and 66 versus long days: 125, 85 and 66 ng ml^{-1}). Prolactin concentrations were significantly modified in MI ewes during the first period of long days at the end of which time prolactin concentrations fell markedly (the interaction between time and treatment and the difference with the HSA ewes were significant). This indicates that the prolactin response of the MI ewes to photoperiod was modified as early as the first immunization, at a time when the antisera titre are still relatively low (Figs 3 and 4).

Fig. 3. Weekly plasma prolactin concentrations (mean $\pm$ SEM) from two groups of six intact Ile-de-France ewes each of which had received three series of immunizations (Imm 1, 2 and 3) against (a) human serum albumin or (b) melatonin. (▨) Short days; (□) long days.

Progesterone concentration in relation to ovulatory activity

Progesterone measurements, which allow the estimation of dates of ovulations, demonstrated that the ovulatory activity of HSA ewes was stimulated by short days (interval between the change from long days to short days and first ovulation, mean $\pm$ SEM, 49.0 ± 3.1 , 59.8 ± 6.3 and 52.5 ± 1.6 days, for the three photoperiodic cycles) and inhibited by long days, leading to a consistent duration of ovulatory activity (53.5 ± 7.1 and 41.5 ± 10.8 days), and high synchrony between ewes (Figs 5 and 6).

In contrast, ovulatory activity of MI ewes, after a response close to that of HSA ewes after the first change from long days to short days (interval to first ovulation 40.8 ± 8.6 days), showed a complete desynchronization of ovulatory activity relative to changes in photoperiod. One ewe was always cyclic, while others showed irregular alternations between activity and rest independent of the changes in the photoperiodic signal, making it difficult to distinguish periods

of ovulatory cyclicity from periods of anovulation clearly (Figs 5 and 6).

The frequency of ovulatory cycles per week and per group (Fig. 5) showed that the ovulatory activity of females in the HSA group was influenced by alternation between long and short days, while MI ewes demonstrated erratic ovulatory activity after the first period of long and short days.

Discussion

In the study described here, active immunization against conjugated melatonin resulted in the development of high antibody titres in treated ewes, which were in the range of, or higher than, values previously reported. The large variability between ewes has frequently been reported in immunization studies (sheep: Arendt *et al.*, 1981; Lincoln and Almeida, 1981; Foldes *et al.*, 1983; goats: Foldes *et al.*, 1992; deer: Ataja *et al.*, 1990).

Fig. 4. Individual plasma prolactin concentrations from two groups of six intact Ile-de-France ewes each of which had received three series of immunizations (Imm 1, 2 and 3) against melatonin (MI group) or human serum albumin (HSA group). (▨) Short days; (□) long days.

The high titres obtained in all MI ewes after the second and third immunization and the specificity of the antibodies demonstrate the efficiency of the melatonin conjugate used here in producing antibodies that immunoneutralize melatonin in the blood circulation.

However, in contrast to these previous reports, the MI ewes in the study reported here lost their ability to respond to photoperiodic changes.

Plasma prolactin concentrations of the MI ewes clearly indicate that this hormonal secretion did not respond to the

Fig. 5. Number of cyclic ewes ovulating per week in two groups of six Ile-de-France ewes each of which had received three series of immunizations (Imm 1, 2 and 3) against (a) human serum albumin or (b) melatonin. (▨) Short days; (□) long days.

abrupt light changes to which the animals were exposed. There have been reports concerning immunization against melatonin and prolactin secretion. In rats, immunization against melatonin caused a significant reduction in prolactin values, although the diurnal rhythm persisted (Niles *et al.*, 1977). In ewes, prolactin concentrations remained unaffected (Arendt *et al.*, 1981), and in red deer, prolactin plasma concentrations were increased (Ataja *et al.*, 1990). We conclude that the MI ewes could no longer respond to the photoperiodic changes to which they were submitted.

In HSA ewes, the ovulatory activity was influenced by the photoperiodic shifts as described by Karsch *et al.* (1984) and Ravault and Thimonier (1988), while the MI ewes demonstrated erratic variations in ovulations after the first short-day period. Reproductive activity in MI ewes was no longer influenced by photoperiod; this is in contrast to previous findings. As mentioned above, immunized ewes (Arendt *et al.*, 1981) and rams (Lincoln and Almeida, 1981) maintained under a natural photoperiod, did not show clear-cut changes in

reproductive seasonality. The photoperiodic conditions of our experiment (artificial abrupt changes between long days and short days) compared with the conditions described in the other reports (natural daylength), are probably responsible for the observed discrepancy between the results. Under conditions of natural daylength, the absence of a response to melatonin immunization could be attributed to the fact that, in the absence of a perception of photoperiodic changes, these animals were exposed to other environmental cues (temperature, social cues from other animals) to time their breeding season in the year. In the present study, these cues were either completely absent (social contact with control ewes), or attenuated (temperature) as animals were maintained in two separate rooms under attenuated ambient temperature changes.

Although the pineal gland of sheep is located deep in the brain, it is situated outside of the blood-brain barrier, and secretes melatonin via the Galen vein into the general circulation. It is likely that the immunoneutralization of melatonin took place in the general blood circulation as the

Fig. 6. Individual plasma progesterone concentrations in two groups of six intact Ile-de-France ewes each of which had received three series of immunizations (Imm 1, 2 and 3) against melatonin (MI group) or human serum albumin (HSA group). (■) Short days.

immunoglobulin probably did not cross the blood-brain barrier. Such a suggestion is consistent with the fact that the restoration of blood melatonin concentrations to night-time values by subcutaneous delivery of melatonin caused the same

effects as exposure to short days (Bittman *et al.*, 1985). However, the results presented here do not distinguish between the parts of the ewe brain (in particular between the pars tuberalis (Williams and Helliwell, 1993) and the

mediobasal hypothalamus (Malpaux *et al.*, 1993)) where melatonin exerts its control of reproductive activity.

It is possible that the antibodies bind melatonin during the night and liberate it during the day, so that the ewes were in a similar situation to those treated with constant infusions of melatonin, as with a subcutaneous implant.

Whatever the mechanism of action, the results reported here show that in ewes active immunization against melatonin can modify the perception of artificial photoperiodic changes.

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References

- Arendt J, Symons AM and Laud C (1981) Pineal function and photoperiod in the ewe *Les Colloques de l'INRA* **6** 219–229
- Ataja AM, Wilson PR, Hodgson J, Hoskinson RW, Purchas RW, Varela-Alvarez H and Barry TN (1990) Responses in venison production to grazing pastures based upon perennial ryegrass or annual ryegrass and to immunisation against melatonin *Proceedings New Zealand Society of Animal Production* **50** 279–285
- Bittman EL, Karsch FJ and Hopkins JN (1983a) Role of the pineal gland in ovine photoperiodism: regulation of seasonal breeding and the negative feedback effects of estradiol upon luteinizing hormone secretion *Endocrinology* **113** 329–336
- Bittman EL, Dempsey RJ and Karsch FJ (1983b) Pineal melatonin secretion drives the reproductive response to daylength in the ewe *Endocrinology* **113** 2276–2283
- Bittman EL, Kaynard AH, Olster DH, Robinson JE, Yellon SM and Karsch FJ (1985) Pineal melatonin mediates photoperiodic control of pulsatile luteinizing hormone secretion in the ewe *Neuroendocrinology* **40** 409–418
- Foldes A, Hoskinson RM, Scaramuzzi RJ, Hinks NT and Maxwell CA (1983) Modification of sheep pineal β -adrenoreceptors by some gonadal steroids but not by melatonin *Neuroendocrinology* **37** 378–386
- Foldes A, Hoskinson RM, Baker P, McDonald BJ, Maxwell CA and Restall BJ (1992) Effect of immunization against melatonin on seasonal fleece growth in feral goats *Journal of Pineal Research* **13** 85–94
- Kann G (1971) Dosage radioimmunologique de la prolactine plasmatique chez les ovins *Comptes Rendus de l'Académie des Sciences Paris* **272** 2808–2811
- Karsch FJ, Bittman EL, Foster DL, Goodman RL, Legan SJ and Robinson JE (1984) Neuroendocrine basis of seasonal reproduction *Recent Progress in Hormone Research* **40** 185–232
- Lincoln G and Almeida OFX (1981) Melatonin and the seasonal photoperiodic response in sheep *Les Colloques de l'INRA* **6** 231–251
- Malpaux B, Daveau A, Maurice F, Gayraud V and Thiéry JC (1993) Short day effects of melatonin on LH secretion in the ewe: evidence for central sites of action in the mediobasal hypothalamus *Biology of Reproduction* **48** 752–760
- Niles LP, Brown GM and Grota LJ (1977) Effects of neutralization of circulating melatonin and N-acetylserotonin on plasma prolactin levels *Neuroendocrinology* **23** 14–22
- Ortavant R, Pelletier J, Ravault JP, Thimonier J and Volland-Nail P (1985) Photoperiod: main proximal and distal factor of the circannual cycle of reproduction in farm mammals *Oxford Reviews of Reproductive Biology* **7** 305–345
- Ravault JP and Thimonier J (1988) Melatonin patterns in ewes maintained under skeleton or resonance photoperiodic regimens *Reproduction, Nutrition, Développement* **28** 473–486
- Saumande J, Tamboura D and Chupin D (1985) Changes in milk and plasma concentrations of progesterone in cows after treatment to induce super-ovulation and their relationships with number of ovulations and embryos collected *Theriogenology* **23** 719–731
- Thimonier J (1978) L'activité ovarienne chez les bovins. Moyens d'étude et facteurs de variation *Annales de Médecine Vétérinaire* **122** 81–92
- Tillet Y, Ravault JP, Selve C, Eeving G, Castro B and Dubois MP (1986) Conditions d'utilisation d'anticorps spécifiques pour la visualisation immunohistochimique de la sérotonine et de la mélatonine dans la glande pinéale du mouton *Comptes Rendus de l'Académie des Sciences Paris* **303** 77–82
- Williams LM and Helliwell RJA (1993) Melatonin and seasonality in the sheep *Animal Reproduction Science* **33** 159–182
- Woodfill CJ, Robinson JE, Malpaux B and Karsch FJ (1991) Synchronization of the circannual reproductive rhythm of the ewe by discrete photoperiodic signals *Biology of Reproduction* **45** 110–121