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Morphological Correlates of Functional Recovery in the Chicken Inner Ear After Gentamycin Treatment

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ABSTRACT

Newly hatched chickens were allowed to survive 6, 10, 15, and 20 weeks after 10 days of gentamycin sulfate treatment. Ultrastructural studies of hair cells and nerve terminals in the auditory receptor organ, the basilar papilla, were carried out with transmission and scanning electron microscopes. Attention was paid to absolute sensory cell (hair cell) numbers, stereocilia maturity and orientation, and reinnervation within a band 100 μm wide centered 1,100 μm from the basal end of the avian cochlea.

Sensory cell numbers were equivalent to those of untreated control animals within the study area in the earliest survival group. Both immature and mature appearing hair cells were identified throughout the recovery period. However, the ratio of mature to immature hair cells gradually increased to exceed 95% at 20 weeks. Stereocilia bundle reorientation also occurred throughout the study period. Orientation was often abnormal at 6 weeks, but by 20 weeks more than 95% of the regenerated hair cells were aligned within normal limits established in the control ears. Hair cell differentiation occurring at 10–15 weeks was associated with degeneration of the afferent nerve receptor complexes commonly observed in 6 week survivors. These complexes were replaced by one or two small bouton shaped efferent terminals per cell. At 20 weeks, two or three chalice shaped vesiculated terminals were observed per cell in both the gentamycin treated and control ears.

On the basis of these observations normal physiological activity would be predicted at 20 weeks following gentamycin treatment, at which time sensory cell repopulation, maturation, reorientation, and innervation approximates the normal anatomical condition.

Key words: hair cell regeneration, ototoxicity, stereocilia orientation

It is now generally accepted that the avian inner ear is capable of regenerating new hair cells following acoustic trauma and ototoxic drug injury. Restoration of the hair cell population, however, does not immediately result in full recovery of function. Whereas Cruz et al. ('87), Duckert and Rubel ('90), and Girod et al. ('91) identified an increase in hair cell number in the avian cochlea 2 weeks after aminoglycoside drug treatment, functional recovery was delayed by 16–20 weeks (Tucci and Rubel, '90). We speculated that physiological immaturity or incomplete innervation was responsible for the delay. We also reported rapid repopulation of sensory cells in the proximal (basal) one-third of the basilar papilla in chicks allowed to survive 7–28 days after 10 days of gentamycin treatment (Duckert and Rubel, '90). Under close scrutiny with transmission and scanning electron microscopy, however, the cells appeared immature, even after 4 weeks. The following features were characteristic of the regenerated sensory epithelium at this time: 1) the cells did not contain a normal complement of organelles, resulting in a characteristic staining pattern that was less dense than that of mature cells; 2) the regenerated hair cells had a globular rather than a "low-squat pitcher" shape; 3) the cells contained myelin figures, which probably represented disorganized internal mitochondrial membranes, suggesting ongoing ototoxic effect; 4) stereocilia number and development approximated the control condition, but the orientation of the bundles was less ordered; and 5) the receptoneural junction maturation was incomplete and the pattern of innervation differed from age-matched controls.

In the present study, animals were allowed to survive for more prolonged periods after gentamycin treatment in an effort to correlate functional recovery with the evolution of morphological maturity. Whereas our earlier observations

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described the more obvious features of cellular recovery, the object of this investigation was also to provide more quantitative analyses of the cell population, stereocilia orientation, and innervation.

MATERIALS AND METHODS

Twenty-four newly hatched chickens were divided into four experimental groups of six birds. An additional group of six birds served as controls. Experimental animals were injected with 50 mg/kg/day of gentamycin sulfate for 10 days. Injections were given subcutaneously once a day. The control animals received no gentamycin. After the last injection, the experimental groups were allowed to survive 6, 10, 15, or 20 weeks before they were killed. Control animals were appropriately age-matched at the time of death. An additional four birds were injected with the same dose of gentamycin sulfate. These birds and four age-matched controls were also allowed to survive for periods of 6, 10, 15, or 20 weeks prior to death (one control and one experimental animal at each age). This group of animals was designated for special tangential sectioning of the cochlea to evaluate synapse formation on the newly regenerated hair cells.

Following killing by an overdose of sodium pentobarbital, the cochleas were perfused via the round window with 2.5% glutaraldehyde (in 0.1 M PBS). The basilar papilla was then removed by standard microdissection techniques (Rubel and Ryals, '82). The specimens were postfixed in 1% osmium tetroxide for 1 hour. From the experimental group of 24 birds plus six controls, 30 ears were prepared for transmission electron microscopy and 30 for scanning electron microscopy. The remaining four experimental birds and four age-matched controls were all prepared for transmission electron microscopy. Figure 1 shows the region of the basilar papilla that was examined in detail. The area was restricted to a band 100 μm long, parallel to the long axis of the papilla, which began 1,100 μm from the basal end of the cochlea (1,500 Hz region). This region and the region previously studied after acoustic trauma (Girod et al., '89) were the same. Only the abneural two-thirds of the band which contained the short hair cell was studied. The tall hair cells were restricted to the neural one-third lip of the basilar papilla and were excluded from study. The short hair cell region was divided into two equivalent zones (A and B) as shown in Figure 1, which corresponded to the neural (superior) and abneural (inferior) short hair cell populations, respectively.

Specimens prepared for transmission electron microscopy were dehydrated in alcohol series and embedded in Spurr's resin. After hardening, 24 experimental ears and six control ears were reoriented and remounted for sectioning parallel to the transverse axis of the basilar papilla. Thick sections were made beginning at the basal end. At 1,100 μm from the basal end the entire width of the 100 μm band was thin sectioned; however, only every 10th section was collected for examination. The remaining eight experimental ears and their corresponding controls were reoriented after hardening of the resin so that serial thin sections could be taken in a plane parallel to the basilar membrane. These hair cells were axially sectioned to permit both quantitative and qualitative analysis of the receptoneural junctions. Fifty to 75 hair cells were available for examination per section. Once placed on grids, all sections were stained with alcoholic uranyl acetate and lead citrate before examination with the JEOL-100 PB transmission electron microscope.

Twenty-four experimental and six control ears were processed for scanning electron microscopy by ethanol dehydration followed by microdissection to remove the tectorial membrane. Final dehydration was accomplished with the graded alcohol series and then critical-point drying. Specimens were mounted on aluminum stubs, covered with pure gold, and examined with a JEOL 355 or a JEOL 6300F electron microscope.

Methods of quantification

Apical surface measurements. Sensory cell apical surface measurements were obtained in individual experimental and control ears at 6 and 20 weeks. Enlargements of scanning photomicrographs containing 60–150 cells each at magnification of more than ×6,000 were measured by a Zeiss Videoplan Interactive Image Analysis system. The surface area of each hair cell in the sample area was determined. These data were analyzed by a one-way ANOVA and individual group means were compared by the Fisher PLSD test.

Stereocilia orientation measurement. The tallest row of stereocilia at the top of the bundle was used to define the long axis of the bundle according to a line drawn parallel to this row. The orientation of this line is perpendicular to the morphological polarity of the bundle (Tilney and DeRosier, '86). At low magnification (×500), a line drawn along the long axis of the bundle and a line drawn along the longitudinal axis of the papilla subtended an angle that could be measured and used to describe the amount of stereocilia bundle rotation.

We used two methods to analyze the variations in bundle rotation. The most conservative method was simply to quantify stereocilia orientation in the experimental ears as the percentage of bundles whose alignment met criteria established in normal animals. Normal limits of stereocilia bundle rotation according to location of cell on the avian papilla have been reported by other investigators. According to Manley ('90), cells lying at the neural and abneural margins of the avian papilla have a long axis of the stereocilia bundle oriented almost parallel (±20°) to the edge, and the axis of more centrally located cells is rotated toward the apex. The amount of rotation varies according to the distance of the cell from the proximal end. At 1 mm from the proximal end of the papilla, the maximum rotation is 45° (Tilney et al., '87). We measured the bundle rotation of each cell in the study area per zone in 6 control ears. A baseline rotation of 5° and 25° was selected for zones B and A, respectively, and the range of normal variability ±20° reported by Manley ('90) was used to establish a criterion of normal bundle rotation in each zone. The variability (±20°) adequately accounted for the normal rotation of the bundle toward the apex from an orientation parallel to the papilla at the extreme abneural edge to a maximum of 45° at the junction of the abneural two-thirds and neural one-third of the basilar papilla. These limits of bundle rotation in zones A and B (25° ± 20° and 5° ± 20°, respectively) were sufficiently generous to describe the hair cell orientation in the control ears with complete confidence (i.e., *all* hair cells from normal animals fall within this range). The application of these standards to the aminoglycoside-treated animals provided an estimate of the number of stereocilia bundles with grossly abnormal orientation. Stereocilia bundle rotation was measured in this way on 4–6 experimental

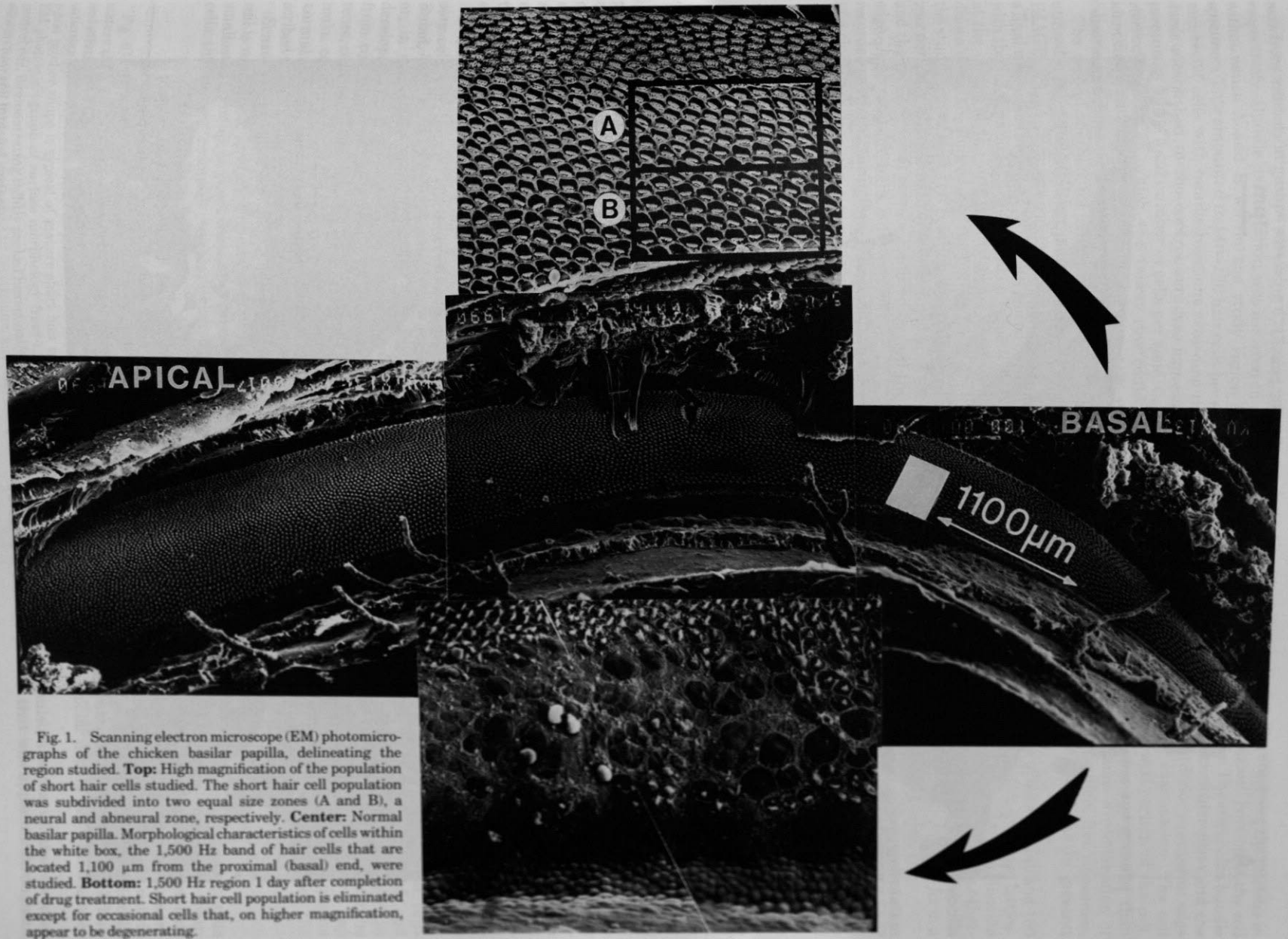

Fig. 1. Scanning electron microscope (EM) photomicrographs of the chicken basilar papilla, delineating the region studied. **Top:** High magnification of the population of short hair cells studied. The short hair cell population was subdivided into two equal size zones (A and B), a neural and abneural zone, respectively. **Center:** Normal basilar papilla. Morphological characteristics of cells within the white box, the 1,500 Hz band of hair cells that are located 1,100 μm from the proximal (basal) end, were studied. **Bottom:** 1,500 Hz region 1 day after completion of drug treatment. Short hair cell population is eliminated except for occasional cells that, on higher magnification, appear to be degenerating.

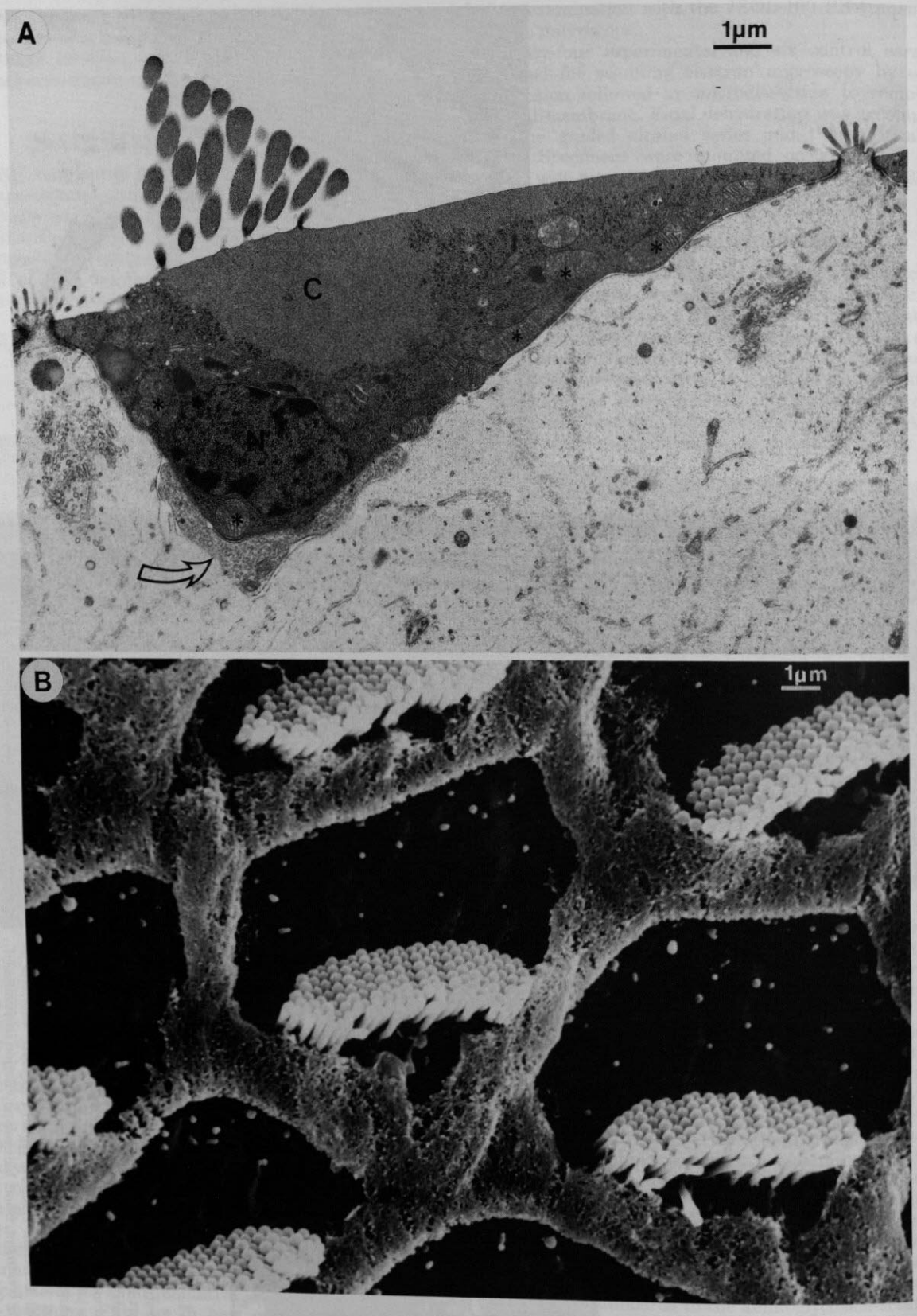

Figure 2

ears from animals in groups surviving 6, 15, and 20 weeks, respectively. Bundle axis rotation was assessed in all cells within zones A and B within the study region in each specimen. This amounted to 140–170 hair cells per ear. The number of cells with bundle rotations exceeding the standards established in the control ears was expressed as a percentage of the total number of cells per zone. Cells with stereocilia tufts in which the tallest row could not be identified and cells with multiple bundles were rated as "disoriented."

The second method for computing bundle orientation utilized a computer assisted image analysis system (NIH, IMAGE, V.1.44). Scanning electron micrographs printed at a final magnification of ×1,000 were placed on a data tablet with the inferior edge of the papilla aligned with the x-axis at the origin of the ordinate (y-axis). A cursor was then drawn along the long axis of the bundle for each hair cell in the study region. Hair cell rotation was considered zero if it was perfectly aligned with the inferior edge of the papilla, and the tallest row of stereocilia faced toward the inferior edge. Any rotation in a counterclockwise direction was recorded as a positive angle (+1° to +180°) and any rotation in a clockwise direction was given a negative value (−1° to −179°). In addition the computer automatically computed the distance (in μm) from the center of each bundle to the inferior edge of the papilla. This distance was computed by constructing the line perpendicular to the inferior edge that intersected the midpoint of each stereocilia bundle.

RESULTS

General observations—Normal condition

The following data and discussion portray a maturation process for the newly regenerated short hair cells. References are made throughout the following text to varying degrees of hair cell maturity, manifested throughout the recovery or regenerative process. The maturation cycle after gentamycin treatment is very similar to normal embryonic development (Cotanche and Sulik, '84; Cotanche and Corwin, '91) and the endstage, fully developed short hair cell is very similar to the normal condition.

Some morphological characteristics of regenerating sensory cells are described in our earlier paper (Duckert and Rubel, '90) and are used here as indices of cell immaturity or, in some cases, debility. The contrasts between the developing cells from experimental ears and mature cells from control ears include differences in cytoplasmic contents, cell shape, surface specialization, and innervation. To make comparison possible, a clear understanding of the normal cell morphology is necessary and it is briefly described for this reason.

Sensory cells. The apical surface of a normal short hair cell is not round. It is multifaceted and asymmetrical. The smallest dimension in most cases approximates 10 μm. The cells themselves resemble squat pitchers (Fig. 2A). The cuticular plate is located centrally, overlying the nucleus in the deepest part of the cell. The "spout" portion of the cell faces the superior edge of the papilla. The stereocilia bundle is located eccentrically within the cuticular plate and the alignment varies predictably from inferior to superior edge within the 1,500 Hz band under examination. The cell nucleus is contained within the basal portion of the cell. The cytoplasm is more electron dense and granular than adjacent supporting cells and contains a combination of smooth and rough endoplasmic reticulum, free ribosomes, vesicles, and mitochondria, which are found in greatest numbers in the cell basal region.

Stereocilia. The bundle consists of between 140 and 160 stereocilia, packed hexagonally in a staircase shaped latticework (Fig. 2B). In the area under investigation, kinocilia are not seen. The surfaces of mature hair cells are otherwise smooth except for occasional microvilli. The stereocilia are 0.2 μm in diameter and constricted at the base to form a rootlet at the insertion into the cuticular plate.

Stereocilia orientation. Stereocilia bundle orientation is nearly parallel to the edge of the papilla at the abneural edge, but becomes more oblique centrally as the bundles rotate toward the apex, as shown in Figure 3A. In the area under scrutiny, bundle orientation measured in six control ears was 5° ± 20° in the abneural zone (Zone B) and 25° ± 20° in the neural zone (Zone A). The gradual increase in orientation angle from the inferior to the superior zone was accounted for by the variability (±20°) that has been reported elsewhere (Manley, '90). A maximum of 45° of stereocilia bundle rotation toward the apex in the superior zone (Zone B) was observed.

Innervation

Afferent. In the basal end of the cochlea, including the area under study, afferent terminals on mature short hair cells are not present (Manley, '90). However, since they can be identified during regeneration and are present in the apical half of the normal papilla, their description is included. Afferent nerve terminals on short hair cells are generally small boutons. The cytoplasm is granular and nonvesiculated, and contains mitochondria and neurofilaments. Synapses are characterized by patches of membrane thickenings in close apposition. The presynaptic cytoplasm contains a synaptic ball approximately 0.2 μm in diameter surrounded by vesicles.

Efferent. Efferent nerve terminals on short hair cells within the study area are large and chalice shaped, and almost entirely enclose the basal end of the cell, as shown in Figure 2A. They contain many small vesicles and numerous mitochondria. The synaptic area is characterized by a gap bridged by fine filaments opposed by a subsynaptic cisterna within the hair cell. In the basal end of the cochlea the terminals number one to three per short hair cell.

Experimental observations

In a pilot study, the basal portion of the basilar papilla was examined 1 day after termination of the drug treatment to ensure complete degeneration of the short hair cells. Whereas the scanning electron microscope demonstrated consistent elimination of the short hair cells within the 1,500 Hz area (Fig. 1), the damage to the tall hair cells was less predictable. It is for this reason that the tall hair cell bearing region of the basilar papilla has been excluded from this study.

Inspection of the repopulated areas of the cochleas revealed a damage pattern that was apparent throughout

Fig. 2. **A:** Transmission EM photomicrograph of normal (control) mature short hair cell characterized by "stairstep" stereocilia bundles and efferent chalice nerve terminal (arrow). Cell contents include the cuticular plate (C) and numerous mitochondria (asterisks). The low, squat pitcher shape is typical of mature short hair cells. **B:** Scanning EM photomicrograph of surface morphology from 1,500 Hz region of control ear, demonstrating tightly packed hexagonally arranged stereocilia on hair cell lumenal surfaces.

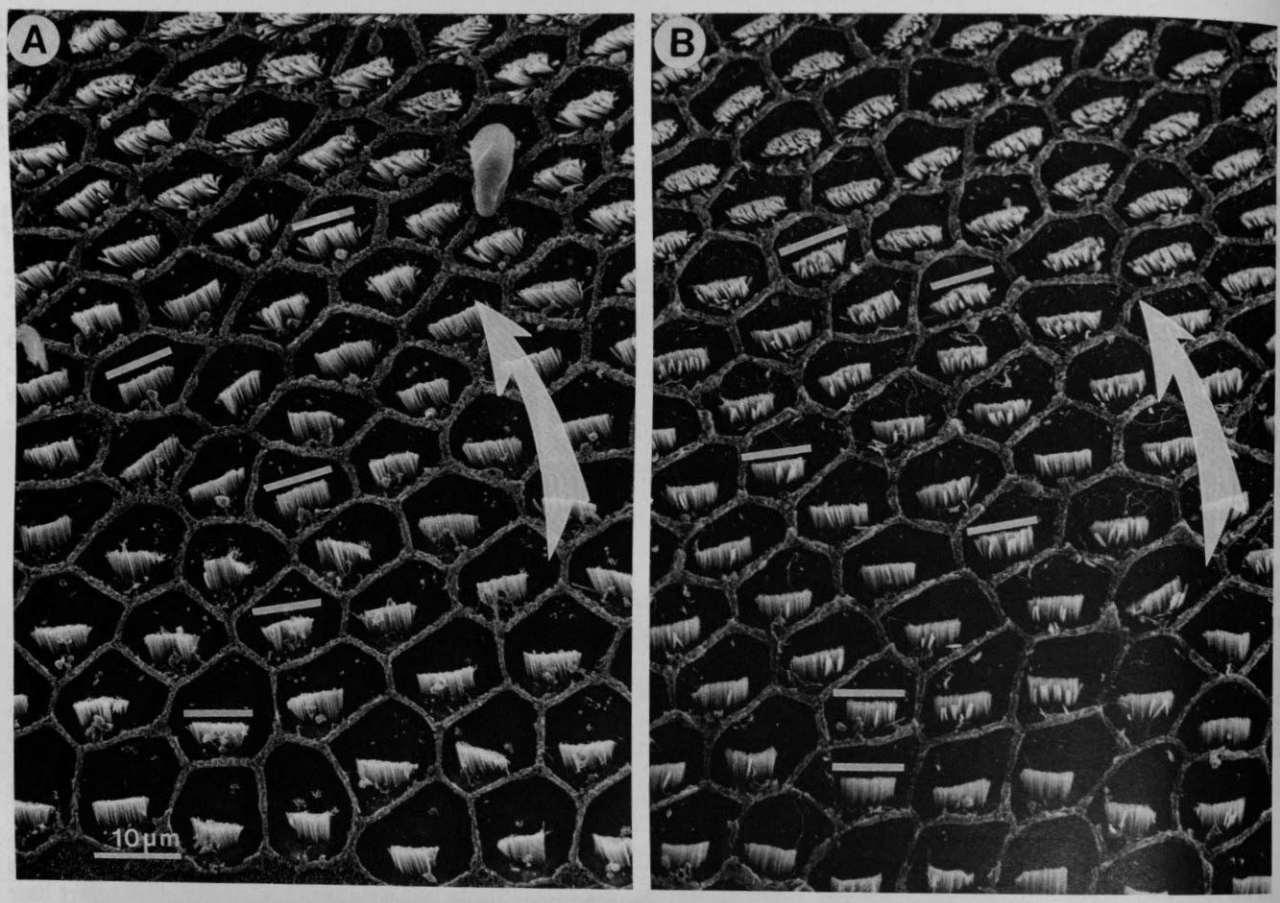

Fig. 3. **A:** Scanning EM photomicrograph of study area from control ear, showing orientation of stereocilia bundles. The bundle orientation is parallel to the inferior lip of the papilla at the abneural edge (inferior) and is more oblique centrally (superior). White bars represent the long axis of the bundle, which is determined by a line drawn along the uppermost row of stereocilia. Arrow indicates direction of bundle rotation from inferior to superior. **B:** Study area from ear of a 20 week experimental animal. The axis rotation (arrow; toward the apex) corresponds to the normal condition shown in A.

the recovery period. The areas were characterized by a heterogeneous population of mature and immature appearing hair cells within an elliptical strip of the papilla extending from the basal tip to its approximate midpoint. As the survival period increased and the hair cell population approached maturity, the borders of the damaged area receded and became less discrete. This process is depicted in Figure 4. In all cases the 1,500 Hz region, also shown in Figure 4, overlapped the damaged area.

Sensory cells. In the 6 week survivors, newly erupted sensory cells were present in both zones (A and B) of the basilar papilla. The population was very heterogeneous, consisting of both mature and immature cells. Hair cell counts from within the area under investigation indicated that sensory cell density was equivalent to that of an untreated control by 6 weeks (Fig. 5). Qualitative differences in cell size were most apparent in the experimental ears from this survival period. The degree of variability was demonstrated by quantitative assessment of short hair apical surface areas in a representative individual experimental ear and a control ear at 6 weeks. In the experimental ear, sensory cell apical surface measurements varied from 7 to 98 sq μm^2 (mean: 24 μm^2), and small, newly erupted immature cells predominated. In contrast, the hair cell population of a 6 week control bird in the same area was much more uniform; apical surface measurements ranged from 34 to 71 μm^2 (mean: 53 μm^2). The mean differences were statistically significant ($P < .01$). The distribution of hair cells according to size from these two specimens is graphically shown in Figure 6, along with similar data from experimental and control specimens from 20 week groups. Figure 7 also demonstrates the variable surface morphology from an experimental ear at 6 weeks compared to the uniform cell surfaces from a control ear. "Giant" hair cells with apical surface diameters approaching 15–20 μm were distributed randomly across the surface of the papilla. In contrast to later survival periods, these were observed in greatest numbers in the 6 week survivors and were not observed after 15 weeks. Whereas the apical surfaces of the sensory cells from the control ear were closely packed, those from the gentamycin-treated animals were disorganized and loosely arranged. The area between adjacent cells was occupied by extensions of supporting cells.

At later stages of recovery, the number of sensory cells in the area examined remained similar in the gentamycin-treated and control animals. However, sensory cell apical surfaces progressively became more uniform in size and configuration. By 20 weeks the cells assumed the closely packed mosaic pattern typical of the control ears.

In transmission electron microscopy, the cell maturation cycle observed throughout the 20 week recovery period in these ears was qualitatively identical to the developmental

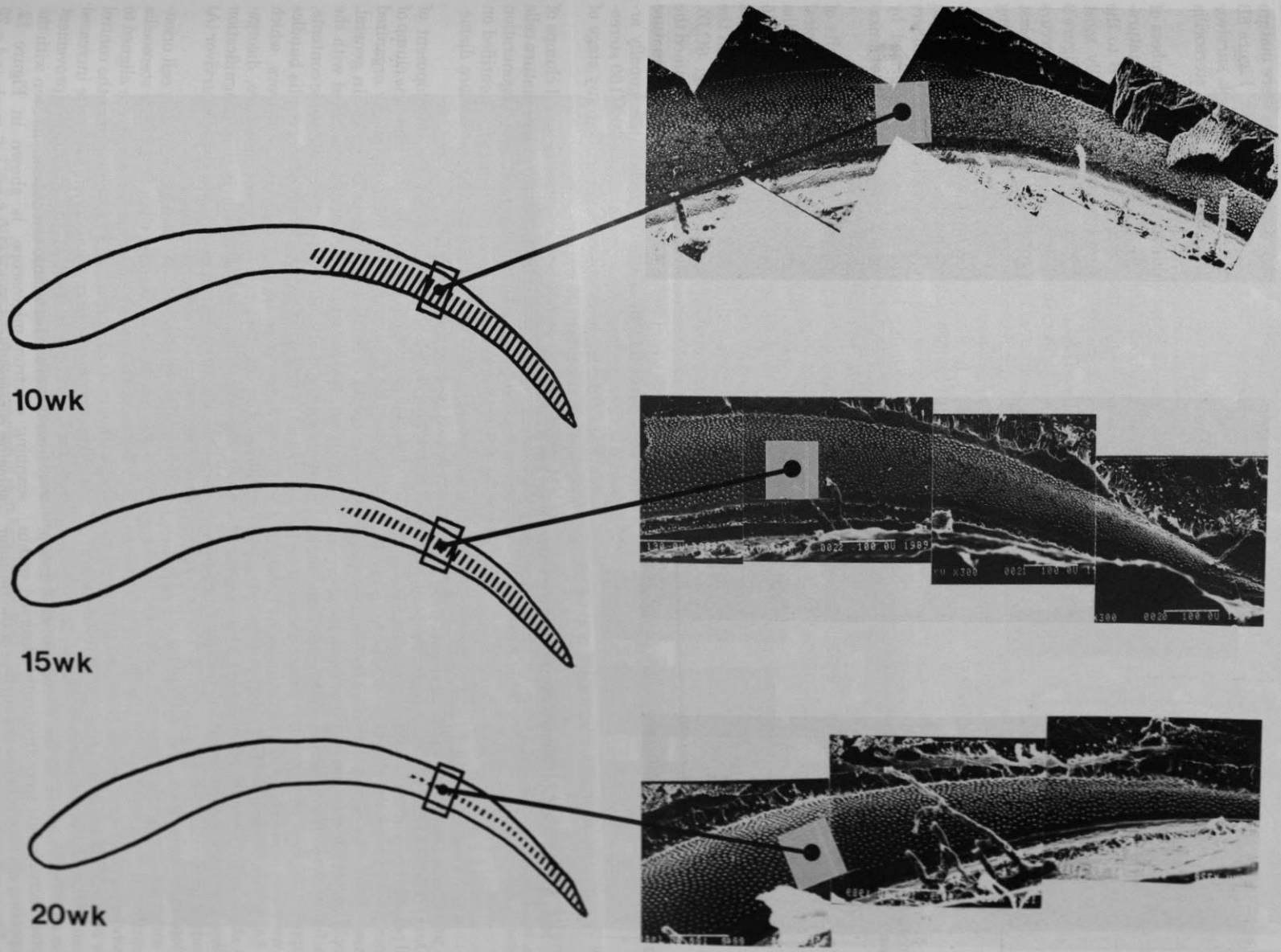

Fig. 4. Schematic representation of damage patterns correlated to age-matched specimens from 10, 15, and 20 week survivors. The damage areas diminish in size as animal survival time increases. Bracketed areas are the 1,500 Hz region, which corresponds to the 100 μm study band (white mask). The band contains only short hair cells.

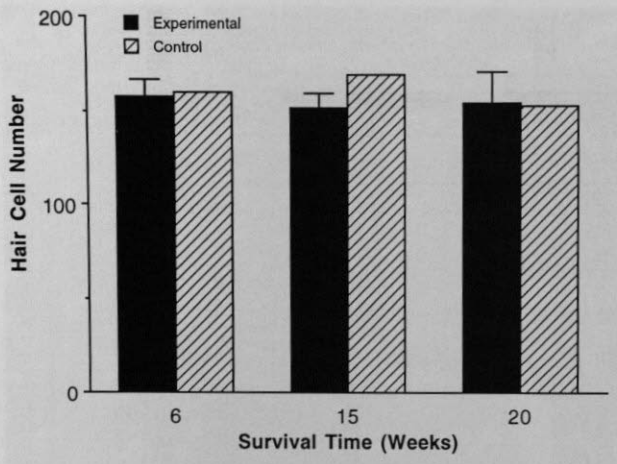

Fig. 5. Mean hair cell counts from six experimental ears each at 6, 15, and 20 weeks. Bars indicate standard deviation. Hair cell counts from 3 age-matched controls are included for comparison.

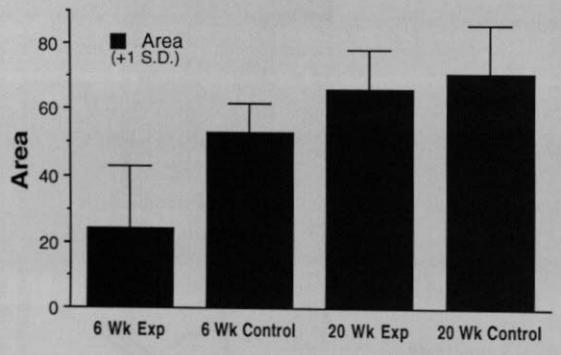

Fig. 6. Mean hair cell apical surface areas (in μm^2) from two 6 week survivors, two 20 week survivors, and age-matched controls. The bars indicate standard deviation of the values. Zones A and B combined.

process previously identified at 7–28 days post-drug treatment (Duckert and Rubel, '90). In animals from each of the survival periods cell maturity could be assessed on the basis of cell morphology and cytoplasmic contents. More primitive sensory cells were characterized by less densely staining cytoplasm, which reflected a relative paucity of organelles, including smooth and coarse endoplasmic reticulum, Golgi apparatus, and mitochondria (Fig. 8A). The cell shapes were also globular, round, or fusiform, as shown in Figure 8A, in comparison to the "squat pitcher" shape of mature short hair cells. As cells matured, the number of organelles increased and the cytoplasm became more granular, leading to increased staining density. The cytoplasm of developing cells also contained osmiophilic dense bodies which sometimes resembled myelin figures. These were also identified at 7–28 days and are believed to be evidence of mitochondrial degeneration (Duckert and Rubel, '90). A number of these inclusions are shown in the cytoplasm of a short hair cell from a 6 week survivor (Fig. 15A).

In the ears from animals in the 6 week survival group, unerupted sensory cells were frequently observed within the supporting cell matrix below the surface of the reticular lamina. These cells were present in cochleas throughout the 20 week recovery period, but were seen only rarely in the long-term survivors. Erupted sensory cells of varying degrees of maturity were also present from 6 to 20 weeks. At 6 weeks, about half of the erupted sensory cells were mature appearing, compared to almost all at 20 weeks. Figure 8B shows a mature appearing cell from a 10 week survivor characterized by a full complement of organelles, stereocilia bundle, and efferent nerve terminal.

Longer survival times equated to increasing numbers of mature cells in both Zones A and B. However, a maturational gradient was clearly apparent from Zone B to the more central Zone A; mature cells were initially most abundant along the inferior Zone B of the papilla. Figure 9 shows the redistribution of mature hair cells that occurs over time in each zone. It is apparent that Zone A is the last area to be totally reconstituted with a mature appearing sensory cell population.

Stereocilia. Immature hair cells were identified by undeveloped apical surface specializations, which by comparison to later recovery periods, were present in greatest numbers at 6 weeks. Cellular maturation was characterized by progressive differentiation of the stereocilia and organization of the bundle, as described by Cotanche ('87a).

Newly erupted cells possessed centrally located tufts of stereocilia, which were located concentrically on the apical surface. In the most immature sensory cells, stereocilia were difficult to differentiate from microvilli. Microvilli were maintained on the apical surface of some sensory cells in addition to stereocilia, which were differentiated on the basis of their increased width and length (Fig. 8C,D). Stereocilia bundles were loosely organized and assumed the shape of a tube, a comma, or a tuft (Fig. 10A). More mature cells were identified by a tightly packed hexagonally arranged staircase shaped bundle containing 140–160 stereocilia (Fig. 10B). No kinocilia were present at any stage of development, from 6 to 20 weeks.

Multiple bundles and cuticular plates or small clumps of damaged stereocilia were characteristic of less mature cells or, alternatively, aborted development and degeneration (Fig. 10C). Multiple bundles were frequently identified on giant sensory cells, which possessed apical surface diameters in excess of 15 μm (Fig. 10D).

Stereocilia development paralleled the development of the cuticular plate. Cells possessing more than one clump of stereocilia also possessed more than one poorly organized cuticular plate early in development (Fig. 8D). In general, the surface specialization development coincided with the maturation and reorganization of the cytoplasmic contents, previously described. The population of stereocilia bundles was most heterogeneous in the 6 week survivors, which exhibited the greatest percentage of immature, disorganized bundles. Figure 11 shows a higher magnification SEM from the study region in a typical 6 week survivor (A) and an age-matched control ear (B).

Stereocilia orientation. At 6 weeks, the hair cell orientation was not random. Greater than 80% of the stereocilia bundles measured in the experimental ears were aligned to within the limits of normal, $\pm 20°$ established in the control ears. The number of normally aligned hair cells increased over time. An appreciation of the gradual improvement over time in stereocilia bundle orientation, as seen with the scanning electron microscope, is shown in Figure 12, showing representative examples from a 6 week and a 20 week experimental ear. At 20 weeks, hair cell orientation was nearly equivalent to that of the age-matched control

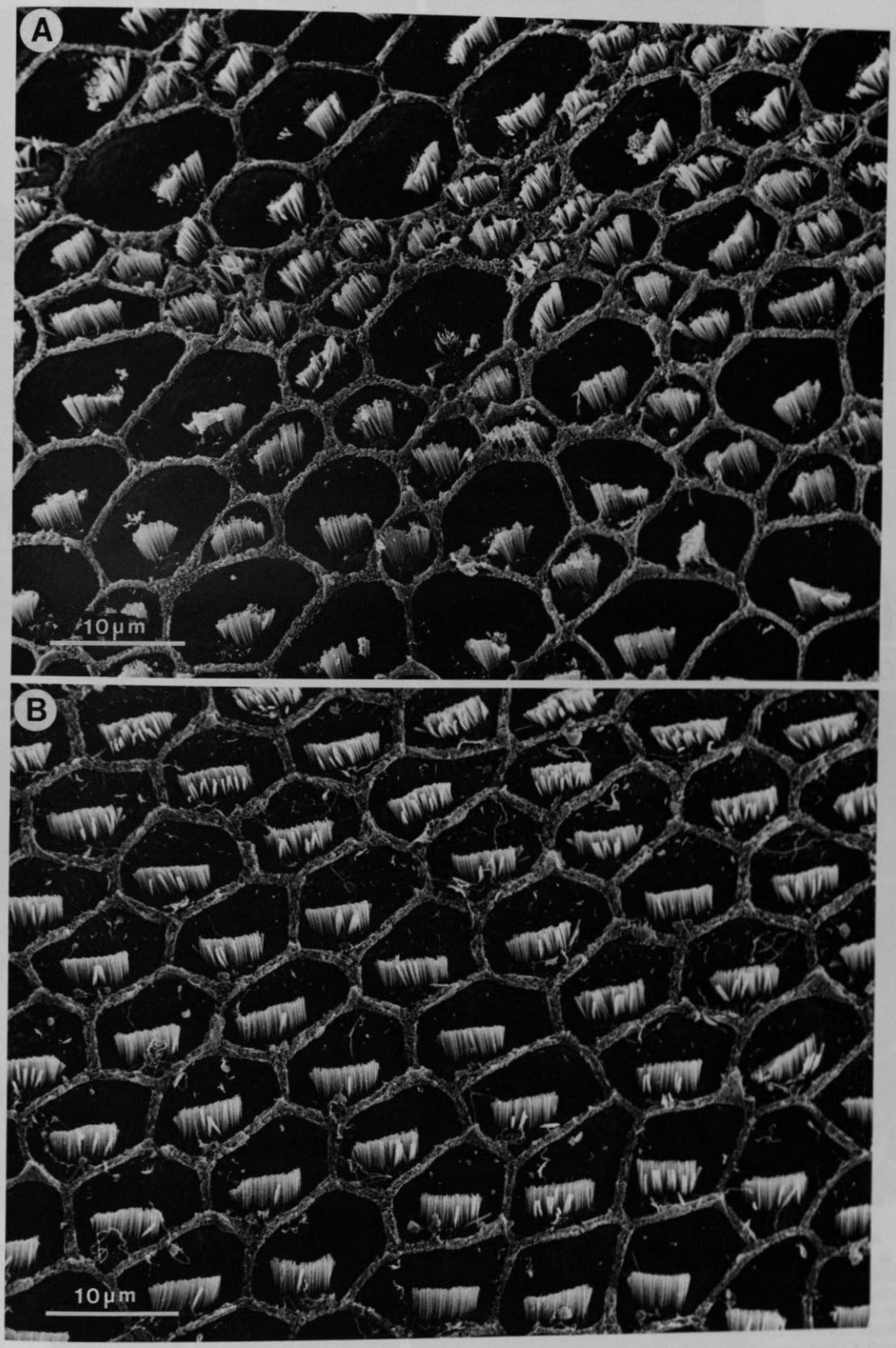

Fig. 7. **A:** Scanning EM photomicrograph of short hair cell population 6 weeks after termination of drug treatment, demonstrating a wide range of apical surface dimensions. The majority of the cells are immature with apical diameters of about 5 μm. **B:** Short hair cell population in 6 week control animal. Note the uniform apical surface dimensions.

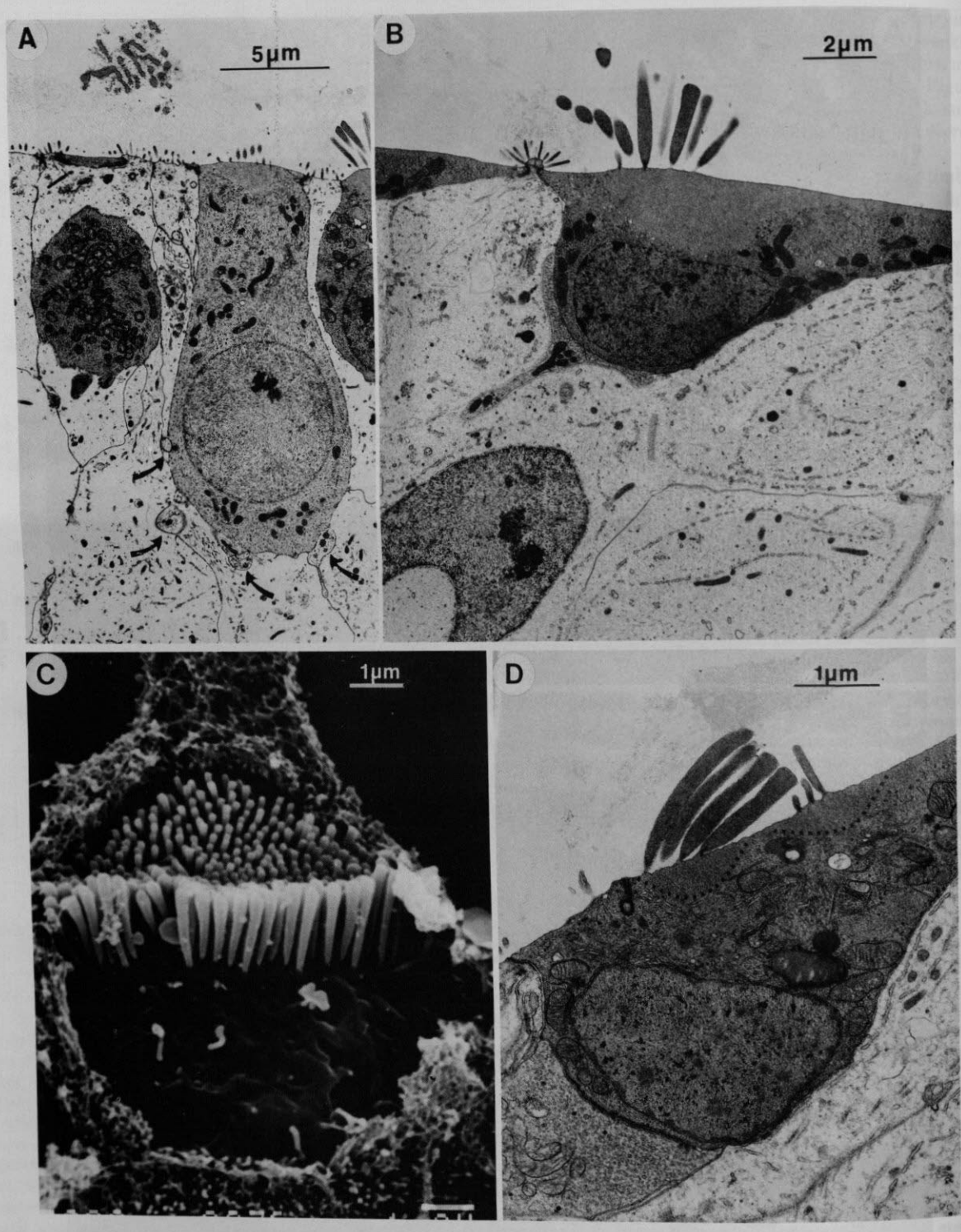

Fig. 8. **A:** The immature regenerated short hair cell from a 6 week survivor is characterized by pale cytoplasmic staining and fusiform shape. Multiple afferent terminals (arrows) are identified. Note that the apical surface is less than ½ of the diameter of a mature cell. **B:** Mature appearing short hair cell in 10 week survivor. Nerve terminal is a chalice shaped efferent. The basally located nucleus is characteristic of a mature cell. **C:** Regenerated short hair cell from animal 20 weeks after treatment termination with retained microvilli. The stereocilia bundle is poorly organized and centrally located, characteristic of an immature cell. **D:** This hair cell from a 10 week survivor has normal appearing stereocilia as well as a "dwarf" cluster based in a separate cuticular plate (outlined). The nerve terminal is a large bouton-shaped efferent, which is a common anatomical feature at 10 weeks.

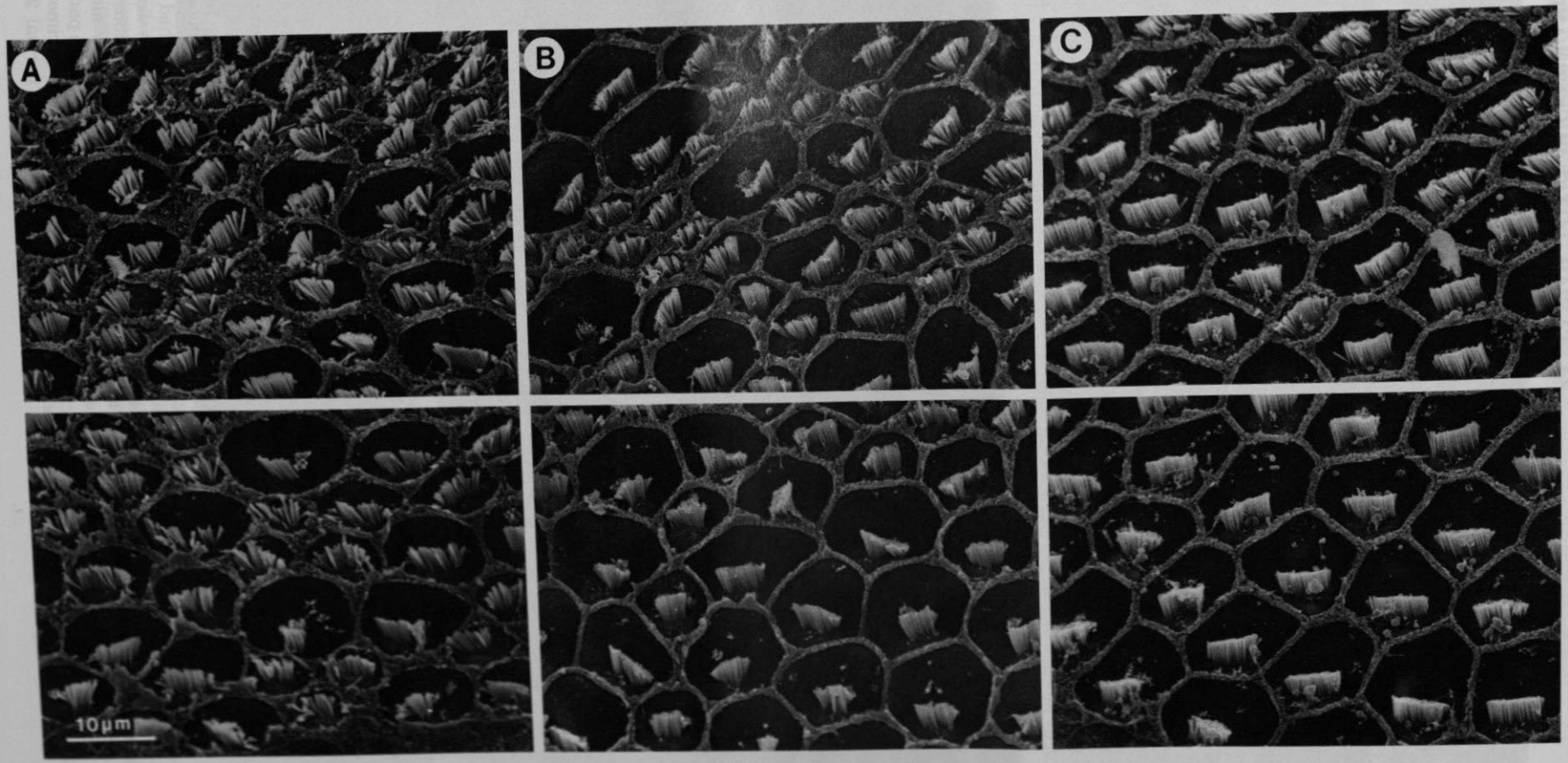

Fig. 9. Representative photomicrographs of study area from 3 experimental ears: 6 weeks (**A**), 15 weeks (**B**), and 20 weeks (**C**). Each is divided into a superior (Zone A) and inferior (Zone B) zone. Zones A and B are displayed in the upper and lower panels, respectively. Note the gradual reduction in the number of immature cells, identified by small apical cell surfaces, over time. Also note the larger number of immature cells that are located in the superior zone (Zone A) compared to the inferior zone (Zone B), especially apparent at 15 and 20 weeks.

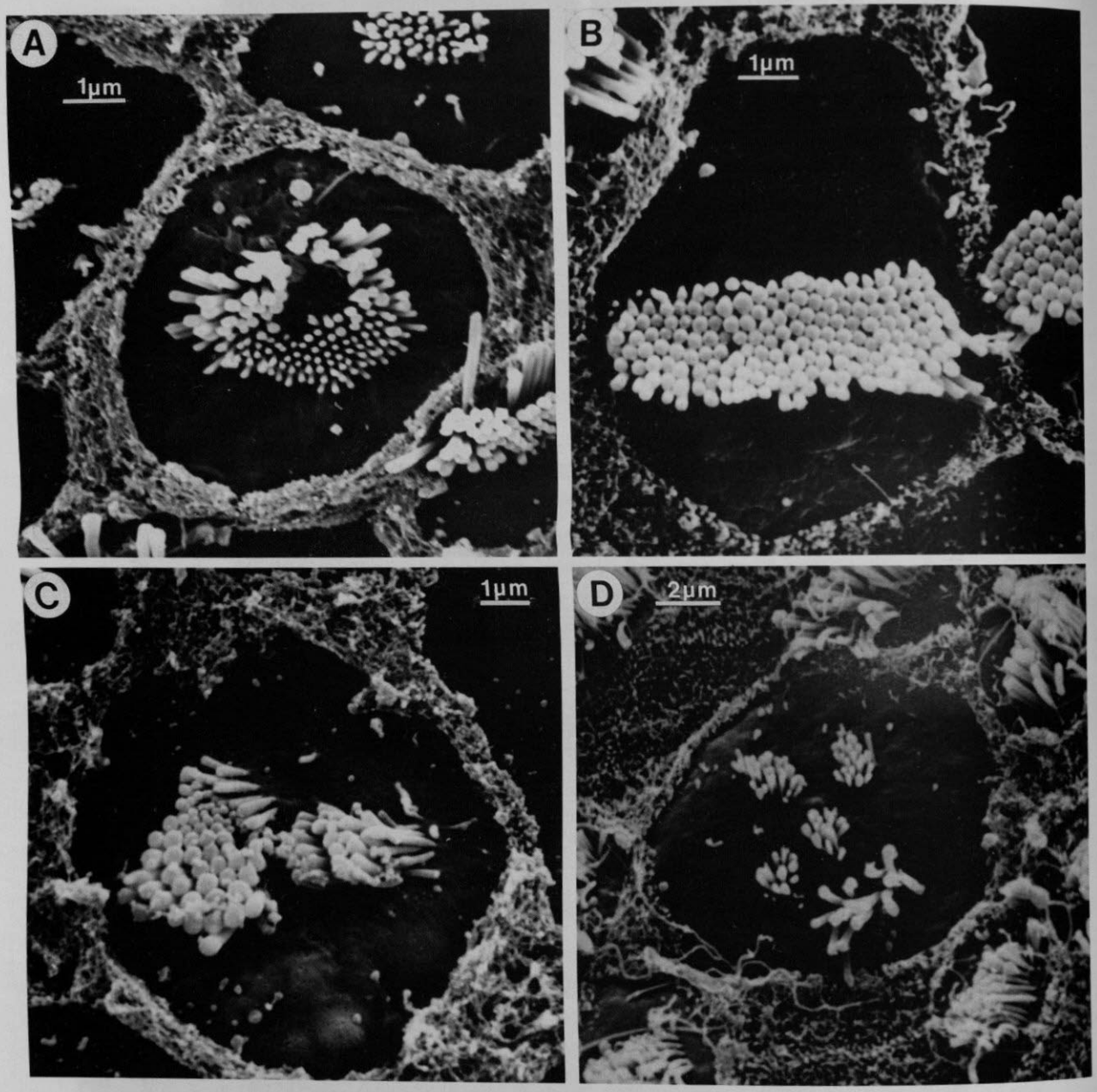

Fig. 10. **A:** Immature hair cell from 15 week survivor, characterized by an abnormal stereocilia alignment. The stereocilia and microvilli cannot be differentiated. The bundle polarity cannot be determined. **B:** Mature appearing hexagonal array of stereocilia in 20 week survivor. The eccentric position of the bundle is characteristic of a mature hair cell. **C:** "Giant" cell at 6 weeks with collapsed clumps of stereocilia. This may represent an ongoing ototoxic drug effect. **D:** "Giant" cell from 6 week survivor with multiple stereocilia bundles. This may represent an abortive attempt at regeneration. Note the small immature hair cells at the periphery.

ears. As noted above, bundles in the superior Zone (A) are normally oriented at a more oblique angle to the longitudinal axis of the papilla, while those in the inferior zone (B) are aligned parallel to it (Fig. 3A). This "orientation" gradient is fully established at 20 weeks (Fig. 3B).

The percentage of normally oriented hair cells also differed according to zone over time. Stereocilia bundles were aligned in greater numbers earlier in the regeneration process along the inferior border (Zone B) than in the superior region (Zone A). The number of bundles aligned to within normal limits in Zone A ranged from 70–80% in the experimental ears at 6 weeks, compared to a range of 80–100% in Zone B. By 15 weeks 95–100% of the hair cells in Zone B (inferior) possessed stereocilia bundles with rotations within normal limits. However, in the superior region (Zone A) 15–25% of the cells still had abnormally oriented bundles. By 20 weeks the bundles were normally oriented in 95–100% of the cells in both regions.

These trends are also observed in the more detailed analysis of bundle rotation (Fig. 13). In each panel of Figure 13 the results from a representative experimental animal are compared with the data from a normal 22 week old

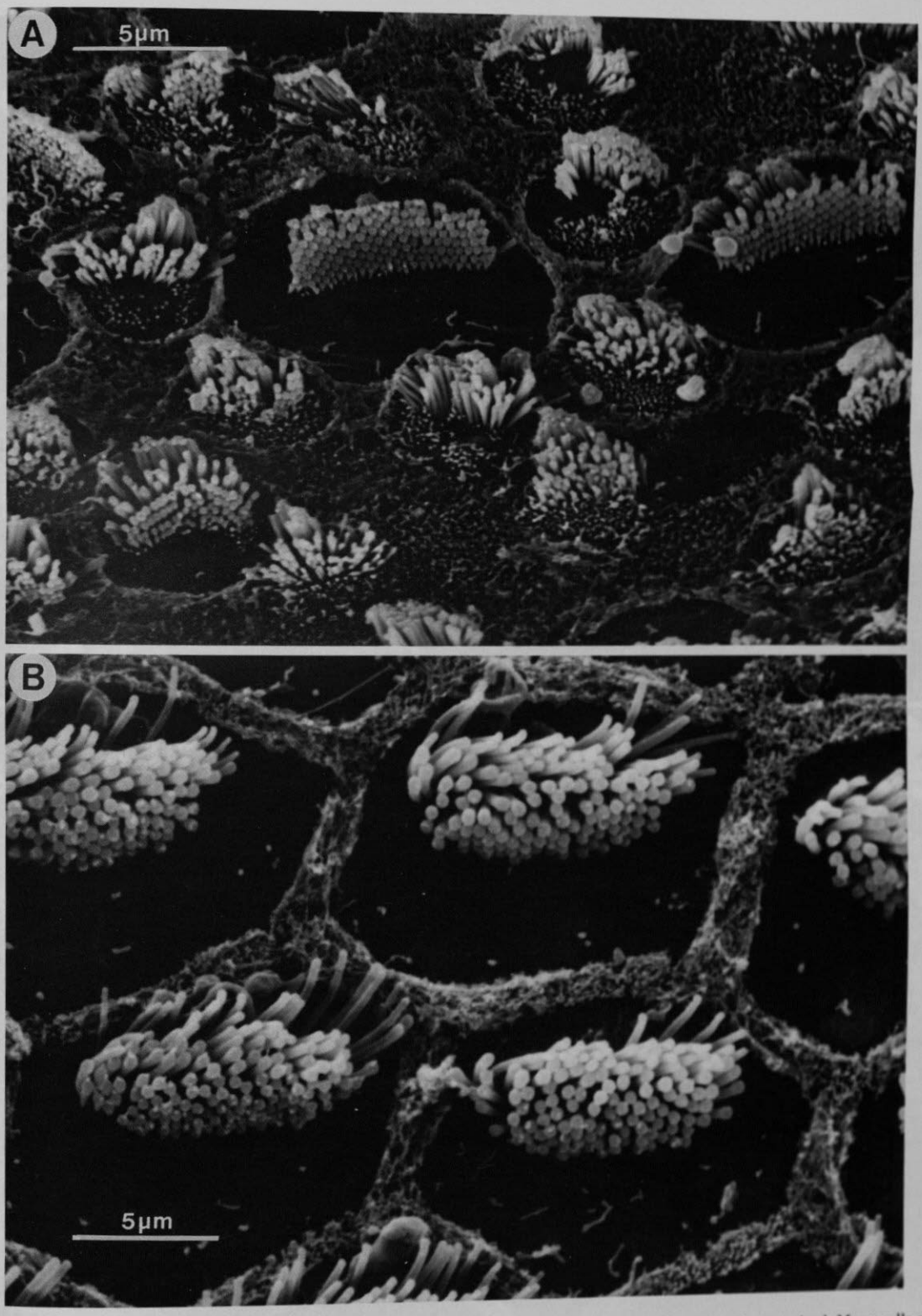

Fig. 11. **A:** Early repopulation of the papilla at 6 weeks is characterized by a heterogeneous population of hair cells of various sizes identified by immature stereocilia bundles. With few exceptions, the bundles appear as tufts and are poorly organized. Many cells are "disoriented." **B:** Short hair cells from age-matched control animal. The cells possess well organized appropriately oriented stereocilia bundles.

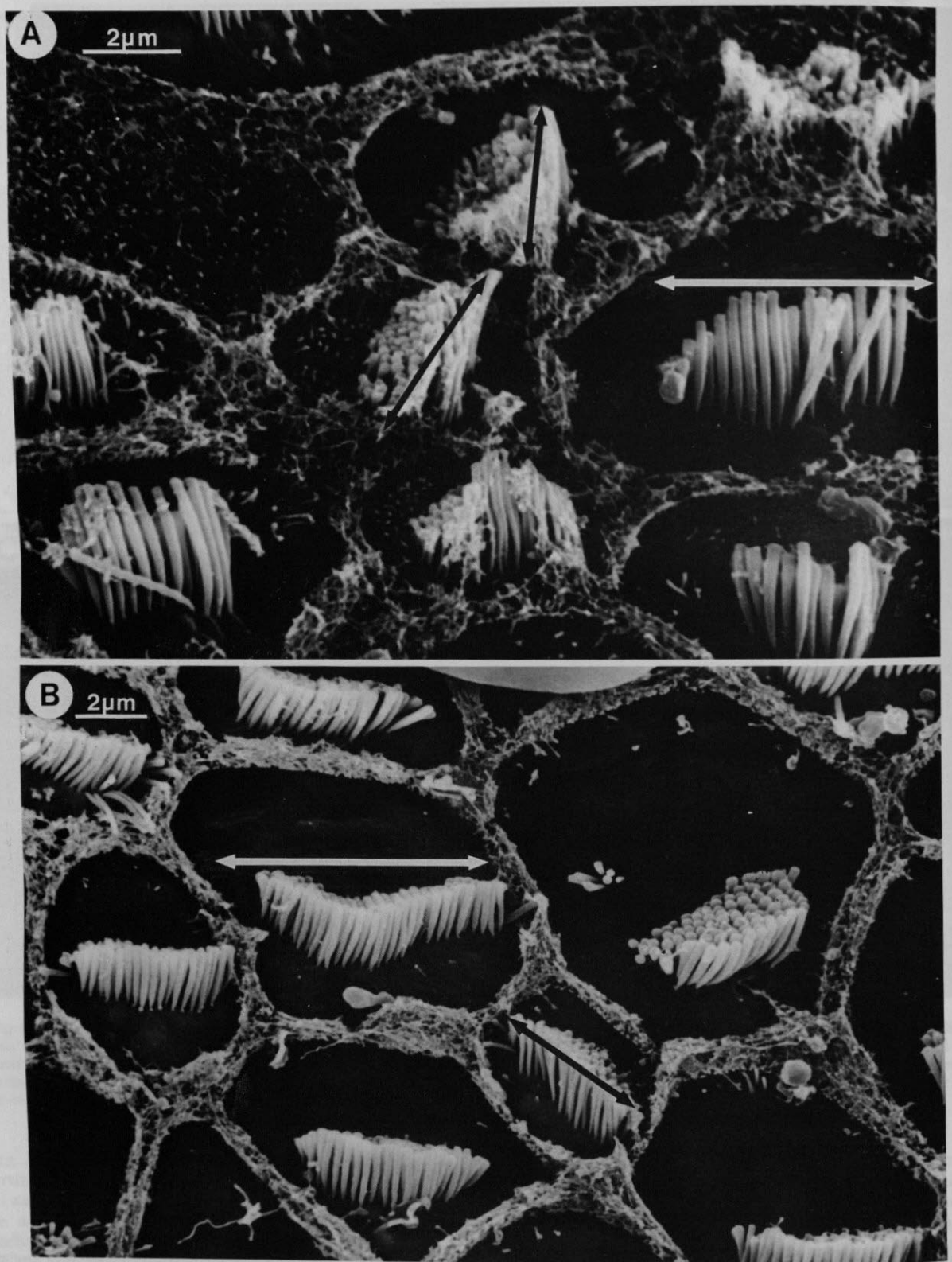

Fig. 12. **A:** At 6 weeks hair cell disorientation is common and the stereocilia bundle axis (black arrows) of these two immature hair cells is about 90° from the normal axis (white arrow). **B:** At 20 weeks almost all stereocilia bundles are oriented to within 20° of the normal axis (white arrow). Only occasional stereocilia bundles are observed to exceed the normal limits of axis rotation. The long axis of the disoriented cell is indicated by the black arrow.

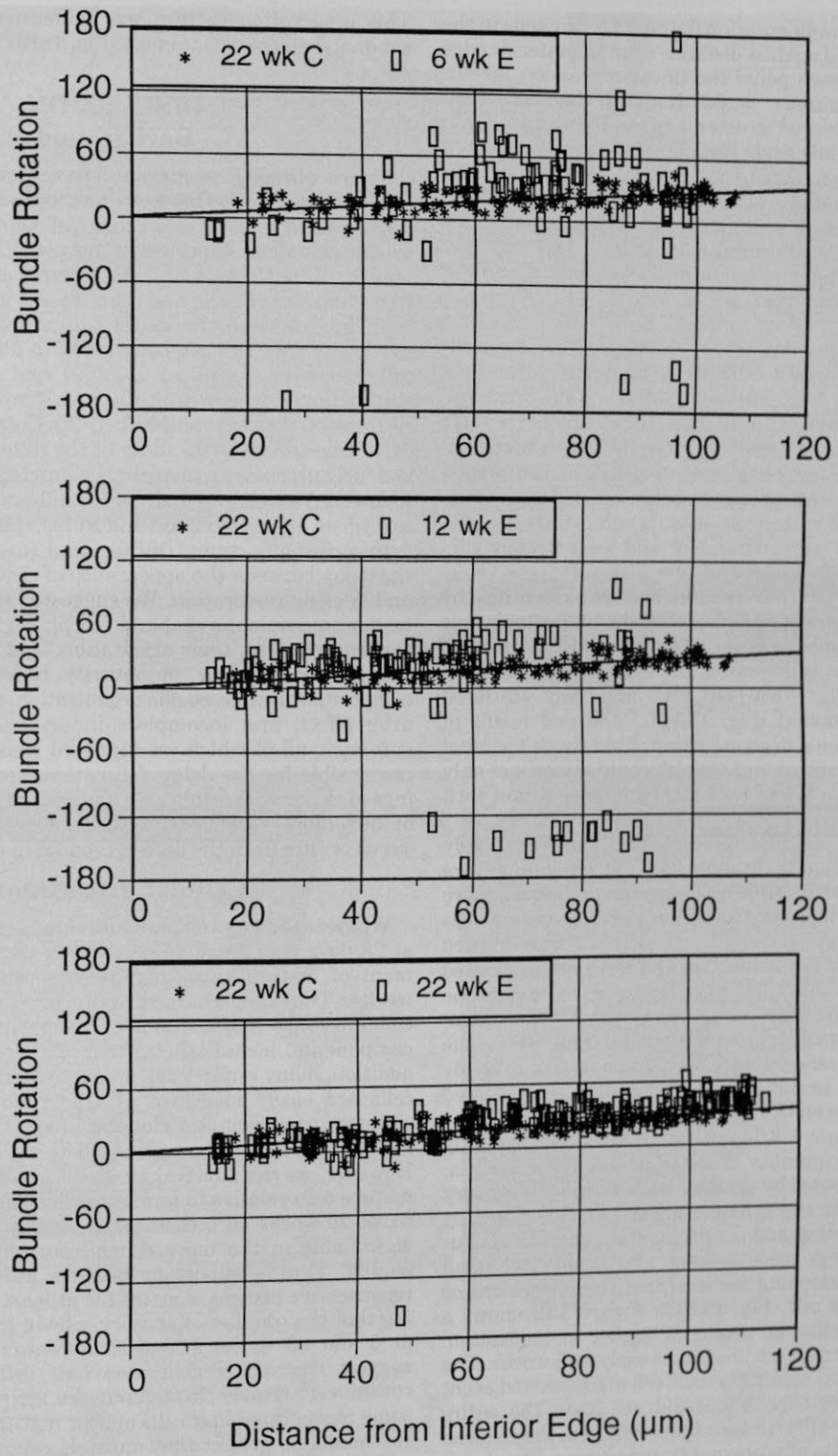

Fig. 13. Stereocilia bundle rotation versus distance from the inferior edge is shown for 80–120 hair cells in the 1,500 Hz short hair cell region. In each panel the data from a normal 22 week control animal (C) are compared to data from a single experimental animal (E) at the indicated survival time. Zero degree rotation indicates that the stereocilia bundle long axis is parallel with the inferior edge of the sensory epithelium, and the taller of two stereocilia is toward the superior side of the cell (staircase faces inferior edge).

control chicken. Bundle rotation for 80–120 hair cells in the study area is plotted against distance from the inferior edge of the papilla. In each panel the linear regression for the control animal is plotted (r = .64). It is clear that the 6 week survival animal showed greater scatter than any of the others (r = .16), while aside from one cell, which appeared turned 180° from normal, the 22 week survival animal showed a normal pattern of bundle rotations (r = .65). An animal studied after 12 weeks survival (middle panel) had a number of cells that appeared rotated by ~180° but was otherwise intermediate between the 6 week animal and the 22 week survivors (r = .29).

Innervation

Afferent terminals. Immature short hair cells of experimental animals 6 weeks after drug treatment often possessed more than four afferent endings (Fig. 8A). The terminals were small boutons that contained a granular cytoplasm and few organelles. The terminals were accompanied by membrane specializations consisting of a thickened synaptic membrane interposed between the cell membrane and a synaptic ball within the sensory cell cytoplasm. The balls were 0.2–0.3 μm in diameter and were usually surrounded by a row of vesicles.

Short hair cells, which were more mature as identified by stereocilia development, cell morphology, and cytoplasmic contents, tended to have fewer afferent terminals than the immature cells. In some cases, the cytoplasm within the nerve terminal was "washed out" and only scattered mitochondria remained (Fig. 14A,B). The end result of what may have been a degenerative process was a terminal empty of cytoplasmic contents, which could sometimes only be identified as an afferent terminal by its association with a synaptic ball.

At 10–15 weeks survival, afferent terminals were rarely seen in a mature appearing short hair cell. Only immature hair cells possessed multiple bouton endings. A much more common finding in cells from this survival period was multiple isolated synaptic balls. These balls were located within the sensory cell cytoplasm and were not associated with nerve terminals, but were adjacent to membrane surfaces opposed by supporting cells (Fig. 14C). The membrane was thickened in a circumscribed zone along the apposition of the sensory cell and the supporting cells. By 20 weeks, afferent terminals had been replaced or displaced by efferent endings on the mature short hair cells.

Efferent terminals. Efferent nerve terminals typically contained a large number of vesicles and mitochondria. They were not opposed by synaptic balls within the sensory cell cytoplasm, but the synaptic areas were identified by membrane thickening and a subsynaptic cisterna. Vesiculated nerve endings were present at 6 weeks as small boutons. Serial sectioning revealed that there were one or two terminals per cell (Fig. 15A,C). Figure 15B shows a typical boutonal efferent ending at higher magnification. Subsynaptic cisterna were present as early as 6 weeks. The cisterna were found within the hair cell cytoplasm adjacent to the synaptic membrane, but did not cover the entire membrane. As the efferent terminals enlarged, the cisterna overlaid larger areas of postsynaptic membrane. The distribution and configuration of these terminals persisted through the 15 week survival period. By 15 weeks, chalice shaped efferent terminals were observed with increasing frequency on mature appearing short sensory hair cells. At 20 weeks the mature sensory cells were innervated by one to three large chalice shaped nerve processes (Fig. 16A). This innervation pattern was indistinguishable from the age-matched control animals (Fig. 16B).

DISCUSSION

Background

Neuroepithelial regeneration in response to trauma was once controversial. However, hair cell and supporting cell regeneration in the avian cochlea following acoustic trauma or ototoxic drug exposure is now well documented (Cotanche, '87b; Cruz et al., '87; Corwin and Cotanche, '88; Ryals and Rubel, '88), and more recent investigations have been designed to better define the regenerative process. In particular, efforts have been made to identify the sensory cell precursor (Girod et al., '89) and to categorize the maturational process that ultimately results in functional restoration (Duckert and Rubel, '90; Cotanche and Corwin, '91). A better understanding of the differentiation process is clearly necessary to explain the functional delay observed following recovery of hair cell numbers provoked by ototoxic drug exposure (Tucci and Rubel, '90; Girod et al., '91).

In a previous study (Duckert and Rubel, '90), we noted the delay between the appearance of regenerated hair cells and hearing restoration. We suggested that the delay may be due to developmental and morphological deficiencies in the sensory cells, their orientation, and their innervation. We defined cochlear immaturity based on cytoplasmic composition and stereocilia organization, evidence of chronic drug effect, and incomplete innervation in the longest survivors, all of which we supposed were, at least in part, responsible for the delayed functional recovery. The findings of this earlier study are pertinent to the interpretation of our more recent data. References to 7 and 28 day survivors are from the data presented in this early study.

Cellular maturation

Whereas sensory cell maturation was notably incomplete at 28 days post-termination of drug treatment, a complement of mature appearing sensory cells is present at 6 weeks. They are characterized by a low-squat pitcher cellular shape and a complete complement of subcellular components, including cuticular plate and stereocilia. In addition, many sensory cell precursors and more immature cells are easily identified at this time by their lighter staining cytoplasm and globular shape. The population of regenerated hair cells was very heterogeneous at 6 weeks. However, as the survival times increases, the number of mature cells relative to immature cells also increases. Even so, at 20 weeks an occasional immature hair cell was still identifiable in the more central portion, Zone A, of the papilla. This is important because it indicates that the regenerative process is active for at least 20 weeks. Recalling that the number of sensory cells in the region sampled at 6 and 20 weeks is essentially equivalent, this would suggest that the regenerated hair cell population may continue to recycle throughout the recovery period. Since some regenerated hair cells appear mature by 6 weeks, it is reasonable to predict that multiple generations of sensory cells may be regenerated and exhausted before the recovery period has ended. The possibility that the heterogeneity of the sensory cell population may be due to prolonged drug effect will be discussed later.

Maturation of the sensory cells appears to progress in a gradient from the inferior to the more central zone of the basilar papilla. This maturation pattern is in keeping with

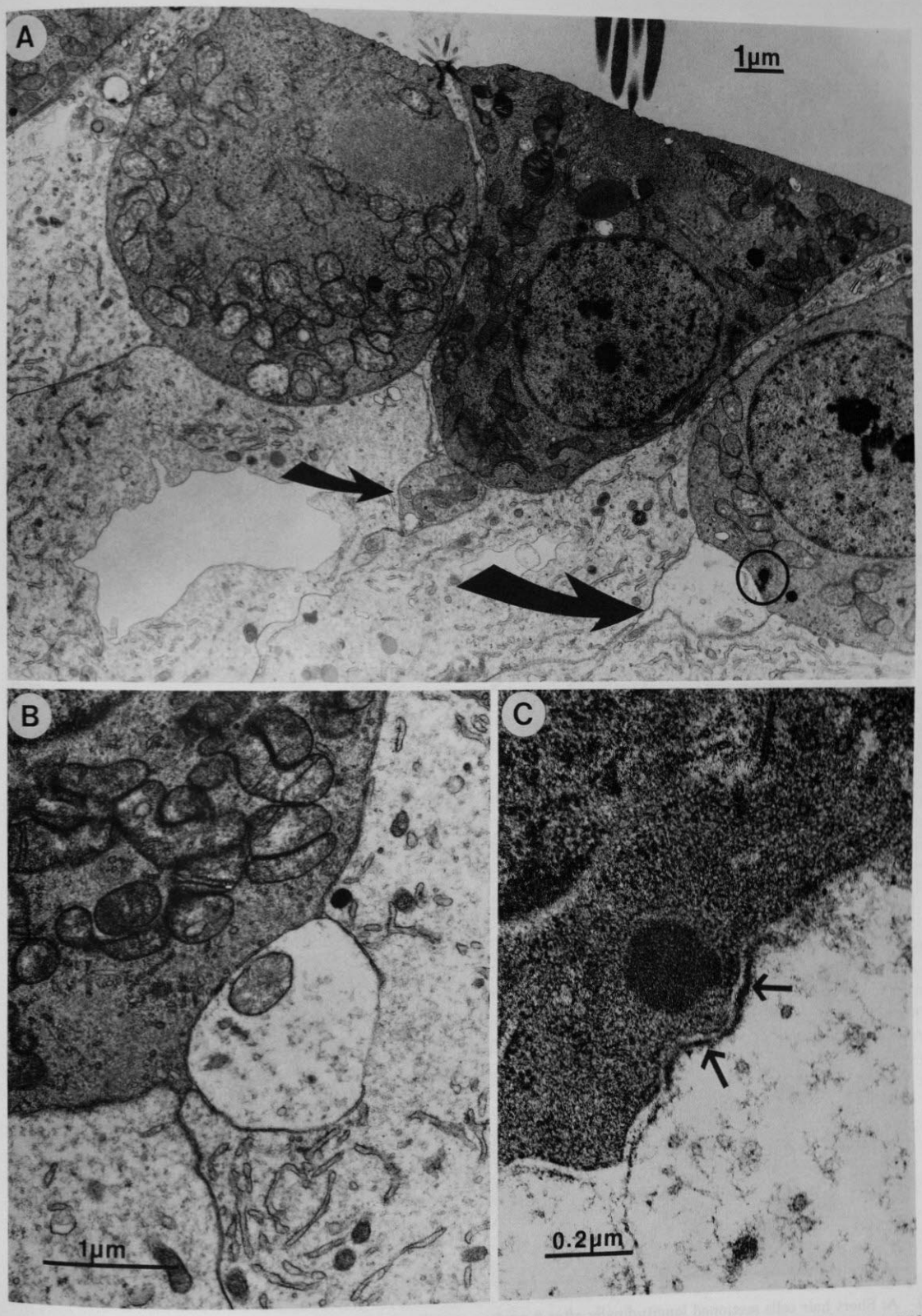

Fig. 14. **A:** A mature appearing short hair cell from a 10 week survivor possesses a bouton shaped efferent terminal (small arrow). The cell has assumed a pitcher shape and the cytoplasm stains more densely in contrast to the less mature neighbor hair cell. At its base is an afferent terminal "ghost" (large arrow) identified by the synaptic ball (circled). The cell shape remains globular, and the cytoplasm is less densely stained than its mature neighbor, but it is still easily differentiated from the supporting cells. **B:** Higher magnification of "depleted" afferent terminal. The terminal is bouton shaped and is adjacent to the base of a sensory cell. **C:** Higher magnification photomicrograph of "widowed" synaptic ball adjacent to supporting cell projection. Membrane thickening is apparent (arrows). No nerve terminal is present. This was determined by serial sectioning. Synaptic vesicles are not well seen.

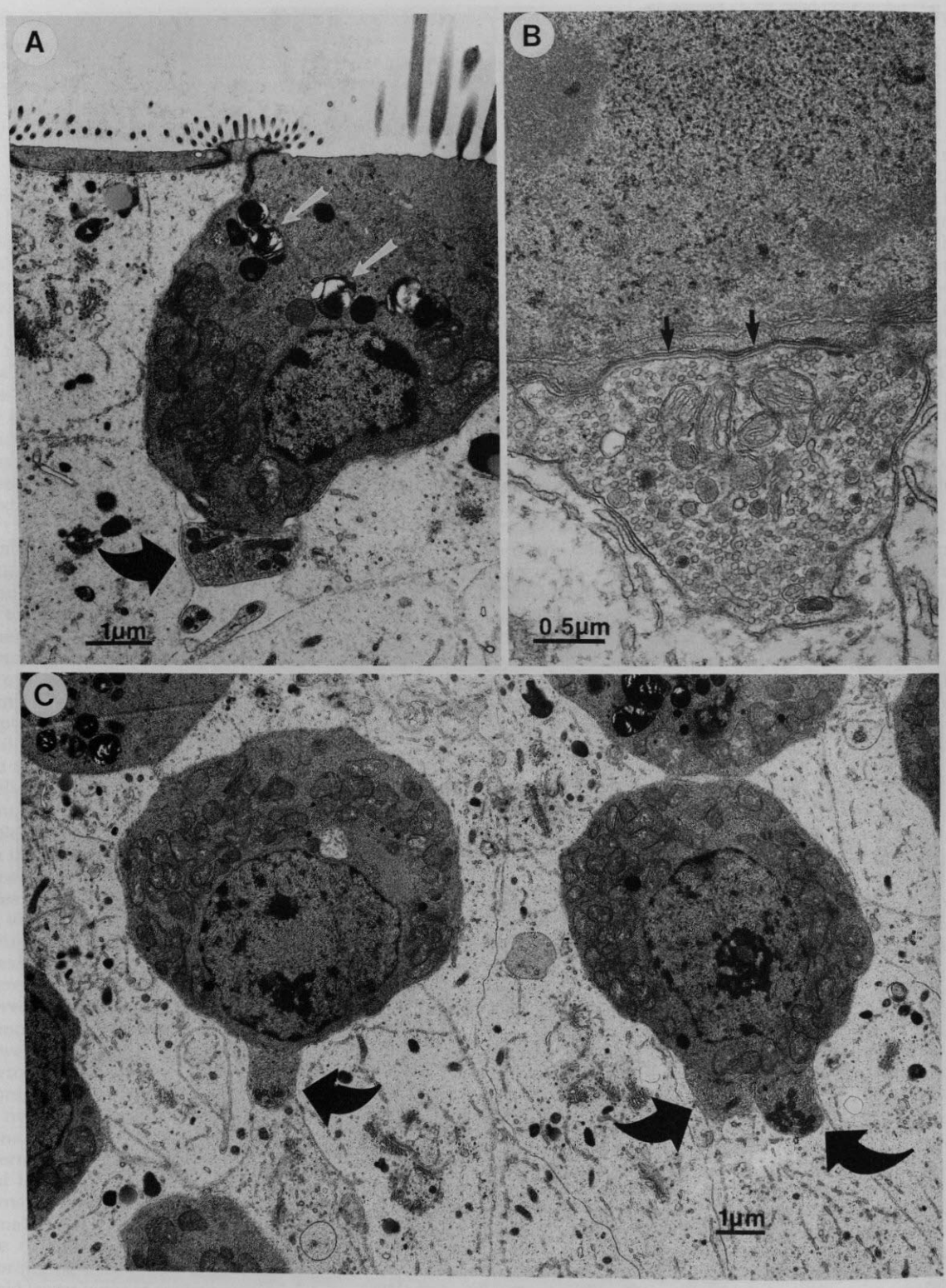

Fig. 15. **A:** Short hair cells sectioned longitudinally after 6 weeks possess one or two bouton shaped efferent terminals (black arrow). This cell contains a number of dense bodies (white arrows). These myelin figures are located in the apical part of the cell and probably evidence an ototoxic effect. **B:** Higher magnification of bouton shaped efferent terminal in 6 week survivor. Arrows indicate subsurface cisterna. The terminal contains numerous vesicles. The cisterna does not cover the entire synaptic membrane. **C:** Efferent terminals (arrows) opposed to hair cells that are sectioned axially. The terminals are bouton shaped and number 1–2 per cell, as determined by serial sectioning.

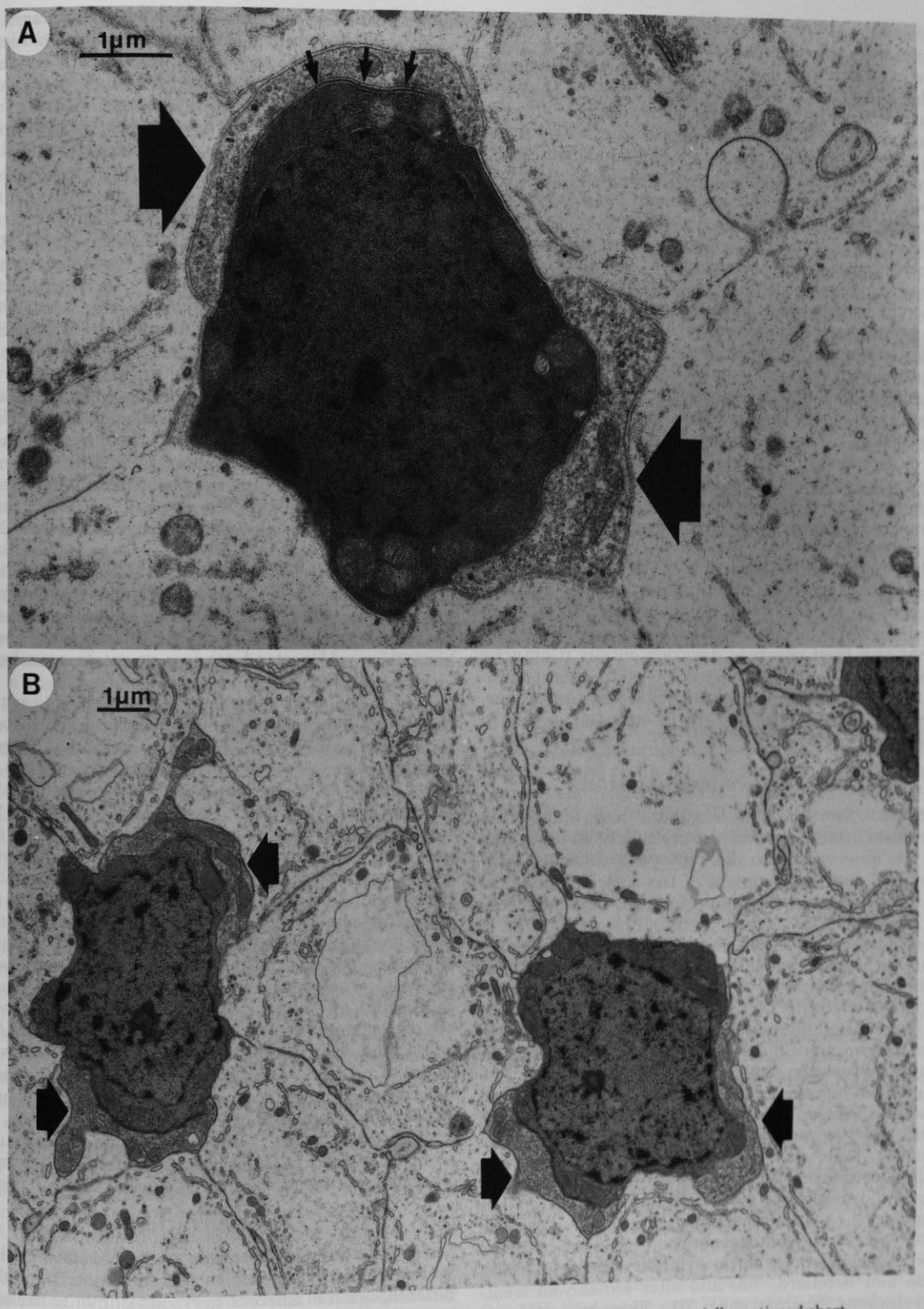

Fig. 16. **A:** Two chalice shaped efferent terminals (large arrows) opposed to an axially sectioned short hair cell from 20 week experimental animal. Subsynaptic cisterna is well developed (small arrows). **B:** Efferent terminals numbering two per cell (arrows) opposed to axially sectioned short hair cells from 20 week control animal.

avian embryogenesis, but is in distinct contrast to hair cell differentiation in the mammalian cochlea, which progresses from the inner hair cells to the outer hair cells (Bredberg, '68; Rubel, '78). Girod et al. ('89) have suggested that one potential precursor cell population is located adjacent to the inferiormost border of the sensory epithelium. After noise induced damage to both the sensory and supporting cells, these inferiorly based hyaline epithelial cells appear to spread out superiorly, and then proliferate and differentiate to repopulate the basilar papilla. According to this scheme, we would expect cell maturation in the central zone first. On the other hand, in the present study the supporting cell population remained intact after gentamycin treatment. In this case, if an inductive mechanism is initiated at the inferior border we might expect a maturational gradient from inferior to superior, repopulating the central zone last with mature hair cells, as we observed. However, if this scenario does occur, the later postmitotic cells would have to migrate through the supporting cell matrix to the more superior regions. Alternatively, organ support cells underlying the more superior regions (Girod et al., '89; Raphael, '92) may provide the later-maturing hair cells. A series of pulse labeling studies will be needed to fully evaluate the precursors and migratory patterns that lead to this gradient of maturation.

Cellular maturation is not limited to changes in cell contents, cellular organization, or shape, but also includes development of apical surface modifications. That apical surface maturation coincides with the cytoplasmic reorganization has also been observed developmentally (Cotanche and Sulik, '84). It is not unexpected, therefore, that stereocilia maturation and orientation, as will be discussed later, also proceed in a gradient from the inferior to superior in the basilar papilla.

Luminal surface modifications

Stereocilia. Stereocilia development and maturation were complete in a minority of sensory cells at 28 days. The process was observed to parallel embryonic differentiation and coincided with development of the cuticular plate, as occurs during normal embryological development (Cotanche and Sulik, '84; Tilney and DeRosier, '86). At 6 weeks, we also observed a disproportionate number of immature to mature appearing stereocilia bundles. This condition is reversed at 20 weeks, when stereocilia bundle maturity appears uniform. Clearly, the appearance of the mature hair cell bundle and coincidental cytoplasmic reorganization are two more manifestations of cell maturity, in anticipation of functional recovery.

The cyclical hair cell degeneration and regeneration predicted on the basis of morphological variability seem all the more likely given the variability in the stereocilia development. Cells with small apical surface areas and immature stereocilia tufts and cells possessing giant apical surfaces and disorganized stereocilia bundles are frequent contemporaries, especially early at the survival periods. The latter pattern of cell degeneration has been observed after noise exposure in chick cochleas (Cotanche, '87b; Consillas and Rebillard, '88) and after kanamycin treatment in chickens (Hashino et al., '91). These two cell types would appear to be on the opposite end of the regeneration-degeneration spectrum and provide evidence for ongoing cytotoxic effects.

Additional evidence for a prolonged ototoxicity affecting the newly regenerated hair cells is provided by the presence of the dense inclusions in the cytoplasm area of the regenerated hair cells. These were also observed in 28 day survivors and could represent mitochondrial degeneration (Duvall and Wersäll, '64) or lysosomes that would be responsible for eliminating mitochondrial debris from the cytoplasm (Ylikoski, '74). These types of cytoplasmic inclusions are also seen after kanamycin treatment of the chick cochlea (Hashino et al., '91).

Stereocilia bundle orientation

At 28 days after aminoglycoside treatment, bundle orientation was less ordered than in the normal condition, but was not quantified. At 6 weeks after drug treatment, bundle reorientation had progressed, and greater than 80% of the newly regenerated hair cells were aligned to within the normal limits. The method by which the stereocilia bundle orientation occurs is presently unclear, but the process follows the inferior-to-superior gradient of hair cell maturation on the papilla. Simply stated, the more mature sensory cells are more likely to possess mature appearing stereocilia bundles that are appropriately aligned with the longitudinal axis of the papilla. It was previously noted in the 7 to 28 day survivors that kinocilia were not present. Similarly, kinocilia were not observed throughout the 6–20 week survival period examined here. It is possible that kinocilia were destroyed during the dissections, but that seems unlikely since they were not observed on very short, immature bundles. If kinocilia are responsible for determining cellular polarity (Tilney et al., '88), then the absence of kinocilia would portend random orientation, unless some other mechanism is involved.

During ontogeny, synaptogenesis is occurring at the time of cellular reorientation and maturation (Hirokawa, '78; Whitehead and Morest, '85). It is attractive to speculate that the ingrowth of nerve fibers may have some trophic effect on the cell polarity, as has been suggested in the rat (Pirvola et al., '91). That innervation is necessary for hair cell orientation seems unlikely, however, based on data provided by Corwin and Cotanche ('89), who demonstrated that denervated cochleas that developed after transplantation to chorioallantoic membranes of host embryos showed normal development of the hair cell orientation pattern.

Cotanche and Corwin ('91) recently reported their observations on regenerated hair cell reorientation after noise exposure. They suggested that mechanical traction might be exerted on the stereocilia bundles by the developing tectorial membrane, resulting in cell reorientation. Alternatively, the kinocilium could make the contact with the tectorial membrane. Since the tectorial membrane is destroyed after acoustic trauma, regeneration of the membrane could promote reorientation of the hair cells, as they suggested. However, after ototoxic drug exposure, the tectorial membrane is not damaged, nor does it regenerate, leaving its contribution to bundle orientation unclear.

Innervation

The normal embryogenic sequence of sensory cell innervation in the avian cochlea remains controversial. Early differentiation of afferent nerve attachments to immature and even unerupted regenerated sensory cells was observed as early as 1 day after 5 days of gentamycin treatment (Duckert and Rubel, '90). This observation would support the theory that synaptogenesis precedes hair cell differentiation (Whitehead and Morest, '85), in contrast to the opposing view that hair cell differentiation precedes inner-

vation (Hirokawa, '78; Cotanche and Sulik, '84; Corwin and Cotanche, '89). The present observations in long-term survivors would confirm that, while synaptogenesis does occur early in the development of the sensory cell, the process of re-innervation is quite complex, and evolves throughout the 20 week recovery period.

Pujol and colleagues ('80) have stressed that differentiation of efferent synaptic endings follows afferent differentiation during embryogenesis in mammals and this finding extends to birds (Fermin and Cohen, '84). The same sequence is clearly seen in the regenerating hair cells. Immature appearing sensory cells that possessed synaptic complexes were commonly observed in apposition to afferent nerve fibers. Our observations are similar to those of Pujol et al. ('80): many of the afferent endings do not survive as the hair cell matures and either are displaced or degenerate during the process. What relationship, if any, exists between the cell differentiation, stereocilia maturation and orientation, and the process of innervation cannot be determined by this study. All that can be said is that these processes are temporally correlated. The appearance of what would appear to be degenerating afferent terminals on sensory cells has been described during mid and late synaptogenesis in the chick by Whitehead and Morest ('85), who concluded that more synaptic endings are formed than ultimately survive. The result was the appearance of many synaptic bodies within sensory cells that were unopposed by afferent nerve endings. At 10–15 weeks after drug treatment we also observed many synaptic bodies in sensory cells that were also unopposed by nerve terminals. Similarly, it is possible that these synaptic balls were in essence widowed as a result of afferent nerve terminal degeneration. On the other hand, these observations are not definitive. Hirokawa ('78) has concluded that the synaptic bodies that are devoid of contact with afferent nerve endings on the 14th day of embryogenesis are virginal.

The fact that afferent terminals were rarely seen on the more mature short hair cells in excess of 15 weeks after gentamycin treatment is consistent with the findings of Manley ('90) and Fischer et al. ('91), who observed no afferent contacts on short hair cells in the basal half of the normal chick basilar papilla. That afferent terminals are routinely seen on immature hair cells is therefore noteworthy. While it is tempting to propose that the association between developing hair cells and the afferent terminals represents a reciprocal induction interaction, we have insufficient data to support such a conclusion. It is equally possible that hair cell and nerve terminal development is initiated coincidentally by a common inductive mechanism.

Efferent terminals were also observed occasionally during the development of the sensory cell as early as 7 days after gentamycin treatment. At 6 weeks, the number of efferent terminals per cell was low, but increased as cochlear maturation progressed. The more immature terminals were smaller and in the shape of boutons, but matured to large, chalice shaped processes, which also increased in number to 1–3 per cell. A similar innervation ratio was described by Ryals et al. ('91) 4 months after exposure of quails to noise. While they reported that the number and configuration of the efferent terminals recovered to normal at 4 months, they also noted slightly more afferent junctions than normal. This observation provides indirect evidence that afferent degeneration may ultimately occur as a result of the regeneration process. It is probably not coincidental that the efferent sensory cell innervation appears to be complete and equivalent to the normal control animals at 20 weeks, when functional recovery is also nearly complete.

CONCLUSION

Regenerated short hair cells morphologically similar to those of age-matched controls were present at 6 weeks after drug treatment and not before 4 weeks, as previously reported. The theory that regenerated cells undergo degeneration or are aborted because of ongoing drug effect during the recovery phase is the subject of ongoing investigations. Other features of the maturation process, including stereocilia bundle orientation and innervation, are not complete until 20 weeks. The pattern of reinnervation is complex and appears to follow embryogenesis; however, the mechanisms responsible for reestablishing hair cell orientation and innervation also need to be identified. The potential influences of latent drug effects have been alluded to, but it is not yet apparent whether modifying the drug schedule will alter the rate of the regenerative process.

In summary, no single developmental feature appears to be solely responsible for the functional recovery that evolves over a 20 week period. Changes in internal cell structure, apical surface, cell orientation, and synaptogenesis are all in part responsible. However, which of these may be the more limiting factor, and to what degree these processes are dependent or independent of one another, are issues to be resolved in the future.

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