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PARAMETERS OF GROWTH IN THE EMBRYONIC AND NEONATAL CHICK BASILAR PAPILLA

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Abstract

The growth of the basilar papilla in the chick cochlear duct was studied utilizing light, scanning, and transmission electron microscopy. The ages of the cochleae investigated ranged from embryonic day 6 to post-hatching day 7. The changes in the length and width of the basilar papilla as well as the establishment of its spatula-like shape were correlated with the maturation of the hair cells' apical surfaces and the changes in the cellular organization of the sensory epithelium. The histological reorganization of the distal hair cell nuclei was concomitant with the broadening of the distal region of the basilar papilla and occurred at a later stage than the reorganization of the proximal hair cell nuclei. Since the stereociliary bundles on all the hair cells are differentiated quite early, it appears that the delayed reorganization of the distal nuclei is associated with anatomical constraints on the cochlear duct, rather than a later differentiation of the distal sensory epithelium. A clear understanding of how growth of the cochlear duct influences both the distribution of hair cells on the basilar papilla's surface and the cellular organization in the sensory epithelium is critical to future studies correlating ultrastructural development with functional maturation of the auditory system.

KEY WORDS: Sensory Organs, Auditory System, Cochlear Duct, Basilar Papilla, Hair Cells, Developmental Biology, Stereocilia, Morphogenesis, Chick, Tonotopic Organization

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Introduction

The relationship between the functional and structural development of the auditory system has generated considerable interest in recent years. This interest has stemmed from the apparent paradox between the basal to apical morphogenesis of the cochlear tissues [16,21] and the low to high frequency development of auditory function [20,27]. In the mature cochlear duct, low frequencies are transduced in the apical region of the duct while high frequencies are transduced in the basal region [2]. It is still unclear how low frequency tones are perceived by immature animals, since the apical region of the cochlea is presumably not yet differentiated when low frequency tones are first processed. There is some evidence that the hair cells in the basal region initially transduce low frequency sounds, but they gradually shift their characteristic frequencies to higher tones as the cochlea matures [18]. As yet, no ultrastructural explanation exists for the shift in tonotopic organization during development, but there is speculation that it is due to either a change in the mechanics of the basilar membrane or a change in the ultrastructural characteristics of the stereocilia [18,25].

The chick basilar papilla provides an advantageous model system for examining these important questions relative to the development of auditory function. The sound spectrum of birds is fairly similar to that of man (compared to other non-mammalian vertebrates) and the uncoiled, easily accessible cochlear duct facilitates evaluation of functional and ultrastructural investigations. Until recently, studies on the morphogenesis of the chick cochlear duct were limited. Now several laboratories are investigating this problem from both a functional and an ultrastructural standpoint. Rubel and co-workers have made several important advances in the evaluation of developing auditory function [17,18,19]. For the past two years, our laboratory has been using scanning (SEM) and transmission (TEM) electron microscopy to study early embryonic structural changes in the chick cochlear duct. We demonstrated that the onset of hair cell differentiation begins in the distal (apical)

region of the chick basilar papilla and moves proximally (basally) with age [6,7]. This pattern parallels the apical-to-basal direction of terminal mitosis which has been seen in the acoustic ganglion cells of chick embryos [8]. Hair cell differentiation occurs at a much earlier embryonic age than has previously been reported for the avian cochlea [3,10] and, together with the findings of D'Amico-Martel [8], bring into question the theory of basal to apical cochlear development in the chick. Tilney and co-workers [24,25,26] have studied the ultrastructural development of embryonic and neonatal chick cochleae. They have demonstrated that the mature number of hair cells (approximately 11,000 per basilar papilla) exists as early as embryonic day 10, two days before the onset of auditory function [20,27]. These new findings on the ultrastructural development of the basilar papilla necessitate a reexamination of morphological and cytological maturation in the avian cochlear duct as these factors have significant implications for the development of auditory function. This paper describes the overall growth in the length and width of the developing chick basilar papilla and correlates this growth with the changes in the cellular organization of the sensory epithelium.

Materials and Methods

Freshly fertilized eggs of the White Rock variety were obtained from Pittsboro Hatchery, Inc., Pittsboro, N.C. and incubated in a laboratory incubator at 37°C and 40% humidity. Cochlear ducts of appropriate ages were obtained by extracting the chick embryos from the eggs and staging them according to the criterion of Hamburger and Hamilton [9]. Inner ears used in this study ranged in age from embryonic day 6 through hatching (embryonic day 21) and up to post-hatching day 7. Only those developmental stages which represent major morphological changes will be described in the Results section, however. Embryos were sacrificed by decapitation while hatchlings were anesthetized with ether before decapitation. The heads were immediately placed in the appropriate primary fixative (see below) and the initial dissections of the cochleae were begun. In the initial dissections, the labyrinth was isolated from the surrounding tissue of the cranial cavity and the superior portion was dissected away to permit access of the fixative to the scala media. The tympanic membrane was removed, the columella extracted and the round window punctured to allow further penetration of the fixative into the scala vestibuli and scala tympani.

Preliminary fixation of cochlear tissues for SEM was carried out in 2.5% glutaraldehyde in 0.1 M Sorensen's buffer at pH 7.4 and 4°C. After 24 hours in the primary fixative, the cochlear duct was extracted from the inferior labyrinth and placed in 2.5% glutaraldehyde in a buffered 0.2 M ethylenediaminetetraacetic acid (EDTA) solution at pH 7.4 [1]. This procedure decalcified the lagenar otolith (preventing it from crumbling and contaminating the distal region of the basilar papilla) and allowed the matrix of the tectorial membrane to be removed more easily and without excessive damage to the stereocilia. After decalcification the cochlear tissues were post-fixed for one hour in 2% osmium tetroxide in 0.1 M Sorensen's buffer at pH 7.4 and 4°C. The tissues were dehydrated up to 70% alcohol, at which point the tegmentum vasculosum was dissected away and the tectorial membrane was removed by squirting a stream of 70% alcohol across the surface of the basilar papilla from a syringe fitted with a 27-gauge needle. From this point the tissues were dehydrated through 100% alcohol and critical point dried in a Balzers CPD10 with CO_2 as a transition fluid. Tissues were mounted on aluminum stubs with double-stick tape and sputtercoated with gold/palladium in a Polaron E5100 sputtercoater. The cochlear tissues were viewed with a JEOL JSM-35 scanning electron microscope at an accelerating voltage of 15 kV.

Primary fixation of tissues for light microscopy (LM) and TEM was carried out in 4% glutaraldehyde in 0.1 M cacodylate buffer at pH 7.4 and 4°C with 4% sucrose added to increase the osmolality (to approx. 600 mosm.). Initial dissections were similar to those described for SEM. After 24 hours the cochlear ducts were dissected out of the inferior labyrinth and placed in a buffered 0.2 M EDTA solution with 4% glutaraldehyde at pH 7.4. When the otolithic portion of the lagena was decalcified the cochlear duct was post-fixed for 1 hour with 2% osmium tetroxide in 0.1 M cacodylate buffer at pH 7.4 and 4°C. The tissues were stained en bloc at room temperature with 2% uranyl acetate in 50% ethanol for 20 minutes and subsequently dehydrated through a graded series of alcohols up to 100%. The tissues were transferred to propylene oxide and embedded in a mixture of Polybed 812 (Polysciences, Inc.) and araldite (modified after Mollenhauer [14]). Thin (60 nm) sections were cut on a Sorvall MT-2B ultramicrotome, placed on uncoated copper grids and stained with 2% uranyl acetate [30] for 20 minutes followed by 0.4% lead citrate [28] for 30 seconds. The sections were viewed with a JEOL 100-B transmission electron microscope at an accelerating voltage of 60 kV.

Sections for light microscopy were cut on a Sorvall JB-4A microtome at a thickness of 2-3 μm. The sections were stained with either toluidine blue or methylene blue-azure II and viewed with a Nikon Biophot light microscope.

Measurements of the length and width of the basilar papilla were made from SEM micrographs of cochleae at numerous developmental stages. Critical point drying in preparation for SEM routinely causes a greater shrinkage of the tissues than that which normally occurs in LM or TEM processing [29]. Accordingly, the measurements obtained in this study will not be equivalent to those obtained in other LM or TEM morphometric studies of the avian cochlea. The difference in hydration between embryonic and neonatal tissues probably results in a variable amount of shrinkage across developmental stages. However, we feel that the variability in shrinkage of the dense otocyst epithelium is insignificant relative to the general overall growth patterns we have described.

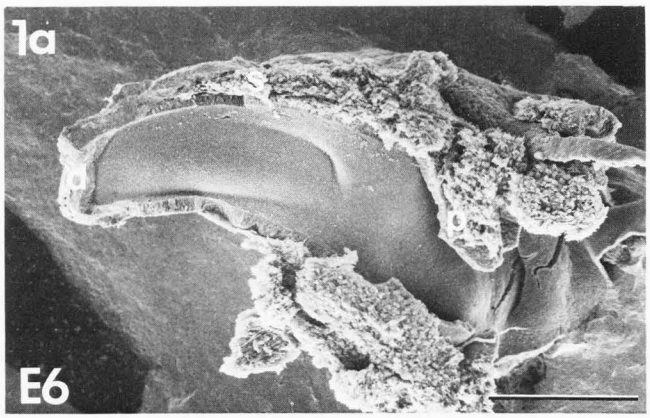
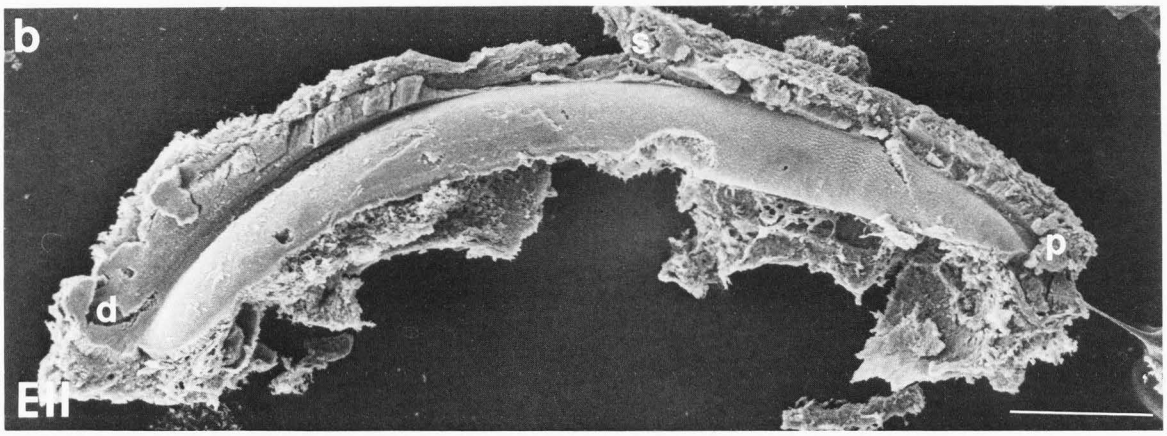
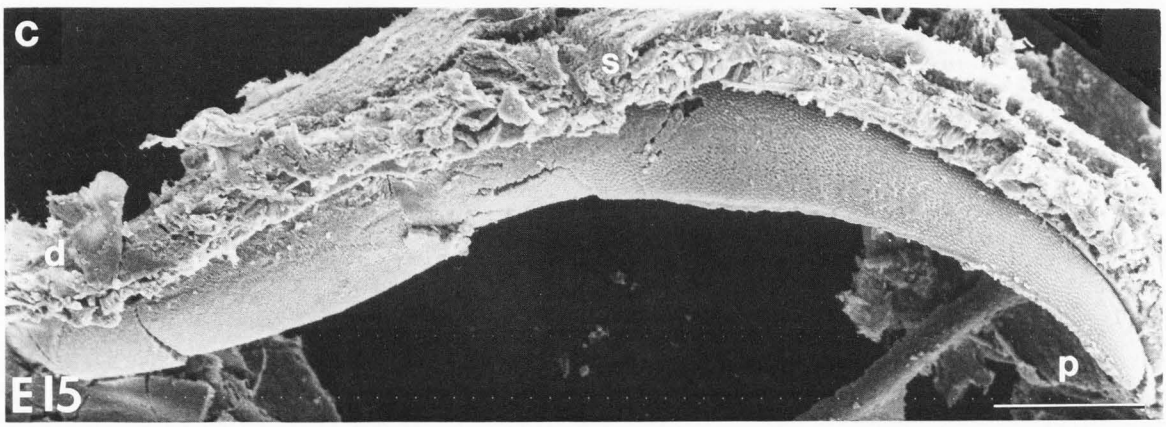
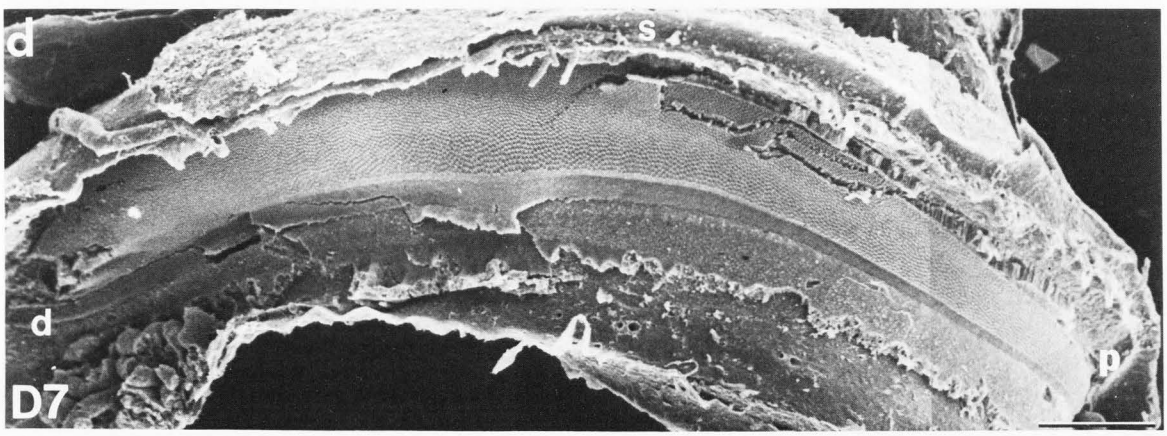


Figure 1 Scanning electron micrographs of the apical surface of the basilar papilla on a)embryonic day 6; b)embryonic day 11; c)embryonic day 15; d)post-hatching day 7. d= distal tip; p= proximal tip; s= superior edge of the basilar papilla. Bar= 200 μm.







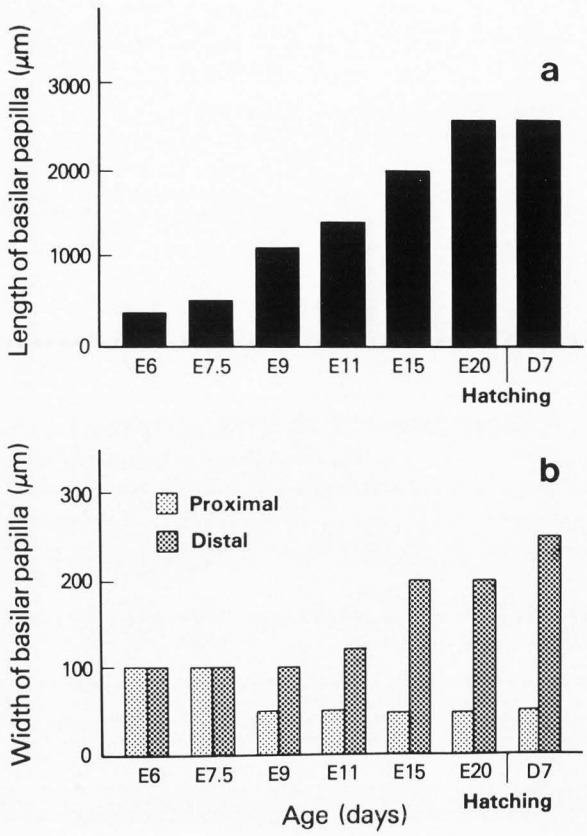


Figure 2 A graphic representation of the growth in the length and width of the basilar papilla. a)changes in the length of the basilar papilla with time; b)changes in the width of the proximal(P) and distal(D) regions of the basilar papilla with time.

Terminology used in describing the avian cochlear duct is adapted from Takasaka and Smith [22].

Results

Growth of the Basilar Papilla

At embryonic day 6 the basilar papilla has just begun to differentiate from the epithelium on the medial wall of the otocyst. It is a flattened, rectangular bed of cells which is approximately 350 μm long and 100 μm wide (Fig. 1a). Beginning at this stage and continuing up through post-hatching day 7 the basilar papilla undergoes a ten-fold increase in its length while the width in the distal region increases roughly two and one-half times. In the proximal region, the width decreases to a mature dimension of approximately 50 μm. The growth in the length and width of the basilar papilla from embryonic day 6 to post-hatching day 7 is summarized in Figure 2.

By embryonic day 11 the basilar papilla has reached a length of approximately 1400 μm. The width has increased to roughly 120 μm throughout all but the very proximal extent of the epithelium. In the most proximal region the width tapers bluntly down to approximately 50 μm. At this stage the basilar papilla is beginning to develop a lateral curvature, with the edge resting on the superior fibrocartilaginous plate being convex (Fig. 1b). The surface of the basilar papilla has acquired a more rounded contour than it had on embryonic day 6.

On embryonic day 15, the basilar papilla clearly exhibits the spatula-shaped outline which is characteristic of the adult structure (Fig. 1c). Its length has increased to approximately 2000 μm and the widened distal region has expanded to roughly 200 μm across. The proximal region tapers down gradually to a width of 50 μm. The basilar papilla at this stage has a pronounced lateral curvature, with its superior edge being convex. In addition, the papilla has acquired a second, longitudinal curvature in which the apical surface of the epithelium is convexly arched. These curvatures have been thought to represent a phylogenetic step toward the coiling seen in the mammalian cochlea [23]. However, this does not appear to be a correct interpretation since the convex curvature of the superior (neural) edge of the basilar papilla is opposite to the concave curvature of the modiolar (neural) edge of the mammalian organ of Corti.

The basilar papilla on post-hatching day 7 is over 2600 μm long and is approximately 250 μm wide in the distal region (Fig. 1d). The narrow, tapered proximal region has a width of roughly 50 μm. The combination of the lateral and longitudinal curvatures in the basilar papilla results in a twisting of the epithelium so that the apical surface of the proximal region lies in a plane oriented at approximately 90° to the apical surface of the distal region.

Changes in the Apical Surface of the Hair Cells

The hair cells undergo a significant increase in their surface areas as the cochlear duct matures (Fig. 3). This growth, however, is not uniform along the length of the basilar papilla. As early as embryonic day 9 the proximal hair cells have a larger surface area than the hair cells in the distal region (Fig. 3a,c). The larger surface area of the proximal hair cells is maintained throughout development. They eventually have a surface area which is up to 4 times larger [24] than that of the distal hair cells (Fig. 3b,d). However, there is a larger number of hair cells across the width of the distal region (40-50) than across the proximal region (6-10) which accounts for the characteristic spatula shape of the mature basilar papilla. During the early stages of development, when the basilar papilla has a uniform width, the smaller but more numerous distal hair cells are packed more densely on the surface of the epithelium than are the larger proximal hair cells (Fig. 4).

While the surface areas of the maturing hair cells increase, the surfaces of the adjacent supporting cells are gradually diminished so that in the mature bird the supporting cells form thin, microvillus-covered collars around each hair cell (Fig. 3b,d).

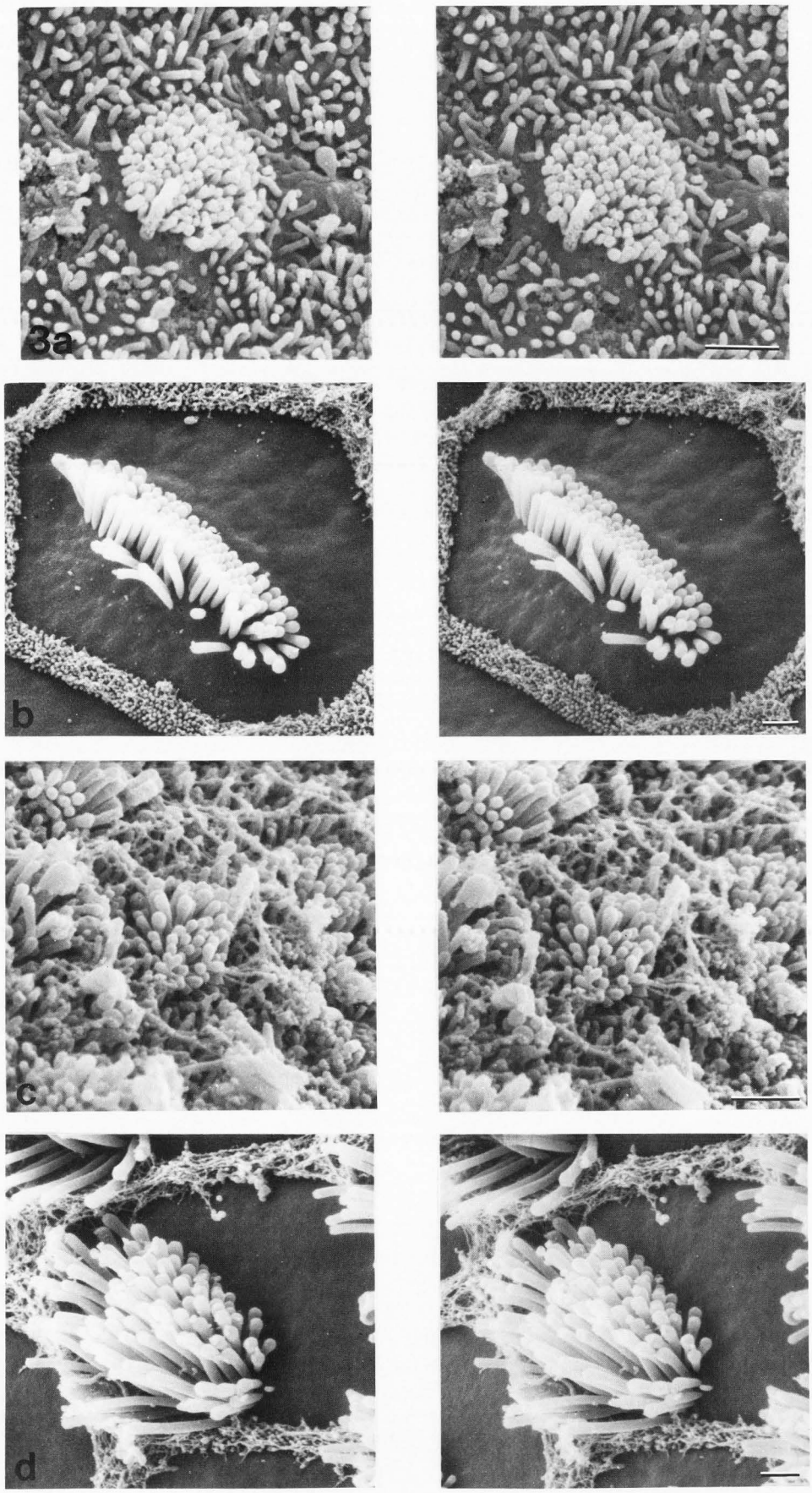
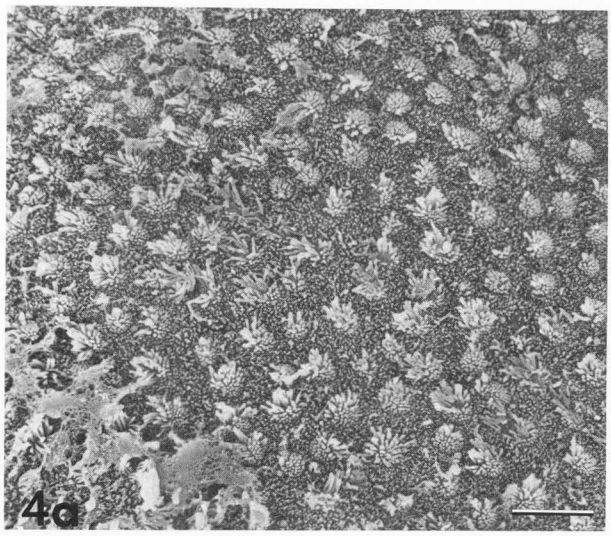


Figure 3
Scanning electron stereomicrographs of the apical surfaces of hair cells in the basilar papilla.
a)proximal hair cell, embryonic day 9;
b)proximal hair cell, post-hatching day 7;
c)distal hair cell, embryonic day 9;
d)distal hair cell, post-hatching day 7.
Bar= 1 μm.



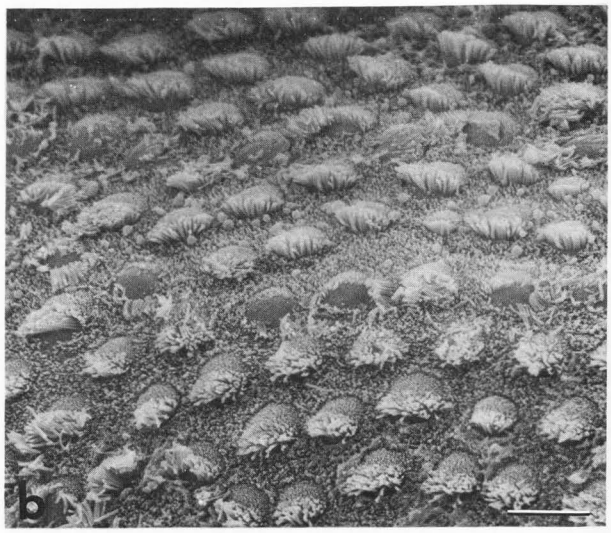
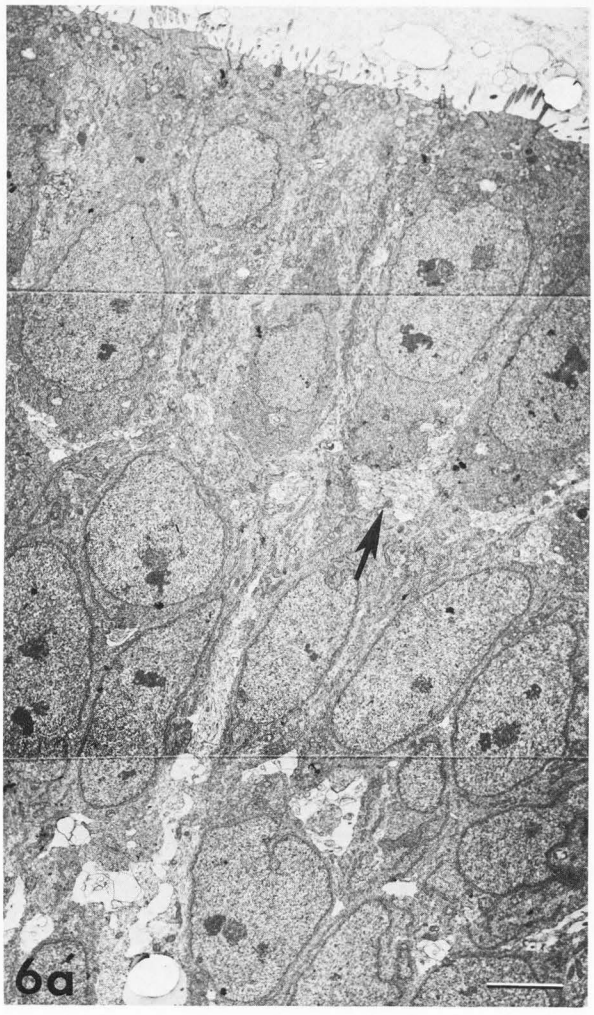


Figure 4 Scanning electron micrographs of the distal(a) and proximal(b) surfaces of the basilar papilla. The distal hair cells have smaller surface areas and are more closely packed on the surface than are the proximal hair cells. Bar= 5 μm.

(Fig. 5 on the facing page).



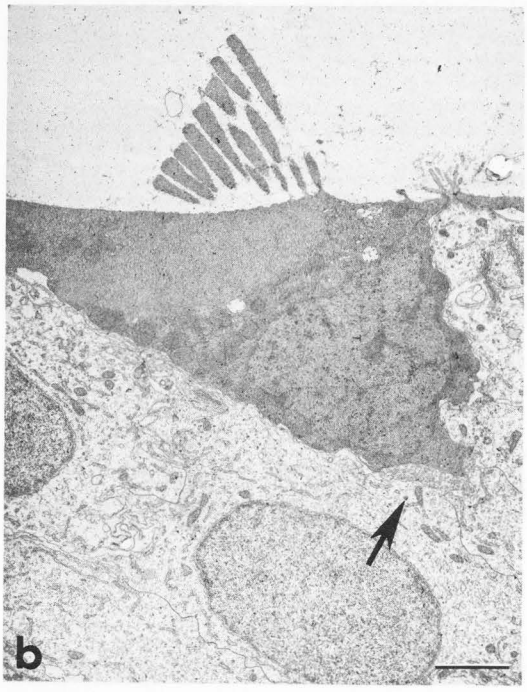


Figure 6 Transmission electron micrographs of the apical portion of the basilar papilla epithelium. a)proximal region, embryonic day 9; b)proximal region, post-hatching day 7. On embryonic day 9 the hair cells are organized in a single layer in the apical portion of the epithelium and are separated from the underlying supporting cells by the terminal processes of the cochlear nerve fibers (arrows). By post-hatching day 7 the cytoplasm of the hair cells stains darkly and individual hair cells are separated by the apical processes of the supporting cells. The base of the hair cell is in contact with an efferent nerve terminal (arrow). Bar in a)= 2 μm; Bar in b)= 1 μm.

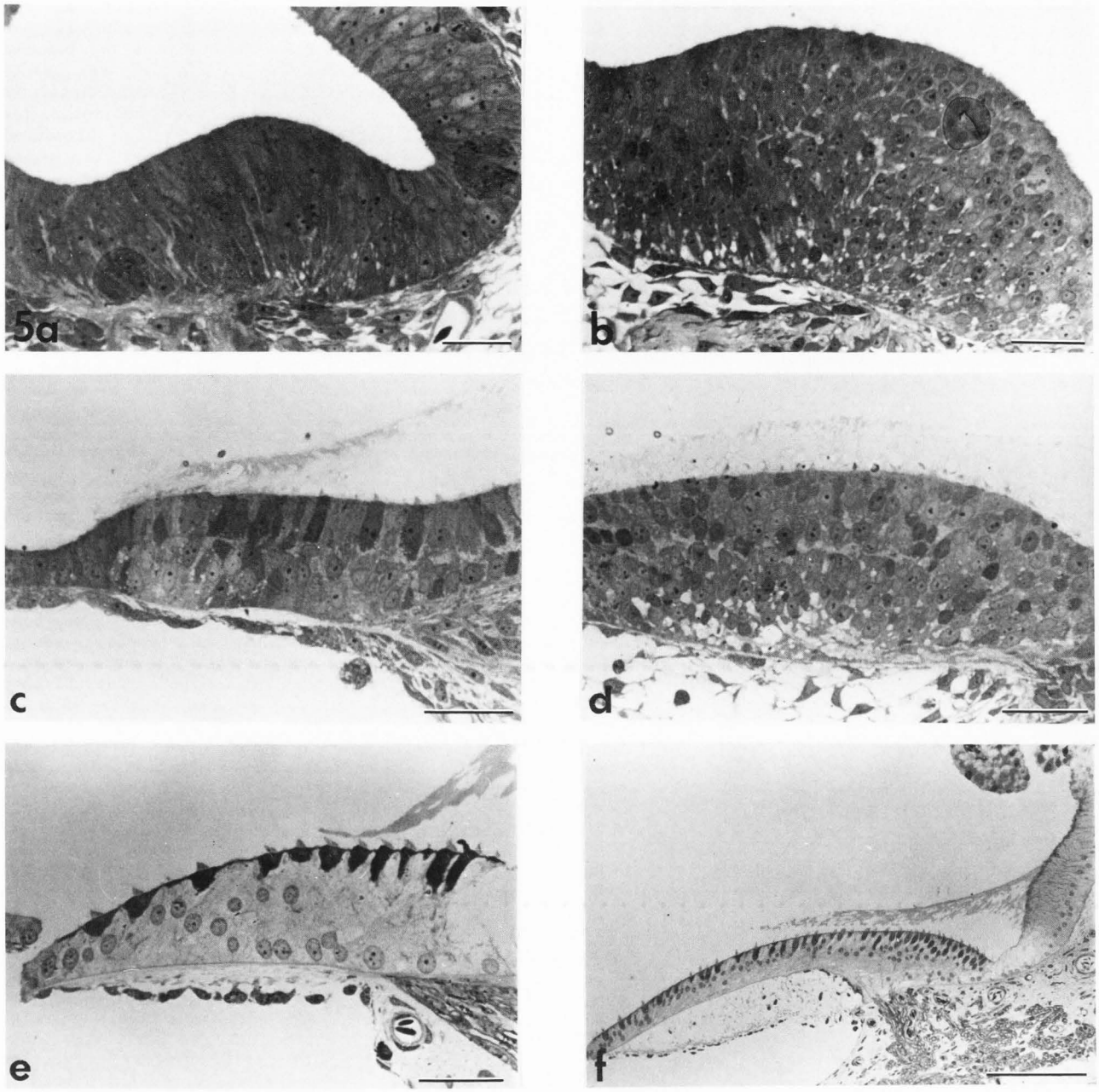


Figure 5 Light micrographs of cross-sections through the proximal and distal regions of the basilar papilla at three developmental stages. a)proximal region, embryonic day 7.5; b)distal region, embryonic day 7.5; c)proximal region, embryonic day 11; d)distal region, embryonic day 11; e)proximal region, post-hatching day 7; f)distal region, post-hatching day 7. As early as embryonic day 7.5 there are many more nuclei in the distal region than there are proximally. The organization of the distal hair cells into a single, apically-placed layer is coincidental with the increased width of the distal region. Bar in a-e= 25 μm; Bar in f= 100 μm.

Changes in the Organization of the Basilar Papilla Epithelium

The sensory epithelium of the basilar papilla is composed of hair cells and supporting cells, both of which are derived from the homogeneous epithelium in the medial wall of the otocyst. At early stages (such as embryonic day 7.5, Fig. 5a,b), the basilar papilla is organized as a pseudostratified epithelium. All of the cells rest on the epithelium's basal lamina and extend up to its apical surface. The cell nuclei occupy a majority of the tissue and are located at all levels. No clear distinction is seen between the nuclei of the hair cells and those of the supporting cells. The number of

nuclei in a cross-section of the tissue increases regularly along the length of the basilar papilla, with many more nuclei present at distal than at proximal locations (Fig. 5a,b).

By embryonic day 11, the epithelium of the proximal basilar papilla is clearly stratified, with the nuclei of the hair cells occupying a single layer in the apical portion (Fig.5c). The somata of the hair cells no longer consist primarily of nuclei. Instead, there are predominant regions of supra- and infra- nuclear cytoplasm. The bases of the hair cells have lost their association with the basal lamina and now lie in the middle region of the tissue, where they are contacted by the terminal processes of the afferent cochlear nerve fibers (Fig. 6a). Below this synaptic region, the nuclei of the supporting cells are organized in two or three layers. The bases of the supporting cells rest on the basal lamina of the epithelium while the apical portions project up between the hair cells to reach the apical surface of the basilar papilla.

In the distal region of the embryonic day 11 basilar papilla the hair cells are segregated in the apical portion of the epithelium, separated from the more basally located supporting cell nuclei by a layer of ingrowing cochlear nerve cell processes (Fig. 5d). The abundant nuclei of the distal hair cells are organized in three or four layers which lie just below the apical surface of the epithelium. There is very little cytoplasmic space between the numerous hair cell nuclei and the supra- and infra- nuclear cytoplasm of the individual hair cells is not apparent.

The hair cells in both the proximal and distal regions of the post-hatching day 7 basilar papilla are organized in a single layer in the apical portion of the epithelium (Fig. 5e,f). The hair cells stain very darkly and are separated from one another by the apical extensions of the supporting cells (Fig. 6b). The proximal basilar papilla is narrow and contains only about 10 hair cells in cross-section (Fig. 5e). The distal region is broad and arched and has over 40 hair cells across its width (Fig. 5f).

Discussion

Experimental analysis of functional development in the chick cochlear duct must take into consideration the dynamics of growth in the basilar papilla. The increase in the length and width of the basilar papilla is dependent upon changes in the size of the hair cells it contains. The basilar papilla has been shown to have the same number of hair cells on embryonic day 10 as it does on post-hatching day 10 [26]. Accordingly, we can surmise that the growth results primarily from an expansion of the surface areas of a proliferatively static population of hair cells. This is in contrast to some other hair cell systems, such as those in the fish and the frog, where growth of the sensory epithelium is associated with a continuous increase in the number of hair cells [4,5,13,15]

The expansion of the hair cells' surface areas in the chick basilar papilla is not uniform, however. The embryonic basilar papilla is not a "miniature" version of the adult structure which simply grows uniformly until it reaches the proper size. Up to embryonic day 11, it is rectangular and lacks the broadened distal end and the tapered proximal tip which are characteristic of the adult. Yet the hair cells are distributed on the surface of the epithelium just as they are in the adult, with 40-50 across the distal region and only 6-10 across the proximal region. This distribution is achieved by having small surface areas on the distal hair cells and by packing them very tightly on the surface of the epithelium (see Figs. 3 and 4). The number of hair cells in the distal region is so large and their packing on the surface is so dense that the nuclei of these cells are distributed in layers in the apical portion of the epithelium (see Fig. 5d).

In the proximal region of the embryonic day 11 basilar papilla, the number of hair cells is greatly reduced, compared to the distal region, and the individual hair cells are sparsely distributed across the papilla's surface. Consequently, the proximal hair cells have large surface areas and their nuclei are organized in a single layer in the apical portion of the epithelium (see Figs. 4 and 5a). The bony labyrinth, which surrounds the cochlear duct, expands laterally and dorsally as the chick's skull increases in size (our unpublished observations). This allows the cochlear duct to expand its width and length and, by embryonic day 15, the basilar papilla has achieved its mature, spatula-like shape. At this stage, the distal hair cell surfaces are still tightly packed, but their nuclei are now organized in a single layer just below the apical surface of the epithelium.

The delayed reorganization of the distal region of the basilar papilla deceptively suggests a proximal to distal wave of hair cell differentiation. However, examination of the apical surfaces of the hair cells with SEM demonstrates that the stereocilia are well differentiated as early in development as embryonic day 7.5 and that the full population of hair cells is present at least two days before the onset of auditory function [6,7,20,26,27]. Since the stereociliary bundles are the actual transducers of mechanical stimuli [11,12], their presence throughout the entire basilar papilla at the onset of hearing implies a functional capacity. This function may be far from mature but at the very least the distal hair cells cannot be considered as "not yet differentiated". The delay in definitive receptor organization in the cells of the distal region may be related to the anatomical confines of the developing temporal bone around the cochlear duct. The electrophysiological function of the distal hair cells may be equivalent to that of the proximal cells, regardless of their constricted environment.

The non-uniform expansion of the basilar papilla's apical surface and the early establishment of a stable population of hair

cells present a problem in evaluating the development of tonotopic organization in the chick cochlear duct. As the basilar papilla increases in length and widens in the distal region, the position of a single hair cell presumably changes relative to the proximal and distal ends of the sensory epithelium. This would cause an apparent shift in the tonotopic organization of the basilar papilla because the location of that cell's characteristic frequency would move in relation to the overall length of the basilar papilla. However, the hair cell itself would not have shifted its position relative to the other hair cells surrounding it. Tilney and Saunders [24] provided a solution to the problem of locating individual hair cells when they outlined the precise gradient in the length, width and number of stereocilia on the hair cells along the basilar papilla. Thus, it is possible to identify particular hair cells by the characteristics of their stereocilia and to continually monitor their position during studies of hearing development.

Our present knowledge of how individual hair cells and their stereocilia develop in combination with our information relative to their positional changes during growth provides us with structural information which will enable us to investigate the mechanisms responsible for development of auditory function in greater detail.

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Discussion with Reviewers

E.R.Lewis: You note that hair cell proliferation in the chick cochlea seems to stop by embryonic day 10. Prior to that time, does hair cell proliferation take place more-or-less uniformly over the sensory surface, or is it concentrated at the edges of the surface (as reported in text references 4,5 and 13)?.

Authors: Hair cell proliferation on embryonic days 6-9 moves in a distal-to-proximal pattern along the length of the basilar papilla. At these early stages the proliferation covers the whole width of the epithelium (see text reference 5).

E.R.Lewis: Do you find anything analogous to the "immature" hair cell types reported by Lewis and Li [13] from the growing edges of bullfrog inner ear sensory surfaces?

Authors: Yes. In the later stages of development there are a few immature hair cells along the inferior edge and distal tip of the basilar papilla. They are not, however, seen in the mature cochlea.

M.Anniko: Is it possible to visualize a gradient in surface maturation on individual hair cells?

Authors: Yes, the stereocilia exhibit a graded development, with those closest to the kinocilium growing faster than those furthest from the kinocilium. Also, there is evidence that some of the shortest stereocilia are lost during the growth and rearrangement of the stereociliary bundle (see text reference 26).

M.Anniko: In several species an apical-to-basal gradient in cytodifferentiation of individual hair cells is observed. Is there any indication of such a feature also in the chick?

J.C.Saunders: Stereocilia maturation may present a distorted view of developmental events in the whole receptor cell. Indeed, stereocilia could be mature and even functional, but if cell depolarization does not lead to transmitter release there will be no neuronal transmission. Is there any evidence that the apical and basal poles of the cell mature at the same time?

Authors: The stereocilia on the apical cell surface differentiate before it is possible to discriminate between the basal regions of the hair cells and the adjacent supporting cells. It is important to understand that this is the opposite of earlier reports of avian hair cell development which assumed that the hair cells differentiated from the synapse upward (text references 3,10).

T.R.Van De Water: Are you suggesting that the place of best frequency is not changing, but the relationship of landmarks to it are, due to overall growth of the basilar papilla, and that the place is maintained throughout development if one localizes on individual hair cell areas?

Authors: Yes, since the growth of individual cell surfaces and consequently, the growth of the basilar papilla is not uniform this possibility must be considered.

T.R.Van De Water: In the mouse, acoustic ganglion cells leave the proliferative cell cycle in a base-to-apex sequence, whereas in chick embryos this pattern is reversed, with acoustic ganglion cells withdrawing from the cell cycle in an apex-to-base sequence. In lieu of your own observations on patterns of sensory cell maturation in the chick, do you attribute any significance to this reversal of acoustic ganglion cell birth date pattern?

Authors: The hair cells in the mouse Organ of Corti leave the proliferative cell cycle in an apex-to-base sequence [31] which is similar to the ultrastructural sequence that I see in the development of chick hair cells. Unfortunately, no one has yet studied terminal mitosis in the chick basilar papilla or the early development of stereocilia in the mouse Organ of Corti, so I can't make a direct comparison. The distal

acoustic ganglion cells in the chick are derived from the otic placode (text reference 8) and this may explain why they follow the same pattern as the hair cells.

J.C.Saunders: You show the length of the papilla to be the same between E20 and Day 7. Is length fully developed at this time?
Authors: Tilney et al (text reference 26) show that while the length is roughly equivalent on E20 and Day 7 (2.6 mm), the basilar papilla reaches a mature length of 3.2 mm by Day 30.

J.C.Saunders: How do you calculate length from SEM photos in older specimens when the proximal tip curvature becomes pronounced?
Authors: The length of all but the curved proximal end is calculated from one micrograph and then the specimen is tilted so that the proximal region can be measured. A specific landmark is used to coordinate the two micrographs and a total length is obtained.

M.Anniko: Do you have any evidence that the future hair cells pass their terminal mitosis more close to the lumen than e.g. supporting cells?
Authors: We have no information at all on terminal mitosis in the chick basilar papilla.

M.Anniko: Is there any indication, as observed with SEM, that the stiffness of individual sensory hairs changes during cytodifferentiation and maturation of hair cells?
Authors: No, not that we have seen.

J.C.Saunders: What do you think the functional advantage is of packing hair cells so densely at the distal end of the papilla? Threshold sensitivity or frequency resolution (tuning curves) do not get better in the chick below 1.0-1.5 kHz.
Authors: We have no idea. However, the organization of the low frequency region resembles that of more phylogenetically primitive ears which function primarily in the low frequency range. Even in mammals the apical, low frequency region of the cochlea often expands to 4 or 5 rows of outer hair cells.

Additional Reference

31. Ruben RJ (1967) Development of the inner ear of the mouse: a radioautographic study of terminal mitoses. Acta Otol Suppl 220, 1-44.