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Pineal gliosis and gland ageing. The possible role of the glia in the transfer of melatonin from pinealocytes to the blood and cerebrospinal fluid

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SUMMARY

Using immunohistochemical approaches applied to 6 μm-thick sections from the pineal glands of cows with ages between 1 and 7 years, we studied the expression of GFAP, vimentin and S-100 and of β-tubulin. The latter compound appeared in both pinealocytes and in some nerve fibres. In both younger and older cows, vimentin was expressed in the somata of cells localised in peripheral zones of the glands and in fibres with a bead-like aspect that coursed deep into the centres of the glands, as well as in pericapillary cells, apparently in the endothelia of vessels and, like GFAP and S-100, in a thick network of prolongations located between the basal side of the epithelial cells of the pineal recess and pinealocytes, sometimes reaching the ependymal lumen. The network of glial prolongations formed a space with a reticulated aspect interposed between the pinealocytes and the ependymal epithelium. The thickness of this network was especially striking in the older cows. The expression of GFAP and S-100 was also observed in the somata and the cytoplasmic prolongations of cells distributed throughout the gland that surrounded the pinealocytes. In cows with ages of 4 and 7 years the pineal gland showed a pronounced degree of gliosis that isolated the pinealocytes from one another and from the vascular bed. Additionally, all three glial markers were expressed in cells whose somata and cytoplasmic prolongations configured spaces with a trabecular aspect, very poor in connective tissue, which coursed throughout the glands to reach the reticulated space, in especially thin neighbouring zones of the epithelium of the pineal recess. The results suggest a decisive role of the pineal glia in gland ageing, in the secretion of melatonin, and in the configuration of the vascular and ependymal pathways that the hormone must follow from pinealocytes to its target organs.

Key words: Reticulated space – Trabecular space – Glia – Melatonin secretion

INTRODUCTION

Melatonin has been implicated in the uptake of the intracellular free radicals produced during cellular metabolism that are responsible for tissue damage and the ageing of cells and tissues. This substance is secreted by the pineal gland in a rhythmic photodependent fashion (Quay, 1963) mediated by the binding of the noradrenalin secreted by pineal synaptic terminals to α and β adrenoreceptors (Klein et al., 1983; Sugden and Klein, 1984) located in the cell membrane of pinealocytes.

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These cells express strong immunoreactivity for β-tubulin, a protein located in neuronal microtubules, but not demonstrable in glial cells (Draverova et al., 1998; Alexander et al., 1991; Lee et al., 1990; Katsetos et al., 1998). Together with the population of melatonin-secreting pinealocytes, the pineal gland also contains a network of glial cells with GFAP, S-100 and vimentin-like immunoreactivity and vimentin, whose number and distribution varies according to the species (Wartenberg, 1968; Wallace et al., 1969; Karasek y Hansen, 1982) and to the age of each animal (Borregón et al., 1993; Redondo et al., 2001). Based on their content in those proteins, pineal glial cells have been identified as astrocytes. It has been proposed that astrocytes may participate in many processes affecting the functioning of the CNS, such as neuron support and isolation, the regulation of the ionic composition of the extracellular space (Orkand et al., 1966; Philippi et al., 1996; Kager et al., 2000; Walz, 2000), the formation and maintenance of the blood-brain barrier (Janzer and Raff, 1987; Wright, 1989; Rubin and Staddon, 1999), limitation of the diffusion of neurotransmitters in the synaptic cleft (Cardinali, 1999), replacement gliosis (Miller et al., 1986; Kimelberg and Norenberg, 1989; O´Callaghan et al., 1995), the metabolic regulation of GABA precursors (Kaufman and Driscoll, 1992), and the modulation of the biosynthetic activity of pinealocytes. It is also known that the synaptic-like microvesicles present in pinealocytes contain L-glutamate, L-aspartate and GABA (Gereau et al., 1995; Winder et al., 1996; Redecker, 1999; Morimoto et al., 2003; Levi and Raiteri, 1993), whereas in vimentin-positive cells the presence of glutamate (Pabst and Redecker, 1999; Yatsushiro et al., 2000) and GABA (Redecker et al., 1996; Redecker, 1998) transporters together with different gabaergic (Schon et al., 1975) and glutaminergic (Mick 1995; Pabst and Redecker, 1999) receptors has been detected.

The presence and distribution of the pineal glia have been studied by many authors using different techniques. Here we explored the distribution of GFAP, S-100 and vimentin-immunoreactive glia in the pineal glands of cows, placing special emphasis on the relationship between the glia and melatonin-secreting pinealocytes, the vascular bed and the epithelium of the pineal recess in the gland, and on the possible role of the glia in the mechanisms of melatonin secretion and transport to the vascular bed and the lumen of the third ventricle.

Materials and Methods

As study material we employed the pineal glands of ten cows, with ages ranging between one and seven years distributed thus: 3 of one year of age, three of 4 and 4 of 7 years of age. Two of the animals of each group were sacrificed between 22:00 and 23:00 h, all by a humane bolt to the brain after electrode stunning, at the Municipal Slaughterhouse in Salamanca (Spain) and Valdivia (Chile). After sacrifice, the glands were removed by craniotomy and were divided into portions by sectioning transverse to the major axis of the gland or by sagittal and horizontal cuts, all of 0.5 cm. These were fixed by immersion over 24 hours in Bouin's solution at room temperature. The fragments were dehydrated in an increasing graded alcohol series, after which they were embedded in paraffin following the usual techniques. Finally, the blocks were cut in 6 μm-sections.

The sections obtained from the different glands were used for study with immunocytochemical techniques, employing avidin-biotin complex (ABC). Prior to incubation with the primary antisera, all sections were treated with 3% hydrogen peroxide in TPBS for 30 min and then incubated in normal 30% goat serum in TPBS, also for 30 minutes, at room temperature.

Study of the glia was accomplished by incubating sections from each gland in anti-vimentin (SIGMA) at 1/50, anti-GFAP (DAKO) at 1/400 and anti-S-100 (DAKO) at 1/800 antisera for 24 hours at room temperature. Neighbouring sections of the different glands were incubated in anti-β-tubulin serum, followed by the procedure described above. After incubation, the sections were washed in PBS and then incubated in biotin at a dilution of 1/50 in PBS for 30 minutes at room temperature. After three washes in PBS, the sections were incubated in avidin at a dilution of 1/ 50 in PBS for 30 minutes at room temperature. The reaction was developed with 3-3´ diaminobenzidine (Sigma) according to the method of Graham and Karnovsky (1966) or with the BIOMEDA fast red tablets chromogen kit, which uses fast red as the chromogen. Contrast staining was accomplished with Weigert haematoxylin.

In some sections, a double staining was performed to carry out simultaneous study of β-tubulin and GFAP. In this case, the sections were first incubated in anti-β-tubulin and the immunoreaction was developed with 3-3´ diaminobenzidine. Then, after a prolonged wash with PBS the sections were incubated with anti-GFAP, using 4-chloro-naphthol to develop the reaction. Control

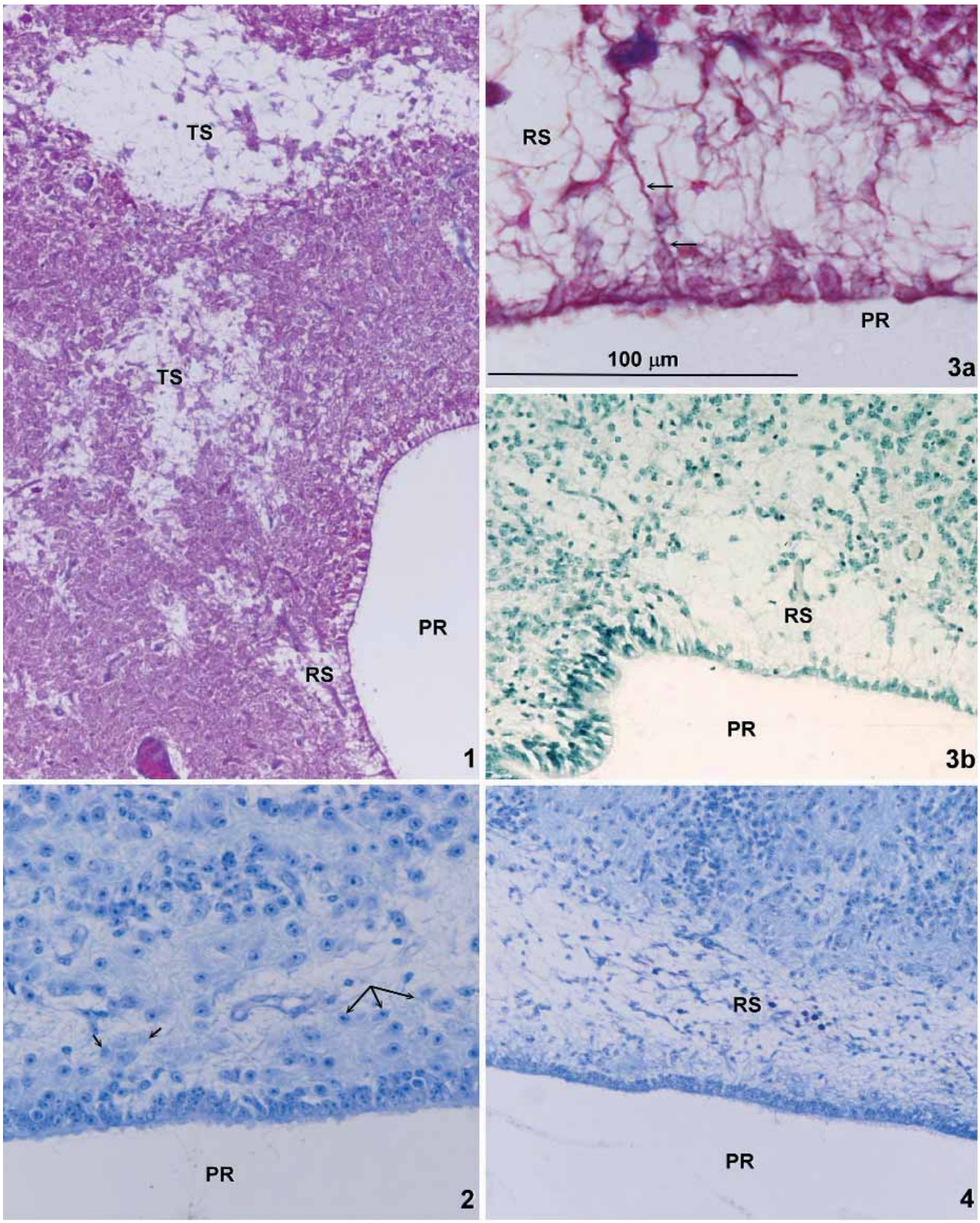


Fig. 1. Section of a pineal gland from a 1-year-old cow, stained with Mallory-Azan. Note the ependymary epithelium of the pineal recess (PR) and the trabecular spaces (TS) located in the glandular parenchyma and their apparent relationship with the subependymary reticulated stratum (RS). x 20.

Fig. 2. Section of a pineal gland from a 1-year-old cow, stained with toluidine blue. The section shows the aspect of the ependymary epithelium, formed at this level by cuboidal cells. Note the subependymary arrangement of groups of pinealocytes (arrows) that do not contact the lumen of the pineal recess (PR). x 40.

Fig. 3. Pineal gland from a 1-year-old cow, showing the aspect of the ependymary epithelium of the pineal recess (PR) and of the reticulated stratum (RS). In 3a (x 63), it is possible to appreciate the extreme thinness of the epithelium, the presence of cells with long cytoplasmic prolongations that contribute to defining the reticulated stratum (arrows), and that this latter separates the pinealocytes from the epithelium. In 3b (x 20), note the different structuring of the epithelium of the pineal recess (PR), which is very thin in the medial zone, and of prismatic cells in the lateral portions of the PR. Stain: Mallory-Azan (3a) and haematoxylin (3b).

Fig. 4. Pineal gland from a 1-year-old cow, stained with toluidine blue. Note the aspect of the reticulated stratum (RS) interposed between the pinealocytes and the epithelium of the pineal recess (PR), which is formed by abundant fibres and nuclei and cell bodies, affording it a reticulated aspect. x 20.

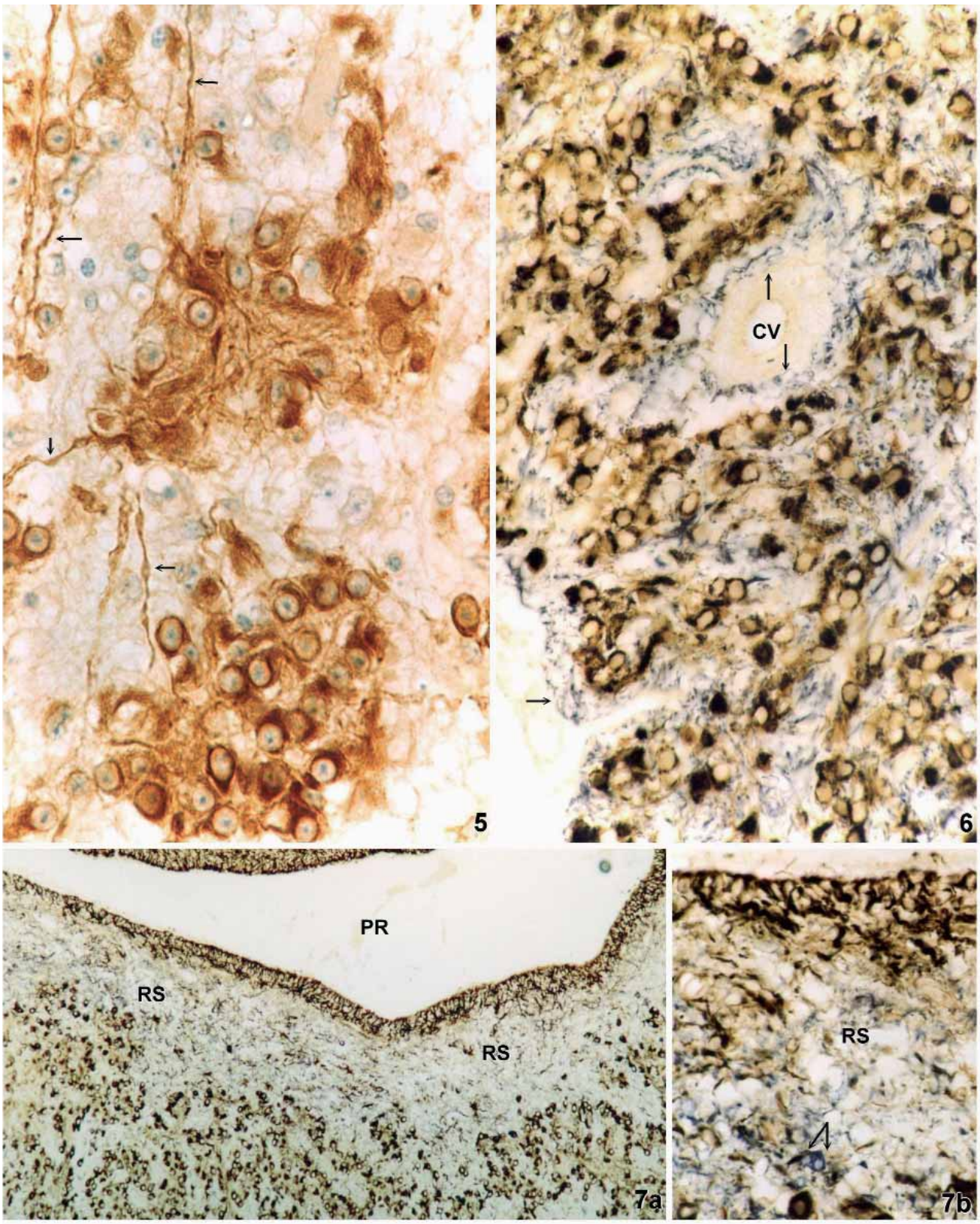


Fig. 5. Pineal gland from a 4-year-old cow incubated in anti-β-tubulin serum. Note the intense immunoreactivity seen in groups of pinealocytes and in long fibres with a straight course (arrows). Stain: ABC complex and 3,3' diaminobenzidine. Contrast staining: Weigert haematoxylin. x 63.

Fig. 6. As in the previous figure, but subjected to double staining, incubating first in anti-β-tubulin serum and then in anti-GFAP serum. The first reaction was developed with 3,3' diaminobenzidine and the second one with 4-chloro-1-naphthol. The immunoreactivity for β-tubulin is localised to the bodies of the pinealocytes while the GFAP-immunoreactive material is located in a network of cytoplasmic prolongations that course through and surround the capillary vessels (CV). x 40.

Fig. 7. As in the previous case. The micrographs show juxtaependymary portions of the pineal gland. In 7a (x 20), the following can be seen: a) the ependymary epithelium limiting the pineal recess (PR), which shows a large number of fibres arranged in a palisade among the epithelial cells, which show intense immunoreactivity for β-tubulin and that seem to emerge from small cell bodies arranged at the pineal pole of the epithelium; b) the reticulated stratum (RS), interposed between the epithelium and the β-tubulin-positive pinealocytes, in which numerous β-tubulin-immunoreactive fibres can be seen. In 7b (x 40), note that next to the β-tubulin-positive fibres there are others showing immunoreactivity to GFAP. This latter is also present in some of the cell bodies (arrows) seen in the tangle of fibres mentioned above.

reactions were performed omitting either the primary antiserum or the anti-rabbit IgG-conjugated biotin or the peroxidase-conjugated avidin from the reagent sequence. The Graham-Karnovsky (1966) reaction was also performed to investigate the possible existence of endogenous peroxidases.

To visualise the connective tissue, some sections were stained with the Mallory blue technique (Mallory, 1900), which employs aniline blue, acid fuschin and orange G as synaleptic dyes. To obtain a broad view of the epithelium of the pineal recess, some sections were stained with 0.5% toluidine blue.

The sections were photographed with a Zeiss Axioplan microscope and with a microscope Nikon Eclipse 90i endowed with an automatic measuring system.

Results

1. *Staining with synaleptic techniques*

The Mallory technique revealed that the conjunctive tissue appears as a layer surrounding the gland, out of which emerge thin, incomplete septa that become thicker as they approach the perivascular spaces. Moreover, the pinealocytes and the glial somata and cytoplasmic prolongations appeared stained with acid fuschin, the existence of irregular spaces distributed throughout the gland being striking. These extended to the vicinity of the pineal recess (Fig. 1). The limits of such spaces were large cells with long, thin prolongations interspersed between the lumen of the spaces and the clusters of pinealocytes.

The pineal gland contacted the lumen of the third ventricle through the ependymal epithelium, which did not show any single pattern of organisation throughout its extent. Both with Mallory blue and with toluidine blue and Mayer haematoxylin, used as contrast in the immunocytochemical techniques, we observed three different patterns of organisation of the epithelium. Thus, it was mostly made up of cubic cells, whose basal pole was in contact with clusters of pinealocytes (Fig. 2) and, with no transition, we observed a very large degree of narrowing of the epithelium, which was reduced to a thin layer formed by very flat cells; these gave off thin prolongations that continued with the cytoplasmic prolongations of the glial cells of the pineal parenchyma (Fig. 3a). Additionally, in the lateral portions of the pineal recess, the epithelium had a pseudostratified aspect and was formed by very long prismatic cells (Fig. 3b). Next to the basal pole of the cubic and prismatic epithelial cells, there were capillary vessels and a network of prolongations of fibres that were scarcely stained with any of the three above-mentioned dyes and that, overall, formed a layer with a reticulated appearance interposed between the epithelium of the recess and the secretory pinealocytes (Figs. 1, 3a, 3b and 4).

2. *Reaction to β-tubulin*

β-tubulin-immunoreactive material was observed in the somata of all the pinealocytes (Fig. 5) present on the surface of the sections and in long, isolated fibres passing through the pineal gland, coursing through the interpineal spaces and present in the neighbourhood of the vessels. When a section was incubated first in an anti-β-tubulin serum and then with an anti-GFAP serum, we observed that the anti-b-tubulin immunoreactivity was located in the pinealocytes whereas the anti-GFAP immunoreactivity appeared in populations of glial cells (Fig. 6) Additionally, an intense degree of β-tubulin immunoreactivity was seen in many cells arranged in parallel among the epithelial cells throughout the pineal recess and in cytoplasmic prolongations, which from the epithelium were irregularly distributed in the reticulated stratum located between the epithelium of the recess and the pinealocytes (Fig. 7a). In sections subjected to double staining, we observed that this stratum was configured by both β-tubulin-positive fibres and by somata and cytoplasmic prolongations of glial cells showing immunoreactivity for S-100, GFAP and vimentin (Fig. 7b).

3. *General aspect of glial distribution*

S-100- and GFAP-immunoreactive material was found in the somata and cytoplasmic prolongations of cells that overall configured a dense network extending throughout the pineal parenchyma. Its strongest expression was seen at the periphery of the gland (Fig. 8), around the vessels (Fig. 11) and in areas close to the pineal recess –the reticular stratum (Fig. 9). This immunoreactivity was more evident in the pineal glands of the older animals. Likewise, S-100-like immunoreactivity was seen in the nucleus of some glial cells (Figs. 10a and 10b), Also, in intermediate zones of the sections, abundant immunoreactive cells were observed, whose shape was not consistent with the typical aspect of astrocytic glial cells. The former cells were arranged in small clusters interspersed among the pinealocytes and prolongations of typical glial

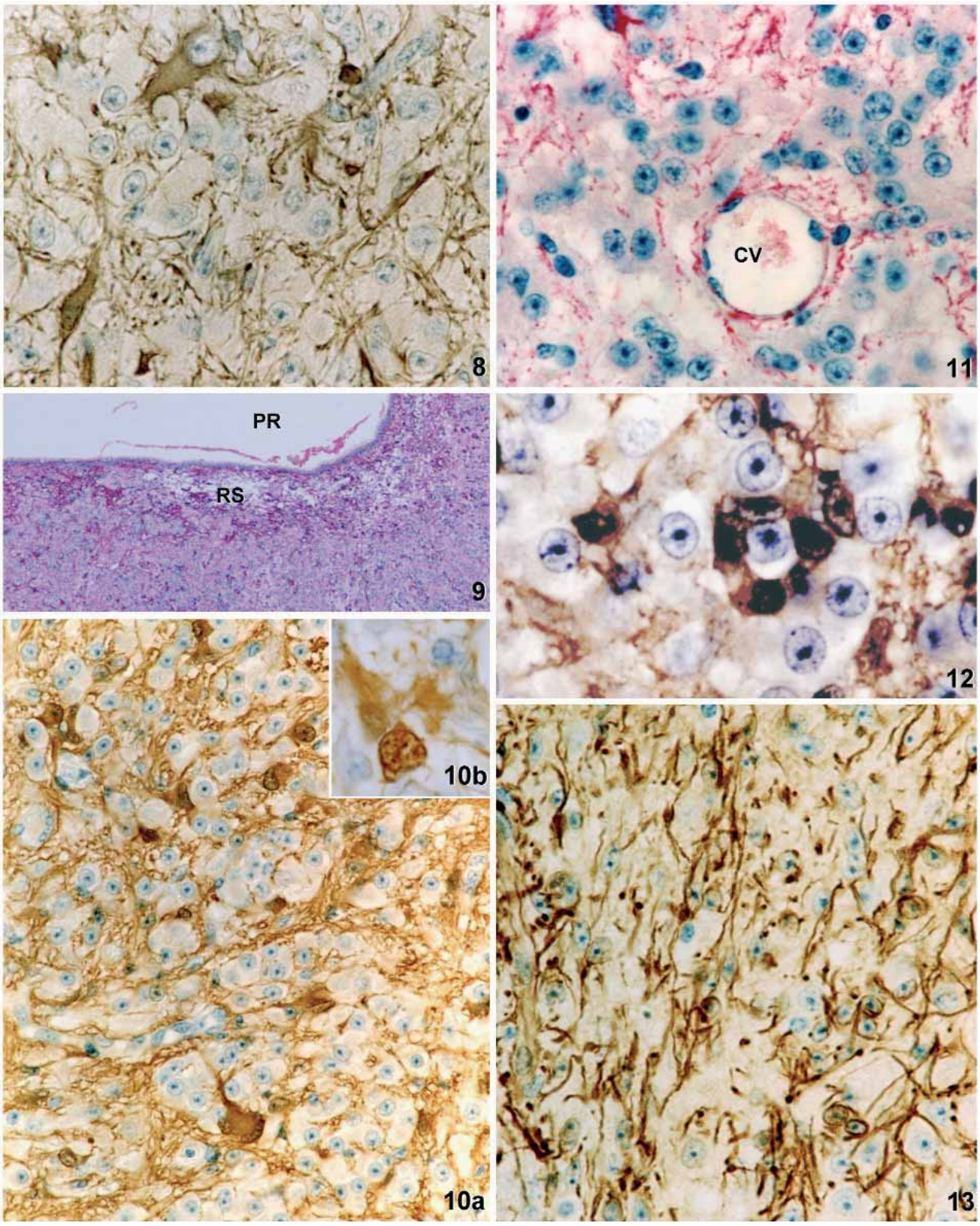


Fig. 8. Centre of the pineal gland of a 4-year-old cow. The section was incubated in anti-GFAP serum. The immunoreactivity is seen both in the cell body and in the cytoplasmic prolongations of glial cells, surrounding the pinealocytes. x 63.
Fig. 9. Juxtaependymary zone showing the glial component of the reticulated stratum (RS), which is especially striking in this zone. Primary antiserum: anti-GFAP; stain: Fast Red. x 10.
Fig. 10. Pineal gland of a 4-year-old cow. The section was incubated in anti-S-100 serum. 10a (x 40): general aspect of a central portion of the pineal gland showing several glial cells and a profuse network of cytoplasmic prolongations surrounding cloisters of pinealocytes or isolated pinealocytes. 10b: detail (x 100) of one of the cells, showing that the immunoreactivity is also present in the cell nucleus.
Fig. 11. Centre of the pineal gland of a 4-year-old cow. The immunoreactivity was visualised using Fast Red and appears localised in fibres surrounding capillary vessels (CV). x 63.
Fig. 12. Section from the same animal after incubation in anti-S-100 serum. Several cells can be seen to surround a pinealocyte. Note the peculiar morphology of these cells and the immunoreactivity present in the cytoplasmic prolongations. x 100.
Fig. 13. Juxtacommissural portion of a section of the pineal gland of a 4-year-old cow, incubated in anti-vimentin serum, showing numerous immunoreactive fibres. x 40.

cells. Sometimes, four or more cells of this type were found surrounding pinealocytes (Fig. 12).

Regarding vimentin immunoreactivity, this was mainly seen in peripheral zones of the gland (Figs. 13, 14a and 14b) in the form of a network of prolongations, sometimes with a bead-like aspect. It also appeared in the soma of some cells with different shapes and sizes (Fig. 14b). Vimentin expression decreased towards the central portions of the sections (Fig. 15). Also, vimentin-like immunoreactivity was especially striking in the neighbourhood of the union of the gland with the habenular commissure and areas close to the pineal recess (Fig. 16).

Moreover, cells expressing all three proteins –GFAP, S-100 and vimentin– were arranged in palisade form, leaving an empty central space, while their cytoplasmic prolongations were buried in the glandular parenchyma or contacted the glia of the trabecular spaces (see below) (Figs. 17a and b). These clusters and the spaces delimited by them were larger in size in the cells appearing close to the pineal recess.

4. *Relationships of the glia with the pinealocytes*

Somata of glial cells and their cytoplasmic processes, which showed an intense reaction to GFAP and S-100, completely or partially surrounded the pinealocytes, both in the peripheral and in the central portions of the gland. In the latter case, the pinealocytes appeared surrounded by thin glial prolongations while in the intermediate and peripheral portions of the gland the glial prolongations or the somata themselves surrounded each pinealocyte, affording a thick glial network interspersed between neighbouring pinealocytes, which were isolated from one another (Fig. 19). This pattern became even more striking as the age of the animal at the time of sacrifice increased (Fig. 20).

Regarding vimentin, the trajectories of the cytoplasmic prolongations were very evident both in longitudinal sections and in sections transversal to the axis of the gland, and vimentin was particularly prominent in peripheral zones and scarce in the central regions of the gland (Figs. 13, 14 and 16).

5. *Relationship of the glia with pineal vessels*

An intense immunoreactivity for vimentin was seen in the pineal capillary endothelia and in some pericapillary cells (Figs. 18a and 18b). Together with the immunoreactivity for vimentin, when working with both anti-GFAP and anti-S-100 antisera we observed that the immunoreactive material was located in some cellular somata and in many cytoplasmic prolongations of the glial cells, forming a network of one or several layers in thickness around the capillary vessels (Fig. 21). In animals with an age of 4 or 7 years, this network was especially marked and occasionally surpassed 20-30 μm in thickness (Fig. 22). Groups of cytoplasmic prolongations approached this perivascular network, their origin being cells located in neighbouring zones. Thus, the pinealocytes were isolated from the perivascular space by the intense glial proliferation.

6. *Relationships of the glia with the epithelium of the pineal recess*

The epithelium of the pineal recess was very thin and was mainly formed by cubic or flat cells, although in the lateral portions of the recess it was possible to observe a prismatic epithelium composed of very elongated cells with their major axis oriented perpendicular to the recess, sometimes with a stratified aspect. In intimate relationship with the basal pole of the central zone of the epithelium of the recess, there was a profuse glial network showing immunoreaction for GFAP, S-100 and vimentin (Fig. 23a). This projected towards deeper zones of the gland and surrounded the pinealocytes, while some fibres coursed parallel to the epithelium or seemed to reach the lumen of the ventricle (see Fig. 16). The network of glial somata and prolongations from the reticulated stratum (see Fig. 10) was interposed between the pinealocytes and the ependymal epithelium.

With respect to the lateral zones of the recess, none of the three markers was present in the epithelium, only a fine subependymal network of glial prolongations being observed in the case of GFAP and S-100, while vimentin was mainly appeared located around the subependymal vessels (Fig. 23b).

In contrast to what was observed with the three labels described, the aspect of the epithelium of the recess changed dramatically when the sections were incubated with anti-B-tubulin serum, since this protein was strongly expressed in elongated cells arranged parallel to the rest of the cells of the epithelium and extending from the lumen of the recess to the subependymal vascular bed and the reticular space. Closely arranged towards the ventricular lumen, the β-tubulin-positive cells showed a slight thickening, causing them to enter into contact with one another, thus forming a structure similar to a thin barrier directly related to the lumen of the third ventricle. The nucleus of these cells, not often seen, seemed to be located within that

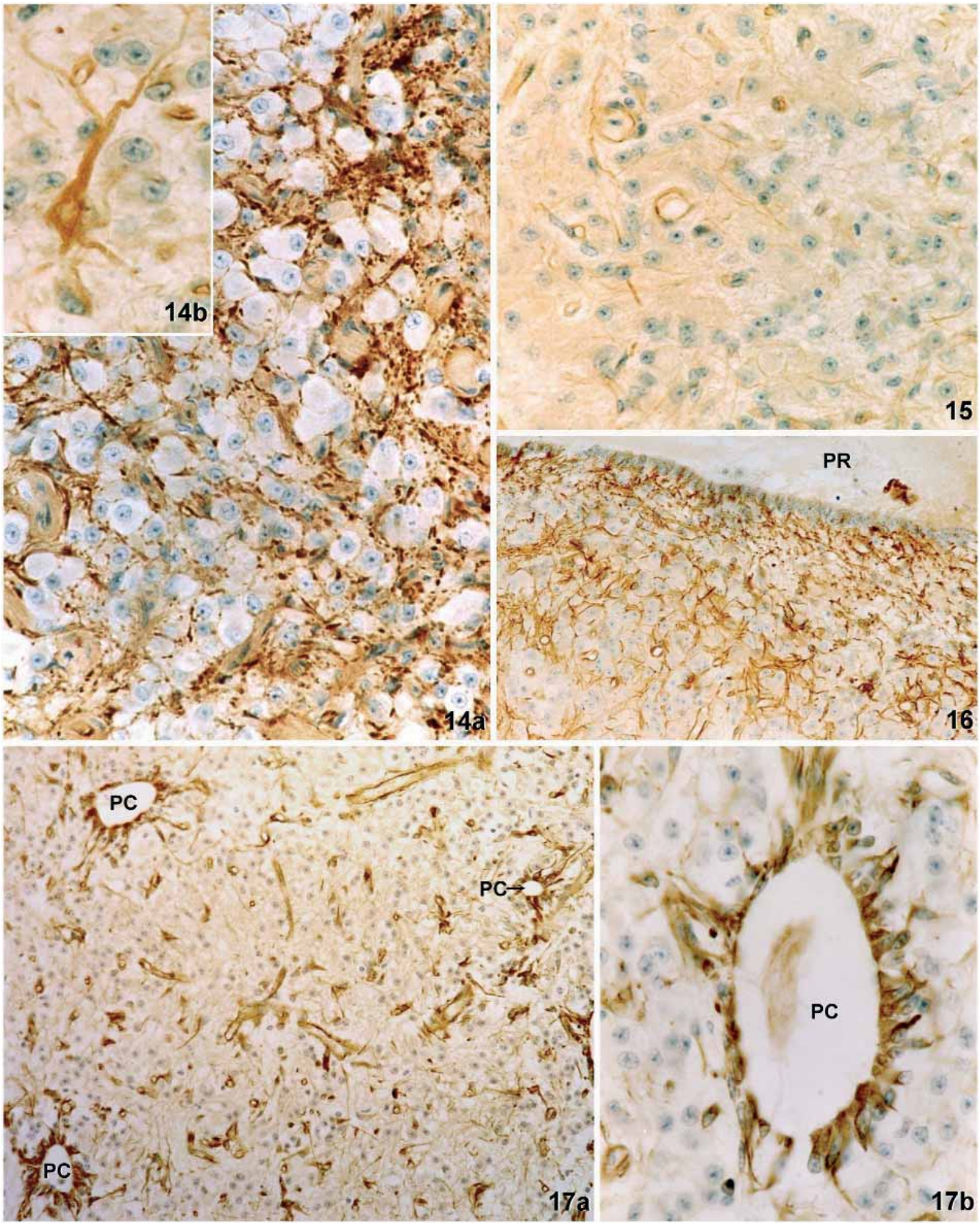


Fig. 14. 4-year-old cow. 14a: (x 40) peripheral portion of the pineal gland and the vimentin-like immunoreactivity appears in numerous fibres cut transversally and arranged around pinealocytes. In 14b (x 63), it is possible to see a glia-like immunoreactive cell with long, thick cytoplasmic prolongations.
Fig. 15. Here, the image was taken of the central zone of the gland. Note the scant vimentin-like immunoreactivity confined to fine trajectories of the cytoplasmic prolongations and the absence of immunoreactive cell bodies. x 40.
Fig. 16. Juxtaependymary portion close to the habenular commissure of the pineal gland of a 4-year-old cow. The section was incubated in anti-vimentin serum. In the zones most distant from the epithelium of the third ventricle, the vimentin-like immunoreactivity appears in fibres surrounding vessels and pinealocytes, while in the juxtaependymary portion proper it appears in fibres arranged in areas superimposable over the reticulated stratum (not visible in this figure), many of which are arranged among the cells of the epithelium. PR= Pineal recess. x 20.
Fig. 17. 4-year-old cow. The organisation of three cavity-like structures whose wall is formed by cells with immunoreactivity for vimentin (17a, x 20). This immunoreactivity is also seen in fibres with a perivascular disposition and others that are related to pinealocytes and the bodies of small cells. Figure 17b (x 40) shows a higher-power magnification of one of the cavity-like structures, where it is possible to observe the palisade arrangement of the vimentin-positive cells, with some spaces free of labelled cells among the glial cells demarcating the cavity.

small periluminal thickening. Cells expressing β-tubulin appeared throughout the epithelium of the recess, including its pseudostratified lateral portions, in contrast to what was found for GFAP, S-100 and vimentin (compare Figs. 23a and 23b with Fig. 24).

7. *Trabecular spaces*

There appeared to be a slight sign of incomplete lobulation of the parenchyma due to the presence of conjunctive tissue only at the periphery of the glands. This appeared surrounding vessels or, on other occasions, forming thin septa, which from the perivascular spaces were distributed among the clusters of pinealocytes. A truly remarkable observation was the existence of spaces delimited by somata and cytoplasmic prolongations of glial cells expressing immunoreactivity for vimentin, S-100 and GFAP (Figs. 25 and 26a). These spaces had a trabecular structure and contained scant, dispersed β-tubulin-immunoreactive pinealocytes (Fig. 26b), and they were arranged throughout the glandular parenchyma from the vertex to the lateral and medial portions of the pineal recess, becoming confused there with the meshes of the reticulated stratum, such that the lumens of both -trabecular spaces and reticulated stratum- were only separated from the lumen of the ventricle by a thin layer of very fine epithelial cells that projected to the glial prolongations delimiting them, the existence of conjunctive fibres in their structure not being revealed with the Mallory blue stain.

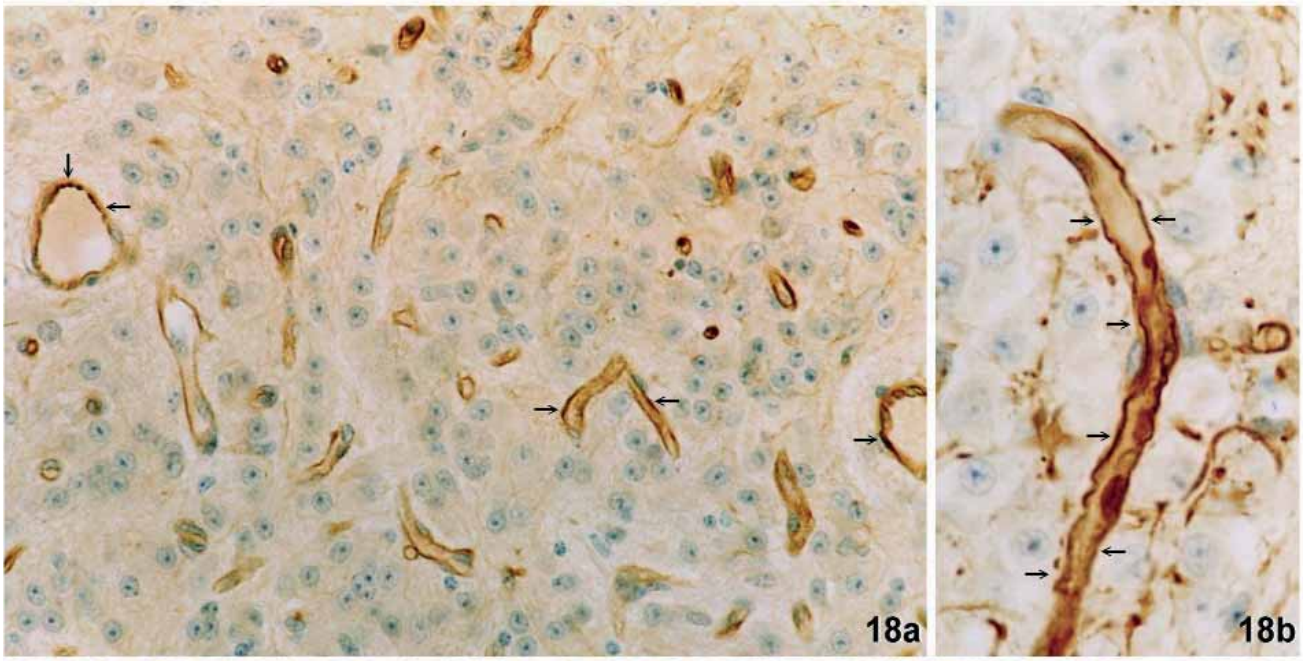
However, converse to the situation in the trabecular spaces, in which there only seemed to be glial cells, in the reticulated stratum β-tubulin-expressing fibres were seen together with these cells and their cytoplasmic prolongations (see Figs. 7a, 7b and 10).

Discussion

The pineal gland is an epithalamic structure that is derived from the dorsal portion of the roof of the third ventricle. It was originally a photoreceptor organ but later on in phylogenetic evolution it became a neuroglandular structure, formed by pinealocytes that synthesise melatonin and serotonin through a network of thin sympathetic terminations coming from neurons located in the superior cervical ganglion, by cholinergic fibres, by capillaries, and by an important number of interstitial cells.

It is known that the secretion of melatonin by pinealocytes decreases as from puberty (Sack et al., 1986; Iguchi et al., 1982; Karasek, 2004; Srinivasan et al., 2005) and it has been proposed that the decrease in melatonin secretion could be related to processes of cellular damage, ageing, and the degeneration of neurons with a periependymal disposition. Although the mechanism through which the pineal gland decreases its capacity to produce and secrete melatonin is not well known, the process of pineal calcification has been considered as a very important factor in this. Also, over the past decade it has been suggested that melatonin would act as a scavenger of the free radicals produced during cellular metabolism, which could be involved in cellular and tissue ageing and could even affect the life span of the individual. Starting out from these postulates, one question that must be addressed is as follows: What leads melatonin production to decrease in the pineal gland? In other words, which factors are involved in the ageing of the pineal gland? This work was designed precisely with a view to observing what takes place in the pineal gland of young cows with ages between 1 and 7 years (in terms of humans, this would correspond to ages of between 3 and 21 years), and we focused our attention on the development of the glia. Among the above-described interstitial cells, it was possible to recognise microglial cells together with a population of macroglial cells typified as being as astrocytes owing to their content in proteins such as GFAP, S-100 and vimentin. Although the presence of these glial cells in the pineal gland has been known for many years (Cajal, 1904, 1911; Del Rio Ortega, 1932), their functional significance remains to be fully elucidated.

To conduct the present study we chose the pineal gland of cows for the following reasons: 1) the abundance of glial cells; 2) because the presence of melatonin in pinealocytes has been demonstrated using immunocytochemical methods (Carvajal et al., 2004), and 3) because the system responsible for generating melatonin in primates is more similar to that of ungulates than that of rodents. Here, we used β-tubulin as a marker of pinealocytes (Draverova et al., 1998; Alexander et al., 1991; Lee et al., 1990; Katsetos et al., 1998), observing with double staining that the cells of glial lineage did not express immunoreactivity for that protein but that it did express immunoreactivity for GFAP, S-100 and vimentin. Once this had been confirmed, we attempted to establish the pattern of distribution of the glial cells, focusing on the relationship between these and melatonin-secreting pinealocytes, the vascular bed -formed by abun-



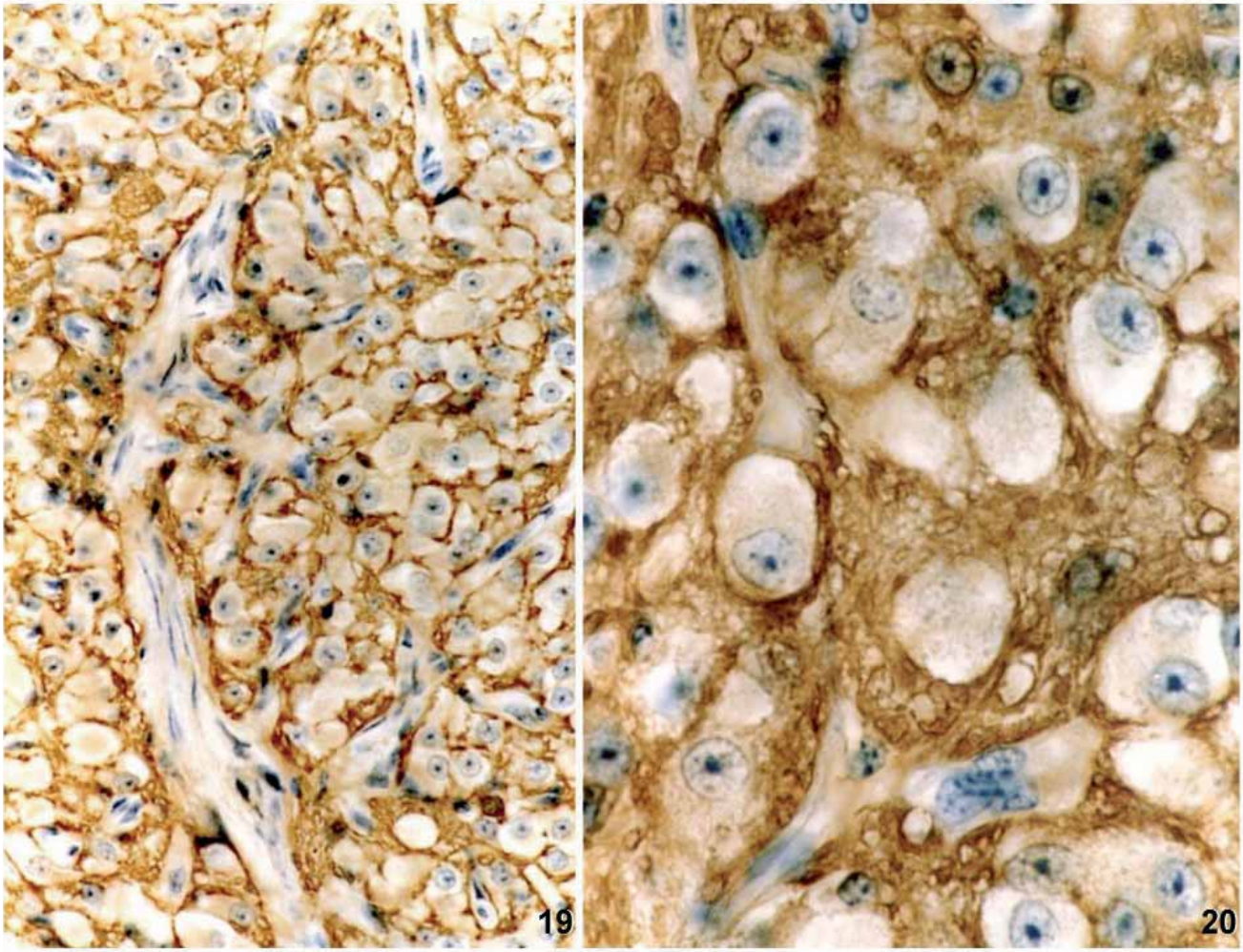


Fig. 18. Central portion of the pineal gland of a 7-year-old cow, incubated in anti-vimentin serum. In 18a (x 40), the immunoreactivity seems to be located in the walls of the vessels (arrows). Figure 18b shows one of these at higher magnification (x 63). It may be seen that the vimentin-like immunoreactivity is located in the wall of the vessel, while some cells with a pericapillary disposition do not show immunoreactivity.
Fig. 19. Central section of the pineal gland of a 7-year-old cow, incubated in anti-GFAP serum. Note the strong development of the glia surrounding the pinealocytes and separating them from one another. x 40.
Fig. 20. The same case but now with a peripheral portion of the gland showing the complete isolation of the pinealocytes determined by glial proliferation. x 100.

dant non-fenestrated cells- and the ependymal epithelium of the pineal recess, which separates the pineal gland from the cavity of the third ventricle. In other words, we studied the relationships between the glia and melatonin-producing cells, on the one hand, and the relationship between the glia and the structures interposed along the route that the substance must travel to its target organs, on the other. In fact, melatonin can be identified in both the vascular tree and in the CSF (Skinner and Malpeaux, 1999; Tricoire et al., 2002), which explains the suggestion of a dual pathway –vascular and ependymal- for melatonin to reach its target nerve tissues. According to our results, in the configuration of both pathways the glia plays an important role.

In general, it may be stated that the immunoreactivity for GFAP, S-100 and vimentin was always present in the pineal glands of all the cows studied here, although the pattern of distribution of such immunoreactivity varied considerably when we compared sections of pineal glands incubated with anti-GFAP and anti-S-100 sera and those incubated in anti-VIM serum. Thus, whereas with the first two antisera immunoreactivity appeared in many somata and in a profuse network of cytoplasmic prolongations of glial cells distributed throughout the gland, vimentin was only expressed in some cells whose somata showed a variable morphology, both in the cytoplasmic prolongations of glial cells located at the periphery of the gland and, more specifically, in the neighbourhood of the glandular areas related to the junction with the habenular commissure and others related to the epithelium of the epiphyseal recess and to the capillary walls. In the medial portions of the gland, the immunoreactivity for vimentin only appeared in thin cytoplasmic prolongations of the glia. Although vimentin and GFAP may coexist in the same cell (López-Muñoz et al., 1992), which is interpreted as a sign of glial immaturity (Voigt, 1989), the differences in the topographic distribution of S-100 and GFAP expression, on one hand, and of vimentin, on the other, suggest not only the existence of variations due to the age of the animals but also different functions attributable to each glial cell. Since the present study was carried out in animals with ages between 1 and 7 years –distant in time from both the embryonic period and old age- we are unable to provide any data that might shed light on the functional significance of the glia as regards to the expression of the three proteins addressed here.

Pinealocytes are known to secrete and synthesise melatonin after challenge by different stimuli, three mechanisms being most closely related to such activity. The first depends on the light-darkness regime, and it is mediated by the release of noradrenalin by the pineal sympathetic terminals induced by the lack of light (Quay, 1963; Moore, 1978). More recently, reports have been made of the presence of peptides able to affect the melatonin-generating system, such as VIP (Yuwiler, 1983; Simonneaux et al., 1990; Shomerus et al., 1996), pituitary adenylate cyclase-activating polypeptide (Simonneaux et al., 1993), and acetylcholine (Shomerus et al., 1995; Yamada et al., 1998). The second is governed by the existence of a system of microchannels -gap junctions- that allow the exchange of information between neighbouring pinealocytes, which in turn facilitates the diffusion of stimuli from one pinealocyte to another or from a pinealocyte to a glial cell (Ceiciura and Karakowski, 1991; Berthoud et al., 2000). The third, probably related to the other two although this remains obscure, is related to the intrinsic activity of the pineal glia. In this sense, although the possible actions of the glia in the synthesis of melatonin remains to be elucidated, two observations suggest an important role for glial cells in both biosynthetic activity and in the secretion and transfer of melatonin to the vascular bed or elsewhere. Thus, the large number of glial cells, on one hand, and the absence of fenestrated capillaries in the cow pineal gland, on the other (Moller and Baeres, 2002), should be taken into account when assessing the interactions between glia and pinealocytes and also the pathway(s) through which melatonin must travel to reach its target organs.

Regarding the first mechanism described above, emphasis should be placed on the proposed ability of the glia to govern the availability of neurotransmitters in the synaptic cleft since although there are no synapses (in the proper sense) between synaptic terminals and pinealocytes, the noradrenalin released by such terminals must bind to pinealocyte membrane receptors (Klein et al., 1981; Zats, 1978) in order to induce melatonin secretion. In light of the existence of a profuse glial network surrounding pinealocytes, noradrenalin availability may be governed not only by the ability of sympathetic fibres to release this substance but also by the ability of the glial network to regulate the amount of that neurotransmitter in the intercellular spaces (Cardinali, 1999; Bezzi et al., 2004). It has also been reported that the glia would regulate the ionic composition of the extracellular space (Orkand et al., 1966; Philippi et al., 1996; Kager et al., 2000; Walz, 2000), which in view

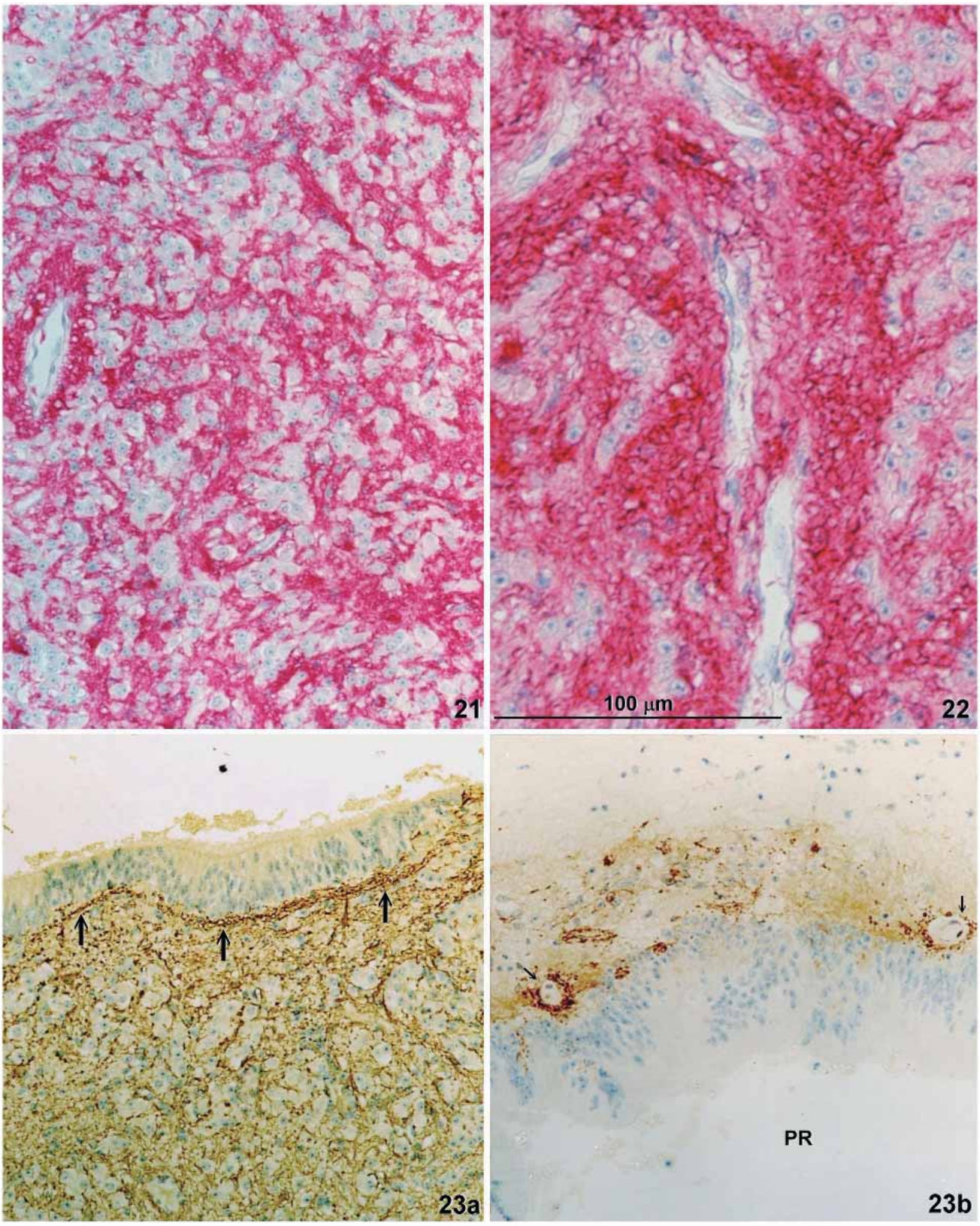


Fig. 21. Section of the pineal gland of a 7-year-old cow, incubated in anti-GFAP serum. The extraordinary expression of glia is seen throughout the glandular parenchyma. The immunoreaction was visualised with Fast Red. x 40.
Fig. 22. In the same group of animals, the glial proliferation present throughout the gland is especially striking around the vascular bed, forming a thick mesh that separates the pinealocytes from the vessels. Scale bar: 100 µm.
Fig. 23. Section of the pineal gland of a 4-year-old cow, incubated in anti-GFAP (23a) and anti-vimentin (23b) sera. Note that for both antisera the immunoreactivity is completely negative in the thick epithelium of the recess, with a pseudostratified aspect of the lateral portions of the pineal recess. By contrast (23a), GFAP immunoreactivity is profuse throughout the surrounding parenchyma and is concentrated in a prominent subependymary network (arrows). In turn, the reaction for vimentin is mainly seen around subependymary vessels (23b, small arrows). x 20.

of the important role of Ca^{2+} in such processes would strongly modify the synthesis and release of melatonin (Sugden et al., 1986, 1987; Muñoz Barragán et al.,.1990; López Pizarro et al., 1989a, b; Muñoz Barragán et al., 1997).

Furthermore, the profuse network of glial cells -an authentic gliosis- surrounding the pinealocytes of cows of 4 or 7 years of age (i.e., quite young) could hinder or prevent interactions between pinealocytes through the interposition of glial prolongations between them. This gliosis suggests the existence of severe repercussions on the exchange of information between neighbouring pinealocytes through gap junctions, which could lead to maladjustment in melatonin release, leading to the decline in the secretion of this substance observed after puberty. It has also been proposed that a type of direct dialogue could occur between the glia and pinealocytes since the existence of gap junctions between both cellular lineages has been described, although the meaning of this dialogue as regards the secretion of melatonin remains obscure (Ceiciura and Karkowski, 1991; Berthoud et al., 2000).

In this sense, four substances also appear to be involved in the interactions between glia and pinealocytes: D-aspartate, L-glutamate, acetylcholine and GABA. The first two strongly inhibit the release of melatonin and have been detected in pinealocytes (Yamada et al., 1996; Yatsushiro et al., 1997). Glutamate is exocytosed to the extracellular space through a mechanism initiated by acetylcholine, after which glutamate binds to mGlutR3 receptors of the cell membrane of pinealocytes, either to exert its action or to be sequestered by the glia to be bound to mGlutR 2, 3 and 5 receptors present in glial cells (Morimoto et al., 2003; Pabst and Redecker, 1999) and increase the proliferative capacity of these (Amos and Chesler, 1998).

Another substance that participates in the glia-pinealocyte relationship is GABA. In fact, the enzymes responsible for the synthesis (glutamic acid descarboxylase) and catabolism (GABA-transaminase) of GABA have been found in the pineal gland of mammals (Ebadi et al., 1984, 1986; Ebadi and Govitrapong, 1986a, b; Rosenstein et al., 1990; Ebadi, 1993; Kanterewicz et al., 1993), and the existence of a pineal GABAergic system that would inhibit the release of melatonin through a paracrine mechanism has been proposed (Rosenstein et al., 1990). More recently, Sakai et al. (2001) have described the presence of GABAergic terminals contacting pinealocytes in the pineal glands of mammals, and Echigo and Moriyama (2004) have demonstrated a vesicular GABA transporter in astrocytes and macroglial cells, suggesting that the amino acid can be stored and secreted by glial cells, to establish a functional connection with pinealocytes and hence to regulate melatonin secretion. The interplay established between L-glutamate and the subsequent release of GABA from glial cells could be crucial for a finely-tuned control of melatonin secretion induced by noradrenalin, as reported by Echigo and Moriyama (2004) in the rat. Accordingly, although it seems certain that glial proliferation would interfere in the relationships between pinealocytes, the existence of gap junctions between glia and pinealocytes would favour the exchange of information between both cellular lineages, mediated by GABA, aspartate and glutamate. The issue lies in knowing in which direction this exchange of information flows, which could also affect the decline in melatonin secretion observed as ageing progresses (Iguchiy col., 1982; Reiter, 1995; Zeitzer, 1999).

Apart from the possible role of the glia in the mechanisms of melatonin secretion, another aspect that merits attention is that of the arrangement of the glial cells in the pathway to be followed by the hormone from the moment when it is secreted until it reaches the blood or the CSF. In our hands, the immunoreactivity for GFAP and S-100 appeared in the form of a network of cytoplasmic prolongations surrounding the capillary vessels. This pattern of immunoreactivity for these two antisera became more striking as the age of the cows increased, and it was very highly expressed in the seven year olds animals. Thus, with advancing age the glia seemed to establish a barrier between the melatonin-secreting pinealocytes and the pericapillary space. In contrast to what has been reported for other parts of the CNS in which astrocytic glial cells emit foot-like prolongations around the vessels, in the case of the cow pineal gland the cytoplasmic prolongations were very long and branched around the perivascular spaces. This arrangement suggests that not only may the secretion of melatonin be mediated by glia but also that this latter also governs the arrival of the hormone in the pericapillary, space and hence the lumen of the vessels. In any case, the possible role -if any- of the glia in regulating the transfer of melatonin remains to be determined, as is the case of the mechanism through which the hormone reaches the blood, crossing the non-fenestrated vascular endothelia. In this sense, it is interesting to recall some of the images offered

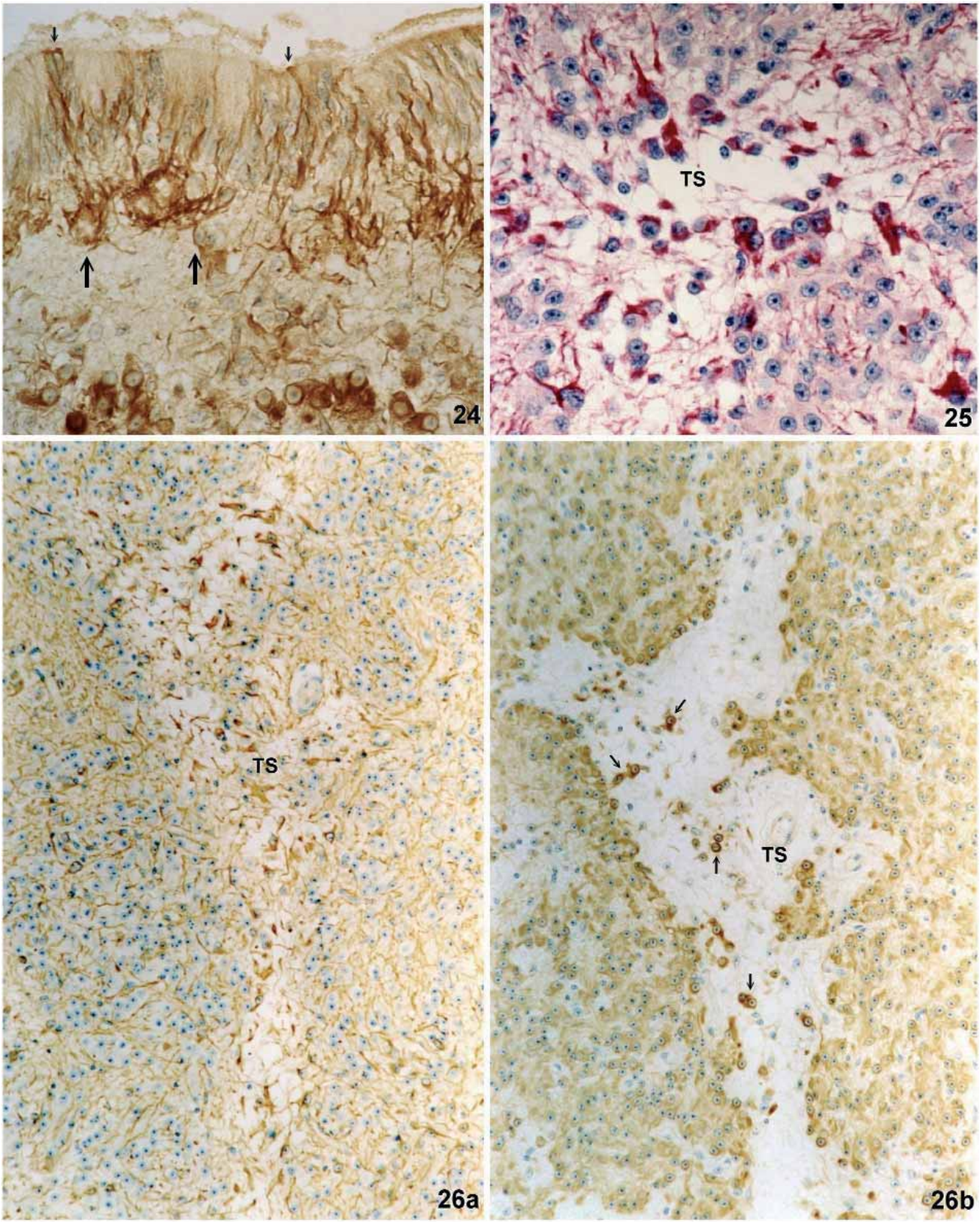


Fig. 24. Section of the pineal gland of a 4-year-old cow, but now incubated in anti-β-tubulin serum. An intense degree of immunoreactivity to this antiserum is seen at the basal pole of the epithelium (large arrows) and in the reticulated stratum, from where prolongations emerge that cross the epithelium to reach the lumen of the ventricle, forming small bulbs (small arrows) that sometimes contact one another. x 63.
Fig. 25. Section of the pineal gland of a 4-year-old cow, incubated in anti-GFAP serum. The reaction was developed with Fast Red. Note how among the groups of pinealocytes there are cell bodies and cytoplasmic prolongations which, overall, configure a space with a trabecular aspect. x 40.
Fig. 26. Sections of the same animals, showing the aspect of the trabecular spaces (TS). Note the presence of some nuclei belonging to cells that are not immunoreactive for GFAP and the extent of the spaces (26a). x 20. In 26b an adjacent section incubated now in anti-β-tubulin serum is showed. Whereas the immunoreactivity can be seen in almost all the pinealocytes located around the trabecular space, it only appears in isolated pinealocytes within those spaces and the glial cells are not labelled. x 20.

by Carvajal et al. (2004) in which it is possible to note melatonin-like immunoreactive material in the endothelial cells of some pineal capillaries.

Regarding the pathway that melatonin must follow from the pineal gland to its peripheral target tissues, and in particular to its neural targets, the findings of Young et al. (1984) and those of Reppert et al. (1979) suggest that melatonin would follow a long trajectory involving the pineal vessels, the vein of Galen, the sinus rectus, and the internal jugular vein, returning to the brain through the carotid arteries and their branches and finally reaching the target nerve centres. This pathway involves the persistence of melatonin in the vascular tree over a relatively long time. Since it has been demonstrated that the concentration of melatonin in the internal jugular vein is up to sevenfold higher than the mean in the internal carotid artery, but much lower than that present in the CSF, Rollag et al. (1978) have proposed that melatonin would reach its neural targets through the CSF present in the cerebroventricular system. Indeed, some authors (Reppert et al., 1979; Rollag et al., 1978; Perlow et al., 1981; Reppert et al., 1982; Arendt et al., 1977; Bruce et al., 1991) have reported melatonin levels in the CSF similar to or even higher than those seen in blood (Hedlun et al., 1977; Kanematsu et al., 1989; Shaw et al., 1989). Similarly, Skinner and Malpaux (1999) observed cyclic variations in melatonin levels in the CSF, with very elevated values during the dark period, suggesting that owing to its highly lipophilic and liposoluble nature the hormone would be diffused through nervous tissue.

All the above data support the hypothesis of the existence of a ventricular pathway that would allow contact between melatonin and its neural targets rapidly and at optimum concentrations. The results obtained here allow us to confirm that hypothesis, at least as regards the path followed by melatonin from the moment it is secreted by pinealocytes to when it reaches the CSF in the third ventricle, a pathway in which glial cells seem to be involved: first, owing to the relationships of the glia with pinealocytes -as mentioned above; second, owing to the existence of the intricate system of trabecular spaces delimited by the glial cells, and third owing to the arrangement of the glia with respect to the epithelium of the pineal recess. Tricoire et al. (2002) have suggested that melatonin would reach the ventricular lumen through the epithelium of the pineal recess. The existence of pinealocytes in direct contact with the ventricular lumen due to the absence of ependymal epithelium has also been proposed (Hewing, 1982). We did not observe this but we did note accumulations of pinealocytes separated from the ventricular lumen by ependymal epithelium. However, in most of the recess the pinealocytes were separated from the epithelium by a dense network of glial prolongations expressing vimentin, GFAP and S-100, which together with the β-tubulin-positive cells located in the epithelium of the recess- configured what we have designated the reticular stratum, as documented in the present images. All the foregoing suggests that the glia must play a hitherto unknown but very important role in allowing melatonin to enter into contact with the ependymal epithelium. In other words, glial plasticity could modulate the location, timing, and amount of melatonin that must reach the basal pole of the epithelial cells before entering the ventricular lumen.

Further, the very high expression of B-tubulin in many cells arranged in the epithelium of the pineal recess, which owing to their aspect could be tanycyte-like cells, suggests an important (albeit imprecise) role for such cells as regards both the arrival of melatonin at the ventricular lumen and the passage of substances from the CSF to the inside of the pineal parenchyma.

Focusing on the trabecular spaces, very few conjunctive fibres were observed when the sections were stained with the aniline-blue of the Mallory stain, these fibres being relegated to the perivascular spaces and to thin septa coursing from such spaces to the periphery of the gland or vice versa. In contrast, these trabecular spaces were delimited by somata and prolongations of glial cells that expressed all three markers - vimentin, GFAP and S-100-, always intimately related to pinealocytes. Since these spaces extended irregularly throughout the glandular parenchyma and reached- either directly or through the reticular stratum- particularly thin zones of the pineal recess, the images suggest that the melatonin secreted by a large number of pinealocytes may reach those zones in large quantities, which would account for the high concentration of melatonin present in the CSF (Hedlund et al., 1977; Kanematsu et al., 1989; Shaw et al., 1989)

Finally, we remark on the special arrangement of some cells immunoreactive for S-100, GFAP and vimentin that appeared clustered in the form of a palisade delimiting a space of greater or lesser dimensions; such clusters of cells have been termed “rosettes” by Regodon et al. (2006)

cow foetuses of 200 days of age. Those authors considered that the "rosettes" were formed by epithelial cells similar to those of the epithelium of the pineal recess and not by cells of the glial lineage. Other authors have also described these structures along the embryonic development of sheep (Anderson, 1965), bovines (Brack, 1962; Anderson, 1965) and rat (Calvo and Boya, 1981) as being formed by undifferentiated cells. In our view, such cells, precisely because they contain the three above-mentioned antigens, should be classified as glial cells, as previously suggested by García Mauriño and Boya (1992), although those authors defined them as interstitial cells. The fact that on some occasions a continuity was observed between the space delimited by the cells and the trabecular spaces suggests the existence of some type of relationship between both types of structure.

In conclusion, from our work it seems possible to infer that pineal gliosis hinders or prevents functional relationships between neighbouring pinealocytes, may hamper the arrival of melatonin at the vascular bed, while the glia may be involved in the formation of the pathway that the hormone must follow until it reaches the CSF in the third ventricle. In sum, our results suggest that pineal gliosis could be related to the ageing of the pineal gland, which would be reflected in a decrease in the levels of melatonin in blood and the CSF.

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