Postnatal neurogenesis in the cow pineal gland: an immunohistochemical study

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Summary. In the pineal gland of cows and rats structures designated rosettes have been described both during embryonic development and in adult animals. In order to investigate the possible nature of the cells comprising such structures, in the present work we studied the pineal glands from 10 cows of one- or four-years-old using conventional immunocytochemical and confocal microscopy techniques. As markers of glial cells, we used anti-vimentin (Vim) and glial fibrillary acidic protein (GFAP) and anti-S-100 sera, and the pinealocytes were labelled with β-III tubulin. As a marker of stem cells, we used an antinestin serum, while an anti-PCNA serum was employed to label proliferating cells. To explore the neuronal nature of some cells of the rosettes, we used an anti-SRIF serum. The rosettes were seen to be present throughout the glandular parenchyma and displayed a central cavity surrounded by cells, most of which expressed all or just some of the above glial labels and nestin, although there were also some rosettes with cells that expressed β-III tubulin and other cells that expressed SRIF. Likewise, in the cells of the rosettes the cell nucleus showed strong expression of PCNA. Confocal microscopy revealed that the walls of the rosettes contained cells that coexpressed Vim/S-100, Vim/GFAP and Vim/nestin. The number of rosettes was significantly greater in the animals of one year of age with respect to the four-year-old cows. The present findings allow us to suggest that rosettes are evolving structures and that most of the cells present in their walls should be considered stem cells, and hence responsible for the postnatal neurogenesis occurring in the pineal gland of cows.

Key words: Pineal, Neurogenesis, Rosettes, Immunohistochemistry, Stem cells

Introduction

In recent years a considerable body of information has been collected to suggest that the central nervous system (CNS) is not a static structure but that it preserves a certain neurogenic capacity after birth (Bennett et al., 2009; Alvarez-Buylla and Lim, 2004; Doetsch et al., 1999, Doetsch, 2003a,b). In fact, neurogenesis after birth has been demonstrated in the fish brain (Byrd and Brunjes, 2001; Zupanc, 2001; Otteson and Hitchcock, 2003; Zupanc et al., 2005; Zupanc and Horschke, 1995; Johns and Easter, 1977) and in those of lizards (García-Verdugo et al., 2002) and turtles (Russo et al., 2004) and birds (Álvarez-Buylla et al., 1990a,b) from a cellular subtype of neurogenic radial glia. Also, using immunocytochemical techniques Omura (2007) and Omura et al. (2003, 2004) demonstrated the presence of proliferating cell nuclear antigen (PCNA) in retinal rod cells of two nocturnal fishes -Anguilla japonicus and Paralichthys olivaceus- and in the pineal organ of the rat.

In adult mammals, neurogenic capacity is present in specific zones of the CNS, such as in the subventricular zone of the lateral ventricles and in the subgranular layer of the dentate gyrus of the hippocampus (Seri et al., 2001, 2004, 2006; Lie et al., 2004). From in vitro studies, it is known that in these cerebral zones there are niches of neural stem cells, in which self-renewal and the differentiation of progenitors to neural cells are determined by the local environment and intrinsic cellular signals (Temple, 2001; Shen et al., 2004; Barkho et al., 2006). It has been proposed that neuronal stem cells would derive from germinal astrocytes (Lendahl et al., 1990; Doetsch et al., 1999; Doetsch, 2003a; Imura et al., 2003; Garcia et al., 2004; Wiese et al., 2004), characterized by expressing proteins typical of intermediate filaments (nestin, glial fibrillary acidic protein -GFAP- and vimentin). It has also been demonstrated that if such cells are cultured, they group...
together to form neurospheres and clusters, whose cells may differentiate into neurons or into glia, depending on the growth factor added to the culture medium. Sometimes, the neurospheres show a cavity (Lazzari et al., 2006), in which case the term rosette is used to refer to such structures. Since most cells present in neurospheres, clusters and rosettes express nestin, GFAP and vimentin (Lim et al., 2010), these proteins can be considered stem cell markers. Consistent with this, Bennet et al. (2009) found stem cells in different circumventricular organs, including the subventricular zone of the rat pineal gland.

Moreover, in fresh pineal tissue fixed for histomorphological studies cavitary structures have been described in the pineal gland of the embryos of cows (Brack, 1962; Anderson, 1965) and sheep (Anderson, 1965), these being termed rosettes. Later, Calvo and Boya (1981), working with the pineal glands from rat embryos, and García Mauriño and Boya (1992), working with the pineal glands of adult rabbits described similar structures, their walls being composed of cells expressing GFAP, while Regodón et al. (2006) observed rosettes in the pineal gland of cow embryos, proposing that their walls would be formed by pinealocytes. Other authors (Gómez Esteban et al., 2008), using an anti-vimentin serum, have found rosettes in the parenchyma of the pineal gland of cows at least one year after birth.

Here we used immunohistochemical techniques applied to sections of the pineal glands of cows with ages of 1 and 4 years to detect the expression of vimentin, GFAP and S-100 as markers of glial cells; nestin- as a marker of stem cells, and PCNA as a marker of proliferating cells, with a view to gaining further insight into the nature and meaning of rosettes as possible sites of the location of stem cells with neurogenic capacity after birth.

We also used 4’6-diamino-epsilon-phenylindole (DAPI) (Schweizer and Nagi, 1976; Stöhr et al., 1978, 1980; Eastman et al., 1980; Buys and van der Veen, 1982), long used for the demonstration of AT-rich DNA segments in nuclear chromatin.

Materials and methods

Six animals of one year of age and four animals of four years of age were used. The animals were obtained from the municipal abattoirs of Valdivia (Chile) and Salamanca (Spain), and were slaughtered by a humane bolt to the brain. Immediately after sacrifice, the pineal glands were removed and divided into portions following a horizontal plane and fixed in 5% paraformaldehyde in 0.1 M phosphate buffer, pH 7.2, or in Bouin fluid. After fixing and the corresponding washes, the portions obtained were subdivided into three portions, following a horizontal plane, for paraffin embedding. Then, all the portions were cut into 6-µm sections and, after deparaffinization and hydration, these were washed thoroughly with PBS for incubation in different antisera following various methods, depending on the technique to be used.

Avidin-biotin-peroxidase technique (ABC), following the procedure of Hsu et al. (1981).

Prior to incubation in the primary antiserum the sections were treated with 0.3% H2O2 in methanol for 30 minutes to inhibit endogenous peroxidases and then in 10% normal goat serum for 30 min (DAKO). After a thorough wash in PBS for 10 min. the sections were incubated in the following antibody: antimouse-vimentin (1:50, Sigma), antirabbit-GFAP (1:400, DAKO), antirabbit-S-100 (1:800, DAKO) all for the demonstration of glial lineage cells; antirabbit-β-III tubulin (1:500, Abcam) to investigate the presence of neurons or pinealocytes; anti-somatostatin S-14 (1:500, DAKO,) to investigate the presence of SRIF secreting cells; antirabbit-nestin (1:200, Chemicon,) to check for the possible existence of stem cells, and antipineal-PCNA, (proliferating cell nuclear antigen) to locate cells in the proliferating phase (1:500, DAKO). After incubation in the various primary antisera, the sections were washed repeatedly with PBS and then incubated in biotin bound to an IgG antirabbit or antimouse serum (Sigma, 1/30 for 1 hour) followed by a wash in PBS for incubation in Avidin proxidase conjugate (Sigma, 1/30 for 1 hour). The immunoreaction was detected with 3’3’-diaminobenzidine, following the procedure of Graham-Karnovsky (1966). All sections were contrasted with Weigert haematoxilin for later study and, where appropriate, they were photographed with a Nikon-Eclipse microscope. As a reaction control, sections in which the primary antiserum or any of the other links in the ABC chain were absent were used. As a positive PCNA immunoreaction control, sections of pineal gland of three rats of two days of age were used; at this time there are still proliferating cells in this species. The animals were sacrificed by anaesthesia with sodium Pentothal.

Confocal microscopy

For the immunofluorescence study we used the same primary antisera as with ABC, followed by incubation of the sections with a fluorochrome bound to an immunoglobulin. In these cases, a confocal microscope was used to demonstrate the possible existence of the colocalization of different antigens in the same cell line. The following combinations were used: antimouse-vimentin antibody (1:200, Sigma) and antirabbit-β-III tubulin (1:500, Abcam); anti-vimentin and antirabbit-GFAP (1:400, Sigma); anti-vimentin and antirabbit-S-100 (1:800, Chemicon-Millipore); and anti-vimentin and antirabbit-Nestin (1:200, Chemicon-Millipore). All primary antibodies were diluted in PBS with 10% normal goat serum (Sigma).

In all cases, the following procedures were carried out: a) after deparaffinization, the sections were washed with PBS (5 washes of 1 min each); b) incubation in
normal 10% goat serum for 30 min; c) incubation with primary antibody at 4°C overnight in a refrigerator; d) 5x1 min washes in PBS; e) incubation in secondary antiserum (goat anti-mouse IgG conjugated with Alexa 488 fluorochrome, 1:1000, Molecular Probes-Invitrogen, which emits in green); f) washing in PBS as above; g) incubation of the second primary antiserum, for 6 hours in a refrigerator; h) washing in PBS; i) incubation in the second secondary antiserum (goat anti-rabbit IgG conjugated with Alexa 594 fluorochrome, 1:1000, Molecular Probes-Invitrogen, which emits red fluorescence) for three hours, and then four washes with PBS (5 min each). For nuclear staining, sections were incubated for 5 min at room temperature (RT) after the last wash with DAPI (300 nM, Invitrogen).

Assessment of rosette numbers

The rosette count was performed by dividing each section of the pineal gland into three parts: the juxtasubependymal deep zone, the body of the gland and the apex. The study was performed using four pineal glands from one-year-old animals and four from animals of four years of age. Whereas the unit area studied in the body of each gland was 100,000 µm², in both the juxtasubependymal zones and in the apex the surface analyzed was much smaller (10,000 µm² per gland) owing to the imprecise nature of the limits of both zones. The statistical study was carried out with Student’s t test for small groups of animals, taking as a reference the total number of rosettes present in each of the zones into which the gland was divided.

Results

Immunocytochemical studies performed with the ABC technique

For the description of our results we took as a reference horizontal sections that divided the gland into two portions: a superior or dorsal portion, and another inferior or ventral one. These sections allowed us to differentiate the following parts in the pineal glands: a) the ependyma of the pineal recess of the third ventricle; b) the subependymal or hypependymal space, located between the ependyma and the glandular parenchyma. Fibres coursed towards this space from the habenular commissure; c) regarding the commissural fibres, we considered the commissural or juxtaconnusional zones, the latter referring to the zone of the glandular parenchyma related to the commissural fibres. All these parts were encompassed within the deep zone of the pineal gland. d) the pineal gland itself.

Our study focused on the location and cellular characterization of the rosettes in the parts described above, although we also studied the distribution of pinealocytes and glial cells differentially. Thus, the use of an anti-ß-III tubulin serum following the ABC method allowed us to detect the presence of numerous pinealocytes arranged in longitudinal accumulations, separated from each other by spaces that did not show any immunoreactivity for that antiserum (Fig. 1A). When we used anti-vimentin, anti-GFAP, or anti-S-100 sera, we observed cells with an irregularly shaped soma, round orcoma-shaped- out of which coursed some prolongations arranged around the pinealocytes, in the spaces remaining among the accumulations of these, and in the perivascular spaces (Fig. 1B,C). In view of the expression of the three proteins, these cells were designated glial cells.

It should be noted that both in the ependyma and in the subependymal space we found cells expressing vimentin and GFAP. In the ependyma, these cells were arranged in a palisade with respect to the ependymocytes, projecting from the subependymal vessels to the lumen of the third ventricle. In the subependymal space vimentin- and GFAP-positive cells were seen isolated or forming groups, often related to vessels (Fig. 2A).

Focusing on the study of rosettes, we observed that these formations showed a peculiarity, differentiating them from any other glandular structure. This was the fact that they were organized in such a way that the cells integrating them delimited a cavity; that is, they were cavitory structures (Figs. 2-4).

A second peculiarity of the rosettes refers to the morphology of the cells forming them, since these latter were sometimes cubic or prismatic and sometimes displayed, or not, cytoplasmic prolongations that departed from the cellular soma in a direction opposite to the lumen of the cavity (Fig. 2B,C).

Rosettes were abundant in the juxtasubependymal zone (Fig. 2A) and in the body of the ventral-most portions of the gland. Within the body they were more abundant in the medial portions of the gland than in the apex of the gland (see Table 1).

When the sections were incubated in anti-ß-III tubulin, as well as many pinealocytes we also found ependymal cells expressing this protein. Sometimes, the ependyma showed very prominent differences in thickness, since it was formed either by cubic cells, where the immunoreactivity appeared, or by very flat and non-immunoreactive cells (Fig. 3A). Additionally, the ependyma was invaginated into the subependymal space, where it was possible to observe two structures with a rosette-like aspect; these were formed of cells similar in shape and also in the β-III tubulin.

<table>
<thead>
<tr>
<th>Age</th>
<th>Number of glands</th>
<th>Pineal body (100,000 µm² per gland)</th>
<th>Juxtasubependymal (10,000 µm² per gland)</th>
<th>Apex</th>
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<tr>
<td>1 year</td>
<td>4</td>
<td>25.2±8.04</td>
<td>15.0±4.6(*)</td>
<td>8.2±2.5(*)</td>
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<tr>
<td>4 years</td>
<td>4</td>
<td>10.5±4.6</td>
<td>7.2±2.9</td>
<td>5.2±2.7</td>
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<tr>
<td>(*)</td>
<td>P&lt;0.01</td>
<td>P&lt;0.03(*)</td>
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Fig. 1. Composite of three images taken from the pineal gland of a one-year-old cow. A corresponds to a section of the pineal gland incubated in anti-tubulin III serum; many pinealocytes (P and similar cells) strongly express the protein and are arranged among non-immunoreactive corridors (IpS). A vessel is marked with V. B corresponds to a section from the same gland incubated with anti-vimentin antiserum, where it is possible to visualize different glial cells distributed among the secretory pinealocytes. C was taken from a section of the same gland incubated in an anti-GFAP serum. Note the central part of the image the soma of a glial cell, out of which course several cytoplasmic prolongations (arrows) arranged among the pinealocytes.
Fig. 2. Composite of three micrographs of the pineal gland from a one-year-old cow. \textbf{A} corresponds to a zone of the gland incubated with anti-vimentin serum in which the following can be seen: a) the ependyma (Ep), showing cells expressing vimentin that run along the ependymocytes until they reach the lumen of the third ventricle (III-V), on one hand, and towards the subependymal space, on the other; b) the subependymal zone or space (SuS), in which there are somata and cytoplasmic prolongations expressing vimentin; c) the juxtasubependymal zone of the pineal parenchyma, in which it is possible to see several rosettes (black arrows) whose walls are formed by vimentin-expressing cells. \textbf{B} shows a zone of the ependyma (Ep) belonging to a section next to the previous one incubated in antiGFAP serum, where it is possible to note the radial arrangement of the positive GFAP cells arranged among the others ependymocytes. In the subependymal space (SuS) it is also possible to observe isolated cells or cells forming groups (black arrows) and others arranged around vessels (V) that strongly express GFAP. \textbf{C} shows two rosettes, one cut in the sense longitudinal to the major axis and the other transversally to this axis. In both cases, almost all cells forming their walls express vimentin and the vast majority show long cytoplasmic prolongations arranged among the pinealocytes. The red arrows depict one of the prolongations and the asterisks denote the lumen of the rosette.
Fig. 3. Composite of five images taken of the pineal gland of a four-year-old cow, incubated in anti ß-III tubulin serum. A shows: a) the ependyma, which exhibits an invagination towards the subependymal space, formed by cells expressing ß-III tubulin (gross arrow); b) in the subependymal space it is possible to observe two rosettes, one marked with a small arrow and the other marked with a. Both rosettes express ß-III-tubulin, although it is not possible to rule out the possibility that other invaginations of the ependyma might be involved, like the one mentioned above. In juxtasubependymal areas there is another rosette marked with b whose cells do not express ß-III tubulin. B corresponds to the part of the image marked with a, at higher magnification, whereas C corresponds to the rosette marked with b. Next to the rosette shown in C there is a group of cells with smaller nuclei and with scant cytoplasm. The a-a1 axis seems to show that a section at that level would show a cluster or section tangential to the wall of another rosette. D and E show juxtasubependymal zones whose cells express B-III tubulin.
immunoreactive material they expressed (Fig. 3A,B). In contrast, the cells of another similar structure, located in the glandular parenchyma close to the juxta-subependymal zone, did not express that protein (Fig. 3A,C). Additionally, in images D and E of Figure 3, it is possible to note many pinealocytes expressing β-III tubulin.

Sometimes the sections did not include the lumen of the rosette itself, cutting its cell wall tangentially. In this case, we observed a small accumulation of cells other than pinealocytes, which were either in continuity with a rosette or separated from it, forming clusters (Fig. 3C).

Most of the rosettes observed were formed by cells expressing the three markers of glial cells: vimentin, GFAP or S-100 (Figs. 2-4), although as explained below regarding the images obtained with confocal microscopy and double labelling some differences were seen in the distribution of the immunoreactive material for each of the above antisera. It should be noted that the non-cavitary side of the cells gave off long cytoplasmic prolongations that contacted other glial cells, which appeared to be migrating from the walls of one of such structures to the surroundings of vessels and of pinealocytes or contacting other rosettes. Striking images of this are shown in Fig. 4 (A-G). Accordingly, despite the observation of the isolated presence of some cells that were non-immunoreactive for the substances long considered to be glial markers, it may be concluded that the wall of the vast majority of the rosettes were formed by astrocytic glial cells.

However, we sometimes found a very few rosettes whose cells expressed β-III-tubulin (Figs. 2.5A). These rosettes were located in the juxtaependymal portions or among the fibres of the habenular commissure, where there were also similar structures expressing vimentin or GFAP (Fig. 5B). Moreover, although now in the glandular body, we observed a few rosettes whose cells contained somatostatin-like immunoreactive material (Fig. 5C), while in zones close to them there were accumulations of SRF-immunoreactive cells (Fig. 5E).

Also, working with the ABC complex technique, but now using anti-nestin antisera at 1:200 dilution (a high dilution for the antiserum used), we observed that the expression of this protein was limited to the bodies of cells dispersed throughout the glandular parenchyma (Fig. 6A) and others located on the wall of the rosettes and in some cytoplasmic prolongations of the same (Fig. 6B,C). When the antinestin serum was used at lower dilutions (range 1:50) the immunoreactivity also appeared localized in all the pinealocytes.

When the sections were incubated in an anti-PCNA antiserum, most of the nuclei of the cells expressing the antigen were located on the walls of the rosettes, in clusters or in isolated cells distributed throughout the pineal parenchyma (Fig. 7A). As a positive control for the PCNA immunoreaction, we incubated sections of rat pineal gland from animals with an age of two days in the same antiserum. The nuclei of many pineal cells also showed immunoreactivity for the antigen (Fig. 7B).

Study based on confocal microscopy

Sections of cow pineal gland were used for confocal microscopy studies with a view to studying -with fluorescence techniques- the colocalization of immunoreactive cells and the possible existence of double labelling.

Vimentin- β - III tubulin

The immunoreaction for β-III tubulin was very striking and appeared localized in the cytoplasm of the pinealocytes in the form of red fluorescence. By contrast, the vimentin-like immunoreactive material was observed in the form of green fluorescence, located in the cytoplasm and cytoplasmic prolongations of many of the cells making up the rosettes and of other cells that formed small clusters or appeared isolated among the pinealocytes.

Regarding rosettes, we observed that most of them were made up of cells mainly expressing vimentin, although there were others that expressed β-III tubulin, a protein present in all the pinealocytes. However, we failed to note the colocalization of both labels in any of the cells of the rosettes. In contrast, we sometimes detected isolated cells in the pineal parenchyma in which vimentin and β-III tubulin seemed to coexist. Also, it was striking to observe that the walls of many rosettes contained some cells that did not express either vimentin or β-III tubulin. All these observations are depicted in figure 8, comprising two series of three images obtained from the pineal gland of one-year-old cows (Fig. 8A-C) and cows of four years of age (Fig. 8D-F) respectively.

Vimentin-S-100

From the immunohistochemical point of view, the pattern of distribution of the cells of the walls of the rosettes is complex. Indeed, many cells of the rosette wall expressed vimentin; others, less abundant, expressed S-100, and only some expressed both proteins. Moreover, the difference in the expression of both antigens was very marked in the case of the glial cells dispersed throughout the glandular parenchyma; these were very numerous in the case of S-100 and very scarce as regards vimentin. An example of this can be seen in figure 9. Thus, on comparing images A and B in that figure it is possible to note differences in the distribution of both markers, together with the existence in the wall of the rosettes, and sometimes in the glandular parenchyma, of cells coexpressing vimentin and S.-100 (Fig. 9C).

Vimentin-GFAP

As in the previous case, the immunoreactivity for vimentin was localized in the cells making up the rosettes and in isolated cells in the glandular parenchyma. Also, vimentin-like immunoreactive
Fig. 4. This figure shows 7 images belonging to sections of the pineal gland from a one-year-old cow incubated with anti-vimentin (A-C) and a cow of four years of age incubated in anti-GFAP (D-G). Note in A that one of the cells displays a cytoplasmic prolongation coursing towards a neighbouring vessel (black arrow). The grey arrows show groups of vimentin-positive cells separated from the rosette. B shows a rosette whose cells express vimentin. The black arrow shows a vimentin-positive cell that seems to be separating from the walls of the rosette. The green arrow points to a space among vimentin-positive cells in the wall of another rosette. C and D show rosettes expressing vimentin and GFAP, respectively. Note how vimentin- or GFAP positive prolongations course out from the rosettes, moving away from them (black arrows) and reaching positive cells located around vessels (red arrows), while the green arrows show breaks in the walls of the rosette. E corresponds to a section of the pineal gland from the same animal incubated in anti-GFAP antiserum in which several rosettes can be seen. The two rosettes marked with R1 and R2 are shown at higher magnification in F (R1) and G (R2). In both images it is possible to see that not all the cells of the wall express GFAP (the green arrows point to the non-immunoreactive zones). Grey arrows point to GFAP-positive cells separate from the rosettes.
Fig. 5. Composite of four images taken from a section of the pineal gland of a four-year-old cow. A corresponds to a section incubated in anti-ß-tubulin-III serum in which it is possible to observe both the habenular commissure (hc) and the pineal parenchyma. In the latter, numerous pinealocytes expressing the protein can be seen (P). The rosette located in the habenular commissure (arrow) is formed by cells that also express ß–III tubulin. B was taken from a section incubated in anti-GFAP serum, showing the habenular commissure (hc), and in this latter two rosettes, marked with arrows, whose walls are formed by GFAP-expressing cells can be seen. The pineal parenchyma is marked with a P. C corresponds to a section incubated with anti-somatostatin serum (anti-S14). In the pineal parenchyma there is a rosette in whose wall it is possible to discern four cells expressing somatostatin, while the remaining cells of the rosette do not contain somatostatin-like immunoreactive material. D was taken from a section incubated in anti-somatostatin S-14. Note the presence of several cells that strongly express SRIF-like immunoreaction distributed throughout the glandular parenchyma.
Fig. 6. This contains three images from a section of the pineal gland of a one-year-old cow incubated in anti-nestin serum, at a dilution of 1/200. Image A shows nestin-positive cells distributed throughout the glandular parenchyma. B, taken from the same section, shows two rosettes in whose walls nestin-expressing cells can be seen. A similar picture is seen with the rosette shown in C. Note that other nestin-positive cells are located at some distance from the rosette.
material appeared in the subependymal and juxtacommissural zones. Moreover, GFAP-like immunoreactive material was located in the rosette walls, although the number of cells labelled with this antiserum was lower than that observed for vimentin. Moreover, many of the rosette cells showed a double reaction (observed in yellow). On the other hand, in the glandular parenchyma there were numerous somata and cytoplasmic prolongations of cells that were GFAP-positive but did not express vimentin and were distributed among the pinealocytes and the surroundings of vessels. This is reflected in the images of the last three

Fig. 7. This shows two images. A corresponds to a section close to that of the images in the previous figure, incubated in anti-PCNA serum. The long black arrows show several rosettes formed by cells whose nuclei express that protein, and the small black arrows depict some of the many PCNA-positive cells scattered through the cellular parenchyma. B corresponds to a section of rat pineal gland of two days of age, used as a positive control of the immunoreaction. The image shows numerous cell nuclei expressing PCNA, some of which seem to belong to cells forming part of a rosette (arrows), while most of them appear isolated or forming small clusters in the glandular parenchyma.
Vimentin-nestin

The expression of nestin varied considerably, depending on the dilution of the antiserum. When dilution was low (range 1:50), labelling was seen in both the pinealocytes and in the glial cells. As the dilution was raised (1:200), the immunoreactivity only appeared in the somata of glial cells dispersed among the pinealocytes and in cells on the walls of the rosettes. In the latter case, it was possible to observe nestin-immunoreactive cells among the vimentin-positive cells, whereas in others a double vimentin-nestin-positive reaction was observed (Fig. 10).

DAPI

The use of DAPI selectively detected both the nuclear membrane and the nuclear heterochromatin of the pineal cells. A discrete blue fluorescence was observed in the nuclei of the pinealocytes, while it was very strikingly expressed in the nuclei of the cells making up the rosettes. (Fig. 11). Also, the nuclear reaction for DAPI was also especially prominent in groups of ependymocytes, which appeared forming folds or pockets opened towards the subependymal space or towards the third ventricle (Fig. 11A,B).

Regarding the number of the rosettes.

A significant difference (p<0.01) was found between the number of rosettes present in the bodies of the pineal glands of one-year-old (25.25±8.04) and four-year-old cows (10.5±4.04). We also observed a significant difference (p<0.03) in the number of rosettes detected in the juxtasubependymal areas in the pineal glands of one-year-old (15±4.6) and four-year-old cows (7.2±2.9),

**Fig. 8.** This shows six images corresponding to two confocal microscopy series applied to a section of the pineal gland of a one-year-old cow, incubated successively in vimentin and β-III tubulin. In the first series (A-C), it is possible to observe a rosette whose wall is formed by cells that express vimentin (A) and β–III tubulin (B, white arrows). In the second series (D–F), at lower magnification, it is possible to see a rosette whose walls strongly express vimentin and some cells expressing β-III tubulin (white arrows). Vimentin-like immunoreactivity also appears in small groups of cells and fibres distributed through the glandular parenchyma surrounding the rosette. The grey arrows point to cells with double labelling (F).
Fig. 9. This figure shows four series of images obtained from sections incubated successively in vimentin and S-100 (A-C), and vimentin and GFAP (D-L). The first series shows numerous cells expressing vimentin (green) in the walls of the rosette (R) located in the centre of the image, while the other cells express S-100 (ova and white arrows). The grey arrows point to cells in which both labels are present. In the second, third and fourth series it is possible to see the differences in the expression of vimentin (green) and GFAP (red). There are some cells that coexpress both labels and others that express only either vimentin or GFAP, both in the rosettes and in the surrounding glandular tissue. Thus, D-F show a rosette composed almost exclusively of vimentin-positive cells, some of which display cytoplasmic processes (white arrows). The small grey arrow signals the soma of a cell coexpressing vimentin and GFAP, while in the gross grey arrows point out isolated cells expressing only vimentin. G-I of the third series show a rosette whose wall displays cells that coexpress vimentin and GFAP, while others express only vimentin. Note also that both vimentin and GFAP are expressed differentially in the somata and cytoplasmic prolongations of isolated cells in the glandular parenchyma. J, K and L, belonging to the fourth series, show a rosette whose cells differentially express vimentin (white arrow) and GFAP (grey arrow). Additionally, a group of cells isolated in the cytoplasm express only GFAP (a).
respectively. No significant differences were found between the number of rosettes found at the apex of the pineal glands of either group of animals. Since there is no defined anatomical structure that would have allowed us to obtain an accurate measurement either of the juxtasubependymal area or of the apex, the statistical study can only refer to the total number of rosettes present in 10,000 µm² of each of these two zones. Whereas a significant difference was noted (p<0.05) between the rosettes observed at the juxtaependymary zone (15±4.6) and the apex (8.2±2.5) of the one-year-old animals, we failed to observe significant differences in the number of rosettes present in juxtaependymary zone and the apex of the four-year-old animals. These data are shown in Table 1.

Discussion

Rosettes have been described in the pineal gland during the embryonic development of bovines (Anderson, 1965; Brack, 1962) and sheep (Anderson, 1965), although in those works the nature of the cells forming them was not specified. Other authors have reported the presence of rosettes in the pineal glands of rat embryos (Calvo and Boya, 1981) and in those of adult rabbits (García Mauriño and Boya, 1992). In both cases, the authors observed that the walls of the rosettes were formed by GFAP-positive cells, such that they assessed them as being interstitial cells. Later, Regodón et al. (2006), working with phosphotungstic acid-haematoxilin and immunohistochemical techniques, revisited the issue and described rosettes in cow fetuses of 200 days as structures made up of cells with an epithelial aspect, similar to the ependymal cells of the pineal recess of the third ventricle. Since none of the cells of the rosettes present in this phase of embryonic development expressed GFAP, those authors concluded that the structures were formed by melatonin-secreting pinealocytes. However, some of us (Gómez Esteban et al., 2008) have reported that most of the cells of the rosettes present in the pineal glands of one-year-old cows expressed vimentin, such that they were designated glial cells.

In view of the diversity of techniques used by the above authors and the disparity of the interpretations offered to explain the meaning of the cells forming the rosettes, we first focused our study on checking the existence of these structures after birth and then on gaining a better knowledge of their nature. To accomplish this, we worked with ABC immunohistochemical techniques applied to sections of pineal glands from cows of 1 and 4 years of age. Our first confirmed observation in this work was that rosettes were always present in all the glands studied, although their numbers were significantly lower in the older animals. Additionally, the number of rosettes varied not only with age but also as a function of the zone of the gland studied. Thus, although the total number of rosettes counted in the body of the gland was significantly

Fig. 10. This figure shows a series of three images taken from a section of the pineal gland of a one-year-old cow, incubated successively in anti-vimentin and anti-nestin. Note the coexistence of both labels in isolated cells and others forming a rosette (R).
greater than that found in zones neighboring the subependymal space (the juxtasubependymal zone), upon comparing the dimensions of the surfaces of both parts we noted that the number of rosettes was proportionally higher in the juxtasubependymal zone than in the body of the gland.

Fig. 11. This shows four images obtained from a section of the pineal gland of a one-year-old cow, incubated in DAPI. A shows a) the ependyma (Ep); b) the subependymal space (SuS), and c) a zone of the glandular parenchyma. The white arrows show structures that seem to be rosettes. Note that the fluorescence is very marked in the Ep and in isolated cells inside the SuS. The zone shown in a white box is depicted at higher magnification in B and shows that the nuclei of that zone of the Ep and of some SuS cells exhibit a very intense DAPI reaction. C corresponds to a portion of the body of the pineal gland, in which it is possible to see the intense reaction for DAPI in the cells that form the rosettes. In D, taken at high magnification, shows a rosette (white arrows) whose cellular nuclei strongly express DAPI. Grey arrows point out some isolated cells with low DAPI reaction.
Regarding the nature of the cells forming the walls of the rosettes, a striking finding was the coincidence of three observations. The first was that most of the rosette cells found in the subependymal space and in neighboring zones (the juxtasubependymal zone) expressed β-III tubulin, a protein that is present in the cytoplasm of the pinealocyte cytoskeleton and of neurons, but not glial cells (Lee et al., 1990; Alexander et al., 1991; Draverova et al., 1998; Katsetos et al., 1998). The second observation was that in certain zones of the ependyma there were many β-III tubulin positive cells. Thirdly, ependymal invaginations rich in β-III tubulin-expressing cells coursed towards the subependymal space. These data seem to confirm the opinions of Lazzari et al. (2006) and Regodón et al. (2006) regarding the notion that these structures derive from the ependyma.

Another aspect meriting comment refers to the cellular composition of the rosette walls, the only aspect regarding which until now there has been some controversy. According to our results, the rosettes are not only the sources of pinealocytes, as suggested by Regodón et al. (2006), or of interstitial cells, proposed by Calvo and Boya (1981) and García Mauriño and Boya (1992), or of glial cells, as proposed by us (Gómez Esteban et al., 2008). Rosettes are highly complex structures made up of cells that express different markers. Thus, in the rosettes observed in zones close to the subependymal space or even within the habenular and posterior white commissures, we observed cells expressing β-III tubulin together with others expressing vimentin or GFAP. In turn, in the rosettes located in the glandular tissue close to the subependymal space (the juxtasubependymal zone) the cells mainly expressed vimentin or GFAP. The cells of the rosettes located in the glandular body and apex mainly expressed vimentin, GFAP or S-100. On remarking mainly twice, we are attempting to convey the idea that the same rosette may contain cells expressing β-III tubulin as well as other cells expressing glial markers, or even cells expressing somatostatin.

The complexity of rosettes can be better understood using confocal microscopy, which allows work to be carried out with two labels simultaneously. Thus, we observed that in the walls of the rosettes there were cells expressing vimentin and others expressing β-III tubulin. In other rosettes there were vimentin-expressing cells together with others expressing GFAP and other rosettes whose cells coexpressed vimentin and GFAP or vimentin and S-100.

These data can be interpreted in the sense that rosettes would be the source of pinealocytes and of glial cells and that some stage in the maturation process of the glia may be occurring in the walls of the rosettes. This would be tantamount to suggesting that the rosettes present in the pineal gland of cows are structures related to processes of neurogenesis and post-natal cellular differentiation.

Different authors have published abundant information about the origin of the Central Nervous System, which seems to arise from a small number of neuroepithelial cells defined as stem cells, because during embryo development they are able to generate different cell types, such as neurons or glia (Doetsch et al., 1999; Doetsch, 2003a,b; Alvarez-Buylla and Lim, 2004). In teleosts, neurogenic capacity persists after birth from a cellular subtype of radial neurogenic glia (Omura et al., 2003, 2004; Zupanc, 2001; Zupanc and Horschke 1995), and also in zebra-fish (Zupanc et al., 2005), in trout (Omura, 2007), and in the dente gyrus of primates (Eriksson et al., 1998; Gould et al., 1999a,b), with a significant increase in the number of neurons (Boss et al., 1985). From in vitro studies it is known that in some zones of the brain of adult mammals, such as the subventricular space of the lateral ventricles and the subgranular layer of the hippocampus, cells with neurogenic capacity persist, these cells having the characteristics of astrocytic glia, such as GFAP expression (Seri et al., 2001; Lie et al., 2004). Also, in the circumventricular organs of the rat —including the pineal gland— cells have been described that express GFAP, vimentin and nestin, such that they should be considered an important source of neurons after birth (Bennett et al., 2009). In this sense, it has been demonstrated that the selective destruction of GFAP-expressing cells involves a loss of neurogenic capacity in the brains of this species (Doetsch et al., 1999; Imura et al., 2003; García et al., 2004), while if GFAP-positive cells persist they group together, forming neurospheres or clusters, which some authors have also called rosettes because they have a cavity inside them (Lazzari et al., 2006). The cells of these structures may differentiate to neurons or glia, depending on the growth factor added to the culture medium. Since all the cells present in the clusters express nestin, GFAP and vimentin, these proteins are considered to be markers of stem cells.

By analogy with the findings of all the above authors, it could be suggested that the cells of the rosette walls would in principle be stem cells and that these would give rise to pinealocytes, glial cells and neurons during the ontogenic development of cows; not only during embryonic development but also after birth, at least during the first few post-natal years. In this sense, it would appear that in the pineal gland of cows aged one and four years a process of post-natal neurogenesis occurs, similar to that described by Lie et al. (2004) and Seri et al. (2001, 2004, 2006) in the allocortex of the hippocampus.

Nevertheless, the authors mentioned in the previous two paragraphs, together with Namiki and Tator (1999), Chouaf-Lakhdar et al. (2003) and Giljarov (2008), consider that in order to define a cell as a stem cell an essential requirement is that it should express nestin. With the ABC technique we found that many of the cells present in the walls of the rosettes expressed nestin. Moreover, many somata of cells dispersed throughout the parenchyma clearly expressed nestin immunoreactivity. When we used double labelling techniques,
we observed that most rosette cells, and sometimes even all of them, coexpressed vimentin and nestin. A similar picture was seen for the isolated cells distributed throughout the parenchyma. This suggests the existence of processes of emigration of cells from the glial lineage from the walls of the rosettes to become distributed throughout the glandular parenchyma, and that some of the steps in cellular differentiation may occur in the glandular parenchyma, depending on the events occurring in the environment of the immunoreactive cells.

The coexistence of vimentin and nestin in rosette cells supports the existence of a process of post-natal neurogenesis in the cow pineal gland. However, what is the purpose of this? It seems that it would be designed to produce pinealocytes and glial cells. This would require the existence of a process of cellular proliferation. To demonstrate this, we used DAPI, since it has been suggested by different authors (Schweizer and Nagi, 1976; Eastman et al., 1980; Stöhr et al., 1978, 1980; Buys and van der Veen, 1982) that DAPI could label segments of DNA rich in A-T. Our images clearly suggest that the nuclei of ependymocytes, and especially the nuclei of rosette cells, express a very strong reaction for this compound, such that the technique could be considered at least complementary to immunohistochemical methods to assess the existence of cells with proliferating activity, which in particular are located in the rosettes, as we observed with PCNA. In fact, we observed strong expression of PCNA both in the nucleus of many isolated cells located in the neighbourhood of such rosettes and on many other cells dispersed throughout the glandular parenchyma. According with Wildemann et al. (2003) and Bolton et al. (1994) this supports the possible existence of an important proliferative activity that is not limited to the walls of rosettes but also occurs in the cells located around them. Since our histological sections only provide static images taken at a given time, we cannot state categorically, but only suggest, that cells would emigrate from the walls of the rosettes and eventually finish their differentiation in the glandular parenchyma.

Accordingly, are rosettes the only source of pinealocytes and glial cells? All the evidence suggests that this would not be the case. We also found rosettes whose walls displayed cells expressing somatostatin, a hormone present in groups of cells dispersed through the glandular parenchyma and whose presence has been known for some time (Brownstein et al., 1975; Pelletier et al., 1975). With the two images shown in our photos, we suggest that rosettes may also be a source of neurons that, in this case, express somatostatin. All the foregoing data suggest that rosettes are very complex structures, in principle formed by pluripotential cells with the ability to differentiate into pinealocytes, neurons, or glial cells. In other words, in theory these structures would be formed by neural stem cells that at a given moment differentiate into somatostatin-secreting neurons, melatonin-secreting pinealocytes, or glial cells, depending on the gene expression that is occurring at each moment or on the environmental factors occurring within the pineal gland.

We also have to mention the existence in the ependyma and subependymal space of many vimentin- and GFAP-positive cells, which were either arranged in a palisade among the ependymocytes or formed small clusters in the subependymal space. It is precisely the subependymal space of other parts of the CNS where different authors have collected vimentin-β GFAP- and nestin-positive cells with a view to performing in vitro assays, concluding that these cells would be responsible for post-natal neurogenesis in animals of different species, including mammals (Boss et al., 1985; Doetsch et al., 1999; Seri et al., 2001; Imura et al., 2003; García et al., 2004; Lie et al., 2004; Lazzari et al., 2006). The existence in the ependyma and the subependymal space of the cow pineal gland of many cells expressing GFAP and vimentin suggests that the process of post-natal neurogenesis in the cow pineal gland might not be reduced to the walls of the rosettes and that the radial ependymal glia might also be involved.

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References


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