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Short Report

Immunohistochemical and ultrastructural study of interstitial cells during postnatal development of the sheep pineal gland

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The pineal originates as an outgrowth of the roof of the diencephalon in all vertebrate species. This evagination arises between the habenular commissure anteriorly (or rostrally) and the posterior commissure and subcommisural organ posteriorly (or caudally). Three cell types have been defined in mammal pineal gland parenchyma: pinealocytes or principal cells, supportive or interstitial cells and pigmented cells. Numerous terms have been used to designate the second cell type, including interstitial cells, type II pinealocytes, glial cells and astrocytes.

Sheep pineal gland interstitial cells were studied during postnatal development using ultrastructural and immunohistochemical techniques. 18 merino sheep were studied from 1 month to > 2 years of age. Animals were divided in three age-groups: group 1 (1, 3 and 6 months of postnatal development), group 2 (9 months, 1 year and 2 years) and group 3 (> 2 years). A second cell type was observed which differed from pinealocytes and showed uniform ultrastructural characteristics similar to those of astrocytes in the central nervous system. Pineal interstitial cells started to show signs of functional activity evident in the vascular tropism detected for these cells and relating to exchange of substances between the pineal parenchyma and blood vessels, at around 9 months and 2 years of age.

Adult sheep pineal glands were sliced parasagitally after 1 hour in Carnoy's fluid. One of the two portions thus obtained was fixed in 10% neutral formalin saline and processed by paraffin-embedding methods. Sections 4 μm thick were cut and stained with phosphotungstic acid hematoxylin (PTAH) for detection of glial-type cells.

ExtrAvidin peroxidase staining (EAS) was carried out on deparaffinized pineal samples for detection of glial fibrillary acidic protein (GFAP), the main protein component of intermediate astrocyte filaments.

The other half of each pineal gland was used for ultrastructural analysis. Tissue blocks were immersed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2), and embedded in epoxy resin. Ultrathin sections were cut, stained with colloidal gold (10 nm) for detection of GFAP positive cells.

PTAH positive cells were morphologically similar in the three postnatal age-groups, differences being purely quantitative (more numerous in group 2). Notably large oval nuclei were distributed uniformly throughout the gland surface (Fig. 1).

Quantitative differences between members of the same age-group were negligible and no significant sex-related differences were found. The following descriptions therefore apply to groups as a whole. The interstitial cells of group 1 displayed two distinctive features: 1.- they were less numerous than pinealocytes; and 2.- they displayed a clearly perivascular arrangement. The rounded nucleus contained nucleolus composed of a highly electron-dense nucleolonema surrounding a fibrillary core. Cytoplasm was less electron-dense and contained fewer organelles than that of pinealocytes. Endoplasmic reticulum was mostly granular. Lysosomes with clearly-defined limiting membrane were observed in perinuclear cytoplasm, together with diplosomes and ribosomes. The most characteristic feature of these cells was the presence of very long processes with abundant microfilaments.

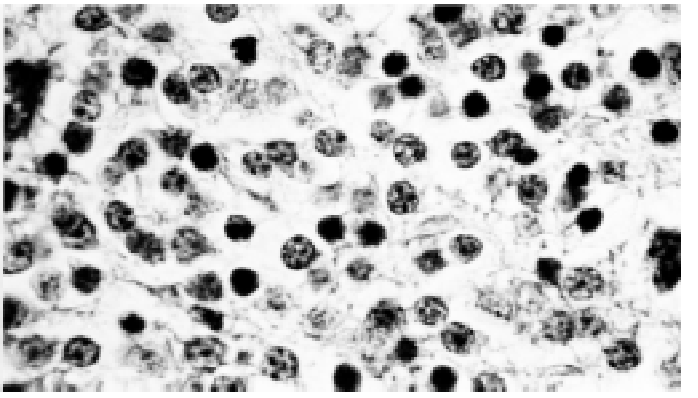

Fig. 1. Interstitial cell nuclei *are small, dense an ovoid. PTAH x 350.*

In group 2, their histological features were similar to those described earlier, except for: 1.- Greater electron-density of nucleoplasm and cytoplasm, and greater abundance of cytoplasmic organelles, particularly Golgi complexes, mitochondria and granular endoplasmic reticulum. 2.- Cytoplasmic processes displayed a larger number of microfilaments and a moderate presence of gap junctions in terminal clubs. Ultrastructural characteristics of interstitial cells in group 3 were similar to those of the previous group, except for:

1.- fewer microfilaments in cytoplasmic processes and

2.- fewer gap junctions in terminal clubs.

GFAP positive cells were observed in pineal glands of all three age-groups studied. In all groups, these cells were distributed uniformly over the whole gland surface, generally in perivascular locations (Fig. 2). Morfologically, they displayed a marked resemblance to central nervous system astrocytes, with a small, ovoid, electron-dense and negative-staining nucleus, surrounded by an intensely-staining cytoplasm. A small number of cytoplasmic processes were visible, arranged both longitudinal and transversely

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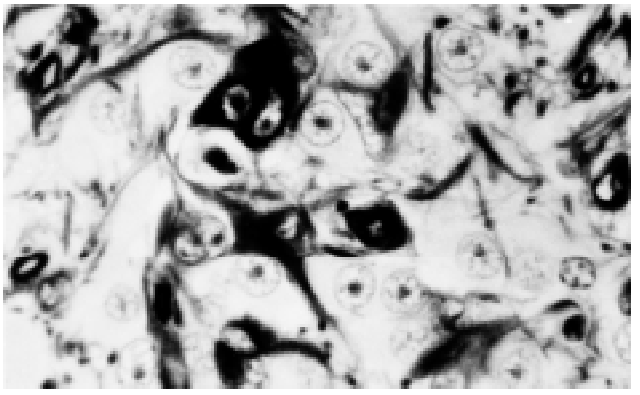

Fig. 2. GFAP+ cells *with long cytoplasmic processes. EAS x 350*

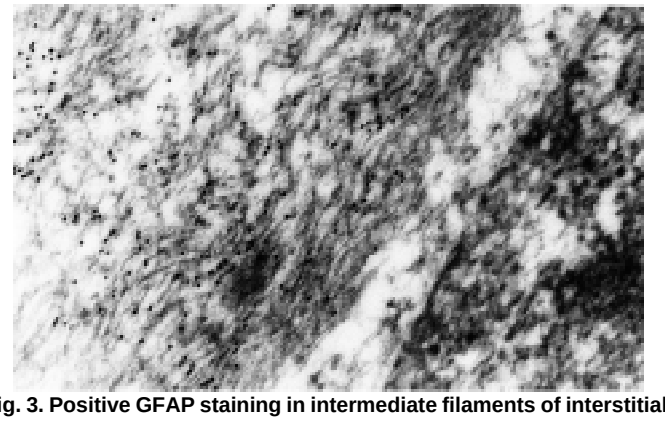

Fig. 3. Positive GFAP staining in intermediate filaments of interstitial cells. *IEM x 30,000.*

with regard to pinealocytes. Group 3 glands contained a larger number of cytoplasmic processes than those of groups 1 and 2; processes in this group also displayed greater variety in terms of both diameter and orientación.

From 1 months through to 2 years of postnatal development (group 1 and 2) immunoreactivity for colloidal gold labelling expression of GFAP showed a progressive increase. Staining intensity decreased considerably in group 3. In postnatal development, colloidal gold labelling revealed expression of GFAP by cells whose morphology closely resembled that described ultrastructurally for the interstitial cells. These cells were observed in perivascular locations, exhibited ovoid and/or elongated non-staining nuclei, and strong cytoplasmic positivity, with clear affinity for the microfilaments of cytoplasmic processes (Fig. 3). Adjacent pinealoblast remained immunonegative.

The results obtained indicate the existence of a second cell type (interstitial cells), in addition to pinealoblasts, during the postnatal development of sheep pineal gland. Light microscopy highlighted the resemblance between these cells and the second cell type described in the pineal gland of other species. However, ultrastructural homogeneity rendered impossible the detection of possible subtypes. Histochemical and immunological tests were thus performed with a view to overcoming this limitation and at the same time enhancing our poor knowledge of this second cell type. The first step was to determine the presence of glial-like cells by PTAH immunostaining. GFAP is widely considered a valid label for the detection of astrocyte development and more particularly as evidence of the presence of astrocytic cells at a certain stage of maturity (Valentino *et al.,* 1983). The second step was thus to determine GFAP espression.

Comparison of the results of these two tests (PTAH and GFAP) showed that of all glial (PTAH positive cells), only a proportion were positive to GFAP. The number of PTAH positive cells increased from 1 to 24 months of age. Thre was a parallel increase in the numerical density of GFAP positive cells; these, however, were always less numerous than PTAH positive cells, indicating that some glial cells (PTAH+) were not GFAP positive; this suggests the possibility of immature astrocytes.

On the basis of the morphometric and immunohistochemical results obtained, two conclusions were drawn regarding the second cell population in sheep pineal glands during postnatal development: 1.- There was no clear correspondence reaction between the number of PTAH positive cells and the number of GFAP positive cells. 2.- The interstitial cells population is in fact a combination of glial-astrocyte cells at varying stages of maturity (López-Muñoz *et al.,* 1992).

Ovine pineal gland interstitial cells appear to show signs of functional activity in animals slaughtered at between 9 months and 2 years of age (group 2). Arguments supporting this hypothetical functional role include: a) The relationship between GFAP positive cells and blood vessels (Borregón *et al.,* 1993; Boya and Calvo, 1993). b) The relationship between interstitial cells and nerve fibers. Sites of specific contact between the two were observed here, and were more abundant in the most morphologically active glands (group 2). The precise functional significance of this relationship is not known, although it has been suggested that nerve fibers might be involved in interstitial cell development; indeed, these cells do not develop in the absence of innervation (Calvo and Boya, 1983).

This hypothetical functional role, relating to the exchange of substances between the pineal parenchyma and the bloodstream (Xu Zang *et al.,* 1985), would complete the support function (similar to that of astrocytes in the CNS) traditionally attributed to these cells. The irregular arrangement of type II cells interspersed throughout the pineal parenchyma may represent morphological evidence of this support function (López-Muñoz *et al.,* 1992).

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