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THE AMPULLAE OF THE INNER EAR IN THE LIZARD *PODARCIS S. SICULA*. ULTRASTRUCTURAL ASPECTS

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Abstract

The inner ear ampullae of the lizard *Podarcis s. sicula* were studied to determine better the ultrastructure of ampullar epithelial cells.

Our study confirmed that the ampullae of the membranous labyrinth of this lizard are similar to those of other vertebrates in their ultrastructural aspect.

Moreover, our observations revealed a special type of dark cells, restricted to a small area of the *crista*. They appeared similar to type II sensory cells and showed a dark, finely granular cytoplasm, containing numerous mitochondria and ribosomes, extensive Golgi apparatus and abundant glycogen.

The morphology of these cells suggests that they may be special sensory cells, or different stages of sensory cells, probably implied in the *crista* cell turnover described for some vertebrate groups.

Key Words: Ampullae, inner ear, lizard, hair cell, dark cell, *septum cruciatum*.

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Introduction

The ampullae of the membranous labyrinth in lizards morphologically resemble those of other vertebrates (De Burlet, 1934; Hamilton, 1964): a simple squamous epithelium lines the ampullary lumen; the ampulla shows specializations on the *crista* and in the areas of the *plana semilunata*.

Differently from what was hypothesized in the past, turn-over phenomena of degenerated cells have recently been demonstrated, under normal and/or traumatic conditions, for the sensory epithelium of *cristae* (Jørgensen and Mathiesen, 1988; Rubel *et al.*, 1991; Weisleder and Rubel, 1993).

Although the surface structure of the ampulla of the semicircular canals was studied by several authors (Watanuki *et al.*, 1970; Ishiyama and Keels, 1971; Harada, 1981; Lim and Anniko, 1985), there is a dearth of scanning electron microscopy (SEM) observations on reptilian ampullae.

This study was aimed at a better determination of the typical ultrastructure of ampulla cells in *Podarcis s. sicula* by SEM and transmission electron microscopy (TEM) and at seeking correlations between cell morphology and potential functions.

Materials and Methods

Twenty adult lizards *Podarcis s. sicula* were collected in localities around Naples, Italy. Animals were subjected to ether anaesthesia, followed by intracardiac perfusion with 2.5% glutaraldehyde in 0.1 M phosphate buffer pH 7.4, and then sacrificed. Temporal bones were removed and immersed in the same fixative for 4 hours at 4°C. After microdissection of the vestibular end organ and mechanical removal of cupular material, the specimens were rinsed in the same buffer and post-fixed in 1% OsO_4 for 2 hours at 4°C. Samples were rinsed several times in the buffer, dehydrated, embedded in Epon 812 resin, and sectioned in a Super Nova Leica Ultratome for TEM studies. Ultrathin sections were placed on copper grids and counterstained with uranyl

acetate and lead citrate. Specimens were observed and photographed in a Philips CM12 electron microscope.

Thin sections for observations by light microscopy were stained with 1% toluidine blue.

SEM preparations were postfixed in 1% OsO_4 for 2 hours at 4°C, dehydrated in 60% ethanol, taken through a graded sequence of ethanol-Freon mixture to 100% Freon, and then critical-point dried. Immediately after drying, the tissues were attached to stubs with double-coated tapes and coated with gold and carbon (about 30 seconds, 15 cm from the source, 40 kV, 2-3 mA). Observations were carried out with a Cambridge Stereoscan 250 MK III.

Results

Semi-thin transverse sections of the *crista ampullaris* observed by light microscopy showed the sensory epithelium merging into the transitional cell and the dark cell areas (Figure 1).

SEM observations showed that the *cristae* of the anterior and posterior semicircular canals had a well-developed *septum cruciatum*, perpendicularly projecting from the ridge of the *crista* (Figure 2A). The lateral *crista* lacked this *septum*, but was furnished with two pockets on each side of the ridge.

The *crista* sensory area showed sensory cells surrounded by supporting cells (Figure 2B). The kinocilia of sensory cells varied in length depending on their location on the *crista*, and the stereocilia were compact bundles with a gradual increase in length of the individual stereocilia (Figure 2B). Globular structures were present in the subcupular region, mainly on the surface of supporting cells (Figure 2C); these cells may be responsible for secreting this globular material on the outward region of the *crista*.

The polarity of sensory cells was determined by the orientation of the kinocilium: in the *cristae* of the anterior and posterior semicircular canals, the kinocilium projected towards the semicircular canal; in the *crista* of the lateral semicircular canal, the kinocilium was, instead, directed towards the utricle.

The central region of the *crista* included between the two protrusions of the *septum cruciatum* was smooth, lacking stereo- or kinocilia, with small hexagonal cells (Figure 3A). In the *septum* region, the diameter of these cells increased, and sparse tufts of microvilli appeared among them (Figure 3B).

In the area of the dark cells, microvilli tufts (Figures 3C and 3D) were regularly located between their large supporting cells. Small microvilli projecting from the luminal surfaces marked the polygonal outlines of the supporting cells (Figure 3C).

Hexagonal or pentagonal cells with varying size and

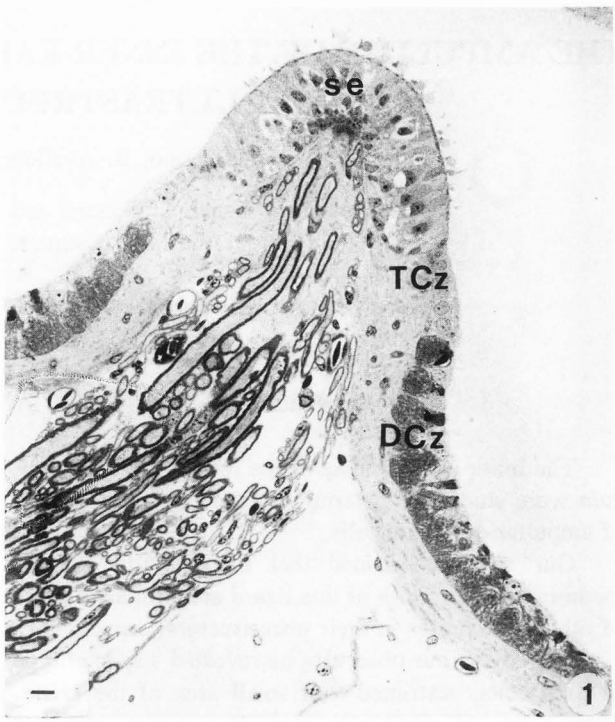
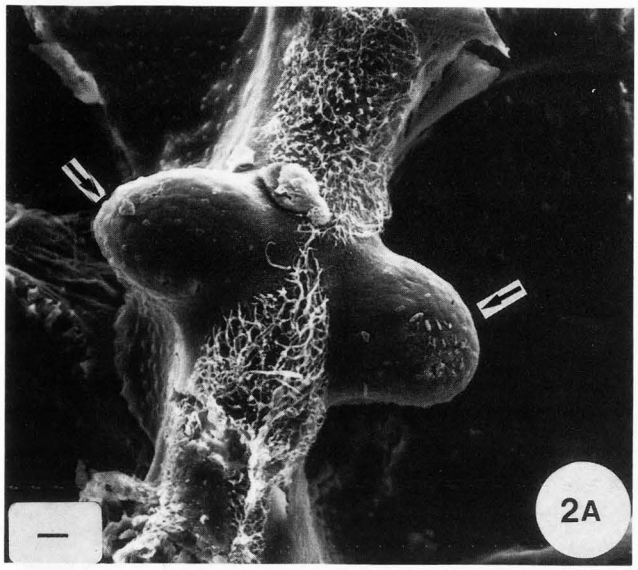


Figure 1 (above). Light micrograph of *Podarcis s. sicula*. Transverse section of the *crista ampullaris*. Sensory epithelium (se), transitional cell zone (TCz) and dark cell zone (DCz). Bar = 10 μm.

Figure 2 (on the facing page, column 1). Scanning electron micrographs of *Podarcis s. sicula*. **(A)** *Crista* of the anterior semicircular canal. Note *septum cruciatum* (arrows) projecting perpendicularly from the summit of the *crista*. Bar = 10 μm. **(B)** Sensory area of the *crista*. Sensory cells surrounded by supporting cells. Note the kinocilia (k) and stereocilia (s) graduated in length. Bar = 1 μm. **(C)** Sensory area of the *crista*. Globular structures (g s) in the subcupular layer on the surface of supporting cells. Bar = 1 μm.

Figure 3 (on the facing page, column 2). Scanning electron micrographs of *Podarcis s. sicula*. **(A)** Sensory area of the *crista*. Central smooth portion of the *crista*, lacking both stereo- and kinocilia, included between the two protrusions of the *septum cruciatum*. Note the small hexagonal cells. Bar = 1 μm. **(B)** *Septum cruciatum*. Note the sparse tufts of dark cell microvilli (arrow). Bar = 10 μm. **(C)** Dark cell area. Microvilli tufts (M) regularly located between the polygonal outlines of their large supporting cells. Note the small microvilli (arrow) projecting from the luminal surfaces of the supporting cells. Bar = 1 μm. **(D)** Higher magnification of microvilli tufts (M). Bar = 100 nm.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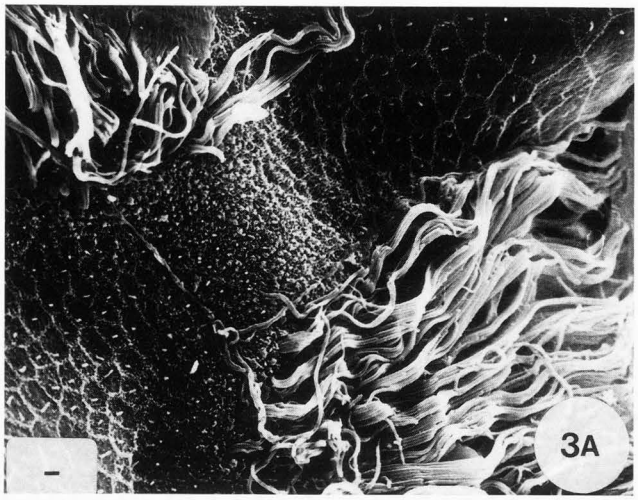
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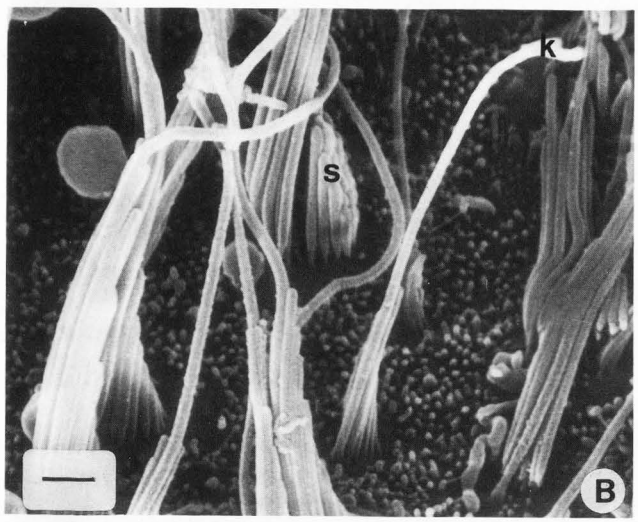
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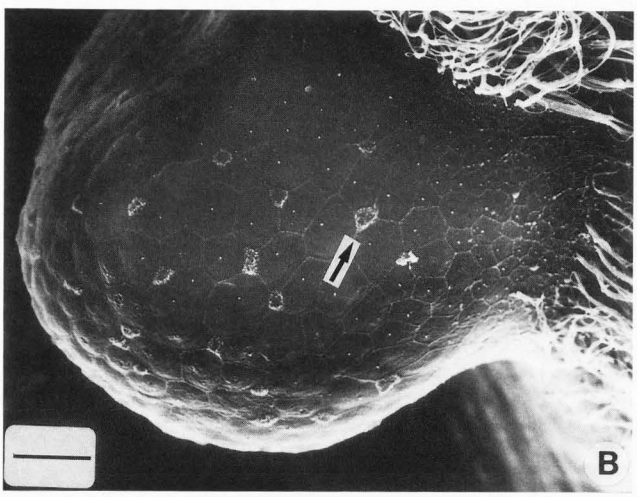
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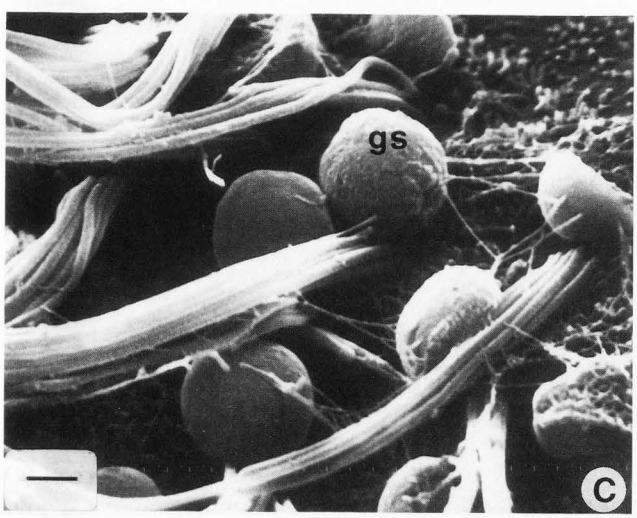
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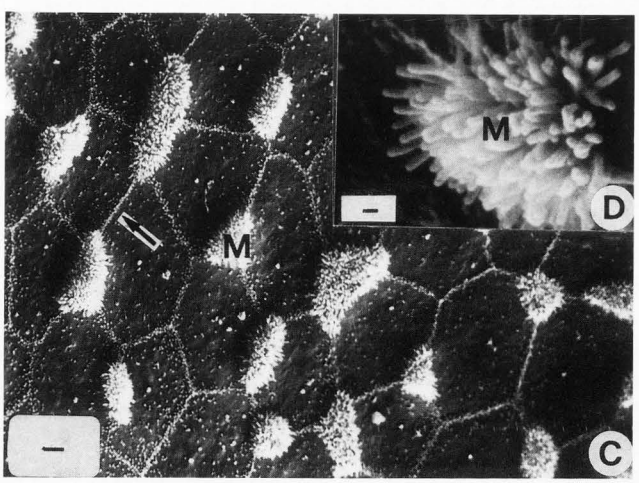
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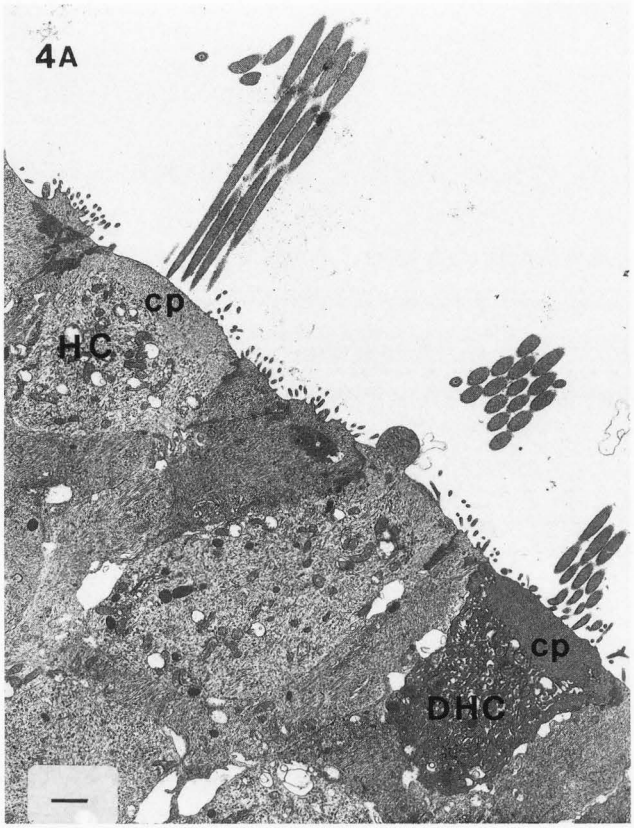
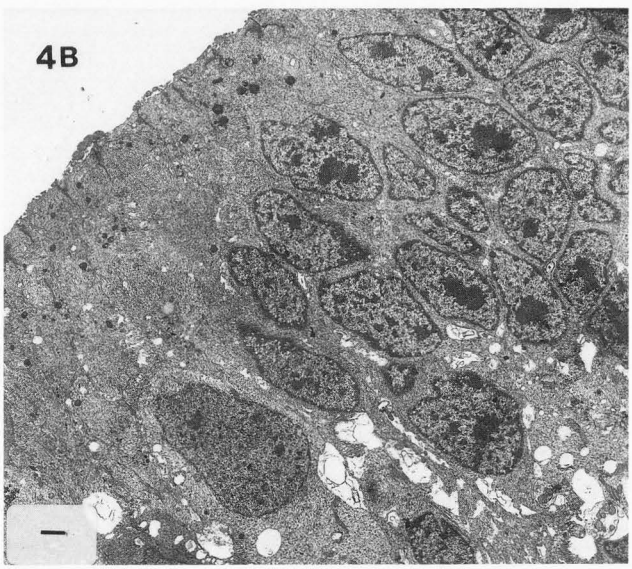




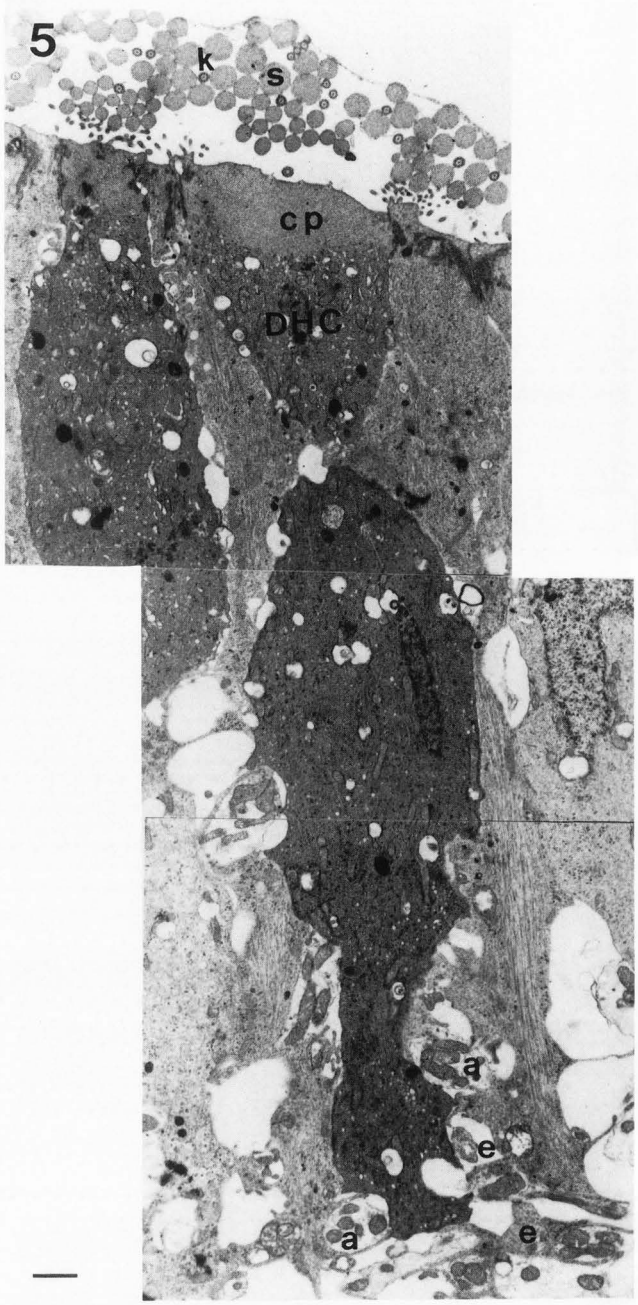
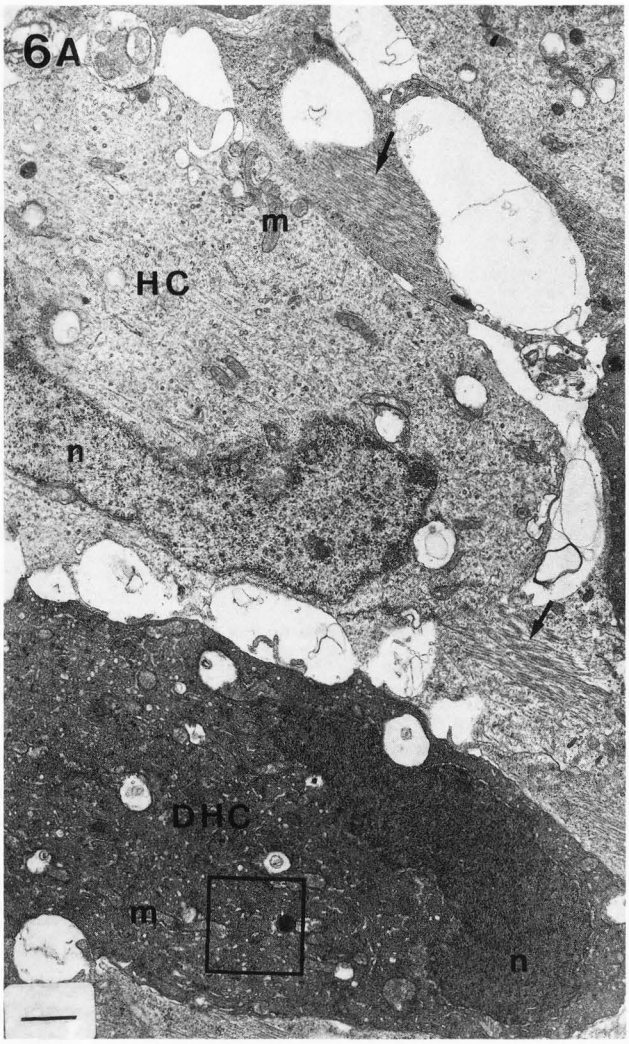
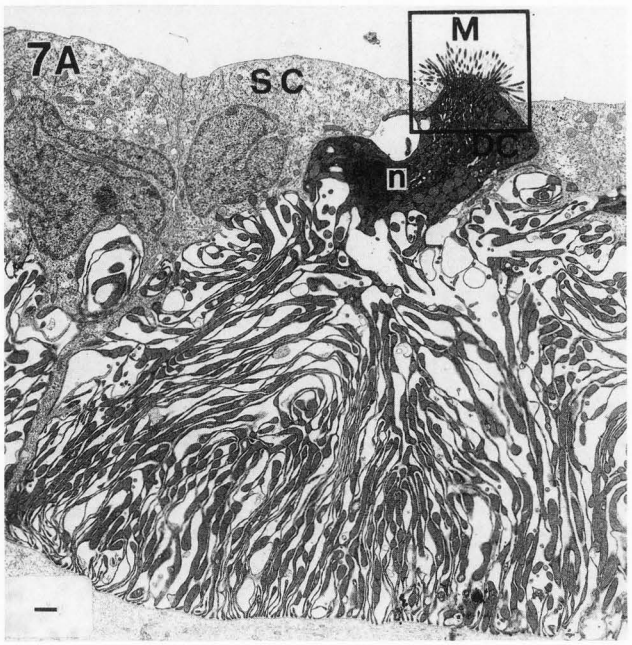
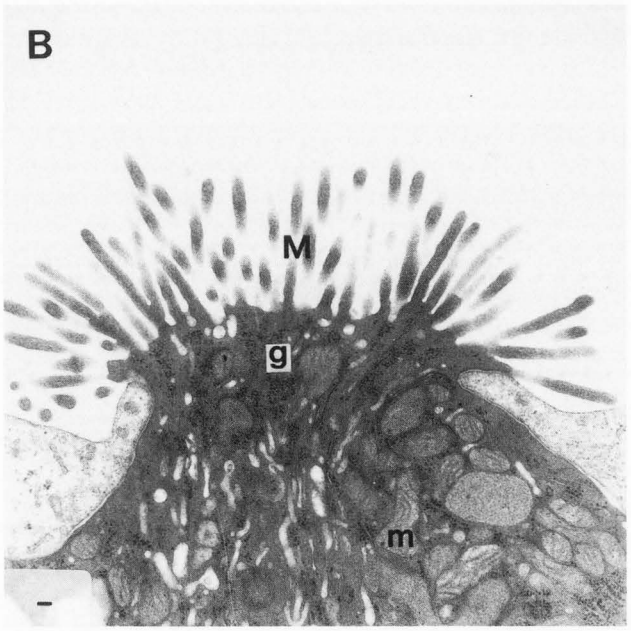


Figure 4 (column 1). Transmission electron micrographs of *Podarcis s. sicula*. Bars = 1 μm. **(A)** Sensory area of the *crista*. Dark hair cell (DHC). Hair cell (HC); cuticular plate (cp). **(B)** "Zone of nuclei" characterized by several small cells close to one another.

Figure 5 (column 2). Sensory area of the *crista*. Dark hair cell (DHC) with rare synaptic buttons, both afferent (a) and efferent (e); cuticular plate (cp); kinocilium (k); stereocilia (s). Transmission electron micrograph. Bar = 1 μm.







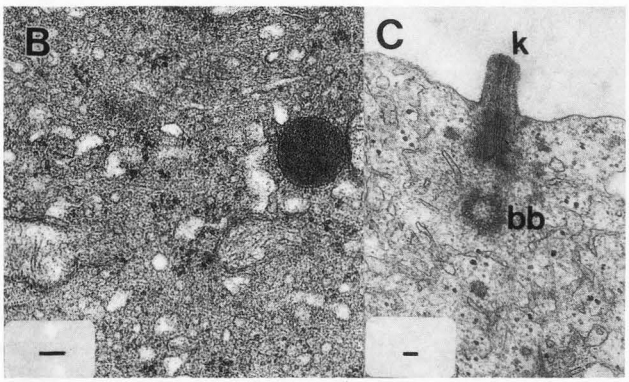


Figure 7. Transmission electron micrographs of *Podarcis s. sicula.* (**A**) *Crista ampullaris*. Dark cell (DC) associated with its supporting cells (SC). Microvillous tuft (M); nucleus (n); note the extensive infoldings of the basal and lateral cell membranes. Bar = 1 μm. (**B**) Enlarged view of the framed area in A. Note the dark, finely granular cytoplasm. Microvillous tuft (M); glycogen (g); mitochondria (m). Bar = 100 nm.

Figure 6 (column 1). Transmission electron micrographs of *Podarcis s. sicula.* (**A**) Sensory area of the *crista.* Portion of a hair cell (HC); portion of a dark hair cell (DHC); nucleus (n); mitochondria (m). Note the supporting cells with extensive arrays of parallel microtubules (arrows). Bar = 1 μm. (**B**) Higher magnification of the framed area in A. Note an osmiophilic body. Bar = 100 nm. (**C**) *Crista ampullaris*. Portion of a supporting cell furnished with a short kinocilium (k) showing an evident basal body (b b). Bar = 100 nm.

a bulging nucleus characterized the last portion of the ampullary epithelium.

TEM observations, confirming these results, also showed further cell details. Hair cells (HC) were located at the summit of the *crista* in the luminal part of the sensory epithelium, and they never extended to the basal membrane. Two major types of hair cell populations (HC I and HC II according to Wersäll, 1956) were found: HC I, bottle-shaped and enclosed in a nerve chalice; and HC II, cylindrical and innervated by terminal buttons.

A third type consisted of dark hair cells (Figures 4A and 5). They were found in the central region of the *crista* near the *septum cruciatum*, adjacent to an area that we defined as "zone of nuclei" (Figure 4B) because of the presence of several small cells adjacent to one another. The cytoplasm of the dark hair cells contained numerous mitochondria and ribosomes, extensive smooth endoplasmic reticulum and Golgi apparatus, as well as abundant glycogen (Figure 6A). Less dense particles of unknown nature were distributed in the cells, giving the cytoplasm a stippled aspect (Figure 6B). Some osmiophilic bodies of about 0.20-0.35 μm (Figure 6A and 6B) in diameter were also present. The nuclei were spindle- or kidney-shaped and were oriented along the major axis of the cell (Figures 5 and 6A). Rare synaptic buttons, both afferent and efferent, were observed in association with these cells (Figure 5). Dark hair cells in the *crista* of lateral semicircular canal did not show a preferential localization.

As with HC I and HC II cell types, dark hair cells were also apically equipped with two types of sensory hairs (Figures 4A and 5): a single kinocilium arising from a basal body with the ordinary 9:2 patterns of microtubules; and a bundle of stereocilia. The stereocilia (Figures 4A and 5) decreased in height stepwise and proportionally with the increasing distance from the kinocilium; they were composed of a fine material basally condensed into a rootlet. This structure was rooted in the "cuticular plate," a homogeneous structure which occupies the apical part of the hair cell, excluding the area surrounding the basal body.

Hair cells were surrounded by supporting cells in which the cytoplasm showed extensive arrays of parallel microtubules extending lengthwise (Figures 5 and 6A). In some supporting cells, TEM showed a short kinocilium and a basal body (Figure 6C).

TEM observations also showed the presence of dark cells (Figures 7A and 7B) with a complex structure located near the *crista* and the *plana semilunata* and in the *septum cruciatum*. These microvillous cells were associated with their supporting cells and showed a dark, finely granular cytoplasm, typical tufts of microvilli (Figures 7A and 7B) projecting into the lumen of the labyrinth and extensive infoldings of the basal and lateral cell membranes (Figure 7A), which were filled with mitochondria, ribosomes and glycogen. The cells were constricted towards the lumen of the ampulla, with a small portion of cytoplasm extending between supporting cells and bulging into the lumen (Figure 7A).

The supporting cells of the dark cells were similar to those found in the adjacent sensory areas: they contained few mitochondria, scattered (but abundant) ribosomes and polysomes, considerable endoplasmic reticulum as well as an extensive Golgi apparatus and showed lateral and basolateral interdigitations with other supporting and microvillous cells.

The transitional cells of the *crista* were morphologically very similar to the supporting cells of the dark cells, and seemed to form a *continuum* with the latter.

The multilayered epithelium of the *plana semilunata* showed few microvilli and numerous secretion granules.

Discussion and Conclusions

The ampullae of *Podarcis s. sicula* are similar in overall morphology to those of other lizard species (Miller, 1966; Jørgensen, 1975). The occurrence of two main hair cell types, HCI and HCII, confirms the observations carried out by Vinnikov *et al* (cited by Jørgensen, 1975) in lizards.

Two types of nerve endings, both afferent and efferent, are present, as already reported for other lizard species (Jørgensen, 1975).

Moreover, our observations revealed a novel type of dark hair cells which are restricted to a small area of the *crista* close to the *septum cruciatum* in the vertical semicircular canals.

These cells are not numerous and share some cytoplasmic characteristics with the microvillous dark cells; they lack, however, laminar infoldings of the basal plasmalemma and microvillous tufts.

Dark hair cells also resemble the sensory cells of type II in that they have a cylindrical shape and possess a "cuticular plate," as well as a kinocilium furnished with a basal body, and stereocilia. Like type I and II sensory cells, they never reach the basal membrane and occupy the luminal portion of the epithelium.

The morphological appearance of dark hair cells suggests that they may be special sensory cells or, as an alternative, different functional stages of sensory cells.

However, synaptic buttons have rarely been observed in connection with these cells, and this may indicate that they are not mature sensory cells. In this respect, their location, the area referred to as "zone of nuclei," is noteworthy. We speculate that the "zone of nuclei" is involved in the physiological phenomena of cell turn-over connected with the renewal of degenerated

or dead cells of the *crista*. Such phenomena have already been described for some vertebrate groups after injury caused by ototoxic substances or acoustic trauma (Raphael, 1992, 1993; Duckert and Rubel, 1993). These cells may be either dormant stem cells or committed cells dividing under particular conditions. The cell turn-over hypothesis is supported by literature data on the regeneration of injured post-mitotic ear sensory cells by transdifferentiation of other cells (supporting cells) (Raphael, 1992; Weisleder and Rubel, 1993). Moreover, Rubel *et al* (1991) showed that the cytoplasm of the regenerated hair cells in the avian inner ear is more electron dense.

As far as microvillous cells are concerned, their general morphology, investigated by Hamilton (1965), suggests that they are possibly involved in ion transport and maintenance of electrolyte balance in the endolymph. Harada and Tagashira (1981) showed that the calcium content of the otoconia located on the surface of the dark cells is reduced to varying degrees. These findings suggest that dark cells are involved in otoconia metabolism and endolymph formation (Kawamata *et al*, 1986; Harada and Takumida, 1990). The function of their supporting cells is less clear, although some structural similarities exist between these supporting cells and microvillous cells.

Finally, the *planum semilunatum* is doubtlessly active in some phases of endolymph production. Indeed, autoradiographic evidence (Dohlman *et al.*, 1959) suggests that *planum* cells are involved in transport and formation of the acid mucopolysaccharide component of the endolymph. The presence of several secretion granules might support this hypothesis.

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Discussion with Reviewers

A. Campos: Ethanol can lead to the appearance of precipitates in the specimens. What has your experience been? Have you processed specimens with acetone instead of ethanol?

Authors: In previous work on the same material we used both alcohol and acetone, and realized that results were similar. Therefore, we continued to use alcohol.

A. Campos: What is the relationship between the dark cells you describe and the numerous osmiophilic mitochondria-rich cells involved in secretory or filtering functions such as the cells of the convoluted segment of the kidney, the interlobular duct of the salivary gland, or the *stria vascularis*?
Authors: We describe two cell types: dark cells and dark hair cells. The first ones morphologically resemble both those of the convoluted segment of the kidney and those of the interlobular ducts of the salivary glands; the other type did not show any infoldings of the basal and lateral cell membranes.

M.L. Wiederhold: Could you comment on the functional significance of the *septum cruciatum*. Do the side lobes anchor, or restrict the movement of the cupula?
Authors: The *septum cruciatum*, when sufficiently prominent, may possibly influence the hydrodynamics of the endolymphatic flow towards the two portions of the *cristae* which the *septum* itself serves to divide. The existence of this projecting structure parallel to the main endolymphatic stream will, no doubt, make its frictional effect larger. According to Dohlman this structure may act as a stabilizer for the cupulae of the vertical canals during horizontal movements.

M.L. Wiederhold: In Figure 2C, is it clear that the globular structures are only on the supporting cells? Several globules in 2C and one in 2B look as if they are at the base of the stereociliary bundle, on the surface of the sensory cells. If so, one would question the hypothesis that the globules are material secreted by supporting cells.
Authors: The globular structures in TEM sections are generally apocrine secretions of supporting cells. Fixation can, sometimes, displace some globules in SEM specimens.

M.L. Wiederhold: Were all hair cells in each *crista* oriented in the same direction? If so, how do their orientations compare to those in mammals?
Authors: Our work shows unidirectional orientation of the hair cells; polarization in each crest is comparable to that described for mammals.

M.L. Wiederhold: In Figures 4 and 5A, what causes all of the vacuoles, both within and between cells? Does this indicate inadequate fixation?
Authors: Dark hair cells, which show a much vacuolized cytoplasm, probably express a functional stage which responds to fixation in a different manner as compared to the other cells in the sensory epithelium.

M.L. Wiederhold: You suggest that the dark cells might be "immature" and that the specimens were "adult." How large were the specimens and do you know how old they might have been, relative to the rate of maturation and life expectancy of this species? Do older specimens of *Podarcis* have larger *cristae* or maculae than young adults, as in fish? If so, this might indicate a continuous production of hair cells, possibly from pre-existing supporting cells.
Authors: Our experimental material was made of 18-month adult specimens and, therefore, in about n the middle of their life cycle, which lasts an average of three years. Older *Podarcis* specimens show more massive *cristae* and this, as you suggest, may indicate a continuous production of hair cells; this production may possibly originate, according to the literature, from pre-existing supporting cells.

M.L. Wiederhold: Does not the tremendous proliferation of cell surface, represented by the many foldings in Figure 6A, argue strongly in favor of the dark cells being involved in endolymph secretion?
A. Campos: On the basis of your data, would you suggest a relationship between sensory cells and cells involved in endolymph regulation?
Authors: The tremendous proliferation of cell surface, represented by many foldings, as is also found in the cell of the convoluted segment of the kidney, in the cells of the interlobular duct of the salivary gland, and in the *stria vascularis*, is typical of cells having a large water and ionic flow; this strongly suggests the dark cells are involved in endolymph secretion, but, based on morphological characteristics, appears unlikely for the dark hair cells.