0030-6665/93 $0.00 + .20

CURRENT CONCEPTS IN HAIR CELL REGENERATION

Larry G. Duckert, MD, PhD, and Edwin W Rubel, PhD

Hair cell regeneration following various mechanical or chemical insults to the inner ear has become the subject of intense scientific research. In the past, it was generally accepted that postembryonic production of hair cells is limited to cold-blooded vertebrates, as is the ability to restore damaged populations of hair cells. In contrast, it was believed that birds and mammals lost the ability to regenerate hair cells as part of the evolutionary process. In both cases, the hair cells that populate the inner ear are produced early in embryogenesis.[14,25] Although some mitotic activity may continue, hair cell regeneration was not anticipated. This is in distinct contrast to a continuous production of hair cells in the saccule of fishes as well as regeneration of hair cells in the lateral line of amphibians.[2,20,21]

Recent attention to the inner ear of the bird was promoted by observations of hair cell repopulation after sensory cell elimination by ototoxic drugs.[7] In an experiment intended by Cruz et al[7] to examine the time course of hair cell degeneration in the chick cochlea after administration of aminoglycoside, neonatal chicks were given gentamicin injections for 10 days and were observed for periods ranging from 1 to 32 days. Following sacrifice, hair cell counts revealed that after 10 days of aminoglycoside treatment, there was nearly total elimination of the hair cells in the basal part of the cochlea. A week later, however, hair cell counts in the same region revealed partial repopulation of the sensory cells. Hair cell degeneration as a result of the ototoxic drug exposure progressed from the basal to apical portions of the cochlea, followed by a sequential recovery of the hair cell counts to normal.

These observations were subject to interpretation. Although it was

From the Department of Otolaryngology–Head and Neck Surgery, University of Washington, Seattle, Washington

attractive to consider that the new hair cells were being produced to replace those destroyed by the aminoglycoside, other interpretations were also entertained. It could also be possible that the hair cells were severely damaged or dedifferentiated by the ototoxic drug and then subsequently recovered.

In a contemporary study, Cotanche[5] examined the neonatal chick cochlea following acoustic trauma. The original purpose of this investigation was to study age differences and position of damage produced by sound overstimulation.[16,24,27] Nevertheless, hair cell regeneration was serendipitously observed a few days after the noise exposure. The scanning electron microscope enabled observations at a subcellular level to be made, and Cotanche[5] noted that the apical surface specializations or stereocilia of the suspected newly regenerated hair cells closely resembled the surface anatomy of immature hair cells during normal embryonic development.

These two studies clearly suggested that hair cell repopulation after exposure to either ototoxic drugs or noise was a realistic possibility and not merely a reflection of hair cell recovery. Subsequent studies made use of tritiated thymidine (^{3}H thymidine) to confirm that the repopulation of the cochlea was due to hair cell regeneration. The radioactive label is incorporated into replicating DNA during S phase of the cell cycle. It remains in the progeny cell nuclei postmitotically and as such is an index of cell division in the presence of the label. Labeled cells are identified by autoradiography.

Ryals and Rubel,[26] among others, used this technique to establish that noise damage to the avian cochlea induced stem cell mitosis and subsequent production of new hair cells as well as support cells. The birds used in their study were sexually mature quail, which were exposed to intense pure tone 1500-Hz noise, effectively destroying the hair cells in the middle portion of the cochlea. Following sound exposure, one group of birds was treated with ^{3}H thymidine for 10 days. The remaining birds were allowed to survive for 10, 30, or 60 days. The cochleas were then sectioned and examined by autoradiography or serially sectioned, and the hair cells counted. At the end of the recovery period, the hair cell population was restored to near normal (Fig. 1). Examination of the cochleas from birds treated with the ^{3}H thymidine demonstrated both labeled hair cells and labeled support cells, thus providing clear evidence of hair cell production by cell division and differentiation. In a parallel study by Corwin and Cotanche,[4] similar observations were made following hair cell destruction in the cochlea of neonatal chicks by acoustic trauma. They also used ^{3}H thymidine to demonstrate labeling of hair cells and support cells during the recovery phase.

The findings of these studies were significant, in that they provided substantive documentation for hair cell production by mitotic activity within a "quiescent" population of precursor cells. Jørgenson and Mathiesen[13] also reported proliferation and hair cell differentiation in adult budgerigars, again with ^{3}H thymidine. This study was noteworthy in that it was the vestibular epithelium, not the cochlea, that was examined using autoradiography to demonstrate labeled hair cells and supporting cells.

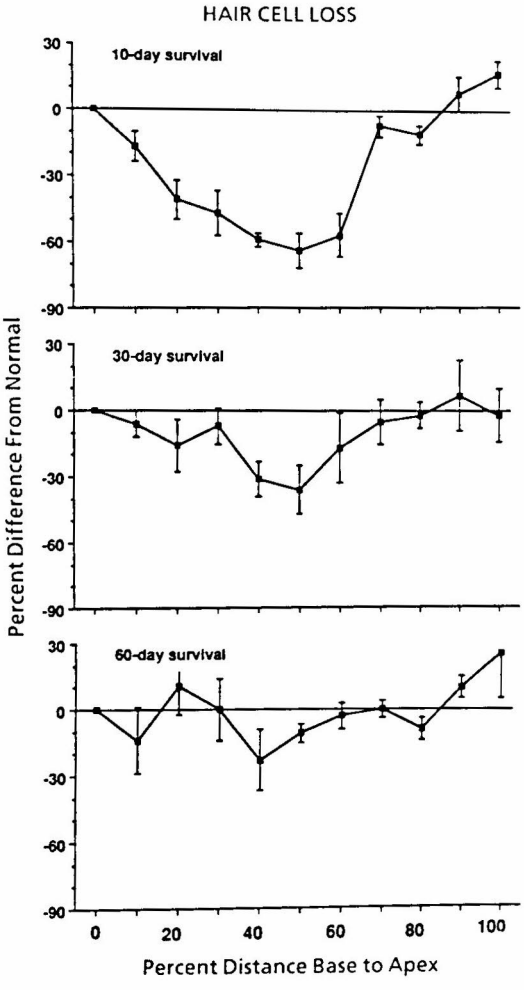

Figure 1. Regeneration of quail hair cells. The mean percentage difference in hair cell number compared with normal controls (N = 6), after acoustic trauma for (*A*) 10-day, (*B*) 30-day, (*C*) 60-day survival times. Normal hair cell number in controls is shown by a straight line at 0 along the cochlea from base to apex. Average percentage difference in hair cells from normal (± 1 SEM) is shown in 10% intervals along the cochlea from base to apex at each survival time (*N* = 6, 5, and 3 for 10-day, 30-day, and 60-day survival, respectively). (*From* Ryals BM, Rubel EW: Hair cell regeneration after acoustic trauma in adult Coturnix quail. Science 240:1774–1776, 1988; with permission.)

Similar observations by Roberson et al[23] indicated that there is a low level of continual turnover of vestibular hair cells in the neonatal chick.

In summary, although the concept of hair cell regeneration in the vertebrate population is not a new one, its discovery in the bird cochlea and vestibular organs has raised many questions regarding the inductive and regeneration potential of cells previously believed to be terminally differentiated. What specific implications this may have for the human condition has become the subject of many ongoing research projects designed to examine the process of hair cell regeneration in the bird cochlea in more detail, in an effort to transfer this knowledge to mammals, including humans. The remainder of this article is devoted to a more detailed description of the hair cell precursor population and the ultrastructural properties of the newly regenerated hair cells.

PRECURSOR POPULATION

Having established that repopulation of the avian cochlea is a result of a stem cell replication and subsequent differentiation of daughter cells, the next issue to be clarified is the identity of the stem cell population. Balak and associates[1] first addressed this problem in the lateral line organ of the axolotl, where he confirmed that supporting cells in the center of the receptor organ are progenitors of regenerated hair cells. Presson and Popper[21] have also identified resident embryogenic-like neuroepithelial cells that give rise to new vestibular hair cells in the stato-acoustic organs of fish.

If we consider a tangential section of the avian cochlea (Fig. 2), the likely precursors may be (1) remaining hair cells that dedifferentiate and become mitotically active, (2) supporting cells beneath the hair cells, (3) epithelial cells that are found either adjacent to the neural or abneural borders of the hair cell population, or (4) an unidentified stem cell population whose progeny migrate into the receptor epithelium following induction.

The goals of an experiment performed by Girod et al[12] were the

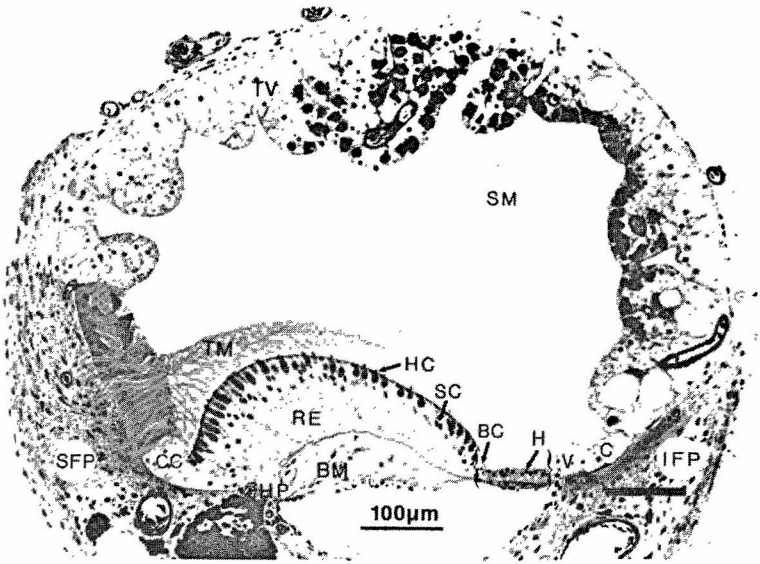

Figure 2. Light microscope photograph of a transverse section through the inner ear of the chicken. The receptor epithelium (RE: also called the *basilar papilla*) lies on the basilar membrane (BM) and is composed of hair cells (HC), supporting cells and unmyelinated terminal portions of cochlear nerve fibers. Two types of supporting cells are present in the receptor epithelium: organ supporting cells (SC) and border cells (BC). Hyaline cells lie inferior to the receptor epithelium (H) and extend inferiorly toward the inferior fibrocartilaginous plate (IFP). Vacuole cells (V) and cuboidal cells (C) rest on the inferior fibrocartilaginous plate. CC, clear cells; TM, tectorial membrane; SM, scala media; TV, tegmentum vasculosum; HP, habenula perforata; SFP, superior fibrocartilaginous plate. (*Adapted from* Rubel EW, Ryals BM: Patterns of hair cell loss in chick basilar papilla after intense auditory stimulation. Acta Otolaryngol 93:31–41, 1982; with permission.)

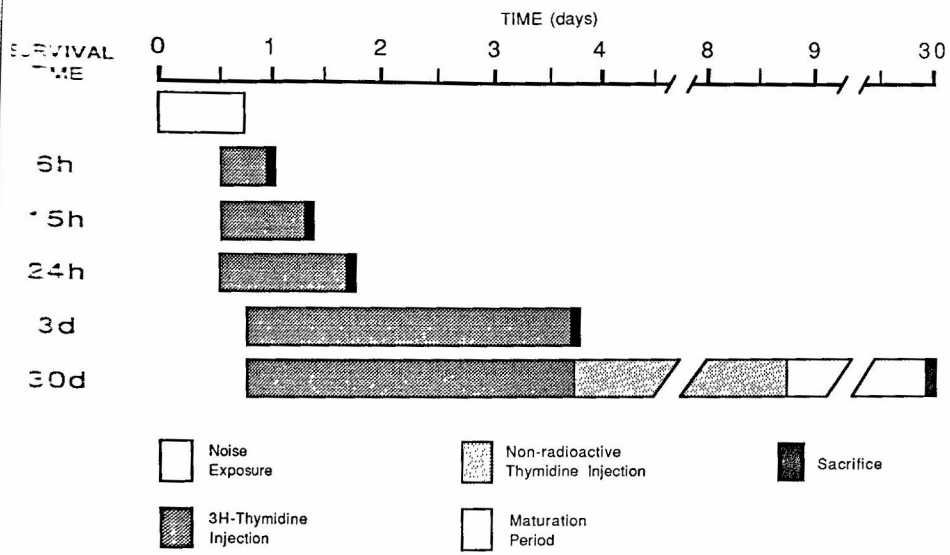

Figure 3. Schematic representation of the experimental design for "pulse labeling" experiment. The animals were divided into five subgroups based on survival time following the completion of the noise exposure. Tritiated thymidine labeling was initiated 12 hours into the noise exposure for the early survival groups ($\leq$ 24 hours) and on completion of the noise exposure for the longer survival groups ($\geq$ 3 days). $N = 4$ in each group. (*From* Girod DA, Duckert LG, Rubel EW: Possible precursors of regenerated hair cells in the avian cochlea following acoustic trauma. Hear Res 42:175–194, 1989; with permission.)

identification of the first cells to become mitotically active following acoustic damage and to follow the fate of their progeny. Neonatal chicks were exposed to an intense pure tone (120 dB sound pressure level [SPL] 1500 Hz) for 18 hours, as represented by the schematic in Figure 3. Either during (after 12 hours) or immediately after the tone exposure, the animals received injections of ^{3}H thymidine and were divided into survival groups of 6, 16, 24, or 72 hours. A final group of chicks was given an abundance of "cold thymidine" following 3 days of ^{3}H thymidine. These chicks were sacrificed 30 days after noise exposure. Following sacrifice, the ears were prepared for autoradiography or scanning electron microscopy (SEM). As shown in Figure 4*A*, the receptor epithelium 6 hours after noise exposure was severely damaged, and none of the cells in the sensory epithelium or its vicinity is labeled. Fifteen to 24 hours after noise exposure, rapid proliferation of the undifferentiated epithelial cells at the inferior edge of the receptor epithelium is observed (Fig. 4*B*), and many of these cells are labeled. With time, the single cell layer becomes two to three cells deep, and labeling is abundant (Fig. 5*A*). In addition, some of the labeled cells are recognized as immature hair cells on the basis of apical surfaces that possess the stereocilia in contact with the lumen. By 3 days following noise exposure, many labeled new hair cells and support cells are observed toward the inferior side of the receptor epithelium (Fig. 5*B*). The new hair cells that are characterized by more densely staining cytoplasm

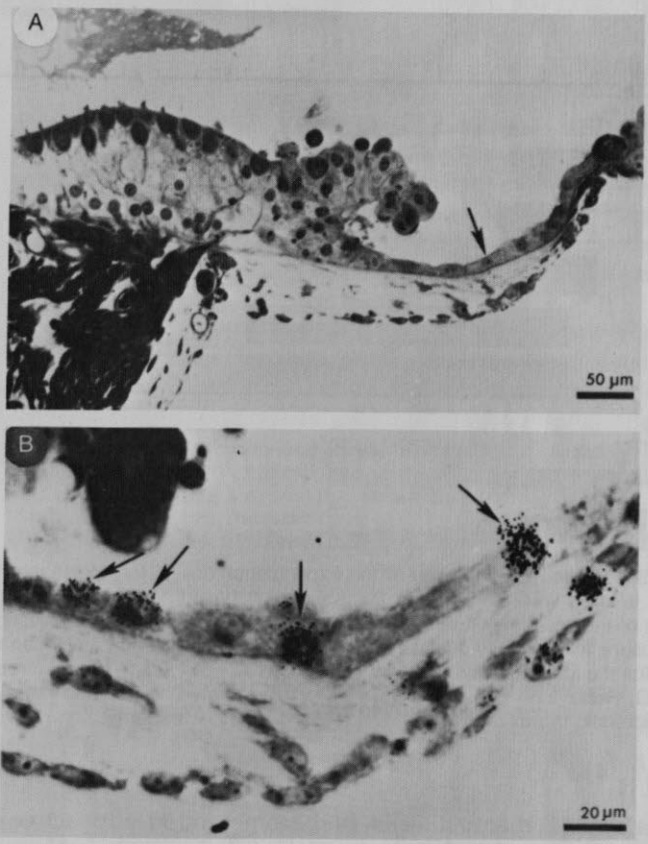

Figure 4. *A*, Transverse light microscopic sections through the 1500 Hz region of the chick cochlea 6 hours after completion of noise exposure, demonstrating extensive hair cell and supporting cell loss at the inferior edge of the sensory epithelium. Note the thin monolayer of cells spreading to cover the basilar membrane (*arrow*). (*From* Girod DA, Duckert LG, Rubel EW: Possible precursors of regenerated hair cells in the avian cochlea following acoustic trauma. Hear Res 42:175–194, 1989; with permission.) *B*, Inferior border of the sensory epithelium in the 1500 Hz region of experimental cochlea, 15-hour survival. Labeled nuclei (*arrows*) indicating mitosis within the cellular monolayer. (*From* Girod DA, Duckert LG, Rubel EW: Possible precursors of regenerated hair cells in the avian cochlea following acoustic trauma. Hear Res 42:175–194, 1989; with permission.)

than the adjacent supporting cells bear a tuft of stereocilia extending from the apical surface and a basal process that extends down to the basilar membrane (Fig. 6*A*). A scanning photomicrograph taken of the cochlea surface at the same time provides high-magnification examination of the surface ultrastructure of the new cells (Fig. 6*B*).

Labeled hair cells from the 30-day survival group, which were proliferated during the 3 days following noise exposure, cannot be distinguished from normal hair cells at the light microscope level. As shown by the scanning electron microscope, the surface morphology of the cochlea has been largely restored but has not recovered its normal mosaic pattern

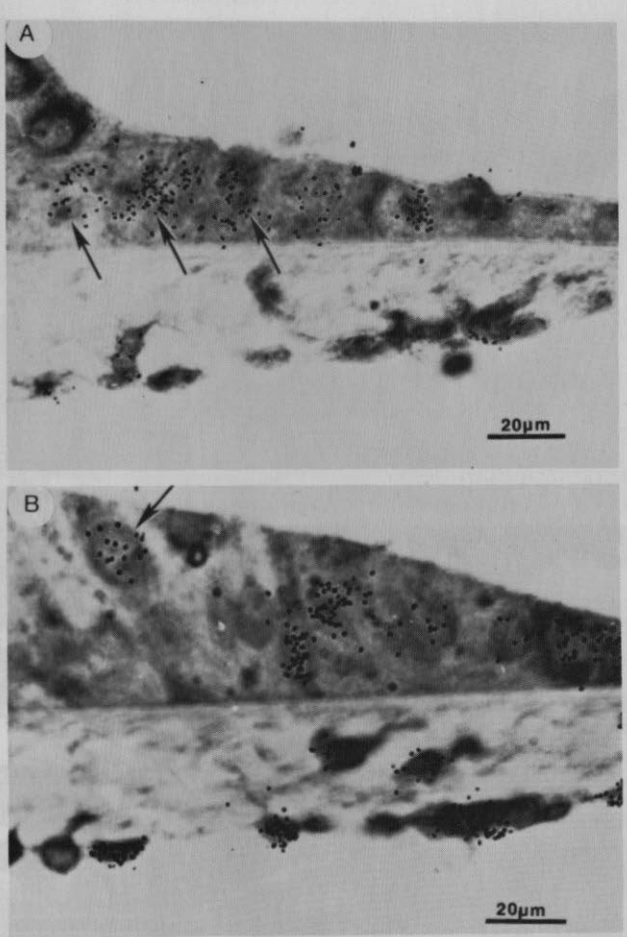

Figure 5. Inferior border of the sensory epithelium in the 1500 Hz region of experimental cochleas. *A*, 24-hour survival; multiple labeled nuclei (*arrows*) in the now stratified epithelium covering the basilar membrane. *B*, 3-day survival after inferior hair cell loss. Labeled regenerated hair cell (*arrow*) with lightly staining cytoplasm and a large round nucleus adjacent to the region of active proliferation. Plane of focus is on the overlying silver grains, putting the cells partially out of focus. (*From* Girod DA, Duckert LG, Rubel EW: Possible precursors of regenerated hair cells in the avian cochlea following acoustic trauma. Hear Res 42:175–194, 1989; with permission.)

(Fig. 7). The organization and orientation of the hair cells also remain abnormal at this time. In summary, the process by which the hair cell population is restored appears to be initiated by a proliferation of support cells near the inferior margin of the cochlea that spread out to cover the area that has been damaged. As the cells proliferate, they form a pseudostratified epithelium, and the nucleus becomes more apically located within the cell. Further differentiation then occurs as the cells assume the morphology characteristic of mature hair cells. In the process of maturation, morphologic changes occur within the cytoplasm and along the

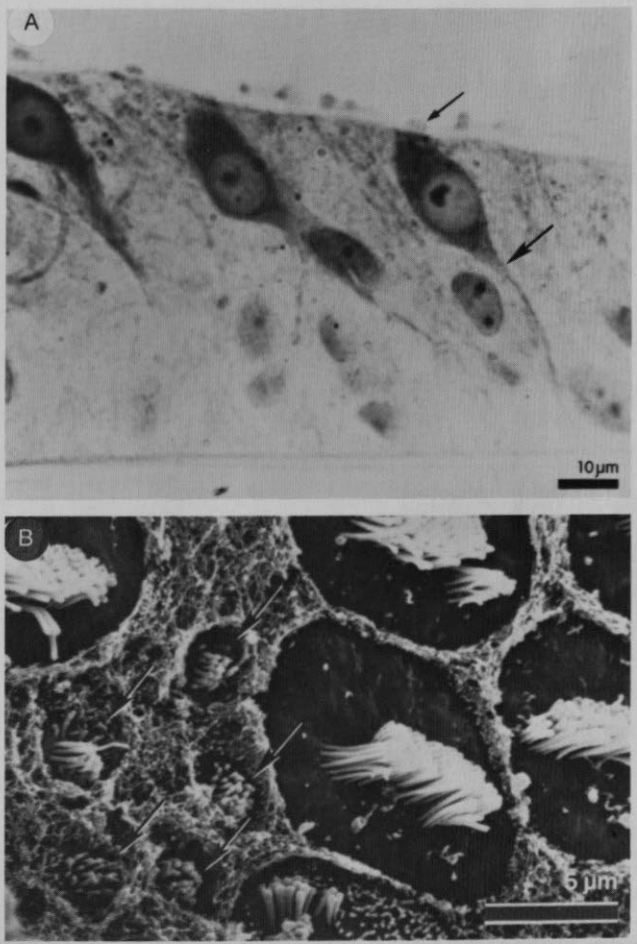

Figure 6. *A*, Immature regenerated chick hair cells seen 3 days after noise exposure. Plane of focus is now on the cells and not on the overlying silver grains. The regenerated hair cells have a unique appearance including a tall spindle-shaped cell body with lightly stained cytoplasm, a large, round nucleus, and very short stereocilia (*small arrow*). Processes seen at the cell bases are probably trailing cytoplasmic processes showing the path of migration from the basilar membrane to the lumen (*large arrow*). *B*, Scanning electron micrograph of the sensory epithelium in the 1500 Hz region of the chick cochlea at 3-day survival. Contrast the stereocilia of newly regenerating hair cells (*arrows*) to the adjacent mature hair cell. (*From* Girod DA, Duckert LG, Rubel EW: Possible precursors of regenerated hair cells in the avian cochlea following acoustic trauma. Hear Res 42:175–194, 1989; with permission.)

luminal surface. (These modifications are described in more detail in the following discussion of the ultrastructural properties of the regenerated hair cells.)

The ultrastructural characteristics of possible hair cell precursors, which are found in the inferior region of the receptor epithelium, have been studied in more detail.[19] In the inferior region of the cochlea (abneural), several rows of cells, termed border cells, separate the organ supporting cells from adjacent hyaline cells (Fig. 8). The border cells

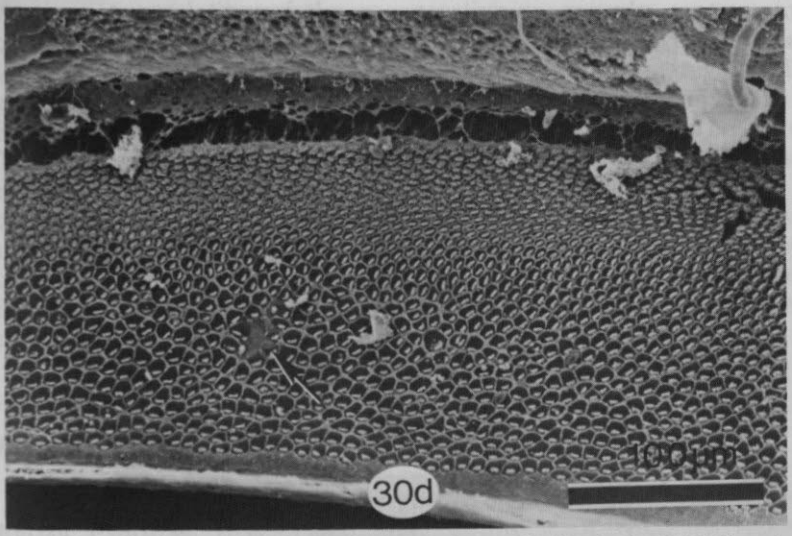

Figure 7. Scanning electron micrograph of the sensory epithelium in the 1500 Hz region of the chick cochlea at 30-day survival. Note the mild disorganization of the normal hair cell mosaic pattern and the very small residual scar (*arrow*). (*From* Girod DA, Duckert LG, Rubel EW: Possible precursors of regenerated hair cells in the avian cochlea following acoustic trauma. Hear Res 42:175–194, 1989; with permission.)

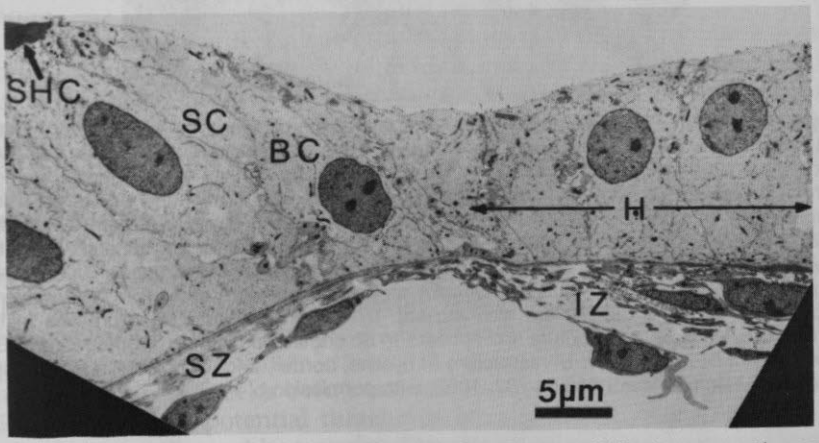

Figure 8. Inferior region of the chick basilar papilla and the neighboring supporting cells. Section is taken from the high-frequency region of the cochlear duct from a 17-day-old chick. A short hair cell (SHC) is on the left. At the inferior edge of the papilla, a few cells—the border cells (BC)—separate the sensory region from the hyaline cells (H). SC = organ supporting cells; SZ = superior zone of the basilar membrane; IZ = inferior zone of the basilar membrane. (*From* Oesterle EC, Cunningham DE, Rubel EW: Ultrastructure of hyaline, border, and vacuole cells in the chick inner ear. J Comp Neurol 318:64–82, 1992; with permission.)

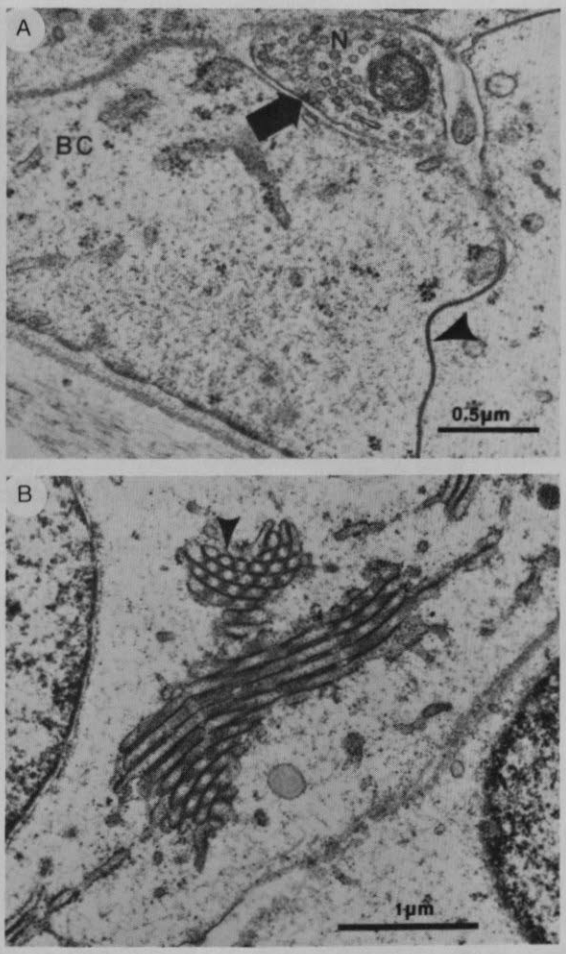

Figure 9. *A*, Section from the high-frequency region of the cochlea from a postnatal 17-day-old chick showing a junctional complex between a nerve fiber (N) and a border cell (BC). Synaptic specializations are present: A small presynaptic density in the neural element (*arrow*) is associated with numerous clear synaptic vessels. Arrowhead points to a low magnification of a gap junction between adjacent border cells. *B*, Unusual structure in hyaline cells: a striated rough tubular aggregate in the cytoplasmic matrix of a hyaline cell from the low-frequency region of the cochlea from a 22-week-old chicken. The structure is composed of parallel arrays of striated tubules. Circular profiles (*arrowhead*) derived from transverse sections through the tubules are also present. Rough endoplasmic reticulum lies in the immediate vicinity of the structure and appears to be continuous with it. (*From* Oesterle EC, Cunningham DE, Rubel EW: Ultrastructure of hyaline, border, and vacuole cells in the chick inner ear. J Comp Neurol 318:64–82, 1992; with permission.)

appear unspecialized, but even so, they can be easily distinguished on the basis of morphology from the adjacent supporting hyaline cells. Synaptic specializations may be observed between nerve fibers and the border cells (Fig. 9*A*). To the extent these cells may be potential precursors for regenerated hair cells, it is possible that this innervation may be involved in the

initiation or regulation of the regenerative process. In contrast to the border cells, the hyaline cells are quite specialized. Of particular interest, the cell cytoplasm is characterized by striated tubular aggregates. These aggregates consist of stacks of up to 31 tubules arranged in parallel (Fig. 9*B*). They may run in one direction or they may run at various angles up to 90 degrees to each other. These aggregates are seen only in the hyaline cells and are not present in the adjacent border or supporting cells. Synapses are also observed between nerve elements and the hyaline cells. The function of the tubular aggregates is unknown. As in the case of the border cell, however, it is attractive to speculate that the neuronal hyaline cell junctions may play some induction role in the hair cell regeneration process.

HAIR CELL REGENERATION AND HEARING RECOVERY

Given that avian hair cells are able to regenerate following acoustic or ototoxic exposure, it is of fundamental importance that the functional status of these hair cells be established. Certain technical difficulties have interfered with attempts to establish function following high-intensity noise exposure, because this insult also disrupts the integrity of the tectorial membrane.[6] In addition to loss and regeneration of the tectorial membrane, other morphologic changes may also result from trauma, making direct structure-function correlations difficult. For example, Ryals et al[28] have shown loss of ganglion cells following noise exposure, even though hair cells have regenerated. It is also characteristic of noise exposure to produce incomplete sensory cells degeneration, so it is unclear whether functional recovery is a result of hair cell repair or hair cell regeneration.

Some of these technical problems can be avoided by using ototoxic drugs, which can predictably produce a complete loss of tall and short hair cells on the basal 25% of the cochlea according to dose and duration of administration. This being the case, any sensory cells identified within this area of the cochlea following drug administration must be newly regenerated hair cells. It logically follows, therefore, that any recovery of function must also be due, at least in part, to the hair cell regeneration.

Two experiments have been undertaken to study the functional properties of regenerated hair cells. In both cases, neonatal chicks were given 10 days of gentamicin (50 mg/kg/day). In the first experiment, one subgroup of animals was tested after 5 days of aminoglycoside. The other groups were tested either after 10 days of treatment or were allowed to survive 1 to 24 weeks before sacrifice. In this first study, by Tucci and Rubel,[32] evoked potential thresholds were recorded from electrodes implanted near the cochlear nuclei. The stimuli were tone bursts at frequencies ranging from 250 to 5000 Hz. Separate groups of 8 to 16 chickens each were studied at the following survival times: 5 days of aminoglycoside treatment plus 1 day recovery, 10 days of treatment plus recovery times of 1 day, 1 week, 2 weeks, 3 weeks, 4 weeks, 5 weeks, and 16 to 20 weeks. In addition to the electrophysiologic measurements, the ears from

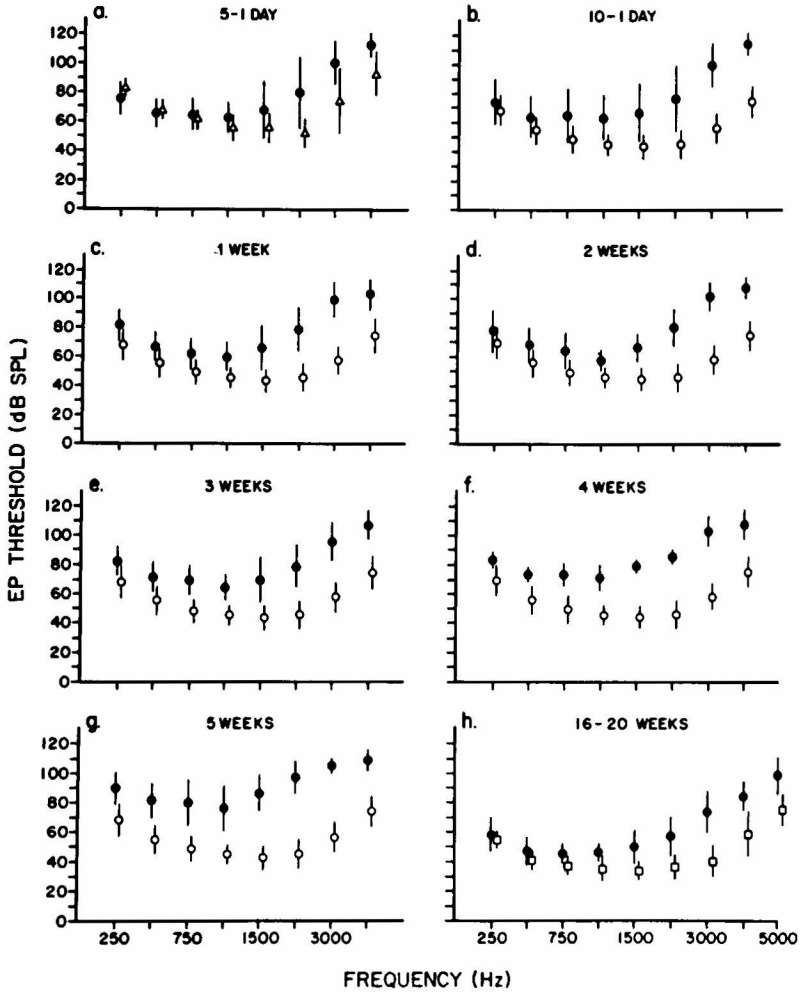

Figure 10. Evoked potential thresholds in dB SPL (mean (± 1 SD) for experimental (filled circles) and control (open symbols) animals at the survival intervals indicated following gentamicin treatment. (*From* Tucci DI, Rubel EW: Physiological status of regenerated hair cells in the avian inner ear following aminoglycoside ototoxicity. Otolaryngol Head Neck Surg 103:443–450, 1990; with permission.)

selected animals were processed for either scanning or transmission electron microscopy. These morphologic observations are discussed in the following section. The results are summarized in Figure 10. In each panel, the mean evoked potential threshold is shown as a function of the stimulus frequency for both gentamicin-treated and control animals at the indicated survival times. At day 1, significant hearing loss was restricted to frequencies above 1.5 kHz. From 1 to 4 weeks, the threshold shifts extend into the middle frequencies and eventually involve the low frequencies (500 Hz) at 4 weeks. At 5 weeks after aminoglycoside treatment, signifi-

cant potential threshold shifts are seen throughout the frequency range examined.

These physiologic measurements are consistent with the morphologic changes observed during the same period using the scanning electron microscope. One day following 5 days of aminoglycoside treatment, there is essentially complete loss of hair cells in the basal 20% of the cochlea (Fig. 11). That the hearing loss measured 1 day after 5 and 10 days of aminoglycoside treatment is not greater may be due to the fact that the tone bursts are not absolutely "place specific" with regard to the site of cochlear damage. Certainly, the picture of the cochlea 4 weeks after treatment is consistent with the threshold elevation observed (Fig. 12). The hair cell regeneration is incomplete at this time, and stereocilia orientation is in disarray. The cell population appears to consist of immature, mature, and degenerating cells. From 16 to 20 weeks after treatment, hearing recovery is apparent. As we will show later, by approximately 20 weeks, the normal cochlear surface morphology is also largely restored.

If the protracted recovery period seems disproportionate to the morphologic recovery observed with the scanning electron microscope, at least with respect to hair cell counts, then it remains a possibility that functional recovery is delayed by other morphologic abnormalities, such as ganglion cell or central nervous system abnormalities. In the second study, Norton and collaborators[18] endeavored to determine whether the protracted recovery time was due to hair cell or neural dysfunction. They recorded

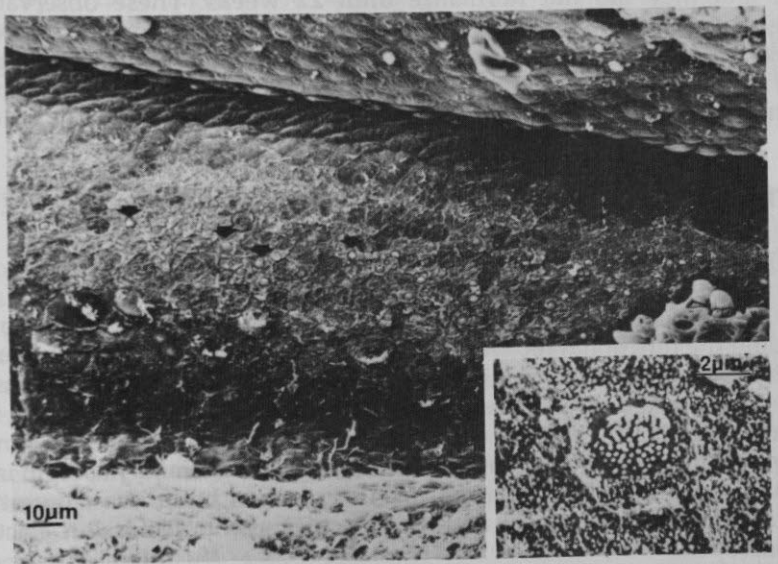

Figure 11. Scanning electron micrograph of proximal (basal) portion of the basilar papilla, approximately 400 μm from the tip, 1 day following 5 days of aminoglycoside treatment. Newly erupted hair cells are identified by stereocilia tufts (*arrows*). *Insert*: Higher magnification scanning electron photomicrograph of newly regenerated hair cell identified by sensory hair tuft. (*From* Duckert LG, Rubel EW: Ultrastructural observations on regenerating hair cells in the chick basilar papilla. Hear Res 48:161–182, 1990; with permission.)

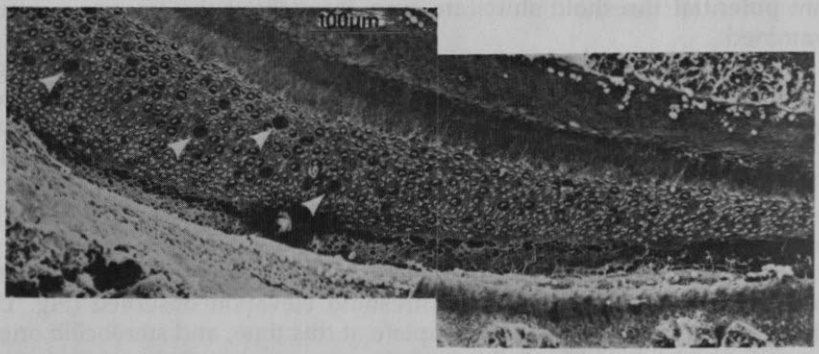

Figure 12. Scanning electron micrograph of the proximal one third of the reticular surface at 28 days following gentamicin treatment. Numerous "giant" hair cells (*arrows*) are randomly distributed across the surface. Large areas are devoid of normal hair cells and the mosaic pattern is disrupted. (*From* Duckert LG, Rubel EW: Ultrastructural observations on regenerating hair cells in the chick basilar papilla. Hear Res 48:161–182, 1990; with permission.)

otoacoustic emissions and auditory evoked potentials from neonatal chickens that had received gentamicin injections for 10 days. Five weeks following treatment, hair cell function as determined by evoked otoacoustic emissions and evoked potentials was still abnormal. By 8 to 14 weeks, the otoacoustic emissions were similar to those controls, whereas the auditory evoked potentials of the experimental animals were still abnormal, and they did not normalize until 22 weeks. These observations suggested that hair cell recovery does precede neural recovery by several weeks. This study, in conjunction with the first study,[32] strongly suggests that the regenerated hair cells are functional and relay information to the central nervous system.

ULTRASTRUCTURAL PROPERTIES OF REGENERATED HAIR CELLS

Early Phase

Having identified the apparent disparity between hair cell repopulation and the functional recovery in the avian cochlea following gentamicin treatment, further investigation was undertaken to examine the sensory cell population during maturation. Duckert and Rubel[9] theorized that examination of the sensory cells at a subcellular level would identify morphologic deficiencies that would help explain their physiologic inactivity. Their study was organized in two parts. In both cases, investigational instruments were the scanning electron and transmission electron microscopes.

In the first experiment, 20 newly hatched chickens were divided into three groups of 6 animals each and two control subjects. One group of six animals was injected with gentamicin sulfate, 50 mg/kg/day for 5 days, and the animals were sacrificed 1 day after the injection period. The

remaining animals received the injections for 10 days. Following the injection period, a group of 6 animals was allowed to survive 7 days and another group 28 days before sacrifice. The control animals received no gentamicin. At the end of the survival periods, the animals were sacrificed, and the temporal bones were processed for either scanning or transmission electron microscopy.

Sensory Cells

The proximal or basal portion of the cochlea was examined 1 day following 5 days of aminoglycoside treatment to ensure that hair cell degeneration was complete. Apical surfaces of newly regenerated sensory cells possess sensory hair tufts that are longer and larger in diameter than surrounding microvilli. At this stage, the primitive sensory cells are irregular and elongated. Unerupted cells could be identified from surrounding supporting cells by their more granular and dense-appearing cytoplasm (Fig. 13*A*). More mature cells contain greater numbers of mitochrondria, clusters of ribosomes, microtubules, and vesicles.

From 7 to 28 days post-treatment, erupted and unerupted cells are present along the proximal portion of the cochlea. More mature cells are characterized by a denser-staining cytoplasm within which the nucleus has assumed a more basal position. Erupted cells are identified by unique globular shape. By comparison, unerupted cells remain more irregular or cylindrical (Fig. 13*B*).

By 28 days, regenerated cells repopulate the proximal one third of the surface of the basilar papilla. Various stages of maturation based on the size and development of the apical surfaces are present. Few, if any, of the newly regenerated hair cells at this time resemble the squat pitcher so characteristic of the mature short sensory hair cell.[30]

Stereocilia

Immature tufts of stereocilia are seen with a scanning electron microscope as early as 1 day after termination of drug treatment (Fig. 14*A*). The diameter of the apical surface of the newly erupted sensory cell is small, averaging only 2 to 3 μm. Stereocilia are arranged concentrically on the surface. Within 7 days, the stereocilia tufts differentiate into staircase-shaped bundles that contain stereocilia of varying lengths. The development of the stereocilia bundles is paralleled by development of the cuticular plate. By 28 days, stereocilia organization has matured to closely resemble the normal control condition. The packing is tighter, and the stereocilia are arranged hexagonally (Fig. 14*B*). The bundles contain between 150 and 170 stereocilia, as has been described in the normal chick cochlea (Fig. 14*C*).[31]

Bundle orientation was determined on the basis of a line drawn perpendicular to its axis. At 28 days, no effort was made to quantify the degree of bundle or hair cell orientation; however, it was our qualitative assessment that orientation was quite disrupted.

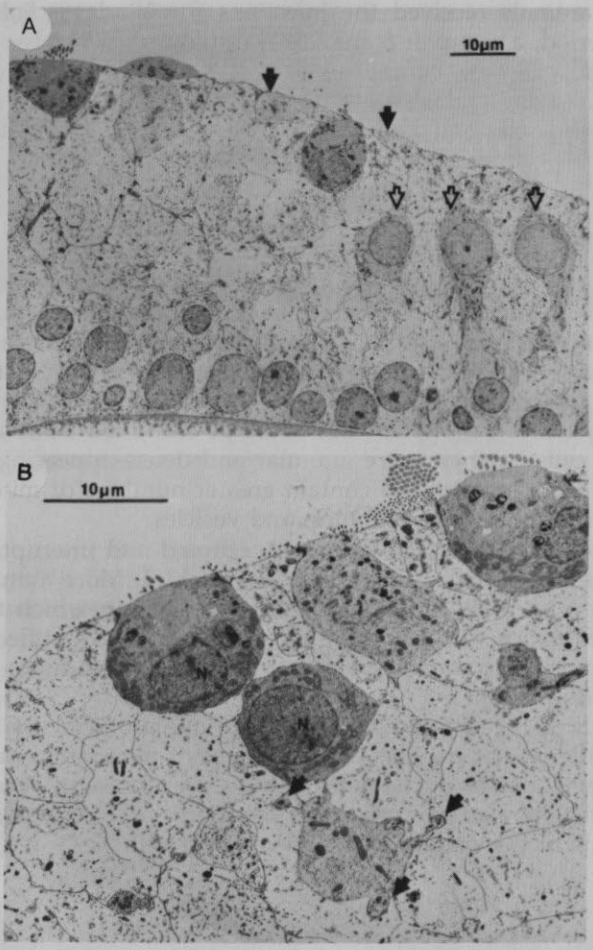

Figure 13. *A*, Transmission electron micrograph showing immature sensory cells (*open arrows*) below the luminal surface (*solid arrows*) of the basilar papilla where they are surrounded by supporting cells. The primitive hair cells are distinguished from the supporting cells by a granular and darker staining cytoplasm. *B*, Transmission electron photomicrograph showing erupted and unerupted regenerated hair cells 28 days after gentamicin treatment. The cells at the surface are rotund with a basally located nucleus (N). Arrows indicate nerve fibers. (*From* Duckert LG, Rubel EW: Ultrastructural observations on regenerating hair cells in the chick basilar papilla. Hear Res 48:161–182, 1990; with permission.)

Innervation

Nerve endings observed 1 day after drug injection are generally small boutons. Opposing these boutons are membrane specializations characteristic of afferent terminals. These consist of thickenings of the neural membrane opposed by a synaptic ball structure within the hair cell (Fig. 15*A* and *B*). At 7 days, both unerupted and erupted sensory cells are associated with these afferent terminals. At 28 days after treatment, as many as 4 to 5 afferent terminals oppose the base of some regenerated

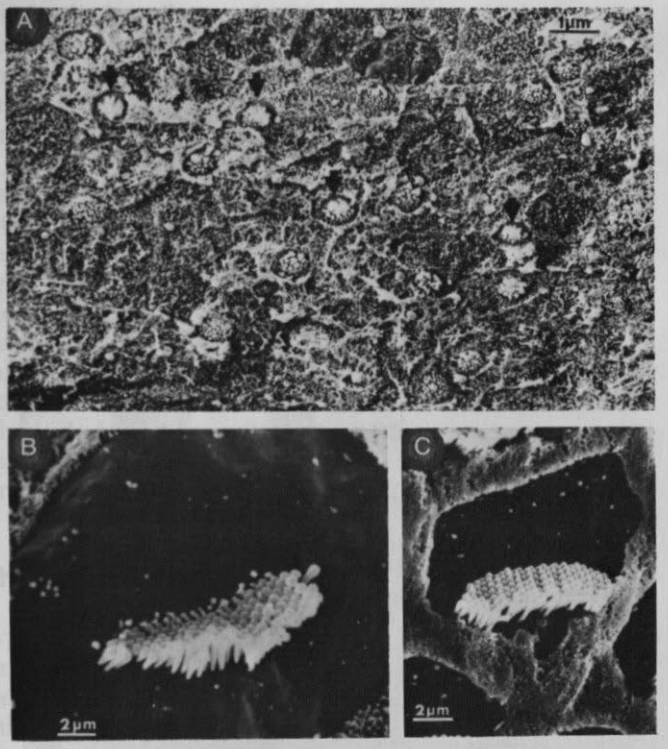

Figure 14. *A*, Scanning electron micrograph of reticular surface 1 day following termination of gentamicin treatment. Early stereocilia are differentiated from the surrounding microvilli on the basis of the increased width and length. Arrows indicate stereocilia tufts. *B*, At 28 days post-termination of gentamicin treatment, the stereocilia bundle is located centrally 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 *C*). *C*, Apical surface of short sensory hair cell from control animal showing hexagonally packed lattice of stereocilia. (*From* Duckert LG, Rubel EW: Ultrastructural observations on regenerating hair cells in the chick basilar papilla. Hear Res 48:161–182, 1990; with permission.)

hair cells, and efferent terminals are exceedingly rare. It is noteworthy that although afferent synaptogenesis has been reported early in the course of hair cell differentiation during embryogenesis,[34] efferent terminals on mature short hair cells at the basal end of the cochlea are generally not seen in the normal postembryonic animal.[17]

Efferent terminals that are more typically seen opposed to the basal portion of short hair cells in the normal condition were still infrequently seen at 28 days. When present, these vesiculated nerve terminals were small and bouton-shaped (Fig. 15*C*).

Structure and Function

These observations of hair cell maturation within the first 28 days of termination of drug treatment clearly document the recovery of cellular

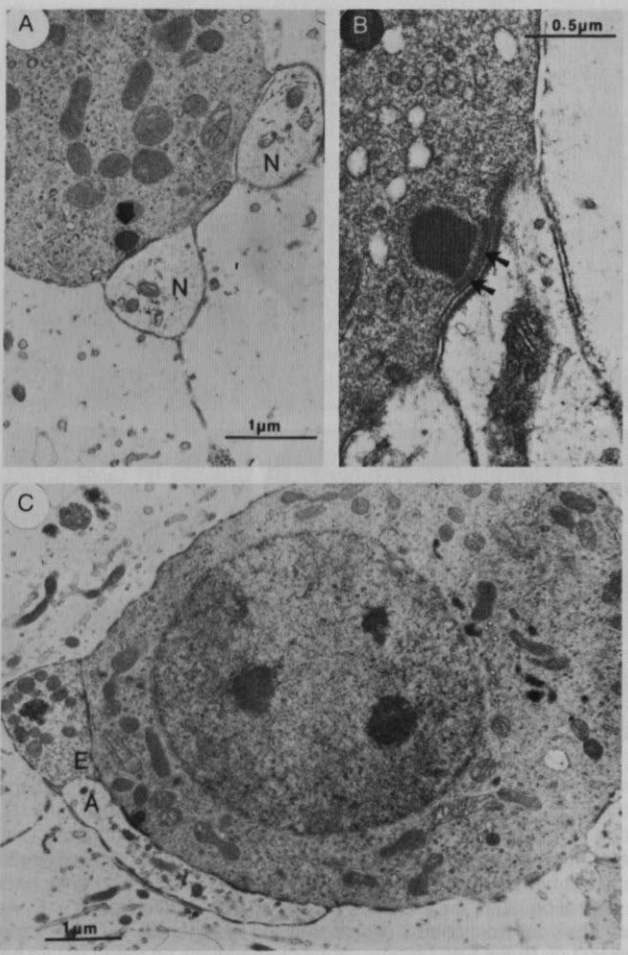

Figure 15. *A*, Small bouton afferent nerve endings (N) opposed to basal portion of regenerated sensory hair cell 1 day post-termination of gentamicin treatment. Arrow indicates synaptic complex consisting of synaptic ball and vesicles. *B*, High magnification of synaptic complex in a regenerated hair cell 1 day following termination of gentamicin treatment. Note row of vesicles surrounding synaptic ball. A granular synaptic membrane (*arrows*) is found between the ball and the cell membrane. *C*, Transmission electron photomicrograph showing both afferent (A) and efferent (E) nerve terminals opposed to the basal portion of an unerupted primitive sensory hair cell 7 days following termination of gentamicin treatment. The efferent terminal contains multiple small round vesicles in addition to mitochrondria. A synaptic complex opposes the afferent terminal. (*From* Duckert LG, Rubel EW: Ultrastructural observations on regenerating hair cells in the chick basilar papilla. Hear Res 48:161–182, 1990; with permission.)

numbers previously seen at the light microscope level. The considerable morphologic variability can easily account, however, for the incomplete functional recovery at this stage of development. Although the hair cells are easily distinguished from supporting cells at 28 days, they carry the stigmata of immaturity, including an irregular globular shape and incom-

plete stereocilia bundle development. Additionally, because bundle orientation appears grossly abnormal, efficient transduction of basilar membrane movements into receptor potentials is probably not possible at 28 days. Incomplete peripheral re-innervation is also a potential source of functional delay, and replacement of afferent innervation by the mature pattern of efferent innervation is probably necessary before full function can be restored. We conjectured that restoration of function would follow completion of the maturation process, which in light of the preceding observations would extend beyond 28 days following termination of drug treatment.

The early phase investigation was followed by a second study in which animals were allowed to survive for more prolonged periods after gentamicin treatment, in an effort to further correlate the evolution of morphologic maturity with functional recovery. These observations, which included more quantitative analyses of the cell population, stereocilia orientation, and innervation, were the subject of the second ultrastructural investigation by Duckert and Rubel.[8]

Late Phase

To complete this investigation, 24 newly hatched chickens were divided into four experimental groups of 6 birds. An additional group of 6 birds served as controls. The experimental animals were injected with gentamicin according to the same protocol as used previously. Following injection, the experimental groups survived 6, 10, 15, and 20 weeks before sacrifice. The control animals were appropriately age-matched. Following sacrifice, the ears were prepared for scanning and transmission electron microscopy. The area of examination corresponded approximately to the area previously described during the early phase of regeneration.

Cellular Morphology

The cellular maturation cycle observed through the 6 to 20 weeks of recovery was qualitatively identical to the developmental process previously identified at 7 to 28 days. In animals from each of the survival periods, cell maturity was assessed on the basis of cell morphology and cytoplasmic contents.

Although there remained few qualitative differences in hair cell maturation, quantitative assessment of the short hair cell surface areas demonstrated a progressive trend toward uniformity at later stages of recovery. This trend is shown graphically in Figure 16. With a scanning electron microscope, this trend to greater uniformity of apical surface areas from 6 to 20 weeks is obvious. By 20 weeks, the cells have assumed the closely packed mosaic pattern typical of the control ears (Fig. 17).

Stereocilia

Morphologic changes in the apical surface specializations of the newly regenerated hair cells throughout the later recovery periods parallel

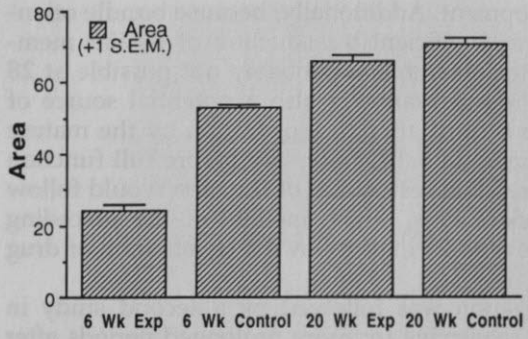

Figure 16. Graphic representation of mean hair cell apical surface areas in two 6-week survivors and two 20-week survivors. The bars indicate standard error of the mean. (*From* Duckert LG, Rubel EW: Morphological correlates of functional recovery in the chicken inner ear following gentamicin treatment. J Comp Neurol 331:75–96, 1993; with permission.)

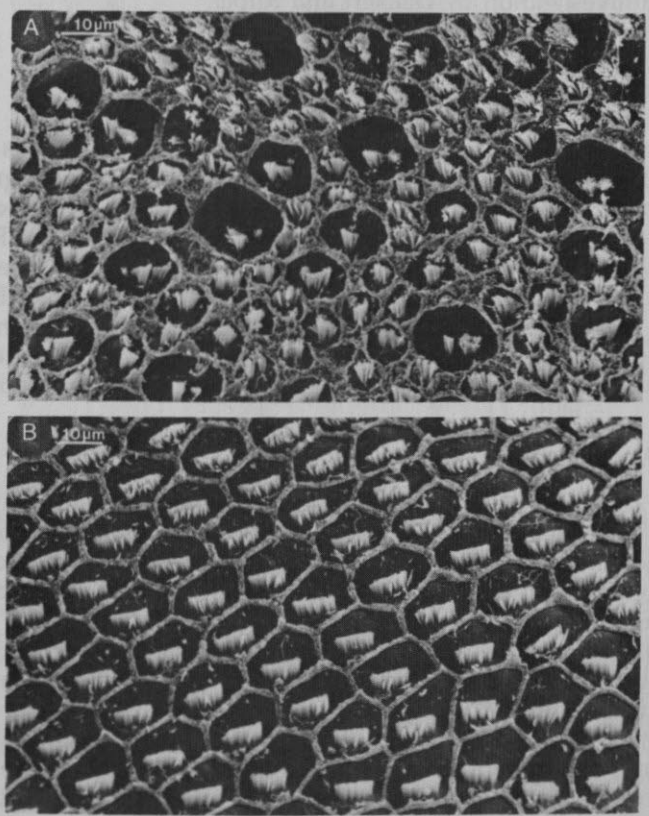

Figure 17. *A*, Scanning electron photomicrograph of short hair cell population 6 weeks after termination of drug treatment demonstrating a wide range of apical surface dimensions. The majority of the cells are immature with apical diameters of about 5 μm. *B*, Short hair cell population in 6-week control animal. Note the uniform apical surface dimensions. (*From* Duckert LG, Rubel EW: Morphological correlates of functional recovery in the chicken inner ear following gentamicin treatment. J Comp Neurol 331:75–96, 1993; with permission.)

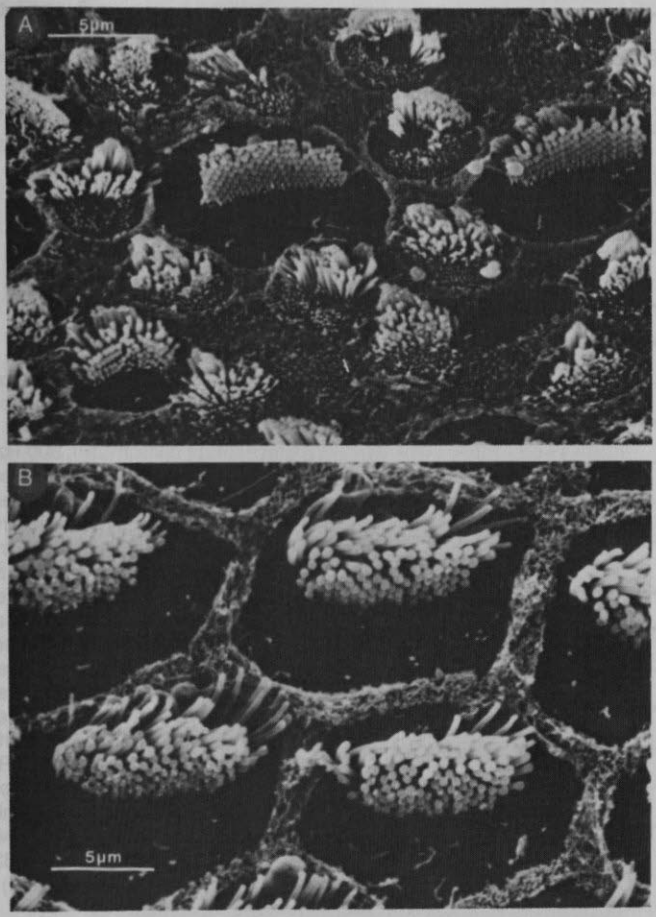

Figure 18. *A*, Early repopulation of the papilla at 6 weeks is characterized by a heterogeneous population of hair cells of various sizes identified by immature stereocilia bundles. With few exceptions, the bundles appear as tufts and are poorly organized. Many cells are "disoriented." *B*, Short hair cells from age-matched control animals. The cells possess well-organized, appropriately oriented stereocilia bundles. (*From* Duckert LG, Rubel EW: Morphological correlates of functional recovery in the chicken inner ear following gentamicin treatment. J Comp Neurol 331:75–96, 1993; with permission.)

those described from 7 to 28 days. The population of stereocilia bundles is most heterogeneous in the 6-week survivors, which exhibit a greater percentage of immature disorganized bundles, whereas the stereocilia bundles at 15 to 20 weeks are typically well organized and are characterized by a normal-appearing hexagonal array (Fig. 18).

In the early phase, stereocilia bundle orientation is extremely nonuniform. For purposes of quantitative assessment of bundle orientation during the late phase, it was necessary to measure bundle rotation of each individual hair cell at each survival period after having identified the bundle axis. Bundle orientation was computed using a computer-assisted image analysis system, which provided an accurate assessment of axis

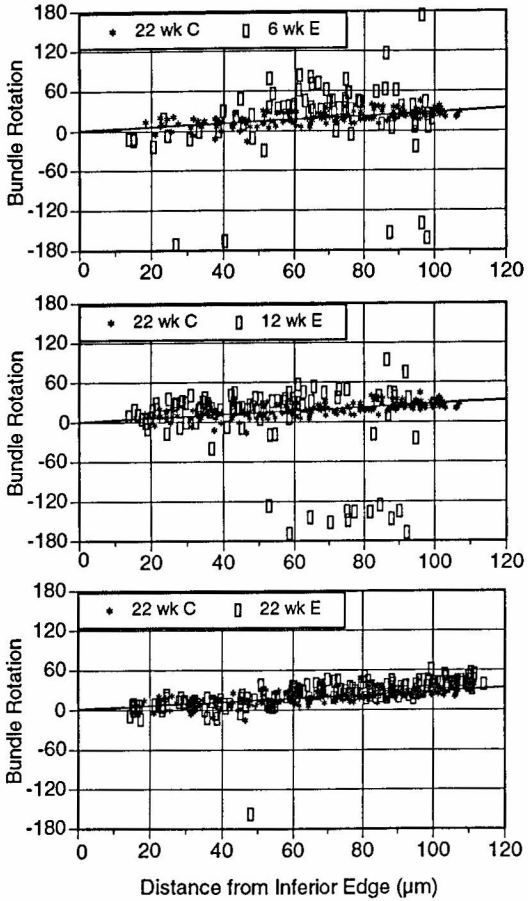

Figure 19. Stereocilia bundle rotation versus distance from the inferior edge is shown for 80 to 120 hair cells in the 1500 Hz short hair cell region. In each panel the data from a normal 22-week control animal is compared with data from a single experimental animal (E) at the indicated survival time. Zero-degree rotation indicates that the stereocilia bundle long axis is parallel with the inferior edge of the basilar sensory epithelium, and the tallest row of stereocilia is toward the superior side of the cell (staircase faces inferior edge). (*From* Duckert LG, Rubel EW: Morphological correlates of functional recovery in the chicken inner ear following gentamicin treatment. J Comp Neurol 331:75–96, 1993; with permission.)

orientation relative to the inferior edge of the papilla. A more detailed description of this method of analysis is beyond the scope of this discussion and is described elsewhere.[9] Normal limits for stereocilia bundle rotation relative to cell location on the basilar papilla have been previously established.[17] The progress of reorientation of stereocilia bundles to approximate the normal ranges is graphically plotted in Figure 19. In each panel, the results from a representative experimental animal are compared with the data from a normal 22-week-old control chicken. Bundle rotation for 80 to 120 hair cells from an equivalent area at each survival period is plotted against a standard reference point. It is clear that the 6-week survival animal shows greater scatter than the other survivors. Aside from one cell, the 22-week survival animal shows a normal pattern of bundle rotation, and an animal after 12 weeks' survival demonstrates an orientation pattern intermediate between the 6-week and 22-week survivors.

Innervation

The later stages of hair cell maturation are characterized by a transition from afferent innervation to efferent innervation. Throughout the recovery process, more mature short hair cells are associated with fewer afferent terminals. In some cases, the nerve terminals appear washed out (Fig. 20*A*). This possibly represents a degenerative process resulting in a

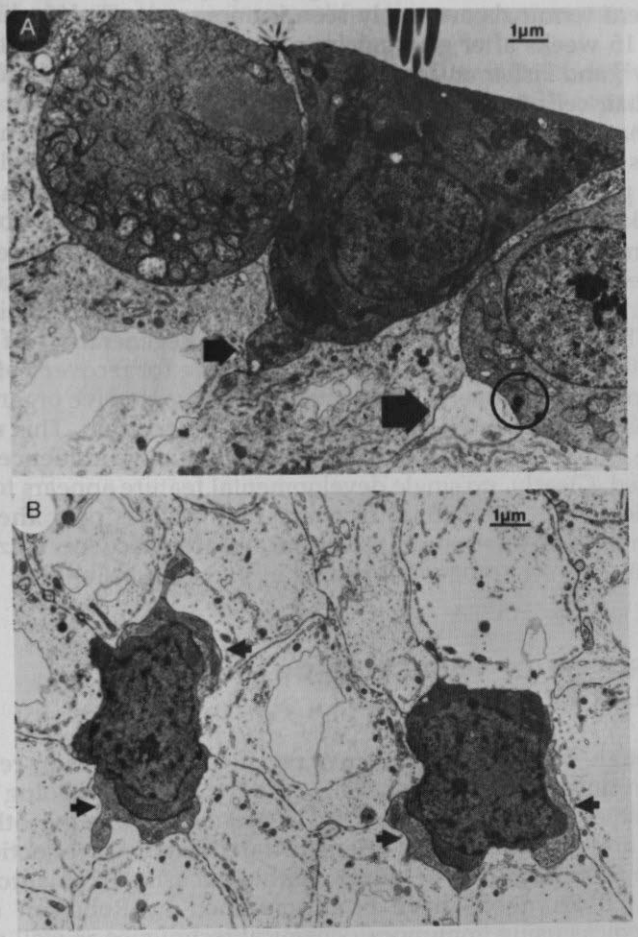

Figure 20. *A*, A mature appearing short hair cell from a 10-week survivor possesses a bouton efferent terminal (*small arrow*). The cell has assumed a pitcher shape, and the cytoplasm stains more densely in contrast to the less mature neighbor hair cell. At the base of the less mature cells is an afferent 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*, Two chalice shaped efferent terminals (*large arrows*) opposed to axially sectioned short hair cells from 20-week experimental animal. Subsynaptic cisterna are well developed (*small arrows*). (*From* Duckert LG, Rubel EW: Morphological correlates of functional recovery in the chicken inner ear following gentamicin treatment. J Comp Neurol 331:75–96, 1993; with permission.)

terminal empty of cytoplasmic content. By 10 to 15 weeks, afferent terminals are rarely seen in the mature-appearing short hair cells. By 20 weeks, afferent terminals are replaced or displaced by efferent endings on the mature sensory cells. Serial sectioning revealed that there are one or two large, chalice-shaped terminals per cell (Fig. 20*B*). This innervation pattern is indistinguishable from that in the age-matched control animals.

The differentiation of efferent synaptic endings following afferent differentiation is also observed during embryogenesis.[10,22] The observation that afferent terminals are rarely seen in more mature short hair cells in excess of 15 weeks after gentamicin treatment is consistent with findings of Manley[17] and Fisher and colleagues,[11] who observed no afferent contact on short hair cells in the basal half of the normal chick basilar papilla. It appears that the afferent terminals present early in development degenerate, as has been observed during synaptogenesis by Whitehead and Morest.[34] The progressive increase in efferent terminals to the normal number at 20 weeks is probably not related to functional recovery by coincidence.

In summary, repopulation of the avian cochlea following termination of drug treatment consists of two phases. The early phase is characterized by regeneration and maturation of the individual hair cells, which may or may not be influenced by extrinsic factors. Regeneration of a critical number of hair cells is not singularly responsible for recovery of function. The later phase of recovery is characterized by progressive organization of stereocilia bundle orientation and efferent re-innervation. This may occur on a more global scale as a result of extrinsic inductive influences as yet to be identified. Clearly, no single developmental feature appears to be solely responsible for the functional recovery that evolves over the 20-week period. Changes in internal cell structure, apical surface specialization, cell orientation, and synaptogenesis are all in part responsible.

REGENERATION OF HAIR CELLS ON THE VESTIBULAR EPITHELIUM

Although the avian cochlea may regenerate hair cells in response to damage produced by acoustic trauma or ototoxic drugs, ongoing proliferation of hair cells is not characteristic of this organ. On the other hand, Jørgensen and Mathiesen[13] have demonstrated ongoing production of hair cells in the postnatal avian vestibular epithelium using DNA autoradiography. Their observations have been confirmed by Roberson et al[23] in postnatal chicks using immunocytochemical techniques as well as autoradiographic methods. The avian vestibular system also responds to the effects of aminoglycoside toxicity by producing new hair cells. In a study by Weisleder and Rubel,[33] newly hatched chickens were injected daily with streptomycin sulfate, 600 mg/kg/day for 7 days. On the fifth day, these animals and age-matched control chicks received either injections of ^{3}H thymidine for 3 days or injections of 5-bromo 2-deoxyuridine (BRDU) for 5 days. BRDU, a thymidine analogue, is incorporated into the DNA of dividing cells; however, it is antigenically distinct from the normal thymidine, and can be recognized by a monoclonal antibody and labeled im-

munocytochemically. The animals were sacrificed 1, 20, or 60 days after the last injection of the markers. The vestibular organs were sectioned and processed for immunocytochemistry or autoradiography.

One day after the last drug injection, clear evidence of vestibular toxicity was present. At this time, BRDU immunostaining and tritiated thymidine autoradiography revealed mitotic activity in the supporting cell layer of all organs in both treated and untreated animals. The treatment group had a much larger number of labeled nuclei than the untreated group (Fig. 21). The increase in proliferation was commensurate with the degree of damage.

By 20 days, the damage to type I cells and, to a lesser degree, to type II cells is still apparent. The number of type II hair cells is increased, and

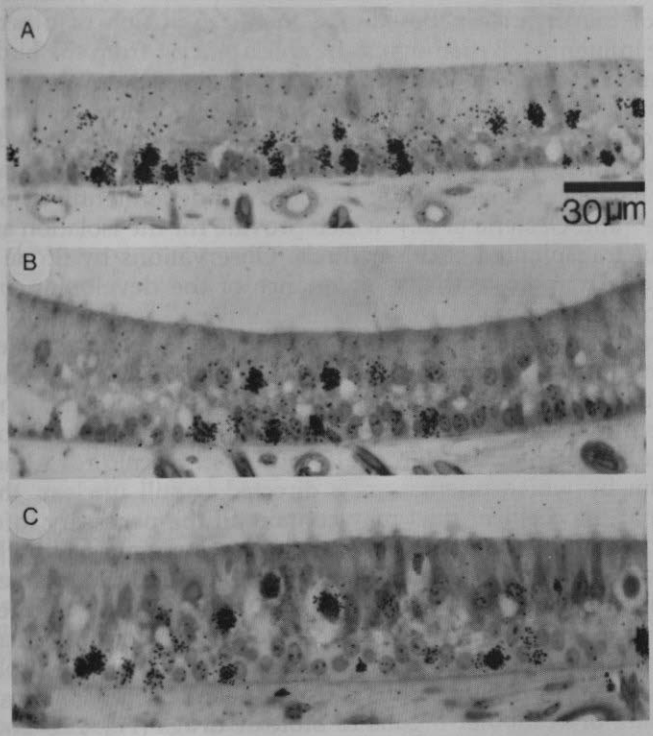

Figure 21. Vestibular hair cell regeneration following streptomycin toxicity. *A*, Histologic signs of streptomycin lesion include loss of type I hair cells and an important but less severe damage to type II hair cells. In this utricle processed for tissue autoradiography, numerous labeled cells can be identified. *B*, Twenty-day survival. Signs of regeneration include the reappearance of several type II hair cells and a few type I hair cells. In this micrograph, several supporting cells and type II hair cells have labeled nuclei. *C*, Sixty-day survival. The anatomy of the utricles from treated animals approximates that of untreated birds. All three cell types normally present in the vestibular sensory epithelium can be easily identified. Labeled nuclei can be found on the three cell types. (*From* Weisleder P, Rubel EW: Hair cell regeneration in the avian vestibular epithelium. Exp Neurol 115:2–6, 1992; with permission.)

many of these cells are labeled as are adjacent supporting cells. Some of the type I cells are labeled but others are not.

At 60 days, the tissue has essentially recovered. Labeled type II and type I hair cells as well as supporting cells are identified, and the normal complement is restored.

The authors concluded from these observations that the avian vestibular system is able to generate hair cells not only on an ongoing basis but also in response to aminoglycoside toxicity. All cell types can be replaced, including the supporting cells.

HAIR CELL DIFFERENTIATION IN ORGAN CULTURES

Ability to observe hair cell differentiation in vitro provides the investigator with some clear advantages relative to the in vivo condition. Development of the organ can be studied to the exclusion of other external inductive influences. By removing the chick otocyst from the bird, Corwin and Cotanche[3] were able to show that chick hair cells developed position-specific properties in the absence of neural inputs. Moreover, in a purely autonomous organ culture, the investigator can manipulate and monitor the biologic mileu to assess its impact on cell differentiation. Organ culture methods have been developed and reported independently by Stone and Cotanche[29] and Oesterle et al,[35] which provide for the isolation and maintenance of transplanted cochlear ducts. Observations by Stone and Cotanche[29] clearly indicate that maintenance of the developing embryonic chick cochlea can be achieved in this manner. These authors have reported on specific aspects of sensory cell development in culture, including stereocilia development and orientation. That stereocilia increase in number and length in culture suggests that these processes are regulated by factors intrinsic to the cochlear duct. Similarly, they have reported that stereocilia bundle reorientation is also observed in culture. Although the mechanism by which orientation is directed is still unclear, these data suggest that the cochlear duct may contain all the necessary information for this to occur. Oesterle and colleagues[35] have maintained both mature cochleas and vestibular organs in culture. In addition, they have demonstrated the ability to operate this tissue in vitro. Organs cultured in the presence of ^{3}H thymidine show evidence of label, indicating that mitotic activity is maintained (Fig. 22*A*). Similarly, uptake of the proliferation marker by sensory and supporting cells has been observed in vitro following exposure of vestibular organs to ototoxic drugs (Fig. 22*B* and *C*).

CONCLUSION

The wealth of information reported by numerous investigators has made a significant impact on our understanding of the regenerative capacity of the avian cochlea and vestibular organs. Considering that until recently the process was unidentified, it may be years until the impact of

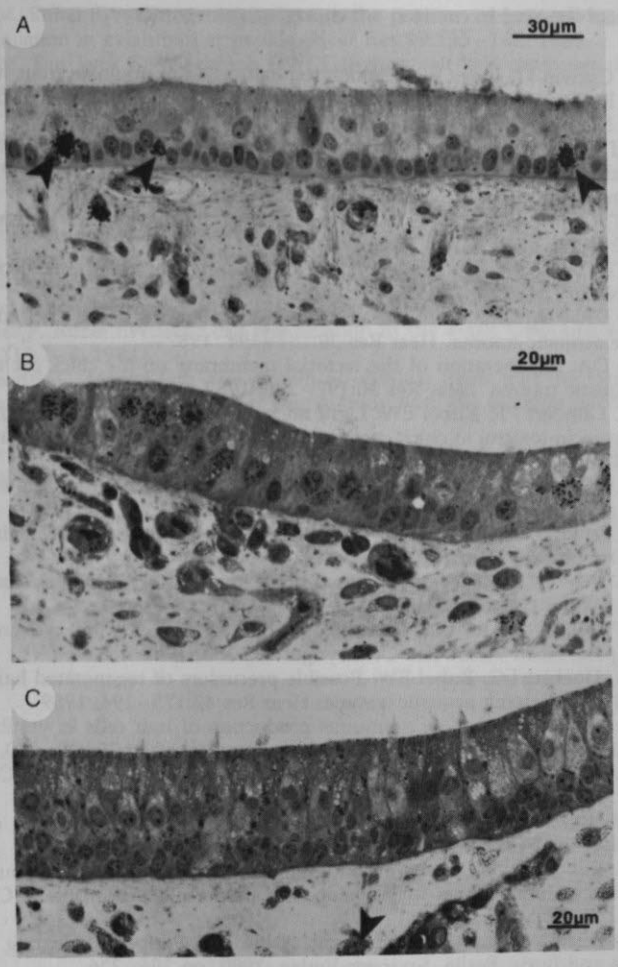

Figure 22. *A*, Micrograph of a normal utricle from an 11-day-old posthatch chick that was cultured for 3 days. Roller-tube culturing techniques were used, and the culture media consisted of Basal Medium Eagle and Earl's Balanced Salt Solution supplemented with 25% horse serum and ^{3}H thymidine, a cell proliferation marker. The normal architecture of the organ is maintained, and hair cells and supporting cells are present. The nuclei of several supporting cells adjacent to the basal lamina are labeled (*arrowheads*) demonstrating the birth of these cells in the cultures and that mitotic activity is maintained in the cultures. *B*, Lagenar macula from a drug-damaged 13-day-old posthatch chick that was damaged in vivo (600 mg/kg streptomycin injected once a day for 6 days) and cultured for 2 days. Hair cells and numerous supporting cells are present in the sensory receptor epithelium. Many cells are labeled by the ^{3}H thymidine. *C*, Lagenar macula from a normal control animal. Hair cells and supporting cells are present in the sensory epithelium, none are labeled in this section. (Courtesy of E. C. Oesterle.)

this observation is fully appreciated. One implication, however, is clear. If similar regenerative capabilities exist in the mammalian cochlea, which are otherwise blocked or dormant, elucidation of the mechanisms that induce hair cell regrowth in the avian cochlea may also culminate in the ability to stimulate hair cell regeneration in the human.

References

1. Balak KJ, Corwin JT, Jones JE: Regenerated hair cells can originate from supporting cell progeny: Evidence from phototoxicity and laser ablation experiments in the lateral line system. J Neurosci 8:2502–2512, 1990
2. Corwin JT: Postembryonic growth of the macula neglecta auditory detector in the ray, *Raja clavata*: Continual increases in hair cell number, neural convergence, and physiological sensitivity. J Comp Neurol 217:345–356, 1983
3. Corwin JT, Cotanche DA: Development of location-specific hair cell stereocilia in denervated embryonic ears. J Comp Embryol 288:529–537, 1989
4. Corwin JT, Cotanche DA: Regeneration of sensory hair cells after acoustic trauma. Science 240:1772–1774, 1988
5. Cotanche DA: Regeneration of hair cell stereociliary bundles in the chick cochlea following severe acoustic trauma. Hear Res 30:181–194, 1987
6. Cotanche DA: Regeneration of the tectorial membrane on the chick cochlea following severe acoustic trauma. Hear Res 30:197–206, 1987
7. Cruz RM, Lambert PR, Rubel EW: Light microscopic evidence of hair cell regeneration after gentamicin toxicity in chick cochlea. Arch Otolaryngol Head Neck Surg 113:1058–1062, 1987
8. Duckert LG, Rubel EW: Morphological correlates of functional recovery in the chicken inner ear after gentamicin treatment. J Comp Neurol 331:75–96, 1993
9. Duckert LG, Rubel EW: Ultrastructural observations in regenerating hair cells in the chick basilar papilla. Hear Res 48:161–182, 1990
10. Fermin LD, Cohen GM: Development gradients in the embryonic chick's basilar papilla. Acta Otolaryngol (Stockh) 97:39–51, 1984
11. Fischer FP, Brix J, Manley GA: Morphological gradients in the innervation of the chick basilar papilla. Abstr Assoc Res Otolaryngol 483:153, 1991
12. Girod DA, Duckert LG, Rubel EW: Possible precursors of regenerated hair cells in the avian cochlea following acoustic trauma. Hear Res 42:175–194, 1989
13. Jørgensen JM, Mathiesen C: Continuous production of hair cells in vestibular sensory organs, but not in the auditory papilla. Naturwissenschaften 75:319–320, 1988
14. Katayama A, Corwin JT: Cell production in the chicken cochlea. J Comp Neurol 281:129–135, 1989
15. Lippe WR, Rubel EW: Development of the place principle: Tonotopic organization. Science 219:514–516, 1983
16. Lippe WR, Rubel EW: Ontogeny of tonotopic organization of brain stem auditory nuclei in the chicken: Implications for development of the place principle. J Comp Neurol 237:273–289, 1985
17. Manley GA: The peripheral hearing organ of birds. *In* Peripheral Hearing Mechanisms in Reptiles and Birds. Berlin, Springer-Verlag, 1990, pp 206–226.
18. Norton S, Tucci D, Rubel EW: Comparison of acoustic and neural responses from avian ears following gentamicin. Presented at Midwinter Meeting of the Association for Research in Otolaryngology, St. Petersburg, FL, 1990
19. Oesterle EC, Cunningham DE, Rubel EW: Ultrastructure of hyaline, border, and vacuole cells in chick inner ear. J Comp Neurol 318:64–82, 1992
20. Presson JC, Popper AN: A ganglionic source of new eighth nerve neurons in a post-embryonic fish. Hear Res 46:23–28, 1990
21. Presson JC, Popper AN: Possible precursors to new hair cells, support cells, and Schwann cells in the ear of a post-embryonic fish. Hear Res 46:9–22, 1990
22. Pujol R, Carlier E, Lenoir M: Ontogenic approach to inner and outer hair cell function. Hear Res 2:423–430, 1980
23. Roberson DW, Weisleder P, Bohrer PS, et al: Ongoing production of sensory cells in the vestibular epithelium of the chick. Hear Res 57:166–174, 1991
24. Rubel EW, Ryals BM: Development of the place principle: Acoustic trauma. Science 219:512–514, 1983
25. Ruben RJ: Development of the inner ear of the mouse: A radioautographic study of terminal mitoses. Acta Otolaryngol 220:1–44, 1967
26. Ryals BM, Rubel EW: Hair cell regeneration after acoustic trauma in adult *Coturnix* quail. Science 240:1774–1776, 1988

27. Ryals BM, Rubel EW: Ontogenic changes in the position of hair cell loss after acoustic overstimulation in avian basilar papilla. Hear Res 19:135–142, 1985
28. Ryals BM, Ten Eyck B, Westbrook DW: Ganglion cell loss continues during hair cell regeneration. Hear Res 43:81–90, 1990
29. Stone JS, Cotanche DA: Hair cell differentiation in the developing chick cochlea and in embryonic cochlear organ culture. J Comp Neurol 314:614–625, 1991
30. Takasaka T, Smith CA: The structure and innervation of the pigeon's basilar papilla. Ultrastruct Res 35:20–65, 1971
31. Tilney LG, Saunders JC: Actin filaments, stereocilia, and hair cells of the bird cochlea. 1. Length, number, width distribution of stereocilia of each hair cell are related to the position of the hair cell on the cochlea. J Cell Biol 96:802–821, 1983
32. Tucci DL, Rubel EW: Physiological status of regenerated hair cells in the avian inner ear following aminoglycoside ototoxicity. Otolaryngol Head Neck Surg 103:443–450, 1990
33. Weisleder P, Rubel EW: Hair cell regeneration in the avian vestibular epithelium. Exp Neurol 115:2–6, 1992
34. Whitehead MC, Morest DK: The growth of cochlear fibers and the formation of their synaptic endings in the avian inner ear: A study with the electron microscope. Neuroscience 14:277–300, 1985
35. Oesterle EC, Tsue TT, Reh TA, Rubel EW: Hair cell regeneration in organ cultures of the postnatal chicken inner ear. Hear Res, in press, 1993

Address reprint requests to

Larry G. Duckert, MD, PhD
Department of Otolaryngology
Head and Neck Surgery RL-30
University of Washington
1959 Pacific NE
Seattle, WA 98195