# Genetic variability in melatonin secretion originates in the number of pinealocytes in sheep

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## Abstract

Genetic variability in plasma melatonin concentrations in ewes results from variations in pineal weight. This study investigated whether it is due to a difference in the number of pinealocytes, or in their size. Two groups of lambs were assigned before birth to being extremes (18 High and 21 Low) by calculating their genetic value on the basis of the melatonin concentrations of their parents. Lambs were bled from 1 week of age until 14 weeks of age. Pineal gland, brain and pituitary weights, length and width of the brain, and length of the hypothalamus were recorded. A significant effect (ANOVA) of genetic group ($P<0{\cdot}05$) and age ($P<0{\cdot}05$) was detected on mean nocturnal plasma melatonin concentrations, as soon as the first week after birth (mean ± S.E.M.; High: $51{\cdot}7 \pm 10{\cdot}7$ vs Low: $31{\cdot}9 \pm 3{\cdot}2$ pg/ml). There was no difference between the two genetic groups in any of the brain parameters measured, but the pineal glands of the High group were heavier and contained significantly more pinealocytes (High: $27{\cdot}8 \pm 2{\cdot}4$ vs Low: $21{\cdot}0 \pm 2{\cdot}4 \times 10^6$; $P<0{\cdot}05$) than those in the Low group. The mean size of pinealocytes did not differ between the two genetic groups. Thus, the genetic variability in nocturnal plasma melatonin concentrations in sheep is expressed by 1 week of age and higher levels of secretion are the consequence of larger pineal glands containing a greater number of pinealocytes.

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## Introduction

In mammals, the pineal gland secretes melatonin, which transduces the neural photoperiodic information received by the retina into a hormonal message which is read by a large variety of tissues in the whole organism (Arendt 1995). Melatonin is synthesized and released into the general circulation with a marked day–night rhythm characterized by low or undetectable concentrations during the day and increases many fold at night (Bittman *et al.* 1983). A large variability in nocturnal plasma melatonin concentrations among individuals has been described in several mammalian species, including humans (Arendt 1995) and sheep (Malpaux *et al.* 1987, 1988).

In sheep, it has been clearly shown that nocturnal plasma melatonin concentrations is a very stable and highly repeatable characteristic within each ewe (Chemineau *et al.* 1996), and estimation of the heritability coefficient of this trait showed that this between-individual variation in melatonin secretion is under strong genetic control (Zarazaga *et al.* 1998*a*). Similar findings were obtained in humans concerning melatonin in urine (Wetterberg *et al.* 1983). While the physiological significance of this genetic variability in melatonin secretion is unknown, investigations conducted regarding their physiological origin have demonstrated that such a variability comes from differences in the synthesis of melatonin from the pineal gland (Zarazaga *et al.* 1998*b*). More specifically, this ability of the pineal gland is not related to enzymatic activity per mg of tissue, but to the size of the pineal gland, which can vary from 30 mg to more than 200 mg (Coon *et al.* 1999). However, despite a well-established characterization of 'high secretors' and 'low secretors' of melatonin in adult sheep, we do not know whether this is due to differences in the number, or in the size, of pinealocytes in the pineal gland.

In the literature, results are scarce about the existence of a relationship between melatonin secretion and pineal size and/or tissue composition of the pineal gland. In rats, no correlation was detected between serum melatonin and pineal size (Vollrath & Welker 1984), and 'classical' books on the pineal gland detailing the anatomy of the gland (Kappers & Pévet 1979, Vollrath 1981) do not raise the question of such a relationship. In other endocrine organs,


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there is evidence that an increase in secretory activity may be due to an increase in the number of secretory cells (Shimokawa *et al.* 1996, Francis *et al.* 2000), or to an increase in cell size (Fouquet *et al.* 1983, Couillard *et al.* 2000), or to changes in secretion rate without changes in cell size or number (Heath *et al.* 1996).

Considering that the sheep pineal gland is composed primarily of pinealocytes (>80%; Arendt 1995), it is possible that the genetic differences in plasma melatonin concentration among ewes occur either because the larger pineal glands of the animals secreting more melatonin contains a greater pinealocyte number, or because pinealocytes are larger, or both.

Regarding the onset of melatonin secretion in life, although a night-time increase in plasma melatonin concentrations has been reported to occur in sheep fetuses (Yellon & Longo 1987, Zemdegs *et al.* 1988) as a direct consequence of melatonin secretion from the maternal pineal gland (Yellon & Longo 1988, McMillen & Nowak 1989) and in the newborn lambs by 1–6 weeks of age (Rodway *et al.* 1985, Claypool *et al.* 1999), it is not known whether the genetic difference in plasma melatonin concentrations previously described in adult ewes is already present soon after birth, or whether it is progressively acquired with age.

Thus, in the present study, we used two extreme groups of lambs genetically selected on the basis of their parent's plasma melatonin concentrations to: (1) determine how early in life the genetic variability in plasma melatonin concentrations is established and (2) investigate whether the genetic difference in melatonin secretion is related to a difference in the number of pinealocytes or in their size.

## Materials and Methods

The experimental procedure reported in this study was carried out at the Institut National de la Recherche Agronomique, Research Center of Nouzilly, France, in accordance with the authorization for animal experimentation no. A37801 of the French Ministry of Agriculture.

### *Animals, blood sampling and slaughtering*

The study was conducted with 39 spring-born male lambs (mean ± S.E.M birth date, 7 March ± 4 days; range, 3–14 March). The Ile-de-France breed used in this study results from crossbreedings performed in France between 1830 and 1900 between the English Dishley (Leicester) and Merinos Rambouillet (imported from Spain in the 18th century) breeds (Perret 1986). The Ile-de-France breed was chosen because it was known that the large variability in the nocturnal plasma melatonin concentrations among ewes (Chemineau *et al.* 1996) is under a strong genetic control (Zarazaga *et al.* 1998*a*,*b*). It is therefore a good model to study the genetic plasma melatonin variability in mammals (Chemineau *et al.* 2001). The whole Ile-de-France flock from which the experimental lambs were obtained is a large flock of about 2500 animals which is divided into six different families. At regular intervals sires are purchased from various private external flocks and are introduced to prevent inbreeding and maintain genetic connections with the national French scheme of genetic improvement of the Ile-de-France breed.

The experimental lambs were chosen from about 400 lambs born at the same time of the year (mid March), in which the genetic value – defined as the sum of the average effects of the genes an individual carries (Falconer 1989) – was calculated on the basis of the endogenous nocturnal plasma melatonin concentration of their parents, previously determined at the June and December solstices (Zarazaga *et al.* 1998*a*). The 39 lambs were chosen and assigned before birth to two extreme groups referred to as Low ($n=21$) and High ($n=18$) groups, on the basis of differences in their genetic value. Lambs of the Low group were the progeny of nine sires and lambs of the High group were the progeny of 11 sires. Immediately after birth, lambs were removed from their mothers, maintained in artificial suckling with reconstituted milk until weaning at 7 weeks of age, and kept under a lighting regime of 16 h light : 8 h darkness (lights on from 0600 to 2200 h) throughout the experiment. Beginning at birth, and until lambs were slaughtered at an average of 14 weeks of age, lambs were weighed weekly to monitor their growth.

Blood (3 ml) was collected from all lambs at weekly intervals from 1 to 7 weeks of age, then at weeks 9, 11 and finally during the week of slaughtering at week 14 of age. At each sampling session, four blood samples were taken at hourly intervals during the night (from 2300 to 0200 h), and then during the following day (from 1000 to 1300 h). At night, blood samples were collected under a dim-red light (<1 lx at 20 cm) with care taken to avoid any direct illumination of the animal's eyes (using a rag to cover animal's eyes during sampling). All blood samples were obtained by venepuncture of jugular vein and plasma was immediately separated by centrifugation at 3000 r.p.m. for 20 min and stored at –20 °C until assayed for melatonin.

Lambs were slaughtered at 14 weeks of age, during the day, and the brain, pineal and pituitary glands were removed and weighed rapidly. Length and width of the brain and length of hypothalamus were also measured. Carcass weight was recorded 24 h after slaughter. For each lamb in the two genetic groups, pineal gland was used for the morphometric study in order to determine the number and size of their pinealocytes.

### *Tissue preparation and morphometric analysis*

Pineal glands were fixed in paraformaldehyde (4%) for 24 h, dehydrated in a graded series of alcoholic solutions, cleared in butanol, and embedded in paraffin wax. The

tissues were cut into 8 μm thick sections and stained with 0·1% cresyl violet.

Pineal volumes (*Vo*) were estimated by the Cavalieri principle (Gunderson 1986) according to the formula $Vo=\Sigma ah$, where *a* is the area in $\mu m^2$ of every 10th section (8 μm thickness) and *h* is the distance in μm between the sections used to determine *a*.

Analysis of the number and individual area of pinealocytes was performed using the SAMBA 2005 Image Analyser (System for Analytical Microscopy in Biological Application, ALCATEL TITN Co., Massy, France). Light microscope images were input into the analyser through an objective with a × 40 magnification, and a camera. A preliminary experiment, performed on a single pineal gland, demonstrated that the number and surface of pinealocytes were homogeneous between sections performed at various levels of the gland (same means and same variances). Thus, within each pineal, two sections, randomly selected at the level of the middle of the gland, were used in the morphometric analysis. On each section, 5350 $\mu m^2$ fields, selected according a grid covering the entire section, were analysed. Threshold cut-off for detection of pinealocytes was 10·4 $\mu m^2$. Accordingly, a total of approximately 1150 pinealocytes per pineal gland were counted and their surfaces measured. The total number of pinealocytes (*n*) from each pineal was estimated from the formula: *n*=(Number of pinealocytes per field/Volume of the field ($\mu m^3$)) × Volume of the gland ($\mu m^3$). The mean volume ($\mu m^3$) for the pinealocytes from each pineal gland was determined, assuming that the shape of the pinealocyte was spherical.

### *Melatonin assay*

Melatonin concentrations were quantified in duplicate aliquots of 100 μl of plasma by the RIA of Fraser *et al.* (1983) using an antibody first raised by Tillet *et al.* (1986). The sensitivity of the assay was 4 pg/ml. Mean intra-assay coefficient of variation, estimated by assaying three plasma pools (low, medium and high concentrations of melatonin) in duplicate every 100 unknown samples, was 5·8%. Mean inter-assay coefficient of variation for the same three plasma pools was 8·7% (two assays).

### *Statistical analysis*

Statistical analyses of the data were performed by ANOVA (Super Anova, Statistical Software, Inc., Berkeley, CA, USA). For melatonin data, an effect of the genetic group and an effect of age was included in the analysis. All melatonin measurements were log-transformed, to correct for heterogeneity of variance, and plasma samples with values below the sensitivity of the RIA were arbitrarily assigned the limit of detection (4 pg/ml of plasma) for statistical analysis. The Pearson correlation coefficient $R^2$ was used to assess correlations among pineal parameters and between pineal parameters and plasma melatonin concentrations, after combining the two groups of lambs. Multiple and step-to-step regressions (STATVIEW, Abacus Concept, Berkeley, CA, USA) were used for the descriptions of relationships among pineal weight and number and/or volume of pinealocytes. A value of 0·05 for *P* was taken as significant. Group data are presented as means ± S.E.M.S.

## Results

### *Melatonin concentrations*

A clear mean day/night (D/N) difference in plasma melatonin concentrations with higher values at night was evident by 1 week of age in the two genetic groups (mean ± S.E.M; High group, D: 5·0 ± 0·4 vs N: 51·7 ± 10·7 pg/ml, $P<0·01$; Low group, D: 4·9 ± 0·3 vs N: 31·9 ± 3·2 pg/ml, $P<0·01$). From 1 week to slaughtering, no difference was found between the two genetic groups in mean daytime plasma melatonin (mean ± S.E.M.; 5·0 ± 0·2 pg/ml). However, ANOVA revealed a significant effect of the genetic group ($P<0·05$) and of age ($P<0·05$) on the mean night-time plasma melatonin concentrations, which were consistently lower in group Low compared with group High (Fig. 1). This difference was detected as soon as the first week after birth (Low: 31·9 ± 3·2 vs High: 51·7 ± 10·7 pg/ml) and was maintained until lambs were slaughtered at 14 weeks of age (Low: 125·0 ± 15·8 vs High: 162·9 ± 14·0 pg/ml).

### *Body growth and slaughtering measurements*

No significant difference was observed on body growth between the two genetic groups. Body weight increased ($P<0·01$) with age. The average weekly liveweight gain from birth to slaughtering was 2·5 ± 0·2 kg/week.

Among the brain parameters measured at slaughter (Table 1), only pineal weight was significantly different between the two genetic groups, with the mean pineal weight higher in the High group than in the Low group whereas no differences were detected between the High and Low groups for total brain weight, pituitary weight, length and width of the brain, and length of hypothalamus. Carcass weight of lambs did not differ between groups (21·5 ± 0·3 kg).

### *Pineal parameters*

Morphometric analysis of pineal glands for the two genetic groups revealed that lambs in the High group had a significantly larger gland volume (High: 55·3 ± 5·5 vs Low: 39·9 ± 4·6 $mm^3$; $P<0·05$) and a significantly higher total number of pinealocytes (High: 27·8 ± 2·4 vs Low: 21·0 ± 2·4 × $10^6$; $P<0·05$) compared with those in the

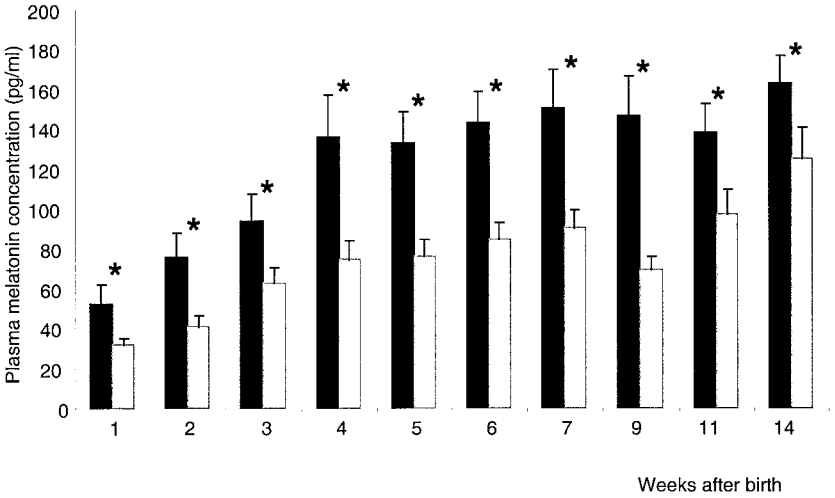


**Figure 1** Mean ± S.E.M. nocturnal plasma melatonin concentrations from genetically extreme lambs (High, filled bars, $n=18$ and Low, open bars, $n=21$), sampled four times during the night, at weekly intervals, from 1 to 7 weeks of age, and at 9, 11 and 14 weeks of age. * indicates significant difference between groups ($P<0{\cdot}05$).

Low group (Fig. 2). In contrast, no significant difference was found on mean individual volume of pinealocytes between the High and Low groups ($185{\cdot}5 \pm 9{\cdot}9$ and $166{\cdot}3 \pm 6{\cdot}8\ \mu m^3$ respectively). An illustration of the pinealocytes is given in Fig. 3.

Pineal weight was highly correlated with pineal volume ($R^2=0{\cdot}86$, $P<0{\cdot}001$), with total number of pinealocytes ($R^2=0{\cdot}79$, $P<0{\cdot}001$), and with volume of pinealocyte ($R^2=0{\cdot}24$, $P<0{\cdot}01$; Fig. 4). Multiple regression indicated that the number of pinealocytes ($P<0{\cdot}0001$) and the volume of pinealocytes ($P<0{\cdot}05$), when combined, significantly contributed to explain more than 80% of the total variance of the pineal weight. Step-to-step regression analysis revealed that about 63% was due to pineal weight and 17% to the volume of pinealocytes. Mean nocturnal plasma melatonin concentrations at slaughtering, at 14 weeks of age, were also significantly correlated with the number of pinealocytes ($R^2=0{\cdot}29$, $P<0{\cdot}001$), with pineal volume ($R^2=0{\cdot}34$, $P<0{\cdot}001$) and with the weight of the pineal gland ($R^2=0{\cdot}24$, $P<0{\cdot}01$). No significant correlation was detected between plasma melatonin concentrations and pinealocyte size.

## Discussion

The present study clearly shows that the two groups of extreme lambs differed by the number of pinealocytes that were counted within their pineal gland, rather than by the size of their pinealocytes. At slaughter, at 14 weeks of age, the lambs of the High group, selected on the basis of the plasma melatonin concentration of their parents, had 32% more pinealocytes in their pineal gland than the lambs of the Low group. Conversely, the size of the pinealocytes did not differ between the two groups.

However, when pooling the two genetic groups, a significant correlation between volume of pinealocytes and pineal weight existed. This apparent contradiction with

**Table 1** Mean values ( ± S.E.M.) of brain parameters measured at slaughtering at 14 weeks of age in the Low ($n=21$) and High ($n=18$) Ile-de-France lamb groups

| | **Low group** ($n=21$) | **Difference between groups** | **High group** ($n=18$) |
|---|---|---|---|
| **Parameter** | | | |
| Pineal weight (mg) | 88·1 ± 7·7 | $P<0{\cdot}05$ | 118·1 ± 9·9 |
| Pituitary weight (mg) | 471·9 ± 27·3 | N.S. | 443·7 ± 31·6 |
| Brain | | | |
| Total weight (g) | 75·0 ± 0·9 | N.S. | 74·4 ± 1·1 |
| Length (mm) | 52·4 ± 0·8 | N.S. | 52·3 ± 0·5 |
| Width (mm) | 42·1 ± 0·6 | N.S. | 42·2 ± 0·4 |
| Hypothalamus length (mm) | 11·1 ± 0·2 | N.S. | 11·5 ± 0·3 |

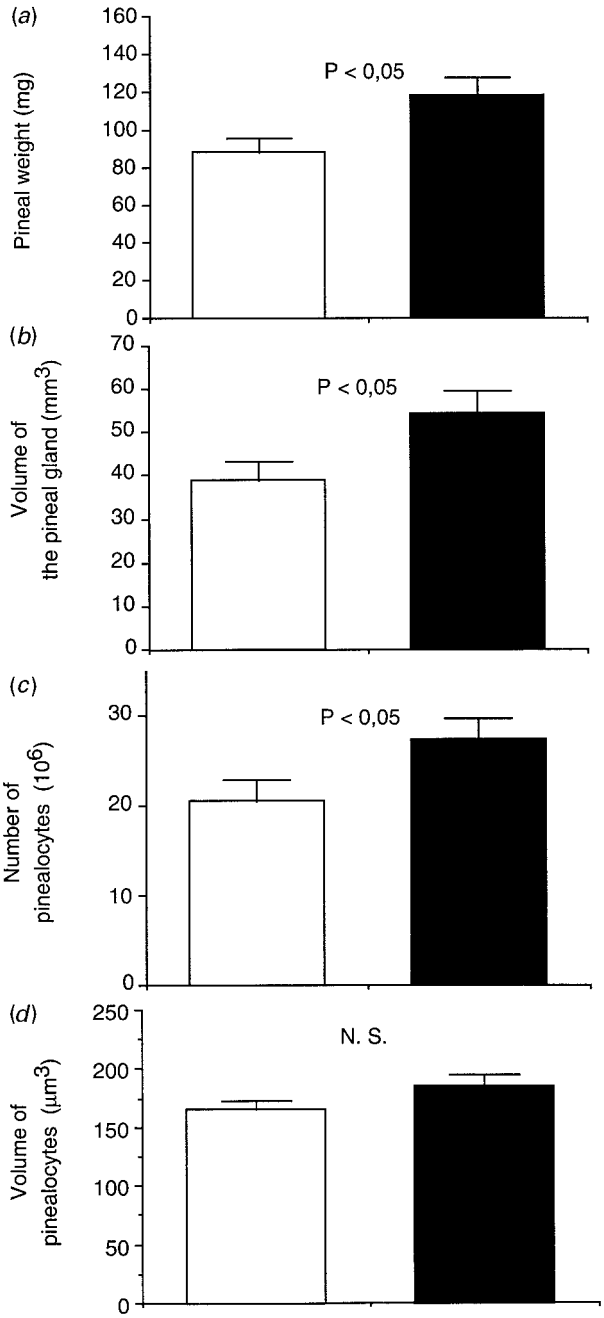


**Figure 2** Pineal weight (a), pineal volume (*b*), number of pinealocytes (c) and volume of pinealocytes (*d*) from genetically extreme lambs (High, filled bars, $n=18$ and Low, open bars, $n=21$) slaughtered at 14 weeks of age. Data are means ± S.E.M.S.

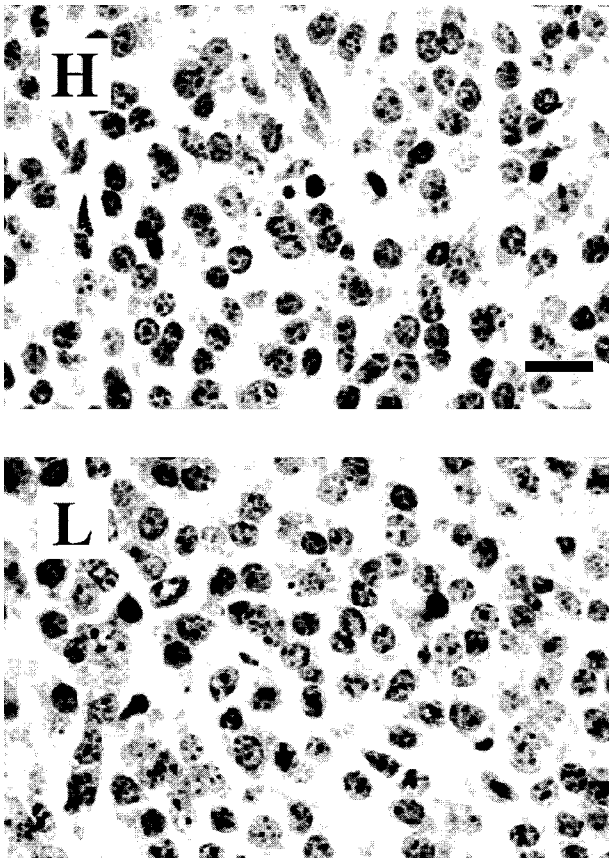


**Figure 3** Illustrative examples of pinealocytes from genetically extreme lambs (High, $n=18$ and Low, $n=21$) slaughtered at 14 weeks of age. Bar=20 µm.

the results obtained when comparing the two genetic groups may indicate that pineal weight of lambs also partly depends on the volume of pinealocytes. By referring to the multiple and step-to-step regression analyses, we can assume that the variation in the number of pinealocytes may explain about 80%, and the volume of pinealocytes about 20%, of the total variation in pineal weight.

Thus, the difference between the two genetic groups of extreme lambs regarding their pineal weight at slaughter, already found in a previous experiment (Coon *et al.* 1999), originated from a higher multiplication rate of pinealocytes rather than an enlargement of the same number of cells. This difference in number of pinealocytes was observed at slaughter, at 14 weeks old. As attested by the substantial increase in mean melatonin plasma concentration from 1 to 14 weeks of age in the present study, already observed in previous publications (Claypool *et al.* 1989), it is highly probable that pineal glands of young-born lambs continue their development in the first weeks of life. We can wonder if the difference between the two groups regarding the number of pinealocytes was already present at birth, or if the difference is acquired progressively during the first weeks of life. The existence, in the present experiment, of a difference in plasma melatonin concentrations as early as less than 1 week after birth, strongly suggests that the lambs of the High group had a higher

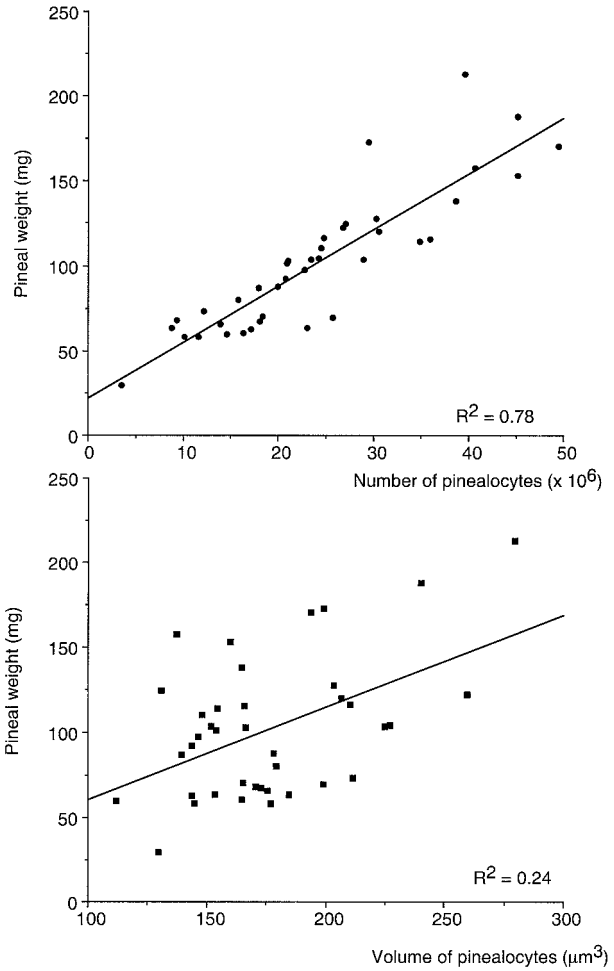


**Figure 4** Individual relationship between number of pinealocytes and pineal weight (upper graph) and volume of pinealocytes and pineal weight (lower graph) in genetically extreme lambs (both groups of lambs were combined) slaughtered at 14 weeks of age.

number of pinealocytes at birth, compared with the number present in the Low group.

As the difference between these two groups is due to the effects of alleles of specific genes (because animals were selected on the performances of their parents), we can suggest that the genes controlling the multiplication of pinealocytes probably act during embryonic or fetal development, when the pineal gland is at the beginning of its development. In vertebrates, the pineal develops from the diencephalon, and in the human species has been identified as soon as the second month of gestation, when the embryo is 6–9 mm long (Binkley 1988). Unfortunately, the identification of these genes will probably not be easy because the specific alleles producing the difference between groups may be expressed and act during a limited period of time during embryonic or fetal development. This was demonstrated for homeobox genes (Gehring 1993), for example, by modifying the yield of multiplication of the stem cells at the beginning of pineal differentiation.

Interestingly, in our study, only pineal weight was different between the two extreme groups of lambs, whereas the other simple brain measurements, such as brain weight, pituitary weight, or brain size, did not differ between the two groups of animals. This may suggest that the specific genes controlling multiplication of pinealocytes could be specific to the pineal gland or the diencephalon, and may not be involved in the general development of the whole brain. However, as these structures are not linked embryologically with the development of the pineal gland, we cannot fully exclude that the genetic differences in pineal size are not associated with other changes in brain characteristics; more detailed measurements on structures linked to the diencephalon would be necessary to ascertain this conclusion.

The absence of observed difference in body growth between the two groups of lambs raises the question of the biological consequence(s) of this genetic variability in pineal weight/melatonin secretion. To our knowledge, only a few studies have addressed this question. Regarding seasonal breeding, a trait known to be under melatonin control, in a limited number of ewes it was suggested that the relative day/night melatonin ratio could be related to the date of onset of the ovulatory activity (Chemineau *et al.* 1993, Zarazaga *et al.* 1996), but this relationship needs to be confirmed on a larger set of ewes. In other species, studies were more focused on duration of melatonin secretion than on amplitude, with the exception of the relationship between melatonin amplitude and ageing in humans (Touitou *et al.* 1981, Sack *et al.* 1986, Wetterberg *et al.* 1993, Kripke *et al.* 1998, Touitou 2001) and primates (Aujard *et al.* 1998, Roth *et al.* 2001). In this latter case, melatonin amplitude was considered as a good marker of ageing processes. In this regard, sheep may constitute a good model for the genetic control of plasma melatonin in mammals.

In conclusion, these results show for the first time (a) that the genetic differences previously observed in adult sheep are detected in young lambs as soon as the first week of life, and (b) that a high secretion of melatonin is due directly to a hyperplasia of the pineal gland that is associated with an increase in pinealocyte number. The identification of genes controlling pineal size (which is in progress in our laboratory) could be of interest for other mammalian species, including humans.

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