

# Immunohistochemical study of the pineal astrocytes in the postnatal development of the cat and dog pineal gland

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**Abstract:** The expression of glial antigens vimentin (VIM) and glial fibrillary acidic protein (GFAP) is described in the pineal gland of cats and dogs from the first postnatal days to adulthood. VIM immunopositive cells were observed from the first postnatal days in both species. GFAP expression starts from the second postnatal week. In adults, a notable population of stellate cells immunopositive for GFAP and VIM was found dispersed throughout the gland. According to their immunocytochemical profile, these cells could be identified as astrocytes.

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## Introduction

The presence of astrocytes in the pineal gland of men and large mammals was described 50 years ago [Rio-Hortega, 1932; Bargmann, 1943]. The identification of these cells as astrocytes relies upon their stellate shape when stained with silver impregnation methods. Electron microscopic studies further demonstrated the existence of cells containing numerous cytoplasmic filaments in the pineal gland of various mammal species [i.e., cow and lamb: Anderson, 1965; cat: Duncan and Micheletti, 1966; Wartenberg, 1968; monkey: Wartenberg, 1968; fox: Karasek, 1983; dog: Calvo et al., 1988a, 1990b]. In the rat, pineal cells with filaments are restricted to the pineal stalk [Luo et al., 1984; Calvo and Boya, 1985].

Recently, the presence of antigens characteristic of glial cells has been shown in the second pineal cell type in several mammalian species [Moller et al., 1978; Lowenthal et al., 1982; Huang et al., 1984; Schachner et al., 1984; Zang et al., 1985; Calvo et al., 1988b; Borregón et al., 1992]. This finding has led to the naming of this cell as the "glial pineal cell" instead the former nonspecific terminology of interstitial cell. However, some issues remain unclear. Thus, the differentiation of this

second pineal cell type in having glial features shows a wide interspecies variation. At the ultrastructural level, filament-rich cells, similar to astrocytes are evident in species with a deeply located pineal gland [type A or AB; for review see Vollrath, 1981]. To date, the presence of glial fibrillary acidic protein (GFAP) positive cells has been shown in only a minor number of these species [Moller et al., 1978; Lowenthal et al., 1982; Zang et al., 1985]. In domestic carnivores, as the cat and dog, the pineal gland is deeply located [type AB according to Vollrath, 1981] and contains cells filled with cytoplasmic filaments [Duncan and Micheletti, 1966; Welser et al., 1968; Calvo et al., 1988a, 1990b]; however, the presence of GFAP<sup>+</sup> cells in the pineal gland of these species has never been demonstrated.

On the other hand, a variability in the antigen pattern expressed by the pineal glial cells has been reported. Thus, in rodents such as the rat, glial pineal cells express antigens characteristic of immature astrocytes such as vimentin (VIM) [Schachner et al., 1984; Calvo et al., 1988b; Borregón et al., 1992], whereas GFAP immunolocalization is restricted to cells located in the proximal portion of the gland and the pineal stalk [López-Muñoz et al., 1992a,b]. Immunohistochemical studies on glial pineal cells in mammals other

than the rat, are largely focused in GFAP localization and no data on vimentin have been published.

In the present work, the presence of VIM and GFAP in the astrocytes of the cat and dog pineal glands is described during the postnatal development to adulthood.

### Materials and methods

Fourteen dogs and 19 cats were used. Animals were kept under natural light conditions (approximately 40°N latitude). Animals were killed under pentobarbital anesthesia at 1100 between March and July. Age intervals were 3, 5, 7, 10, 15, 30, and 60 days. In previous light and electron microscopic studies [Calvo et al., 1990a,b], we have demonstrated that the dog pineal gland reaches its adult morphology at 45–60 postnatal days. No previous studies on postnatal development have been published for the cat pineal gland. Thus, 1- and 2-year-old cats of both sexes and an additional 7-year-old female cat were used to investigate the adult period. Two animals (one of each gender) were studied in each age interval. A tissue block including the entire pineal gland and the adjacent brain tissue was quickly removed and fixed in methacarn (60% methanol, 30% cloroform, 10% glacial acetic acid) for 12–14 hr at 4°C. After paraffin embedding, tissue blocks were oriented sagitally and 7-μm thick serial sections were cut and mounted on chromagel coated slides for immunolabeling.

A peroxidase-antiperoxidase (PAP) method for GFAP as well as an indirect immunoperoxidase method for VIM were carried out according to Taylor [1986]. The following antisera were applied: polyclonal rabbit immunoglobulins to bovine GFAP and monoclonal mouse immunoglobulins to human VIM diluted 1:300 and 1:50, respectively (both from Dakopatts, Glostrup, Denmark). After diaminobenzidine reaction, a nuclear counterstaining with haematoxylin (25 sec) was applied. Negative controls consisted in the substitution of the primary antisera for nonimmune serum of the same species as well as for an irrelevant antibody. Positive controls consisted of brain or cerebellum sections; furthermore, the immunostaining of astrocytes, ependyma and subcommisural organ of the brain tissue adjacent to the pineal gland served as an intrinsic positive control.

### Results

#### Dog

*Week 1.* The morphology of the dog pineal gland during the postnatal development has been de-

scribed previously [Calvo et al., 1990a]. VIM immunostaining showed positivity in the pineal capsule and the blood vessels within the gland. Ependymal cells lining the pineal recess and the dorsal surface of the gland are strongly immunolabeled (Fig. 1). Out of the limits of the gland, the strong labeling of the meninx and the subcommisural organ, which is well developed in this species, deserves mention. In the neighboring brain parenchyma VIM<sup>+</sup> radial glia are still observed.

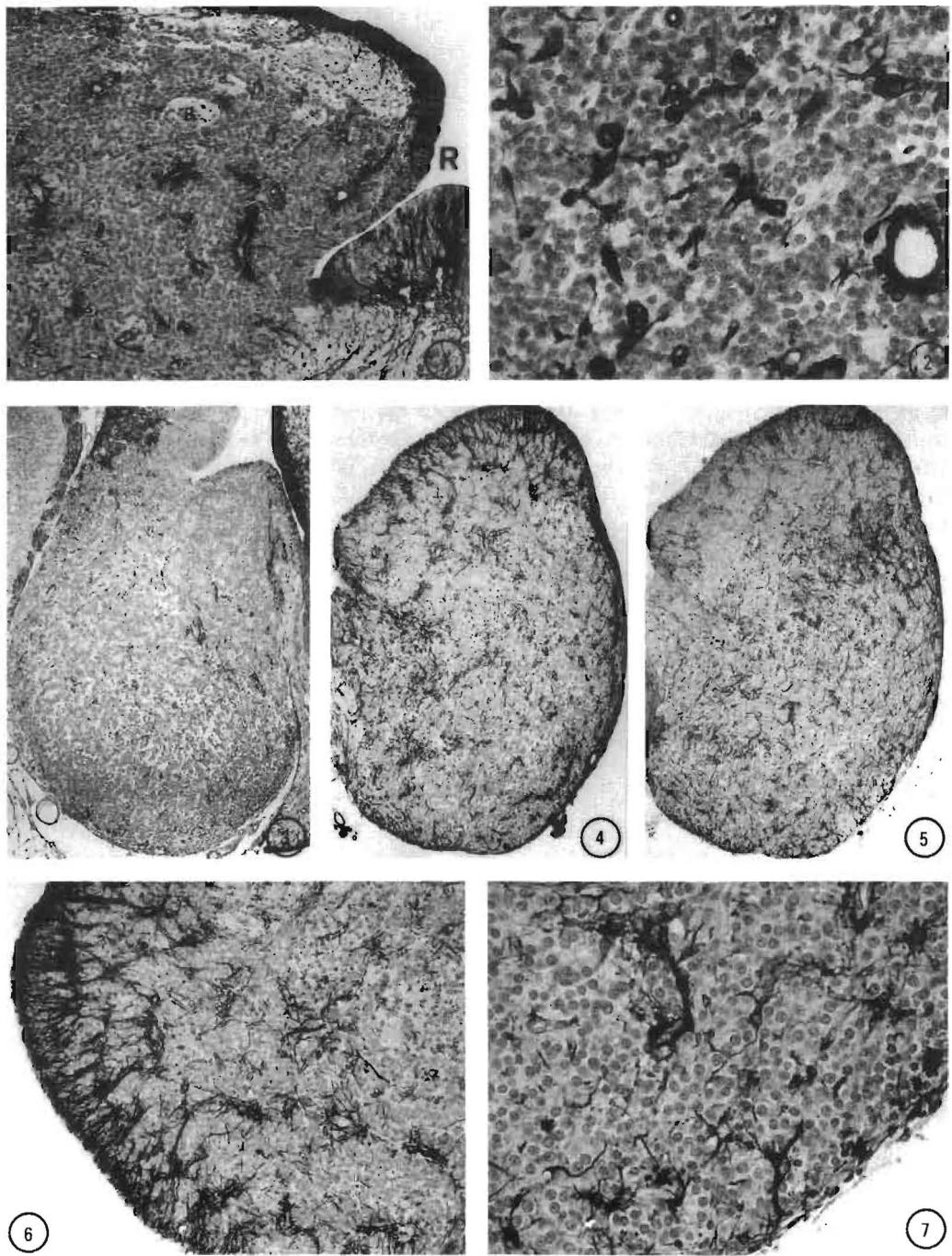
Very scarce VIM<sup>+</sup> somata and processes mainly located around blood vessels are observed within the pineal parenchyma (Fig. 1). GFAP immunolabeling is not present at this stage.

*Week 2.* There is an increase in the number of VIM<sup>+</sup> perivascular somata and processes. Moreover, these same structures are also evident far from the blood vessels, within the parenchymal cell cords (Fig. 2). Although VIM<sup>+</sup> structures are widely dispersed throughout the gland, they seem to be more abundant in the distal tip of the gland. GFAP immunolabeling also showed positive somata and processes throughout the gland, though in lesser numbers when compared with VIM (Fig. 3).

*Months 1–2.* A notable increase in the number of immunopositive structures for VIM (Fig. 4) and GFAP (Fig. 5) is now observed dispersed throughout the gland. The ependyma is strongly VIM<sup>+</sup> (Fig. 6). Ependymal cells exhibit long basal processes that penetrate deeply within the pineal parenchyma. These processes often reach the vicinity of the pineal blood vessels (Fig. 6). Only some scattered ependymal cells were immunopositive for GFAP. In the pineal parenchyma, star-shaped astrocyte-like cells were found to be positive for both VIM (Fig. 6) and GFAP (Fig. 7). In addition, very abundant VIM<sup>+</sup> and GFAP<sup>+</sup> cell processes appeared throughout the pineal parenchyma displaying a tendency to form clusters around pineal blood vessels (Fig. 6), as well as reaching the surface of the gland (Fig. 7).

#### Cat

*Week 1.* The ependyma lining the pineal recess and the dorsal surface of the gland were intensely immunopositive for VIM (Fig. 8). In addition, VIM<sup>+</sup> cell processes were seen in the pineal parenchyma beneath the ependyma. These processes ended in the vicinity of the pineal blood vessels (Fig. 8). Although these processes also showed GFAP immunopositivity, only a part of ependymal cells were GFAP<sup>+</sup> (Fig. 9). Small clusters of VIM<sup>+</sup>



*Fig. 1.* Male dog, 3 days old. Technique for VIM. Immunopositivity of ependymal cells near the pineal recess (R). In the pineal parenchyma, VIM<sup>+</sup> structures can be observed in perivascular location.  $\times 175$ .

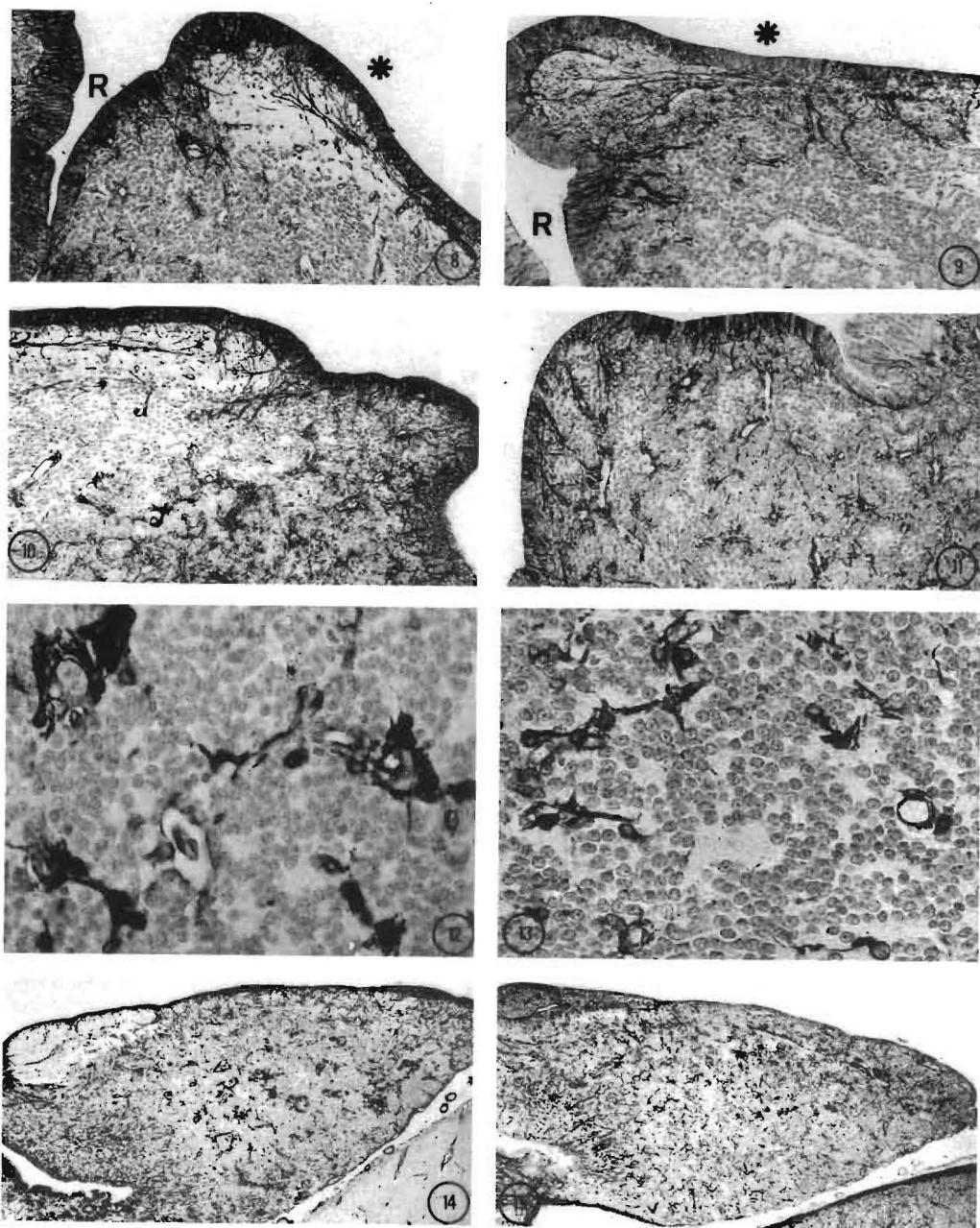
*Fig. 2.* Male dog, 10 days old. Technique for VIM. Immunostained cells located near the blood vessels and within the parenchymal cell cords.  $\times 400$ .

*Fig. 3.* Female dog, 15 days old. Technique for GFAP. Immunopositive structures in scarce numbers and dispersed throughout the gland first appear.  $\times 75$ .

*Figs. 4 and 5.* Male dog, 2 months old. Low magnification of two consecutive sections of the pineal gland immunostained for VIM (Fig. 4) and GFAP (Fig. 5). Abundant immunopositive structures are observed throughout the gland.  $\times 80$ .

*Fig. 6.* Male dog, 2 months old. Technique for VIM. Higher magnification of Figure 4 showing the immunostaining of ependymal cells and their basal processes. Immunopositive cell processes are also seen in the pineal parenchyma.  $\times 160$ .

*Fig. 7.* Female dog, 2 months old. Technique for GFAP. Immunopositive stellate cells. Some processes end on the pineal surface.  $\times 320$ .



*Figs. 8 and 9.* Female cat, 5 days old. Proximal region of the pineal gland with the pineal recess (R) showing the ependyma immunostained for VIM (Fig. 8) and GFAP (Fig. 9). All ependymal cell somata are VIM<sup>+</sup> (Fig. 8), whereas very few of them are GFAP<sup>+</sup> (Fig. 9). Few immunostained structures are seen in the pineal parenchyma. Asterisk (\*) = dorsal surface of the pineal gland.  $\times 150$ .

*Figs. 10 and 11.* Male cat, 15 days old. Proximal region of the pineal gland immunostained for VIM (Fig. 10) and GFAP (Fig. 11). Increase of immunostaining of ependymal cells. Appearance of abundant immunopositive structures in the pineal parenchyma.  $\times 120$ .

*Fig. 12.* Female cat, 10 days old. Technique for VIM. Perivascular cell clusters.  $\times 420$ .

*Fig. 13.* Male cat, 15 days old. Technique for VIM. Immunostained cells close to blood vessels and dispersed throughout the parenchymal cell cords.  $\times 380$ .

*Figs. 14 and 15.* Male cat, 2 months old. Low magnification of two consecutive sections of the pineal gland immunostained for VIM (Fig. 14) and GFAP (Fig. 15). Very abundant immunopositive structures located throughout the gland.  $\times 45$ .

and GFAP<sup>-</sup> perivascular cells were observed in the remainder of the pineal gland.

**Week 2.** An increase in the number of VIM<sup>+</sup> and GFAP<sup>+</sup> cell processes beneath the ependyma was seen (Figs. 10,11). Moreover, these structures extended along the dorsal surface of the gland. This dorsal extension appeared simultaneously with a similar extension of the ependymal layer towards the pineal tip. The presence of perivascular VIM<sup>+</sup> cell clusters is still evident, some of them showing a large size (Fig. 12). Furthermore, isolated VIM<sup>+</sup> cells located within the parenchymal cell cords far from the vessels were also evident (Fig. 13). From postnatal day 7, GFAP<sup>+</sup> somata and processes dispersed throughout the gland first appeared. The number of these structures increased progressively with age.

**Months 1–2.** A large number of VIM<sup>+</sup> and GFAP<sup>+</sup> cells were observed (Figs. 14, 15) Ependymal cells and their basal processes still exhibit a strong VIM immunolabeling (Figs. 16, 18). The ependymal lining extends along the dorsal surface of the gland, reaching the pineal gland tip (Figs. 14, 18). Very few scattered ependymal cells displayed GFAP<sup>+</sup> (Figs. 17, 19). Within the pineal parenchyma, GFAP<sup>+</sup> star-shaped cells showed several processes forming a network among the pinealocytes (Figs. 17, 19, 20). VIM immunostaining showed many positive cells, although the stellate morphology is less evident when compared with GFAP (Figs. 16, 18). At this stage, some perivascular VIM<sup>+</sup> cell clusters are still observed (Figs. 16, 18).

**Adult.** No major differences were observed. In the oldest animal studied (7 years old), large clusters of GFAP<sup>+</sup> and VIM<sup>-</sup> cell processes were found. These structures resembled glial scars (Figs. 21, 22).

No sex-related differences were observed in any species or stage studied.

## Discussion

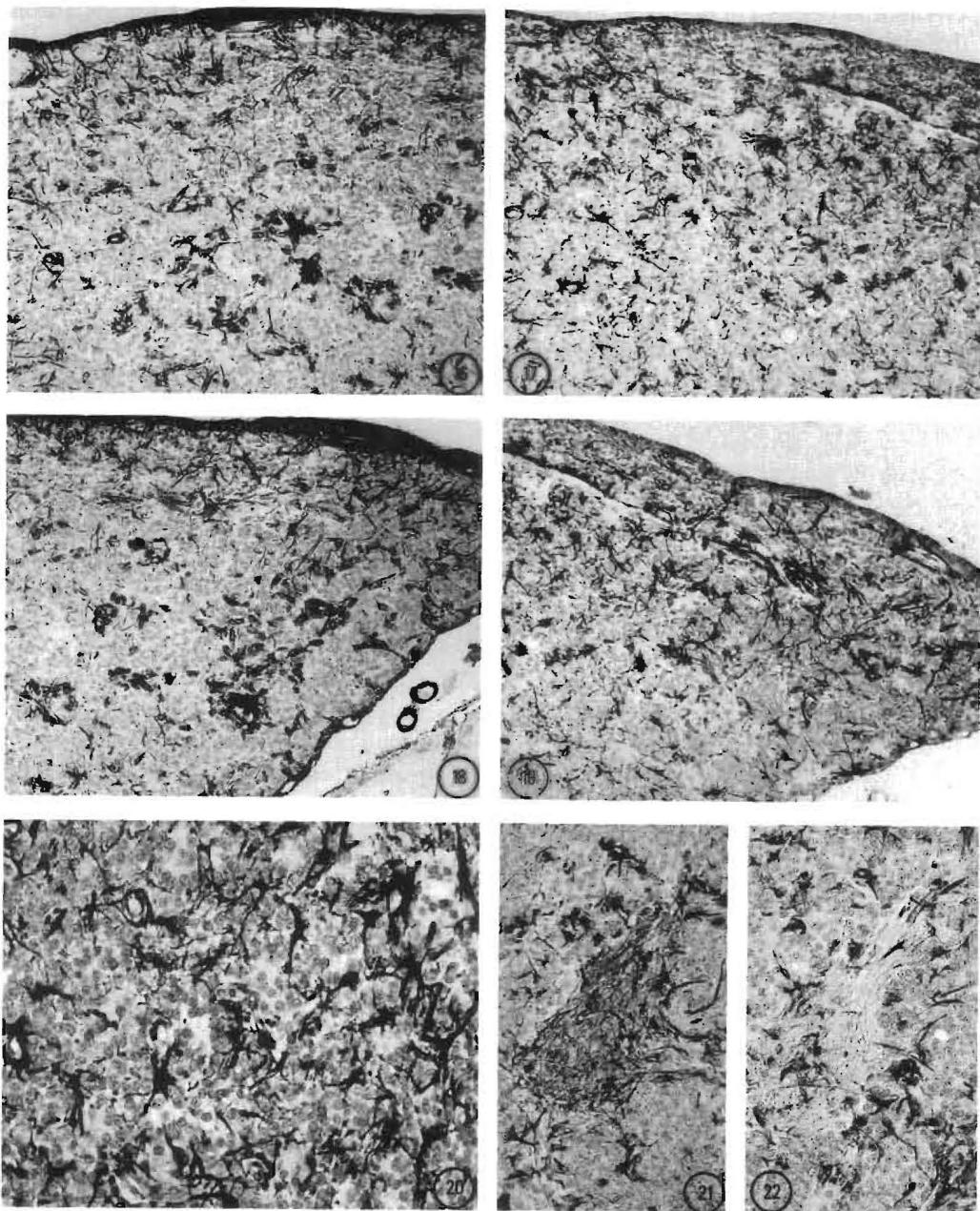
According to our results, the cat and dog pineal glands exhibit numerous GFAP<sup>+</sup> stellate cells. Previous ultrastructural studies have shown the presence of abundant cytoplasmic filaments in the second pineal cell type of both species [Duncan and Micheletti, 1966; Welser et al., 1968; Calvo et al., 1988, 1990b]. Taken together, these results suggest an astrocytic nature for the second pineal cell type in the cat and dog. Our study has also demonstrated the presence of abundant VIM<sup>+</sup> cells in the pineal gland of these species. VIM has been considered an

immunocytochemical marker for the second pineal cell type in several species, specially in rodents [Huang et al., 1984; Schachner et al., 1984; Calvo et al., 1988b; Borregón et al., 1992, López-Muñoz et al., 1992b]. The findings reported here show for the first time the presence of this intermediate filament in the pineal gland of the cat and dog.

The method used in this work does not allow us to exclude the possibility that VIM and GFAP are expressed in different cell types. However, according to our results, both antigens showed a similar immunostaining pattern in the two species tested. On the other hand, the coexpression of VIM and GFAP has been previously reported in the second pineal cell type of the rat [López-Muñoz, 1992b]. Therefore, it seems likely that VIM and GFAP would be expressed in the same cell type in the cat and dog, as occurs in the rat pineal gland.

Several consequences can be inferred from the expression of VIM in the second pineal cell type of the cat and dog. As mentioned before, GFAP expression can be considered as evidence of the astrocytic nature of this cell type. VIM expression in these cells does not exclude this hypothesis. Thus, although VIM was initially described as a marker for mesenchymal-derived cells, glial precursors (radial glia and immature astrocytes) are VIM<sup>+</sup> and GFAP<sup>-</sup> [Schnitzer et al., 1981; Voigt, 1989]. During the astroglial maturation, GFAP expression appears while VIM is lost. This shift in the antigenic expression occurs during the first 2 postnatal weeks in rodents [Pixley and De Vellis, 1984]. The immunohistochemical pattern of the second pineal cell type in the cat and dog is similar to that observed in developing astrocytes during the transitional period, i.e., VIM<sup>+</sup> and GFAP<sup>+</sup>. This finding can be interpreted as this pineal cell type presents a delay of maturation if compared with astrocytes in the central nervous system (CNS).

However, VIM immunopositivity in the pineal gland remains unchanged even in the oldest animals studied. This is in contrast with the absence of VIM immunolabeling of astrocytes in the CNS of adult animals. Nevertheless, some exceptions occur, and the expression of VIM and GFAP may persist in mature astrocytes of some areas (cerebellum, large myelinated tracts) [Schnitzer et al., 1981, Calvo et al., 1990c]. The immunocytochemical profile of the second pineal cell type in the cat and dog is thus equivalent to these CNS astrocytes. Therefore, VIM positivity does not seem to reflect immaturity or incomplete maturation of these cells, but more likely it may reflect the existence of a different tissular environment to that of most of the regions of the adult CNS.



*Figs. 16 and 17.* Male cat, 2 months old. Higher magnification of Figures 14 and 15 showing the dorsal surface of the pineal gland immunostained for VIM (Fig. 16) and GFAP (Fig. 17). All ependymal cells are strongly VIM<sup>+</sup> (Fig. 16) whereas only very occasional GFAP<sup>+</sup> ependymal cells could be observed (Fig. 17). Numerous star-shaped immunoreactive cells can be seen in the pineal parenchyma, mainly with GFAP technique. (Fig. 17). In VIM-stained section (Fig. 16), some perivascular cell clusters still remain.  $\times 165$ .

*Figs. 18 and 19.* Male cat, 2 months old. Higher magnification of Figures 14 and 15 showing the distal tip of the pineal gland immunostained for VIM (Fig. 18) and GFAP (Fig. 19). The ependymal layer reaches the distal tip of the gland (Fig. 18). Perivascular VIM<sup>+</sup> cell clusters are still more prominent than in the dorsal region.  $\times 165$ .

*Fig. 20.* Female cat, 2 months old. Technique for GFAP. Immunopositive cells with processes located among pinealocytes.  $\times 360$ .

*Figs. 21 and 22.* Female cat, 7 years old. Two consecutive sections showing a large cluster of processes positive for GFAP (Fig. 21) and negative for VIM (Fig. 22).  $\times 220$ .

## Postnatal development of cat and dog pineal astrocytes

If this hypothesis is assumed, pineal glands of carnivores and other large mammals may provide an interesting model to study the astrocytic maturation. Thus, the pineal gland of cats and dogs would allow for the study of the development and the characteristics of astrocytes without the influence of some factors present in the CNS such as direct contact with neurons or presence of the blood-brain barrier.

The early development of GFAP and VIM immunoreactivity in the pineal gland of the cat and dog is similar to that observed in rodents [Li and Welsh, 1991; Borregón et al., 1992]. Thus, VIM is expressed from very early postnatal days, while GFAP expression starts from the second postnatal week. However, in later stages of development they differ. In the cat and dog, GFAP<sup>+</sup> cells are dispersed throughout the entire pineal gland, showing a similar pattern to VIM. In contrast, GFAP staining in rats displays positive cells only in the proximal part of the gland. According to these results, our study confirms that the second pineal cell type in those species, as the cat and dog, with a deep located pineal gland, has a higher astrogli differentiation.

In the cat and dog, almost all the dorsal surface of the pineal gland is covered by the ependyma of the suprapineal recess [Fleischhauer, 1964; Calvo et al., 1990a]. Ependymal cells show long basal processes that penetrate deeply within the pineal parenchyma. These processes have been previously reported in the cat by Fleischhauer [1964]. Our findings further support the presence of well developed processes, both in the cat and dog. These processes are more evident applying the immunolabeling for VIM. The basal processes of the ependymal cells may have a physiological role in the interchange of substances between the cerebrospinal fluid and the pineal parenchyma. Fleischhauer [1964] has shown that dyes injected in the subarachnoid space diffuse fast into the pineal parenchyma in cats. The interactions between the brain and the pineal gland through the cerebrospinal fluid have been reviewed by Vollrath [1981].

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