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The Pineal Region in the Opossum, *Didelphis virginiana*

I. Ultrastructural Observations

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Summary. In the pineal region of the opossum, *Didelphis virginiana*, two types of cells predominate: 1) pinealocytes, and 2) fibrous astrocytes. Pinealocytes are characterized by the presence of prominent Golgi bodies, numerous clear and dense-cored vesicles, sensory cilia (9+0), vesicle-crowned rods, and condensation of a material that was always associated with the rough endoplasmic reticulum. In addition, two other cell types are occasionally seen. These include 1) neuron-like cells, and 2) darker staining cells of unknown identity. The endoplasmic reticulum of the darker staining cells is typically expanded and filled with an amorphous substance. Although the pineal region is small in size, the present findings suggest that pinealocytes in this species are metabolically active cells displaying a secretory function. Moreover, the presence of sensory cilia (9+0) and vesicle-crowned rods indicates that pinealocytes of the opossum are phylogenetically related to the photoreceptor cells found in the pineal organ of lower vertebrates.

Key words: Pineal region – Opossum – Ultrastructure.

Comparative studies of the pineal organ in different classes of vertebrates have demonstrated varying morphological features related to the phylogenetic transition from a photoreceptive organ to a secretory gland (Oksche, 1975). Moreover, there is ultrastructural evidence that mammalian pinealocytes are homologues of the photoreceptor cells of lower vertebrates, having evolved along a sensory cell line (Collin, 1971; Oksche, 1971). This hypothesis is supported by the presence in reptiles and birds of intermediate cell types termed "secretory rudimentary photoreceptor cells" (Collin, 1969) or "pseudosensory cells" (Vivien-Roels, 1970). With differentiation, pinealocytes of placental mammals have been shown to resemble these rudimentary sensory cells (Zimmerman and Tso, 1975).

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Relatively few morphological studies are available on the pineal of more primitive, non-eutherian mammals (Delahunt et al., 1975; Hofer et al., 1976; Jordan, 1911; Kenny and Scheelings, 1979). The purpose of the present study, therefore, was to examine the fine structure of the pineal region in a marsupial, the American opossum, *Didelphis virginiana*. Considering its phylogenetic position, being derived from ancestral stock between reptiles and placental mammals, the ultrastructure of pineal cells in this species will provide additional information on functional evolutionary changes in the pineal gland of vertebrates.

Materials and Methods

Tissue from nine adult, male opossums (1.8–2.3 kg) was utilized in this study. Animals were anesthetized with 64 mg/kg sodium pentobarbitol and perfused via the left ventricle with 0.89% saline containing 0.5% procaine hydrochloride followed by 1500–2000 ml of 1% paraformaldehyde, 1.25% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) with 1% sucrose. The brains were removed and placed in an additional volume of fixative for 12–16 h. One brain was embedded in paraffin, sectioned in a sagittal plane, and stained with luxol fast blue and cresyl violet. The entire pineal region was removed intact from the remaining brains, postfixed in 1% osmium tetroxide in phosphate buffer, dehydrated in acetone, and embedded in Epon. Sections from each of these tissue blocks were cut at 1 μm thickness, stained with toluidine blue and examined with the light microscope. For electron microscopy, ultrathin sections (60–80 nm) were stained with uranyl acetate and lead citrate and examined with either a RCA EMU-3F or Hitachi 11-B electron microscope.

Results

The pineal region in the opossum comprised a layer of cells bordering the third ventricle anterior to the habenular commissure and coursing posteriorly to form a recess between the habenular and posterior commissures (Fig. 1). In the present study, both the extent of the recess and thickness of the pineal varied among specimens. Small bundles of myelinated nerve fibers passed through the posterior regions of the pineal (Fig. 2A). Ventrally, the pineal cells were continuous with ciliated ependymal cells

Two predominant cell types were identified at the light microscopic level by differences in the staining intensity of their nuclei (Fig. 2A). Cells with darker, generally ellipsoid-shaped nuclei represent glial elements. Lighter-staining cells had nuclei that were usually round and contained a prominent nucleolus. These cells, which are here referred to as pinealocytes, extended to the ventricular surface and were sometimes seen to form rosettes (Fig. 2B).

A third cell type was less frequently encountered in the vicinity of the myelinated nerve fiber bundles. The nucleus of this cell was slightly larger than that of pinealocytes and contained little heterochromatin (Fig. 2A).

Electron microscopic observations confirmed that the cells with darker-staining nuclei are glial cells, specifically fibrous astrocytes. The nucleus of these cells contained peripheral heterochromatin and sometimes exhibited an irregular nuclear envelope (Fig. 3). Large bundles of microfilaments were present in the cytoplasm. Other organelles were sparse and included mitochondria, Golgi bodies, and occasional rough endoplasmic reticulum. Processes from these astrocytes extended between pinealocytes and their processes.

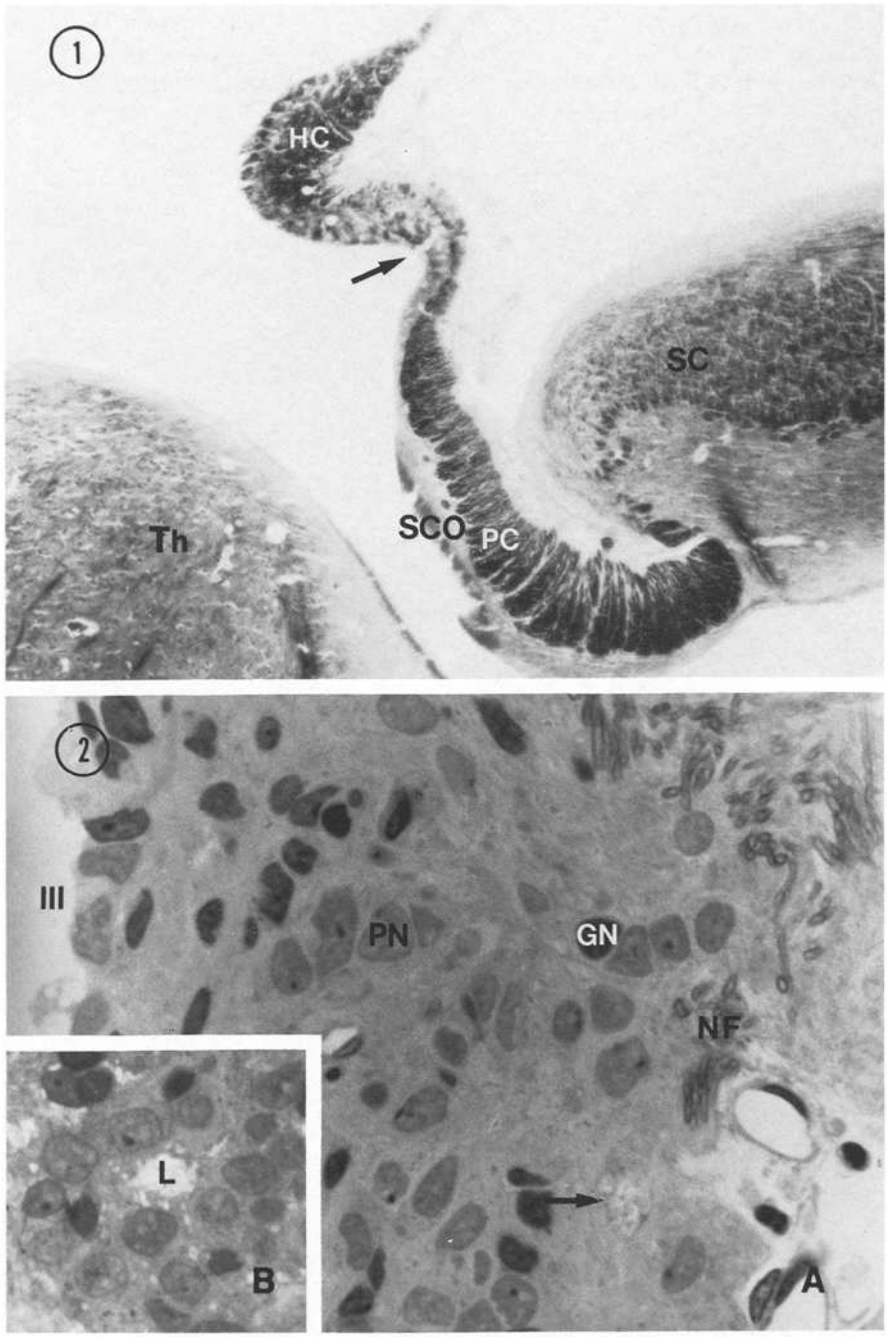


Fig. 1. Sagittal section (anterior to the reader's left) of pineal region demonstrating the pineal recess (*arrow*). *HC* habenular commissure; *PC* posterior commissure; *SC* superior colliculus; *SCO* subcommissural organ; *Th* thalamus. × 40

Fig. 2. A Higher magnification of pineal region. Pinealocyte nuclei (*PN*), glial-cell nuclei (*GN*), and myelinated nerve fibers (*NF*) are identified. A cell with a large, vesicular nucleus (*arrow*) is seen in the posterior region of the pineal. *III* third ventricle. × 720. **B** Pinealocytes arranged in a rosette around a small lumen (*L*). × 720

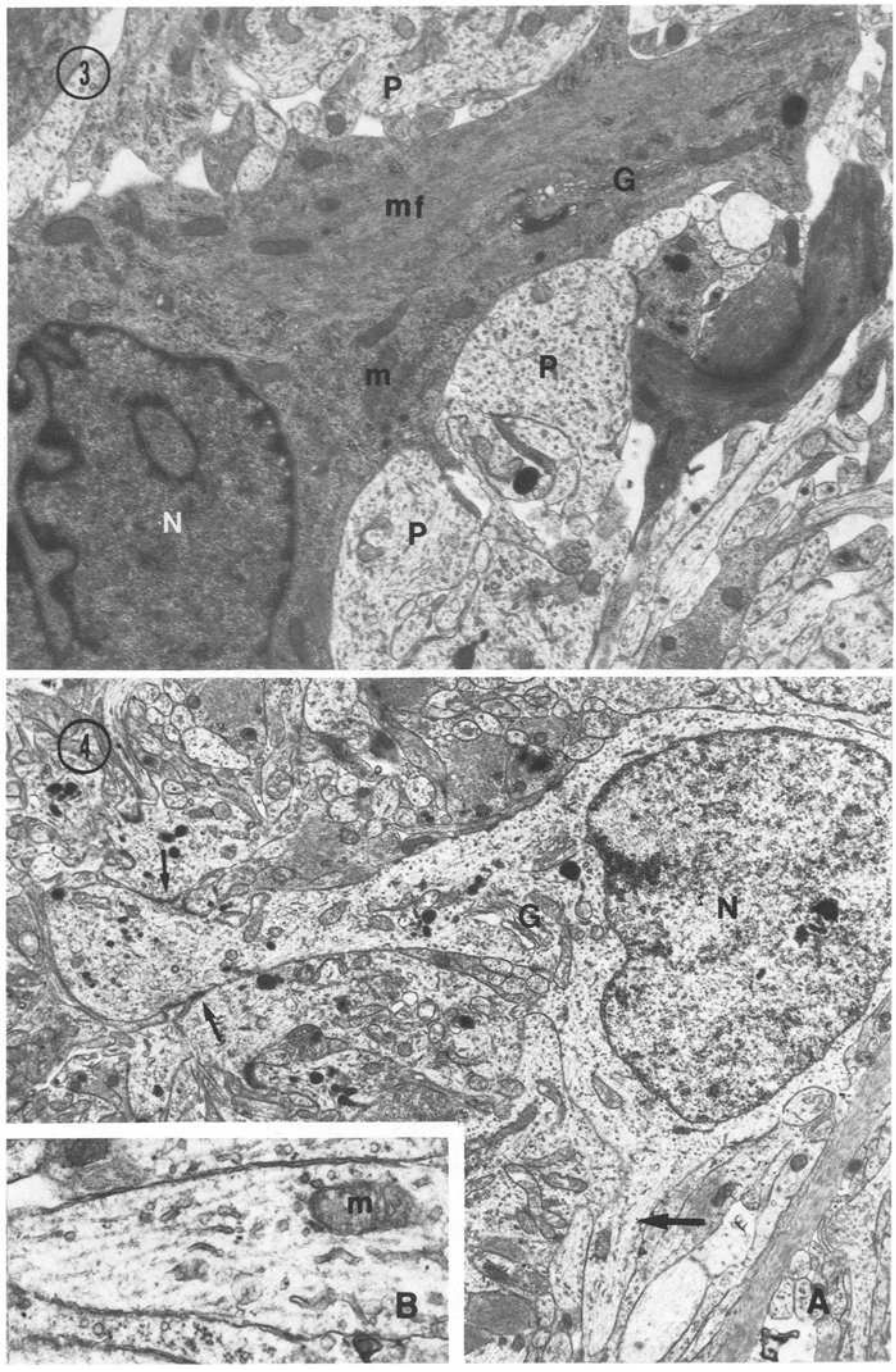


Fig. 3. A fibrous astrocyte situated between several pinealocyte-processes (*P*). *G* Golgi body; *m* mitochondria; *mf* microfilaments; *N* nucleus. × 10,500

Fig. 4. A A pinealocyte with its basal nucleus (*N*) and expanded apical portion forming junctional complexes with adjacent pinealocytes (*small arrows*). A thin process is seen extending from the basal region of the cell (*large arrow*). *G* Golgi body. × 7000. **B** Higher magnification of basal process; *m* mitochondria. × 26,000

The nuclei of pinealocytes contained little heterochromatin and showed slight indentation of the nuclear envelope. Although the shape of pinealocytes varied, in certain planes of section these cells were found to be dumbbell-shaped, the nucleus located basally, the supranuclear region being constricted, and the apical portion being expanded (Fig. 4A). Apical expansions from other pinealocytes converged in various regions of the pineal forming numerous junctional complexes with one another. In some cases a small lumen was formed where the pinealocytes converged (Fig. 5). These aggregations most likely correspond to cellular extensions from the pinealocyte rosettes observed with the light microscope.

Organelles within the cytoplasm of pinealocytes included mitochondria, lysosome-like inclusions resembling lipofuscin granules and multivesicular bodies, numerous polysomes, microtubules, and several Golgi bodies. The Golgi zones were extensive, displaying dilated saccules surrounded by both clear and dense-cored vesicles, 80–110 nm in diameter (Fig. 6). Vesicles were distributed throughout the cytoplasm, occasionally being open to the extracellular compartment (Fig. 7). A second population of clear vesicles (40–60 nm in diameter) was common and found in various regions of the cytoplasm (Figs. 8, 16). The smaller, clear vesicles were also associated with synaptic ribbons (vesicle-crowned rods) present in pinealocytes (Figs. 8, 9).

Cilia were found in a number of pinealocytes (Figs. 10–12). When cut in cross section, these cilia exhibited a 9+0 arrangement of microtubules typical of sensory cilia. In one case a cilium was seen in the region where apical portions of pinealocytes converged (Fig. 5).

Both rough and smooth endoplasmic reticulum was present in pinealocytes, although neither was extensive. In many pinealocytes a dense material was observed either between adjacent cisternae of the rough endoplasmic reticulum (Figs. 13, 15, 16), or between the endoplasmic reticulum and the nuclear envelope (Fig. 14). Concentric arrangements of the endoplasmic reticulum (Fig. 15) as well as other configurations (Fig. 16) were observed. Portions of the endoplasmic reticulum formed subsurface cisternae, which sometimes faced one another in adjacent pinealocytes (Fig. 17).

Extending from the basal region of pinealocytes were one or more processes containing microtubules, mitochondria, clear vesicles (40–60 nm in diameter), and occasional dense-cored vesicles (Figs. 4B, 18). These processes, which resembled dendrites, coursed throughout the parenchyma of the pineal, sometimes running in small bundles (Fig. 18). At the ventricular surface, pinealocyte processes containing clear vesicles were sometimes arranged in layers (Fig. 19). Junctional complexes were not observed between adjacent pinealocytes bordering the ventricle.

A clear demarcation existed ventrally between pinealocytes and ependymal cells (Fig. 20). The ependymal cells exhibited typical cilia (9+2) and numerous microvilli along their apical surface. Junctional complexes were present between adjacent ependymal cells and pinealocytes.

Two other cell types were identified with the electron microscope. The first had a large, round nucleus with little heterochromatin and a prominent nucleolus (Fig. 21). This cell type is classified here as a neuron-like cell and is believed to correspond to the cell type observed with the light microscope in the vicinity of the myelinated nerve bundles (Fig. 2A). Organelles in the cytoplasm were generally sparse. The second cell type had a round or oval nucleus with heterochromatin

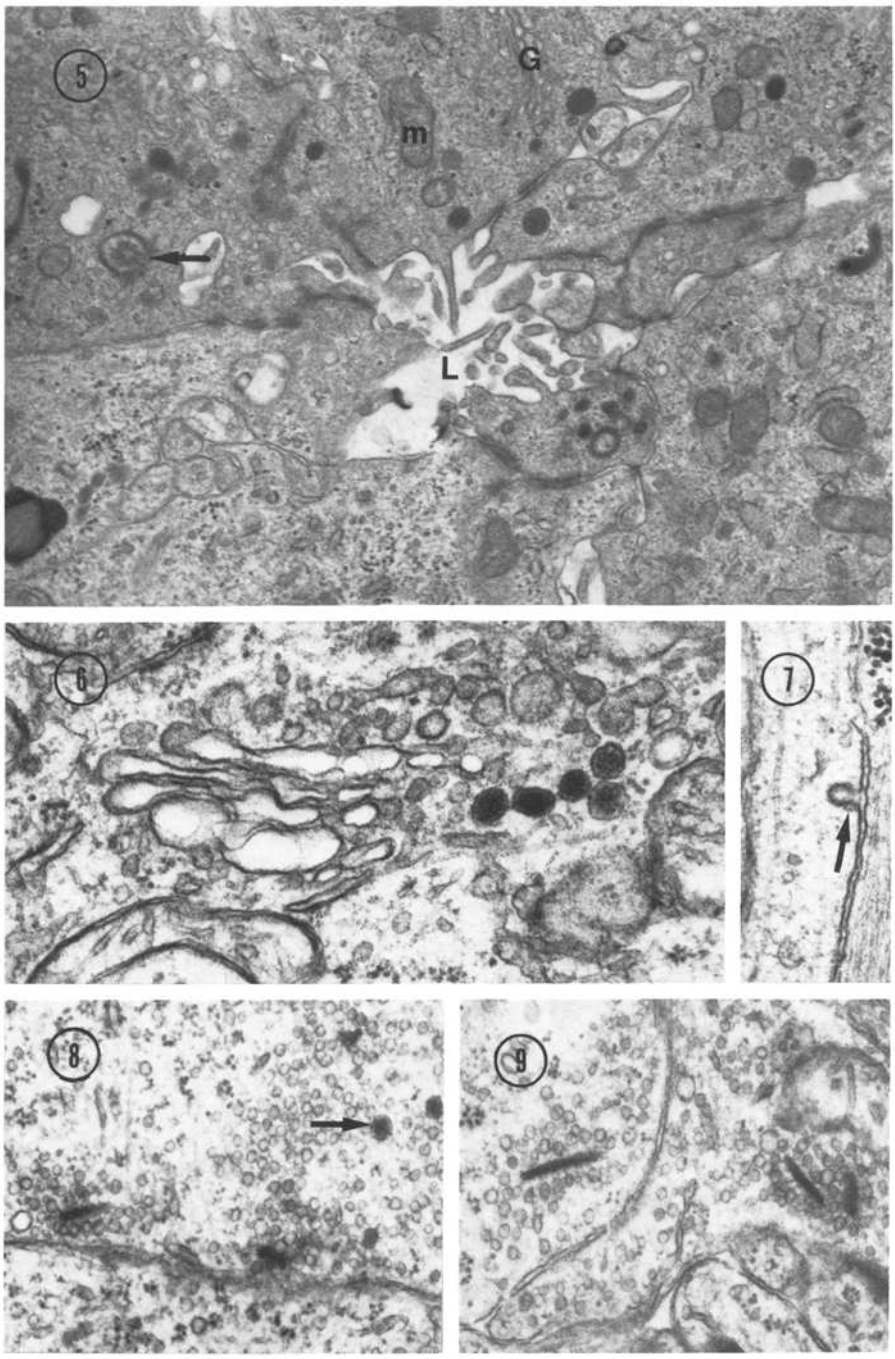
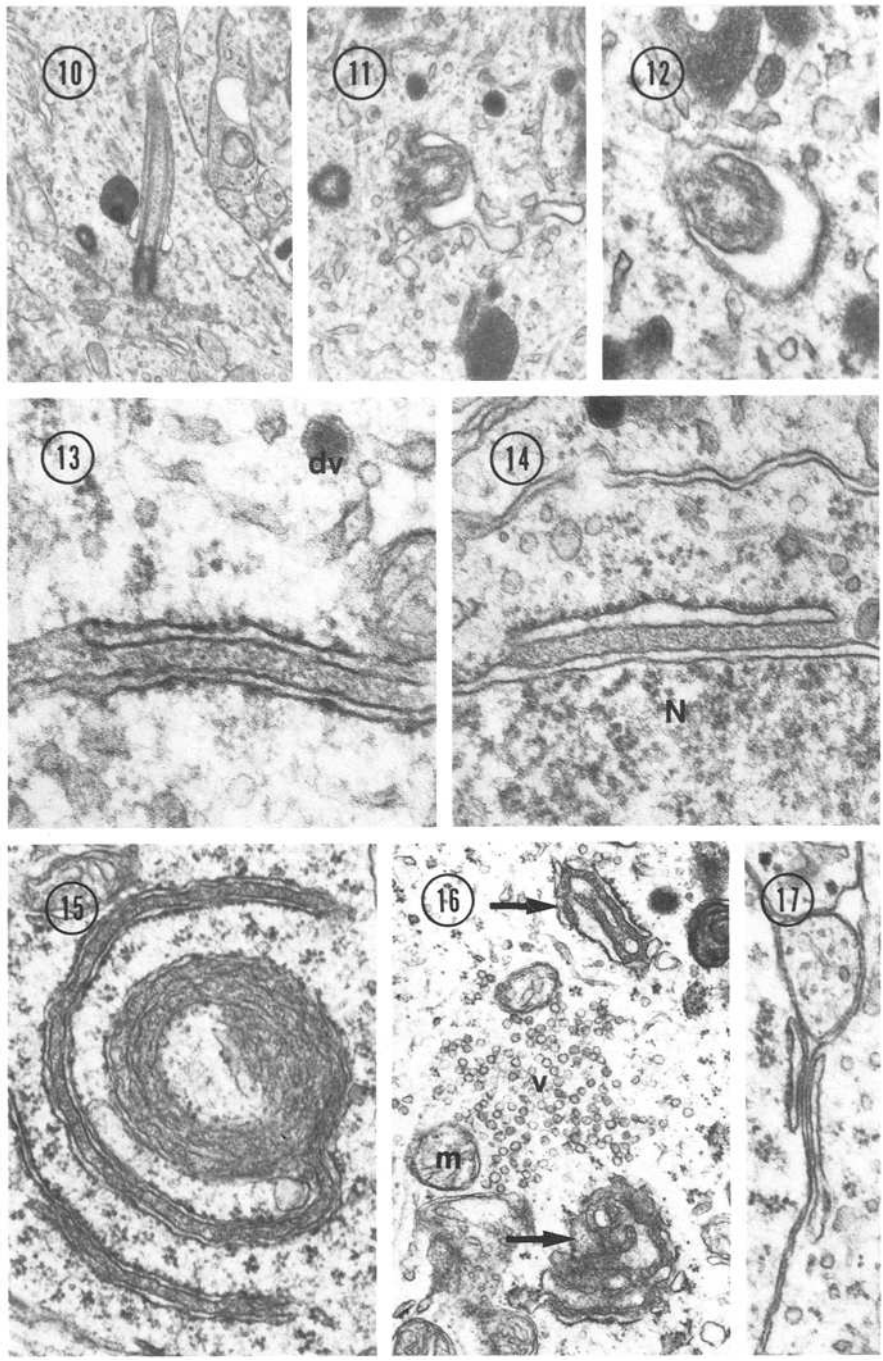


Fig. 5. Portions of several pinealocytes converging to form a small lumen (*L*). The cells are connected by numerous junctions. A cilium is seen in one cell (*arrow*). *G* Golgi body; *m* mitochondria. × 20,300

Fig. 6. Golgi body of pinealocytes associated with both clear and dense-cored vesicles. × 45,000

Fig. 7. A clear vesicle (*arrow*) continuous with the plasma membrane of a pinealocyte. × 39,000

Figs. 8 and 9. Examples of vesicle-crowned rods found in the cytoplasm of pinealocytes. Numerous clear vesicles and occasional dense-cored (*arrow*) vesicles are seen. × 39,000



Figs. 10–12. Cilia exhibiting a 9+0 arrangement of microtubules. ×4500, ×32,500, ×52,000

Fig. 13. A dense granular material situated between cisternae of rough endoplasmic reticulum in a pinealocyte. A dense-cored vesicle (*dv*) is also seen. ×86,000

Fig. 14. Dense material between rough endoplasmic reticulum and nuclear envelope of pinealocyte. *N* nucleus. ×45,000

Fig. 15. Concentrically arranged membranes of rough endoplasmic reticulum. ×39,000

Fig. 16. A cluster of small, clear vesicles (*v*) in the cytoplasm of a pinealocyte. Specializations of the rough endoplasmic reticulum (*arrows*) are also seen; *m* mitochondria. ×26,000

Fig. 17. Rough endoplasmic reticulum forming subsurface cisternae in adjacent pinealocytes. ×45,500

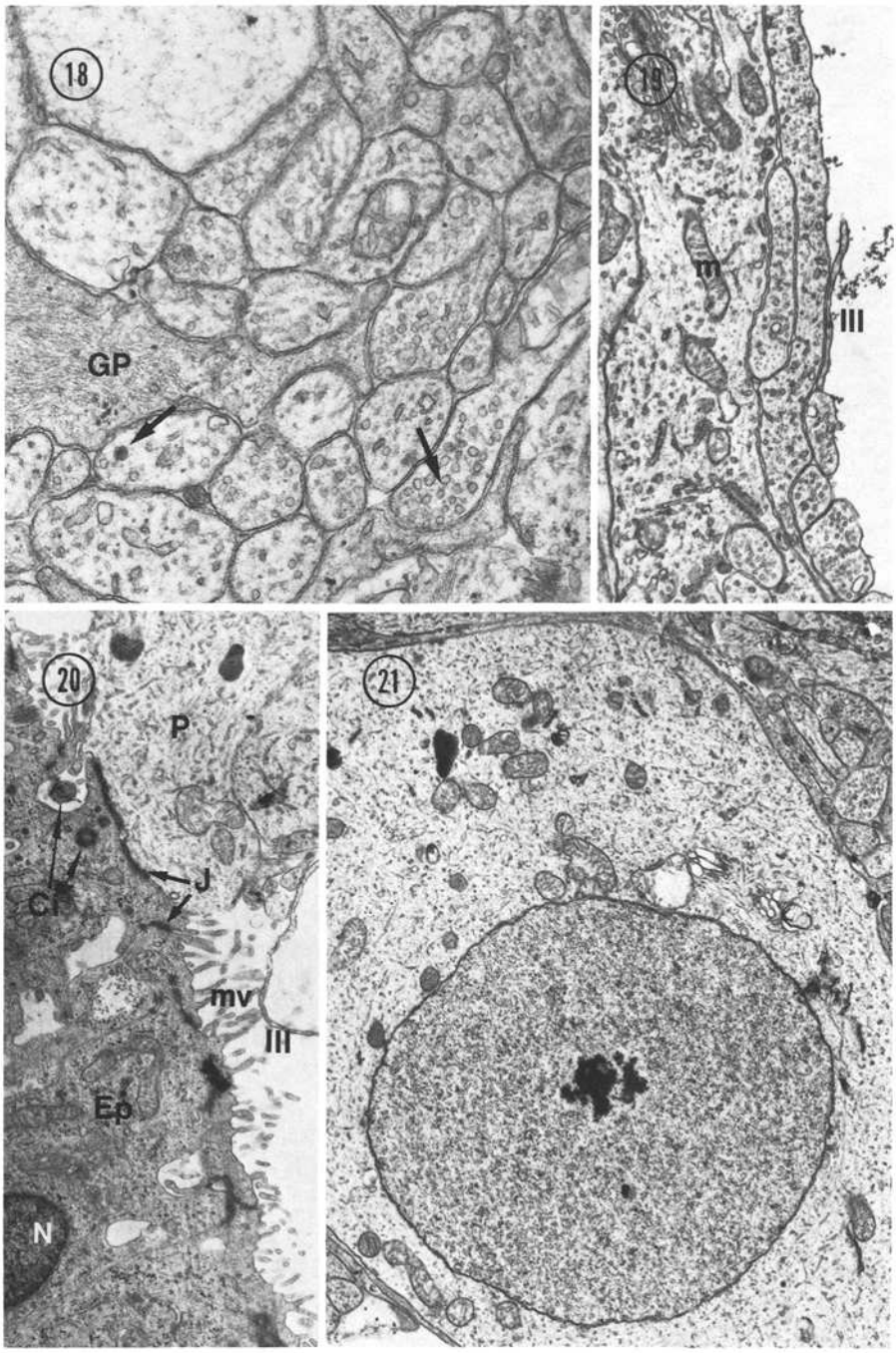


Fig. 18. A bundle of pinealocyte processes containing microtubules as well as clear and dense-cored vesicles (*arrows*). *GP* glial-cell process. ×25,000

Fig. 19. Overlapping processes of pinealocytes bordering the third ventricle (*III*); *m* mitochondria. ×14,500

Fig. 20. Transitional zone between a pinealocyte (*P*) and ependymal cells (*Ep*). *Ci* cilia; *J* junctions; *mv* microvilli; *N* nucleus; *III* third ventricle. ×12,000

Fig. 21. Neuron-like cell. ×8700

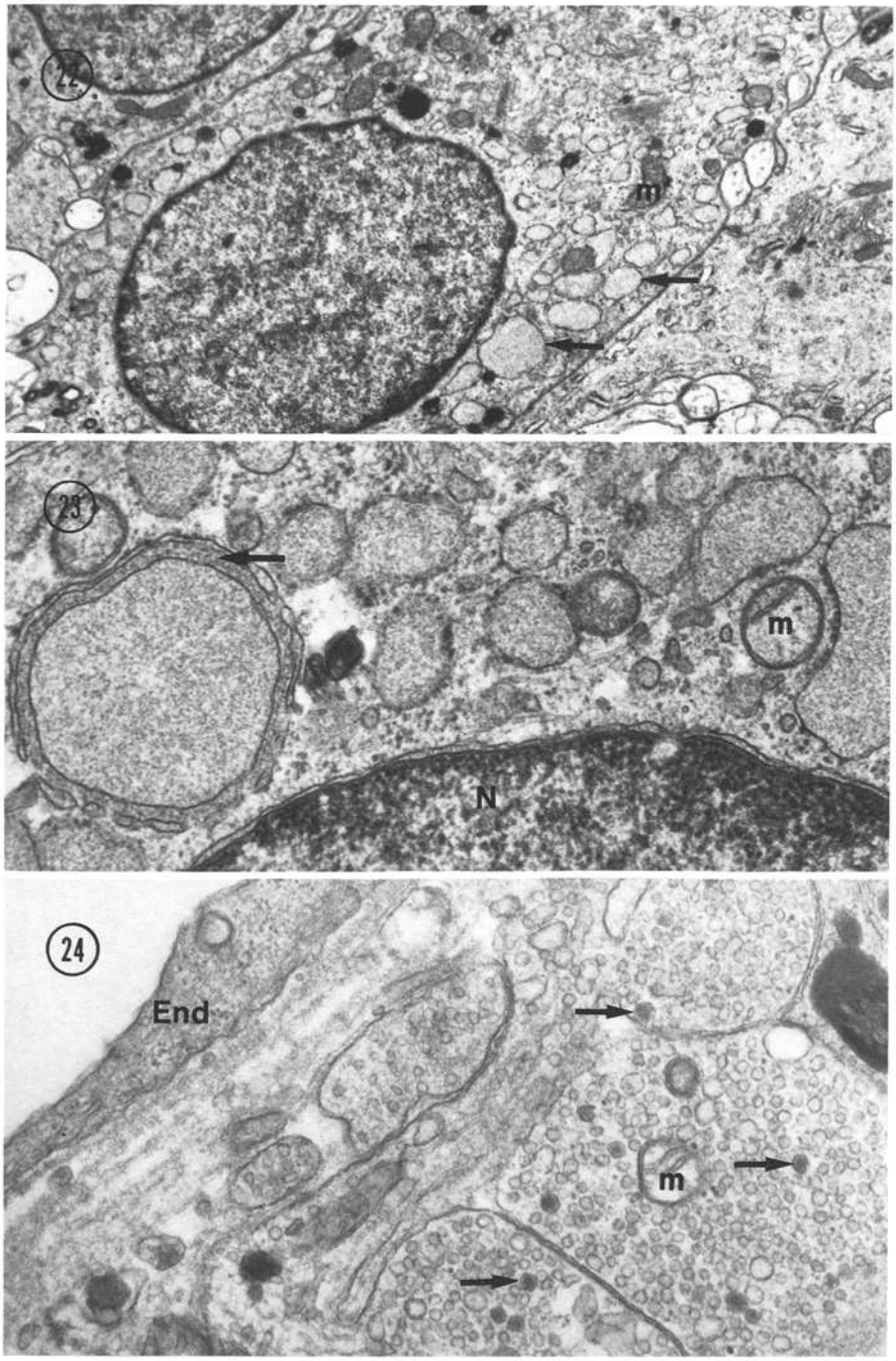


Fig. 22. A "dark" cell containing dilated rough endoplasmic reticulum filled with a fine precipitate (*arrows*). × 10,500

Fig. 23. Higher magnification of the cytoplasm of a "dark" cell. A dense material is also seen between cisternae of the endoplasmic reticulum (*arrow*). *N* nucleus. × 39,000

Fig. 24. Portions of several terminals found within the pericapillary space. The terminals are filled with clear vesicles and occasional dense-cored vesicles (*arrows*). *End* endothelial cell. × 39,000

dispersed throughout the nuclear matrix and concentrated along the nuclear envelope (Fig. 22). Characteristically, the cytosol was more electron dense than that of pinealocytes. The rough endoplasmic reticulum of these cells was dilated and filled with a fine, amorphous substance (Figs. 22, 23). A more dense material was also present between cisternae of the endoplasmic reticulum (Fig. 23), a characteristic similar to that seen in pinealocytes. Dense-cored vesicles, cilia, and vesicle-crowned rods were not observed in these cells.

Endothelial cells of capillaries coursing through the pineal were of the non-fenestrated, continuous variety. The pericapillary space was extensive and contained a number of cell terminals filled with both clear and dense-cored vesicles, 40–60 nm in diameter (Fig. 24).

Discussion

On the basis of its morphology, the pineal region of marsupials has long been thought of as a rudimentary structure (Jordan, 1911). As pointed out by Kenny and Scheelings (1979), the formation of a pineal recess resembles the early stages of embryogenesis of the pineal gland in eutherian mammals. This is further supported by the observation in this study that pinealocytes formed rosettes, reminiscent of the follicles present in the developing pineal gland of rodents (Clabough, 1973; Kappers, 1960). It appears from this study that proliferation of pinealocytes in the opossum does not occur to the extent present in eutherian mammals. Of interest in this regard are reports of a "deep" pineal that forms a small recess and borders the ventricular surface in the hamster (Hewing, 1978; Sheridan and Reiter, 1970), the deer mouse (Quay, 1965), and the collared lemming (Quay, 1978). In the hamster, division of the superficial and deep pineal is complete by the second post-natal week, although the two structures remain connected by a thin pineal stalk (Sheridan and Walker, 1975). It is conceivable that this "deep" pineal corresponds to the pineal recess found in marsupials.

Although the pineal region is poorly developed, the ultrastructure of pineal cells in this metatherian mammal has many features in common with the pineal gland of vertebrates in other classes. The observation that pinealocytes in the opossum are dumbbell-shaped and exhibit cilia with a 9+0 arrangement of microtubules as well as vesicle-crowned rods suggests that these cells are homologous to the photosensory cells of lower vertebrates. Cells belonging to the "sensory cell line" are typically polar in shape and contain sensory cilia (9+0) that form the membranous saccules comprising the outer segments of those cells functioning in photoreception (Collin, 1971). Vesicle-crowned rods in these cells presumably form synaptic contacts with dendrites from ganglion cells present in the pineal organ (Oksche, 1971). Sensory cilia and synaptic ribbons persist in the non-photoreceptive pinealocytes of placental mammals (Pévet and Collin, 1976), although their function, particularly of the vesicle-crowned rods, is not clearly understood. Polarization of pinealocytes in higher mammals also occurs during differentiation (Zimmerman and Tso, 1975) as well as in adults (Pévet and Collin, 1976).

Pinealocytes in eutherian mammals are known to function in the synthesis and secretion of a number of active compounds under the control of sympathetic

innervation (Kappers, 1976). Although the site of synthesis and mechanisms of secretion of these compounds are not known, many authors are of the opinion that Golgi vesicles, and in particular the dense-cored vesicles, contain important secretory products (see Pévet, 1977). The numerous clear and dense-cored vesicles observed in pinealocytes of the opossum correspondingly suggest that these cells have intense secretory functions. Moreover, the size of vesicles in the terminals found within the pericapillary spaces are similar to those of adrenergic nerve endings (Matsushima and Reiter, 1977) indicating that sympathetic innervation may play an important role in regulating the metabolism of the pineal in this marsupial.

The most generally accepted pathway by which secretory products from the pineal gland might reach their respective targets is the vascular supply (Kappers, 1976). Fenestrations in the capillary endothelial cells of most species provide at least a morphological substrate for the transport of substances across the endothelial cell wall. In the American opossum the capillaries are not fenestrated, indicating that the blood-brain barrier may be intact in the pineal region. However, the observation that vesicles are open to the extracellular compartment together with the absence of any junctional complexes between pinealocytes at the ventricular surface suggests the possibility for flow of materials between pinealocytes and the cerebrospinal fluid. The question of whether this represents the process of secretion or uptake cannot be answered at this time. In the brush-tailed opossum, Delahunt et al. (1975) reported large vacuoles in those cells comprising the central zone of the pineal recess, again suggesting either secretion into, or uptake from, the cerebrospinal fluid. Similar vacuoles were not observed in the present study.

Additional evidence that pinealocytes in this species are synthetically active is the presence of a dense material between cisternae of the rough endoplasmic reticulum. A similar condensation of material has been reported in pinealocytes of a bat (Pévet et al., 1977) and a hibernating ground squirrel (McNulty and Dombrowski, 1980). Although the functional significance of this material is not known, it is noteworthy that both the bat and hibernating squirrel normally experience prolonged periods of darkness, a condition that is known to enhance metabolic activity of the pineal gland. This specialization of the endoplasmic reticulum is not peculiar to pinealocytes, having also been reported in developing neurons (Buschmann, 1979), which are actively involved in protein synthesis. The formation of subsurface cisternae might be indicative of communication between adjacent pinealocytes through the exchange of ions or metabolites (Anderson, 1965). They have been observed in a number of mammals (see Pévet et al., 1976), being particularly prevalent in synthetically active pinealocytes.

It is not clear whether those cells having a dense cytoplasm and dilated endoplasmic reticulum filled with an amorphous substance represent a separate population of pinealocytes as have been described in a number of eutherian mammals (Pévet, 1977). On the one hand, the presence of a dense material between cisternae of the endoplasmic reticulum is a specialization that is common to pinealocytes of the opossum as well as some eutherian mammals (Pévet et al., 1977; McNulty and Dombrowski, 1980). However, dense-cored vesicles, cilia, and vesicle-crowned rods, which are characteristic of pinealocytes, were not observed in this cell type. Nevertheless, the abundant material within the cisternae of the rough

endoplasmic reticulum indicates that this population of cells is intensely active in the synthesis of proteinaceous compounds, possibly for export outside the cell.

Likewise, the functional significance of the neuron-like cells in the region where myelinated nerve processes passed through the pineal parenchyma is not known. However, their presence together with the large number of dendritic processes within the pineal suggest the possibility of intrapineal nervous connections. Using silver impregnation, Kenny and Scheelings (1979) described an extensive intrapineal nervous network as well as nerve fibers passing through the pineal parenchyma connecting the habenular and posterior commissures in several species of marsupials. These authors also reported the presence of neuron-like cells along the periphery of the organ. A more detailed study of the innervation and synaptic organization of the opossum pineal will be presented in a forthcoming paper.

In conclusion, the result of the present study suggest that pinealocytes in the opossum are metabolically active cells having secretory functions similar to those in eutherian mammals. Moreover, ultrastructural similarities between pinealocytes in this marsupial and the photoreceptor cells of "lower" vertebrates support the concept that pinealocytes in this methatherian mammal evolved along a "sensory cell line" (Collin, 1969)

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