Postnatal maturation of parenchymal cell types in sheep pineal gland: an ultrastructural and immuno-electron-microscopic study.°

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SUMMARY

Ultrastructural and immunohistochemical techniques were used to study the pineal parenchyma of 18 Merino sheep at postnatal ages ranging from 1 month to > 2 years. Animals were arranged in three age-groups, each containing six sheep (three male and three female): group 1 (1, 3 and 6 months of postnatal development), group 2 (9 months, 1 year and 2 years) and group 3 (> 2 years of postnatal development). 2 cell types were distinguishable in the pineal-gland parenchyma: pinealocytes and interstitial cells. Pinealocytes showed morphological evidence of functional activity, with considerable development of the organelles involved in protein synthesis, especially from 9 months to 2 years of age. From 3 years of age onwards, there was evidence of cell decline. The developmental pattern of interstitial cells was similar to that of pinealocytes. The vascular tropism observed suggests that these cells in addition to their classical function as support cells may also have a more functional role as a selective barrier in the exchange of substances between pineal parenchyma and blood vessels. The remainder of the gland was composed of a stroma rich collagen containing- especially in group 2-, abundant non-fenestrated capillaries and non-myelinated nerve fibers. A number of factors point to the intense functional activity of the ovine pineal gland during postnatal development. These include: a high degree of innervation and vascularisation; numerous gap junctions between pinealocytes, between interstitial cells and between the two cell types; morphological evidence of secretory activity in pinealocytes; and finally the vascular tropism of interstitial cells. This morphological evidence was most manifest from 9 months to 2 years of age; it would thus appear that the gland is most active in this age-group.

KEY-WORDS: pinealocytes - interstitial cells - ultrastructure - pineal gland - sheep.

RÉSUMÉ

Maturation postnatale des différents types cellulaires du parenchyme de la glande pinéale ovine : étude ultrastructurale et immuno-électronique. Par: S. REGODÓN, J. MASOT, A. FRANCO et E. REDONDO.

Des techniques ultrastructurales et immuno-histochemiques ont été utilisées afin d’étudier le parenchyme de la glande pinéale sur 18 brebis Merinos, dont l’âge postnatal est compris entre un mois et 2 ans. Ces animaux furent classés par groupes d’âges, chacun d’eux contenant 6 brebis (3 mâles et 3 femelles): premier groupe (1 mois, 3 et 6 mois de développement postnatal), second groupe (9 mois, 1 an et 2 ans) et troisième groupe (> à 2 ans de développement postnatal).

Deux types cellulaires ont été différenciés : les pinéalocytes et les cellules interstitielles. Les pinéalocytes manifestent une évidence morphologique en ce qui concerne leur activité fonctionnelle, avec un développement considérable des organelles impliquées dans la synthèse protéique, spécialement à l’âge de 9 mois à 2 ans. A partir de 3 ans le déclin cellulaire est évident. Le développement des cellules interstitielles est similaire à celui des pinéalocytes. Le tropisme vasculaire observé, suggère que ces cellules à part leur fonction classique de support pourraient également jouer un rôle plus important comme barrière sélective dans l’échange de substances entre le parenchyme et les vaisseaux sanguins. Le reste de la glande est composé d’un tissu riche en collagène, contenant, spécialement dans le groupe 2, d’abondants capillaires non-fénétrés ainsi que des fibres nerveuses non-miélisées. De nombreux éléments marquent l’intense activité fonctionnelle de la glande pinéale ovine pendant le développement postnatal. Cela inclue : un haut degré d’innervation et de vascularisation; de nombreuses zones d’union entre les pinéalocytes, entre les cellules interstitielles et entre les deux types de cellules; évidence morphologique de l’activité sécrétrice dans les pinéalocytes et finalement le tropisme vasculaire des cellules interstitielles. Cette évidence morphologique s’étant manifestée à partir de l’âge de 9 mois à 2 ans, ce qui permet de supposer que la glande est plus active dans ce groupe d’âge.


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Introduction

The pineal gland originates as an outgrowth of the roof of the diencephalon in all vertebrate species. This evagination arises between the developing habenular commissure anteriorly (or rostrally) and the posterior commissure and subcommissural organ posteriorly (or caudally) [24]. The pineal gland, in all vertebrate species, is composed of pinealocytes and interstitial cells [3, 15].

In mammals, the pineal gland is known to be involved in the photoperiodic regulation of endogenous biological rhythms [22]. In light-dependent animals, melatonin secretion by the pineal gland is essential to the photoperiodic response. Among such mammals the sheep, given its seasonal polyestrus, is an excellent model for investigating the relationship between changes in pineal gland structure and seasonal variations in melatonin production [9, 18, 20, 21].

Any approach to the study of pineal gland physiology requires detailed knowledge of the morphological changes taking place during prenatal and postnatal development. Research in this area includes studies by JORDAN [1], ANDERSON [14] and the present authors [10, 18, 19, 20, 21]. However, this research addresses prenatal development; the literature contains no reference to studies of postnatal development of the ovine pineal gland. In view of this paucity of information, and given the importance of the subject, the present paper describes the ultrastructural morphology of pinealocytes and interstitial components of sheep pineal gland during postnatal development. It is hoped that this report on the ultrastructural maturation of parenchymal cell types in sheep pineal gland will serve as a basis for future neuroendocrine research.

Material and methods

A) ANIMALS

18 Merino sheep ranging from 1 month to > 2 years in age were used for this study. Sheep were arranged in three age-groups, each containing six animals (3 males, 3 females): group 1 (1, 3 and 6 months of postnatal development), group 2 (9 months, 1 year and 2 years old) and group 3 (> 2 years of postnatal development). Grouping followed current physiological and zootechnical criteria.

Animals were exposed to natural photoperiodicity and given free access to food and water; they were tranquilized with an intramuscular injection of 0.5 mg/100 Kg body weight of propionyl phenothiazine, and euthanized by jugular vein administration of 2 g sodium thiopental as a 20% aqueous solution at 10.00 A.M. hours between February and June.

B) ELECTRON MICROSCOPY (EM)

Pineal glands were fixed in ice-cold 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2) with or without the addition of 3% sucrose. After washing in buffer, tissue blocks were postfixed for 2 hr in 1% osmium tetroxide in 0.1 M cacodylate buffer (pH 7.2), dehydrated through alcohol series and embedded in epoxy resin. Ultrathin sections were cut, stained with lead citrate and uranyl aceate, and examined with a Jeol Jem 100 S-X electron microscope.

C) IMMUNOHISTOCHEMICAL ELECTRON MICROSCOPY (EM)

Ultrathin sections cut from blocks obtained for electron microscopy were stained with colloidal gold for detection of (glial Fibrillary acidic protein) GFAP-positive cells. Sections were treated with a saturated aqueous solution of sodium metaperiodate and then incubated in a moist chamber at 37°C with TRIS-buffered saline (TBS) 0.05 M (pH 7.6) and 1% bovine serum albumin (Sigma Aldrich Química, Madrid, Spain). Sections were subsequently incubated with primary antibody (rabbit anti-bovine GFAP, diluted 1:1,000) at 37°C for 3 hr. After incubation, sample sections were washed three times in TBS 0.05 M, pH 7.6. Finally, they were incubated with diluted (1:50) anti-rabbit IgG, 10 nm gold conjugate (Biocell, UK) for 60 min in a moist chamber at room temperature and contrasted with lead citrate and uranyl acetate.

Results

A) ULTRASTRUCTURAL EXAMINATION

Quantitative differences between members of the same age-group were negligible, and no significant sex-related differences were found. The following descriptions therefore apply to groups as a whole.
B) GROUP 1 (1, 3 AND 6 MONTHS' POSTNATAL DEVELOPMENT)

Two cell types were distinguishable in the pineal gland parenchyma in this group: pinealocytes and interstitial cells.

Pinealocytes (Fig. 1 to 4) presented ovoid nucleus (Fig. 1) with dense homogeneous chromatin (Fig. 2) and peripheral densifications (Fig. 1). A nucleolus was frequently observed. Cytoplasm was lightly electron-dense (Fig. 1) and contained highly-developed organelles. The well-developed Golgi complex usually presented numerous small cistern stacks located in a large area near the nucleus (Fig. 3). Rough and smooth endoplasmic reticulum and free ribosomes were found throughout the cell. Scattered microtubules and abundant lipid droplets (Fig. 3) and pigment granules were visible. The latter displayed highly-heterogeneous morphology and electron-density (Fig. 2).

Pinealocyte processes, arranged in a clearly perivascular manner, contained abundant microtubules, some mitochondria and scattered reticulum elements (Fig. 4). These processes terminated in very large bulbous endings filled with light vesicles (Fig. 4).

The second visible cell-type, termed interstitial cells [10], displayed two distinctive features: 1) they were less numerous than pinealocytes; and 2) they followed a clearly perivascular arrangement. The rounded nucleus contained granular chromatin scattered throughout a clear nucleoplasm (Fig. 5). The characteristic nucleolus was composed of a highly electron-dense nucleolonema surrounding a fibrillary core. Cytoplasm was less electron-dense and contained fewer organelles than that of pinealocytes. Endoplasmic reticulum was mostly granular, and cisternae had fairly narrow lumina. Lysosomes with clearly defined limiting membrane were observed in perinuclear cytoplasm, together with diplosomes and ribosomes. Pigment granules of varying shape and electron-density were occasionally observed (Fig. 6, 7).

The most characteristic feature of these cells was the presence of very long processes with numerous microfilaments (Fig. 5, 7). Interstitial cell processes were in close contact with pinealocyte processes (Fig. 4).

Pineal gland vascularisation consisted in capillaries composed of one or two endothelial cells, whose cytoplasm contained the usual organelles: mitochondria, Golgi complex, rough and smooth endoplasmic reticulum, multivesicular bodies and both endocytic and exocytic vesicles. A slim, continuous capillary basal membrane was visible (Fig. 1), but displayed no distinctive characteristics. Capillaries were not fenestrated (Fig. 1, 8), and the relatively narrow pericapillary space contained non-myelinated nerve fibers (Fig. 8), and projections from pinealocytes and interstitial cells.

Non-myelinated nerve fibers were arranged around capillaries, between pineal parenchyma cell projections (Fig. 8). Specific contact areas were frequently observed between nerve fibers and the terminal clubs of interstitial cell processes (Fig. 8).

Stroma surrounding the perivascular space appeared to be composed of a tiny amount of collagen fibers (Fig. 1).

C) GROUP 2 (9 MONTHS, 1 YEAR AND 2 YEARS)

Pinealocytes displayed significant differences with respect to those of the previous group. Nuclei (Fig. 10) presented a variety of shapes: oval, rounded and elongated. The nuclear membrane showed patent pores as well as diaphragms. A prominent nucleolus with clearly-defined nucleolomema was visible, adhering to the nuclear envelope with chromatin densifications (Fig. 9). Cytoplasmic organelles included well-developed Golgi complexes with associated light and dense-cored vesicles (Fig. 11); these vesicles were seen in the pinealocyte soma and also occurred in processes. Oval or elongated mitochondria were scattered throughout the cytoplasm (Fig. 11); elongated mitochondria were characterized by a dense matrix and longer tubular cristae, whereas the more numerous oval mitochondria were both larger and less electron-dense. Both types were often present within the same cell. Rough endoplasmic reticulum (ER) was more abundant than smooth ER (Fig. 11). Cytoplasmic organelles especially Golgi complexes, rough endoplasmic reticulum, mitochondria and ribosomes were more numerous than in the previous group. Cytoplasm contained lipid droplets, glycogen granules and pigment granules of varying size, shape and electron-density (Fig. 11).

Pinealocyte processes and endings contained the usual organelles, including light vesicles, dense cored vesicles, mitochondria, lysosomes and numerous microtubules and gap junctions (Fig. 12). All bulbous endings of pinealocyte processes ended in the pineal parenchyma instead of in the perivascular space.

Both pigmented and non-pigmented interstitial cells were observed. Their histological features were similar to those described earlier, except for:

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**Figure 9.** — Female sheep pineal gland. 9 months old. Pinealocyte with large nucleus, prominent nucleolus, loose chromatin and slightly electron-dense hyaloplasm. Moderate organelle development. EM x 15,000.

**Figure 10.** — Female sheep pineal gland. 1 year old. Pinealocyte adjacent to an interstitial cell process (IP). EM x 20,000.

**Figure 11.** — Male sheep pineal gland. 1 year old. Pinealocyte cytoplasm (detail) showing cisternae and smooth endoplasmic reticulum (ER) vesicles (arrows); Golgi complex; mitochondria with laminar crests; glycogen granules (asterisks) and liposomes. EM x 15,000.

**Figure 12.** — Male sheep pineal gland. 2 years old. Gap junctions in the terminal clubs of pinealocyte processes. EM x 10,000.

**Figure 13.** — Female sheep pineal gland. 9 months old. Interstitial cell containing microfilaments, lysosomes and pigment granules. EM x 8,000.

**Figure 14.** — Female sheep pineal gland. 1 year old. Immune electronmicroscopic demonstration of GFAP, nonstained nuclei and positive cytoplasm in interstitial cells. IEM x 10,000.

**Figure 15.** — Male sheep pineal gland. 1 year old. Interstitial cell cytoplasmic processes (IP) containing abundant microfilaments and gap junctions (arrow) adjacent to a pinealocyte process (PP). EM x 30,000.

**Figure 16.** — Male sheep pineal gland. 9 months old. Non-myelinated nerve fibers in perivascular space. Specific contact sites between nerve fibers and interstitial cell processes (arrow). EM x 15,000.

• Greater electron-density of nucleoplasm and cytoplasm (Fig. 13).
• Greater abundance of cytoplasmic organelles, particularly Golgi complexes, mitochondria and rough endoplasmic reticulum (Fig. 13).
• Cytoplasmic processes displayed a larger number of microfilaments (Fig. 14).
• Greater abundance of gap junctions in the bulbous endings of cytoplasmic processes (Fig. 15).
• Development of gap junctions with pinealocyte processes (Fig. 15).

Non-fenestrated intrapineal capillaries were more abundant (Fig. 16, 17). Each capillary was composed of 2 or more endothelial cells. Histologically, cytoplasmic organelles in these cells largely resembled those reported for group 1, except that RER, Golgi complexes and mitochondria were more developed (Fig. 17). The capillaries were bounded by a continuous basal lamina. There was an increase in the amount of stroma bordering the vascular space, and collagen microfibrils were numerous (Fig. 17).

Non-myelinated nerve fibers with adielectric electron-dense vesicles were observed in the large perivascular space, intercalated between cytoplasmic projections from pinealocytes and interstitial cells (Fig. 16). Specific contact sites between these nerve fibers and interstitial cell cytoplasmic processes were also detected (Fig. 16).

**D) GROUP 3 (> 2 YEARS OF POSTNATAL DEVELOPMENT)**

Pinealocytes nuclei were smaller, with clustered chromatin clearly visible within a dense nucleoplasm (Fig. 18). A single nucleolus was frequently observed. Cytoplasm was highly electron-dense and contained few organelles (Fig. 18). Golgi complexes and endoplasmic reticulum were particularly sparse. Mitochondria varied in both shape and distribution (Fig. 18, 19), but were frequently detected in association with liposomes (Fig. 19). Lipid droplets were abundant, large and highly electron-dense (Fig. 19).

Cytoplasmic processes were scarce and slender, and microtubules were fairly numerous (Fig. 18). Terminal clubs of cell processes displayed fewer gap junctions. Interstitial cell nuclei were ovoid, with a prominent nucleolus; the clear nucleoplasm contained granular chromatin (Fig. 20, 21). RER cisternae, ribosomes and Golgi complexes were relatively sparse. These cells often contained occasional lipofuscin granules.

Cytoplasmic processes contained fewer microfilaments than in the previous group (Fig. 20, 21), and terminal clubs displayed fewer gap junctions; these interstitial cell processes were intermingled with pinealocyte processes (Fig. 22) nerve fibers in the perivascular space. Pigment granules were infrequently observed in pinealocytes, and virtually absent from interstitial cells.

Non-fenestrated capillaries composed of several endothelial cells, containing a moderate abundance of cytoplasmic organelles, were separated from the adjacent collagen fibers by a slender, continuous basal membrane. Arterioles were also visible (Fig. 23) contained erythrocytes and platelets. The smooth muscle surrounds the endothelial cell. A basal lamina is apparent at the peripheral border of the smooth muscle cell.

Collagen fibers (Fig. 24) and non-myelinated nerve fibers, were visible in connective tissue of perivascular space (Fig. 23). The disposition of nerve fiber was, along cytoplasmic projections pinealocytes and interstitial cells.

**E) IMMUNOHISTOCHEMICAL EXAMINATION**

GFAP-positive cells were observed in the pineal parenchyma of all animals studied. Immunostaining yielded similar results for all groups, and no sex-related differences were detected.

Colloidal gold labelling revealed expression of GFAP by cells whose morphology closely resembled that described ultrastructurally for the second cell population (interstitial cells). These cells were distributed uniformly throughout the gland, mainly located close to blood vessels. The GFAP-positive cells exhibited ovoid and/or elongated non-staining nuclei, (Fig. 7, 14) and strong cytoplasmic positivity, with clear affinity for the microfilaments of cytoplasmic processes (Fig. 14). Both pigmented and non-pigmented interstitial cells stained positively (Fig. 7, 14), while adjacent pinealoblasts remained immunonegative.
Discussion

During postnatal development, ovine pineal parenchyma is composed of two cell types: pinealocytes and interstitial cells.


Our findings indicate that the ultrastructural differences observed between pinealocytes are probably the result of different functional states of the same cell type [9, 13]. This assertion is based on one consideration: the degree of electron-density depends on the abundance of ribosomes, on the presence of electron-dense material associated with rough endoplasmatic reticulum, and on the extent of the rough endoplasmatic reticulum (ER) hypertrophy.

Throughout postnatal development, pinealocyte cytoplasmic processes display a marked vascular tropism. This affinity, reported elsewhere [11, 12, 18, 20] together with the presence of both light and dark vesicles in terminal clubs [4, 11, 12, 18] and of gap junctions [8,11, 12, 18] suggest that pinealocytes may have certain secretory functions. The presence of all the cytoplasmic structures involved in secretory metabolic activity and the greater degree of development of these structures in group 2 pinealocytes, lend morphological support to this hypothetical functionality [3, 18, 20, 24].

Glycogen granules were occasionally observed in pinealocytes, though never in animals more than 1 year old. Glycogen has also been reported during prenatal development of the ovine pineal gland [18]. The gradual disappearance of glycogen during postnatal growth suggests that it is a differential characteristic of cell immaturity [6, 18, 19]. However, CALVO et al. [7], argue that glycogen disappearance is merely evidence of the lability of glycogen to routine techniques.

Synaptic ribbons were not detected during postnatal development, perhaps for the same reasons that they are not encountered in ovine pineal gland ontogenesis [18]:

- Animals were sacrificed during the daytime, while the pineal gland displays an elevated circadian rhythm at night [9,16].
- The abundance of synaptic ribbons is inversely proportional to that of non-myelinated nerve fibers, which were highly abundant here. Similar findings are reported for various carnivores [6, 7]. However, synaptic ribbons are commonly detected in cats [4].

Numerous terms have been used to designate the second cell population in mammalian pineal gland parenchyma, including interstitial cells [9, 10, 13, 17], Type II pinealocytes [5], glial cells [1, 6], and astrocytes [4, 26]. We have opted to use the term «interstitial cells» [10, 18, 19, 20, 21]. Ultrastructurally, these cells tend to be fairly homogeneous, as reported for prenatal development [10]; however, interstitial cells morphology in group 2 shows clearer evidence of functional activity.

Gap junctions between interstitial-cell terminal clubs were most common in animals slaughtered between the ages of 9 months and 2 years. Similar findings are reported for pinealocytes. This would appear to suggest that: 1) gap junctions play an important role in ovine pineal parenchyma, acting as control barriers for the circulation of intercellular fluids [8, 25]; and 2) group 2 pineal glands are the most functionally active, given the abundance of gap junctions between pinealocytes, between interstitial cells and between these two cell types [8].

Both ultrastructurally and in terms of antigen expression of GFAP, interstitial cells displayed characteristics similar to those reported for astrocytes [4, 9, 26]. GFAP positive cell processes were located close to perivascular spaces [13, 23], indicating a possible functional significance of interstitial cells as substrate for the exchange of substances between the pineal parenchyma and the bloodstream [10, 18, 19, 20, 23].

It would be interesting to determine the relationship between interstitial cells and nerve fibers. Site of specific contact were observed between both of them, and were more abundant in the most morphologically active glands (group 2). The precise functional significance of this relationship is not known, although it has been suggested that nerve fibers might be involved in interstitial cell development; indeed, these cells do not develop in the absence of innervation [5].

Prenatal differentiation of cells containing pigmented granules in the developing ovine pineal gland [21] is confirmed during postnatal growth. Pigment granules were detected in both pinealocytes and interstitial cells. Similar findings are reported for pre- and post-natal development in dogs [6]. The existence of pigmented cells as a distinct population from pinealocytes and interstitial cells, as reported in prenatal development of the ovine pineal gland [21] was not observed here during postnatal growth. If these cells play a major role in pigment biosynthesis during prenatal development, it might be asked which population assumes that role postnataally. The answer would appear to be the pinealocytes, for two reasons: 1) the abundant pigment granules detected in these cells; and 2) the heterogeneous nature of these granules in terms of shape, size and electron-density.

Stroma was rich in non-myelinated nerve fibers [4, 7], in collagen microfibrils and capillaries. Stroma generally resembled that observed in sheep embryos at 150 days’ gestation [18].

Intrapineal capillaries were non-fenestrated, and similar to those encountered in prenatal development of sheep [10, 18, 20]; and in postnatal development of carnivores [4, 7]. Their presence during postnatal development bears out the hypothesis put forward by REDONDO et al., [18], who postulated the non-existence of the blood-brain barrier in the developing sheep pineal gland.
In conclusion, the ovine pineal gland displays morphological evidence of endocrine metabolic activity throughout postnatal development. Pinealocytes contained all the cytoplasmic structures involved in secretory mechanisms [3]. Interstitial cells, in addition to their classical support role, may be involved in the exchange of substances between the pineal parenchyma and the bloodstream [10].

Both pinealocytes and interstitial cells were highly active in group 2 animals, particularly in terms of creating gap junctions between cytoplasmic processes. Given that the coordinated functions of the pineal gland depend on the junctional systems existing between pinealocytes, between interstitial cells and between pinealocytes-interstitial cells [8], it is significant that between 9 months and 2 years of age the ovine pineal gland displays the greatest degree of morphological and thus functional differentiation. This is borne out by the intense vascularisation and the abundance of nerve fibers and gap junctions, and greater development of stroma.

From three years onwards, there is morphological evidence of cellular and consequently glandular decline: decreased vascularisation, fewer non-myelinated nerve fibers and gap junctions, and greater development of stroma.

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