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# Embryonic development of the rabbit pineal gland *(Oryctolagus Cuniculus)* (Lagomorpha)

**J.E. García-Mauriño, C. Sicilia, J. Boya and J.L. Calvo**

*Department of Cell Biology. Faculty of Medicine. Complutense University of Madrid, Spain*

SUMMARY

The embryonic development of the rabbit pineal gland was studied from day 12 post conception (E-12) until day 30 (E-30, the end of gestation). The pineal anlage appears on E-12 as a thickening of the neuroepithelium lining the roof of the diencephalon. By E-14, a rostrally-orientated evagination appears in this area. From E-18 the pineal gland grows caudally, acquiring an elongated shape with a distal thickening. Although still small (1.469 mm in length), it shows its final form and anatomical relationships by E-22. On E-30 it is 2.99 mm long and has a volume of 0.123 $mm^3$. Characteristic of the embryonic pineal parenchyma are rosette-like structures formed by type II pinealoblasts with ovoid, heterochromatic nuclei, located around a narrow lumen. These rosettes originate through the intense mitotic activity of the neuroepithelium lining the pineal recess. As development proceeds, the rosettes begin to lose their structure and separate from one another due to the proliferation of cell cords between them. These cords are composed of type I pinealoblasts, which have rounded nuclei and loose chromatin. Melanin granules are first seen on E-16 in type II pinealoblasts. Therefore, the greatest amount of pigmentation is seen in the rosettes, mainly in those located at the enlarged, distal end of the gland. The connective tissue septa, which carry blood vessels, invade the pineal gland from the thin capsule. From E-18 they develop throughout the rest of embryonic life, contributing to the gradual separation of the rosettes.

From the beginning of its development, the pineal gland is associated with large cerebral veins. A large part of the gland ends up occupying an "intravascular" position. We suggest that during embryonic development, the pineal gland may act, at least in the rabbit, as a reference for the location and organisation of large cerebral veins.

**Key Words**: Pineal gland – Embryonic development – Rabbit – Pigment

## INTRODUCTION

The embryonic development of the mammalian pineal gland has mainly been studied in rodents such as the rat (Gardner, 1949; Kappers, 1960; Owman, 1960, 1961; Clabough, 1973; Calvo and Boya, 1981; Ueck, 1986) and hamster (Clabough and Siebel, 1968; Clabough, 1970, 1973), and also in the cow (Hollman, 1963; Anderson, 1965), sheep (Brack, 1962; Redondo et al., 1996; Regodón et al., 1998), mole (Pevet, 1980) and in rare species such as the Indian palm squirrel *Funambulus pennanti* (Haldar-Misra y Srivastava, 1986) and the marsupial *Dasyurus hallucatus* (Ueck, 1986).

The rabbit (Lagomorpha) has an elongated pineal gland [8 mm long in the adult, (Romijn, 1973a)] with a thickened distal end - a shape uncommon in mammals. Its structure, ultrastructure and innervation have been widely studied by Romijn (1973a,b,c; 1975a,b; 1976), Romijn and Gelsama (1976), Romijn et al. (1976; 1977a,b). Romijn (1973a), using light microscopy, described the adult rabbit pineal gland as having a cortex and medulla. The pineal parenchyma is formed by two cell types: type I pinealocytes, with clear cytoplasm and a lobed nucleus, and type II pinealocytes, with oval nuclei and dense cytoplasm commonly containing pigment. The postnatal development of the gland has been described by Kerenyi and von Westarp (1971), and by García-Mauriño and Boya (1992a,b).

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Correspondence to:
Prof. Dr. J. Boya Vegue. Dept. Biología Celular. Facultad de Medicina,
Universidad Complutense. 28040 Madrid. Spain. Fax: 91 3941396

With respect to the embryonic development of the rabbit pineal gland, only one paper, published in 1937 by Turkewitsch, is available. This work describes only three phases of development, and provides little histological information. Since then, the only reference to the rabbit embryonic pineal gland was made by Holmes (1957), who described transplanted pineal glands of 20 and 26 day-old foetuses into the anterior chamber of the eye of adult animals. Moller et al. (1975), however, reported the presence of the foetal pineal nerve in embryos at 23 and 24 days of gestation.

The present report describes a detailed study of the embryonic rabbit pineal gland using light microscopy.

## Materials and methods

A total of forty embryos of pigmented (tawny Borgogne) and albino (New Zealand White) rabbits (gestation period: 30 days) were used. The day of mating was considered as day 0. At embryonic days E-12, E-14, E-16, E-18, E-20, E-22, E-24, E-26, E-28 and E-30, samples of at least four embryos were taken for light microscopic analysis. Brains were fixed in Bouin's solution, embedded in paraffin and serially sectioned (7μ). Two cutting planes were chosen: sagittal and frontal. Sections were stained with haematoxylin-eosin, the Gordon and Sweet silver method for reticular fibres, or the Masson-Fontana method for pigment melanin. Melanin bleaching techniques using potassium permanganate were also performed.

For morphometric studies, a VIDS IV® computerised image analyser was used. The following measurements were made of one of every three sections: the length of the pineal gland in sagittal sections, total surface area, surface area occupied by rosettes (including that occupied by the pineal recess), the surface area of the cell cords or pineal parenchyma not forming part of the rosettes, and the number of cells undergoing mitosis located both in the rosettes and the cell cords (mitoses clearly within the connective tissue septa were excluded). The measurement of the surface area of the cell cords was performed as follows: the area occupied by the rosettes, plus that of the connective tissue septa, was subtracted from the total surface area of the section. The estimation of glandular volume was determined in sagittal sections, multiplying the surface areas of the sections by their thickness.

## Results

### *Appearance of the pineal anlage*

The pineal anlage appears on day 12 of embryonic development (E-12). Modifications appear in an area of the midline of the roof of the diencephalon. There is a discrete thickening of the neuroepithelium, where many degenerated cells are found mixed with others undergoing mitosis. At the same time, in the mesenchyma adjacent to this region, blood vessels with wide lumens develop (Fig. 1).

By E-14, an evagination (0.17 mm in length) appears in the midline of the roof of the diencephalon. This takes the form of a sac with an oblique trajectory orientated rostrally (Fig. 2). It is the only structure that stands out in the mesenchyma surrounding the encephalon, and is situated very superficially. This pineal anlage is composed of a stratified neuroepithelium lining a narrow lumen corresponding to the primitive pineal recess. The cells closest to the lumen are prismatic with heterochromatic, ovoid nuclei displaced towards the base of the cell, leaving a thin cytoplasmic band in the immediate vicinity of the recess (Fig. 3).

Close to the lumen, mitotic cells are abundant. At the base of the neuroepithelium, in the peripheral-most areas of the pineal anlage, cells with round nuclei are seen (Fig. 3). The degenerated cells seen in the last phase are no longer observed. The posterior commissure is seen about 0.5 mm caudal to the pineal anlage (Fig. 2).

The whole external surface of the anlage is closely associated with large, developing blood vessels (Fig. 3). In transverse sections, smaller vessels are visible on both lateral faces of the anlage. These vessels will maintain their position throughout development.

### *DAY 16*

The sac-like evagination emanating from the roof of the diencephalon becomes more pronounced, reaching 0.387 mm in length (Table 1). It also becomes more irregular in contour (Fig. 4). The scarce mesenchyma that surrounds the pineal anlage constitutes the capsule of the growing gland. The anlage follows a rostral, oblique trajectory, as before. The neuroepithelium that covers the lumen of the pineal recess is irregularly folded. Cells close to the lumen have characteristics similar to those described in the previous phase, although the cytoplasmic band close to the lumen of the pineal recess becomes more noticeable (Fig. 5). In these cells, a few small granules of brownish pigment begin to appear (Fig. 5). Mitotic activity increases considerably in this phase (Graphic 2), most (90.9%) dividing cells being located close to the lumen of the pineal recess. In the external-most part of the epithelium covering this cavity, close to the capsule, a few cells with rounded nuclei, loose chromatin and one or two nucleoli are observed (Fig. 5). The posterior commissure is situated immediately caudal to the gland's origin (Fig. 4).

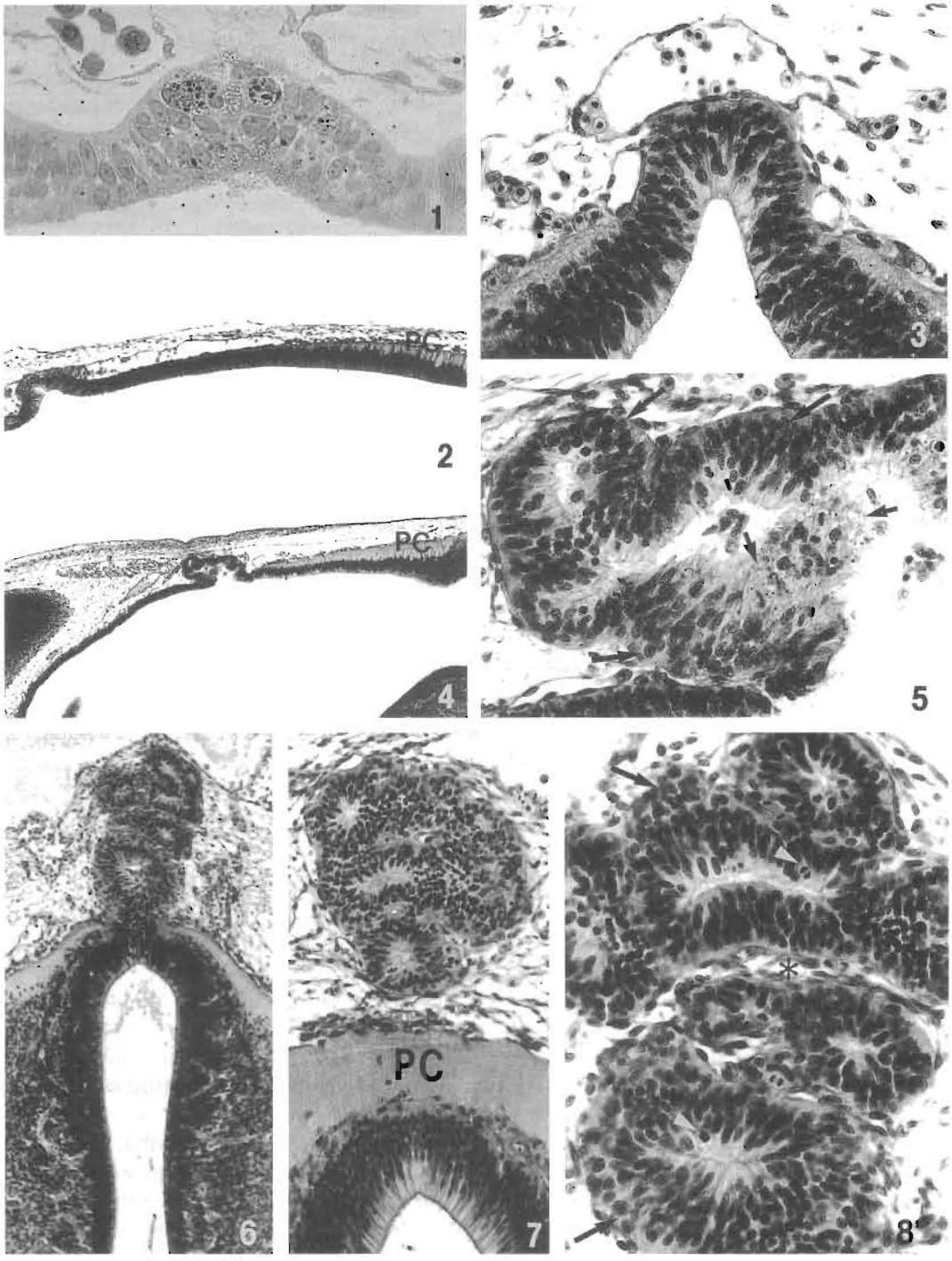



**Fig. 1.-** E-12. Frontal section. Semi-thin section (fixed in 3% glutaraldehyde, embeded in vestopal and stained with silver nitrate). Enlargement of the neuroepithelium of the diencephalon roof where the pineal gland originates. Its appearance is stratified, and some degenerated cells are seen. Note, in the mesenchyma close to the neuroepithelium, the proximity of blood vessels in development, occupied by nucleated erythrocytes. x1000.

**Fig. 2.-** E-14. Sagittal section. HE. Sac-like evagination of the pineal anlage coming from the roof of the diencephalon, distant from the posterior commissure (PC). The narrow lumen of the pineal anlage corresponds to the primitive pineal recess. x40.

**Fig. 3.-** E-14. Frontal section. HE. The pineal anlage is lined by stratified neuroepithelium. Close to the lumen of the pineal recess there is an eosinophil band, a consequence of the confluence of the apical poles of cells closest to the lumen. At the base of the neuroepithelium there are cells with round nuclei. Note the close association of the pineal anlage with blood vessels of variable diameter. x400.

**Fig. 4.-** E-16. Sagittal section. HE. Pineal gland growing rostrally. The lumen of the pineal recess is wider and has a more irregular contour than in the previous phase. Note the proximity of the pineal anlage and the posterior commissure (PC). x40.

**Fig. 5.-** E-16. Sagittal section. HE. Detail of the pineal anlage. The neuroepithelium covering the anlage has an irregular contour. The cells in immediate contact with the lumen have a more noticeable apical cytoplasmic band than in earlier phases. In this area, small granules of pigment can be seen (small arrows). Mitotic activity is abundant close to the lumen. Outside the neuroepithelium, cells with round nuclei and loose chromatin are observed (large arrows). x400.

**Figs. 6 and 7.-** E-18. Frontal sections. HE. In proximal sections (Fig. 6), the association between the immature pineal gland and the diencephalon roof can be appreciated. In Fig. 7, the pineal gland is close to the posterior commissure (PC), demonstrating caudal growth of the gland. The rosettes –seen as irregular rounded structures– are evident. Fig. 6 = x100; Fig. 7 = x200.

**Fig. 8.-** E-18. Frontal section. HE. Abundant rosettes are seen with numerous mitoses (arrowhead). Outside the rosettes are type I

**Table 1.**- Values for length and volume were obtained from sagittal sections. The values are relative data: the global result of measuring sagittal and frontal sections.

| Gestation age | E-14 | E-16 | E-18 | E-20 | E-22 | E-24 | E-26 | E-28 | E-30 |
|---|---|---|---|---|---|---|---|---|---|
| Length (mm) | 0,17 | 0,387 | 0,706 | 0,956 | 1,469 | 1,82 | 2,606 | 2,637 | 2,99 |
| Volume ($mm^3$) | 0,000538 | 0,00409 | 0,0016 | 0,0252 | 0,0372 | 0,06 | 0,057 | 0,095 | 0,123 |
| % Rosette Surface | 41,59 | 45,25 | 45,43 | 34,71 | 31,11 | 29,74 | 19,7 | 20,92 | 11,56 |
| % Cell Cords Surface | 58,41 | 54,74 | 47,29 | 62,35 | 64,2 | 67,53 | 66,94 | 63,63 | 69,04 |
| % Rosette Mitosis | 90,9 | 79,42 | 69,41 | 56,7 | 52,91 | 52,68 | 43,33 | 43,85 | 37,55 |
| % Cell Cords Mitoses | 9,6 | 20,57 | 30,58 | 43,29 | 47,08 | 47,31 | 58,88 | 56,14 | 62,45 |
| Mitosis/100.000 $\mu m^2$ | 19,55 | 22,84 | 23,67 | 18,42 | 20,65 | 15,94 | 9,22 | 7,67 | 5,62 |

**% Area of Rosettes**: Proportion of the section occupied by rosettes with respect to that occupied by cell cords.
**% Area of Cell Cords:** Proportion of the section occupied by cords with respect to that occupied by rosettes. The missing % (that needed for 100% total) is occupied by the connective tissue septa.
**% Mitosis in Rosettes:** The percentage of mitotic events in the section localised in the rosettes.
**% Mitosis in Cell Cords:** The percentage of mitotic events in the section localised in the cell cords.
**Mitosis/100.000 $\mu m^2$:** Total number of mitotic cells in the pineal parenchyma for 100.000 $\mu m^2$ of section surface area.

*DAY 18*

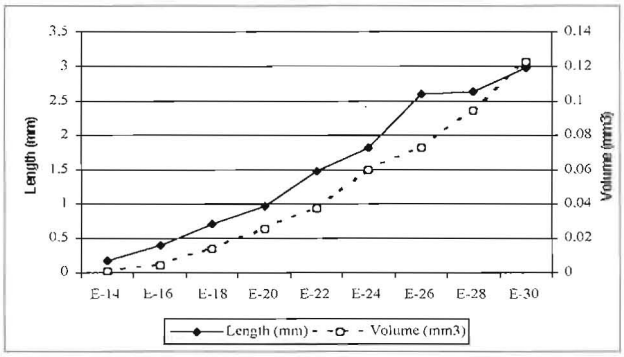
Up to this point, the gland has grown in predominantly the rostral direction, but now starts to follow a caudal path due to the greater growth of the posterior face of the pineal anlage. It becomes elongated, following a straight trajectory 0.766 mm long. Transverse sections reflect caudal growth because of the proximity of the pineal gland to the posterior commissure (Figs. 6 and 7). The wall of the pineal recess forms complex folds resembling to rosettes. These rounded or elongated structures, each with a small lumen surrounded by cells with chromatin organised in small clumps, are similar to those contacting the lumen of the pineal recess in the previous phases. In the cells that border the rosette lumens, small granules of brownish pigment can be observed, especially in the distal-most cells of the gland. Positive staining using the Masson-Fontana technique, and bleaching with an oxidising agent (potassium permanganate) allow this pigment to be identified as melanin. The presence of this pigment, plus the shape of the nuclei and their later development, identifies these cells as type II pinealoblasts. Outside the rosettes lie cells with round nuclei, loose chromatin and one or two nucleoli, corresponding to type I pinealoblasts. These are organised into thin, irregular cell cords (Fig. 8).

In this phase, the greatest density of mitoses per surface area is observed (Graphic 2). Dividing cells are found mainly in the rosettes (61.94%) (Table 1). They are also found, although in a smaller proportion, in the peripheral-most part of the gland, close to the capsule and especially in the most caudal region.

In transverse sections, thin connective tissue septa bearing blood vessels are seen (Fig. 8); outside, large blood vessels are observed.

*DAY 20*

The pineal gland increases in volume, reaching 0.0252 $mm^3$, and elongates, attaining a length




**Graphic 1.**- Variations in length and volume of the rabbit pineal gland during embryonic development. Values were obtained from sagittal sections.

of 0.956 mm (Graphic 1). Dorsally, but closely associated with the gland, large cerebral blood vessels are found, maintaining the relationships established at the beginning of embryonic development.

In the pineal parenchyma, the rosettes are still noticeable, although they occupy a smaller surface area than in earlier phases (34.71% of the section). Although the rosettes generally are variable in form, those located in the proximal zone of the gland tend to be elongated (Fig. 9). In the distal-most regions they are more rounded, and frequently fold over themselves to acquire complex and irregular shapes (Fig. 10). Some of them show loss of structure: a disorganisation of cells that in turn leads to a loss of the rosette lumen. The result is a series of irregular accumulations of type II pinealoblasts. Study of serial sections shows that they are not images of tangentially-cut rosettes. Cell cords of type I pinealoblasts, intermingled with the same of type II pinealoblasts, are found among the rosettes.

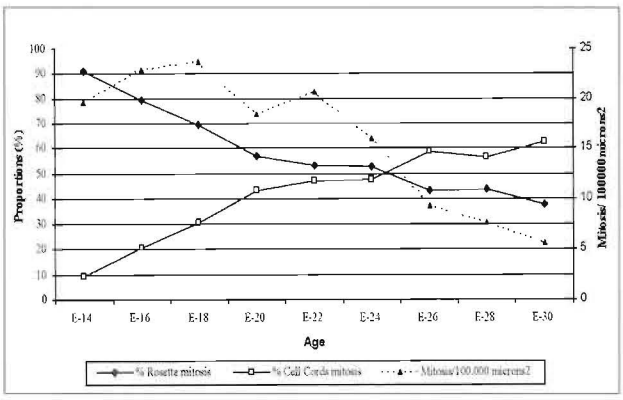
Mitotic activity (mitoses per unit area) is reduced during this phase. However, it tends to be more equally shared between the rosettes (56.7%) and cell cords (43.29%). In the rosettes, mitotic phenomena occur close to the lumen.

Pigment is now very evident, mainly at the distal end of the gland. It is found in the centre of the rosettes, in the cytoplasmic regions of the type II pinealoblasts that line the narrow lumen (Fig. 11). In the distal region itself, the quantity of pigment varies among rosettes. In lower proportions, pigmented cells are also seen in the cell cords (Fig. 11).

*DAY 22*

During this phase the pineal gland acquires its definitive shape. The gland becomes considerably longer, reaching a length of 1.469 mm (Graphic 1), and shows a thickening at its distal end (Fig. 12). At the same time, the gland takes on a slightly sinuous trajectory, especially in the intermediate region. Bearing in mind its anatomical relationships, three zones can be identified: 1) The proximal zone (its dorsal surface is associated with a small extension of the third ventricle –the suprapineal recess– whose covering of ependimary cells rests on the capsule; 2) the intermediate zone (its dorsal face is associated with the medial prosencephalic vein); 3) the distal zone (corresponding to the caudal thickening of the gland which protrudes into the confluence sinuum). Along its whole length, the ventral face is associated with the great cerebral vein, which finally empties into the confluence of the transverse sinuses (Fig. 12).

In the pineal parenchyma, although the rosettes are still abundant, their ratio with respect to the cell cords is reduced (Graphic 3). The




**Graphic 2.-** **Proportion of global mitoses** of rosettes and cell cords with respect to section area. These values are compared with the distribution of total mitotic events in the pineal parenchyma with respect to section surface area.

rosettes in the proximal third are elongated and course parallel to the main axis of the gland (Fig. 13). Their narrow lumens are continuous with the pineal recess, which in turn communicates with the third ventricle. As one approaches the distal region, the rosettes become more complex and irregular, and destructuring is common (Fig. 14). Although the global mitotic activity of the gland undergoes a discrete increase, the ratio of mitotic events between cell cords (47.08%) and rosettes (52.91%) is similar to the previous phase (Graphic 2) (Table 1).

Pigment is found throughout the gland, but there is a clear gradient increasing towards the distal end. This pigment tends to concentrate at the centre of the rosettes, although it can also be seen in isolated cells in the cords (Fig. 15).

*DAY 24*

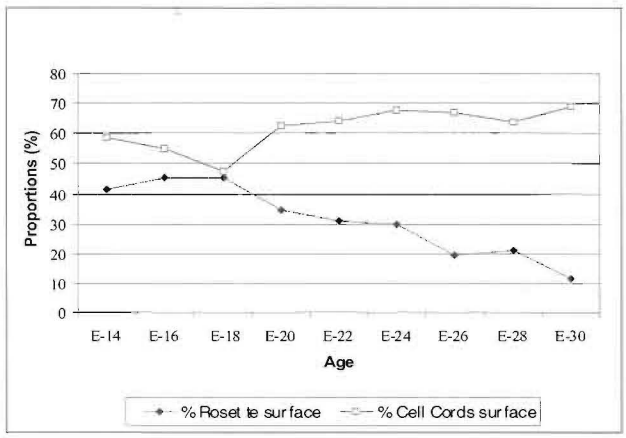
The pineal gland is now 1.82 mm long and has a volume of 0.060 $mm^3$ (Graphic 1). During this phase, there is progressive growth of the cell cords, leading them to occupy 67.53% of the section area. This elicits a gradual separation of the rosettes, which become even more irregular, especially in the distal region (Fig. 16).

Pigment is still abundant, mainly in the rosettes located in the distal portion of the pineal gland.

*DAY 26*

During this phase the gland becomes longer (2.606 mm) and larger (0.073 $mm^3$) (Graphic 1). The anatomical relationships described for day 22 are maintained. The pineal parenchyma is still organised in rosettes formed by type II pinealoblasts. The rosettes at the intermediate third of the gland become ramified and acquire more complex forms, and those of the distal third undergo destructuring and separation owing to the increased development of the cell cords. Mitoses, which previously were more common in the rosettes, are now more frequent in the cell cords (58.88%) (Graphic 2).

Pigment distribution is maintained, being more frequent in the distal third. It is localised mainly in the central part of the rosettes, but now pigmented cells are commonly seen in the cell cords. The latter cells may be type II pinealoblasts that have become isolated as a consequence of rosette destructuring.




**Graphic 3.-** Development of the relative proportions occupied by rosettes and cell cords with respect to total section area. The missing % (needed to make 100%) is that occupied by the connective tissue septa.

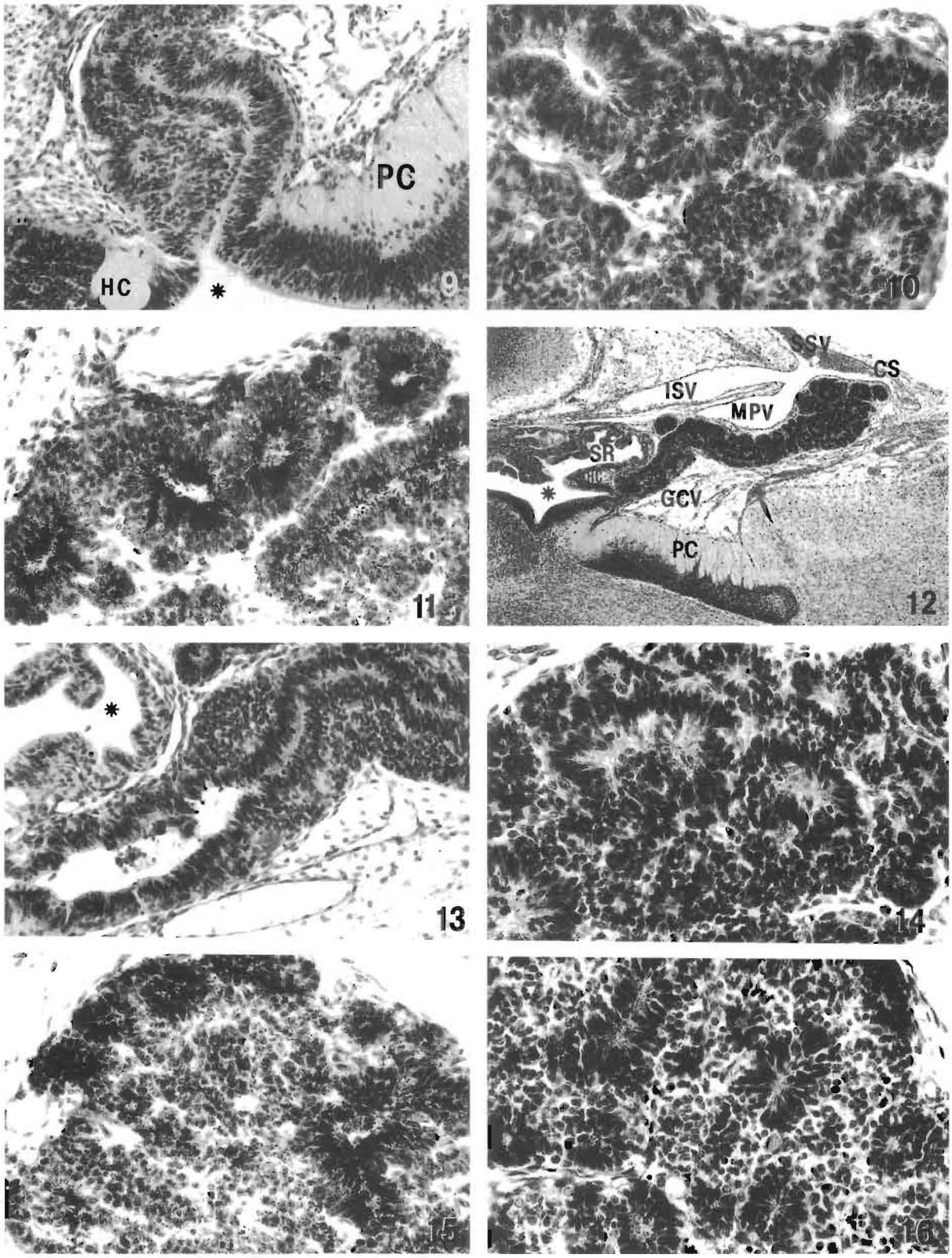



**Fig. 9.-** E-20. Sagittal section. HE. Origin of the pineal gland situated between the habenular commissure (HB) and the posterior commisssure (PC). An elongated proximal rosette has its lumen in contact with the third ventricle. (*): third ventricle. x200.

**Fig. 10.-** E-20. Frontal section. HE. Distal zone. Irregular rosettes. x400.

**Fig. 11.-** E-20. Frontal section. Masson-Fontana method. Distal zone. Rosettes with abundant melanin pigment close to the lumen. Some pigment-containing cells are seen outside the rosettes. x400.

**Fig. 12.-** E-22. Sagittal section. HE. Final form and anatomical relationships of the pineal gland. CS: confluens sinuum; GCV: great cerebral vein; HC: habenular commissure; ISV: inferior sagittal vein; MPV: medial prosencephalic vein; PC: posterior commissure; SR: suprapineal recess; SSV: superior sagittal vein; (*): third ventricle. x40.

**Fig. 13.-** E-22. Sagittal section. HE. Rosettes are seen parallel to the longitudinal axis of the gland, whose lumens communicate with the third ventricle. The wide lumen seen in the figure may be an artefact. (*): suprapineal recess of third ventricle. x200.

**Fig. 14.-** E-22. Sagittal section. HE. Destructured rosettes in the distal zone of the pineal gland. x400.

**Fig. 15.-** E-22. Frontal section. Masson-Fontana method. Cells with melanin granules between the rosettes. x400.

**Fig. 16.-** E-24. Sagittal section. HE. Rosettes of the distal zone separated from one another by wide cell cords. x400. Compare with Fig. 14.

*DAYS 28-30*

At the end of development the pineal gland attains a length of 2.99 mm and a volume of 0.123 $mm^3$ (Graphic 1). Its shape is now the same as in the adult (Fig. 17). In serial transverse sections, the contour of the gland changes depending upon the region. In the proximal third, while the gland is associated with the suprapineal recess, it is flattened (Fig. 18a). In the intermediate third, the pineal contour becomes more irregular (Figs. 18b, c), and finally rounded (Fig. 18d). The distal thickening has an ovoid or slightly triangular contour (Figs. 18e, f).

The close association established with the large blood vessels in previous phases is maintained, and even the capsule of the pineal gland forms an integral part of the vein walls. The distal end of the gland fully invades the lumen where the transverse sinuses meet.

The rosettes become less common in the pineal parenchyma, but are still noticeable. The lumens of those located in the proximal zone are continuous with the third ventricle. The rosettes of the distal zone are frequently destructured. The cell cords develop progressively due to a relative increase in mitosis (62.45%) and the destructuring of the rosettes formed of type II pinealoblasts. The latter cells become incorporated into the cords. This can be appreciated in the gradual increase in the number of pigmented cells inside. The gland's pigment distribution is maintained: much is found at the far end of the gland and less in proximal regions. The pigment is more abundant in the central zones of the rosettes (Fig. 19, inset).

## Discussion

During the embryonic development of the rabbit pineal gland, two periods can be observed: 1) a morphogenetic period lasting from the appearance of the anlage (E-12) until the gland acquires its final form and establishes its definitive anatomical relationships (E-22); and 2) a period of growth in which the gland increases in length and volume, following clearly ascending curves (see graphics). During this period, the stroma and pineal parenchyma become reorganised. The length of the pineal gland at the end of embryonic development is 17.5-fold that of the anlage on E-14. The volume increases 288.6-fold during the same period. Quay (1974) established three phases in the development (both embryonic and postnatal) of the rat pineal gland: the morphogenetic, proliferative, and differentiation stages. During the embryonic development of the rabbit, however, only the first two are seen. No signs are seen, at least by light microscopy, of cellular differentiation. In the rat, these latter phenomena occur during postnatal life.

The earliest signs of the appearance of the pineal anlage on E-12 are a thickening of the neuroepithelium of the roof of the diencephalon, where there is intense mitotic activity mixed with degenerated cells. Abundant mitosis has been described in the hamster (Clabough, 1973) and rat (Calvo and Boya, 1981) pineal anlage. The latter authors have shown that degenerated cells are also seen in 15 day-old embryos. In the rabbit, the mesenchyma close to the pineal anlage develops blood vessels. The relationship between the degenerated (apoptotic) cells, the abundant mitoses in the pineal anlage and the development of blood vessels in the proximal mesenchyma might be explained as follows. Cells undergoing apoptosis in the neuroepithelium of the pineal anlage would release growth factors that are free in the cytoplasm. Basic fibroblast growth factor (bFGF) could be one of these (see Unsicker et al., 1993). As these diffusible growth factors are released, they would act through a paracrine mechanism on neighbouring cells, stimulating them to begin mitosis. Blood vessels would also be attracted to, and develop in the vicinity of adjacent mesenchyma (Gospodarowicz, 1990). In support of this idea, the existence of bFGF has been reported in 18 day-old embryos (González et al., 1990), and in the pineal gland of the postnatal rat (Marín et al., 1994), although the degree of expression varies with age. Further studies are required, however, to confirm this hypothesis.

In all mammals, the pineal gland originates as a sac-like evagination that emerges from the roof of the diencephalon in the caudal direction (see Vollrath, 1981). However, in the rabbit initial growth is rostral. From E-18, the posterior face of the pineal anlage proliferates, growing caudally. Meanwhile, the anterior region hardly develops at all. Turkewitsch (1937) described a double anlage at the point where the rabbit pineal gland emerges: a) an evagination of the roof of the diencephalon; and b) a cellular mass situated immediately rostral to this evagination. According to our results, the rabbit pineal gland does not originate from a double anlage, but from a single one, which initially grows rostrally and later caudally, forming a structure that might look as though it had a double origin. Similar changes in the direction of growth of the gland's longitudinal axis during embryonic development have been described in humans: the major axis is initially vertical but later becomes horizontal (Hülsemann, 1971).

During embryonic development of the rabbit pineal gland, there is a great deal of mitotic activity. It is very intense in the different phases up to E-22, coinciding with the morphogenetic period. After this point, it gradually slows until the end of development. Very few attempts have been made to quantify this activity during embryonic

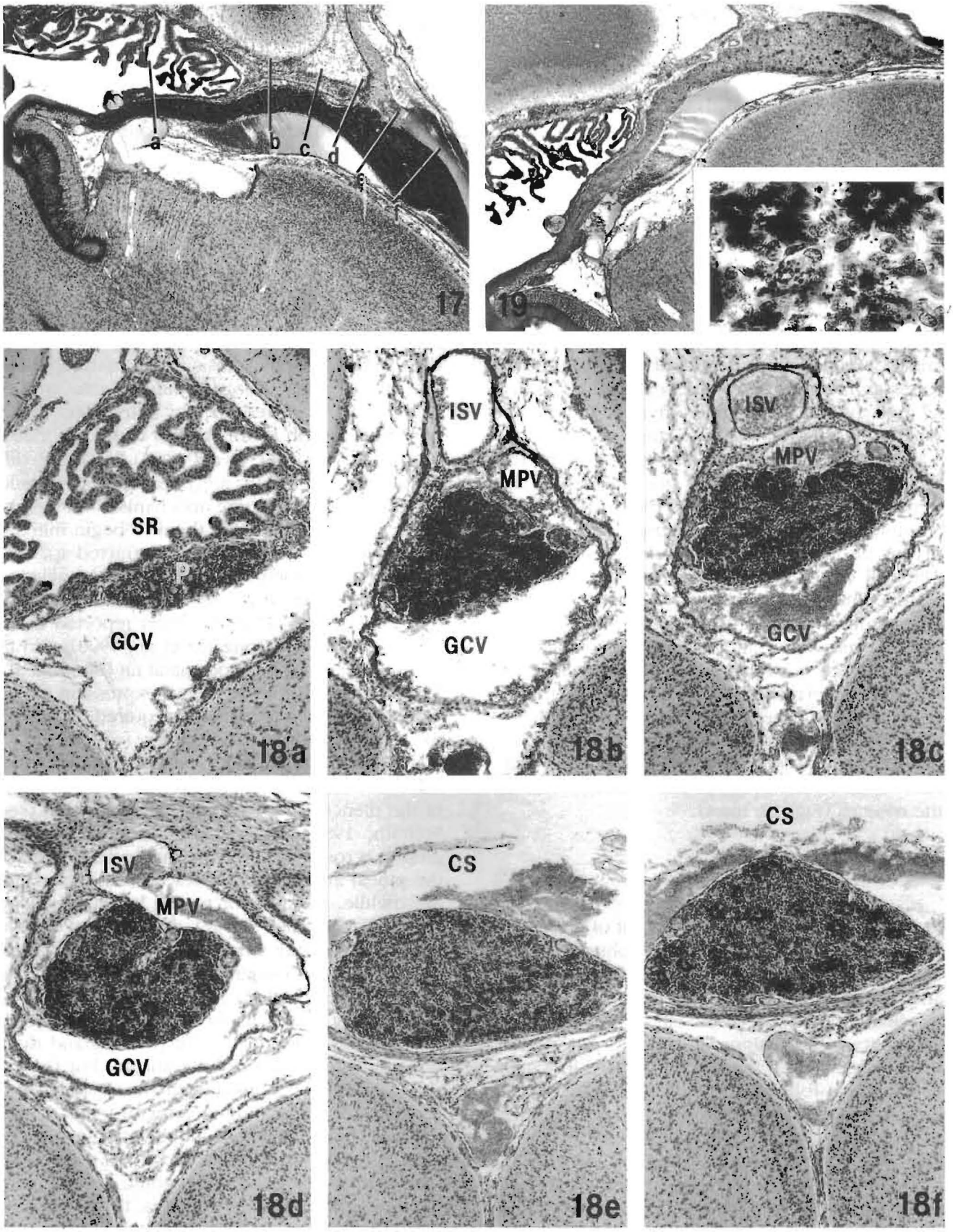



**Fig. 17.**- E-30. Sagittal section. HE. Form and anatomical relationships of the pineal gland at the end of gestation. The different planes of section of Fig. 18 are shown. x40.

**Fig. 18.**- E-30. Frontal sections. HE. Different frontal section planes at different levels (shown in Fig. 17): proximal zone (**a**), intermediate zone (**b, c, d**), and distal zone (**e, f**). Changes in the shape of the gland can be seen. x100. CS: confluens sinuum; GCV: great cerebral vein; ISV: inferior sagittal vein; MPV: medial prosencephalic vein; P: pineal gland; SR: suprapineal recess.

**Fig. 19.**- E-30. Melanin distribution in the pineal gland. Note the scarcity of pigment in the proximal zone, and how this pigment increases distally. x40. Inset: Detail of the pigment-containing cells between the rosettes. x1000.

development. Calvo et al. (2000), using bromodeoxyuridine immunohistochemical techniques to study cellular proliferation during the development of the rat pineal gland, reported intense mitotic activity during embryonic growth. These authors observed maximum activity on E-18, thereafter falling gradually until birth (E-21), and showing a behaviour similar to that seen in the rabbit. According to our results, the fall in mitosis seems to be greater than in the rat. This might be because mitosis was estimated on a surface that increases as development proceeds. Dividing cells are therefore dispersed.

The mitotic events of the pineal parenchyma of the rabbit vary in terms of location and frequency throughout embryonic development. Three stages are appreciable. In the first (E-14 – E-20), the majority of mitosis (90.9%) occur in the wall of the pineal recess and in the rosettes. Later, mitotic activity falls in these structures while increasing in the cell cords. In the second stage (E-20 – E-24), while the mitotic activity of the rosettes is still predominant (52.68%), a balance between that of the rosettes and cell cords is approached. In the third stage (E-26 – E-30), there are more mitoses in the cell cords (62.54%) than in the rosettes (37.55%). If it is accepted that the rosettes are formed of type II pinealoblasts and that the cell cords are mainly of type I, these results indicate a different proliferative behaviour for these two cell types during embryonic development. In the first stages, type II pinealoblasts divide profusely, gradually reducing their activity as development proceeds. Type I pinealoblasts, however, initially multiply more slowly, and then speed up until the end of development, when their activity is greater than that of type II pinealoblasts.

In the pineal gland parenchyma of mammalian embryos, it is common to find structures in the form of follicles or rosettes. These have been described in the rat (Kappers, 1960; Tapp and Blumfield, 1970; Clabough, 1973; Quay, 1974; Calvo and Boya, 1981), hamster (Clabough, 1973), cow and sheep (Anderson, 1965), and in the Indian palm squirrel (Haldar-Misra and Srivastava, 1986). In rabbit embryos, these structures are quite noticeable, as described by Turkewitsch (1937) and Holmes (1957). According to the present results, in the first phases of embryonic development, rosettes occupy a large part of surface area of the section: 45.43% on E-18. From this date onwards, there is a gradual decrease in the area occupied by the rosettes as terrain is surrendered to the cell cords. These data are in keeping with the above-mentioned changes in mitotic activity during embryonic development. The area occupied by the rosettes is also reduced by the invasion and development of the connective tissue septa from the capsule.

In most species, the rosettes disappear at the end of embryonic development. However, in the rabbit they remain for a long time after birth, disappearing only when the animal reaches an age of 30 days (Holmes, 1957; Kerenyi and von Westarp, 1971; García-Mauriño and Boya, 1992a).

The rosettes of the rabbit pineal gland probably arise as a consequence of an accentuated proliferation of the neuroepithelium covering the pineal recess. This early and intense growth of a small gland must lead to the formation of complex and irregular folds. Later, these folds fragment and become independent, forming isolated rosettes, as described in the rat (Calvo and Boya, 1981). Once the rabbit pineal gland reaches its final form, the rosettes show morphological differences: in the proximal zone they are predominantly elongated, while in the intermediate and –especially– distal zones they are more irregular. These changes may be due to a lack of space. The proximal zone is narrow, whereas the others are wider and allow more complex folds. As development proceeds, the rosettes fragment and disintegrate, giving rise to cell cords or cell masses of type II pinealoblasts which originally formed part of the rosettes.

The rosettes can be considered as the source of type II pinealoblasts in the rabbit pineal gland. Owing to the nuclear characteristics and the presence of pigment in these cells, they can be identified as precursors of type II pinealocytes. In the adult gland, pigment has been described only in type II pinealocytes (Romijn, 1973a). The pigment is therefore a marker of type II pinealocytes that allows the rosettes to be understood as the site of origin of these cells, which in turn come from the neuroepithelium of the pineal recess.

The cell cords correspond to the pineal parenchyma between the rosettes. These cell cords are mainly made up of type I pinealoblasts in the first stages of development. Later, type II pinealoblasts are incorporated into the cell cords as the rosettes disintegrate, as revealed by the progressive increase in cord cells.

The origin of these type I pinealocytes can be observed in the first stages of development (E-14), when the pineal anlage is in the form of a sac. These cells are located at the base of stratified neuroepithelium lining the pineal recess, close to the mesenchyma. They are cells with round nuclei and loose chromatin, different from the cells in contact with the lumen, and can be identified as type I pinealoblasts. In the early phases of development, the area occupied by these cells is greater than that taken up by the rosettes. However, by E-18 the intense mitotic activity of the rosettes renders the areas occupied by rosettes and cell cords equal. From this moment, and coinciding with a progressive

increase in mitosis in the cell cords, the rosettes separate and gradually occupy less surface in favour of the cell cords. The progressive development of the connective tissue septa aids this changeover.

The rabbit is one of the mammals with a pigmented pineal gland (Wartenberg and Gusek, 1965; Romijn, 1973b). In the adult, this pigmentation is limited to type II pinealocytes. The first granules of pigment appear on E-16 in the cells lining the pineal recess. As similarly described by Holmes (1957), the amount of pigment in the pineal gland increases strongly as development proceeds. It is localised in the centre of the rosettes, in the apical poles of the type II pinealoblasts, next to the narrow lumen. The positive results of the Masson-Fontana technique and the bleaching caused by oxidising agents such as potassium permanganate identify the pigment as melanin. Melanin is frequently found in the pineal glands of a variety of mammals, although in variable amounts (cat: Calvo et al., 1992, Boya et al., 1995; dog: Zach, 1960, Calvo et al., 1988; horse: Cozzi and Ferrandi, 1984, Cozzi, 1986; bat: Bahtnagar and Hilton, 1994; sheep: Regodon et al., 1998).

During embryonic development, the distribution of this pigment is not homogeneous. The greatest amount is found in the distal part of the gland, but gradually decreases towards the proximal zone. Although the pigment is most common in the rosettes, it can also be seen in some cells making up the cell cords. These cells are type II pinealoblasts that have separated from the rosettes as a consequence of their disintegration.

It is not easy to explain the existence of melanin in the rabbit pineal gland. In studies on the bat pineal gland, Bhatnagar and Hilton (1994) suggested that the pigment might be a phylogenetic vestige that had evolved in a manner similar to the pigmented epithelium of the retina. Photoreceptors have been found in the pineal glands of lower vertebrates and it is believed that in mammals type I pinealocytes are derived from these cells (see Vollrath, 1981). Just as the photoreceptors in the retina are associated with a pigmented epithelium of neuroepithelial origin, in the pineal gland there could be subsidiary pigmented cells for type I pinealocytes.

The existence of pigment in type II pinealocytes might be explained by the fact that these cells belong to the astrocyte family (Romijn, 1973b). Although few astrocytes have been described in the adult rabbit pineal gland, the type II pinealocytes of this species share morphological similarities with those of the rat pineal gland. Calvo et al. (1988) demonstrated the expression of astrocyte markers such as gliofibrilar acidic protein (GFAP), vimentin and S-100 protein in this type of cell. In the rabbit pineal gland, no studies have been performed to demonstrate glial cell markers in type II pinealocytes. However, they do look very similar to those of the rat. In phylogenetically more evolved species, such as the cat, Calvo et al. (1992) have reported the presence of melanin in pineal astrocytes.

From E-18, thin connective tissue septa, derived from the surrounding mesenchyma, are visible in the rabbit embryonic pineal gland. These septa serve as a vehicle for the introduction of small blood vessels into the pineal gland. According to Turkewitsch (1937), these blood vessels penetrate the rosettes. However, in the present study the septa were always seen outside the rosettes; never invading them. The gradual development of the connective tissue septa between the rosettes, together with the growth of the cell cords, helps separate the rosettes as development progresses. During embryonic development, no particular distribution of the connective septa is seen in the rabbit pineal gland. The cortex is established 5 days after birth (García-Mauriño and Boya, 1992a).

The association of the pineal gland with large blood vessels of the brain is apparent in all mammals (see Vollrath, 1981), but not often is it as strong as that seen in the rabbit. The thin capsule that covers the gland actually forms part of the blood vessels with which it is closely in contact. The dorsal and ventral faces in the intermediate and distal zones of the pineal gland are "literally bathed" in blood, as described by Turkewitsch (1937) and Smith (1971). It might be said that the distal thirds of the rabbit pineal gland are "intravascular", as revealed by transverse sections taken in the late phases of development.

From early on in embryonic development it is noticeable that, simultaneous with the first changes in the neuroepithelium (mitosis and degenerated cells), there is a development of blood vessels in the neighbouring mesenchyma. In fact, the identification of these vessels in very young embryos shows where the pineal anlage is. If one accepts the hypothesis that the rabbit pineal gland does release growth factors during embryonic life, these factors could attract blood vessels and stimulate the growth of cerebral vasculature. Thus, the embryonic pineal gland might be said to have a different function to that of the adult. At least in the rabbit, it might help induce the formation of the large cerebral veins, serving as a point of reference for their placement and organisation in the encephalon during embryonic life. The work of Holmes (1957) seems to support this. This author observed that when embryonic pineal glands were implanted in the anterior chamber of the eye, large blood vessels grew around them, but when adult glands were transplanted they produced no vasculature and degenerated.

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