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ON THE PRESENCE OF MELATONIN IN PINEAL GLANDS AND PLASMA OF FOETAL SHEEP

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SUMMARY

Melatonin is shown to accumulate within the foetal sheep pineal gland in the last few days of gestation. The mean ± S.E.M. foetal content was 79 ± 25 pg per gland for 30 foetuses obtained from day 124–145 gestation and 836 ± 286 pg per gland for 21 foetuses judged to be within 5 days of birth. In 5 neonates, the mean pineal content on day 1 was 899 ± 280 pg per gland. This accumulation correlates to a rise in tissue hydroxyindole-O-methyltransferase activity and foetal plasma melatonin. The possible relationship between these parameters and the endocrine changes preceding parturition is discussed. Significant levels of monoamine oxidase activity were found in foetal sheep pineal and the possibility is raised of other indoles (e.g. 5-methoxy tryptophol) being of importance in foetal pineal function.

INTRODUCTION

There is now compelling evidence that the foetal lamb plays a major role in the mechanisms controlling parturition [1]. Extensive studies by Liggins and others have clearly indicated a primary involvement of the foetal hypothalamic–pituitary–adrenal system in the endocrine changes mediating the birth process, but the factors which initiate the functioning of this axis are largely unknown.

Since there is evidence of an interplay between the pineal and pituitary glands, we have examined the functioning of the pineal gland in foetal life to ascertain its relation to changes in adrenal function prior to parturition in the sheep.

We have previously reported changes in hydroxyindole-O-methyltransferase activity (HIOMT) a key enzyme in melatonin synthesis in pineal tissue approaching term [2]. In this study we report on the synthesis and content of melatonin, a putative pineal hormone, in the pineal gland and blood of the foetal lamb.

EXPERIMENTAL

Animals. Foetal sheep (mainly merino or merino cross breed) were obtained from our own mated flocks where the day of conception was known accurately, or in the majority of instances, from the metropolitan abattoirs (SAMCOR). The length and weight of each foetus was determined, plus the adrenal weight. From this data, an estimation could be made of the stage of gestation of foetuses obtained from the abattoirs using prepared nomograms. Pregnancy in the sheep breed studied is of 147–150 days duration.

Pineal tissue and blood samples, obtained by heart puncture, were collected from each foetus within 20 min of maternal death. The samples were transported to the laboratory in ice for analysis. Each gland was weighed in a fresh state and then frozen and held at −10°C for analysis. Blood samples which were collected into tubes containing lithium heparin (12.5 I.U. per ml), were centrifuged and the plasma fractions stored at −10°C.

Reagents. These were of the highest grade possible, and solvents were redistilled before use. Tritiated melatonin (S.A. 26 Ci/mmol) was obtained from New England Nuclear Corporation, Boston, U.S.A. $[^2H]_3$-melatonin, used as an internal reference standard for selected ion monitoring, was synthesised from N-acetylserotonin using deuterated methyliodide as previously described [3]. Indole standards were obtained from Sigma, St. Louis, U.S.A. or synthesised using standard procedures.

Enzyme studies. Hydroxyindole-O-methyltransferase activity (HIOMT) were assayed as previously described [2]. Monoamine oxidase activity was assayed as described by Wurtman and Axelrod[4] after homogenising in 0.15 M KCl. This assay was proven to be valid for sheep pineal tissue with only minor modification.

Assay of melatonin by radioimmunoassay (RIA). For analysis, plasma (2 ml) was extracted with chloroform (8 ml) following mixing with 2 ml of 0.5 M borate pH 10.0. Tissue homogenate representing 5 mg tissue was mixed with 2 ml borate buffer and extracted with 8 ml chloroform. Following extraction, the samples were centrifuged, the aqueous layer removed and the chloroform evaporated at 37°C under N_2. The residue was then taken up and transferred to columns (3.2 × 175 mm) of Lipidex 5000 (Packard Instrument

Co. Ill., U.S.A.) in 0.5 ml of the solvent mixture $CHCl_3$:light petroleum (1:1). The first 0.5 ml eluate was discarded and a further 4 ml solvent added and the eluate discarded. Melatonin was then eluted into assay tubes (12 × 100 mm) and the solvent (5 ml) evaporated at 37°C under a stream of nitrogen.

Full details of the assay procedure will be reported elsewhere [3]. Briefly, the extract was equilibrated with [^{3}H]-melatonin (10,000 c.p.m.) and antibody (1:4000 final dilution) overnight at pH 7.4 in 0.1 M phosphate buffer containing 0.1% NaN_3, 0.15% gelatine and 0.9% NaCl and the free and bound fractions then separated by ppt. with 50% $(NH_4)SO_4$. Known amounts of melatonin (30–1000 pg) were taken through the assay procedure as reference standards.

Reliability criteria. The antibody used was raised in a rabbit to an antigen formed by condensation between N-acetylserotonin and the Mannich adduct of BSA and formaldehyde using the method of Grota and Brown[5].

The antibody raised crossreacted to other N-acetyl indoles to the same extent as previously described [3, 5], the required degree of specificity being dependent on solvent partition and chromatography systems used. The antibody used (R8 17/6) gave 41% binding at a final dilution of 1:4000, and at this dilution non specific binding was 5.5 ± 0.4% (n = 41). The least detectable amount of melatonin was 30 pg and 50% displacement was produced by 225 pg melatonin. Recovery of [^{3}H]-melatonin was 70%, the correlation coefficient between assayed and expected amounts of melatonin (50–500 pg) added to plasma was 0.966. The within and between assay coefficient of variation over the range 100–600 pg was 6% and 10% respectively.

Analysis of melatonin by selected ion monitoring techniques (SIM). In the present report the SIM procedure was used solely to confirm the reliability of the RIA for assay of melatonin in pineal tissues.

Following chromatography [^{2}H]$_3$-melatonin (40 ng) was added to a portion of individual pineal gland extracts as internal reference standard, and after removal of the solvent, the pentafluoro-propionyl (PFP) ester was then formed [6] and the product subject to SIM. The other portion of the extract was subjected to RIA.

The SIM assays were carried out with an AEI MS-30 mass spectrometer as described elsewhere [3]. Quantitation was achieved by monitoring and comparing the molecular ions of the corresponding PFP derivatives of melatonin and [^{2}H]$_3$-melatonin. (M/z 360 and 363 respectively).

Determination of melatonin content of 9 individual pineal glands obtained from day killed sheep by SIM and RIA, showed a correlation for the relationship of 0.986, the RIA tending to slightly under-estimate (≤5%) the content of melatonin i.e. 1.0 ± 0.094 (S.E.M.) ng per mg tissue.

Bioassay of melatonin in pineal tissue. Individual glands or grouped glands from foetuses of similar gestational age were homogenised in 0.1 N NaOH and extracted with chloroform.

The extract was taken to dryness and resuspended in isotonic fish buffer, and this was then injected intraperitoneally (10 μl per fish) into the pencilfish (*Nannostomus beckfordi anomalous*), according to the technique of Reed and Finnin[7]. The bioassay depends upon changes in melanosome distribution of the day band of the fish and the definite end point is judged visually. The assay is highly specific for melatonin [8], and the useful range, in our experience, was 110–500 pg per injected sample. At least 10 fish were used for injection and the lowest melatonin concentration thus detectable in each extract was about 1–2 ng.

Cortisol assay. Plasma cortisol was determined by the competitive protein binding procedure of Bassett and Hinks[9].

RESULTS

Figure 1 summarises the results of the study on foetal pineal and adrenal tissue weights, the enzyme activity and melatonin content of the pineal glands, and the foetal plasma melatonin and cortisol content through the latter part of gestation.

Foetal pineal melatonin content

Bioassay studies. With the bioassay procedure melatonin was not detectable (<200 pg per gland) in 114 extracts prepared from single or grouped pineal glands obtained from foetuses before 141 day of gestation, but was detected in 6 of 34 individual glands obtained in the 6–9 days preceding birth with a range of melatonin content of 1.3–2.4 ng per gland.

RIA studies. With the more sensitive RIA, melatonin was detected in all but 8 of 51 individual foetal glands studied from 124 day of gestation to term and in all pineals from 5 neonatal (day 1) lambs studied.

The mean ± S.E.M. foetal pineal melatonin content was 79 ± 25 pg per gland for 30 foetuses obtained from day 124–145 and 836 ± 286 pg per gland for 21 foetuses judged on the basis of size to be within 5 days of birth. In the 5 neonates studied, the mean pineal content on day 1 was 899 ± 280 pg per gland.

Relationship of pineal melatonin content to HIOMT and monoamine oxidase activity. As shown in Fig. 1, the rise in HIOMT activity occurring over the last few days of gestation corresponded with a rise in pineal melatonin content. The positive correlation between these two events is illustrated in Fig. 2b presenting data for individual lambs in which both the HIOMT activity and melatonin content of pineal tissue was determined. No direct relationship was apparent between the pineal HIOMT activity, or melatonin content, and the MAO activity.

Relationship of pineal melatonin to plasma melatonin content. As is also indicated in Fig. 1, no pattern emerged from studies of changes in mean content of plasma melatonin with gestational age that clearly

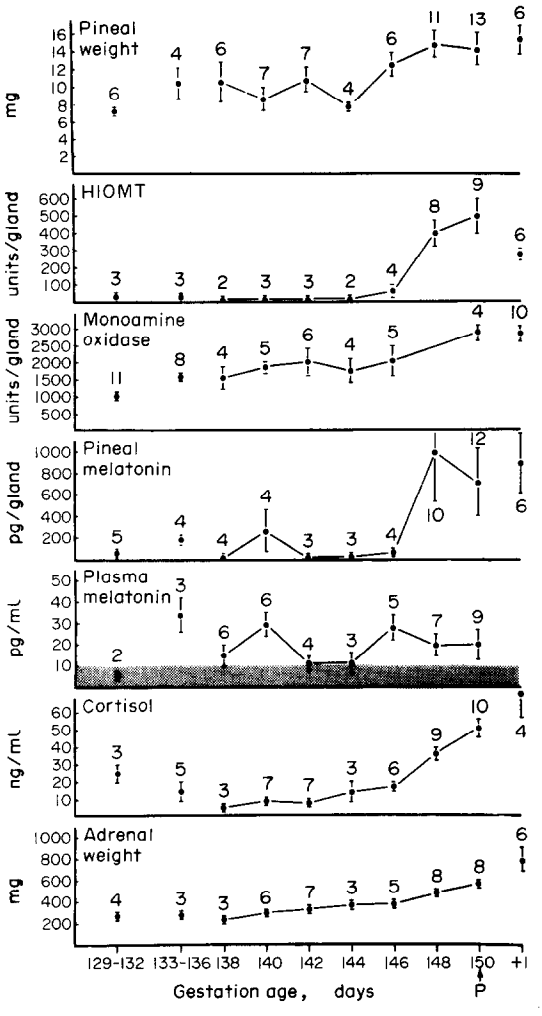

Fig. 1. Mean (±S.E.M.) values for weight, HIOMT activity, monoamine oxidase and melatonin content of the pineal gland, the plasma melatonin and cortisol and adrenal weight of the foetal lamb at various stages of gestation. Enzyme results are expressed as units/gland where 1 unit is equivalent to the formation 1 pmol [^{14}C]-labelled product per 60 min. The hatched area denotes values not differing significantly from zero.

related to changes seen in pineal melatonin content. Albeit, in 30 foetuses where both measurements were made, the pineal melatonin content was below 30 pg per gland in 9 foetuses where no (<10 pg) plasma melatonin was detected and as is seen in Fig. 2a in the remaining foetuses a positive correlation was apparent between plasma and pineal melatonin.

Relationship of pineal melatonin to plasma cortisol content. The relationship of pineal melatonin *vs* plasma cortisol was studied in 35 foetuses with the results shown in Fig. 2c. Plasma cortisol values exceeding 20 ng per ml were used to provide an indication that birth was imminent within 5 days [1]. Of the 21 foetuses in which the plasma cortisol values exceeded this figure, 12 had significantly elevated pineal melatonin content if compared with the foetuses with plasma levels of cortisol below 20 ng per ml. A strong positive correlation between pineal melatonin content and plasma cortisol was evident.

DISCUSSION

Our present studies reveal an accumulation of melatonin in the foetal sheep pineal gland in the last few days of gestation. This data, in conjunction with the early histological investigations of Jordan[10] and recent enzyme investigations [2], clearly suggest that the foetal sheep pineal gland is both functional and can synthesize methoxyindoles towards the end of gestation.

Little data is available from other species concerning the foetal pineal. In the rat, the pineal has been

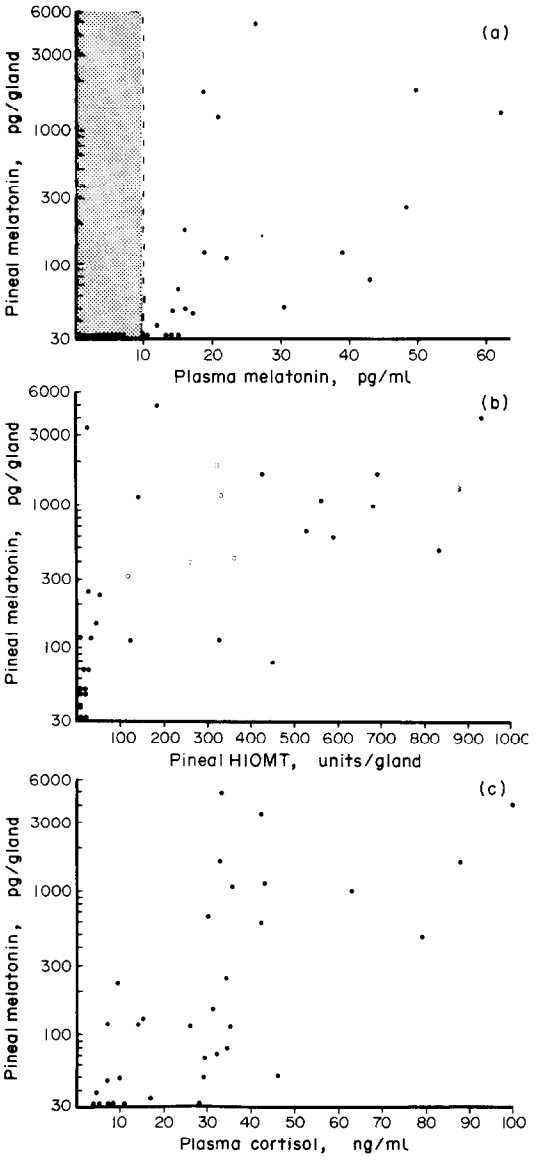

Fig. 2. Concentration of melatonin in foetal (●) and neonatal lamb (○) pineals in relation to (a) plasma melatonin; (b) pineal HIOMT activity; and (c) plasma cortisol. The hatched area in Fig. 2a encloses values not differing significantly from zero.

shown to contain certain enzymes involved in indole synthesis or metabolism such as N-acetyltransferase [11] prior to birth and monoamine oxidase is found in the pineal of the neonate at about 75% of adult values [12]. However, no HIOMT activity was detected until about the seventh day of *post natal* life [12, 13] and tissue levels of serotonin and 5-hydroxy tryptophan decarboxylase were also very low until this time [12], indicating that the production of melatonin in the foetal pineal is unlikely. Other investigations in fact suggest that in the rat the maternal pineal gland dominates and may play an active role in foetal development, if only in setting the rhythmicity of the neonate [14].

The possibility exists that the melatonin in the foetal sheep pineal could itself stem from another source, possibly the maternal pineal. Preliminary studies in our laboratory have shown that the maternal gland remains active during pregnancy, and that melatonin may, as in the rat [15] cross the placenta. Furthermore, other studies using [^{3}H]-melatonin in non-pregnant cats and rats, have shown that exogeneous melatonin may accumulate within the pineal gland [16].

It is also feasible that the melatonin detected may have been formed in other extra pineal tissues. There are several reports indicating that alternative sources of melatonin exist [17–19], and we have recently demonstrated that melatonin is present in the plasma of pinealectomised ewes [3] although the normal circadian rhythms are abolished. However, the strong positive correlation between the levels of HIOMT activity and the melatonin content of the foetal pineal gland, provides compelling evidence that the melatonin is synthesised at least in part within the foetus, as does the positive correlation between pineal and plasma melatonin content.

The function of melatonin in the foetus remains enigmatic, but it seems likely that it has a regulating action on pituitary function in the mature animal [20], and if extrapolation may be made to the foetus, then the pineal gland may influence the endocrine changes prior to parturition.

As was shown, the period of rise in HIOMT activity and melatonin content of the foetal pineal, corresponds to a surge of adrenal activity. There is, however, very little evidence suggesting a positive role for the pineal in the control of adrenal function. Indeed the majority of evidence suggests that it is inhibitory [20].

Alternatively, the functioning of the pineal gland may itself be influenced by the rapid endocrine changes which occur prior to parturition. No data is available to suggest that cortisol or other adrenal corticoids can directly influence pineal gland function and the characteristic preparturient surge of unconjugated oestrogen and prostaglandins in the foetal sheep and ewe occurs only in the last day of gestation [1], that is after the increase in melatonin synthesis.

One distinct possibility, however, is that the changes in pineal synthesis of melatonin may relate to changes in the sympathetic nervous system. It is known for example that there is increased medullary phenyl ethanolamine N-methyltransferase activity over the same period as the rise in pineal HIOMT [21] and pineal function, at least in rats, is markedly influenced by sympathetic input *via* the superior cervical ganglion as well as exogenously administered catecholamines [20]. Thus the increase may simply reflect the development of effective sympathetic innervation and/or adrenal medullary secretion of norepinephrine. It is also known that oestrogens and certain other steroids influence the pineal responses to catecholamines and so the placental steroids may act indirectly on the foetal pineal through modulating sympathetic activity [22].

Another possibility is that the changes in the pineal may relate to preparturient changes in thyroid function [23], but the data concerning the connection between these two glands is ambivalent and more direct studies with the foetus are required.

Albeit, whilst it is interesting to postulate possible roles of the foetal pineal in parturition, until more information is available as to whether melatonin is a significant pineal hormone and more is understood of the interrelationship of the pineal and other endocrine tissues it must be a very speculative and limited exercise. The occurrence of monoamine oxidase activity in foetal sheep and rat [12] pineal tissue for example raises the distinct possibility that other indoles such as 5-methoxy tryptophol may be important in mediating pineal gland function before birth. It has also been shown that the foetal pineal may be innervated by tracts other than those stemming from the sympathetic cervical ganglion [24]. Thus, although the present study confirms that the pineal functions in foetal life, and that changes are occurring immediately prior to birth, the exact significance of these findings remain to be elucidated.

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DISCUSSION

Grumbach. Have you looked at the hypothalamus as well? I am sure you are well aware of the data of David Klein that melatonin suppresses LRF secretion in pituitaries in cultures.

Matthews. No, we have not, but Koslow [17] has shown by SIM techniques that melatonin is present in the rat hypothalamus and, interestingly, that this content remained unaltered up to one month after pinealectomy. If these extra pineal sites of melatonin synthesis do indeed occur and can assume prominence following pinealectomy, this may contribute towards an explanation for the many conflicting reports stemming from investigations of the endocrine function and physiological significance of the pineal gland.