Scanning Microscopy

Volume 9 | Number 4 Article 23

10-19-1995

Elliptical-P Cells in the Avian Perilymphatic Interface of the Tegmentum Vasculosum

C. D. Fermin
Tulane University School of Medicine, fermin@tmc.tulane.edu

D. H. Lee
Ochsner Foundation of New Orleans

D. S. Martin
Tulane University School of Medicine

Scanning Microscopy, Vol. 9, No. 4, 1995 (Pages 1207-1222) 0891-7035/95$5.00+.25
Scanning Microscopy International, Chicago (AMF O'Hare), IL 60666 USA

ELLIPTICAL-P CELLS IN THE AVIAN PERILYMPHATIC INTERFACE OF THE *TEGMENTUM VASCULOSUM*§

C.D. Fermin*, D.H. Lee[1], and D.S. Martin

Tulane University School of Medicine, Department of Pathology & Laboratory Medicine
1430 Tulane Avenue, Room 6720, New Orleans LA 70112-2699
[1]Presently at: Ochsner Foundation of New Orleans

(Received for publication April 23, 1995, and in revised form October 19, 1995)

Abstract

Elliptical cells (E-P) are present at the perilymphatic interface lumen (PIL) of the lagena. The E-P cells often separate from the *tegmentum vasculosum* (TV) and have touching processes that form a monolayer between the K^+ rich perilymph and the Na^+ rich endolymph, similar to the mammalian Reissner's membrane. We examined the TV of chicks (*Gallus domesticus*) and quantitated the expression of anti-S100$\alpha\alpha\beta\beta$ and S100β. There was a 30% increase of S100β saturation in the light cells facing the PIL when compared to other TV light cells. We show that: (**1**) the dimer anti-S100$\alpha\alpha\beta\beta$ and the monomer anti-S100β are expressed preferentially in the light cells and the E-P cells of TV; (**2**) expression of S100β is higher in light cells facing the PIL than in adjacent cells; (**3**) the expression of the dimer S100$\alpha\alpha\beta\beta$ and monomer S100β overlaps in most inner ear cell types, including the cells of the TV, most S100$\alpha\alpha\beta\beta$ positive cells express S100β, but S100β positive cells do not always express S100$\alpha\alpha\beta\beta$; and (**4**) the S100β expression in light cells, the abundant Na^+-K^+ ATPase on dark cells of the TV, and previously demonstrated co-localization of S100β/GABA in sensory cells suggest that S100β could have, in the inner ear, a dual neurotrophic-ionic modulating function.

§This work is dedicated to Prof. G. Dohlman.

Key Words: Elliptical-P cells, *Tegmentum vasculosum*, S-100β, transmission electron microscopy (TEM)-post-immunolabeling, chicken, inner ear, color thresholding, video imaging, objective quantitation.

*Contact for correspondence:
C.D. Fermin, address as above
Telephone number: 504-584-2521
FAX number: 504-587-7389
E-Mail: Fermin@tmc.tulane.edu

Introduction

The avian *tegmentum vasculosum* (TV) and the mammalian *stria vascularis* (SVC) are regarded as the sites of K^+ and Na^+ control for the vertebrate inner ear, because the endolymphatic ionic composition and endocochlear potentials (Salt *et al.*, 1987; Schulte and Schmiedt, 1992; Syka and Melichar, 1985) arise in succession during maturation of the cochlea (Bosher and Warren, 1971; Schulte and Adams, 1989; Yao *et al.*, 1994). Both structures contain cells with dark and clear cytoplasm. Detailed studies have demonstrated that the dark cells of the TV, with lighter cells in the pigeon, formed a mosaic-like pattern (Dohlman, 1968; Ishiyama *et al.*, 1970; Jahnke *et al.*, 1969; Kuijpers *et al.*, 1970). Ishiyama *et al.* (1970) provide a very complete ultrastructural analysis of the TV and review of the classical literature. Most important, however, was Dohlman's observation that addition of NaCl or methylene blue into the endolymph resulted in active uptake of those substances by dark cells of the TV within just 10 minutes (Dohlman, 1968); clearly showing the very active absorptive function of the these cells. Avian dark cells were also described later (Money *et al.*, 1974), and recently, other works (Dallos, 1992; Zenner *et al.*, 1994) discussed their importance for the ionic balance of hair cells' function. It is accepted that the Na^+-K^+ ATPase system in the TV of the chicken cochlea (Kuijpers, 1974; Kuijpers *et al.*, 1970) and the SVC of mammals play essentially identical roles (Dohlman, 1977). It is well established that malfunctioning of the stria contributes to a multitude of vestibular and auditory problems (Belal and Ylikoski, 1980; Kerr *et al.*, 1982; Pauler *et al.*, 1988; Yoon *et al.*, 1991), some of which lead to endolymphatic hydrops.

The role of the light cells and other cells in the TV and SVC is less well understood, despite numerous works on the embryonic development and maturation of the avian TV (Cotanche *et al.*, 1987; Schneider *et al.*, 1987; Yoshihara *et al.*, 1987, 1990). Comparison of the above works and recent observations (Lavigne-Rebillard and Bagger-Sjöbäck, 1992) in the human SVC indicate

Figures 1-8 (on the facing page).

Figure 1. Light micrograph of the *tegmentum vasculosum* (TV) of a 13-days-old (Stage 39) chick embryo. The perilymphatic lumen (P) is shown with small dark elliptical cells (arrows) lining the light cells. In this area of the TV, there are more light than dark cells, and in longitudinal sections of the lagena, at least one cell layer of the TV is formed of light cells. Note that the elliptical cells (arrowheads) are not present in the endolymphatic lumen (E). The TV has many blood vessels (V), and the endothelial cells lining the vessels have the same appearance as the elliptical cells at the light microscopy level. Materials in Figures 1-6 were fixed *in situ* with a tannic acid-glutaraldehyde mixture and embedded in Araldite.

Figure 2. Higher magnification light photo-micrograph of elliptical (E-P) cells (arrows) lining the light cells (L) with large round nuclei (N) at the perilymphatic lumen. This layer of light cells become intermingled with dark cells forming a mosaic pattern toward the endolymphatic lumen where dark cells usually have multiple microvilli-like extensions (see Figure 11).

Figure 3. Elliptical (E-P) cells' nuclei are surrounded by thin cytoplasmic processes that extend bi-directionally from the cell body and remain in register with the basement membrane of the light cells. The ultrastructural appearance of the light cells (L) shown here is altered in methacrylate plastic processed tissues (see Figures 9 and 13), probably due to extraction of the cytosol during dehydration.

Figure 4. The elliptical (E-P) cells' plasma membranes and the cytoplasmic extensions of the light cells (L) remain in register with a prominent basement membrane (asterisk). Such proximity may facilitate the formation of a barrier to maintain certain ions from penetrating into the endolymphatic lumen through the intracellular junctions of the light cells.

Figure 5. The Elliptical (E-P) cells' cytoplasmic extensions can cover more than a 15 μm distance; and at some points the cytoplasmic processes are very thin (arrows). Nonetheless, the processes remain in register with those of the light cells, regardless of distortion of the basement membrane.

Figure 6. There were occasional separations (arrowheads) of the basement membrane (asterisk) from the light cells and the elliptical cells. At those points, the cytoplasmic processes (E-P) and the basement membrane were not equidistant. In the absence of distortion, breakage, or vascularization of the light cells, we assume that such changes were not artifactual but this requires further analysis.

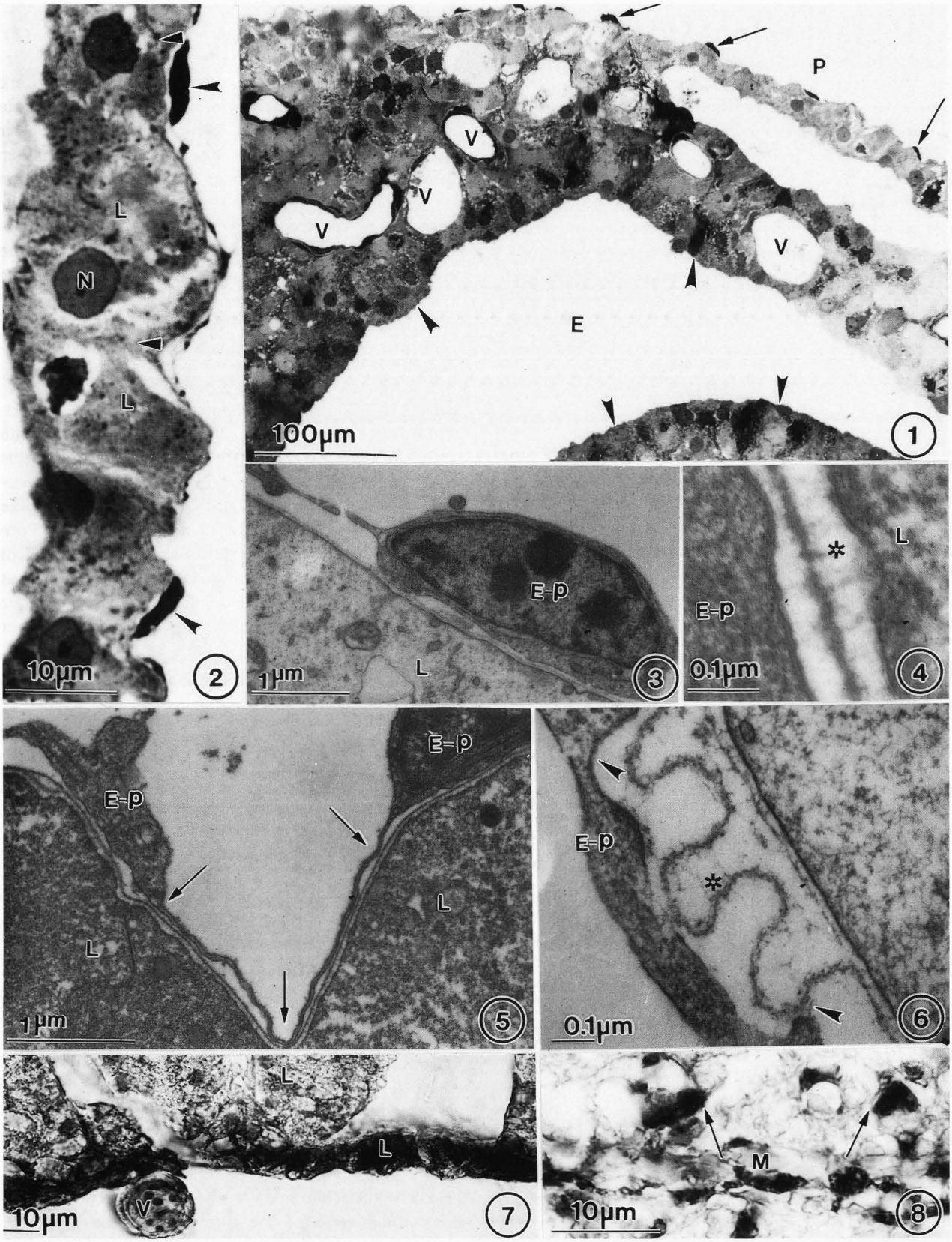
Figure 7. Light micrograph of anti-S100β labeled light cells (L) at the perilymphatic lumen. Note that light cells away from the PIL are only lightly labeled. We believe that the higher intensity label on those light cells at the PIL may be related to certain ionic functions of light cells at the junction of the perilymphatic lumen. Blood vessels (V) are abundant outside the main body of the TV and within it (see Figure 1).

Figure 8. Anti-S100$\alpha\alpha\beta\beta$ positive myelin (M) and glial cells (arrows) in the reticular formation of the chick brain stem. In addition, to preabsorbing anti-S100β with the pure protein as a negative control, we took advantage of the built-in positive control provided by brain, since S100β was originally isolated from central glial cells and those cells should always react with this antibody.

---

that the chick embryo remains as an excellent model for inner ear research. For instance, there is a correspondence between the maturation of avian TV and of the mammalian SVC at the 7th embryonic day and the 12th week respectively, which permits analysis of identical processes in a shorter period of time in aves than in mammals. For this reason, we investigated the TV cell types in the chick and identified small, flattened cells lining the perilymphatic interface lumen (PIL), which we designated as elliptical cells (E-P). We now propose that the E-P cells, together with the TV cells, probably contribute to the well known barrier that exists between the K^+ rich endolymph and the Na^+ rich perilymph by serving a function equivalent to that of mammalian Reissner's membrane. The E-P cells line even the blood vessels at the PIL, and may correspond to mesenchymal cells recently described (Chole and Tinling, 1994; Kikuchi *et al.*, 1994), which could permit selective passage of the perilymph at some locations of the cochlea (Tinling and Chole, 1994). Small cells were illustrated over the TV by Takasaka and Smith (1971) in the pigeon, but were not characterized as forming a monolayer or shown to express calcium binding proteins.

In both, the TV or the SVC there are three important issues deserving attention: (1) The dark cells are undoubtedly the source of sodium pumps; and are, therefore, sensitive to Ouabain-sensitive K^+-dependent activity (Kanoh *et al.*, 1993; Nishiyama *et al.*, 1994), diuretic drugs (Fikes *et al.*, 1994; Rybak *et al.*, 1992; Whitworth *et al.*, 1993), hormones (Julien *et al.*, 1994;


P
V
V
V
V
E
100μm
1
L
N
L
10μm
2
E-p
L
1μm
3
L
E-p
0.1μm
4
E-p
E-p
L
L
1μm
5
E-p
E-p
0.1μm
6
L
L
V
10μm
7
M
10μm
8

Pitovski *et al.*, 1993; Rarey *et al.*, 1991), aminoglycosides (Forge *et al.*, 1992; Park and Cohen, 1982), and other substances (Dieler *et al.*, 1994; Kobayashi *et al.*, 1993; Marcus *et al.*, 1994; Shehatadieler *et al.*, 1994; Spicer *et al.*, 1990; Wangemann and Shiga, 1994). (**2**) The dark cells have many cytoplasmic processes that interdigitate with the processes of the light cells, which selectively express the calcium binding protein and neutrophic molecule S100 (Fermin and Martin, 1995). (**3**) Dark cells and light cells in the chicken cochlea are not segregated from other cells as is the case in the mammalian SVC and the Reissner's membrane cells, but rather are combined into the bulky TV. Thus, the results and interpretations presented here should be evaluated with this in mind. Finally, the data and interpretations shown in this paper preclude dark cells in other organs of the inner ear.

Materials and Methods

Tissue processing

Experiments were approved by the Tulane University School of Medicine's Advisory Committee for Animal Resources. Ten newly hatched white leghorn (*Gallus domesticus*) chicks were anesthetized with an intraperitoneal injection of Nembutal sodium solution (40 mg/kg body weight). Chicks were perfused via the left ventricle for 1 minute with phosphate buffered saline (PBS). After the PBS pre-wash, fixation was achieved by perfusing for 2 minutes with 10% buffered formalin. The head was removed and a slice of the head containing the temporal bone was cut by hand, while immersed in the fixative used for vascular perfusion. Tissues were embedded in paraffin and cut at 8 μm (Fermin and Martin, 1995). Three embryos at E13 (stage 39) were used for comparison to hatchlings.

Immunohistochemistry of paraffin embedded materials

Tissue sections were floated on a water bath, affixed to polylysine coated slides, and processed. All incubations were done at room temperature with tris buffer rinse in between: (**1**) 20 minutes in 1% hydrogen peroxide; (**2**) 10% goat serum for 20 minutes; (**3**) primary anti-S100 rabbit IgG diluted 1:4000-1:9000 in 2% bovine serum albumin-Tris (BSA-TMS/HCL) buffer; (**4**) biotinylated goat-anti-rabbit IgG for 30 minutes; (**5**) streptavidin-horseradish peroxidase (Biogenex Laboratories, San Ramon, CA) for 30 minutes; (**6**) 3'3'-diaminobenzidine tetrahydochloride (DAB) for 5 minutes or 3-amino-9-ethylcarbozole (AEC); and (**7**) counterstain in hematoxylin. Relative concentration of S100β was assessed with color thresholding, a technique that was recently described in detail (Fermin *et al.*, 1992). Contrary to

Figure 9. Post-immuno labeling of anti-S100$\alpha\alpha\beta\beta$ (40 nm diameter particles) at the perilymphatic lumen (P). Note that light cells (L) have the highest number of particles/μm^2 including the nucleus (N), whereas the dark cells (D) have less particles/μm^2. In particular, note that there are virtually no particles over the acellular portion of this view (P), attesting to the antibody-antigen specificity in our preparations. Non-specific interaction known to occur by the action of electrical and static attractions are common with gold labeling, and are impossible to appreciate in high magnification views of the specimens. Cytoplasmic processes that characterize the avian TV dark cells (arrow), and the counterpart marginal cells of the mammalian stria vascularis, are seen. Each process has a mitochondrion that is better illustrated below in Figure 12.

Figure 10. Contrasting the anti-S100$\alpha\alpha\beta\beta$ labeling pattern of light (L) and dark cells processed (arrows) with the endolymphatic lumen (E), one can appreciate that gold particles are present mainly on the light cells processes (arrows). This pattern is a mirror image of the histochemical labeling pattern of Na^+-K^+ ATPase (Yoshihara *et al.*, 1987, 1990) where the processes of the dark cells are exclusively stained, suggesting that dark cells have a very active sodium pump (Rodriguez de Lores Arnaiz, 1992). The predominant label over light cells with anti-S100$\alpha\alpha\beta\beta$ and with S100β as well, suggests that light cells probably house the ionic modulating molecules and that perhaps dark cells pump the ions across the boundaries that separate the endolymph from the perilymph (Dohlman, 1968).

Figure 11. At the endolymphatic lumen, dark cells extend microvilli-like short cytoplasmic processes (arrow) perhaps for increasing the surface areas at those locations (see Figures 1 and 9). It is noteworthy though that the luminal endolymphatic surface of the dark cells (D) is smaller than the light cells (L), but that the surface area of the interdigitations, where the pump action was detected histochemically (Yoshihara *et al.*, 1987, 1990), is greater in the dark than in the light cells (see Figure 10).

Figure 12. Negative control with no gold particles over the cellular or acellular areas. A characteristic heterochromatic dark cell nucleus (DN) is shown and a mitochondrion (arrow) is shown at the end of a cytoplasmic process.

Figure 13. Anti-S100β (20 nm diameter particles) labeled elliptical cell (arrows); and some particles over the non-cellular areas of the embedding media are seen. Note different labeling pattern over light cells (L) with anti-S100β as compared to that of anti-S100$\alpha\alpha\beta\beta$ (Figures 9 and 10).

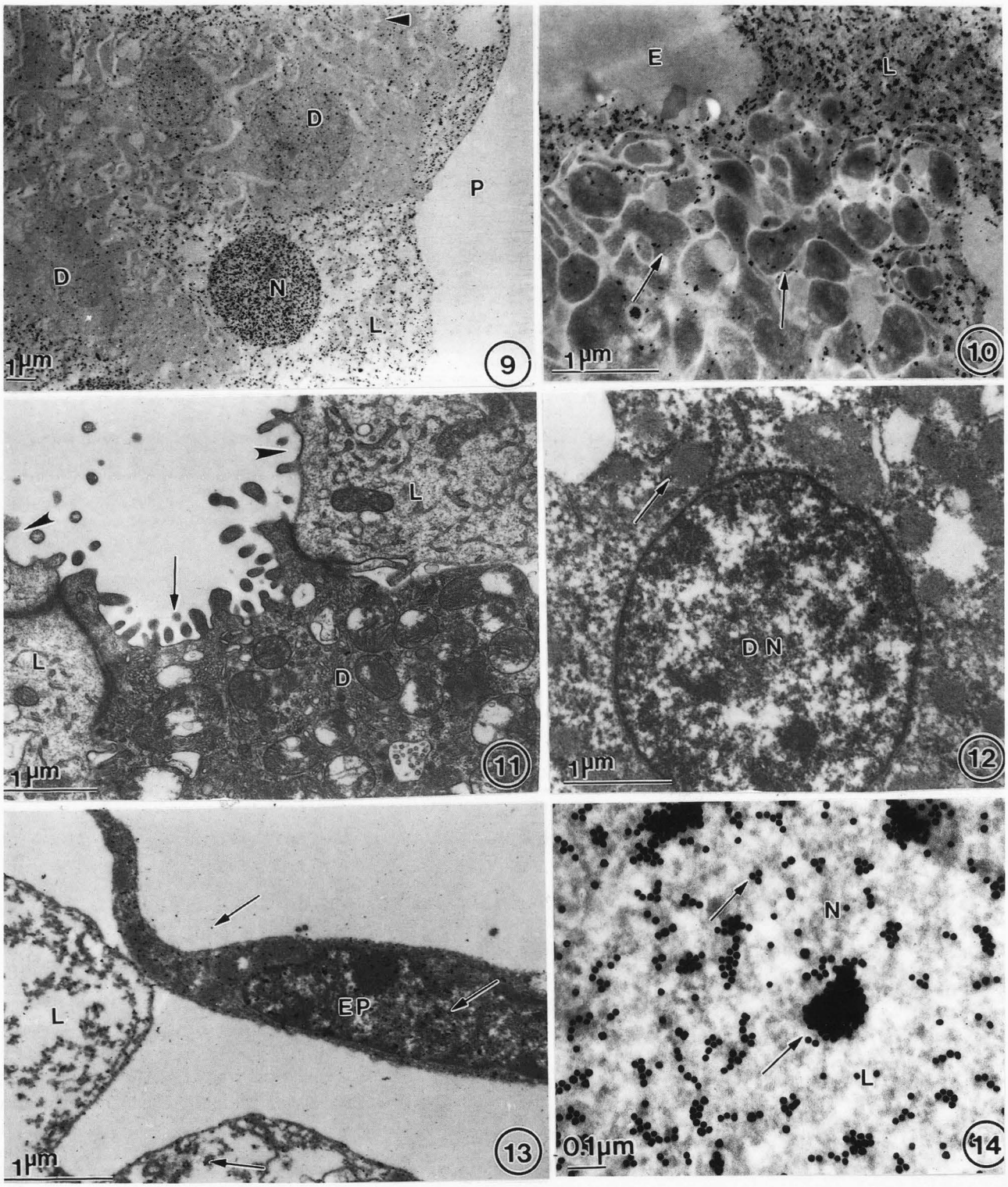


Figure 14. S100$\alpha\alpha\beta\beta$ label over the nucleus of a light cell (20 nm diameter particles). Aggregation of particles occurred mainly over the heterochromatin (arrows). The presence of the dimer S100$\alpha\alpha\beta\beta$ and monomer S100β in the nuclei of glial and Schwann's cells was described, but the significance of S100 in the nucleus is unknown. However, in the vestibular ganglion of the chick, specific expression of S100β was quantitated before (Fermin and Martin, 1995).

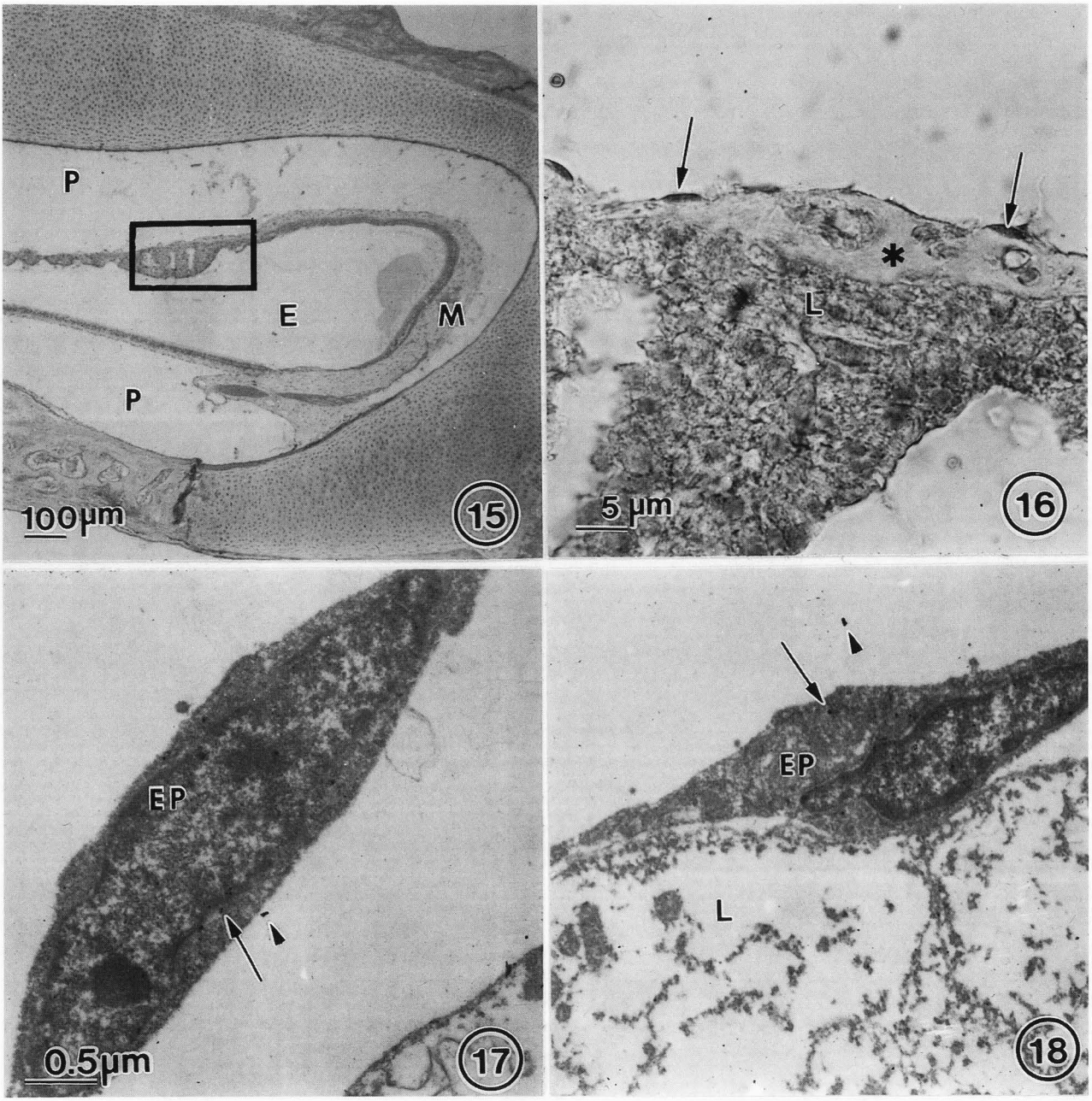


Figure 15. Negative control at the level of the macula lagena (M), the perilymphatic (P) and the endolymphatic (E) lumens counter-stained with hematoxylin. No expression of S100β is present over any cellular component. The area inside the box is enlarged in Figure 16.

Figure 16. E-P cells (arrows) are seen at the perilymphatic interface with mesenchymal matrix (asterisk) separating the E-P cells from the light cells (L) as the E-P cells begin their covering over the hyaline cartridge. No immunoreaction is present, and the denser areas were caused by the hematoxylin counterstain.

Figure 17. E-P cells minimal labeling (arrows) over the cell itself, and over the non-cellular areas. Dirt is indicated by the arrowheads.

Figure 18. E-P cells over light (L) cells. No label over the light cells, and minimal label over the E-P cells is present (arrows). Dirt is indicated by the arrowheads.

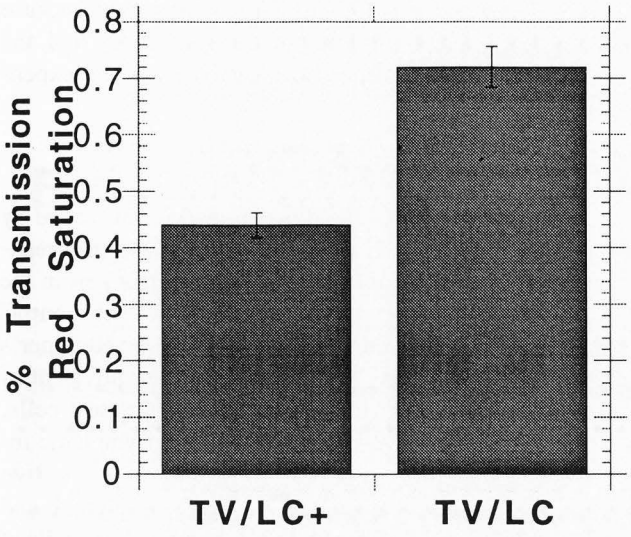


Figure 19. Comparison of the saturation intensity for red threshold as indicator of S100β immunoreactivity over light cells at the perilymphatic interface lumen (PIL), where the elliptical cells are located (TV/LC+), and light cells elsewhere in the *tegmentum vasculosum* (TV/LC). S100β expression over the cells at the PIL is higher (lower percent of light passing through sections) than in other cells. Fifty fields, approximately 100 μm^2 in each one of the 10 chicks processed, were measured along the TV of sections similar to those illustrated in Figure 15.

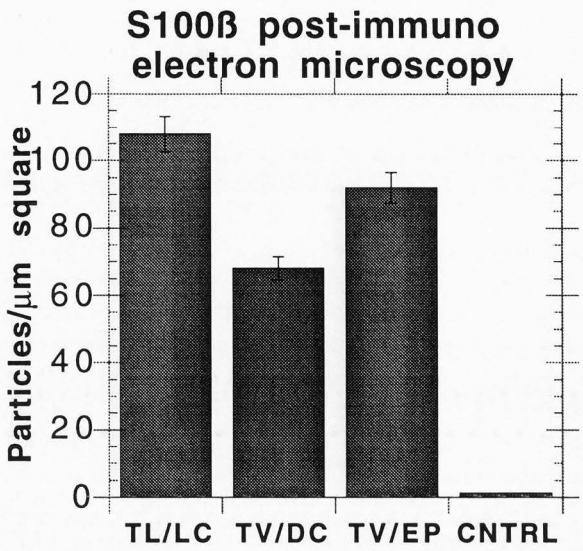


Figure 20. Comparison of the number of gold particles per μm^2 as indication of S100β immunoreaction with light cells (TV/LC), dark cells (TV/DC), elliptical cells (TV/E-P), and control, after omitting the primary antibody or preabsorption with the pure protein. The highest number of particles was counted over the light cells. The number of particles/μm^2 counted over non-cellular areas of the section (mostly plastic or epoxy) was as low as the control illustrated in this bar graph.

---

black and white densitometry, which often cannot separate closely related hues of similar intensities, color thresholding can potentially separate 16 million hues. For instance, demonstrating that a given antibody is present over a tissue section has less scientific validity than assigning to the color (hue) a numerical value, which can subsequently be reproduced objectively without user bias (Fermin *et al.*, 1992).

Controls

Two negative controls were included: (1) omission or replacement of the primary antibody with an unrelated antisera, and (2) pre-absorption of anti-S100β after appropriate titration with pure S100 protein (Sigma, St. Louis, MO). Two built-in positive controls exist in our preparations. First, the brain on the same slide has glial cells that always stain with S100β. Second, Purkinje's cells stain with both S100β and GABA.

Electron microscopy

Animals were perfused via the left ventricle for 1 minute with phosphate buffered saline (PBS). After the PBS pre-wash, fixation was achieved by perfusing for 2 minutes with PBS containing 4% paraformaldehyde and 1% glutaraldehyde. Some specimens were fixed in 8% tannic acid-2.5% glutaraldehyde in 0.2 M sodium cacodylate buffer (pH 7.4). The specimens were rinsed and osmicated in 1% osmium tetroxide, dehydrated with acetone and embedded in Araldite® (Fermin, 1993; Fermin *et al.*, 1990). The head was removed and split along the mid-sagittal plane, the brain was removed, and the temporal bones dissected while immersed in the fixative used for vascular perfusion. Each temporal bone was cut at the level of the utricle and the distal portion of the macula of the lagena under a stereo-microscope. The cochlear structures were micro-dissected, and rinsed with PBS no more than 2 hours after primary fixation of the chicks; the cochleae were then embedded as described before in epoxies or plastics.

Embedding in the epoxy Araldite: Tissues were post-fixed with 1% osmium tetroxide for 1 hour and then dehydrated with 50%, 70% and 80% ethanol (30 minutes each). Infiltration was carried out at room temperature in Araldite:acetone (2:1) for 30 minutes and pure resin for 24 hours. Polymerization was done at 60°C for 24 hours.

Embedding in the plastic LR White®: Tissues were dehydrated with 50%, 70% and 80% acetone (30 minutes each). Infiltration was carried out in LR White: ethanol (2:1) for 30 minutes at room temperature,

followed by pure resin for 24 hours at 4°C. Polymerization was carried out at 60°C for 24 hours.

Immunogold labeling for electron microscopy

Anti-S100$\alpha\alpha\beta\beta$ or anti-S100β rabbit IgG were purchased from Sigma Immunochemicals (St. Louis, MO), and goat anti-rabbit IgG-gold (20 and 40 nm diameter particles) was purchased from E-Y Laboratories, Inc. (San Mateo, CA). Immunogold labeling was achieved using our previously published protocol (Fermin *et al.*, 1994) consisting of following steps: (**1**) Float sections on grids section side down-ward on the following: (**2**) distilled H_2O (1 minute); (**3**) 10% H_2O_2 (15 minutes); (**4**) rinse 2 X 1 minute in distilled H_2O; (**5**) phosphate-buffered (0.01 M, pH 7.2) saline (0.15 M) containing 1% bovine serum albumin (5 minutes); (**6**) PBS containing 1% gelatin (10 minutes); (**7**) PBS containing 0.02 M glycine (5 minutes); (**8**) primary antibody in BSA-PBS containing 0.05% Tween-20 and 0.05% Triton X-100 (24 hours at 4°C) [~ 40 μg protein/ml] = (1:250); (**9**) rinse 5 X 1 minute in BSA-PBS; (**10**) gold probe (1:4) = [~ 2.5 μg protein/ml] in BSA-PBS containing 0.05% Tween-20 and 0.05% Triton X-100 (1 hour); (**11**) rinse 5 X 1 minute in PBS; (**12**) fix with 1% glutaraldehyde in PBS (3 minutes); (**13**) rinse 5 X 1 minute in distilled H_2O; (**14**) counter-stain in saturated uranyl acetate for 1 minute; and (**15**) air or vacuum dry and examine with a Zeiss 109 transmission electron microscope (TEM).

Data analysis

Color thresholding was used to estimate the relative concentration of S100β in the light cells, because it allows fine tuning to hue variations of color reaction products (e.g., brown diaminobenzidine, red phosphatase, blue hematoxylin counterstain, etc.). The V150® color imaging system (ONCOR Imaging Systems, Gaithersburg, MD) has been described in detail (Fermin and DeGraw, 1995, Fermin *et al.*, 1992). The system separates primary and secondary colors based on their hues-saturation-intensity (HSI), allowing a theoretical 16 million color possibilities. We used color thresholding rather than monochrome thresholding, because differences between adjacent pixels are not always easy to separate with systems since they often cannot separate hues of similar intensities (e.g., a dark brown and a dark blue). This is particularly important when trying to count particles (gold, isotope) on a dark background (e.g., dark field and *in situ* hybridization). Red-green-blue imaging based systems are more computationally intense than HSI-based imaging systems. The HSI mode is based on the same principle that the human brain uses to distinguish colors. HSI consists of a three dimensional space within which discrimination of colors is performed without the need of filter selection (Fermin and DeGraw, 1995; Fermin *et al.*, 1992). This is important because filters only select for but not for saturation, and the systems that rely on filters are usually slow and expensive.

Results

The main body of the TV is a massive structure by inner ear standard. It is highly vascularized and separates the scala vestibuli (perilymphatic lumen) from the scali tympani (endolymphatic lumen). However, contrary to the bilayer mammalian counterpart, the Reissner's membrane, the avian TV also includes the equivalent of the SVC and is thus bulky. Flattened elliptical cells, that we called E-P cells here, line the perilymphatic interface lumen (PIL), and may contribute to a selective electrolytic barrier between the K^+ rich endolymph and Na^+ rich perilymph (Figure 1). A layer of light cells at the PIL extends the length of the TV, and is lined by the E-P cells (Figure 2). The E-P cells have heterochromatic nuclei with minimal cytoplasm around them (Figure 3) because the majority of the E-P cytoplasm extends bi-directionally from the cell body for over 15 μm in close register with a thin basement membrane that stains with tannic acid (Figure 4). The association between the E-P cells, the basement membrane, and the light cells seems so intimate that only breakage of the light cells cytoplasm and plasma membrane is able to separate the three structures (Figures 5 and 6).

The light cells immediately adjacent to the PIL and in contact with the E-P cells expressed more S100 protein than other light cells located elsewhere in the TV (Figure 7). In fact, the intensity of the immunoreaction was similar to the built-in positive control that we always process simultaneously and in the same section (Fermin and Martin, 1995). S100β was originally isolated from glial cells and for this reason, myelin, glial cells in the brain (Figure 8). At the electron microscopic level, the light cells at the PIL showed higher numbers of gold particles/μm^2 than cells elsewhere in the TV (Figure 9). The cytoplasmic processes of the light cells and the cytoplasm also had more gold particles than the processes of the dark cells (Figure 10). The preferential distribution of anti-S100-gold particles over the light and elliptical cells, but not over the dark cells, suggest different functions for these cells. Dark cells contain larger numbers of microvilli-like extensions where they face the endolymph (Figure 11). Away from the lumen, the dark cells have mitochondria in each cytoplasmic process. When incubated with pre-absorbed sera there were only a few gold particles (Figure 12). Like the light cells at the PIL, the E-P cells also stained with S100β (Figure 13). The nuclei of light cells next to the E-P cells also stained with large particle aggregates over the heterochromatin (Figure 14). Negative

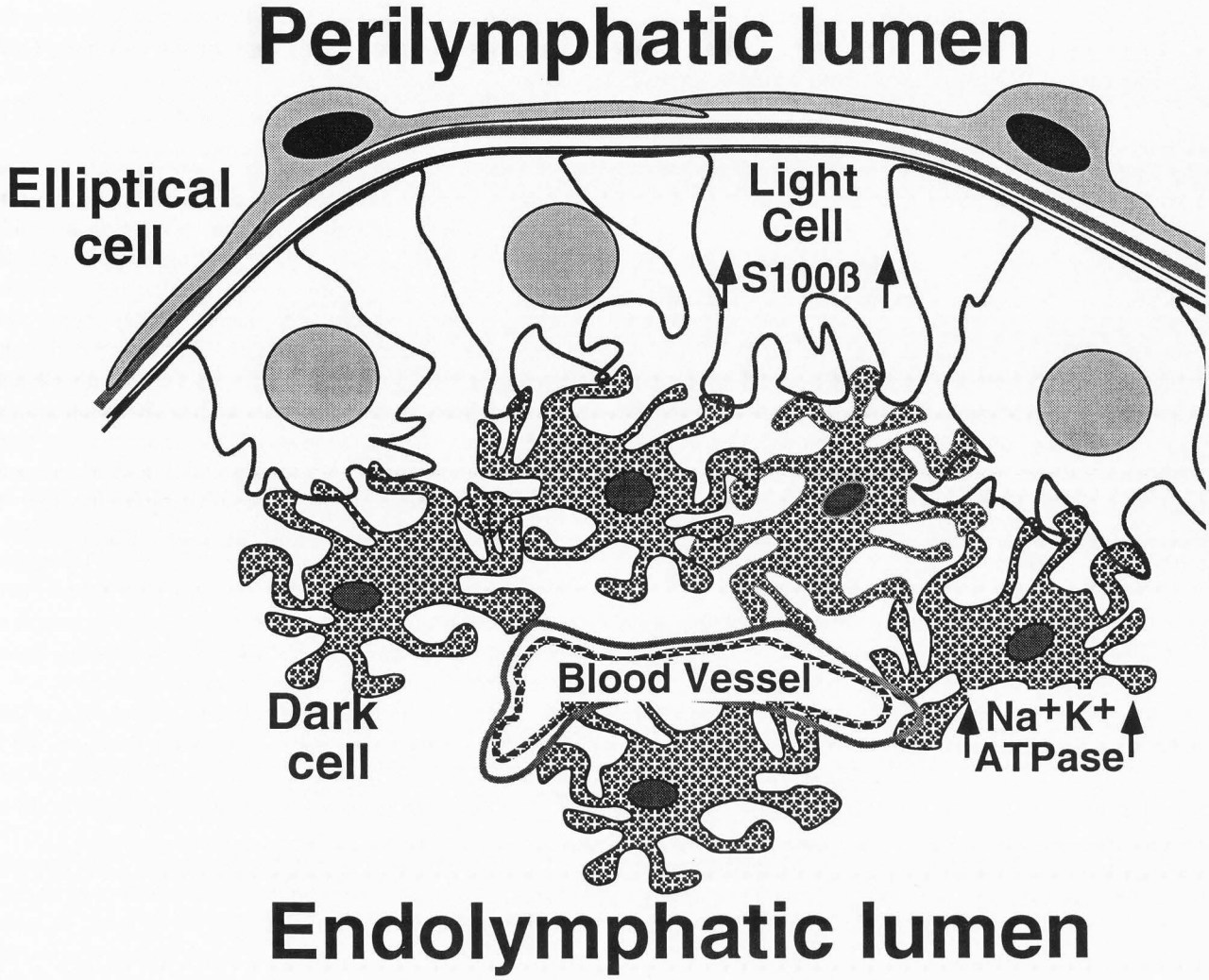


Figure 21. Simplified diagram of dark, light and E-P cells at the perilymphatic interface lumen (PIL) of the *tegmentum vasculosum* in normal chickens. A single layer of light cells located immediately under the E-P cells express large amounts S100β (arrows up). Light cells have short cytoplasmic processes that contact the larger cytoplasmic processes of the dark cells. Dark cells do not express large amounts of S100β, but contain instead a large number (Yoshihara *et al.*, 1990) of Na^+ K^+ ATPase pumps (arrows up). The well known calcium binding and neutrophic functions of S100β in other cells (Fermin and Martin, 1995), and the S100β expression over light cells suggest that light cells could modulate the dark cells absorptive function. In addition, the well-known absorptive function of the TV (Dohlman, 1968) and the demonstrated response of dark cells to Na^+-K^+ ATPase pump depleting drugs (Park and Cohen, 1982; Whitworth *et al.*, 1993), coupled with a demonstrated functional and morphological damage of the TV (Ryals *et al.*, 1995), suggest that the TV's PIL is probably a critical barrier for the homoestasis of the inner ear fluids. Furthermore, the effectiveness of the barrier that the PIL forms is probably enhanced by (as yet demonstrated functional atributes) of the the E-P cell facing the perilymphatic lumen.

---

controls showed no immunoreactivity in any portion of the cochlear duct at the light microscopy level (Figures 15 and 16), or at the electron microscopy level. The number of gold particles counted was below the signal level considered positive (Figures 17 and 18). Quantitation of the relative concentration of the immuno reaction at the light microscopic level and with color thresholding (Fermin and DeGraw, 1995), yielded a measurable difference between the immunolabeling of light cells at the PIL and those elsewhere (Figure 19). Similarly, the number of gold particles/μm^2 was higher in the light cells at the PIL (Figure 20). Figure 21 summarizes our interpretation of the results presented in this paper in relation to previous works.

Discussion

The data of this paper show that flattened elliptical cells (E-P) lining the perilymphatic interface lumen (PIL) of the *tegmentum vasculosum* (TV) formed, in the chick inner ear, a monolayer over the light cells (Figures 1 to 6). Small cells were illustrated on the PIL of the pigeon by (Takasaka and Smith, 1971), but were not described. In addition, the E-P and light cells at the PIL expressed S100 (Figures 7, 9, 10 and 13). The significance of the expression of a calcium binding protein-neurotrophic factor hints at an ionic function of the E-P cells and the light cells. The E-P cells could be similar to the flat cells that form the bilayer mammalian Reissner's membrane, but such assumption requires further analysis. The preferential expression of S100 on the light cells of the TV rather than on the dark cells is interesting, because Na^+-K^+ ATPase is highly concentrated in the dark cells, which have a high absorptive capacity (Dohlman, 1968, 1977). Possible implications of dark and light cells staining affinities will be discussed below.

The morphological arrangement between these two cell types suggests that dark cells, which have abundant sodium pumps (Kanoh *et al.*, 1993; Nishiyama *et al.*, 1994; Schneider *et al.*, 1987; Tencate *et al.*, 1994; Yao *et al.*, 1994; Yoshihara *et al.*, 1987), move electrolytes; whereas the light cells, which have neurotrophic molecules (Fermin and Martin, 1995) could modulate the dark cells' function. Such modulation could be partially provided by neurotrophic molecules. Furthermore, the absence of the elliptical cells from the endolymphatic lumen suggests that the E-P cells are needed at the PIL, but this assumption requires further analysis. The TV has many blood vessels and even though the endothelial cells lining the vessels have the same appearance as the elliptical cells, there seems to be two distinct types of cells. First, the endothelial cells of the capillaries do not stain with S100β and second, the endothelial cells do not extend bilaterally for up to 15 μm distance as E-P cells do (Figure 1).

The E-P cells facing the perilymphatic fluid, to our knowledge, were not shown before as being S100β positive. The monolayer that is formed by these cells could function as a barrier between the TV cells and the perilymph. These cells contain the highest concentration of S100β in hatching TV, at the light and the transmission electron microscopy levels. Like other S100β positive inner ear cells (e.g., Schwann cells), the E-P cells could be derived from the neural crest (Lauriola *et al.*, 1986; Le Douarin *et al.*, 1993; Murphy *et al.*, 1991; Ziller and Smith, 1982). The avian E-P cells may correspond to the basal cells of the stria vascularis. However, flattened avian cells similar in size to E-P cells in other parts of the cochlear duct, but that are not separating the endolymph from the perilymph do not form a monolayer.

Implications of dark, light and E-P cells' location in the TV

The mosaic-like pattern of dark and light cells form a unique sieve through which the endolymph probably passes (Figures 9 and 10). It is conceivable that the dark cells' rich sodium pumps (Rodriguez de Lores Arnaiz, 1992; Varon and Skaper, 1989) move ions across their membrane, while the light cells provide the necessary stimuli for that function. On the other hand, the proximity of the E-P cells and of the light cells at the PIL may facilitate the formation of a barrier to keep ions from penetrating into the endolymphatic lumen, through the intracellular junctions of the light cells. It is possible that the higher intensity labeling pattern of S100 over those light cells at the PIL could be related to, as yet undetermined, ionic functions of light cells at the junction of the perilymphatic lumen. The extent of the E-P cells' coverage is such that even the blood vessels at the PIL are covered with the E-P cells.

Since (Dohlman, 1968) already demonstrated that dark cells have a very active absorptive function and it is now clear that dark cells have extensive sodium pumps, it is reasonable to assume that dark cells move ions across their membranes. The E-P cells may, on the other hand, restrict the passage of ions, but this requires further analysis. The predominant label over light cells with anti-S100$\alpha\alpha\beta\beta$ and with S100β as well, suggests that light cells are strategically intermingled with the dark cells, and may aid dark cells' absorptive function. It is noteworthy that the luminal endolymphatic surface of the dark cells is smaller than the light cells, but the surface area of the interdigitations, where the pump action was detected histochemically (Yoshihara *et al.*, 1987, 1990), is greater in the dark than in the light cells (Figure 10).

In the avian design, the first layer of light cells and the E-P cells may, form a barrier that is probably as effective as the mammalian Reissner's membrane (Dohlman, 1968). A diagram with highly schematic representation of how the E-P cells may stay in register with the uppermost light cells' layer of the TV is shown in Figure 21. At the perilymphatic interface lumen (PIL), the E-P cells may, with the aid of the basement membrane shown in Figure 4, form an effective selective barrier. Such barrier is formed in the mammalian cochlea by the Reissner's membrane bilayer (Kikuchi *et al.*, 1995), which isolates the SVC from the perilymph and may, in effect, aid in the generation of electrogenic potentials there (Shehatadieler *et al.*, 1994).

In the avian TV, dark cells increase in number away

from the first layer of light cells. The dark cells have extensive cytoplasmic processes rich in Na^+-K^+ ATPase activity (Tencate *et al.*, 1994), that interdigitate with the processes of the light cells (Figure 10). Like the mammalian SVC, the avian TV is highly vascularized. Dark cells cytoplasmic extensions may directly contact the vasculature, and the contact may be important in the high electrolytic exchange that one would expect in this organ.

Thus, while the avian TV is bulky, when compared to the mammalian SVC and Reissner's membrane, aves manage to isolate the endolymphatic and perilymphatic compartments very effectively, despite having to compress 32 mm of stria into 7 mm of TV. It remains to be demonstrated that the immuno expression of the S100 monomer and dimer in light cells and the high number of Na^+-K^+ ATPase pumps in the dark cells are related to the dual calcium binding (dimer) and neurotrophic (monomer β) functions of this molecule.

The prospect of such a scenario is intriguing because the inner ear has multiple functions cramped into a confined space. Such design in nature usually favors molecules that are small, multimeric, and can perform two of the most important functions needed for transducing mechanical into electrical energy: (**1**) electrolytic homeostatic control, and (**2**) neuronal modulation of sensory and non-sensory tissues. It happens that S100 can do both. While the sensory hair cells have been the main actors in the stage of inner ear research, it is possible that the cells of the avian TV (or mammalian stria), that balance hair cells' ionic environment (Dohlman, 1968), play a more crucial role (Zenner *et al.*, 1994) than was previously ascribed to them. The presence of an ionic regulator of known neurotrophic activity such as S100β deserves further analysis (Rodriguez-deLores-Arnaiz, 1992; Varon and Skaper, 1989).

Possible functional implications of S100 in the TV

S100 is a multimeric calcium binding (CBP) E-F-hand protein (Andressen *et al.*, 1993; Baimbridge *et al.*, 1992) molecule. The dimer and monomer are found in the central nervous system (CNS) glia, and peripheral nerve Schwann's cells. The dimer S100$\alpha\alpha\beta\beta$ has a wide and non-compartmentalized distribution in adult and embryonic tissues (Bhattacharyya *et al.*, 1992; Hayashi *et al.*, 1991). S100$\alpha\alpha\beta\beta$ is also present in the adult inner ear of mammals (Foster *et al.*, 1994; Igarashi *et al.*, 1991) and fishes (Foster *et al.*, 1993; Saidel *et al.*, 1990). The presence of S100$\alpha\alpha\beta\beta$ in hair cells type I of fishes is not related to our findings, because fishes and amphibians do not have true type I hair cells (Guth *et al.*, 1994). S100$\alpha\alpha\beta\beta$ is expressed in normal (Kligman and Hilt, 1988) and tumors cells, (Hayashi *et al.*, 1991); and the S100β subunit acts as a neurotrophic factor in neuronal tissue (Marshak, 1990; Van Eldik *et al.*, 1991).

It is possible that S100β and other calcium proteins of the E-F-hand family, such as calbindin-D 28K (Dememes *et al.*, 1992; Fermin and Martin, 1995) modulate some function of the inner ear; setting a precedent for including S100β as a good candidate for a multi-functional molecule. In the inner ear, S100 could act as a neurotrophic-like factor and as an ionic modulating molecule. Particularly, because like calbindin and other CBP, S100β is co-expressed with potential neurotransmitters of the inner ear such as, GABA (Meza *et al.*, 1982, 1992, Usami *et al.*, 1987). Previous work related the functions of S100$\alpha\alpha\beta\beta$ to protein phosphorylation, ATPase, adenylate cyclase, and calcium release among other functions (Donato, 1991). As a member of the E-F-hand proteins, the function of the S100β monomer could parallel that of calbindin and parvalbumin, molecules that are closely associated with selected neuronal groups, in particular with GABA-energic neurons (Celio, 1990). Vestibular hair cells in the rat that were positive for GABA and other calcium binding proteins (Braun, 1990; DeFelipe and Jones, 1992; Fermin and Martin, 1995) were shown in the chick to stain with S100$\alpha\alpha\beta\beta$ as well (Fermin and Martin, 1995).

Our interest in S100β started when we noticed that this small but multimeric calcium binding and neurotrophic molecule was expressed in afferent perikarya of the VIIIth cranial nerve of the chick (Fermin *et al.*, 1994). Others showed the molecule in the vestibular hair cells of the rat (Celio, 1986, 1990), but no correlation with ionic controlling cells was made. A molecule with multiple functions is attractive in the ear, which is a unique organ with heterogeneous tissue and cell types; and conduits filled with fluids in which exquisite homeostatic control of Na^+, K^+ and Ca^{++} occurs in birds (Yoshihara *et al.*, 1987) and mammals (Schulte and Adams, 1989; Schulte and Schmiedt, 1992). S100β, like other E-F-hand proteins, could serve the dual purpose of modulating neuronal function and/or development; and as yet unknown, some calcium binding function. Modulation of neurons is needed for proper synaptology to occur, and regulation of calcium is also needed to aid in the formation of otoconial calcium rich membranes (Fermin, 1993). S100β could aid in the maintenance of the exquisite micro-environment of the endolymphatic and the perilymphatic fluids. It is well known that cells facing these fluid-filled reservoirs contain rich Na^+-K^+ ATPase activity (Cotanche *et al.*, 1987; Schulte and Adams, 1989; Yoshihara *et al.*, 1987, 1990). S100β could mobilize intracellular calcium stores in the ear as is suggested to occur in other cells (Barger and Van Eldik, 1992).

Comparison of the saturation intensity for red with color thresholding (Fermin and DeGraw, 1995; Fermin *et al.*, 1992) as indicator of S100β immunoreactivity over light cells at the PIL, where the elliptical cells are located and light cells elsewhere in the TV was done (Figure 19). S100β expression over the cells at the PIL was higher (lower percent of light passing through sections) than in other cells. Comparison of the number of gold particles/μm^2 as indication of S100β immunoreaction over light cells, dark cells, and elliptical cells suggests that the highest number of particles counted over the light cells is perhaps related to stimulatory functions of the light cells. The number of particles/μm^2 counted over non-cellular areas of the section (mostly plastic or epoxy) was as low as the control. The presence of the dimer S100$\alpha\alpha\beta\beta$ and the monomer S100β in the nuclei of glial and Schwann's cells was described before, but the significance of S100 in the nucleus is unknown, even though expression of S100β was quantitated before in the vestibular ganglion of the chick (Fermin and Martin, 1995). Statistical analysis of the data showed that light cells have the highest number of particles/μm^2 including the nucleus, whereas the dark cells had less particles/μm^2. In particular, it is interesting that virtually no particles were observed over the acellular portions of the thin sections, attesting to the antibody-antigen specificity we obtain regularly in our laboratory (Figures 9, 10, and 13). This is an important consideration, because there are non-specific interactions known to occur by the action of electrical and static attractions common with gold labeling and are impossible to appreciate in high magnification views of the specimens as shown in Figures 14 to 18.

We interpreted the relative concentration of S100 detected as a reliable indicator of antigenicity, because we included negative and positive controls in our tissue preparations. In addition to preabsorbing anti-S100β with the pure protein as a negative control, we took advantage of the built-in positive control provided by the brain tissues. S100β was originally isolated from central glial cells and those cells should always react with this antibody (Fermin and Martin, 1995). The immunohistochemical staining pattern observed here with S100 on light cells, is a mirror image of the histochemical labeling pattern of Na^+-K^+ ATPase already reported for dark cells (Yoshihara *et al.*, 1987, 1990). The relationship that may exist between the presence of S100β in light cells and abundant number of sodium pumps in the dark cells still remains to be demonstrated.

Acknowledgments

This work is dedicated to the memory of Prof. G.F. Dohlman, with whom CDF had the privilege of discussing his views of endolymph homeostatic control, and whose early 50's and 60's work on the subject stimulated CDF to study the *tegmentum vasculosum*. Supported in part by NASA NAG1516, the NIH and the Department of Pathology and Laboratory Medicine at Tulane University. Specials thanks to Mr. Diaz for photography work, and C. Trepangnier and M.G. Fermin for correcting the manuscript. The constructive criticism and continuous support of R.F. Garry from the department of Microbiology at Tulane University is greatly appreciated.

References

Andressen C, Blümcke I, Celio M (1993) Calcium-binding proteins: Selective markers of nerve cells. Cell Tis Res **271**: 181-208.

Baimbridge K, Celio M, Rogers J (1992) Calcium-binding proteins in the nervous system. TINS **15**: 303-308.

Barger S, Van Eldik L (1992) S100 beta stimulates calcium fluxes in glial and neuronal cells. J Biol Chem. **267**: 9689-9694.

Belal A Jr, Ylikoski J (1980) Pathologic significance of Meniere's symptom complex. A histopathologic and electron microscopic study. Am J Otolaryngol. **1**: 275-284.

Bhattacharyya A, Oppenheim RW, Prevette D, Moore BW, Brackenbury R, Ratner N (1992) S100 is present in developing chicken neurons and Schwann cells and promotes motor neuron survival *in vivo*. J Neurobiol. **23**: 451-466.

Bosher SK, Warren RL (1971) A study of the electrochemistry and osmotic relationships of the cochlear fluids in the neonatal rat at the time of the development of the endocochlear potential. J Physiol **212**: 739-761.

Braun K (1990) Calcium-binding proteins in avian and mammalian central nervous system: Possible localization, development and possible functions. Prog Histochem Cytochem **21**: 1-64.

Celio M (1986) Paralbumin in most gamma-aminobutyric acid-containing neurons of the rat cerebral cortex. Science **231**: 995-997.

Celio M (1990) Calbindin D-28k and paralbumin in the rat nervous system. Neuroscience **35**: 375-475.

Chole RA, Tinling SP (1994) Bone lining cells of the mammalian cochlea. Hear Res **75**: 233-243.

Cotanche DA, Cotton CU, Gatzy JT, Sulik KK (1987) Ultrastructural and electrophysiological maturation of the chick *tegmentum vasculosum*. Hear Res **25**: 125-139.

Dallos P (1992) Feature article: The active cochlea. J Neurosci **12**: 4575-4585.

DeFelipe J, Jones E (1992) High-resolution light- and electron microscopic immunocytochemistry of co-localized GABA and calbindin D-28k in somata and double bouquet cell axons of monkey somatosensory cortex. Eur J Neurosc **4**: 46-60.

Dememes D, Raymond J, Atger P, Grill C, Winsky L, Dechesne CJ (1992) Identification of neuron subpopulations in the rat vestibular ganglion by calbindin-D 28K, calretinin and neurofilament proteins immunoreactivity. Brain Res **582**: 168-172.

Dieler R, Shehatadieler WE, Richter CP, Klinke R (1994) Effects of endolymphatic and perilymphatic application of salicylate in the pigeon. 2. Fine structure of auditory hair cells. Hear Res **74**: 85-98.

Dohlman GF (1968) Secretion and absorption of the endolymph. In: Third Symposium on the Role of the Vestibular Organs in Space Exploration. SP-152 N. NASA Office of Technology Utilization, Scientific and Techn. Information Division, Pensacola, Florida. pp. 101-123.

Dohlman GF (1977) Experiments on the mechanism of Meniere attacks. J Otolaryngol **6**: 135-156.

Donato R (1991) Perspectives in S-100 protein biology. Cell Calcium **12**: 713-726.

Fermin CD (1993) High resolution and image processing of otoconia matrix. Microsc Res Technique **25**: 297-303.

Fermin C, DeGraw S (1995) Color Thresholding in video imaging. J Anat **186**: 469-481.

Fermin C, Martin D (1995) Expression of S100β in sensory and secretory cells of the vertebrate inner ear. Cel Mol Biol **41**: 213-225.

Fermin CD, Lovett AE, Igarashi M, Dunner K Jr (1990) Immunohistochemistry and cytochemistry of the inner ear gelatinous membrane and statoconia of the chick (*Gallus domesticus*). Acta Anat **138**: 75-83.

Fermin CD, Gerber MA, Torre-Bueno J (1992) Colour thresholding and objective quantification in bioimaging. J Microsc **167**: 85-96.

Fermin C, Lee D, Martin D (1994) Post-embedding TEM signal to noise ratio of S-100. Hear Res **73**: 195-202.

Fikes JD, Render JA, Reed WM, Bursian S, Poppenga RH, Sleight SD (1994) Insensitivity of the chicken embryo to the ototoxicity of aminoglycoside antibiotics and a loop diuretic. Toxicol Pathol **22**: 10-14.

Forge A, Wright A, Davies S (1992) Analysis of structural changes in the stria vascularis following chronic gentamicin treatment. Hear Res **31**: 252-266.

Foster JD, Drescher MJ, Khan KM, Drescher DG (1993), Immunohistochemical localization of S-100 protein in the saccule of the rainbow trout (Salmo-Gairdnerii r). Hear Res **68**: 180-188.

Foster JD, Drescher MJ, Hatfield JS, Drescher DG (1994) Immunohistochemical localization of S-100 protein in auditory and vestibular end organs of the mouse and hamster. Hear Res **74**: 67-76.

Guth P, Fermin C, Pantoja R, Edwards R, Norris C (1994) Hair cells of different shapes and their placement along the frog crista ampullaris. Hear Res **73**: 109-113.

Hayashi K, Hoshida Y, Horie Y, Takahashi K, Taguchi K, Sonobe H, Ohtsuki Y, Akagi T (1991) Immunohistochemical study on the distribution of a and β subunits of S-100 protein in brain tumors. Acta Neuropathol **81**: 657-663.

Igarashi S, Sasaki H, Nkano Y, Ino H (1991) Immunohistochemical examination of S-100 protein and substance P in the inner ear of the rat. Acta Otolaryngol **481**: 163-165.

Ishiyama E, Cutt R, Keels E (1970) Ultrastructure of the *tegmentum vasculosum* and transitional zone. Ann Otol **79**: 998-1009.

Jahnke V, Lundquist P-G, Wersall J (1969) Some morphological aspects of perception in birds. Acta Otolaryngol **67**: 583-601.

Julien N, Loiseau A, Sterkers O, Amiel C, Ferrary E (1994) Antidiuretic hormone restores the endolymphatic longitudinal k^+ gradient in the brattleboro rat cochlea. Pflugers Arch-Eur J Physiol **426**: 446-452.

Kanoh N, Kumoi T, Kobayashi T, Okada T, Seguchi H (1993) Ultracytochemical study of Ouabain-sensitive K^+ dependent p-nitro-phenylphosphate activity in stria vascularis of reserpinized guinea pigs. Acta Otolaryngol **113**: 143-145.

Kerr TP, Ross MD, Ernst SA (1982) Cellular localization of Na^+,K^+-ATPase in the mammalian cochlear duct: Significance for cochlear fluid balance. Am J Otolaryngol **3**: 332-338.

Kikuchi T, Adams JC, Paul DL, Kimura RS (1994) Gap junction systems in the rat vestibular labyrinth: Immunohistochemical and ultrastructural analysis. Acta Oto-Laryngol **114**: 520-528.

Kikuchi T, Kimura RS, Paul DL, Adams JC (1995) Gap junctions in the rat cochlea: Immunohistochemical and ultrastructural analysis. Anat Embryol **191**: 101-118.

Kligman D, Hilt DC (1988) The S-100 protein family. Trends Biol Sci (TIBS) **13**: 437-443.

Kobayashi T, Rokugo M, Takasaka T, Thalmann R (1993) Differential physiologic effects of perfusion of scala tympani versus scala vestibuli in the ischemic cochlea. Acta Oto-Laryngol **113**: 507-511.

Kuijpers W (1994) Na-K-ATPase activity in the cochlea of the rat during development. Acta Otolaryng **78**: 341-344.

Kuijpers W, Houben NMD, Bonting SL (1970) Distribution and properties of ATPase activities in the cochlea of the chicken. Comp Biochem Physiol **36**: 669-676.

Lauriola L, Sentinelli S, Maggiano N, Michetti F, Cocchia D (1986) Glial-like cells in sympathetic neural crest derivatives during human embryogenesis. Detection by S-100 immunohistochemistry. Brain Res **393**: 69-74.

Lavigne-Rebillard M, Bagger-Sjöbäck D (1992) Development of the human stria vascularis. Hear Res **64**: 39-51.

Le Douarin NM, Ziller C, Couly GF (1993) Patterning of neural crest derivatives in the avian embryo - *in vivo* and *in vitro* studies. Dev Biol **159**: 24-49.

Marcus DC, Liu JZ, Wangemann P (1994) Transepithelial voltage and resistance of vestibular dark cell epithelium from the gerbil ampulla. Hear Res **73**: 101-108.

Marshak D (1990) S-100β as a neurotrophic factor. Prog Brain Res **86**: 169-181.

Meza G, Carabez A, Ruiz M (1982) GABA synthesis in isolated vestibulary tissue of chick inner ear. Brain Res **241**: 157-161.

Meza G, Wu J-Y, Lopez I (1992) GABA is an afferent vestibular neurotransmitter in the guinea pig. Immunocytochemical evidence in the utricular maculae. Ann NY Acad Sci **656**: 943-946.

Money KE, Landolt JP, Correia MJ, Lauger J (1974) Anatomical and physiological investigations of the vestibular system of birds. Brain Behav Evol **10**: 212-227.

Murphy M, Bernard O, Reid K, Bartlett PF (1991) Cell lines derived from mouse neural crest are representative of cells at various stages of differentiation. J Neurobiol **22**: 522-35.

Nishiyama S, Okada T, Kabayashi T, Delsaz EG, Seguchi H (1994) Na^+-K^+-ATPase activity in the guinea pig stria vascularis in experimentally-induced endolymphatic hydrops. Histol Histopathol **9**: 205-209.

Park JC, Cohen GM (1982) Vestibular ototoxicity in the chick: Effects of streptomycin on equilibrium and on ampullary dark cells. Am J Otolaryngol **3**: 117-127.

Pauler M, Schuknecht H, White J (1988) Atrophy of the stria vascularis as a cause of sensorineural hearing loss. Laryngoscope **98**: 754-759.

Pitovski D, Drescher M, Kerr T, Dennis D (1993) Aldosterone mediates an increase in [^{3}H] ouabain binding at Na^+,K^+ ATPase sites in the mammalian inner ear. Brain Res **601**: 273.

Rarey K, Peter J, Lohuis B, Wouter-JF T (1991) Response of the stria vascularis to corticosteroids. Laryngoscope **101**: 1081-1084.

Rodriguez-deLores-Arnaiz G (1992) In search of synaptosomal Na^+,K^+-ATPase regulators. Mol Neurobiol **6**: 359-375.

Rybak L, Whitworth C, Weberg A, Vernedra S (1992) Effects of organic acids on the edema of the stria vascularis induced by furosemide. Hear Res **59**: 75-84.

Saidel W, Presson J, Chang J (1990) S-100 immunoreactivity identifies a subset of hair cells in the utricule and saccule of a fish. Hear Res **47**: 139-146.

Salt A, Melichar I, Thalman R (1987) Mechanisms of endocochlear potentials generation by stria vascularis. Laryngoscope **97**: 981-991.

Schneider M, Cotanche D, Fambrough D, Saunders J, Matschinsky F (1987) Immunocytochemical and quantitative studies of Na^+,K^+-ATPase distribution in the developing chick cochlea. Hear Res **31**: 39-54.

Schulte BA, Adams JC (1989) Distribution of immunoreactive Na^+,K^+-ATPase in gerbil cochlea. J Histochem Cytochem **37**: 127-134.

Schulte BA, Schmiedt RA (1992) Lateral wall Na^+,K^+-ATPase and endocochlear potentials decline with age in quiet-reared gerbils. Hear Res **61**: 35-46.

Shehatadieler WE, Richter CP, Dieler R, Klinke R (1994) Effects of endolymphatic and perilymphatic application of salicylate in the pigeon. 1. Single fiber activity and cochlear potentials. Hear Res **74**: 77-84.

Spicer SS, Schulte BA, Adams JC (1990) Immunolocalization of Na^+,K^+-ATPase and carbonic anhydrase in the gerbil's vestibular system **43**: 205-218.

Syka J, Melichar I (1985) The effect of loop diuretics upon summating potentials in the guinea pig. Hear Res **20**: 267-73.

Takasaka T, Smith CA (1971) The structure and innervation of the pigeon's basilar papilla. J Ultrastruct Res **35**: 20-65.

Tencate WJF, Curtis LM, Rarey KE (1994) Na^+-K^+-ATPase alpha and beta subunit isoform distribution in the rat cochlear and vestibular tissues. Hear Res **75**: 151-160.

Tinling SP, Chole RA (1994) Apical cochlear nerve exposed to perilymph in the gerbil and rat. Hear Res **73**: 203-208.

Usami S-I, Igarashi M, Thompson GC (1987) GABA-like immunoreactivity in the chick vestibular end organs. Brain Res **418**: 383-387.

Van Eldik LJ, Christie-Pope B, Bolin LM, Shooter EM, Whetsell WO (1991) Neurotrophic activity of S-100β in cultures of dorsal root Ganglia from embryonic chick and fetal rat. Brain Res **542**: 280-285.

Varon S, Skaper S (1989) The Na^+, K^+ pump may mediate the control of nerve cells by nerve growth factor. In: Oncogenes and Growth Factors. 4th ed. Bradshaw R, Prentis S (ed.). Elsevier, New York. pp: 284-291.

Wangemann P, Shiga N (1994) Cell volume control in vestibular dark cells during and after a hyposmotic challenge. Am J Physiol **266**: C1046-C1060.

Whitworth C, Morris C, Scott V, Rybak LP (1993) Dose-response relationships for furosemide ototoxicity in rat. Hear Res **71**: 202-207.

Yao XF, Tencate WJF, Curtis LM, Rarey KE (1994) Expression of Na^+-K^+-ATPase alpha 1 subunit mRNA in the developing rat cochlea. Hear Res **80**: 31-37.

Yoon TH, Schachern PA, Paparella MM, Le C (1991) Cellular changes in Reissner's membrane in endolymphatic hydrops. Ann Otol Rhinol Laryngol **100**: 288-293.

Yoshihara T, Fermin CD, Igarashi M (1987) Localization of Na^+-K^+-ATPase activity in the *tegmentum vasculosum* of the chick cochlea. Arch Otorhinolaryngol **243**: 401-402.

Yoshihara T, Igarashi M, Fermin C (1990) Ultracytochemical localization of Na^+-K^+-ATPase activity in the *tegmentum vasculosum* of the developing chick cochlea. Acta Otolaryngol **110**: 366-373.

Zenner H-P, Reuter G, Zimmermann U, Gitter A, Fermin C, LePage E (1994) Transitory endolymph leakage induced hearing loss and tinnitus: Depolarization, biphasic shortening and loss of electromotility of outer hair cells. Eur Arch Otorhinolaryngol **251**: 143-153.

Ziller C, Smith J (1982) Migration and differentiation of neural crest cells and their derivatives: *In vivo* and *in vitro* studies on the early development of the avian peripheral nervous system. Reprod Nutr Develop **22**: 153-162.

Discussion with Reviewers

A. Forge: Is the origin of the E-P cells mesenchymal or epithelial?

Authors: We are not sure about the embryological origin of the E-P cells and cannot clarify it with the analyses conducted for this study. However, unlike the basal cells, which can be one-three layers (Forge *et al.*, 1992), the E-P cells were found only as a single layer, by us and those who depicted them earlier such as Takasaka and Smith (1971). The idea is nevertheless intriguing. Furthermore, as our data show now, and other data illustrated in the past (Takasaka and Smith, 1971), the perilymphatic space (scala timpani) under the basilar membrane is also lined by a monolayer. Flattened cells, similar in size to E-P cells, that are not separating the endolymph from the perilymph, do not form a monolayer. It is possible that the arrangement of E-P cells as a monolayer over the light cells of the TV at the scala vestibuli may be significant for the selective passage of molecules through the TV that was so eloquently demonstrated more than 20 years ago (please also see response to Dr. Cohen below) by Dohlman (1968).

J. Park: Could a heavier staining of S100β protein in the light and E-P cells be a developmental phenomenon (albeit late development) or is the same pattern and intensity seen in adult avian ears?

Authors: The data of this paper are based on staining patterns of newly hatched chicks. Figures 1, 2 and 3, from a 13-days-old embryo, were included for comparative purposes only. Other figures are from newly hatched birds. Thus, all comparisons were made between animals of similar age. However, in another paper (Fermin *et al.*, 1995), we showed that expression of S100β on the TV is increased toward the end of embryonic development. The increase is more pronounced after temporary synapses are formed (around stage 42 or 16 days of incubation) and usually remain unchanged for several days after hatching.

J. Park: Although it would be entirely speculative, would the authors care to elaborate (perhaps by a schematic) on the possible interaction of the dual S100 functions in the light and E-P cells with the ATPase activity in the dark cells?

Authors: We hesitate to add more figures to the manuscript, but we would speculate that the short cytoplasmic processes of light cells may serve to activate the sodium pump through the S100β that is present in the processes of the light cells, which touch those of dark cells. The light cells' processes are shorter and thinner than dark cells' processes in which mitochondria are numerous. Other differences of the two cells in the TV are: dark cell cytoplasm is dense, has abundant glycogen and the nucleus is irregular; whereas, the light cell cytoplasm is not dense, has little glycogen, and the nucleus is larger and round with less heterochromatin than the nucleus in dark cells. An association between nerve growth factors (NGF) and the sodium pump has been demonstrated (Varon and Skaper, 1989), and similar association between two adjacent epithelial cells may exist in the inner ear as well. The E-P cells would, under this model, remain as part of a selective barrier that the TV is known to be. In other words, activation of the dark cells' absorptive activity with participation of a calcium binding protein/neurotrophic factor, such as S100β, would not downplay the role of E-P cells as possible selective barrier for passage of electrolytes and other molecules across the monolayer.

Reviewer IV: Do you have any direct functional data to support your speculations about the function of S100 in these cells?

Authors: No functional analyses were preformed in this study. The presence of S-100β in the cells of TV, whose function was already demonstrated, suggests that S-100β may be involved with the absorptive function of the TV already demonstrated decades ago (Dohlman, 1968). We used the **Discussion** to review the already published functions of S-100β and the function of the

TV (Ryals *et al.*, 1995), and assumed that the two may be related. Experiments in progress will demonstrate if this assumption is correct (please see also, Figure 21).

Reviewer IV: The "color thresholding" method is referenced, but it would be useful to know, in this study, what exact parameters, such as, how many cells, etc., were sampled for the data shown in Figure 19?

Authors: Fifty fields of approximately 100 μm^2 from each one of the 10 chicks processed, were measured along the TV of sections similar to those illustrated in Figure 15. The manner in which color thresholding differs from monochrome thresholding has been established and discussed elsewhere (Fermin *et al.*, 1992; Fermin and DeGraw, 1995). Briefly, pixels that contain hue, saturation, and intensity (HSI) for a given color are examined in real time: (**a**) Saturation of pixel colors, and/or (**b**) comparison of pixels (used to determine ratios, but not used in this paper) are calculated without need for using filters. In this paper, however, we just measured the saturation of pixels for color of interest in different cells.

G.M. Cohen: In Figure 4, the basement membrane seems to have separated during fixation. Did the fixation cause this separation? In the living organism, the basement membrane presumably closely laminates itself to the overlying and underlying cells and keeps them in close contact?

Authors: We agree with Dr. Cohen that the fixation used (4% paraformaldehye:1% glutaraldehyde), weaker than standard fixation (3% glutaraldehyde) generally used for TEM, probably did not preserve cells optimally. The fixation and the embedding medium used (Methacrylate plastic LR White) caused extraction of the cytosol and exaggerate intercellular spaces (Fermin *et al.*, 1994) are illustrated in Figures 1-6. Figure 4 shows that there is almost an equidistant space between the basement membrane (BM) and the plasma membrane of the light and E-P cells. It seems that the main portion of the BM is part of the light cells. Figure 6 shows that when mechanical manipulation of the tissue caused the BM to separate (asterisk), the main bulk of it seemed to remain in register with the light cell plasma membrane (lower arrowhead), whereas the places where BM remained attached to the E-P cells (upper arrowhead) were basically denuded. This arrangement suggests that while the BM may be deposited by the light and the E-P cells in cooperation, the light cells may clutch the BM tighter than the E-P cells. While purely speculative at this point, a loose association of the E-P cells with the BM may facilitate their separation during histological processing.

G.M. Cohen: The authors observed that E-P cells cover the blood vessels. How extensive is the coverage within the *tegmentum vasculosum* and outside of it? Could these serve a function of selective permeability as occurs with the blood brain barrier? Is there a typical basal lamina covering the capillaries?

Authors: The monolayer, that the E-P cells form, is very sensitive to fixation, the reason why it was probably ignored before by us and other investigators. We have not conducted scanning electron microscopic analyses to visualize the E-P cells *in situ*, but blood vessels are covered by the monolayer. Thus, we agree with Dr. Cohen that the E-P cells could have a very selective function, which, in some way, may resemble that of the blood brain barrier (BBB). This is certainly an attractive idea that deserves further analysis. It is the potential for such possibilities that stimulated this study in the first place. It is worth remembering that in the true BBB, cerebrospinal fluid (akin to perilymph) is in contact with the basement membrane, whereas in the TV, the E-P cell cytoplasm is. In any event, considering the well demonstrated absorptive function of the choroid plexus in the brain ventricles, and the resemblance of the TV to the choroid plexus, we favor a possible functional correlation between the two, but this assumption requires future analysis.

Additional Reference

Ryals B, Stalford M, Lambert P, Westbrook E (1995) Recovery of noise-induced changes in the dark cells of the quail *tegmentum vasculosum*. Hear Res **83**: 51-61.