

Presence of pigment in the ovine pineal gland during embryonic development

S. Regodón, A.J. Franco, A. Gazquez and E. Redondo

Department of Anatomy and Histology, Faculty of Veterinary Medicine, University of Extremadura, Cáceres, Spain

Summary. Light- and electron-microscopic analyses were used to identify and describe the characteristic features of cells containing pigment in the ovine pineal gland during prenatal development. 72 ovine embryos (36 male, 36 female) ranging in age from 54 to 150 days were used for this study. Cells containing pigment granules were a constant feature in this gland. When samples from the different groups were inspected using the naked eye, the detection of pigment was of 61% in groups I (54 to 67 days of prenatal development) and II (71 to 92 days of prenatal development), 83% in group III (98 to 113 days of prenatal development) and 25% in group IV (118 to 150 days of prenatal development). The morphological features and histochemical properties of the pineal pigment enabled it to be identified as melanin. Several types of pigment granules were ultrastructurally distinguished; these varied in size, shape and location within pineal cell populations. The pigment granules were detected in pinealocytes, interstitial cells and pigmented cells. The largest amount and the widest variety of pigmented granules were found in pigmented cells. The presence of cells containing pigmented granules amongst the cell populations of the developing ovine pineal gland was analysed and compared with that of other mammalian species.

Key words: Pigment, Pineal gland, Embryonic development, Ovine

Introduction

The pineal gland has been extensively studied in domestic mammals through a variety of biochemical, physiological, pharmacological and histological techniques. Most histological studies focus on the postnatal development of the pineal gland in a number of mammalian species like rabbit (Romijn, 1973), cat (Calvo et al., 1992; Boya et al., 1995), dog (Zach, 1960;

Calvo et al., 1988), cow (Santamarina and Meyer-Arendt, 1956; Santamarina, 1958), and horse (Cozzi and Ferrandi, 1984; Cozzi, 1986). Few studies have addressed the ovine pineal gland development (Anderson, 1965) and up until now there is no published research on its prenatal development.

Three classes of pigment are to be found in the pineal gland: melanin, lipofuscin and haemosiderin (Quay, 1974). Melanin has been identified by histochemical methods in the horse (Cozzi and Ferrandi, 1984; Cozzi, 1986), in the dog (Zach, 1960; Calvo et al., 1988), in the cat (Calvo et al., 1992), and in the rabbit (Romijn, 1973). In cattle, it has been chemically isolated and studied using spectrophotometric and cytochemical methods (Santamarina and Meyer-Arendt, 1956; Santamarina, 1958).

This paper describes the histochemical and ultrastructural analysis of cells containing melanin and haemosiderin pigments as well as their distribution within the ovine pineal gland during prenatal development.

Materials and methods

Animals

Seventy-two clinically-healthy ovine embryos at different stages of development were used for this study. Specimens were arranged according to age in four groups with reference to the C-R length and the most relevant histological characteristics detected using light microscopy: group 1 (54 to 67 days of prenatal development), group 2 (71 to 92 days), group 3 (98 to 113 days) and group 4 (118 to 150 days) (Table 1). 36 embryos, male and female, ranging in age from 54 to 150 days, were used for the light microscopic study; the remaining 36, of similar ages, were used for the electron microscopic examination.

In order to obtain embryos at various stages of development, caesarean section was performed after synchronization of estrus, using hormonal techniques and uterine flushing. Fluorogesterone acetate was administered 14 days before introduction of males. Then

Pigment in ovine pineal gland

600IU of pregnant mare serum globulin (PMSG) were inoculated. Caesarian section was performed from day 54 following introduction of males. Animals were tranquilized with an intramuscular injection of 0.5 mg/100 kg of body weight propionyl phenothiazine, and anaesthesia was induced by an intravenous injection of 5 cc of sodium thiopental (4g as a 20% aqueous solution). Once separated from maternal linking, embryos were euthanized by umbilical vein administration of 2 cc of sodium thiopental (4 g as a 20% aqueous solution).

Light microscopy

Samples for light microscopy were fixed in 10% formalin and embedded in paraffin. In order to obtain longitudinal sections of the pineal gland, serial sections (6 μ m thick) were obtained from blocks cut along the sagittal plane. Melanin identification was performed using a method involving hydrogen peroxide bleaching (Rodriguez and McGravan, 1969). For this, one gland from each of the groups was treated in the following manner: 40 ml of pineal gland homogenate diluted in distilled water was added to a test-tube containing 10 ml of 10% hydrogen peroxide and left for 48 h.

The Masson-Fontana silver technique and the Perls' method were also used to demonstrate melanin and haemosiderin pigments respectively. Some sections were stained with haematoxylin and eosin for routine light microscopic examination. Granular size was determined in serial microphotographies of the same resolution using a semiautomatic image analyser (Olivetti M-24, using a VIDS IV program). In every gland 100 granules were studied. Glands were classified according to mean size and electron density of the granules.

Electron microscopy

Pineal glands were fixed in 2.5% glutaraldehyde in 0.1M cacodylate buffer (pH 7.2). After 12 h of fixation at room temperature, tissue blocks were postfixated for 2 h in 1% osmium tetroxide in 0.1M cacodylate buffer (pH 7.2). Samples were dehydrated in graded ethanol solutions (50, 70, 90 and 100%) and finally embedded in epoxy resin. Ultrathin sections were cut, stained with lead citrate and uranyl acetate, and examined in a Jeol Jem 100 S-X transmission electron microscope.

Results

When pineal glands of the different groups were inspected using the naked eye, the detection of the percentages of the pineal glands containing pigment was 61% in groups I (54 to 67 days of prenatal development) and II (71 to 92 days of prenatal development), 83% in group III (98 to 113 days of prenatal development) and 25% in group IV (118 to 150 days of prenatal development).

Hydrogen peroxide bleaching of glandular homogenate revealed gradual discolouration over 48 h of

Table 1. Experimental design and details of age, crown-to-rump length (C-R), and number of specimens.

GROUP	AGE (DAYS)	C-R LENGTH (cm)	No. OF SPECIMENS
I	54 to 67	9 to 14.5	18
II	71 to 92	16 to 24	18
III	98 to 113	26 to 33	16
IV	118 to 150	36 to 42	20

treatment. Loss of stain was more evident in group III, where embryos showed intense evidence of pigment.

Light microscopy

A similar pattern of spatial distribution of cells containing pigment in the pineal gland was recorded in groups I, II and III. Differences detected were purely quantitative, the cells with pigment being more abundant in group III. In all three groups, these cells were observed below the glandular capsule, in the dorsal and ventral regions and in clusters on the surface of the pineal recess (Fig. 1). In group IV (Fig. 4), these cells were observed in the vicinity of the midline of the sagittal plane of the gland. Besides the presence of pigment granules, light microscopy revealed no morphological differences between pigmented and non-pigmented pineal cells.

In embryos from group III, cells containing pigment were also observed outside the pineal gland. These extrapineal pigmented cells were mainly located in the posterior and caudal commissures and in the adjacent meningeal space, and their morphology was similar to that of cells within the pineal gland.

Pigment exhibited a negative reaction to the Perls' staining technique, and reacted positively to the Masson-Fontana silver stain. This positive response was most marked in cells with pigment from group III (Fig. 3). In general terms, pigment granules were rounded or ovoid in shape, dark-brown to blackish in colour and were observed intracellularly (Figs. 2-4).

Cells containing pigment were scarce in group IV (Fig. 4), moderate in groups I (Fig. 1) and II (Fig. 2) and abundant in group III (Fig. 3). Nevertheless, these findings were closely related to gross observations. Vascularization appeared to increase throughout ontogenesis; however, it should be noted that a close link between glandular pigment and vascularization was detected within similar age-groups. Therefore, glands showing a greater degree of pigmentation (group III) were also the most highly-vascularized, while glands belonging to group IV showed very little vascularization.

Electron microscopy

The ultrastructural analysis enabled the classification of the cell types containing pigment. In groups I and II, the pineal gland contained pigment in pinealocytes (Figs. 5-7). From 98 days to 150 days (groups III and IV), the

Pigment in ovine pineal gland

pigment of the pineal gland was contained in pinealocytes (Figs. 10 and 13), interstitial cells (Figs. 8 and 11) and pigmented cells (Figs. 9, 12 and 14).

The ultrastructure of pinealocytes (Figs. 5-7, 10, 13) remained similar throughout embryonic development varying only in terms of changes in the amount of their organelles. These cells were characterised by the presence of one or two typical cytoplasmic extensions, and an oval or lobulated nucleus in which heterochromatin was observed close to the nuclear envelope. One or two nucleoli were present. Cytoplasm was rich in free ribosomes and contained few smooth endoplasmic reticulum cisternae. Both Golgi apparatus and rough endoplasmic reticulum were abundant, though the latter was confined to the perinuclear area. Mitochondria were elongated or oval, with a moderately electron-dense matrix. A few microfilaments were detected. Maculae

adherents were sometimes visible between the cytoplasmic projections of adjacent pinealocytes.

Interstitial cells (Figs. 8 and 11) remained ultrastructurally homogeneous throughout ontogenesis, varying only in their degree of maturity. These cells were located in the perivascular space, and were generally less electron-dense than pinealocytes. Nuclei were small, oval and electron-dense, with a delicate ribbon of chromatin surrounding the nuclear membrane. One nucleolus was visible. The cytoplasm contained glycogen granules and microfilaments. Regarding other cytoplasmic organelles, no significant differences were observed between interstitial cells and pinealocytes.

The ontogenetic maturing pattern of pigmented cells was similar to that described for the other two cell populations (Figs. 9, 12 and 14). These cells contained an ovoid nucleus and abundant heterochromatin clusters.

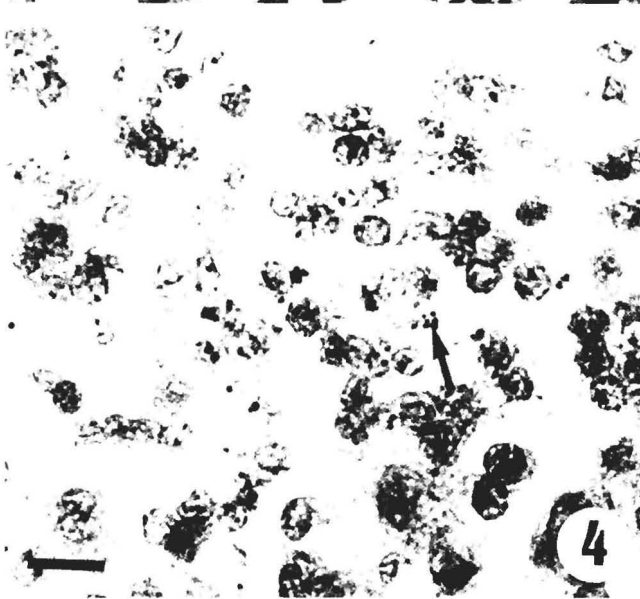
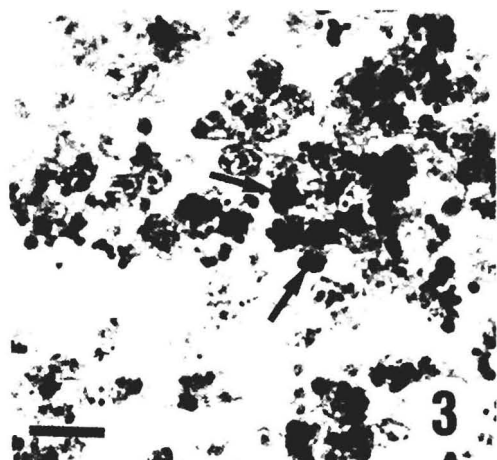
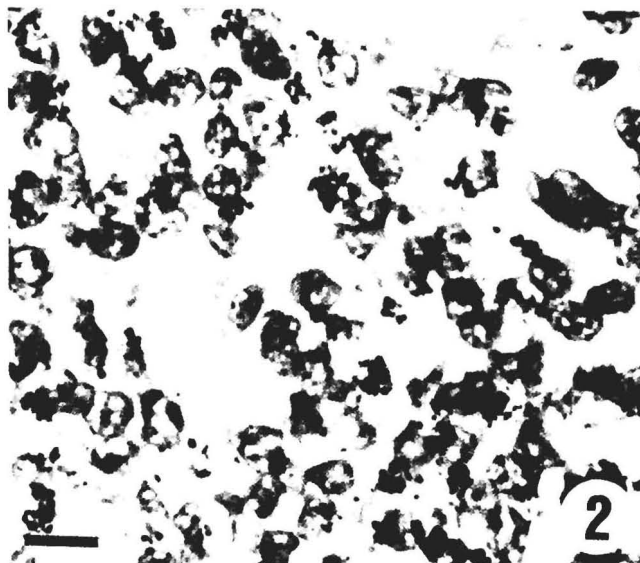


Fig. 1. Sagittal section of pineal gland, embryo 10 cm (group I). Cluster of cells with melanin on the gland surface, showing marked tropism for the subcapsular space and pineal recess. Masson-Fontana (M-F). Bar: 300 μ m.

Fig. 2. Sagittal section of pineal gland, embryo 17 cm (group II). Irregular distribution of pigment with intracellular location. Haematoxylin-Eosin (H-E). Bar: 200 μ m.

Fig. 3. Intensive accumulation of cells containing melanin (arrows). Sagittal section of pineal gland, embryo 26 cm (group III). M-F. Bar: 200 μ m.

Fig. 4. Arrangement of cells containing few melanin (arrow) on the sagittal plane midline, embryo 39 cm (group IV). M-F. Bar: 200 μ m.

Pigment in ovine pineal gland

together with one or two small nucleoli. Cytoplasm contained abundant RER cisternae and mitochondria with electron-dense matrix and a well-developed Golgi apparatus, the latter exhibiting clusters of cisternae and associated vesicles. Centrioles and microtubules were identified in areas adjacent to the Golgi apparatus. Occasional lysosomes and microtubules were observed in the cytoplasm. No microfilaments were visible.

Pigmented granules varied in size, shape and location within pineal gland cell populations. Three types of small granules ($0.7 \pm 0.3 \mu\text{m}$) were distinguished in terms of electron-density, which was either homogeneous (type 1-granule) or lamellar (type 2-granule), or even a mixture of homogeneous and lamellar types (type 3-granule). Large granules ($1.5 \pm 0.5 \mu\text{m}$) were classified into three groups according to the same criteria, and were designated type 4, type 5 and type 6. The location of these different granule types within the pineal gland was as follows: group I contained granule types 1, 2, 3 and 4 in pinealocytes (Figs. 5 and 6). The pinealocytes of group II (Fig. 7) showed granule types 1-6. In group III, all granule types were observed in pinealocytes (Fig. 10), pigmented cells (Figs. 9 and 12) and interstitial cells (Figs. 8 and 11). In group IV, granule types 1-3 were observed in both pinealocytes (Fig. 13) and pigmented cells (Fig. 14); pigment was not detected in interstitial cells from group IV.

Discussion

The staining reactions and the ultrastructural morphology indicate with a fair degree of certainty the presence of pigment during ontogenesis of the ovine pineal gland, which can thus be included in that group of mammals in which pineal pigments have been demonstrated. This group currently includes the rabbit (Romijn, 1973), cat (Calvo et al., 1992; Boya et al., 1995), dog (Zach, 1960; Calvo et al., 1988), cow (Santamarina and Meyer-Arendt, 1956; Santamarina, 1958), and horse (Cozzi and Ferrandi, 1984; Cozzi, 1986). This is the first report of the presence of melanin during ovine embryonic development.

Appreciable amounts of melanin were found (group III). This finding agrees with the results reported in the tree shrew (Hwang, 1982), in nocturnal mammals (Pevet et al., 1977; Pevet and Racey, 1981), in rabbit (Romijn, 1973), in cow (Santamarina and Meyer-Arendt, 1956; Santamarina, 1958), and horse (Cozzi and Ferrandi,

1984; Cozzi, 1986). However, the present findings do not concur with those reported in cats (Calvo et al., 1992) and in dogs (Zach, 1960; Calvo et al., 1988) in which the amounts of melanin detected were relatively small.

Like Calvo et al. (1988, 1992) in dogs and cats, we failed to correlate the amount of pigment and sex of the animals. However a correlation was detected between age and amount of pigment (larger amounts in group III), even though Zach (1960) reported pigmented cells in half the dogs studied and Calvo et al. (1988) observed only a small number of pigmented cells in relation to the total cell number.

The differences regarding the location of pigment in the various cell populations of the pineal gland prompt two questions. First, are pigmented cells a special type of pinealocyte, or are they ultrastructurally distinct? The ultrastructural observations reported here would tend to support Sheridan and Reiter (1973), who defined pigmented cells as a new cell type whose characteristics differ from those of pinealocytes. These authors base their assertions on the absence of microfilaments and the presence of pigment granules in pigmented cells.

On the other hand, the absence in pigmented cells of the two cytoplasmic projections characteristic of pinealocytes would run counter to the hypothesis advanced in the rabbit (Romijn, 1973), in nocturnal mammals (Pevet et al., 1977), in the horse (Cozzi and Ferrandi, 1984), and in the dog (Calvo et al., 1988) and cat (Calvo et al., 1992), in which the pigmented cell is considered as a special type of pinealocyte.

The second question is: if pigment granules have been detected, to a greater or lesser degree, in all pineal gland cell types, which of these cells plays a major role in the biosynthesis of melanin? Two findings appear to confirm the theory that pigmented cells play a fundamental part in melanin biosynthesis: a) the variability of pigment granules detected in these cells, and b) the negligible presence of lysosomes. The activity of pigmented cells during embryonic development would appear to be focused on the production and storage of pigment. Only during postnatal development lysosome digestion would ensure a constant amount of pigment; this has been reported during postnatal development of cat pineal gland (Calvo et al., 1992).

The different types of pigment granules observed in the present study are likely to represent different stages of maturity of a single granule type, as suggested by

Fig. 5. Pineal gland, embryo 10 cm (group I). Pinealocyte containing pigment granule types 1 (1), 2 (2) and 4 (4). $\times 16,000$

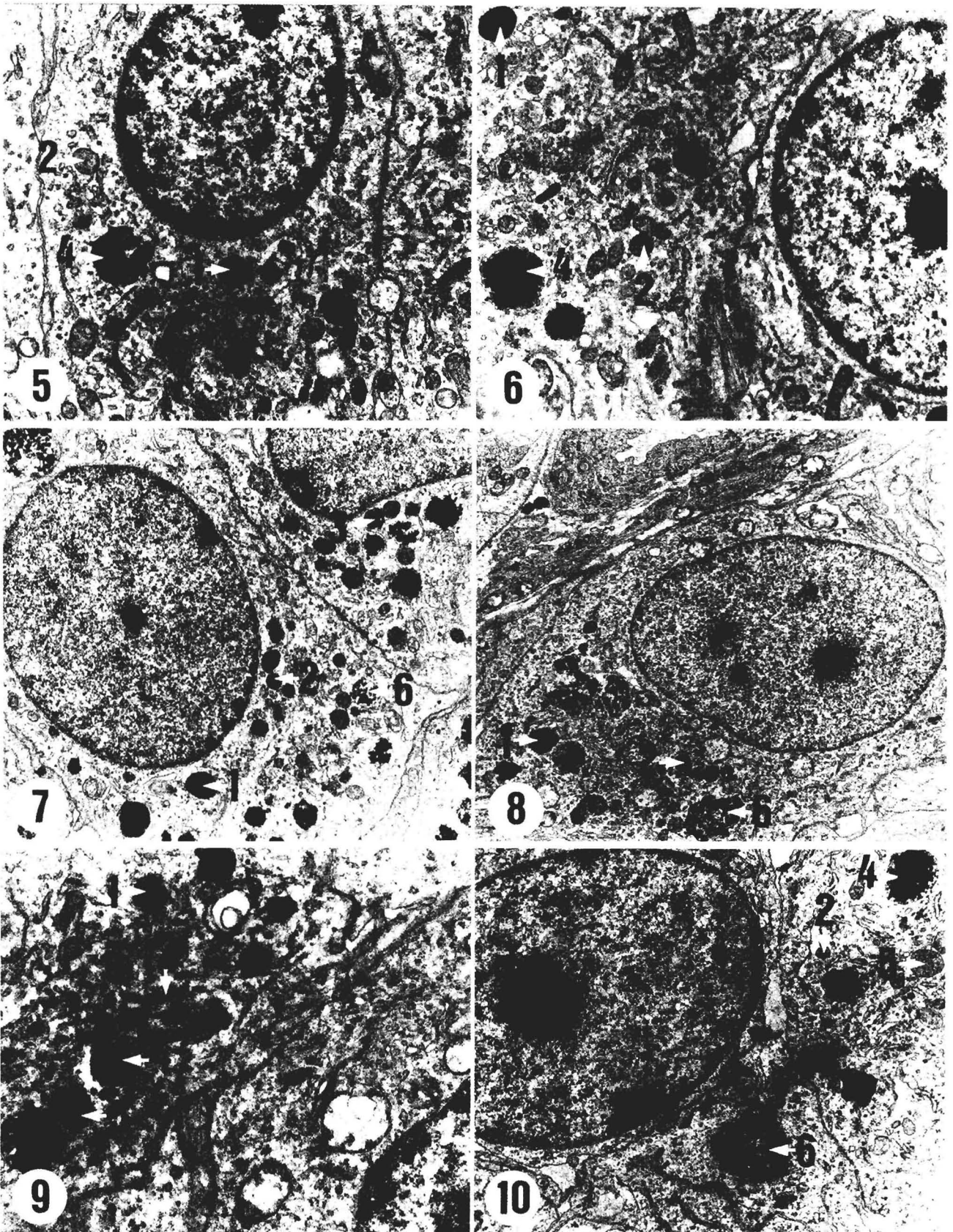
Fig. 6. Pineal gland, embryo 13 cm (group I). Pinealocyte with pigment granule types 1 (1), 2 (2), and 4 (4). $\times 15,000$

Fig. 7. Pineal gland, embryo 20.5 cm (group II). Pinealocytes containing pigment granule types 1 (1), 2 (2), 3 (3) and 6 (6). $\times 10,000$

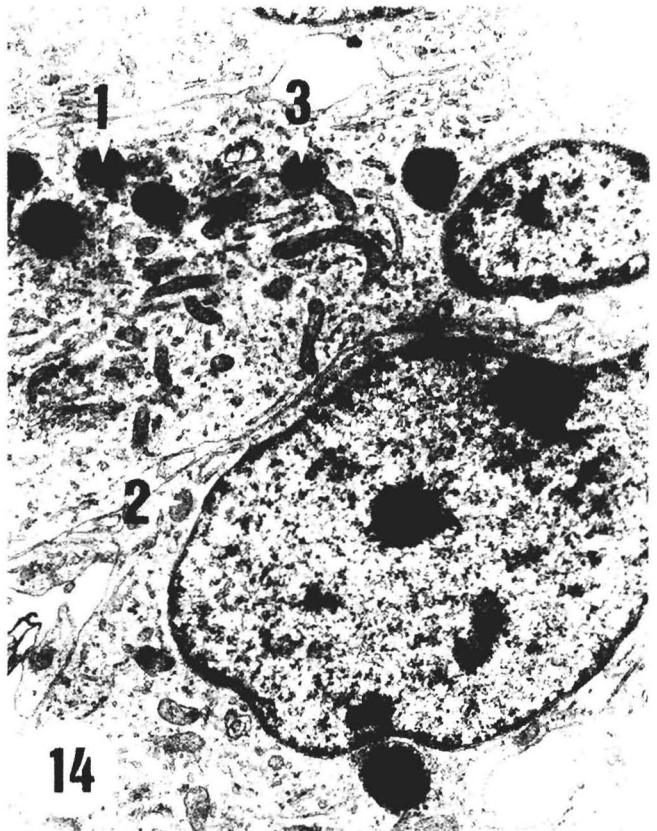
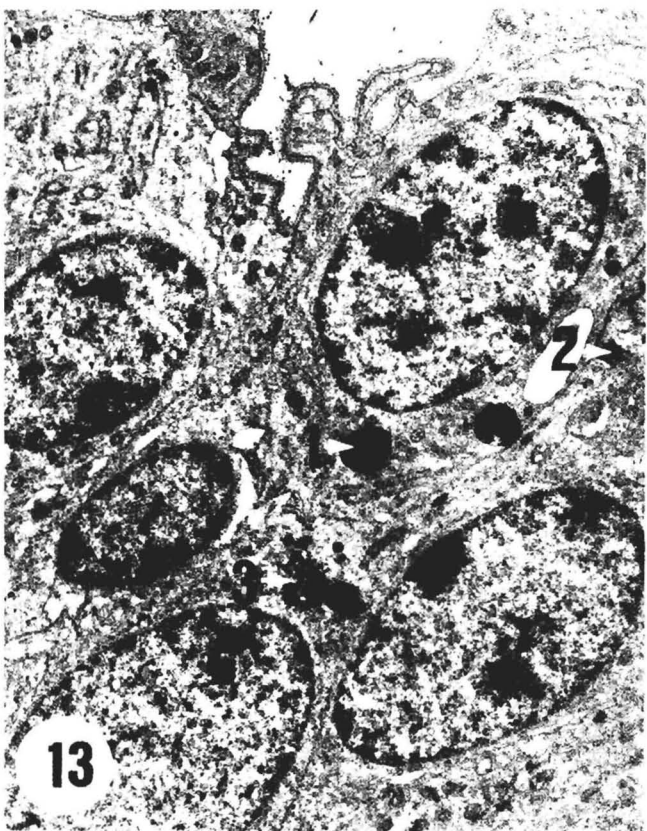
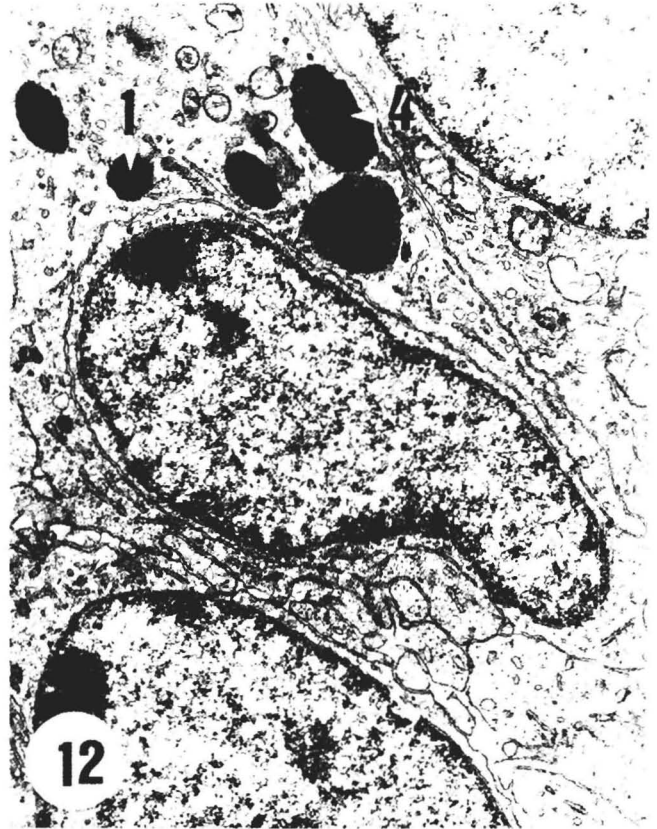
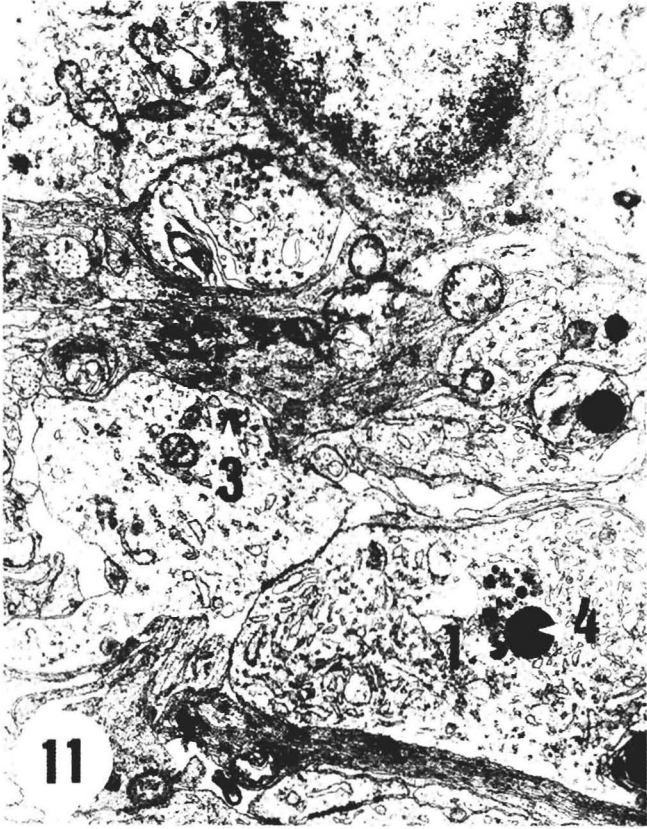
Fig. 8. Pineal gland, embryos 26 cm (group III). Perivascular interstitial cell with pigment granule types 1 (1), 2 (2) and 6 (6). $\times 6,000$

Fig. 9. Pineal gland, embryos 27 cm (group III). Pigmented cell showing granule types 1 (1), 2 (2), 4 (4) and 5 (5). $\times 11,000$

Fig. 10. Pineal gland, embryos 28 cm (group III). Pinealocyte containing pigment granule types 2 (2), 4 (4), 5 (5) and 6 (6). $\times 16,000$



Pigment in ovine pineal gland



Pigment in ovine pineal gland

Fig. 11. Pineal gland, embryos 30.5 cm (group III). Interstitial cell with pigment granule types 1 (1), 3 (3) and 4 (4). x 12,000

Fig. 12. Pineal gland, embryos 30.5 cm (group III). Pigmented cell showing granule types 1 (1) and 4 (4). x 18,000

Fig. 13. Pineal gland, embryos 36 cm (group IV). Distribution of pinealocytes over the pineal recess surface, pigment granule types 1 (1), 2 (2), and 3 (3). x 9,500

Fig. 14. Pineal gland, embryos 40 cm (group IV). Pigmented cell showing granule types 1 (1), 2 (2) and 3 (3). x 17,500

Romijn (1973) for rabbit pineal gland, and subsequently discussed by Calvo et al. (1988) in dogs. The progressive storage of pigment throughout embryonic development would govern both the size and the electron-density, which in turn would give rise to the morphological variations reported in pigment granules (Calvo et al., 1988). The ultrastructure of pigment granules here was broadly similar to that described for other species of domestic mammals. Moreover, granule content closely resembles the black neuromelanin substance described by Moses et al. (1966) and Lukaszuk and Reiter (1975) and later discussed by Calvo et al. (1988).

Despite the fibrillar content observed in some types of pigment granule, no evidence was detected of the striated fibrils characteristic of cutaneous melanocytes reported in horses (Cozzi, 1986). Attention is also drawn to the absence of lipid content in these granules, as reported in dog pineal gland (Calvo et al., 1988), and earlier in human piamater and plexus coroideus (Moses et al., 1966).

A further two questions arise with regard to the biosynthesis of melanin. Do pinealocytes, as Cozzi and Ferrandi (1984) have suggested, play a major role in biosynthesis? Or are they simply the final morphological substrate in which peptide biosynthesis is developed? Our results tend to suggest that the role of pinealocytes in melanin biosynthesis is not a fundamental one, since the pigment has been detected in other cell types. Moreover, there is morphological evidence (intense development of rough endoplasmic reticulum, Golgi apparatus and abundant mitochondria) of substantial protein biosynthesis in one cell type: the pigmented cells. These are also the cells exhibiting the greatest variation in pigment granule populations.

The functional role of the pineal gland in melanin biosynthesis is a matter of some debate. While Quay (1974) has argued that this functional role is very limited, Santamarina (1958) suggests a direct relationship between pigmentation and vascularisation. Our own observations support the latter thesis, since most pigmented glands were highly vascularised (group III).

Moreover, the selective location of pigmented cells observed in the canine pineal gland (Calvo et al., 1988), and the intensive development of cell organelles involved in protein biosynthesis (Cozzi, 1984) clearly agree with the present results, suggesting a possible functional role of the pineal gland in melanin biosynthesis.

Recently, Schraermeyer (1996) hypothesized that pigment cells may use protein matrices from different cellular pathways in melanogenesis: 1) the protein matrix rich in tyrosine residues; and 2) the matrix resulting from lysosomal protein degradation.

In conclusion, the most striking morphological findings in this study were as follows:

a) Confirmation of the prenatal differentiation of cells containing pigmented granules, as suggested by Calvo et al. (1992) in dogs. It should be noted, however, that these authors treat pigmented cells generically, without distinguishing between the various cell populations of the pineal gland.

b) The existence of morphological evidence (i.e. strong development of cellular organelles involved in protein biosynthesis, larger population of pigmented granules) that the role of pigmented cells in the biosynthesis of melanin is more significant than that of pinealocytes and interstitial cells.

c) The absence of pigmented granules in interstitial cells in group IV suggests that the role of these cells in melanin biosynthesis is secondary with respect to that of pigmented cells and pinealocytes.

Further neuroendocrinological studies are required to determine more clearly the functional activity of the pineal gland in melanin biosynthesis, and above all to ascertain the role of pigmented cells as distinct from that of other cell populations in which melanin has also been detected.

Acknowledgements. The authors thank José Luis Sanz Rodrigo and M^o de Carmen González Bravo, of the Pathological Anatomy Unit at San Pedro de Alcántara Hospital, Cáceres; and Germán Fernández of the Faculty of Veterinary Medicine, Cáceres, for technical assistance.

References

- Anderson E. (1965). The anatomy of bovine and ovine pineals. Light and electron microscopic studies. *J. Ultrastr. Res.* 12, 1-80.
- Boya J., Calvo J. and Rancaño D. (1995). Structure of the pineal gland in the adult cat. *J. Pineal Res.* 18, 112-118.
- Calvo J., Boya J., García-Mauriño J.E. and López-Carbonel A. (1988). Structure and ultrastructure of the pigmented cells in the adult dog pineal gland. *J. Anat.* 160, 67-73.
- Calvo J., Boya J., García-Mauriño J.E. and Rancaño D. (1992). Presence of melanin in the cat pineal gland. *Acta Anat.* 145, 73-78.
- Cozzi B. (1986). Cell types in the pineal gland of the horse: ultrastructural and immunocytochemical study. *Anat. Rec.* 216, 165-174.

Pigment in ovine pineal gland

- Cozzi B. and Ferrandi B. (1984). Fine structure and histochemistry of the equine pineal gland, with special reference to the possible functional role of the electron-dense intrapinealocyte bodies. *Clin. Vet.* 107, 337-346.
- Hwang B.H. (1982). Fluorescence and electron microscopic study of the tree shrew pineal organ. *J. Neural Transm.* 53, 193-212.
- Lukaszyk A. and Reiter R.J. (1975). Histophysiological evidence for the secretion of polypeptides by the pineal gland. *Am. J. Anat.* 143, 451-464.
- Moses H.L., Ganote C.E., Beaver D.L. and Schuffman S.S. (1966). Light and electron microscopic studies of pigment in human and rhesus monkey substantia nigra and locus coeruleus. *Anat. Rec.* 155, 167-184.
- Pevet P. and Racey P.A. (1981). The pineal gland of nocturnal mammals. II. The ultrastructure of the pineal gland in the pipistrelle bat (*Pipistrellus pipistrellus* L.). Presence of two populations of pinealocytes. *Cell Tissue Res.* 216, 253-271.
- Pevet P., Kappers J.A. and Voute A.M. (1977). The pineal gland of nocturnal mammals. I. The pinealocytes of the bat (*Nyctalus noctula*, Schreber). *J. Neural Transm.* 40, 47-68.
- Quay W.B. (1974). Pineal chemistry in cellular and physiological mechanisms. ed. Springfield. Ill. Charles C. Tomas. New York.
- Rodriguez H.A. and Mc Gavran M.H. (1969). A modified DOPA reaction for the diagnosis and investigation of pigment cells. *Am. J. Clin. Pathol.* 52, 214-227.
- Romijn H.J. (1973). Structure and innervation of the pineal gland of the rabbit, *Oryctolagus cuniculus* L. II. An electron microscopic investigation of the pinealocytes. *Z. Zellforsch.* 141, 545-560.
- Santamarina E. (1958). Melanin pigmentation in bovine pineal gland and its possible correlation with gonadal function. *Can. J. Biochem. Physiol.* 36, 227-235.
- Santamarina E. and Meyer-Arendt J. (1956). Identification of melanin in the bovine pineal gland. *Acta Histochem.* 3, 1-5.
- Schraermeyer U. (1996). The intracellular origin of the melanosome in pigment cells. A review of ultrastructural data. *Histol. Histopathol.* 11, 445-462.
- Sheridan M.N. and Reiter R.J. (1973). The fine structure of the pineal gland in the pocket gopher, *Geomys bursarius*. *Am. J. Anat.* 136, 363-382.
- Zach B. (1960). Topographie und mikroskopisch-anatomischer Feinbauder Epiphysis cerebri von Hund und Katze. *Anat. Histol. Embryol.* 7, 273-303.

Accepted July 28, 1997