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Ultrastructural observations on regenerating hair cells in the chick basilar papilla *

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This experiment was designed to investigate cellular and subcellular maturational changes in regenerated immature sensory cells and support cells of the chick basilar papilla following gentamycin treatment. Scanning and transmission electron microscopy were used. The experimental animals received one subcutaneous injection of gentamycin sulfate daily (50 mg/kg) for five or 10 days. The animals receiving five days of injection were sacrificed the following day. The remaining animals were allowed to survive either seven or 28 days before sacrifice and preparation for electron microscopy. The initial lesion consisted of total degeneration of hair cells within 500 μm of the proximal tip providing the opportunity to study a 'pure' population of regenerating sensory cells. Sensory cell regeneration could be identified by one day after terminating gentamycin treatment. Early in development sensory cell precursors were morphologically very similar to supporting cells. A density gradient, based on cytoplasmic staining characteristics, was established which increased from cells displaying low density at the base of the supporting cell layer to high density cells at the luminal surface. These changes in density were equated to increases in number of and types of cytoplasmic organelles. In contrast to the empty appearing cytoplasm of the support cell, the cytoplasm of the hair cell precursor contained numerous mitochondria, clusters of ribosomes, and vesicles. As the cell approached the surface, mitochondria became more numerous as did smooth and coarse endoplasmic reticulum and Golgi apparatus. This gradient suggested that determination of the cellular phenotype occurred at the level of the basal membrane followed by migration to the surface, during which time differentiation was characterized by an increase in number and complexity of cellular organelles.

Luminal surface modifications occurred as soon as the cell erupted. The development of stereocilia, rootlet, cuticular plate and cellular polarization followed the normal embryogenetic pattern. At 28 days, stereocilia organization was still incomplete as was the orientation of the bundle. To the extent that proper orientation of hair cells or bundles is necessary for normal transduction, mature function at 28 days would not be anticipated.

Innervation of the presumptive hair cell precursors could be observed one day after treatment, early in the course of hair cell differentiation. Synaptogenesis followed the normal embryogenetic sequence; however, afferent and efferent nerve terminals remained immature appearing at 28 days. This observation may have physiological implications manifested by delay of hearing recovery in these animals.

Stereocilia; Nerve terminals; Gentamycin; Ototoxicity

Introduction

Post embryonic hair cell loss in birds or mammals secondary to acoustic overexposure or ototoxic drugs has generally been thought to be irreversible. However, regeneration of hair cells and supporting cells has been conclusively demonstrated in neonatal chicks (Corwin and Cotanche, 1988) and mature quail (Ryals and Rubel, 1988) following acoustic trauma. Recovery of hair cell numbers has also been observed in the chick basilar papilla following aminoglycoside treatment (Cruz et al. 1987). This observation is not without clinical correlation in the human population. Recovery of hearing following aminoglycoside treatment has been described by a number of authors (Fee, 1980; Moffat and Ramsden, 1977). No histopathological observations, however, are avail-

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able and the mechanism by which this recovery occurs remains undetermined.

The concept of hair cell recovery after ototoxic drug exposure or acoustic over-stimulation has understandably become the subject of much scientific excitement and industry. Not only does the avian cochlea have the capability of regenerating hair cells following injury, but functional hearing recovery follows histological repair (Tucci and Rubel, 1989, 1990). Of note, however, there is a delay of some weeks duration between hearing restoration and the appearance of newly regenerated hair cells. This would suggest that the newly regenerated hair cells are physiologically immature or inappropriately innervated. Since previous investigations have recorded hair cell regeneration at the light microscope level initial examination of the developing hair cells at the subcellular level may help explain the functional delay.

This investigation was undertaken to help answer the question posed by the apparent disparity between hair cell repopulation and functional recovery in the avian cochlea following gentamycin treatment. We theorize that examination of the sensory cell precursor during maturation at a subcellular level will identify morphological deficiencies which may help explain the physiological inactivity. Furthermore, we believe that scrutiny of the immature unerupted sensory and support cells shortly after regeneration is induced will help to identify series of events underlying regeneration and differentiation of a functioning receptor epithelium. In this report we describe observations on the ultrastructure of regenerating hair cells during the first few weeks after ototoxic aminoglycoside treatment. The observations we describe are the most obvious and have been seen in many preparations. More subtle, quantitative changes, comparing the regenerating cells to normal hair cells will be considered in later publications.

Methods

Twenty newly hatched chicks were divided into three groups of six animals each and a control group of two animals *. One group of six animals was injected with gentamycin sulfate 50 mg/kg daily for five days. The animals were sacrificed one day after the injection period. This group was included in order to observe the early ultrastructural events associated with regenerating hair cells. The remaining animals received gentamycin injections for a total of 10 days. Following the injection period two groups of six animals were allowed to survive seven and 28 days before sacrifice. The control animals received no injections. At the end of the survival period the animals were given an overdose of pentobarbital sodium and each cochlea was perfused via the round window with 2.5% gluteraldehyde in 0.1 molar phosphate buffered saline. The temporal bones were removed and the basilar papilla exposed using standard microdissection techniques. The specimens were then placed in 1% osmium tetroxide for one hour followed by another rinse in buffered saline. The right temporal bones were processed for scanning electron microscopy and the left temporal bones were processed for transmission electron microscopy.

The specimens processed for scanning electron microscopy were dehydrated in ethanol followed by final dissection, including removal of the tectorial membrane. Tissue was then dehydrated through graded ethanol series and critical point dried. Specimens were mounted on aluminum stubs and coated with pure gold prior to examination with the JEOL 35C electron microscope (15 kv accelerated voltage). Specimens prepared for transmission electron microscopy were dehydrated in alcohol series and embedded in Epon resin. After hardening the specimens were reoriented and remounted for sectioning along the transverse axis of the basilar papilla. Thick sections were harvested at 50 μm intervals from the proximal tip. At each interval thin sections were prepared. The grids were then stained with alcoholic uranyl acetate and lead citrate before examination with the JEOL-100B transmission electron microscope.

This fixation and dehydration process proved suitable for preservation of all cellular components with minimal distortion except for cell and nuclear membranes. Alternative fixation techniques may be necessary should more precise imaging of these structures be desired.

* Animals were selected at random from a study population made available by D.L. Tucci and E.W Rubel (1989).

Results

Supporting cells

The supporting cell morphology appears to remain constant throughout the period of hair cell maturation. The cells extend from the basilar membrane to the surface of the basilar papilla. The nuclei are found in the basal parts of the cell along the basilar membrane (Fig. 1A). The cell shape varies considerably, however, in general they assume a tubular configuration with irregular cell margins.

The cytoplasm of the supporting cells is very pale and the cells appear empty except for scattered organelles. Clumps of fibrogranular material are scattered throughout the cytoplasm. In some areas this material is clustered around the Golgi apparatus which is the most common cellular organelle (Fig. 1B).

Sensory cells

Examination of the proximal (basal) portion of the basilar papillae at low magnification one day following five days of aminoglycoside treatment suggests that hair cell degeneration was complete from the basal tip for the initial 500 μm (Fig. 2). The apical surfaces of damaged hair cells are occasionally seen. The relatively complete destruction of hair cells at the basal end provides the fortuitous opportunity to study a population of regenerating hair cells in the absence of surviving mature hair cells. Thus, all of the observations noted below are on this portion of the basilar papilla.

Within this region the surface of the basilar papilla is characterized by an irregular mosaic pattern defined by apical surfaces of supporting cells. At higher magnification the apical surfaces of a few newly regenerated sensory cells can be identified by the sensory hair tufts which are longer and larger in diameter than the surrounding microvilli (Fig. 2 insert). Unerupted sensory cell precursors can be identified below the luminal surface of the basilar papilla where they are surrounded by supporting cells (Fig. 3A).

At this stage as well as later stages sensory cell precursors were considered to be unerupted if the cell body and nucleus were contained within the supporting cell matrix well below the surface of the reticular lamina. The presence of cellular extensions to the surface outside the plane of sectioning was also considered, and cannot be entirely discounted. However, we never saw remote, thin, cellular processes leading to the luminal surface from these cells and in several cases the cell boundaries were established by serial section examination. Thus, we believe that cells which we have classified as 'unerupted' do not have extensions leading to the luminal surface.

The primitive sensory cells are irregular and elongated in shape and are distinguished from the supporting cells by a more granular and dense appearing cytoplasm (Fig. 3B). In contrast to the supporting cells, the hair cell cytoplasm contains mitochrondria in greater numbers, clusters of ribosomes, microtubules, and vesicles. The nucleus is located in the center of the cell and is characterized by dispersed chromatin and inapparent nucleoli. These observations, at the electron microscope level, are entirely consistent with observations by Girod et al. (1989) based on light microscopy staining properties following acoustic trauma.

At seven days post treatment fully erupted and unerupted cells are present along the proximal portion of the basilar papilla. As the apical surface of the unerupted cell approaches the surface of the basilar papilla, the nucleus assumes a relatively more basal position, nuclear chromatin becomes more dense and the nucleoli more prominent (Fig. 4A). Cytoplasmic contents assume a more granular appearance and the cells stain more densely in proximity to the surface.

The cytoplasm contains numerous clusters of smooth and coarse endoplasmic reticulum, Golgi apparatus, and mitochrondria (Fig. 4B). Vesicles, both smooth and coated, are common throughout the cytoplasm as are multivesicular bodies. Erupted sensory cells are identified by the densely staining cytoplasm and unique globular shape. The cells are short, ranging in height from 8 to 10 μm. By comparison the more immature cells tend to be less globular and more cylindrical in shape. This suggests that during the maturation process the cells tend to shorten and round up.

At 28 days regenerated hair cells have repopulated the proximal one-third surface of the basilar papillae. Various stages of maturation based on

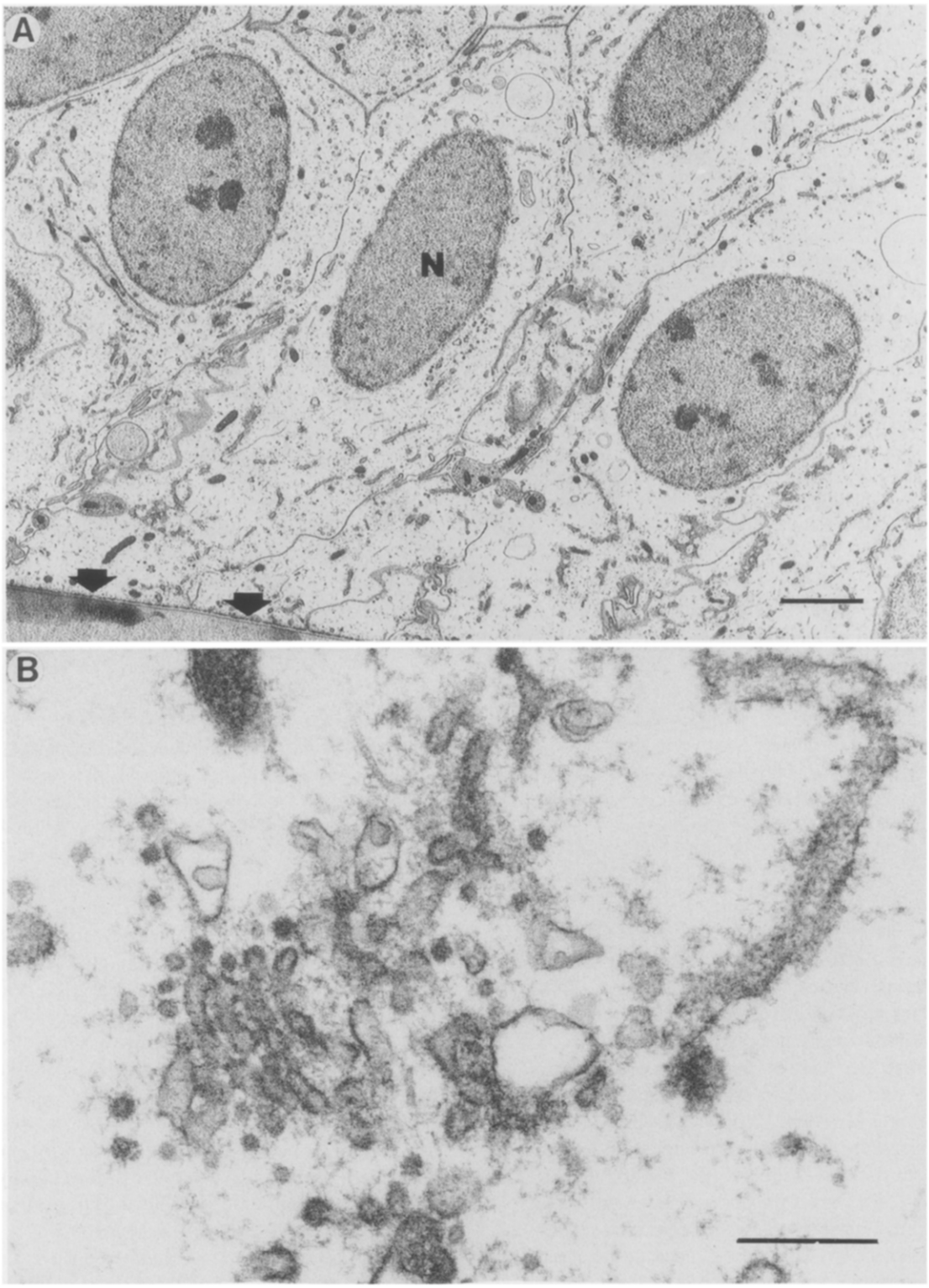


Fig. 1. (A) Supporting cells in the basilar part of the basilar papilla adjacent to the basilar membrane (arrows) N = nucleus. Bar 2 μm. (B) Golgi apparatus within supporting cells cytoplasm. Bar 0.5 μm.

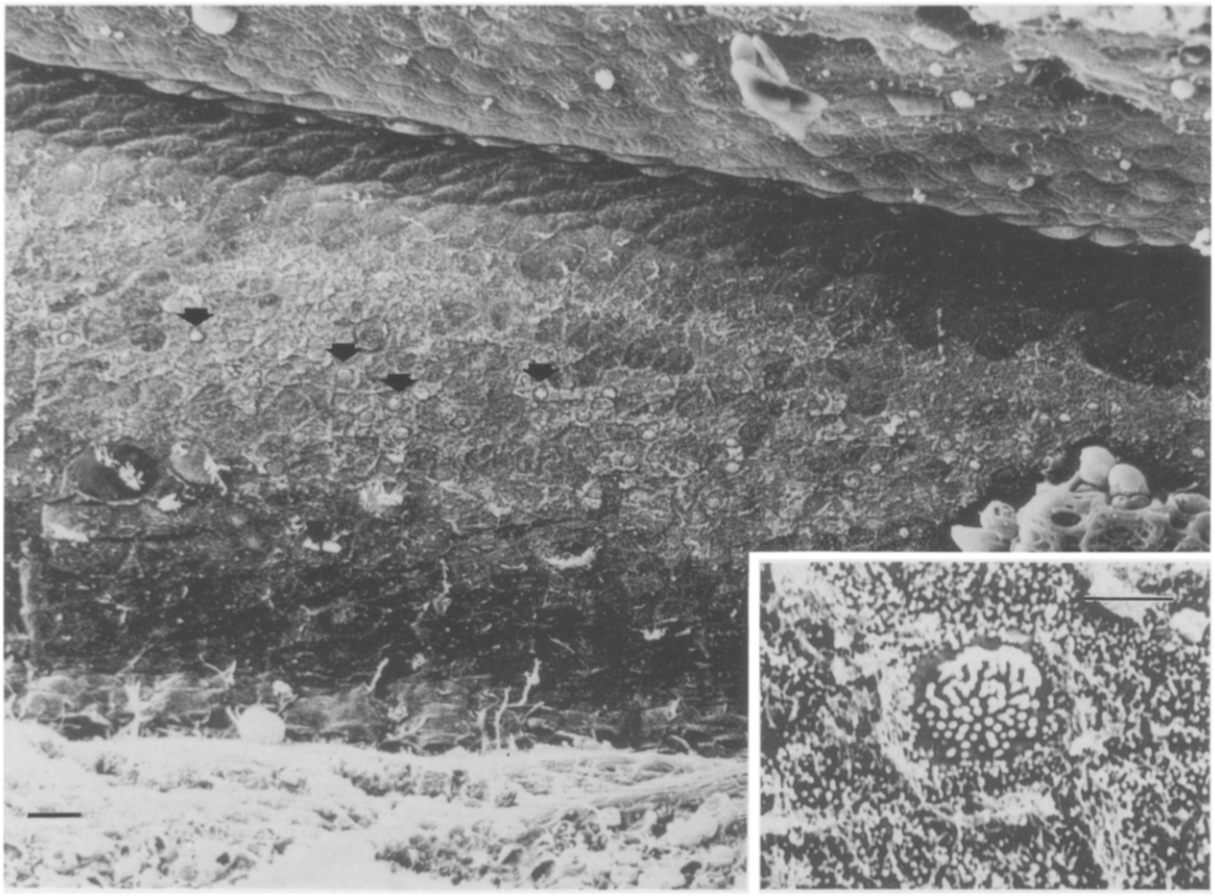


Fig. 2. Scanning electron photomicrograph of proximal (basal) portion of the basilar papilla one day following five days of aminoglycoside treatment, approximately 400 microns from the tip. Newly erupted hair cells are identified by stereocilia tufts (arrows). Bar 10 μm. (Insert). Higher magnification scanning electron photomicrograph of newly regenerated hair cell identified by sensory hair tuft. Bar 2 μm.

the size and development of the apical surface, are represented (Fig. 5). Apical surface diameters range from 3–10 μm. Occasional 'giant' hair cells with apical surface diameters in excess of 15 μm are randomly distributed across the proximal one-third of the reticular surface. Both erupted and unerupted sensory cells, observed with the transmission electron microscope, are frequently found in pairs or couples (Fig. 6). Sometimes, the cells are in contact with each other, although no junctional complexes have been identified. The cells are round with a basally located nucleus. The hair cells contain more densely packed organelles consisting of endoplasmic reticulum with and without attached ribosomes, free clusters of ribosomes, vesicles and Golgi apparatus (Fig. 7A).

Mitochondria are numerous and assume a more basal position within the cytoplasm adjacent to the nucleus. Osmophilic dense bodies may be found scattered throughout the apical cytoplasm. These dense bodies contain an amorphous osmophilic material which is either granular or organized into myelin figures (Fig. 7B). The likelihood that these bodies represent degenerating mitochondria will be discussed later.

Stereocilia

When the stereocilia are examined with the

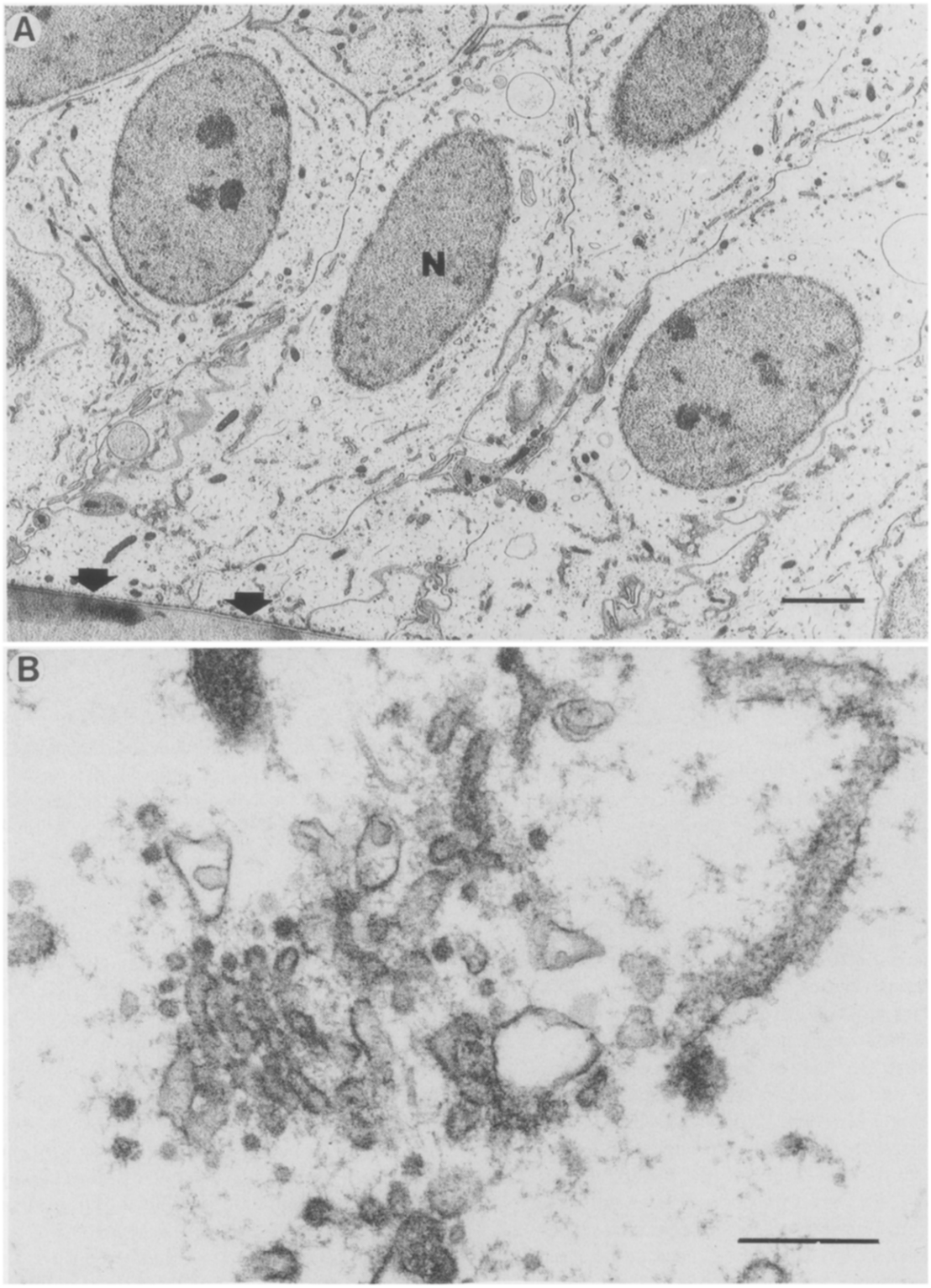


Fig. 3. (A) Transmission electron micrograph showing immature sensory cells (open arrows) below the luminal surface (solid arrows) of the basilar papilla where they are surrounded by supporting cells. The primitive hair cells are distinguished from the supporting cells by a granular and darker staining cytoplasm. B = Basilar membrane. Bar 10 μm. (B) Higher magnification transmission electron photomicrograph of primitive sensory cells. Note association between nerve fibers (arrows) and basal ends of the cells. N = nucleus. Bar 5 μm.

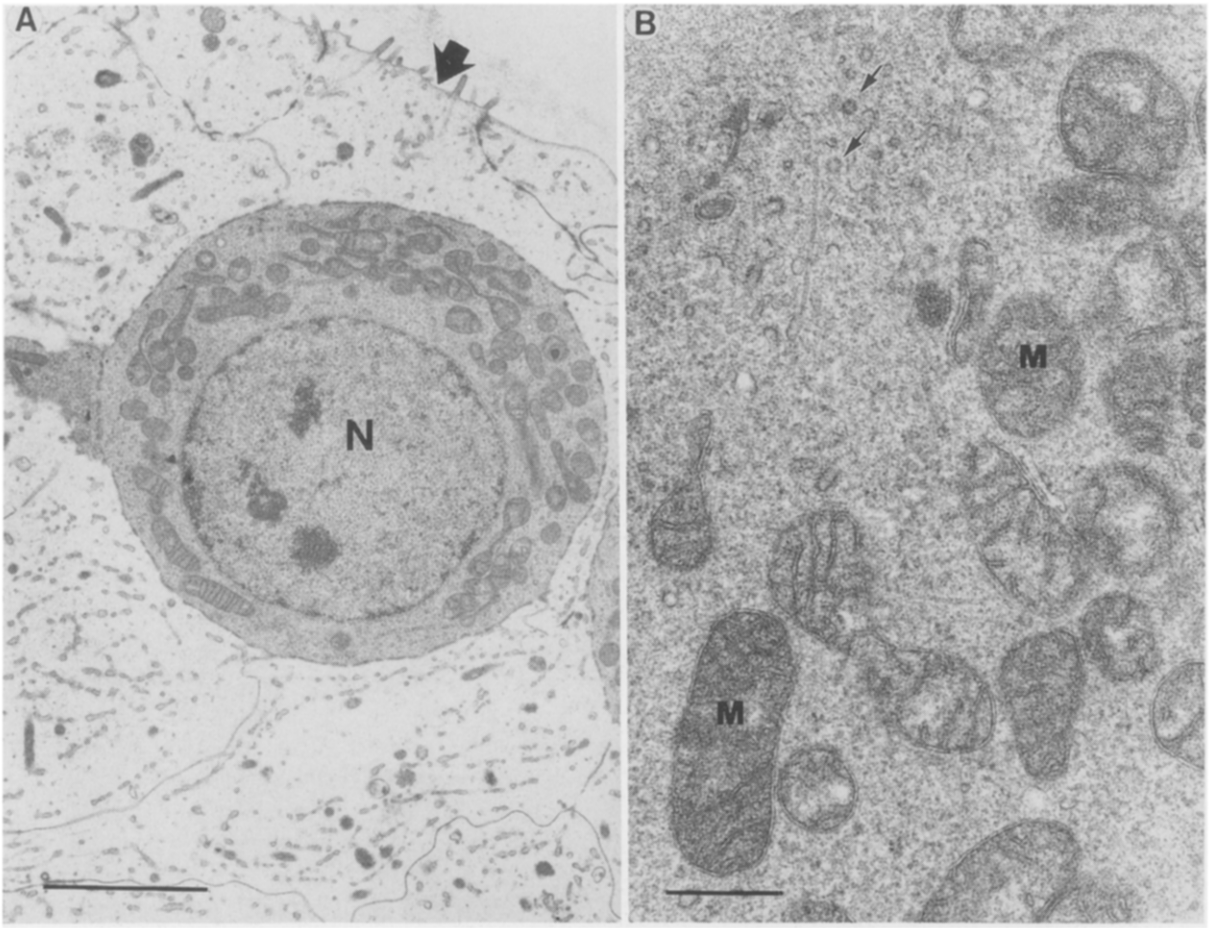


Fig. 4. (A) Unerupted regenerated sensory cell below the luminal surface of the basilar papilla (arrow). The nuclear chromatin has become more dense and the mitochrondria are found in greater numbers in the apical portion of the cell. N = nucleus. Bar 5 μm. (B) The cytoplasm of a primitive sensory cell seven days post gentamycin treatment contains a variety of organelles including vesicles (arrow), clusters of ribosomes, Golgi apparatus and mitochrondria (M). Bar 0.5 μm.

scanning electron microscope one day after termination of drug treatment they are differentiated from the surrounding microvilli on a basis of their increased width and length (Fig. 8A). The diameter of the apical surface of the newly erupted sensory cell is small, averaging only 2–3 μm. The stereocilia are arranged concentrically on the surface (Fig. 8B). No kinocilia were observed and no cellular polarity can be established based on the stereocilia organization. In the most immature sensory cells it is frequently impossible to differentiate microvilli from developing stereocilia with the transmission electron microscope. In rare cases a stereocilia bud, identified by its larger diameter, is observed to erupt from the luminal surface of the newly erupted hair cell (Fig. 8C). In this instance no well developed cuticular plate can be identified and the subsurface cytoplasm is characterized by dense granularity.

At seven days, differentiation of the apical surface has progressed, facilitating hair cell identification with the scanning electron microscope. Stereocilia are much taller and thicker than surrounding microvilli. No kinocilium are seen. The stereocilia vary in length and are loosely packed, from short to tall, into a staircase shaped bundle

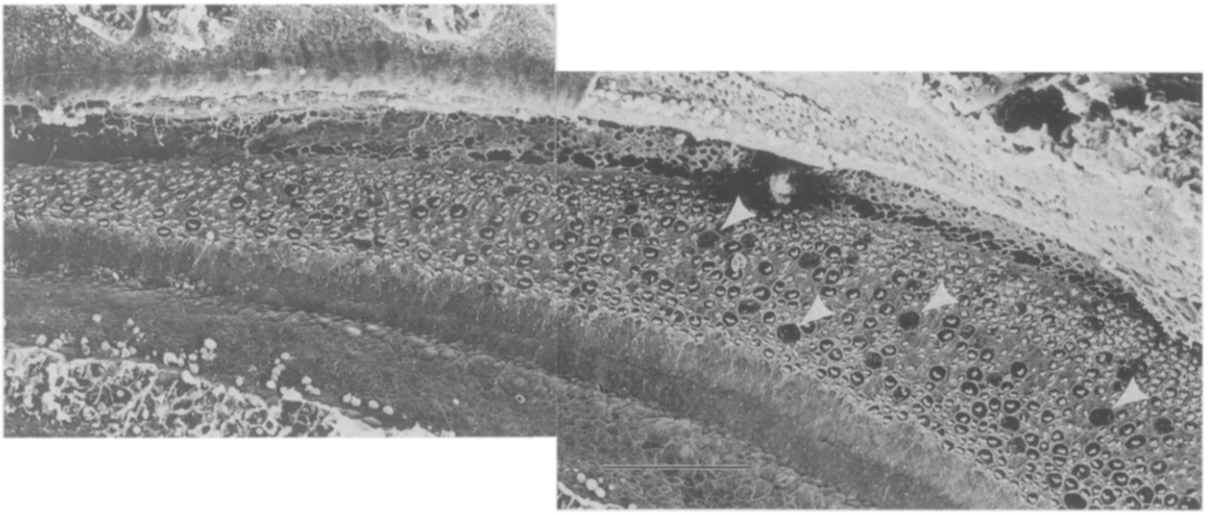


Fig. 5. Scanning electron photomicrograph of the proximal one-third of the reticular surface at 28 days following gentamycin treatment. Numerous 'giant' hair cells (arrows) are randomly distributed across the surface. Bar 100 μm.

(Fig. 9A). The cellular polarity can be identified based on the orientation of the staircase in the absence of kinocilia. It is not uncommon that the apical surface will be covered both by the stereocilia bundle and by microvilli (Fig. 9B), although on the more mature cells the microvilli are absent.

Cuticular plate development parallels the development of the stereocilia. Early in development the cuticular plate is poorly organized and is identified by a matrix of microfilaments into which the stereocilia are embedded. In some cases the early cuticular plate extends down into the cytoplasm to assume a more comma-like shape. A longitudinal section through the stereocilia itself reveals its filamentous organization. In the more immature cells there is no constriction at the base of the stereocilia at its insertion into the cuticular plate (Fig. 9C). The cuticular region and stereocilia are frequently centrally located along the luminal surface of the cell.

At 28 days the stereocilia organization has matured in a larger percentage of the sensory cells. The percentage of apical surface of each cell occupied by the stereocilia bundle decreases as the cells mature and the circumference of the apical end of the cell increases. As in the seven day animals, the stereocilia bundle is centrally located on the apical surface in contrast to the normal sensory cells where the hair bundles are placed off center in the cuticular plate. Also, in more immature cells, the stereocilia are loosely packed. In the larger more mature cells (Fig. 10A) packing is tighter and the stereocilia are arranged hexagonally as in the normal condition (Fig. 10B). The shape of the bundle is also changed from circular to more rectangular. Measurement of the number of stereocilia was difficult where packing was imprecise. An approximation was obtained by counting the stereocilia of four adjacent more mature appearing proximally located hair cells. The bundles contained between 150 and 170 stereocilia as viewed from above with the scanning electron microscope.

Cell orientation can be determined on the basis of each hair bundle axis and a line drawn perpendicular to it. In the normal adult chicken the hair cells are oriented so that the tallest row of stereocilia faces away from the superior edge of the basilar papilla (Tilney et al., 1987). In contrast, in our material hair cell orientation across the proximal epithelium at 28 days is not nearly as well organized as in normal birds. Microvilli are commonly observed on the apical surface in the 28 day survivors although the numbers are reduced in the more mature cells. The cuticular plate has grown more basally and the stereocilia are constricted at their insertion. The microfilamentous core of the stereocilia are well ordered and rootlets

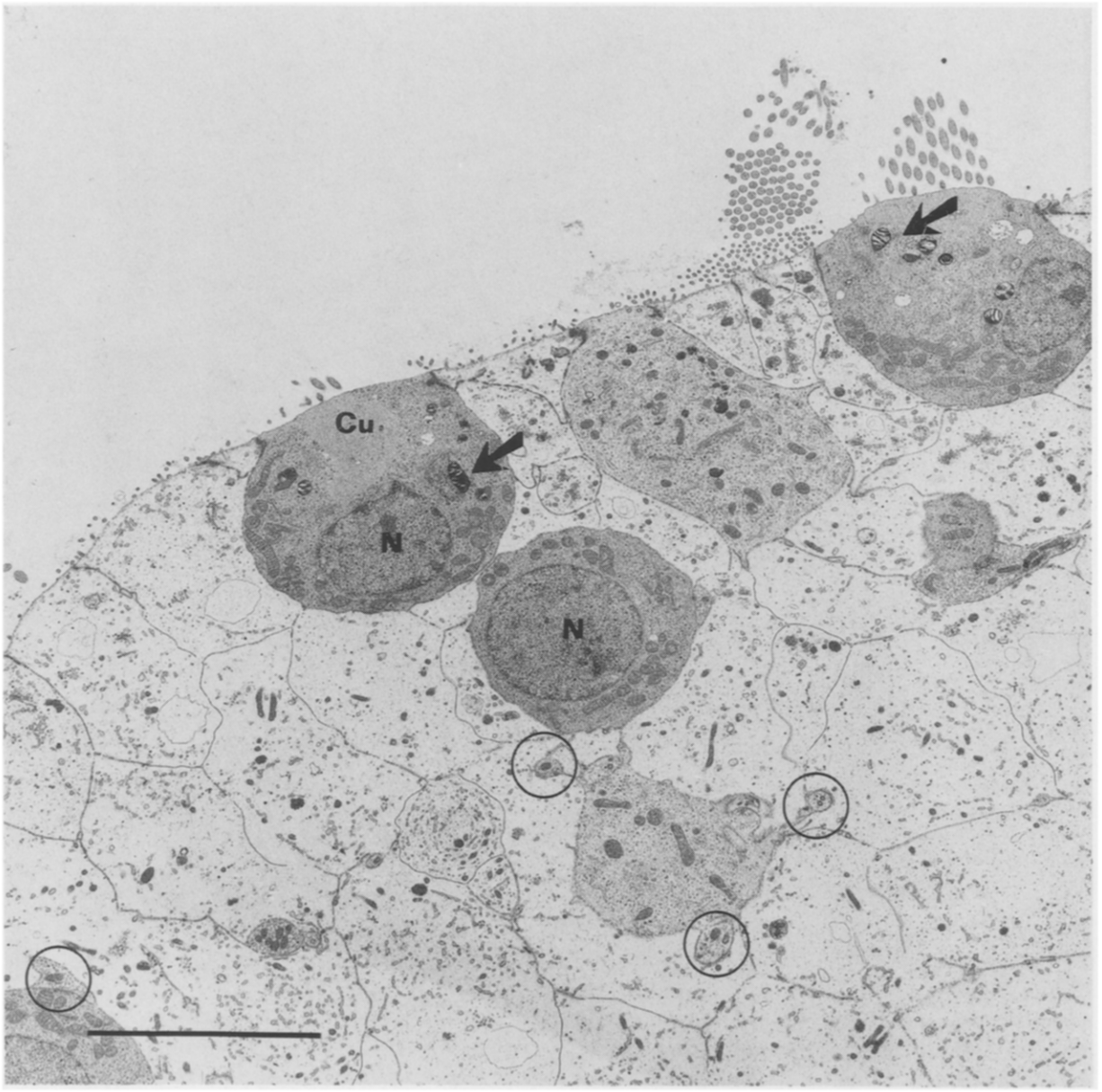


Fig. 6. Transmission electron photomicrograph showing erupted and unerupted regenerated hair cells 28 days after gentamycin treatment. The cells at the surface are rotund with a basally located nucleus (N). Cu, cuticular plate; arrows, myelin figures. Nerve fibers are circled. Bar 10 μm.

extend into the cuticular plate at their insertion (Fig. 10C). In contrast, the cuticular surface of the 'giant' hair cells may be completely devoid of surface specializations except for microvilli or small clumps of damaged stereocilia (Fig. 10D).

Nerve terminals

In the normal animal afferent terminals on the short hair cell may be small and rounded or cup shaped. The endings may make deep invaginations into the cell (Fig. 11). They contain vesicles, tub-

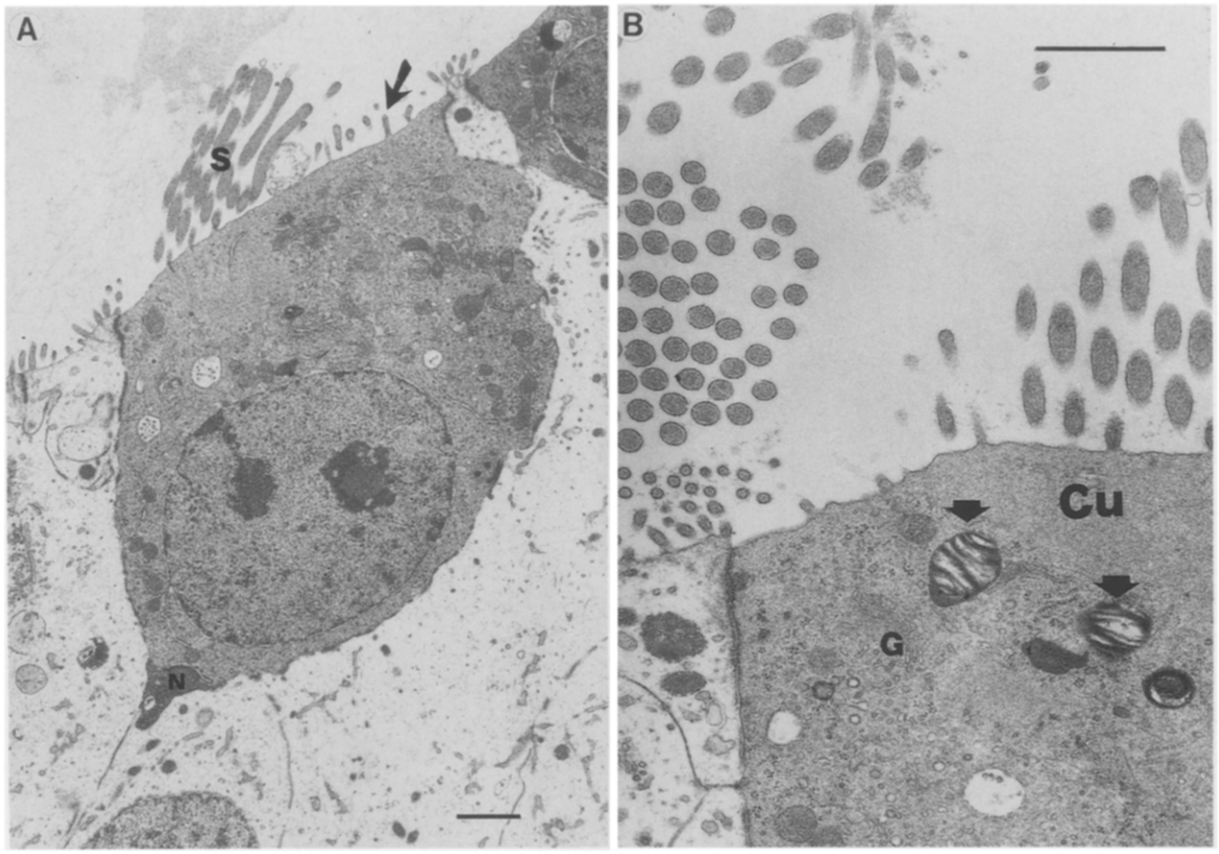


Fig. 7. (A) Regenerated hair cell 28 days after termination of gentamycin treatment. Note the presence of both stereocilia (S) and microvilli on apical cell surface (arrows). The nucleus is basally located while the numerous cellular organelles are apically located. N = nerve terminal. Bar 1 μm. (B) Higher magnification transmission electron photomicrograph of apical portion of newly regenerated hair cell revealing myelin figures (arrow) in apical portion of cell. An early cuticular plate (Cu) is present as are both stereocilia and microvilli. G = Golgi. Bar 1 μm.

ules and mitochrondia as well as synaptic specialization. Synaptic balls 0.2–0.3 μm in diameter surrounded by a single row of vesicles are found adjacent to presynaptic membrane thickenings.

The short hair cell is also provided with vesiculated efferent terminals which may be very large and cup-like. The endings are packed with round vesicles and many mitochrondria. The endings are opposed by sub-synaptic cisternae inside the hair cells.

Afferent terminals

Nerve fibers are found in close association with the basal portions of hair cell precursors by one day after drug injection. The nerve endings are generally small boutons (Fig. 12A). Membrane specializations are restricted to small circumscribed areas where the fiber and the sensory cell membranes are in apposition. The synaptic areas are characterized by thickening of the neural membrane which is opposed by a synaptic ball structure within the hair cell. A granular synaptic membrane is found between the synaptic ball and the cell membrane. The ball is surrounded by a row of vesicles (Fig. 12B). The cytoplasm of the nerve fiber is sparse and contains few organelles, including mitochondria.

At seven days both unerupted and erupted sensory cells can be associated with afferent nerve terminals (Fig. 12C). Synaptic balls are a common feature in addition to the other membrane specializations observed at one day. Twenty eight days

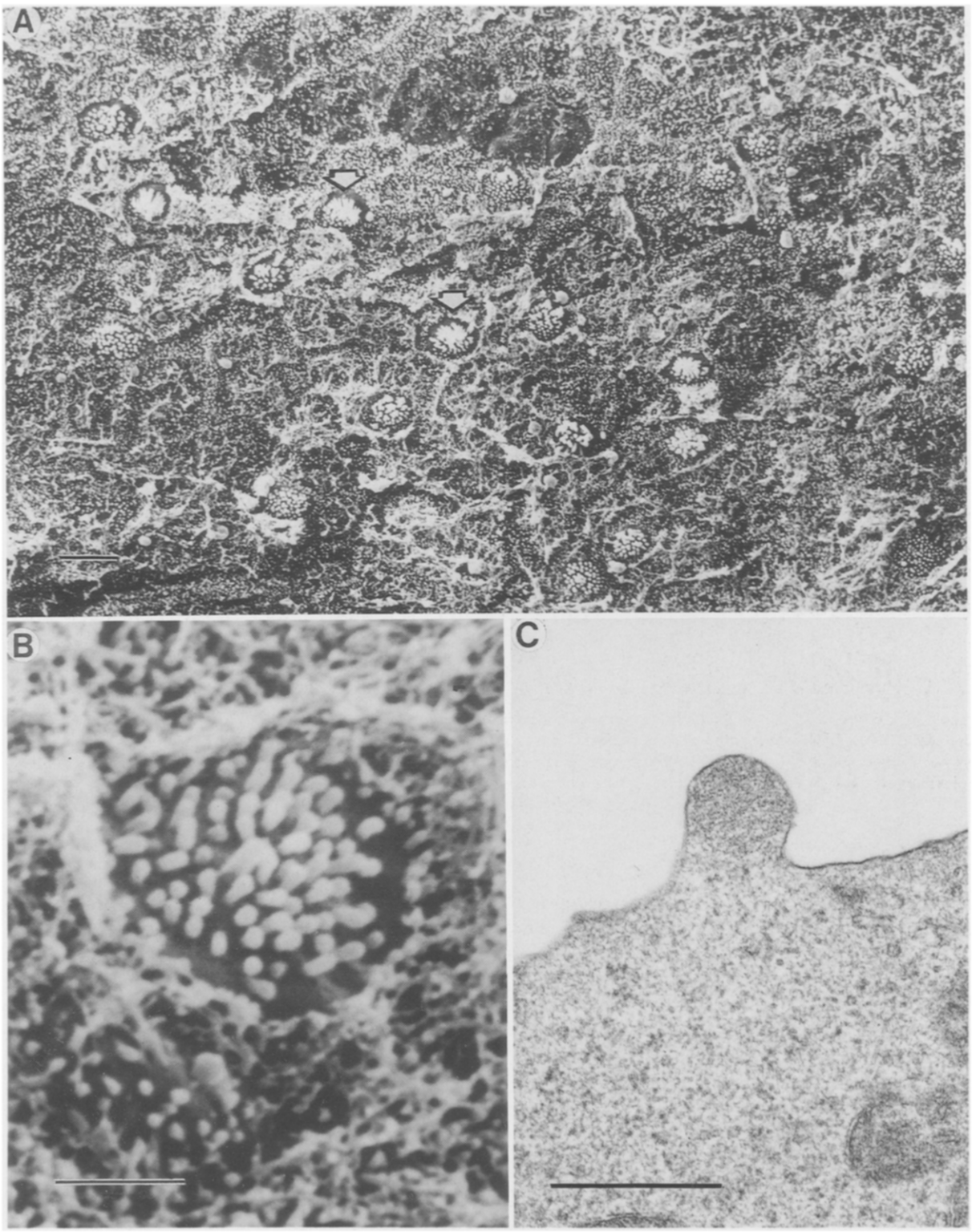
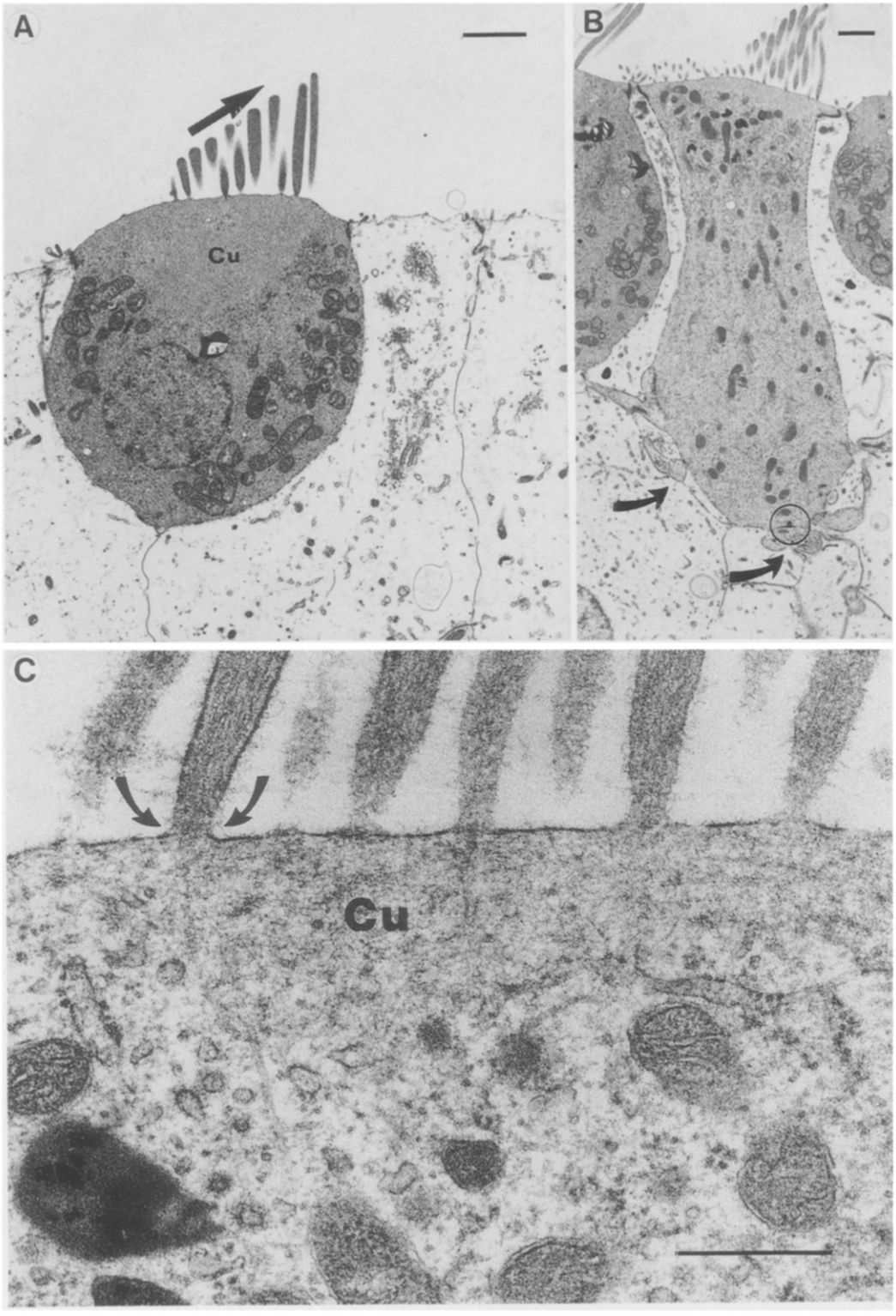


Fig. 8. (A) Scanning electron photomicrograph of reticular surface one day following termination of gentamycin treatment. Early stereocilia are differentiated from the surrounding microvilli on the basis of the increased width and length. Arrows, stereocilia tufts. Bar 2 μm. (B) Higher magnification scanning electron micrograph showing stereocilia tuft. Bar 1 μm. (C) Stereocilia bud erupting from the luminal surface of the newly erupted hair cell. No clearly developed cuticular plate is identified. Bar 0.5 μm.


A
Cu
B
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after termination of treatment afferent terminals are observed on the sensory hair cells in increased numbers. Four to five terminals may oppose the cell base. In general, the endings are small boutons and cup-like endings are seen less often. The synaptic balls are commonly observed.

Vesiculated terminals

Small vesiculated bouton-shaped nerve terminals are seen as early as seven days on unerupted and erupted immature hair cell precursors. The terminals contain multiple small round vesicles in addition to mitochondria and occasional collections of dense osmophilic material (Fig. 13). Synaptic balls are not associated with synaptic areas which are characterized by opposing membrane thickenings. The synaptic gap approximates 200 Å. The terminals are small boutons and are attached directly to the hair cell and not to the afferent endings. Subsynaptic cisterna were not apparent.

Discussion

Background

Neural epithelial regeneration in response to injury is not a revolutionary concept; it occurs by the process of basal cell differentiation in the olfactory organ of many animals. Similar undifferentiated stem cell populations exist in the auditory organs of fish and amphibians, which provide for repopulation of the sensory organ with both support cells and hair cells throughout life (Corwin, 1985; Corwin et al. 1989; Popper and Hoxter, 1984). In contrast, the sensory cell population of the cochlea in mammals and birds is established well before birth and popular dogma has predicted that these cells have no repopulation capacity. To the contrary, hair cell and supporting cell regeneration in the avian cochlea has recently been documented following both acoustic trauma and ototoxic drug exposure (Cruz et al., 1987; Cotanche, 1987a; Corwin and Cotanche, 1988; Ryals and Rubel, 1988). This finding provoked further investigation into the source of the newly regenerated hair cells. Corwin and Cotanche (1988) suggested that the supporting cells or an unidentified stem cell population was responsible for repopulating the sensory organ. Using tritiated thymidine labeling following acoustic trauma, Girod et al. (1989) identified two potential precursor populations active in the regeneration of both hair cells and supporting cells. First, the cuboidal or hyalin epithelial cells, which lie just inferior to the short hair cells, appear to be responsible for recovery of the inferior portion of the sensory epithelium. The process is initiated by cellular migration followed by proliferation (15–24 h after noise exposure) and differentiation (24 h to three days after exposure). In a final maturation phase the regenerated sensory epithelium assumes the cellular characteristics of the adult animal. Second, supporting cells underneath and adjacent to hair cells may serve as the precursor population for more superior hair cells. These supporting cells may be morphologically and functionally different from the inferior supporting cell.

The model used in the present study offers a fortuitous opportunity to examine hair cell regeneration and maturation. Because gentamycin effectively destroys all original hair cells in the basal (proximal) portion of the chick cochlea hair cell precursors and immature hair cells can be identified on the basis of their unique identity without labeling. Having established that sensory cell repopulation of the gentamycin damaged avian cochlea does occur, the regeneration and matura-

Fig. 9. (A) At seven days following termination of the gentamycin treatment differentiation of the apical surface has progressed. The cuticular plate (Cu) is identified. The stereocilia are packed into a staircase shaped bundle (arrow). Bar 2 μm. (B) Primitive hair cell at seven days post termination of gentamycin treatment possesses both stereocilia bundle in a staircase configuration and surface microvilli. Note the fusiform cell shape and the cochlear nerve terminals (arrows). Synaptic complex is also present (circled). Bar 2 μm. (C) Higher magnification transmission electron micrograph of poorly developed cuticular plate (Cu). There is minimal constriction at the base of the stereocilia (arrows) and no rootlet formation. Bar 0.5 μm.

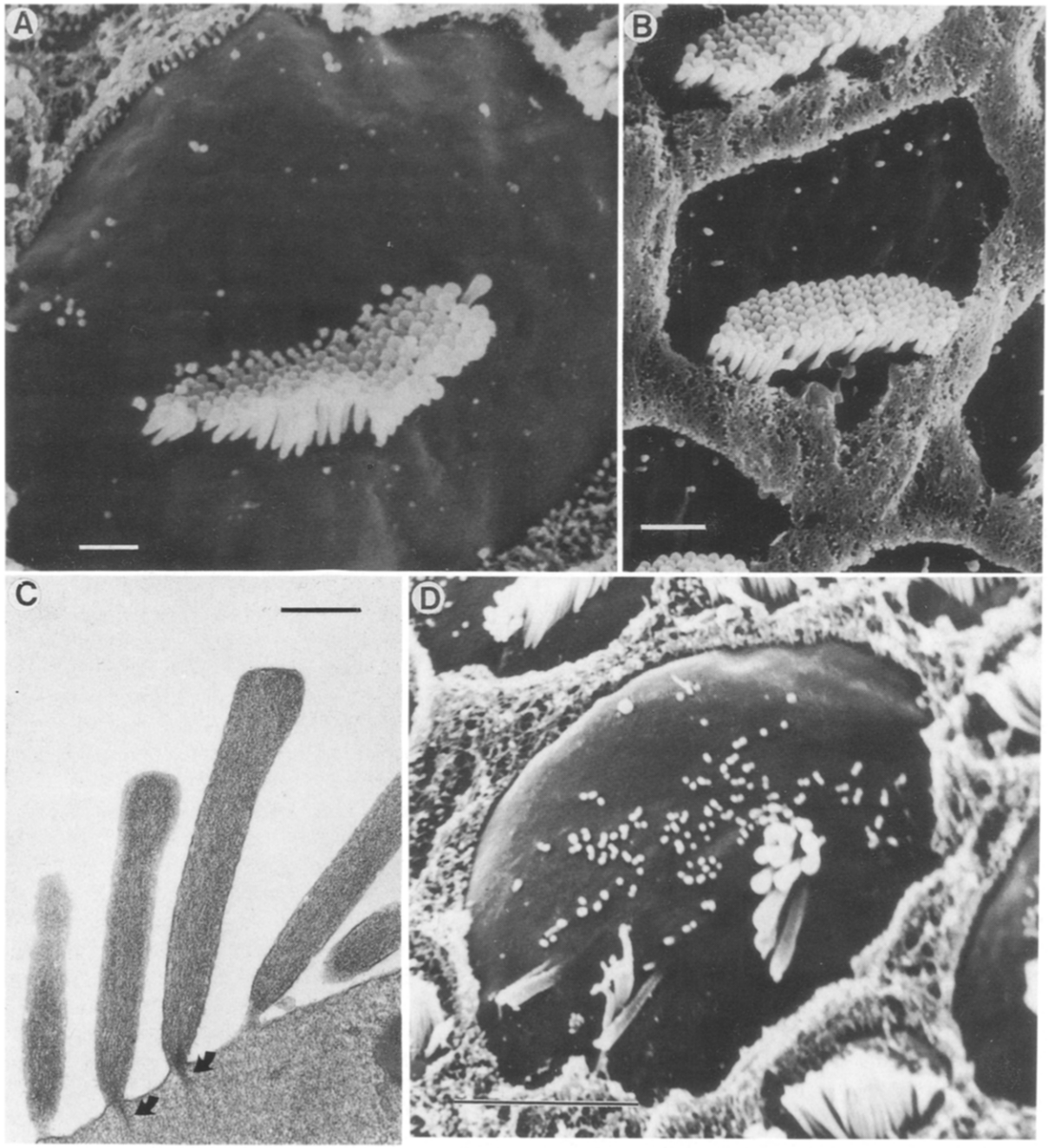


Fig. 10. (A) At 28 days post termination of gentamycin treatment the stereocilia bundle is centrally located on the apical surface of the regenerated hair cell. The staircase orientation is easily recognized. The stereocilia bundle is better organized in the more mature cells and the tightly packed hexagonal lattice configuration observed in normal control animals is seen (see Fig. B). (B) Apical surface of short sensory hair cell from control animal showing hexagonally packed lattice of stereocilia. Bar 2 μm. (C) Transmission electron photomicrograph of more mature stereocilia 28 days after termination of treatment. The stereocilia are constricted as they insert into the cuticular plate (Cu). Arrows identify rootlets. Bar 0.5 μm. (D) Cuticular surface of a 'giant' hair cell. Scattered clumps of disorganized and degenerated stereocilia are seen in addition to microvilli. Bar 5 μm.

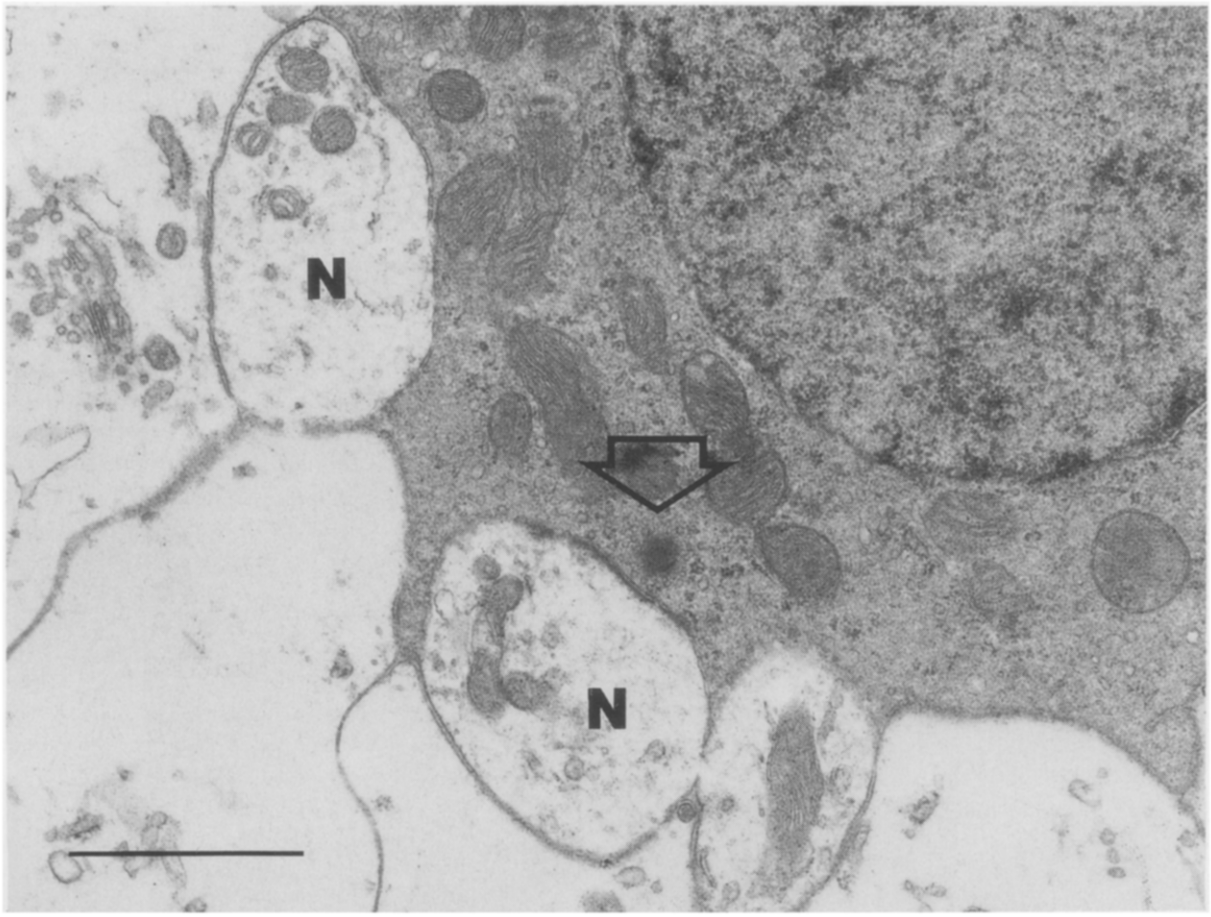
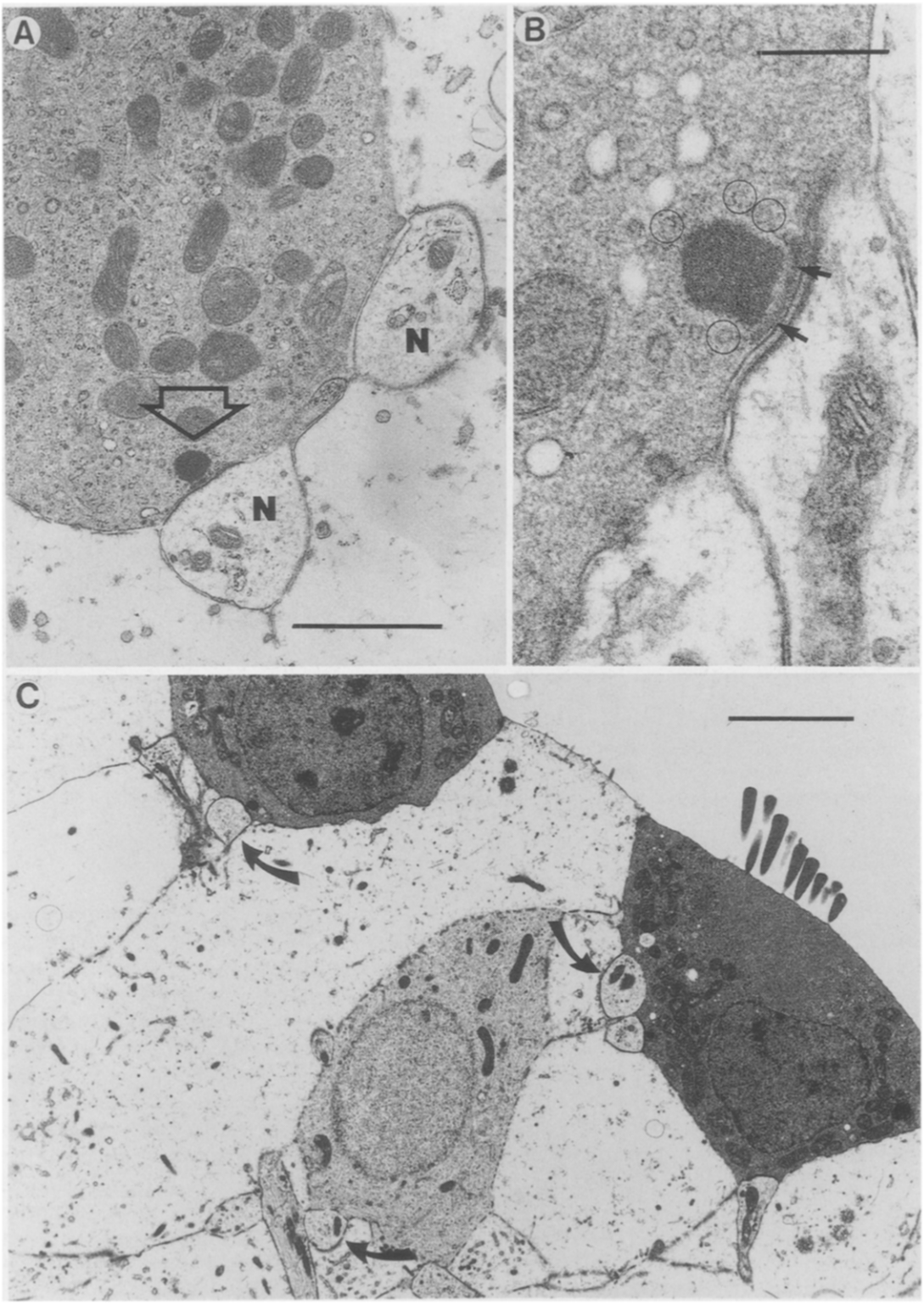


Fig. 11. Afferent cochlear nerve terminals (N) on a short hair cell in a control animal. Arrow, synaptic ball surrounded by vesicles. Bar 1 μm.

tion process will be the initial focus of attention followed by a discussion of the functional correlates and implications.

Sensory cell precursor differentiation

Both erupted and unerupted hair cell precursors can be identified as early as one day after five days of gentamycin treatment has ceased. The unerupted precursors are found within the strata of supporting cells which they closely resemble. The filiform shape and position suggest a migratory process beginning at the basilar membrane and ending at the luminal surface. During the migration cellular morphogenesis is identified by changes in cytoplasmic staining. A distinct gradient is apparent. There is a gradual increase in the density of cytoplasmic staining from cells characterized by low density at the basilar supporting cell layer to cells showing high organelle density at the luminal surface. These changes in density can be equated to increases in the number and type of cytoplasmic organelles. This process will be discussed in greater detail below. Suffice it to say, it is primarily on the basis of differential staining that the precursor of the hair cell can be segregated from the supporting cell early in its development. To the extent that the cells are similar in all other respects it is easy to conjecture a common identity until morphogenesis is induced. The observation that such differentiation occurs throughout the 28 day period suggests the presence of a pluripotential stem population which is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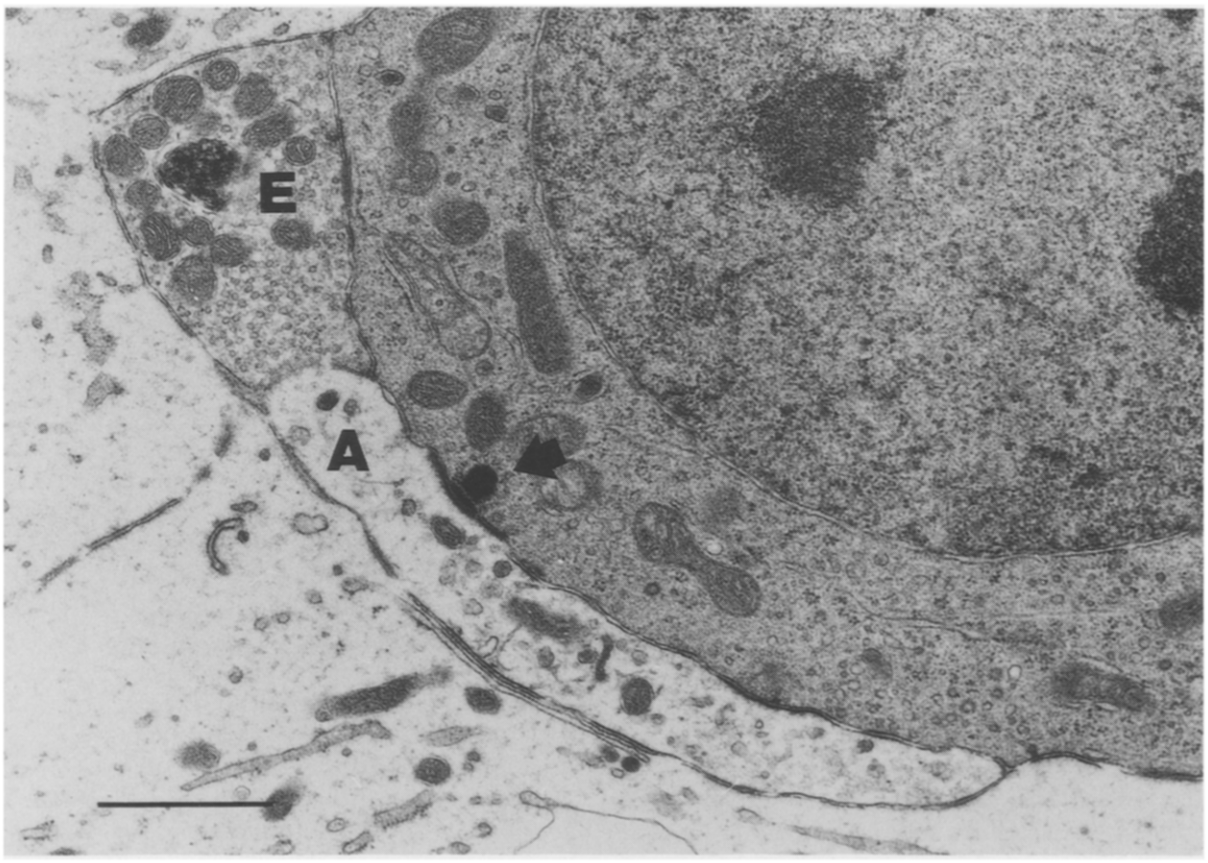

Fig. 13. Transmission electron photomicrograph showing both afferent (A) and efferent (E) nerve terminals opposed to the basal portion of an unerupted primitive sensory hair cell seven days following termination of gentamycin treatment. The efferent terminal contains multiple small round vesicles in addition to mitochrondria. A synaptic complex (arrow) opposes the afferent terminal. Bar 1 μm.

sequentially induced. Aside from the supporting cells, no other histologically distinct basal cell population was identified at one or seven days. The possibility that the hair cell precursor and the supporting cell are identical or at least histologically undistinguishable until migration is induced appears very real. Pulse-labeling studies using tritiated thymidine will be required to clearly delineate the stem cell population and the early stages of daughter cell differentiation.

While the nature of the inductive mechanism is unknown, it is equally true that the means by

Fig. 12. (A) Small bouton afferent nerve endings (N) opposed to basal portion of regenerated sensory hair cell one day post termination of gentamycin treatment. Arrow, synaptic complex consisting of synaptic ball and vesicles. Bar 1 μm. (B) High magnification of synaptic complex in a regenerated hair cell one day following termination of gentamycin treatment. Note row of vesicles surrounding synaptic ball (circles). A granular synaptic membrane (arrows) is found between the ball and the cell membrane. Bar 0.5 μm. (C) Transmission electron photomicrograph of primitive regenerated hair cells seven days following termination of gentamycin treatment. The more mature sensory cells are more densely stained than the unerupted precursor cell below the reticular surface. Numerous afferent nerve terminals (arrows) approximate the basal portion of the both erupted and unerupted sensory cells. Bar 5 μm.

which morphogenesis and migration is terminated is also unknown. At 28 days, unerupted and erupted hair cell precursors were still observed in the sensory cell strata along the luminal surface. The significance of the frequent association between an immature erupted hair cell and an unerupted precursor is unknown. The apparent migration from basal supporting layer to luminal surface appears to occur randomly. However, one cannot totally discount the possibility of a trophic influence on the unerupted precursor by the more mature erupted hair cells.

Cellular maturation

Maturation of the newly generated presumptive hair cell is initiated prior to eruption and continues after the cell has reached the lumen. Morphological changes appear limited to the cytoplasm during migration to the surface, after which the apical end differentiates characteristic surface specializations.

Cell contents. The first hair cell precursors can be distinguished from the adjacent supporting cells by a darker staining cytoplasm. This differential staining characteristic has been identified as a growth parameter in the normal neonatal chick (Cotanche and Sulik, 1985). The supporting cell contains few organelles, with the exception of Golgi apparatus, indicating a low level of metabolic activity and physiologic specialization. In contrast, immature hair cell cytoplasm is characterized by a greater volume of organelles and granularity. Prior to eruption, as the cell approaches the surface, the number of organelles increases and the cytoplasm stains darker. The most attractive interpretation would correlate increasing precursor metabolic activity, maturation and cellular differentiation with cell position along a vertical gradient from basilar membrane to luminal surface. The possibility that the hair cell emerges from the more primitive basilar precursor cell is consistent with this concept.

Cytoplasmic organization. After eruption cellular maturation continues and is characterized by further accumulation of cellular organelles as well as changes in cytoplasmic organization. Early in the course of differentiation the nucleus is located in the central portion of the cell. As the cell matures and approaches the luminal surface the nucleus assumes a more basal position and the organelles relocate apically. Similar cytoplasmic reorganization is observed early in development of the normal neonatal chick basilar papilla (Cotanche and Sulik, 1985). Coincidentally, modification of the apical cell surface is initiated following eruption. The cellular reorganization would seem appropriate to concentrate metabolic efforts in the location of stereocilia and cuticular plate formation.

Cell shape. Maturation is also characterized by changes in cellular shape. The shape of the earliest identifiable hair cell precursor is fusiform. At the time of eruption it is globular. The apparent retraction of the more basal portion of the hair cell is observed during embryogenesis (Whitehead and Morest, 1985) and follows detachment of the precursor from the basal lamina. Nuclear migration follows as differentiation continues. These observations are also consistent with the hypothesis that the hair cell precursor is a basal cell.

Immature sensory cells were easily identified at seven and 28 days by shape and staining characteristics. The globular shape is similar but distinctly different from the shape of the short hair cell typically found in the proximal part of the basilar papilla. These cells have been described to resemble a 'low squat pitcher' (Takasaka and Smith, 1971). The distinctive flared apical lip is probably influenced by a well developed cuticular plate. In contrast, the cuticular plate of the mature appearing sensory cells observed in animals surviving 28 days is less well developed. The extent to which this finding can account for the globular cell shape is unknown, however, it is easy to envision surface changes dependent upon or associated with maturation of subcellular components.

Morphological variability. The more mature hair cells identified in 28 day survivors contain a more complete complement of normal appearing organelles. Among them were frequently found osmophilic inclusion bodies, and myelin figures. These were confined to the supranuclear region of the cell and are probably degenerated mitoch-

rondria. Disorganization of the internal mitochrondrial membranes into myelin figures is a well established and early result of ototoxic drug exposure (Duvall and Wersäll, 1964). In the final stages degeneration into cystic structures is seen. Other possible evidence of continued ototoxic drug affect include 'giant' sensory cells with disorganized or absent stereocilia. This observation and the subapical location of the degenerated mitochrondria supports the hypothesis that the ototoxic drug or a metabolite is accumulated in the endolymph where it continues to exert direct effects on the hair cells which have reached the luminal surface (Wersäll and Hawkins, 1962). The other possibility is that the degenerative changes are the legacy of either primary or delayed injury to surviving hair cells. This seems unlikely, however, given the hair cell decimation observed with the scanning electron microscope immediately after treatment. The hair cells identified at 28 days also carry the stigma of immaturity, including characteristic cellular staining, shape and surface specialization (discussed below).

Luminal surface modifications

Stereocilia. As previously stated specializations of the luminal surface of the hair cell precursor occur only after the cell reaches the lumen. Thereafter, in the recovering cochlea, stereocilia development is defined by a series of morphogenic events which parallel embryonic hair cell differentiation. The normal development of stereocilia in the chick has been described in detail (Cotanche and Sulik, 1984; Tilney and DeRosier, 1986; Tilney et al., 1988a) and the maturation of stereocilia in regenerated hair cells following acoustic trauma has been carefully described by Cotanche (1987a). A detailed discussion of stereocilia growth is beyond the scope of this study. Suffice it to say, that growth occurs rapidly and stereocilia assume a stairstep organization within seven days of treatment termination. These events include, in addition to stereocilia formation, rootlet and cuticular plate formations, stereocilia organization, and cellular polarization. Early stereocilia are already identified in the one day survivor as a condensation of microvilli. It has already been established in the embryonic chick that stereocilia are derived from microvilli and, as such, contain actin filaments. The organization of the stereocilia staircase into a hexagonally packed lattice was first described by Tilney and Saunders (1983). This reorganization has important functional implications as it would seem that proper bundle orientation in the epithelium of the basilar papillae is necessary for transduction. At 28 days a well organized stereocilia bundle is a common feature of more mature regenerated hair cells and cellular orientation can be predicted on that basis. However, the orientation of the bundle or hair cell is clearly less ordered than in the normal condition. Stereocilia counts were performed on a limited number of sensory cells which possessed approximately the same number as normal sensory cells from a corresponding basal location (Tilney and Saunders, 1983). The shape of the bundle is more rectangular than round, and the bundle remains centrally located on the apical surface. In addition, many cells possess microvilli as well as the stereocilia. To the extent that the morphological recovery is not complete at this stage, functional recovery would also not be anticipated.

To what degree the participation of a kinocilium is necessary for the stereocilia bundle orientation is unclear. During embryogenesis stereocilia and kinocilia morphogenesis are temporally associated and by implication the kinocilium may be responsible for cellular polarity (Tilney et al., 1988). In the basal region of the normal mature chicken basilar papilla kinocilium are sporadic at best. However, all hair cells are thought to have kinocilia early in development. One would speculate that the disarray of hair cell orientation observed within 28 days was, in part, due to the uniform absence of kinocilium in the precursor hair cell.

Cuticular plate. The development of the cuticular plate occurs simultaneously with stereocilia formation and in the same sequence as normal embryogenesis. In newly erupted hair cells, as in early embryos (8–10 days), the cuticular plate is undeveloped (Tilney and DeRosier, 1986). Stereocilia insertion into an immature fibrous cuticular plate region is observed at seven days followed by rootlet formation and constriction of the rootlet base which is apparent at 28 days. Relative to the normal developmental process in which cuticular

plate formation is completed at 14 days, the regenerative process is prolonged following gentamycin treatment. The rate at which stereocilia reach maturation is also much faster in the embryo then was observed following ototoxic drug exposure. It may seem intuitive that hair cell regeneration proceeds at a slower rate following cochlear insult in a more mature bird than during embryogenesis. However, observations following noise damage offer evidence to the contrary. Cotanche (1987a) determined that following a severe acoustic insult hair cell regeneration proceeds through the same developmental steps as well as at the appropriate time course as in the embryo. In view of this, the relative developmental delay that we have observed may reflect continued influence of the ototoxic drug. It has been suggested that ototoxic drugs are secreted in the endolymph where they exert a direct toxic effect on the neuroepithelium (Wersäll and Hawkins, 1962). If this is the case, it follows that those cytoplasmic structures in proximity to the luminal surface, including stereocilia and cuticular plate, would be preferentially affected. Such may be the case for the 'giant' sensory cells devoid of stereocilia. Other aspects of cellular maturation, in particular innervation, may be less affected by the ototoxic drug at the luminal surface.

Innervation

Some controversy exists regarding the developmental sequence of nerve–epithelial cell contact and synaptogenesis in the basilar papillae of the chick embryo. It has been suggested, on the one hand, that hair cell differentiation precedes innervation (Hirokawa, 1977; Cotanche and Sulik, 1984; Corwin and Cotanche, 1989). On the other hand, synaptogenesis has been reported very early in the course of hair cell differentiation. Early nerve-epithelial attachments have been observed at embryogenic day five (Whitehead and Morest, 1985). Characteristic functional morphology including synaptic bodies, vesicles, and thickened synaptic membranes is present at 10 days (Hirokawa, 1978; Fermin and Cohen, 1984). That differentiation of afferent attachments occurs earlier and coincidentally with sensory cell maturation is implied by the observations of this study. In fact, synaptogenesis following gentamycin treatment is precocious in this regard since afferent synaptic complexes are observed in immature erupted hair cells at day one. Innervation, therefore, may have occurred during the drug exposure. A possible explanation for this apparent accelerated innervation process may be predicated on early availability of nerve endings. During normal embryogenesis nerve fibers must first cross the basal lamina and spread across the basilar papilla before reaching the proximal epithelial cells. Since it appears that neuronal degeneration does not immediately follow drug induced sensory cell degeneration, a delay imposed by fiber growth would be obviated. This being the case, ready availability of intact nerve fibers to hair cell precursors would promote early receptoneural contact. The appearance of structurally complete afferent synapses on unerupted hair cell precursors by day one suggests that synaptogenesis can occur very early in hair cell development. This observation is compatible with a theory postulating induction of hair cell differentiation by afferent or efferent nerve fibers.

Differentiation of efferent synaptic endings is also evident quite early in the present study. Efferent terminals were not observed at one day, but were present at seven days. During embryogenesis efferent innervation follows afferent differentiation by about five days (Fermin and Cohen, 1984). Our observations suggest a similar sequence may occur during hair cell repopulation. Conclusions in this regard, however, were limited by the relatively small number of efferent terminals identified and the fact that quantitative analyses were not carried out in the present study.

Structure and function

Preliminary observations have indicated that functional recovery does not occur until 16–20 weeks following gentamycin treatment (Tucci and Rubel, 1989; 1990). This seems inconsistent with the observation that cochlear repopulation begins very early after drug treatment. It appears that the recovery in cellular numbers documented at the light microscope level is insufficient to restore function, indicating that a subcellular or physiological immaturity may be responsible for the delay. It is reasonable to expect that the transduction of basilar membrane movements into receptor

potentials can occur only after proper orientation of hair cells and/or their stereocilia bundles. In the embryonic cochlea, hair cell orientation proceeds through a series of coordinated steps until the precise adult pattern is achieved (Cotanche, 1987b). Following acoustic trauma hair cell regeneration and reorganization is also predictable (Cotanche, 1987a). It follows that a similar coordination and orientation process must occur following ototoxic drug exposure before the restoration of hearing.

While the stereocilia disorientation alone would seem incompatible with full recovery of hearing, other sources of functional delay cannot be overlooked. The immature appearance of sensory cell innervation observed at 28 days is clearly suspect. Although afferent terminals are present even before sensory cells erupt they may remain deficient in both size and numbers until 16–20 weeks. It is very likely that the correct pattern of innervation is also necessary before function is restored. In the normal mature animal efferent innervation is generally more prominent on the short hair cells than the tall hair cells (Hirokawa, 1978). In contrast, efferent endings were rare in our material and even at 28 days they did not appear mature.

Further insight into synaptic maturation is provided by recent studies of otoacoustic emissions (Norton et al., 1990). Cochlear emissions may be generated by the hair cell alone while evoked auditory potentials require functional synapses. In this investigation recovery of evoked acoustic distortion products preceded recovery of brainstem evoked potentials by several weeks suggesting that receptoneural junction maturation lags hair cell recovery.

Delay of functional recovery may not be justified entirely by incomplete peripheral innervation. It is also possible that more central neuronal structures are directly damaged by aminoglycoside administration or suffer transneuronal effects due to transient hair cell loss. Ryals et al. (1989) noted continued ganglion cell loss while hair cell regeneration was occurring following noise trauma.

It was noted earlier that cochleas from 28 day survivors demonstrated certain morphological stigma associated with ototoxic drug exposure. More specifically, mitochrondrial degenerative changes raise the possibility of residual aminoglycoside toxicity adversely affecting regenerated hair cells after eruption. Absence of stereocilia or stereocilia disarray on the cuticular surface of sensory 'giant' cells may be another manifestation of delayed ototoxicity. The distinct possibility exists that the drug effect may not be an isolated event in an avian cochlea, but may persist to damage multiple hair cell generations. The coincidental presence of hair cell precursors and more mature but structurally damaged hair cells are testimonial to this effect. If this is the case, a functional delay may be anticipated until the drug effect has dissipated. It may be safe to assume that the drug effect persists in excess of 16 weeks after exposure (Tucci and Rubel, 1989; 1990).

The morphological changes found suspect in the delay of hair cell recovery are gross in comparison to the physiological implications of the multitude of more subtle anatomical alterations which occur during the maturation process. Given the complexity of the regenerative process it is almost certainly the case that functional delay is a result of multiple causes and not isolated developmental obstacles.

The more important questions regarding induction and regulation of cell maturation can only be answered after the process has been completely inventoried. Obviously, additional studies will be necessary to examine each of the various aspects of cellular growth and development if this is to be accomplished.

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