

Hair cell regeneration in the avian inner ear

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Abstract. The postembryonic production of hair cells in fish and reptiles has been known for several decades. Until recently it was assumed that this capacity was absent in the more highly specialized inner ears of birds and mammals. Recent research has shown, however, that birds have the capacity to rebuild a damaged inner ear. Summarized here are studies conducted in our laboratory which address the following questions: (1) Which are the precursors of the regenerated hair cells? (2) Are the new hair cells functional? (3) What are the ultrastructural properties of regenerated hair cells? and (4) Can the level of proliferation be regulated? Both the auditory and the vestibular systems of the avian inner ear were studied. Our results provide some answers to these questions. The implications of the results are discussed.

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The potential for vertebrate hair cells to be proliferated and differentiate throughout life has been recognized for over 50 years (see Corwin et al 1989). For example, Stone (1933, 1937) studied regeneration of lateral line organs after tail amputation in amphibian embryos. He found that regenerated organs were numerically similar to the previous complement and that they were supplied by the migration of precursor cells from the last organ of the proximal tail stump. More recently, a number of groups have investigated both the continued production of hair cells in the saccule of rays and fishes as well as the regeneration of hair cells in the lateral line of amphibians (Balak et al 1990, Corwin 1983, Corwin et al 1989, Presson & Popper 1990a,b).

Until recently, however, it was generally accepted that the postembryonic production of hair cells is limited to cold-blooded vertebrates, as is the ability to restore damaged populations of hair cells. That is, birds and mammals have lost this ability during evolution. In both birds and mammals, hair cells that will normally populate the inner ear are produced relatively early in embryogenesis (Katayama & Corwin 1989, Ruben 1967). While some mitotic activity may continue after this time, the new cells have not been thought to differentiate into hair cells.

Two serendipitous findings suggested that birds were able to restore the population of hair cells after the elimination of embryonically produced hair cells. Cruz et al (1987) performed an experiment intended to examine the time course of destruction of hair cells in the chick cochlea after the administration of an aminoglycoside antibiotic. Neonatal chicks were given gentamicin injections for 10 days and allowed to survive for varying amounts of time ranging from one to 32 days. Cochleas were serially sectioned and the number of hair cells was counted at 100 μm intervals from the base to the apex. Hair cell counts revealed that after the 10-day aminoglycoside treatment there was a nearly total elimination of hair cells in the basal third of the cochlea. A week later the damage had spread to eliminate hair cells throughout the basal two-thirds of the cochlea, but counts at the basal pole revealed that the number of hair cells had been partially restored. After another two weeks the number of hair cells throughout the basal two-thirds of the cochlea appeared to be recovering toward normal.

While these results strongly suggested that new hair cells were being produced to replace those destroyed by the aminoglycoside treatment, other interpretations were considered. For example, it was possible that the aminoglycosides caused deterioration or dedifferentiation of hair cells to the extent that they were unrecognizable in the microscopic sections, and then recovery ensued. Even if new hair cells were taking the place of embryonically produced cells that had been destroyed by the drug, we could not conclude that these were newly produced cells. It was possible that the aminoglycosides or the resulting damage induced support cells to differentiate into new hair cells.

While that study was progressing, Cotanche (1987a) was examining the neonatal chick cochlea by scanning electron microscopy after acoustic trauma. Although the initial purpose of the study was to examine age differences in the position of damage produced by sound overstimulation (see Rubel & Ryals 1983, Ryals & Rubel 1985, Lippe & Rubel 1983, 1985), Cotanche also noted the repopulation of hair cells a few days after the noise damage. Again, this observation is open to several interpretations, including both cell regeneration and the recovery of stereocilia. Cotanche made two additional important observations; the apical surfaces of the cells that appeared to be repopulating the cochlea bore a striking resemblance to immature hair cells, and the sequence of differentiation paralleled the embryonic development of stereocilia.

Taken together, these two studies (Cruz et al 1987, Cotanche 1987a) suggested that the restoration of hair cells was not due to recovery, but represented newly created hair cells or transformed support cells. Three studies (Corwin & Cotanche 1988, Jørgensen & Mathiesen 1988, Ryals & Rubel 1988) then used [^3H]thymidine to label mitotically active cells in the inner ear of young and adult birds in order to prove that new hair cells were being produced. The radioactive tracer is incorporated into replicating DNA during the S phase of the cell cycle. It then remains in the nucleus throughout the life of the cell. If an abundance of radioactive thymidine is introduced into the environment of

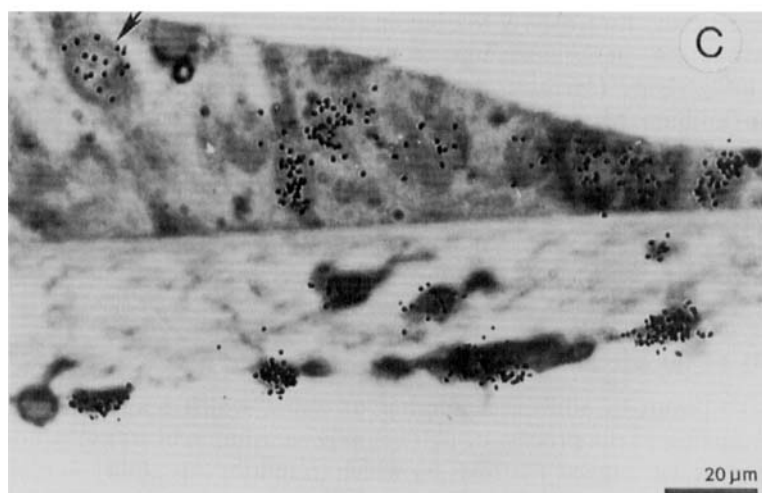
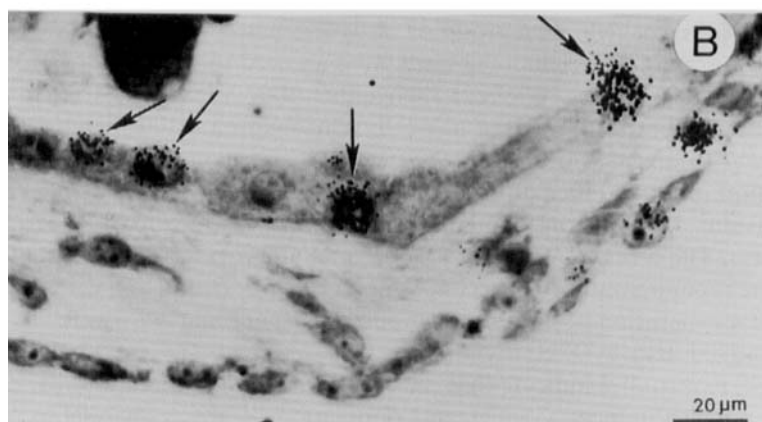
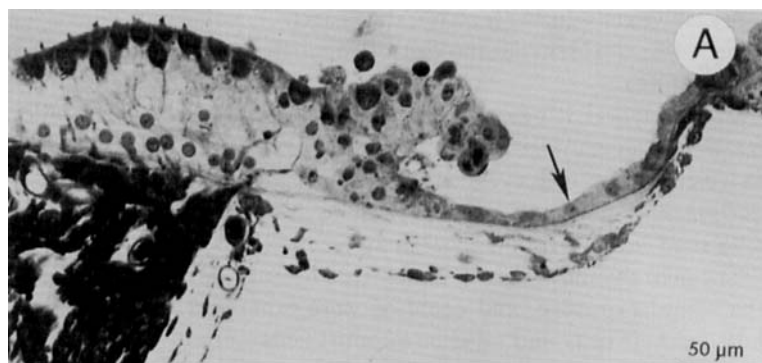
mitotically active cells during S phase, the progeny become radioactively labelled. Cells labelled by [^3H]thymidine can be identified at any subsequent time by autoradiography.

Using this method, Corwin & Cotanche (1988) and Ryals & Rubel (1988) demonstrated that damage to the avian cochlea causes a population of stem cells to re-enter the cell cycle and produce new cells, which subsequently differentiate into new hair cells as well as new support cells. Corwin and Cotanche used intense sound to destroy receptors in the cochlea of neonatal chicks. Tritiated thymidine was administered to 9–13-day-old chicks for 10 days after the sound exposure, and cochleas were processed for autoradiography. Both labelled hair cells and labelled support cells were observed. In a parallel study (Ryals & Rubel 1988) young, sexually mature quail (*Coturnix coturnix*) were exposed to an intense pure tone (1500 Hz) to destroy hair cells in the middle region of the cochlea. One group of birds was given [^3H]thymidine over the ensuing 10 days. The remaining birds were allowed to survive for 10, 30 or 60 days. Cochleas were sectioned; then either they were processed for autoradiography, or the numbers of hair cells were counted at 100 μm intervals from the basal to apical end. Ten days after the noise damage there is a massive reduction in the number of hair cells in the basal half of the cochlea. During the ensuing 50 days, the population of hair cells is restored to near-normal numbers. The birds treated with [^3H]thymidine provided convincing evidence that the repopulation of hair cells is due to the production and differentiation of a new generation of cells. Both support cells and hair cells were labelled in the lesioned area by [^3H]thymidine.

These two studies indicate that, in both neonatal and mature birds, damage to the cochlea induces a 'quiescent' population of precursor cells to re-enter the mitotic cycle. Newly produced cells then differentiate into either hair cells or support cells. Jørgensen & Mathiesen (1988) also reported on the production of new hair cells in the postembryonic inner ear of birds. In this case, normal adult budgerigars (*Melopsittacus undulatus*, Australian parrot) were given [^3H]thymidine, after which the vestibular epithelia were sectioned and processed for autoradiography. Scattered labelled hair cells and support cells were seen in each receptor epithelium. This result, which we have recently replicated in neonatal chicks (Roberson et al 1991), indicates that there is a low level of continual turnover of hair cells and support cells in the avian vestibular system.

Identity of the precursor population

We have begun to address a number of issues which may lead to further understanding of the process of hair cell regeneration in birds in the hope that the answers will suggest methods by which to induce this ability in mammals, including man. One of the foremost questions is the identification of the cellular

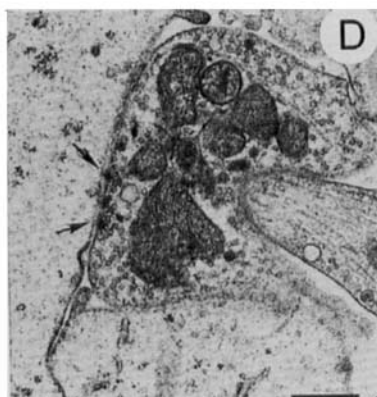
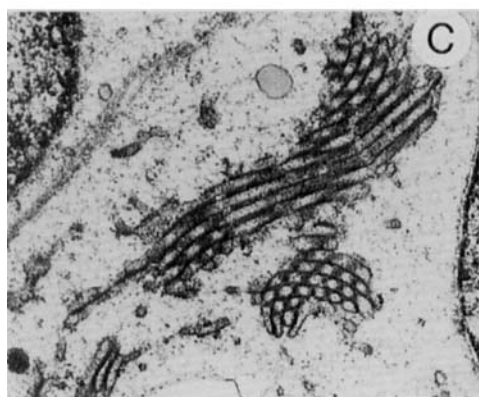
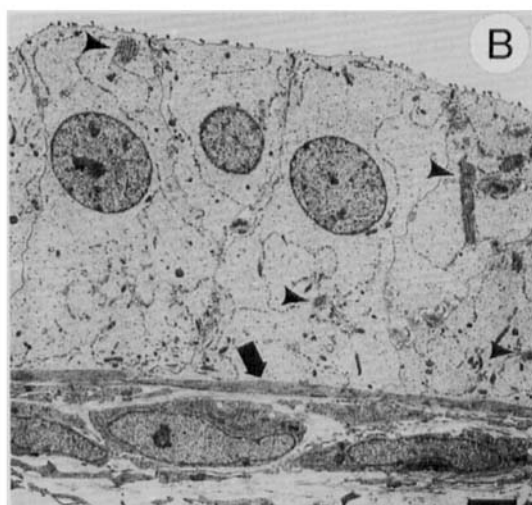
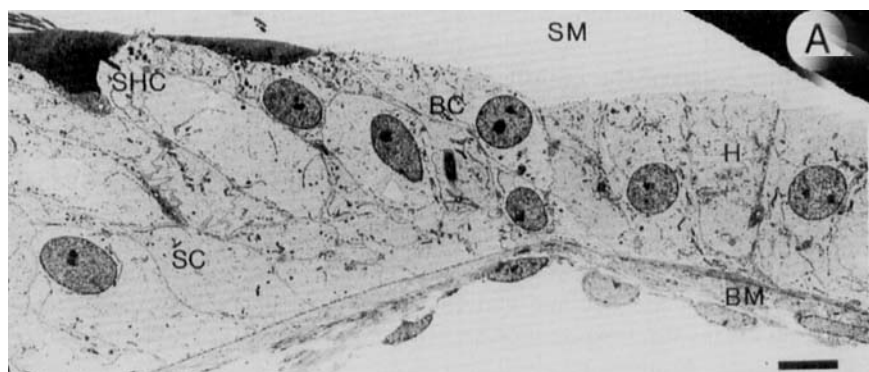


population or populations which re-enter the mitotic cycle and produce new hair cells. This issue has been elegantly addressed by Balak et al (1990) in the lateral line organ of the axolotl, where it was confirmed that supporting cells in the centre of the receptor organ—the internal support cells—are progenitors of regenerated hair cells. In statoacoustic organs of fish, resident embryonic-like neuroepithelial cells give rise to new vestibular hair cells (Presson & Popper 1990b).

In our first experiment, designed to identify hair cell precursors in the avian cochlea (Girod et al 1989), we determined the cells which first become mitotically active after acoustic damage and then followed the fate of their progeny. Neonatal chicks were exposed to an intense pure tone (120 dB SPL, 1500 Hz) for 18 hours. Animals received injections of [^3H]thymidine over survival periods of 6, 15, 24 or 72 hours after exposure to the tone. One group of chicks was given an abundance of 'cold' thymidine after three days of [^3H]thymidine; these chicks were killed 30 days after noise exposure. Cochleas were processed for autoradiography or scanning electron microscopy (SEM). Figure 1A shows the appearance of the receptor epithelium six hours after the noise exposure. The epithelium is severely damaged and most of the short hair cells are extruded. At this time, no cells in the receptor epithelium or its vicinity are labelled. By 15 or 24 hours after noise exposure the undifferentiated epithelial cells lying at the inferior edge of the receptor epithelium are rapidly proliferating (Fig. 1B). As these cells continue to proliferate the single cell lamina is transformed to a layer 2–3 cells deep and labelled nuclei are abundant. Many labelled new hair cells as well as support cells can be observed toward the inferior side of the receptor epithelium three days after the noise exposure (Fig. 1C). New hair cells are easily recognized by the staining characteristics of their cytoplasm, by a thin apical process extending to the lumen already bearing a tuft of immature cilia, and by a basal process extending down toward the basilar membrane. In scanning electron microscopy, the tips of immature stereocilia are clearly recognized.

The ultrastructural characteristics of the potential hair cell precursors identified by Girod et al (1989), namely cells in the area of the hyaline or cuboidal

FIG. 1. Transverse light microscopic sections through the 1500 Hz region of chick cochleas after noise damage (1500 Hz tone at 120 dB SPL for 18 h). A. Sensory epithelium 6 h after completion of noise exposure. The inferior edge of the sensory epithelium is extensively damaged; hair cells and supporting cells are lost. Note the thin monolayer of cells spreading to cover the basilar membrane (arrow). B. Inferior region of the sensory epithelium 15 h after completion of noise exposure. Labelled nuclei (arrows) indicate mitosis within the cellular monolayer. C. Inferior region of the sensory epithelium three days after completion of the sound exposure. Labelled 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, which puts the cells partially out of focus. (A–C from Girod et al 1989.)



cells, have been studied (Oesterle et al 1990) in the normal chick cochlea. The inferior region of the basilar papilla in the chicken cochlea is shown in Fig. 2A. The inferior hair cell, a short hair cell, abuts an organ supporting cell. Several rows of cells, that we have termed border cells, separate this group of organ supporting cells from nearby hyaline cells. The hyaline cells are a row of cuboidal cells which lie inferior to the border cells and extend inferiorly towards the fibrocartilaginous plate. Border cells appear unspecialized, whereas hyaline cells are highly specialized. Dense bundles of filaments are present in the base of hyaline cells from the basal half of the papilla (Fig. 2B), and an unusual structure, a striated rough tubular aggregate, is present in hyaline cell cytoplasm (Fig. 2B and 2C). An intriguing new observation is that synaptic specializations are observed between neural elements and hyaline cells (Fig. 2D) and neural elements and border cells.

Our laboratory is now studying the ultrastructural characteristics of the regenerating receptor epithelium in the chick cochlea. Sound exposure parameters were identical to those of the earlier (Girod et al 1989) study, and chicks were killed 0, 12, 24 or 72 hours after the sound exposure. A consistent picture is emerging from the light and electron microscopic studies of the inferior region of the basilar membrane after noise

FIG. 2. Inferior region of the receptor epithelium and the neighbouring supporting cells in the normal chick cochlea. A. Electron micrograph showing the inferior region of the receptor epithelium in the chick inner ear and the neighbouring supporting cells. A short hair cell (SHC) is on the left. At the inferior edge of the receptor epithelium, a few cuboidal cells, that we have termed border cells (BC), separate the sensory region from the hyaline cells. Hyaline cells (H) are a group of cuboidal epithelial cells which rest on the basilar membrane (BM) and extend up to the scala media space (SM). Organ-supporting cells (SC). Bar, 5 μm . B. Hyaline cells from the high frequency region of the cochlea. Dense networks of fine filaments appear directly adjacent to the basal lamina (arrow). An unusual structure consisting of regular arrays of tubular membrane, a striated rough tubular aggregate, is often observed in apical and/or basal regions of hyaline cells (arrowheads). Bar, 2 μm . C. High magnification of a striated rough tubular aggregate. The tubular aggregate may reach up to 3–4 μm in length and 0.5 μm in diameter. Individual subunits are approximately 94 nm in diameter. Rough endoplasmic reticulum often lies in the immediate vicinity of the tubular aggregate and may be continuous with it. The rough striated tubular aggregate is observed in hyaline cells only; it does not occur in other cell types in and or adjacent to the receptor epithelium. Bar, 0.5 μm . D. Synaptic specializations are observed in nerve fibres juxtaposed to hyaline cells. A nerve fibre opposite a hyaline cell from the low frequency region of the cochlea is shown. Small presynaptic densities ringed by associated synaptic vesicles are present inside the nerve fibre (arrows) and are attached to the cytoplasmic face of the presynaptic membrane. Single densities may be visible or, as shown here, several densities may be clustered together. Analogous presynaptic specializations between neural elements and hyaline cells are observed in all regions of the cochlea; they are most numerous in the high-frequency regions. Bar, 0.5 μm . (D from Oesterle et al 1990.)

damage. Initially, support cells near the inferior margin of the receptor epithelium spread out to cover the area of the basilar membrane that has been damaged. This process involves major changes in the shape of the organ supporting cells and border cells. Minor shape changes may also occur in hyaline cells. The epithelial covering of the basilar membrane is seen as early as we have examined the tissue, at the end of 18 hours of sound exposure. Proliferation of epithelial cells does not appear to be involved in this process, because [^3H]thymidine labelling is not seen in this region six hours after the 18 hours of noise exposure.

The next phase involves rapid proliferation of this epithelial layer, which begins between six and 15 hours after exposure. The proliferating cells lie in the inferior region of the receptor and adjacent to the receptor cell epithelium. These cell types include cuboidal cells, hyaline cells, border cells and support cells under the hair cells. The specific cell type(s) which undergo mitotic activity are now under investigation. These new cells appear to form a pseudo-stratified epithelium and may show nuclear translocation from the basilar membrane to the lumen during mitosis (see Presson & Popper 1990a). Large multinucleated cells are seen 24 hours after exposure (Fig. 3). As cells leave the mitotic cycle, the epithelium thickens and some of the cells begin to differentiate into hair cells. The incipient hair cells can be recognized as they migrate toward the lumen by their relatively greater cytoplasmic staining density, which is associated more with organelles, than surrounding support cells. After the apical tip of the differentiating hair cell contacts the luminal surface, specializations begin to appear; these include small microvilli, immature stereocilia, and a cuticular plate. These and other surface modifications have been described in detail by others (Cotanche 1987a, Cotanche & Corwin 1991, Marsh et al 1990). During the next 30 days the new hair cells mature to the point where they are indistinguishable (except for [^3H]thymidine labelling) from normal hair cells in that part of the cochlea. As discussed later, even such individualized and specialized features as the number and height of stereocilia appear to be replicated in the postembryonically produced hair cells.

In a few cases we have observed hair cell loss limited to a strip of cells lying at the junction between short hair cells and tall hair cells (see Cotanche et al 1987). In these cases we have not observed proliferation of border or hyaline cells, which suggests that another cell population is generating the new hair cells. We have not yet identified this progenitor population; it could be the organ supporting cells, as suggested by Corwin & Cotanche (1988), or a population of stem cells that has heretofore remained unrecognized.

Since most of the damaged animals that have been studied with radioactive thymidine have sustained short hair cell damage, it is not surprising that regeneration of short hair cells has predominated. We have, however,

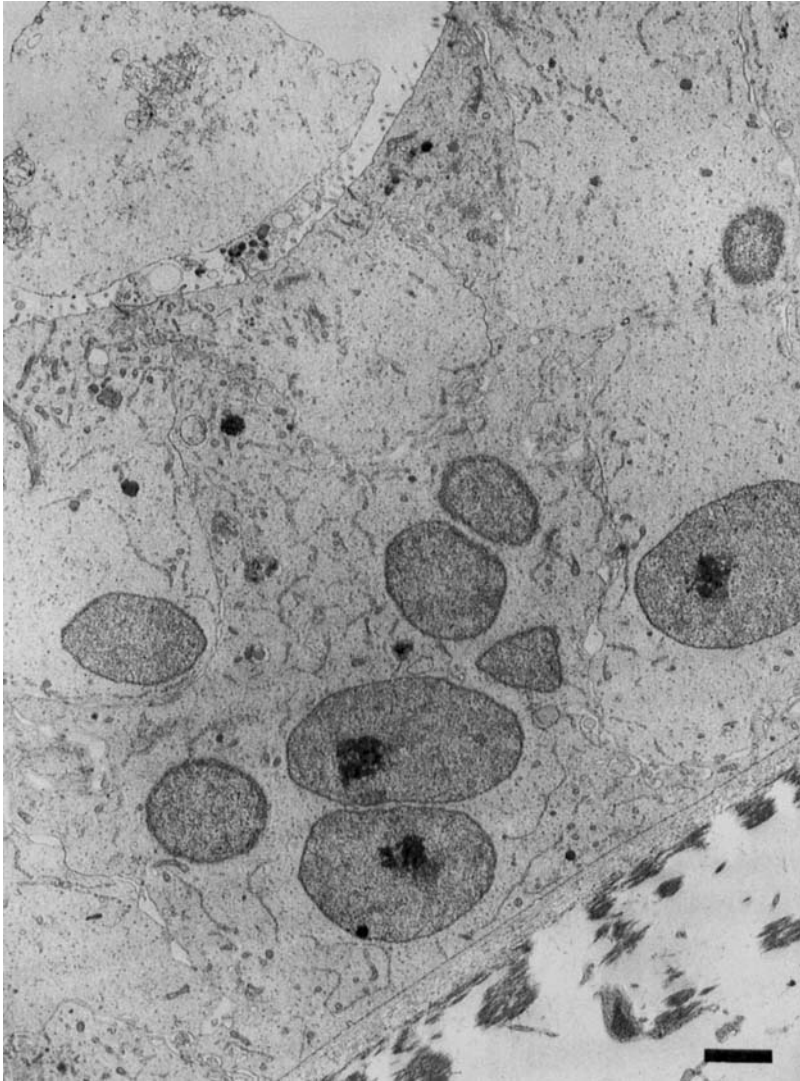
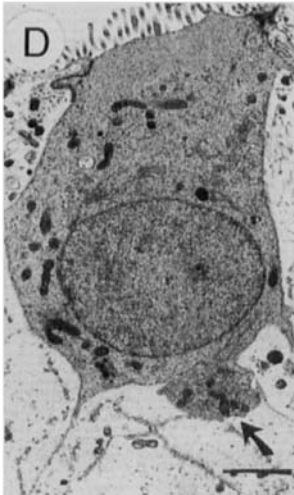
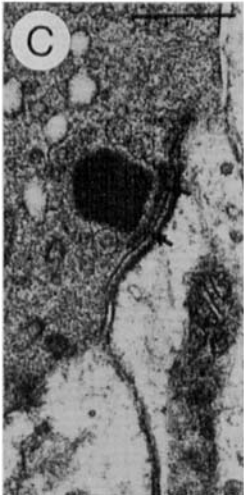
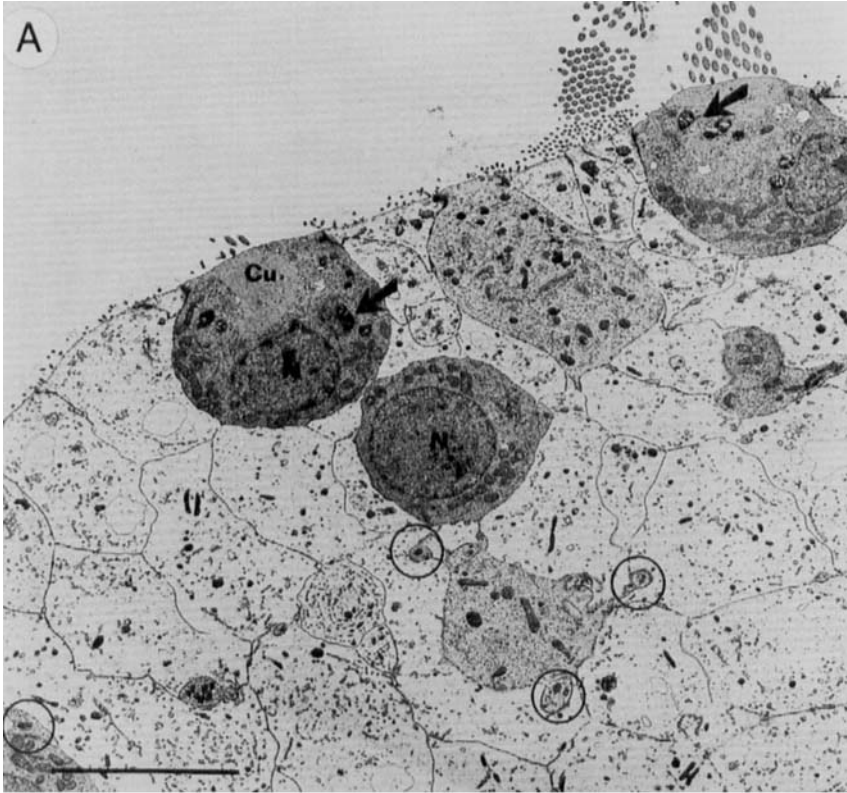


FIG. 3. Electron micrograph of a large multinucleated cell from the 1500 Hz region of a chick cochlea 24 hours after the completion of noise exposure (1500 Hz tone at 120 dB SPL for 18 hours). Bar, 2 μ m.

occasionally seen labelled tall hair cells, suggesting that both types can be produced postembryonically. The few tall hair cells observed were always at an appropriate location, superior to the short hair cells. Early in the regeneration process after noise damage, the tectorial membrane—also destroyed by



overstimulation—is re-formed (Cotanche 1987b). Its reconstitution may be important for the alignment of stereocilia bundles (Cotanche & Corwin 1991).

Do regenerated hair cells restore hearing?

The physiological status of the newly regenerated cells has been studied by Tucci & Rubel (1990) and Norton et al (1990). In the first experiment, tone-burst (250–4000 Hz) auditory evoked potentials (AEPs) were recorded from gentamicin-treated chicks (10 days of subcutaneous gentamicin injections, 50 mg/kg). AEP thresholds deteriorated by 30 to 40 dB until five weeks after treatment. Significant threshold improvements were noted between 16 and 20 weeks at all frequencies tested. In the second study, Norton and collaborators were interested in investigating whether the protracted time course of functional recovery was due to hair cell or neural dysfunction. Evoked otoacoustic emissions (EOEs) and AEPs were recorded from neonatal chickens that had received gentamicin injections for ten days. Five weeks after this treatment, hair cell function (EOEs) and evoked potentials were still abnormal. However, by 8–14 weeks the EOEs of the treated group were not different from those of controls, whereas the AEPs of the injected birds were still abnormal and did not approach normal levels until 22 weeks. The difference suggests that hair cell recovery precedes neural recovery by several weeks. In short, these experiments strongly suggest that the regenerated hair cells are functional and relay information to the central nervous system.

Ultrastructural properties of regenerated hair cells

Our group and other groups (e.g. Cotanche 1987a, Marsh et al 1990, Cotanche & Corwin 1991) have begun to study the ultrastructural properties of the

FIG. 4. Ultrastructural characteristics of regenerated hair cells in drug- and sound-damaged chickens. A. Transmission electron micrograph 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). Note increasing electron density as cells migrate toward surface. Cu, cuticular plate; arrows, myelin figures. Nerve fibres are circled. Bar, 10 μ m. (From Duckert & Rubel 1990.) B. Small bouton afferent nerve endings (N) opposed to basal portion of regenerated sensory hair cell one day after termination of gentamicin treatment. Arrow, synaptic complex consisting of synaptic ball and vesicles. Bar, 1 μ m. C. High magnification of synaptic complex in a regenerated hair cell one day after the termination of gentamicin treatment. Note row of vesicles surrounding synaptic ball (circles). A granular synaptic membrane (arrows) is found between the ball and the cell membrane. Bar, 0.5 μ m. (From Duckert & Rubel 1990.) D. Regenerated hair cell three days after completion of sound exposure (1500 Hz tone at 120 dB SPL for 18 h). Note the efferent terminal at the base of the cell (arrow) and the immature stereociliary bundle and cuticular plate at the apical surface. Bar, 2 μ m.

regenerated sensory epithelium after noise-induced or drug-induced damage. In this section we briefly describe ultrastructural observations on regenerated hair cells after aminoglycoside treatment. More detailed descriptions can be found in Duckert & Rubel (1990). Preliminary observations on the ultrastructural characteristics of regenerated hair cells after sound damage will also be discussed.

The two issues to be addressed here are related to the conclusions we have drawn from our functional analyses. First, it is of interest to determine whether regenerated avian hair cells make synaptic connections with the central nervous system and, if so, the time course over which these connections mature. Second, since in normal animals the characteristics of the stereocilia bundle vary precisely as a function of cochlear location, it is of interest to examine the number and length of the stereocilia and the orientation of stereocilia bundles on regenerated hair cells. These stereocilia properties are thought to be related to functional properties of mature hair cells and their coupling to the tectorial membrane.

Synaptic connections with regenerated hair cells

The principal questions we wish to address here are: (1) When can afferent and efferent terminals be recognized on the regenerating hair cells? and (2) What is their relative maturity? More detailed analyses involving quantitative studies of synaptology are in progress.

In both drug- and sound-damaged birds, immature cells destined to become hair cells can be recognized soon after they begin migrating from the basilar membrane toward the luminal surface. By comparison with the supporting cells, the cytoplasm of regenerating hair cells is more electron dense, as a result of an increase in the number of organelles. As the cells approach the luminal surface this difference increases. A good example is shown in Fig. 4A. The three immature hair cells lined up under the luminal surface show progressively decreasing electron density, but all are more dense than the surrounding support cells. Circled elements in Fig. 4A are nerve fibres in close approximation to an immature hair cell, which has yet to reach the luminal surface or to produce stereocilia.

Figures 4B and C show afferent synaptic terminals on regenerated hair cells. Afferent synaptic complexes on immature cells are seen as early as we have looked in drug-induced lesions in chicks. They are seen one day after the termination of gentamicin treatment but are more numerous at 1–4 weeks. They are found both on cells that have reached the luminal surface (erupted) which are beginning to produce stereocilia, and on unerupted migrating cells. The synaptic complexes appear immature but have the full complement of specializations including synaptic ball, vesicles, presynaptic density and postsynaptic density. The terminals are usually boutons and are less densely packed than on mature cells. Afferent synaptic complexes have not been observed in sound-induced lesions three days after sound exposure.

Vesiculated terminals, which are presumably efferents from the central nervous system, are also seen on both erupted and unerupted immature hair cells, in both sound- and drug-induced lesions. These have not been observed earlier than one week after aminoglycoside treatment, but have been observed three days after sound exposure (Fig. 4D). In both normal and regenerating cochleas they tend to occur most often on short hair cells. Typically they are packed with synaptic vesicles. Often, but not always, subsynaptic cisternae are seen. At early times these endings tend to be boutons, while at later survival times and in normal animals they form flattened cups around the base of the short hair cells.

Thus, after drug insult, both afferent and efferent terminals can be identified early in the regeneration process. We have observed both types of terminals on erupted and on non-erupted cells and all the normal components are present. Further analyses will be required to describe the maturation of synapses, which may be important for understanding the lag between the recovery of hair cell function (emissions) and the recovery of evoked potential thresholds. After noise insult, efferent but not afferent terminals can be identified early in the regeneration process, by three days following sound exposure.

Stereocilia

The maturation of stereocilia in regenerating hair cells has been described by a number of investigators (Cotanche 1987a, Henry et al 1988, Girod et al 1989, Duckert & Rubel 1990). Recently Cotanche & Corwin (1991) showed that the orientation of stereocilia bundles during regeneration after noise trauma in chicks initially varies over approximately 100 degrees. Then, over four days, the bundles became aligned with those of other regenerating cells and with the orientation of surviving hair cells surrounding the lesion. The change occurred 6–10 days after the sound exposure. Although the reorientation of stereocilia bundles after the induction of aminoglycoside ototoxicity in chicks has not been quantified, the same general phenomenon has been seen, but with a much longer time course (Duckert & Rubel 1990). Scanning electron microscopy observations indicate that five weeks after gentamicin treatment the stereocilia bundles throughout the basal two-thirds of the cochlea are disoriented. By 10–12 weeks some reorientation is apparent in the middle region but in the basal and mid-basal regions high variability still predominates. By 22 weeks all but the basal region appears to show consistent bundle orientation.

In the chick cochlea, stereocilia bundles vary systematically in the length and number of elements (Tilney & Saunders 1983). At the basal end the bundle consists of many (200–300) individual stereocilia and the tallest row extends roughly 5 μm from the hair cell surface; toward the apical end the number decreases and the height increases. Development of this gradient has been studied in detail by Tilney et al (1986, 1988). It is of considerable interest to understand

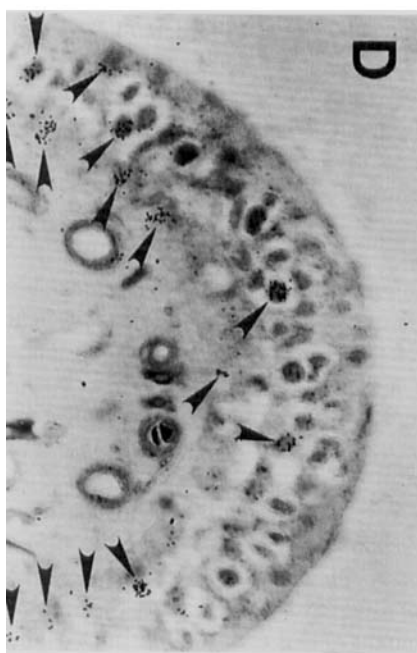
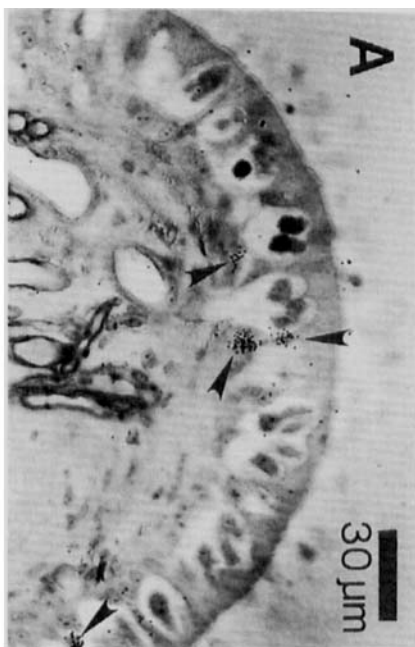


FIG. 5. Up-regulation of hair cell regeneration in the avian vestibular system. Ampullary tissue from chicks killed after a three-day course of $20 \mu\text{Ci/g}$ per day $[^3\text{H}]$ thymidine. Arrowheads point to cells in the sensory epithelium which have incorporated this label. A. Ampulla from a control chick allowed to survive 60 days after $[^3\text{H}]$ thymidine injections. Note the presence and normal complement of nerve calyces, Type I and Type II hair cells, and the single row of supporting cells. Only four labelled cells are seen. B. Ampulla from a chick treated with 600 mg/kg per day streptomycin sulphate for seven days and then given $[^3\text{H}]$ thymidine. Note the absence of all nerve calyces, all Type I and most type II hair cells, and the presence of several rows of supporting cells. Numerous labelled cells are seen. C. Ampulla from a streptomycin-treated chick allowed to survive 20 days after $[^3\text{H}]$ thymidine injections. The nerve calyces, which usually contain several Type I hair cells, can now be observed at the top of the organ. Most, however, contain only one cell. Labelled Type I and Type II hair cells as well as supporting cells are seen. D. Ampulla from a drug-treated chick allowed to survive for 60 days. The tissue has recovered its normal appearance. Numerous labelled cells can still be observed.

the signals underlying the ontogeny of this pattern and it might be expected that these signals are restricted to the embryonic period. We have begun to examine the number and height of stereocilia in regenerated hair cells. Few measurements have been obtained so far, but even cursory observation reveals the striking finding that after 20–25 weeks the normal patterns have been restored. The height of the stereocilia of regenerated hair cells appears identical to that of the remaining hair cells at any given position along the cochlea, and at the basal end the entire complement of new hair cells have short stereocilia. The number of stereocilia has been counted on several mature-appearing regenerated hair cells from the basal region in a 20-week survivor after gentamicin treatment. The number was between 150 and 170 per cell, which is comparable to the number reported by Tilney & Saunders (1983). Without more extensive measurements of stereocilia height and number throughout the length of the cochlea, the precision of this pattern cannot be evaluated, but our observations to date strongly suggest that the signals regulating this pattern persist in the mature avian cochlea. The site of these signals remains to be determined. They may be expressed by the genome of the stem cell population or be obtained from the local environment of the differentiating hair cells.

Regeneration of hair cells in the vestibular epithelium

Jørgensen & Mathiesen (1988) and Roberson et al (1991) demonstrated the ongoing production of hair cells in the postnatal avian vestibular system. Using DNA autoradiography, Jørgensen and Mathiesen were able to label supporting cells and hair cells in the ampullary and otolithic organs of sexually mature budgerigars. Roberson and his collaborators utilized immunocytochemical techniques in addition to autoradiographic methods, and corroborated Jørgensen and Mathiesen's results in chicks.

In a more recent study, we evaluated the effects of aminoglycoside toxicity on the capacity of the avian vestibular system to produce new hair cells (Weisleder & Rubel 1991). Newly hatched chicks received daily injections of streptomycin sulphate (600 mg/kg per day) for seven days. Starting on the fifth day, these animals, and age-matched control chicks, received either injections of [^3H]thymidine for three days or injections of 5-bromo-2'-deoxyuridine (BrdU) for five days. BrdU is a thymidine analogue which is incorporated into the DNA of dividing cells. BrdU is antigenically distinct from normal thymidine and can be recognized by a monoclonal antibody and labelled immunocytochemically. Streptomycin-treated and control chicks were killed one, 20 or 60 days after the last injection of proliferation marker. Vestibular organs were sectioned and processed for immunocytochemistry or autoradiography. The tissue of [^3H]thymidine-injected chicks was used to study morphological characteristics of the regenerated cells; that of BrdU-injected birds was used for purposes of quantification.

One day after the last proliferation marker injection, the tissue from streptomycin-treated chicks shows clear signs of vestibulotoxicity (Fig. 5B). As previously described by Wersäll & Hawkins (1962) and Duvall & Wersäll (1964), these signs include the loss of most Type I hair cells; a drastic reduction in the number of Type II hair cells; and a reduction in the number of myelinated fibres. At this time, BrdU immunostaining and [^3H]thymidine autoradiography reveal mitotic activity in the supporting cell layer of all organs in both treated and untreated animals (Fig. 5A and B). In the drug-treated group, however, the number of labelled nuclei is four-fold greater than the labelling seen in control chicks. Just as the damage to the sensory epithelium is generalized, so is the proliferative activity. Labelled nuclei are observed throughout the organs. There appears to be no specific area of proliferation, as described in other species (Corwin 1981, 1983, 1985). Instead, the pattern of generalized proliferation reported by Jørgensen & Mathiesen (1988) is seen. In both control and drug-treated animals positive nuclei in the supporting cell layer usually occur in pairs. In some instances they are stacked one on top of the other; in other instances they appear side by side.

Twenty days after the last cell proliferation marker injection, the aminoglycoside's damage can still be detected. Few Type I hair cells are present in the sensory epithelium. The number of Type II hair cells, however, has increased. Many supporting cells and several Type II hair cells are labelled (Fig. 5C). Labelled Type II hair cells usually occur on top of labelled support cells. In some cases two labelled hair cells occur side by side on the sensory epithelium. Interestingly, Type II hair cells are found throughout the organs' epithelia, including areas usually occupied only by Type I hair cells.

Finally, in the 60 days survival group the sensory epithelium has recovered its normal appearance. Type I hair cells can now be identified occupying their normal location at the top of the organ. Type II hair cells occupy the skirts of the organ as they do in the untreated animals. Labelled nuclei are found in Type I and Type II hair cells as well as in some supporting cells (Fig. 5D). The normal complement of each cell type appears to have been restored.

Two main conclusions can be drawn from these experiments. First, the avian vestibular system is able to recover from aminoglycoside toxicity. All cell types normally present in the vestibular sensory epithelium can be identified within 60 days of streptomycin treatment. Second, the level of proliferation in the avian vestibular system appears to be dynamically regulated. The level of proliferation in the sensory epithelium increased significantly in response to the drug damage. The observed increase in the number of cells with labelled nuclei is, apparently, an attempt by the organism to compensate for the massive loss of hair cells.

According to Ramprasad et al (1986), the gross and microscopic anatomies of the avian, reptilian and mammalian vestibular systems are essentially similar. For this reason we believe that understanding the process of regeneration in the avian vestibular system may provide important pointers toward the

development of strategies for eliciting hair cell regeneration in the mammalian sensory epithelium.

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DISCUSSION

Fernald: I am a little concerned by your use of the term ‘regulation’ when you find an increased number of mitoses in response to what is clearly an overdose of some drug. I wouldn’t consider that to be regulation. I think this is indicative of the problems that we may have, of distinguishing between the inferences you want to draw from damage experiments, as opposed to tracing natural developmental sequences. The real issue is the regulation of cell division in the normal course of events, as cells turn over slowly. It’s not surprising that you get a massive increase in mitoses in response to widespread drug damage, but I am not sure you would learn from that how cell division is regulated normally.

Rubel: I agree with you, and maybe we don’t want to say ‘regulation’ in the fine sense of the term. I can say, however, from these studies, that either the rate of the cycle, or the number of cells kicked into the mitotic cycle, is increased by causing cochlear damage. Hair cells are lost; we don’t know if it’s the actual loss of hair cells, or some other stimulus, which produces this increase. We think it’s the number of cells that are entering the mitotic cycle that is increased.

Fernald: Let’s turn it around, then, and ask what, in the normal course of events, would be a reasonable regulator of the generation of new hair cells in a normal ear, given normal hair cell loss.

Calof: Is there a turnover of hair cells in the normal avian ear?

Rubel: In the normal avian vestibular system, in all the epithelial surfaces (of which there are six) there is some ongoing, normal turnover. It is, in fact, turnover rather than production of permanent new cells, because there’s not a large increase in the size of the organs. There is no proliferative zone; the proliferation seems to happen all over the receptor epithelium; and there’s not a large increase in size.

Fernald: But with a slow rate of cell division and a slight increase in size, it might be the new generation of cells.

Rubel: It might be, but I would doubt it! We don’t think there’s any significant turnover, or increase in size, or ongoing proliferation, in the avian cochlea.

In many hundreds of sections we have occasionally seen a labelled cell, in normal birds, or in the non-damaged zone of the cochlea in noise-damaged animals; but we don't normally see proliferation. Once in a while Brenda Ryals has seen it, and so have we, but we attribute it to small amounts of damage due to noise in the colony, and things like that.

Ryals: I agree that it seems unlikely that there is hair cell proliferation in the cochlear partition of the normal avian ear. If such proliferation were occurring, we would predict that the number of cells within the partition would increase with age. We have counted cells in serial sections from the basilar papilla in young (three month) and old (3–6 years) quail and do not find any increase in the number of cells. In fact we find a slight decrease (6%) in the number of hair cells in older quail (Ryals & Westbrook 1988). So, although we occasionally see some cells with [^3H]thymidine uptake in normal, non-noise-exposed quail, we feel that these are probably replaced cells rather than added cells (Ryals & Westbrook 1990).

Reasner: Dr Rubel, could you compare the noise levels that you use to induce hair cell damage with the typical sound levels present in the colony room?

Rubel: No, because colony sound levels fluctuate so much. We have used between 110 dB and 125 dB SPL of an intense pure tone (the former level for a longer duration). Our standard level now is 1500 Hz at 120 dB for 18 hours.

Reasner: I think it is relevant to ask to what extent might typical stimuli act on a normal sensory system and be a causal factor in receptor cell turnover.

Rubel: Yes, but I don't know how the normal stimuli in our colony would compare to a farmyard.

Calof: Dr Rubel, I thought your view was that there is a separate precursor population in the avian cochlea, and that it is these cells that are the precursors of both the supporting cells and the hair cells? Therefore, in any of these paradigms where you induce hair cell loss, do you also see a loss of supporting cells? And do you then see the normal level of proliferation in the supporting cell population, or is that increased as well, by sound damage?

Rubel: In this field so far, there are two ways by which cochlear hair cell damage is produced experimentally: either with noise or with aminoglycosides. The changes are very different. Noise induces a loss of hair cells, and also loss of supporting cells. You also see loss of tectorial membrane. With this type of damage, induced by pure tone noise, we find proliferation both of hair cells and supporting cells. We have no evidence suggesting that they derive from different precursor cell populations. So the hair cells and supporting cells could be coming from the same or different precursors; we don't know.

With aminoglycosides, you kill all the hair cells, but there's very little loss of supporting cells. You get hair cells labelled; supporting cells are also labelled. We don't yet know whether equivalent numbers are produced in these two situations. Brenda Ryals has some figures.

Ryals: We find two to three times as many labelled support cells than hair cells, after noise damage. We are looking at the gentamicin-treated birds now.

Burd: You have not done short survival periods, then?

Rubel: We have done continuous labelling and then fixation. We have not done pulse-fix experiments yet.

Corwin: The last time I heard about this work, you distinguished hyaline and cuboidal cells as the sources of regenerated hair cells at the inferior edge of the papilla (Girod et al 1989).

Rubel: That was clearly partially wrong! We can now distinguish a subclass of cells that we call border cells based on their position, inferior to the most inferior short hair cell.

Corwin: That helps, because I wondered what the evidence was for border cells as a distinct subclass.

Rubel: In our first study (Girod et al 1989) we gave thymidine to chicks for various periods after noise damage and then fixed the cochlea. All we can determine from this study is the area of the papilla where we first see labelling. From that, we said that cells in the inferior region were usually the first to be labelled after noise trauma. Then this area becomes several cells in depth, and then we can recognize labelled immature hair cells in the inferior, damaged area.

In these cases, it appeared that the inferior portion of the sensory epithelium was destroyed and all that was left were the hyaline and cuboidal cells. Thus we concluded, perhaps prematurely, that they must be dividing and be the precursors for the new hair cells. At that time we had no cytological markers for the different cell types. We can now recognize the hyaline cells on the basis of the cytological characteristics I've discussed in the chapter. When we looked closer, right after damage, we realized that the marker for hyaline cells (namely, basal actin filaments and cytoplasmic tubular structure) is seldom seen in cells which have recently divided. We have looked for a variety of cytological and antigenic markers of various cell types but have not found any except for those mentioned for hyaline cells.

We have distinguished a group of cells we call border cells because they are positioned between the last short hair cell and the hyaline cells. There are about three cells adjacent to each other and they look like the 'support' cells which surround hair cells, except they do not have microvilli or vesicles at their apical pole. Immediately after damage these cells seem to remain, flatten out and then divide. We never see support cells that are directly under the remaining short hair cells dividing. However, at the margin between the tall and short hair cells, we definitely see support cells dividing. Thus we now feel that there may be specialized subpopulations of cells lying in particular regions of the epithelium which are capable of becoming mitotically active. This is not to say that they are cytologically recognizably different from the rest of the support cells, at this time.

Corwin: Part of my concern is over using the last short hair cell as the criterion for deciding where support cells end and border cells begin. This was the problem with the proposal that hyaline and cuboidal cells were the precursors. It may have looked as if it was these cells because the epithelium had become flattened due to the loss of hair cells. That is, it lost its stratified appearance and therefore now appeared like the unstratified region of the hyaline and cuboidal cells.

Rubel: When the damage is at the inferior edge, support cells are lost as well. Clearly something is spreading out or migrating in, to form the cellular layer covering the basilar membrane.

Cotanche: You still see the hyaline cells very clearly after the noise trauma; there's a clear border where they are, and your labelled cells are some way in from the hyaline cells. They are at least halfway across the basilar membrane from the hyaline cells.

Rubel: However, during the time between the noise exposure and fixation of the tissue, the cells can flatten and spread out. At the light microscope level we could not recognize that the epithelial layer of cells were not hyaline cells, because we had no markers. Now, at the EM level, we can.

Corwin: But how do you define the border cell?

Rubel: We define them as the cells inferior to the last short hair cell, and they reside directly over a structural change in the composition in the basilar membrane that can be clearly identified.

Corwin: Defining the border cell on the basis of that last short hair cell is not valid, because when damage occurs the most inferior hair cells are often lost.

Rubel: You are correct! But location on the basilar membrane is valid. You can also make measurements from the superior edge of the basilar membrane in normal and damaged animals and show that proliferation is usually seen first at about the distance from the superior edge that is consistent with the normal location of the border cells. All of this is indirect, of course; we cannot positively identify the progenitor cells until we have distinct antigenic or cytological markers for all of the cell types and perform closely timed pulse-labelling studies. We are trying to find clear antigenic markers, but have not been successful so far.

Steinberg: In terms of mechanism, have you obtained, or can you obtain, a dose-response curve with noise, and see if the level of noise which produces cell death is the same as the level that produces an increase in mitosis?

Rubel: We haven't done that. There's a significant variability in the amount of hair cell loss that you get with any amount of exposure. Another approach that we are taking now, in collaboration with Doug Cotanche, is to look at various times after aminoglycosides are given, to see whether we start getting proliferation before there's actual hair cell death, or whether proliferation starts while the hair cells are still present.

Calof: What is the mechanism of toxicity of the aminoglycosides?

Rubel: This is not known. There are some good theories.

Fernald: In the noise paradigm, since you are using 1500 Hz, can't you use that to localize along the basilar membrane where you are destroying the hair cells? This might allow a more subtle destructive force, so that you leave part of the membrane intact and destroy part by highly focused sound frequencies.

Rubel: Yes, that is exactly what we do. But that gives us a measure of where *along* the basilar membrane we are destroying cells, not where *across* it. The greatest variability is in where the damage is across (from superior to inferior) the basilar membrane and in the longitudinal extent of damage—not in the central point of damage.

Corwin: Dr Rubel, how do you know that the migrating cells that became innervated were not contacting the luminal surface?

Rubel: Dale Cunningham and Larry Duckert have done serial EM sections on two such cells. They show no processes reaching the lumen. In addition, we have looked at many, many sections; if there was an apical process, I think we would have seen some. We can see cells that are two cell diameters down in from the lumen with no indication of small thin processes going up to the surface, but which are innervated.

Corwin: At what stage is that, and how do you know that those are new cells?

Rubel: After aminoglycoside treatment, all the hair cells that are going to occur in the basal tip of the papilla are new cells. We have destroyed all the original hair cells at the basilar end.

Corwin: You know that 100% were destroyed?

Rubel: We know that about 99% were! And we have seen this innervation in many cells; so I don't doubt that they are new hair cells. We see a progression of the density of cytoplasmic staining as cells migrate up to become hair cells. We haven't followed cells as elegantly as you have in the lateral line, with time-lapse cinematography, so we can't say on that level; but this is a continuous progression, so I have little doubt that these are going to become hair cells.

Cotanche: How do you know they are not going the other way? You see this continual dying of cells after gentamicin; could they be cells that have died and are being resorbed?

Rubel: I can't tell the direction of movement, obviously, but I am taking a best guess on the basis of looking at a lot of electron micrographs.

Lewis: Is there any evidence for or against the notion that some hair cells are produced without a cell division, simply by a direct change in the character of supporting cells?

Rubel: We can't say whether an existing supporting cell turns into a hair cell after damage. The only way to do that would be to inject the animal with aminoglycoside and maintain the [^3H]thymidine label at a high level for a long time. We think that aminoglycosides induce delayed hair cell death. So we would need to see if, after a long period, all the hair cells were labelled. We haven't done that yet.

Burd: Using a fluorescent dye you could label all mature supporting cells that have surface contact on the epithelium, and combine that with tritiated thymidine labelling. You could then determine whether a hair cell was new, because it would be labelled with thymidine, or whether it was pre-existing, because it would be labelled with the dye.

Rubel: Or you could do the reverse experiment—label supporting cells with thymidine when they are really young, and proliferating, and then induce damage when the animals reach adulthood. You would have to keep a [^3H]thymidine-labelled animal for a long time, which is expensive.

Margolis: That wouldn't work; the question is whether a support cell will change into a hair cell.

Burd: A mature support cell, from what Ed Rubel said, should have a process to the surface of the epithelium and you could label it with the dye.

Rubel: Some 'support' cells reach the luminal surface; we have no idea whether all do.

Lewis: I am not so much concerned with the definition of a supporting cell, but just whether you can produce a hair cell without prior cell division.

Rubel: I don't know!

Pujol: Another possibility is that a dedifferentiation of the sensory cell occurs, as recently shown in denervated vestibular epithelium (Favre et al 1989); then a new differentiation follows.

Rubel: We have never seen anything suggesting that a cell with any of the properties of the hair cell was proliferating. It would have to be complete dedifferentiation.

Powers: You discussed the restoration of hearing in your paper. There are a couple of things we have to think about here, though I don't expect there to be answers to these questions. First is the quality of the restored hearing. You showed simply thresholds; when considering regenerating systems we have to ask whether there would be sound discrimination equivalent to or even approaching the level of the normal hearing system, and whether intensity-response functions are normal.

Rubel: That's right; all I have shown are the results of evoked potential studies using tone bursts. Nobody, to my knowledge, has examined frequency selectivity, yet.

Powers: The second point I want to bring up is that evoked potentials, while valuable as far as they go, don't tell us that the animal is hearing. Only its behaviour would tell us that.

Cotanche: I think these noise-exposed birds do hear again. When I was in Charleston I used to keep them in my lab, caged with birds that did hear. When they first came out of the noise they were placed in a cage with unexposed birds. The normal birds would always jump when someone came into the room, whereas the birds that had been exposed to noise would not. After 4–5 days,

the damaged birds ended up responding to sounds. Perhaps they had learned to jump, but I think they were regaining their hearing!

Rubel: There are elegant psychophysical techniques that have been worked out for testing birds; in a couple of years we may see good studies on auditory perception.

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