Hair Cell Regeneration After Streptomycin Toxicity in the Avian Vestibular Epithelium

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ABSTRACT

Recent reports documented the ability of the posthatch avian vestibular epithelia to produce hair cells continually at a low rate. This project was designed to investigate whether, in addition, the chicken vestibular system is capable of regenerating its sensory epithelium in response to a lesion. Aminoglycoside injections were given to young birds in order to damage the vestibular epithelium. Tritiated thymidine injections were used to label cells produced in response to the lesion. Treatment and age-matched control animals were killed at 1 day, 20 days, or 60 days after aminoglycoside injections, and vestibular organs were processed for autoradiography. Our results show that the chicken vestibular sensory epithelium is capable of regenerating hair cells after severe damage. Moreover, the epithelium is capable of complete anatomical recovery. Finally, drug damage increases the pace at which hair cells are replaced, compared to the rate of hair cell turnover in untreated tissue. © 1993 Wiley-Liss, Inc.

Key words: vestibular system, chicken, inner ear, sensory-cell proliferation, repair

Regeneration, the process by which injured tissue is replaced by cells of the same type, has been reported to occur in sensory epithelia of several vertebrate classes. Stone ('37) demonstrated that amputation of a portion of a salamander's tail stimulates regeneration of the tail and replacement of lost neuromasts. In the electrosensory system of weakly electric fish, Zakon ('86) and Fritzsch et al. ('90) demonstrated that receptor organs are regenerated after injury. Oley et al. ('75), and more recently Constanzo ('85), showed that the olfactory system of mammals has a remarkable capacity for neurogenesis and replacement of sensory receptor neurons. In the gustatory system, Olmsted ('20) and Olmsted and Pinger ('36) demonstrated that taste buds degenerate following denervation and then reappear following reinnervation, a finding that has been replicated and extended by Zalewsky ('69) and Hosley et al. ('87). Finally, in the visual system, Keefe ('73) showed that the retina of amphibians is capable of sensory cell regeneration, a finding similar to the one more recently reported by Maier and Wolburg ('79) and Raymond et al. ('88) for the teleost retina.

Previous studies have demonstrated that the potential for hair cell replacement is present in the anamniote ears. Platt ('77), Corwin ('77, '81, '83, '85), Popper and Hoxter ('84, '90), and Presson and Popper ('90) have demonstrated that the ears of sharks, skates, bony fish, and amphibians add new hair cells and supporting cells during postembryonic life. So significant is this cell addition that the popula-

tion of hair cells in the ears of some of these animals increases up to 12-fold between birth and adulthood (Corwin, '86).

Unrecognized for many years, the capacity of birds to regenerate auditory hair cells has recently been documented by several authors. Cruz et al. ('87) utilized cell counts to document hair cell regeneration in the basilar papilla of young chickens after gentamycin treatment. Concurrently, Cotanche ('87) examined the neonatal chick cochlea by scanning electron microscopy after acoustic trauma and noted the repopulation of hair cells a few days after noise damage. Subsequently, Corwin and Cotanche ('88) and Ryals and Rubel ('88) demonstrated that damaged hair cells were replaced by newly produced hair cells. After this initial work, investigators have studied diverse aspects of regenerated auditory hair cells including ultrastructure (Cotanche, '87; Duckert and Rubel, '90, '92), possible precursor cells (Girod et al., '89; Oesterle et al., '92; Raphael et al., '92), and the physiological status of newly regenerated cells (McFadden and Saunders, '89; Tucci and Rubel, '89; Norton et al., '90).

Two recent studies, Jorgensen and Mathiesen ('88) and Roberson et al. ('92), recognized that the avian vestibular epithelium displays an ongoing, low level of cell proliferation and new hair cell differentiation. These reports were unprecedented, since it was previously thought that the vestibular system of birds and mammals stopped producing sensory cells during embryonic development (Ruben, '67). In the present study we investigated whether the chicken vestibular sensory epithelium is capable of regenerating hair cells and of anatomical recovery after a series of streptomycin injections. We also studied whether the pace at which hair cells are regenerated after injury is different from the rate at which cell turnover takes place in the undamaged epithelium.

MATERIALS AND METHODS

A large number of young posthatch chickens (~40) were used to document streptomycin toxicity, proliferation rates, and cellular recovery. The final groups of animals, providing the quantitative data reported below, consisted of six 2 week old White Leghorn chickens (Gallus domesticus) which received daily intramuscular injections of 600 mg kg streptomycin sulfate (Pfeizer, New York, NY) for 7 days and six age-matched control animals. Starting on the fifth day of streptomycin injections, treatment animals and control animals received twice-daily injections of 10.00 mCi kg tritiated thymidine (ICN Radiochemicals, Irvine, CA) for 3 days. The animals were then allowed to survive 1, 20, or 60 days (n = 2 group survival time).

At the end of the survival period the animals were deeply anesthetized with Nembutal, perfused transcardially with 0.9% saline solution containing 20.000 IU heparin liter, and fixed with 3.6% paraformaldehyde 0.3% glutaraldehyde fixative in 0.1 M phosphate buffer (pH 7.4). The birds were then decapitated, and the heads were placed in the fixative solution overnight. Vestibular organs were dissected the next morning. By a lateral-to-medial approach, a small opening was made in the temporal bone 3 mm above and behind the external auditory meatus to expose the lateral semicircular canal. With fine-tip forceps, the osseous labyrinth was elevated and the membranous labyrinth was uncovered. The first organ exposed was the lateral ampulla which after careful removal allowed visualization of the interior of the vestibule. The rest of the structures were removed in the following order: ampulla of the superior canal, utricle, saccule, and ampulla of the posterior canal. Both inner ears were dissected in this manner, yielding approximately 48 otolithic organs and 72 ampullary organs. Occasional organs were destroyed during the dissections. The quantitative data reported below were always obtained from organs of at least two animals. The same trends were seen in all of the organs processed for histological observation.

Dissected organs were placed in buffer solution, postfixed in osmium (1% OsO_4 in 0.1 M $NaPO_4$ buffer) for 1 hour, dehydrated in serial alcohols (35, 70, 95, and 100% ethanol) and propylene oxide, and embedded in Spurr's plastic (Polysciences, Warrington, PA). Semi-thin sections (2.5–3.0 μm) were cut on a JB-4 microtome with glass knives. The sections were mounted on chrom-alum coated acid-washed slides and allowed to dry. Slides were then dipped in Kodak NTB-2 Nuclear Track Emulsion (1:1 dilution; International Biotechnologies, Inc., New Haven, CT) and incubated for 4–8 weeks at 4°C. They were then developed in Kodak D-19 developer for 4 minutes and fixed in Kodak Fixer for 3.5 minutes. The sections were lightly counterstained with 0.01% toludine blue in 1.0% sodium borate and coverslipped with DPX. A cell with five or more silver grains overlying the nucleus was considered labeled (Sidman, '70). In most cases, however, labeled cells had a much larger number of grains (20–30) and distinguishing between labeled and unlabeled cells was clear-cut.

Scanning electron microscopy

A small group of additional animals were examined by SEM. The micrographs presented are from a control animal, a 1 day survival treatment animal, and a 60 day survival treatment animal. Other animals from these groups showed similar results. No attempt was made to quantify the amount or pattern of damage or recovery from the SEM photographs. Following dissection, osmication, and dehydration, the superior ampullae and utricles were processed for scanning electron microscopy. The organs were critical-point dried in CO_2, sputter-coated with Au-Pd, and viewed in a JEOL 840A SEM.

Streptomycin vestibulotoxicity

For a historical account of streptomycin's vestibulotoxicity see Molitor et al. ('46), Farrington et al. ('47), Stevenson et al. ('47), Berg ('49), Rüedi et al. ('49), Hawkins and Lurie ('52), Wersäll and Hawkins ('62), Duval and Wersäll ('64), Igarashi et al. ('66), and Lindeman ('69). To confirm the damage caused by streptomycin, alternate 3 μm thick sections of one ampullary and one otolithic organ of two streptomycin-treated 1 day survival animals were stained with toludine blue and viewed under light microscopy. This analysis was done in order to assess which cell types of the avian vestibular epithelium were affected by the aminoglycoside treatment, and whether the damage was generalized or selective to specific areas of the sensory epithelium.

Hair cell numbers

The average number of hair cells per 100 μm of sensory epithelium in six streptomycin-treated and in four control animals was obtained. Twenty-two 3 μm thick serial sections from one ampulla of two streptomycin-treated animals in each survival group (1, 20, or 60 days survival) and two control animals of the 1 and 60 day survival groups were studied (n = 10 organs). Also, 10 tissue sections (every 24 μm) from one utricle of two streptomycin-treated animals in each survival group (1, 20, or 60 days survival) and two control animals of the 1 and 60 day survival groups were studied (n = 10 organs). Separate counts of Type I and Type II hair cells were done for ampullary organs, while only the total number of hair cells was obtained for utricles. After hair cell counts the length of the sensory epithelium at the level of the basement membrane was measured on each section with the aid of a BioQuant Image Analysis system (R & M Biometrics, Nashville, TN). These data were used to quantify the effects of streptomycin damage. The data also provided answers to the following questions: 1) Are vestibular hair cells replaced after an aminoglycoside lesion; 2) If so, are both types of hair cells replaced; and 3) Are either Type I or Type II hair cells replaced ahead of the other?

Hair cell regeneration

To demonstrate that new hair cells are added to the sensory epithelium as a result of cell proliferation, serial (3

μm thick) sections of three ampullary organs and every eighth tissue section (one in every 24 μm) of two utricles of treatment and control animals from each of the three survival groups were processed for autoradiography. Labeled hair cells and supporting cells were identified microscopically. The criteria for identification of a hair cell were position of the cell body in the sensory epithelium, presence of stereocilia, and location of the cell nucleus. Type I and Type II hair cells were differentiated on the basis of the presence or absence of a nerve calyx. Cells whose nucleus was located in the layer of supporting cells were considered as such.

Cell proliferation rate

The tissue utilized for the aforementioned analysis was also used to compare the relative amount of proliferation that had occurred in the various treatment and survival conditions. Labeled nuclei of hair cells and supporting cells in the ampullary sensory epithelium of treated and untreated birds at all survival times were counted. To compare the relative rates of cell proliferation in treated and untreated animals, 22 tissue sections from the central portion the cristae ampullaris of three treatment animals and three control animals at each survival time were examined. Also, in 10 tissue sections from two utricles per treatment condition and survival time the total numbers of labeled cells were studied. The average number of labeled cells per 100 μm of sensory epithelium was determined. The criteria utilized to determine the presence of a hair cell have already been explained. For this analysis, however, more importance was given to the position of the cell and its nucleus in the sensory epithelium than to the presence of stereocilia. Two factor (treatment × survival time) ANOVAs were used to identify differences between the ratio of labeled cells/100 μm in ampullae and utricles of streptomycin-treated animals and of age-matched control animals.

RESULTS
The avian vestibular epithelium

The peripheral vestibular organs of vertebrates consist of three layers (Iurato, '67). Internally there is nervous tissue which is penetrated by blood vessels. The intermediate layer is a thin basement membrane. The lumenal lining consists of columnar-type epithelium in which nerve endings, supporting cells, and hair cells can be found. Two types of hair cells can be identified in the avian and mammalian vestibular sensory epithelia: Type I and Type II hair cells (Fig. 1). The hair cells are differentiated on the bases of their morphology and their innervation (Wersäll, '56; Jørgensen and Cristensen, '89). Type I hair cells are pear-shaped, with narrow apical regions and wide bases. This type of hair cell is surrounded by a nerve calyx. Type I hair cells are more concentrated in the central part of the macula, the striola, and at the summit of the crista. Type II hair cells have the shape of an elongated cylinder. Their basal surface is in contact with several bouton-type nerve endings. Type II hair cells are found throughout the epithelium of the otolithic organs. In the ampullary organs, they are found on the skirts of the sensory epithelium, but a few are also seen between nerve calyxes. Supporting cells form the basal layer of cells in the sensory epithelium. Under normal conditions there is a single row of supporting cells in the vestibular parenchyma. These cells rest on the basement membrane. The cells have traditionally been described as cuboidal in morphology with an ovoid nucleus located near the base of the sensory epithelium and up to three nucleoli. The cells, however, extend to the free surface of the epithelium in such an irregular and contorted fashion that the full extent of one cell is difficult to see in a section. On the surface of the cell, oriented towards the lumen of the organ, the cells have a number of microvilli and a modified short kinocilium.

Scanning electron microscopy

Figure 2 compares the superior crista ampullaris of an untreated animal with the same structure immediately after streptomycin treatment and after 60 days of recovery. The avian ampullary organs consist of two sensory areas perpendicular to the semicircular canal separated by a nonsensory structure parallel to the canal, the septum cruciato. The surfaces of the sensory areas are covered by an abundance of stereocilia which belong to the underlying hair cells. The surface of the septum cruciato is covered by the apical ends of dark cells which do not bear stereocilia. After 7 days of streptomycin injections (Figs. 2B, 3) this organ is relatively devoid of stereocilia. In addition, at higher magnification (Fig. 3), we are able to see parts of cell bodies of presumably dead hair cells being ejected from the epithelia. In the midst of the lesion, however, the initial signs of regeneration can be appreciated as presumably recently regenerated hair cells are in the process of acquiring their normal features (Fig. 3, insert). Note that although the stereocilia are still shorter than normal, they already display the characteristic "staircase" pattern of height. Figure 2C displays the superior crista ampullaris of a streptomycin-injected animal allowed to survive 60 days. Most normal anatomical features have been recovered; the surface morphology is indistinguishable from that of a normal animal.

Streptomycin vestibulotoxicity and hair cell numbers

The histological signs of streptomycin toxicity and recovery in superior cristae ampullaris are presented in Figure 4. Figure 4A displays the tissue from a control bird. Figure 4B is a section from an animal killed 1 day after the last of seven streptomycin injections. Signs of aminoglycoside toxicity in the vestibular epithelium include virtually complete loss of Type I hair cells, leaving behind empty nerve calyxes, and a marked reduction in the number of Type II hair cells. In some cases, cells without stereocilia, located in the hair cell layer of the epithelium, can be seen in the parenchyma. The row of supporting cells appears not to have sustained damage. In some areas, however, the single layer of supporting cells has been substituted by two or three layers of cells.

Quantitative data on hair cell loss and recovery are shown in Figure 5 and Table 1. The average number (±SEM) of hair cells per 100 μm of sensory epithelium (from lateral to medial) in cristae ampullaris from four control birds is presented in each panel for comparison with data from experimental animals. These normative data were compiled from two organs harvested from 10 day old chicks (the age-matched controls of the 1 day survival treatment animals) and two organs from 70 day old birds (the age-matched controls of the 60 day survival treatment animals). These data were combined since the densities in the two groups did not differ. Plotted against these averages are the data from two streptomycin-treated animals al-
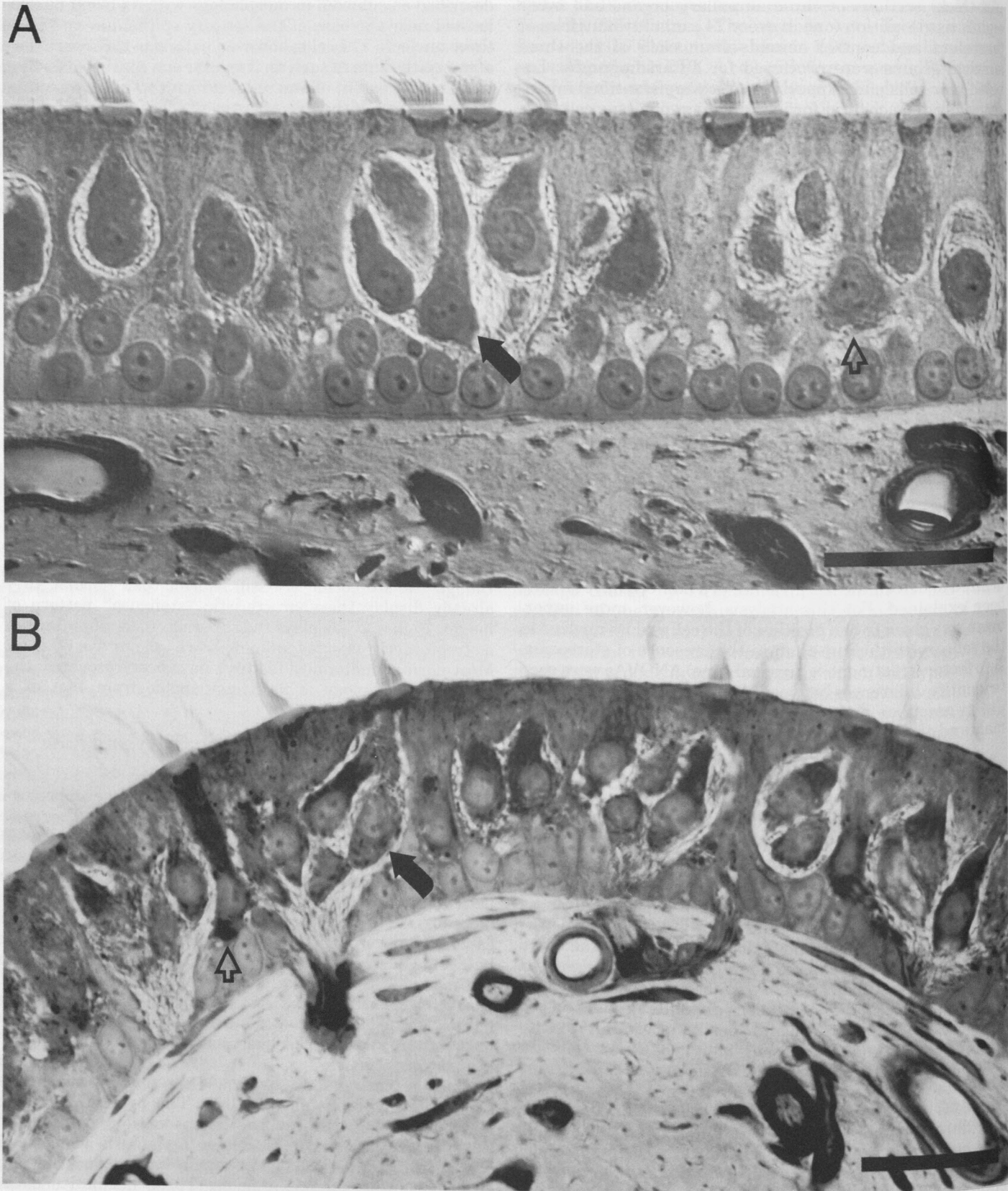
Fig. 1. Photomicrographs of the vestibular sensory epithelium of a 10 day old chick. **A:** saccule. **B:** ampulla. Solid arrows: Type I hair cells; these cells are pear-shaped and enclosed in the nerve ending. Open arrows: Type II hair cells; these cells are cylindrically shaped and receive bouton type innervation. Supporting cells form the basal layer of cells in the vestibular parenchyma. Calibration bars = 20 μm.
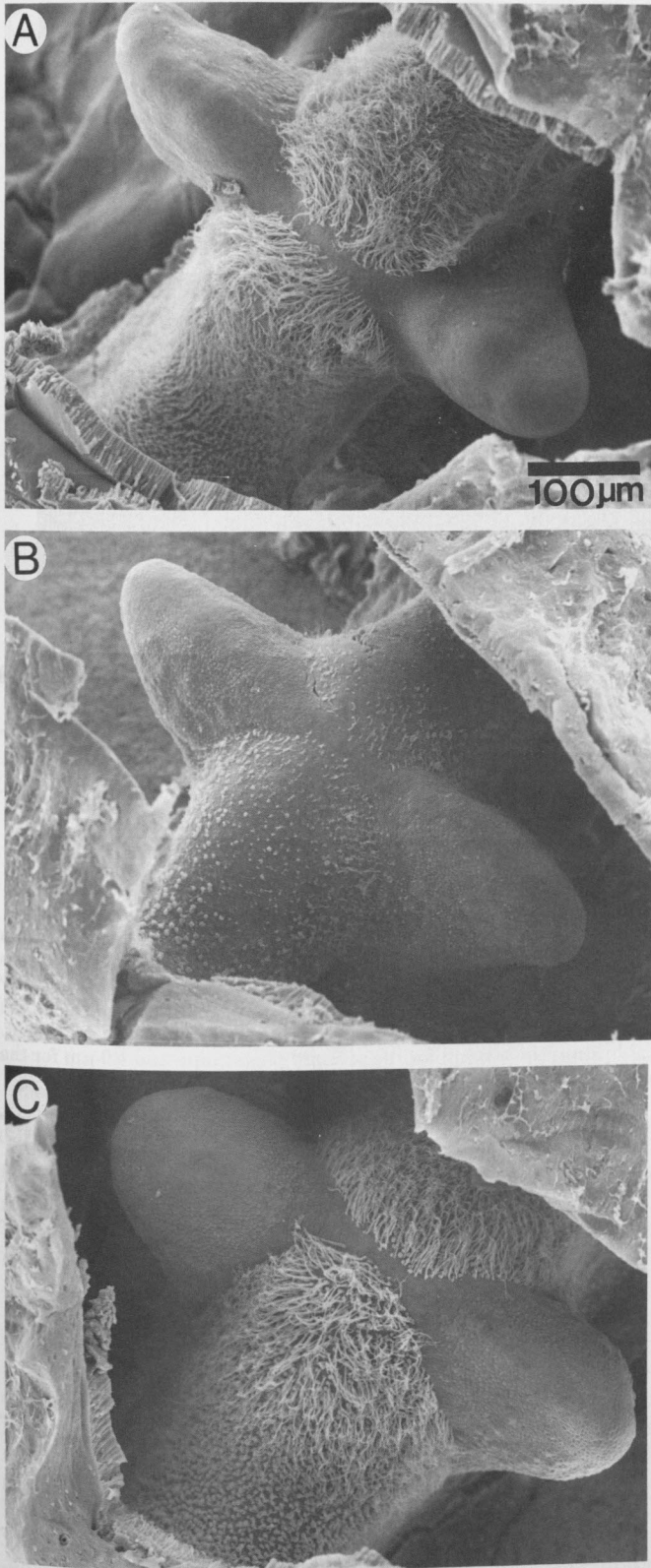
Fig. 2. Scanning electron micrographs of superior ampullae (the cupula of the organs was removed). **A:** Crista ampullaris of a 10 day old control animal. Note the abundance of stereocilia. **B:** Crista ampullaris of a streptomycin-injected animal killed at the end of a 7 day schedule of streptomycin injections. Note the damage to the tissue characterized by the almost complete absence of stereocilia. **C:** Crista ampullaris of an animal allowed to survive 60 days after the aminoglycoside injection schedule. The sensory epithelium has recovered most of its normal appearance.

lowed to survive 1 day, 20 days, or 60 days. As can be seen, control birds have almost six times as many hair cells as treated animals examined immediately after the streptomycin injections. Also apparent from this figure is that the ototoxic lesion to the vestibular sensory epithelium is generalized and homogeneous. Hair cell loss is seen throughout the sensory epithelium. Streptomycin's vestibulotoxic lesion primarily affects Type I hair cells (Table 1). This is reflected by our inability to identify a single example of this cell type in ampullae from aminoglycoside-treated animals at 1 day. The population of Type II hair cells, however, is also significantly affected. In ampullae of 1 day survival control animals there are almost twice as many Type II hair cells as in ampullae of streptomycin-injected birds ($P < .0001$; Table 1).

Twenty days after the streptomycin injections, there are clear signs that recovery is taking place (Fig. 4C); new hair cells can be recognized in the vestibular parenchyma. A striking finding is that hair cells, similar to Type II hair cells, can be found at the summit of the cristae, an area of the epithelium exclusively populated by Type I hair cells in untreated animals. In addition to being able to identify Type II hair cells in the tissue from streptomycin-treated animals, a small number of Type I hair cells can be seen sharing the summit of the cristae with Type II hair cells. Interestingly, at this recovery time the nerve calyxes that we observe contain a single Type I hair cell. Comparisons with tissue from control animals reveal that in untreated vestibular organs each nerve calyx encompasses two to three hair cells. Figure 5B again presents data from the control birds' ampullae plotted with those from two streptomycin-treated animals. The average number of hair cells in the tissue of the two treated birds is 10.01. This number, although approaching the figure from untreated birds, is still significantly less ($P < .0001$; Table 1). The data shown in Figure 5B, thus, provide evidence that 20 days after streptomycin injections hair cells are being replaced. They also show that the process of hair cell replacement is not restricted to any particular area of the sensory epithelium as hair cell numbers increase in all regions. While the number of hair cells in ampullae of streptomycin-treated birds is approaching that of untreated animals, most of the increase is afforded by an increment in Type II hair cells. The average number of Type II hair cells per 100 μm in streptomycin-injected birds is 8.09 while that of untreated animals is 3.17. On the other hand, the number of Type I hair cells in treated birds is less than that of untreated birds by a factor of four. It is thus apparent that the initial response to the streptomycin lesion in the ampullary organs is an increase in the number of hair cells that meet our criteria for designation as Type II.

By 60 days after the last streptomycin injection, the aminoglycoside-treated ampullary tissue looks similar to the tissue from control animals (Fig. 4D). Type I hair cells can be identified inside nerve calyxes at their usual location, the summit of the organs. Moreover, the number of hair cells per calyx has increased. Up to three hair cells can be counted inside a calyx. Although several Type II hair cells can still be seen at the summit of the organ, most occupy their usual location on the skirts of the cristae. The row of supporting cells has recovered its monolayer appearance. Figure 5C and Table 1 show that the differences in total hair cell numbers between experimental and control animals have disappeared. Not only do total hair cell counts
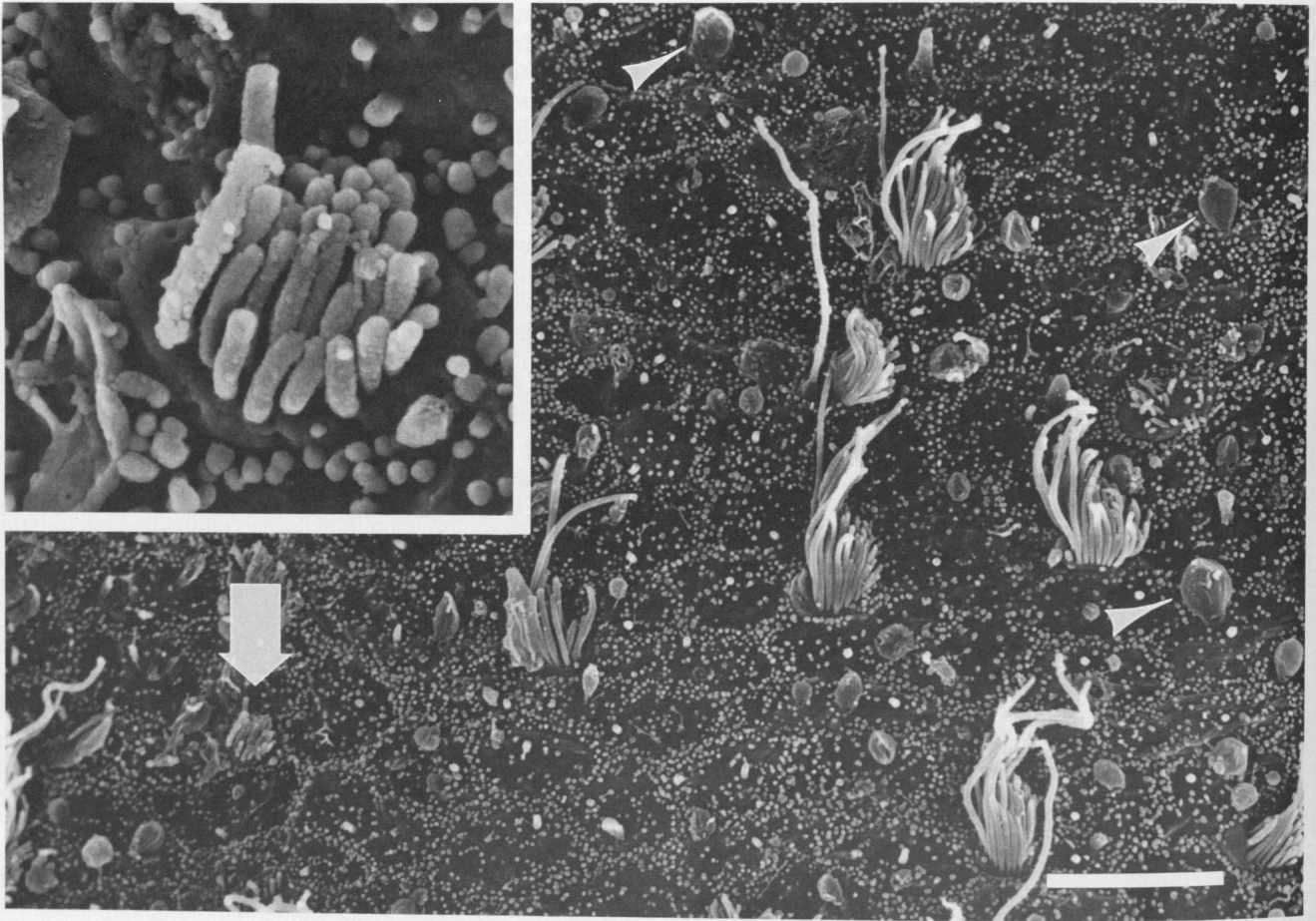
Fig. 3.   Higher power scanning electron micrographs of sensory epithelium from same specimen as shown in Figure 2B. This region is approximately 75 μm from the septum cruciato on the left-hand side of Figure 2B near the summit of the crista. This figure shows that the sensory epithelium is largely devoid of normal hair cells after the aminoglycoside treatment. A few normal appearing stereocilia bundles are seen with an adjacent lengthy kinocilium. In addition, numerous instances of extruded parts of cells are observed (arrowheads), and a small stereocilia bundle from a presumably regenerated hair cell is indicated by the arrow. The **insert** shows a higher magnification view of this bundle in which the stereocilia are beginning to show the characteristic "staircase" pattern and a larger kinocilium is evident. Calibration bar = 5 μm for the primary photograph and 1.0 μm for the insert.

reveal no reliable differences between treated and untreated animals, but most of the normal anatomy of the vestibular parenchyma has been restored. While total hair cell numbers are indistinguishable from control birds, analysis of the tissue reveals a difference in the ratio of Type I to Type II hair cells. In untreated animals, this ratio favors Type I hair cells 2.65:1. In streptomycin-injected animals, however, the ratio favors Type II hair cells 1:1.2 (Table 1). This observation suggests that although most recovery has taken place by 60 days, there may be some fine morphologic remodeling that is yet to occur.

Our observations on utricular organs are similar to those from ampullae (Fig. 6; Table 1). There are, however, some variations worth mentioning. First, the lesion to the sensory epithelium is not as severe as that seen in the ampullae (Fig. 6A,B), the difference being that the population of Type II hair cells appears to have sustained less damage, although the organs are devoid of Type I hair cells. Second, the pattern of recovery appears somewhat different. The total number of hair cells immediately following the damage is, as expected, significantly smaller in treated than in untreated animals. Twenty days after the last streptomycin injection the total number of hair cells is slightly recovered. By 60 days, however, the utricles of treated animals actu-

ally have 20% more hair cells than the tissue of control birds, a difference which is significant ($P < .0001$). Data from serial sections across 30% of the organ from utricles of two experimental and two control animals are presented in Figure 7.

Cell proliferation

Tritiated thymidine injections were used to detect cells traversing the "S" phase of the cell cycle and the progeny of those cells. Quantitative evaluations are provided from counts of labeled supporting cells and labeled hair cells in the sensory epithelium in superior cristae ampullaris of streptomycin-treated and control birds. Total numbers of labeled cells were documented for utricular tissue. Epithelium length measurements were taken at the level of the basement membrane to convert raw counts to density measurements. Again, tissue from six groups of animals were evaluated: three treatment groups (1 day, 20 days, and 60 days) and three groups of age-matched controls. For the ampullary tissue, 22 nonadjacent sections taken from the mid-portion of the cristae ampullaris were evaluated from each organ. Three organs (from at least two animals) were evaluated ($n = 18$). In addition, 10 sections from the mid-
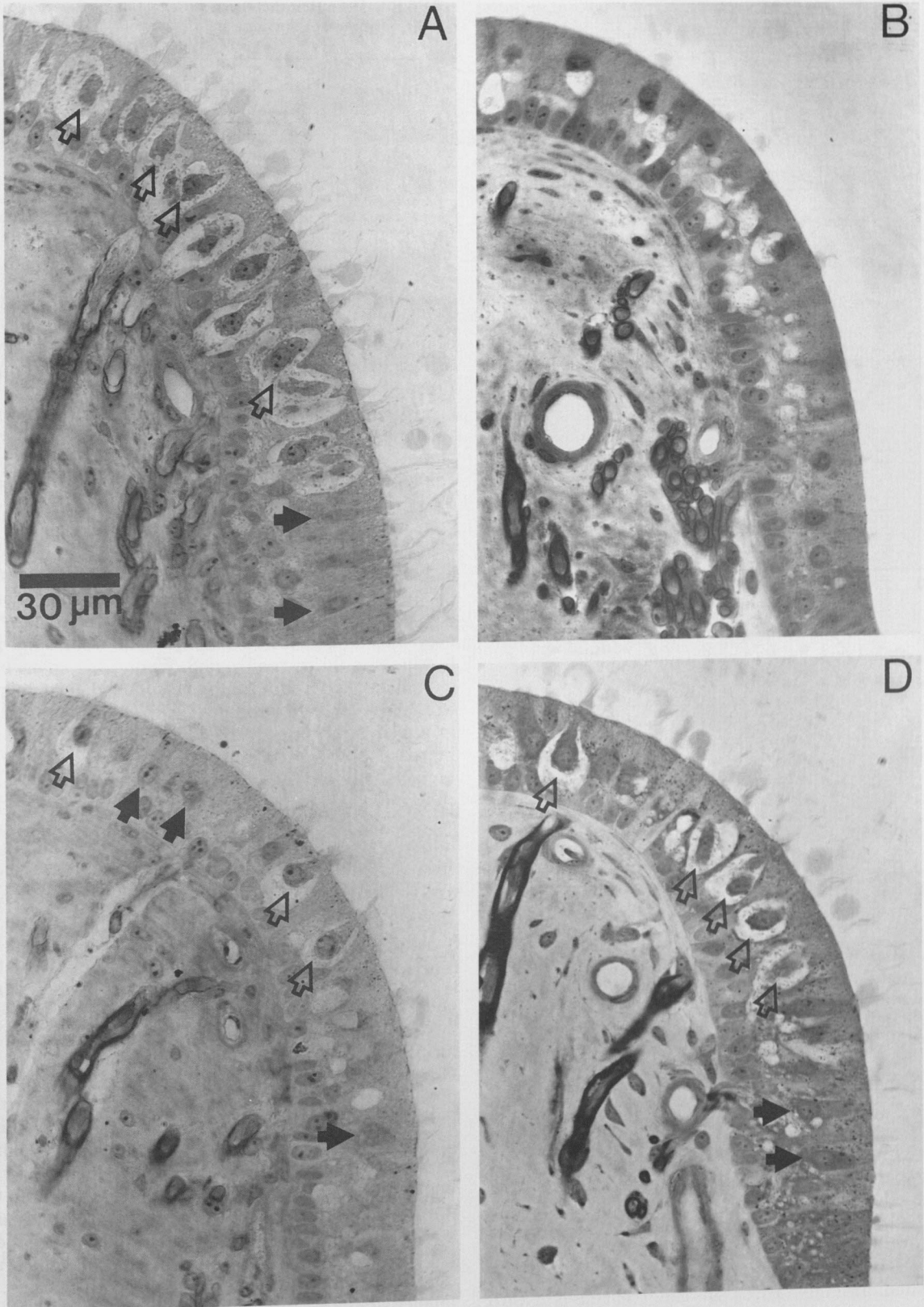
Fig. 4. Photomicrographs showing signs of streptomycin toxicity and recovery in ampullary organs. **A:** Control animal. Type I hair cells, inside nerve calyxes, are found in the upper two-thirds of the organ (open arrows). Type II hair cells are found on the skirts (solid arrows). **B:** One day of survival. Note the absence of Type I hair cells, leaving behind empty nerve calyxes. The number of Type II hair cells is also reduced. **C:** Twenty days of survival. Histological signs of regeneration include the reappearance of several Type II hair cells (solid arrows) and a few Type I hair cells (open arrows). **D:** Sixty days of survival. Most of the normal characteristics of the epithelium have been restored. There are, however, not as many Type I hair cells as in control ampullae (open arrows).
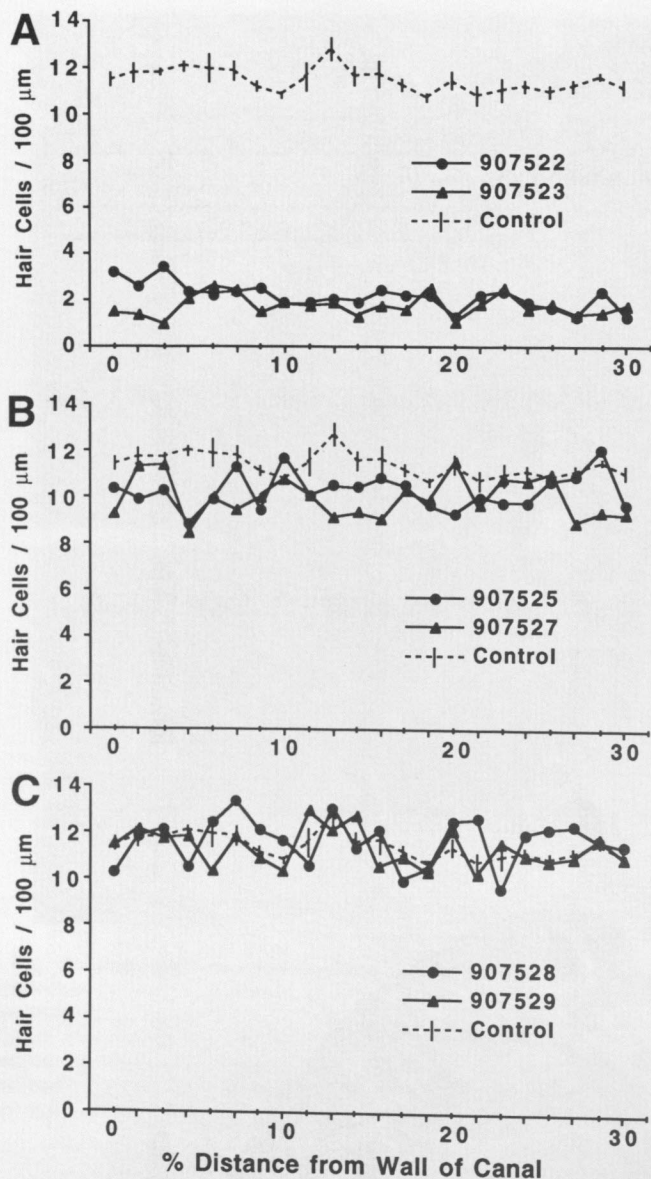
Fig. 5. Total number of hair cells per 100 μm sensory epithelium in serial, lateral to medial sections of cristae ampullaris from control and experimental animals. **A:** One day of survival. **B:** Twenty days of survival. **C:** Sixty days of survival. In each case the averages of four control animals (Control) are plotted against the individual data of two streptomycin-treated animals from the aforementioned survival times (vertical bars = SEM).

TABLE 1. Mean Hair Cell Density (Cells/100 μm Sensory Epithelium) in Superior Cristae Ampullaris and Utricles of Control and Streptomycin-Injected Animals[1]

| | Ampullae | | | Utricles: |
	Total	Type I	Type II	Total
Controls	11.6 (0.1)	8.59	3.17	9.67 (0.2)
1 day survival	1.97 (0.08)	0.0	1.97	7.10 (0.2)
20 day survival	10.01 (0.12)	1.93	8.09	7.37 (0.1)
60 day survival	11.56 (0.1)	5.2	6.37	11.56 (0.3)

[1]Separate counts of Type I and Type II hair cells were made for ampullae. Numbers in parentheses = SEM.

smaller, are also higher in treated animals. These data are presented in Figure 7, where it is apparent that there are almost four times as many labeled cells in the tissue of streptomycin-treated animals than in the tissue of control birds.

Twenty days after the last streptomycin injection, labeled nuclei are again found abundantly among supporting cells. In addition, labeled Type II hair cells are commonly seen in the parenchyma. A new finding is that, occasionally, a labeled Type I hair cell can also be seen in the epithelium (Fig. 8C). Just as in the 1 day survival group, there are many more labeled hair cells and supporting cells in streptomycin-treated tissue than in untreated tissue. Similar to the 1 day survival group, there are more labeled supporting cells than hair cells; the differences are, however, not as prominent (Fig. 9). One additional important finding should be mentioned. There is a small but significant increase in the number of labeled supporting cells in the 20 day survival group as compared to the 1 day survival group of streptomycin-treated animals ($P < 0.001$). This increase, however, is not as robust as that seen for labeled hair cells. In the same animals the number of labeled hair cells increases by over a factor of three during this period ($P < 0.001$). This large increase in the number of labeled hair cells, graphically presented in Figure 9, is also seen in control animals, indicating that during this period hair cells are the predominant cell-type being added to the sensory epithelium.

Sixty days after the last streptomycin injection the tissue from treated animals has recovered most of its normal anatomical features. Analysis of the tissue exposed to the cell proliferation marker reveals the same trend seen in the two previous survival-time groups: streptomycin-treated animals have a larger number of labeled hair cells and supporting cells than untreated animals. There are, however, some variations. Many labeled Type I hair cells can be detected in the epithelium, and the labeled Type I hair cells are seen at the appropriate location in the parenchyma. Between 20 and 60 days the number of labeled hair cells kept increasing steadily in both treated and untreated animals although at a slower pace. Interestingly, at 60 days we observe a small decrease in the number of labeled supporting cells for both treatment conditions. It is conceivable that by 60 days some of the cells that were originally labeled are dying and being replaced by new unlabeled cells.

A two factor ANOVA performed on the total number of labeled cells per 100 μm in ampullae of treated and untreated animals (three organs per treatment group and survival time) revealed significant differences for treatments ($F_{(1,12)} = 52.66$; $P < .001$) and survival times ($F_{(2,12)} = 5.34$; $P < .05$). The test also revealed no interaction between treatments and survival times. These results confirm that the pace at which cells are replaced in

portion of two utricles for each treatment condition ($n = 12$) were evaluated.

In the 1 day survival group, tissue autoradiography reveals mitotic activity both in the supporting cell layer and in the hair cell layer of the vestibular organs in both treated and untreated animals. Birds in the treatment group, however, have a much larger number of labeled nuclei than animals in the control group (see Figs. 8A,B, 9). In addition, in both groups of animals, the vast majority of labeled cells are in the supporting cell layer or within one nuclear diameter. The ampullae of streptomycin-injected birds have an average of 1.7 labeled supporting cells per 100 μm while untreated animals only have an average of 0.44 labeled supporting cells. The figures for labeled hair cells, although
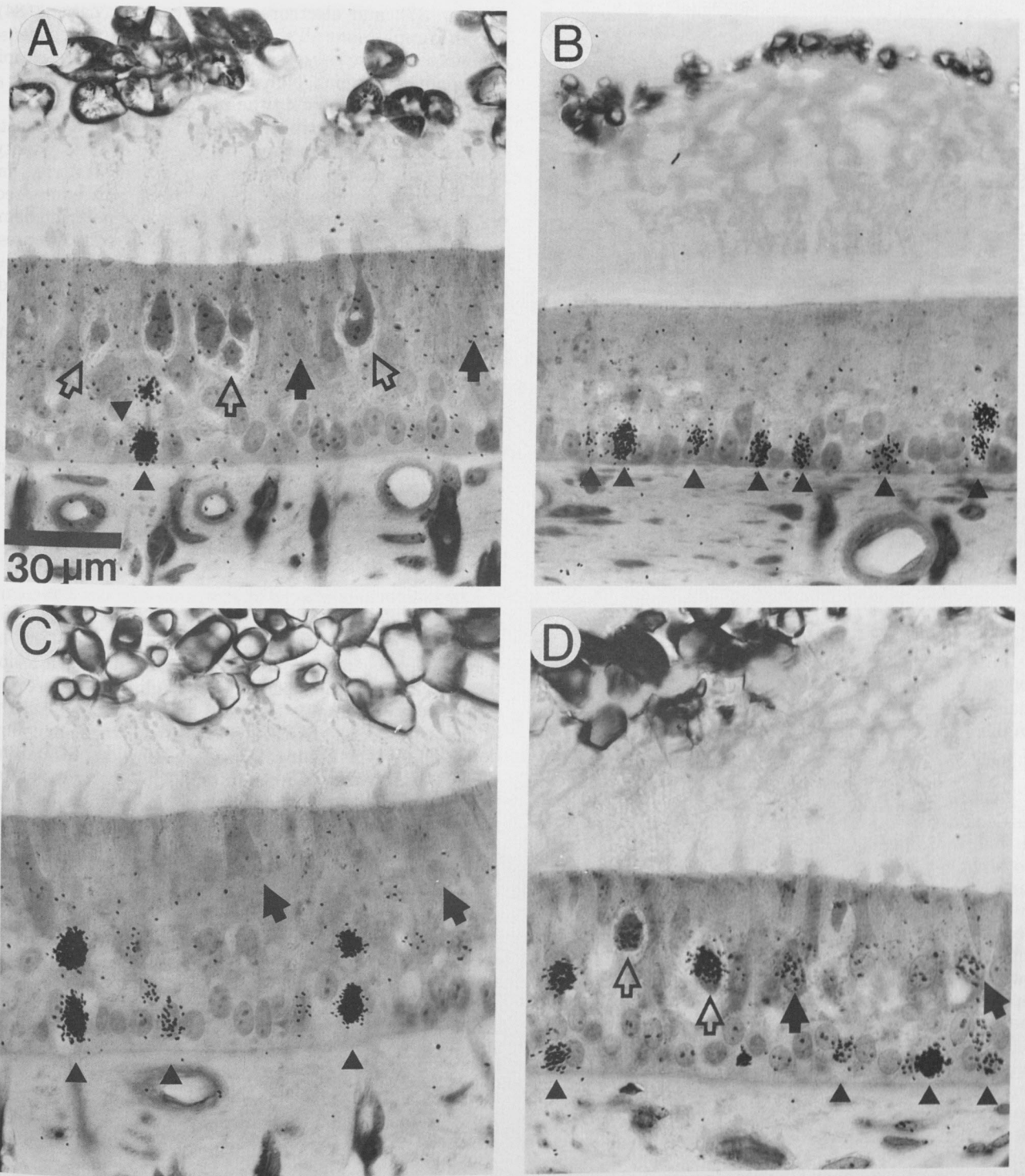
Fig. 6. Signs of streptomycin toxicity and hair-cell regeneration in utricles. **A:** Control animal from the 20 days of survival group. Note the presence of both Type I (open arrows) and Type II (solid arrows) hair cells. Only two cells are labeled by the proliferation marker (arrowheads). **B:** One day of survival. There are no Type I hair cells in the sensory epithelium. Several cells within the supporting cell layer display the label (arrowheads). **C:** Twenty days of survival. The number of Type II hair cells has increased slightly and an occasional Type I hair cell can be seen. The label is displayed both by Type II hair cells (solid arrows) and by supporting cells (arrowheads). These hair cell-supporting cell pairs are a common occurrence in the epithelium. **D:** Sixty days of survival. Several labeled Type I (open arrows) and Type II hair cells (solid arrows) can be seen in the epithelium.

the tissue of aminoglycoside-damaged animals is significantly faster than the level of cell replacement in untreated animals. The results also substantiate that the number of labeled cells increases significantly with time, presumably due to multiple divisions of the labeled precursor cells.

Data from the otolithic organs revealed a trend similar to that seen in ampullary organs. The number of labeled cells in streptomycin-treated animals was always larger than in control birds (Fig. 6). A two factor (treatment × survival time) ANOVA of the total number of labeled cells per
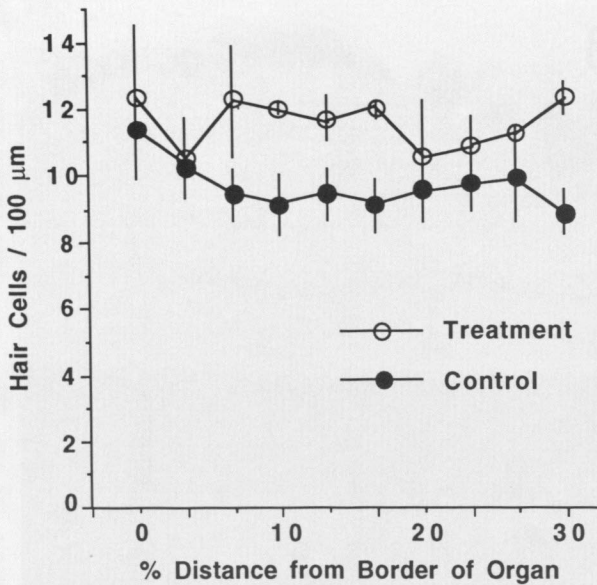
Fig. 7. Hair cell density in the utricle of 60 days of survival treated animals (Treatment) and control birds (Control). Treated animals display a 20% overproduction of hair cells across the epithelium (vertical bars = SEM).

100 μm in utricles of treated and untreated animals revealed a significant value for treatments ($F(1,6) = 9.98$; $P < .05$) but not for survival times, with no interaction between the two factors. Post-hoc pairwise comparisons revealed significantly more labeled cells in treated animals than their age-matched controls at all survival times.

DISCUSSION

As demonstrated first by Jørgensen and Mathiesen ('88) and then by Roberson et al. ('92), there is a low rate of mitotic activity and hair cell differentiation in the postnatal avian vestibular epithelium. The purpose of such addition may be to replace cells that die during an ongoing normal process of cell death and replacement, to increase the number of cells as the animal and the vestibular organs grow, or both. As is discussed below, our data tend to support the first alternative. The results from this investigation demonstrate that the avian vestibular system is also capable of regenerating hair cells after severe damage. This proliferation appears to result in restoration of sensory input to the brain so that the function of the vestibular organs is reestablished. Electrophysiological data indicate that regenerated hair cells do promote recovery of function after aminoglycoside injury. Jones and Nelson ('92) recorded vestibular evoked potentials (VsEPs) at various times after 7 days of streptomycin injections. Their data reveal an initial profound functional vestibular deficit characterized by abolishment of component waves of the VsEPs. One week later, some signs of recovery were apparent and after 60–70 days posttreatment the VsEPs of treated animals were indistinguishable from those of intact birds.

In retrospect, once Jørgensen and Mathiesen ('88) discovered ongoing proliferation in the adult avian vestibular epithelium, the ability of this system to regenerate hair cells could have been predicted. The gustatory (Zalewsky, '69) and olfactory systems (Graziadei, '73) of all vertebrate classes and the visual (Raymond et al., '88), lateral line

(Stone, '37), and electroreceptor systems (Zakon, '84) of fish and amphibians all show ongoing production of sensory elements and also possess the capacity to proliferate rapidly in response to lesions. Nonsensory organs such as liver and bone, and nonsensory epithelia such as the skin and the epithelium of the gastrointestinal tract, also show constant turnover and display a rapid increase in mitotic activity in response to trauma (Cotran et al., '89). In contrast, most neural tissue and heart muscle, both systems where recovery from damage through regeneration has not been demonstrated, also lack the capacity to replicate at low rates. It thus seems likely that the two phenomena, ongoing proliferation and regeneration, are linked, and that the latter does not occur if the former isn't expressed. An exception to this rule may be the avian auditory system, where Jørgensen and Mathiesen ('88) reported not being able to detect ongoing production of sensory elements, while other authors (Cruz et al., '87; Corwin and Cotanche, '88; Ryals and Rubel, '88) demonstrated regeneration after damage. On the other hand, Ryals and Westbrook ('90) as well as a recent study by Oesterle and Rubel ('92) provide evidence that a *very* low rate of ongoing proliferation of support cells does takes place in the avian basilar papilla.

Vestibulotoxic effects of streptomycin

The deleterious effects of streptomycin were recognized soon after its introduction as an antibiotic (Farrington et al., '47). In our experiments, we confirmed that large dosages of this aminoglycoside produce a dramatic lesion in the avian vestibular sensory epithelium. As previously described in mammals (Wersäll and Hawkins, '62; Duval and Wersäll, '64), this lesion consists of a near complete depletion of Type I hair cells and a reduction in the number of Type II hair cells. Our results, although consistent with those of Wersäll and his collaborators, are in contrast with those reported by Park and Cohen ('84). These authors injected chickens of similar age to those used in this experiment with streptomycin sulfate (either 400 mg/kg/day for 30 days or 1,200 mg/kg/day for 15 days) and were not able to detect any hair cell loss in the sensory epithelium. Hair cell regeneration can be ruled out as the reason why these investigators did not detect hair cell loss since animals in the former group were killed 24 hours after the 30th day of streptomycin injections and animals in the latter group 24 hours after the 15th day of injections. As demonstrated here, a 1 day survival period is far too short for complete anatomical recovery. We find this disagreement puzzling, since we consistently see hair cell loss and other signs of damage in streptomycin-injected animals, even in those to which we administer the aminoglycoside for only 2 days (Weisleder and Rubel, '92b).

As previously reported (Igarashi et al., '66; Lindeman, '69), the ampullae are more susceptible to injury by streptomycin than the otolithic organs. The number of Type II hair cells counted in ampullary organs of streptomycin-treated animals was always smaller than that of untreated animals. This difference became smaller at the longest survival time when anatomical recovery approached completion. The lesion to the utricular macula, on the other hand, was not so severe, as demonstrated by the smaller difference in hair cell density between treated and control animals.

Finally, although damage to the vestibular sensory epithelium was generalized, the population of Type I hair cells was much more severely affected than the population of Type II hair cells. In the utricular macula of fish, Yan et al.
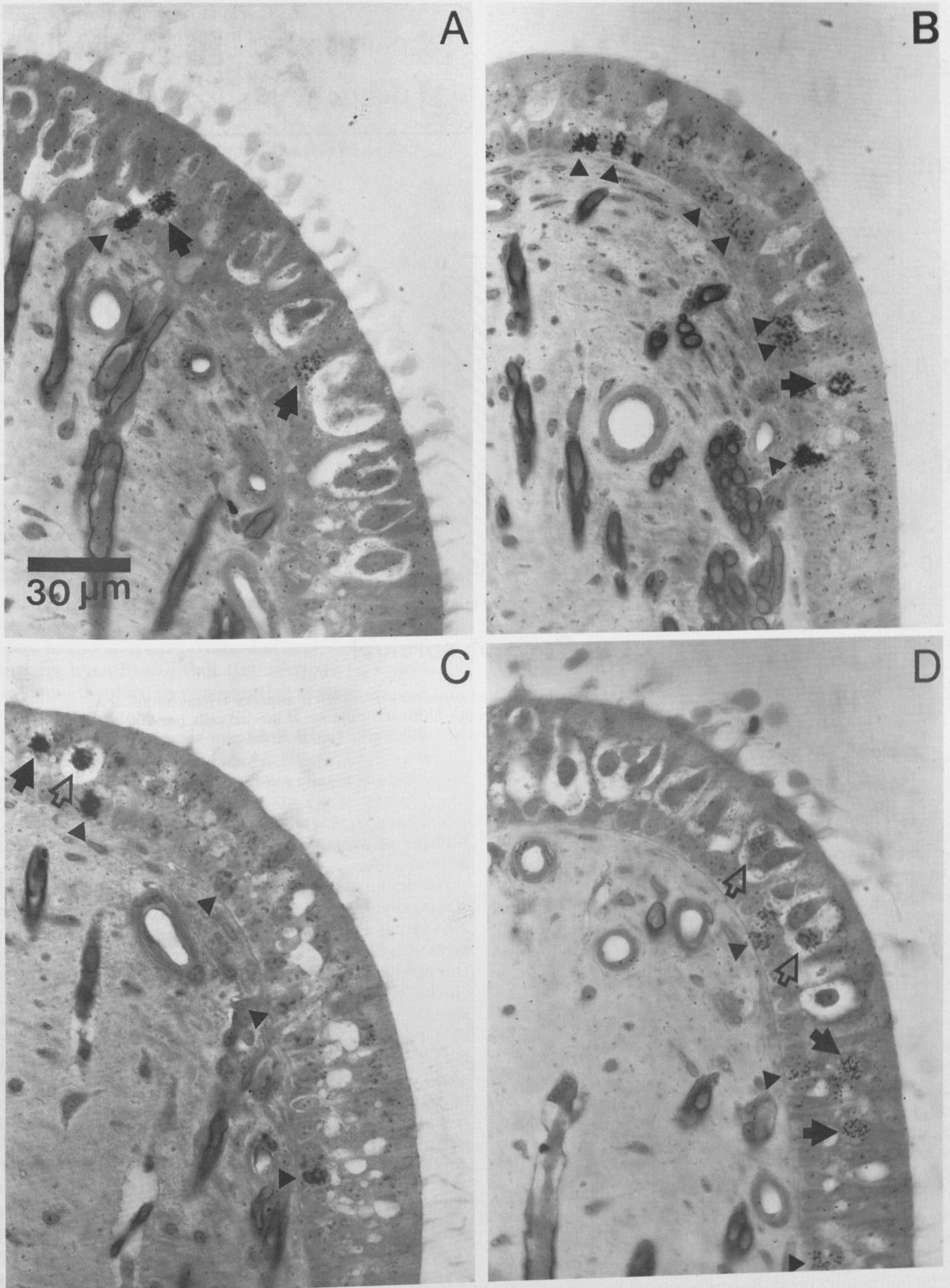
Fig. 8.   Cell proliferation in superior cristae ampullaris. **A:** Control bird from the 20 days of survival group. Two Type II hair cells (solid arrows) and one supporting cell (arrowhead) display the label. **B:** Streptomycin-treated, 1 day of survival. One Type II hair cell (solid arrow) and many supporting cells (arrowheads) have incorporated the proliferation marker. **C:** Streptomycin-treated, 20 days of survival. One Type I hair cell (open arrow), one Type II hair cell (solid arrow), and several supporting cells (arrowheads) display the label. **D:** Streptomycin-treated, 60 days of survival. In addition to the anatomy resembling that of an untreated organ, Type I (open arrow) and Type II (solid arrow) hair cells and supporting cells (arrowheads) are labeled.
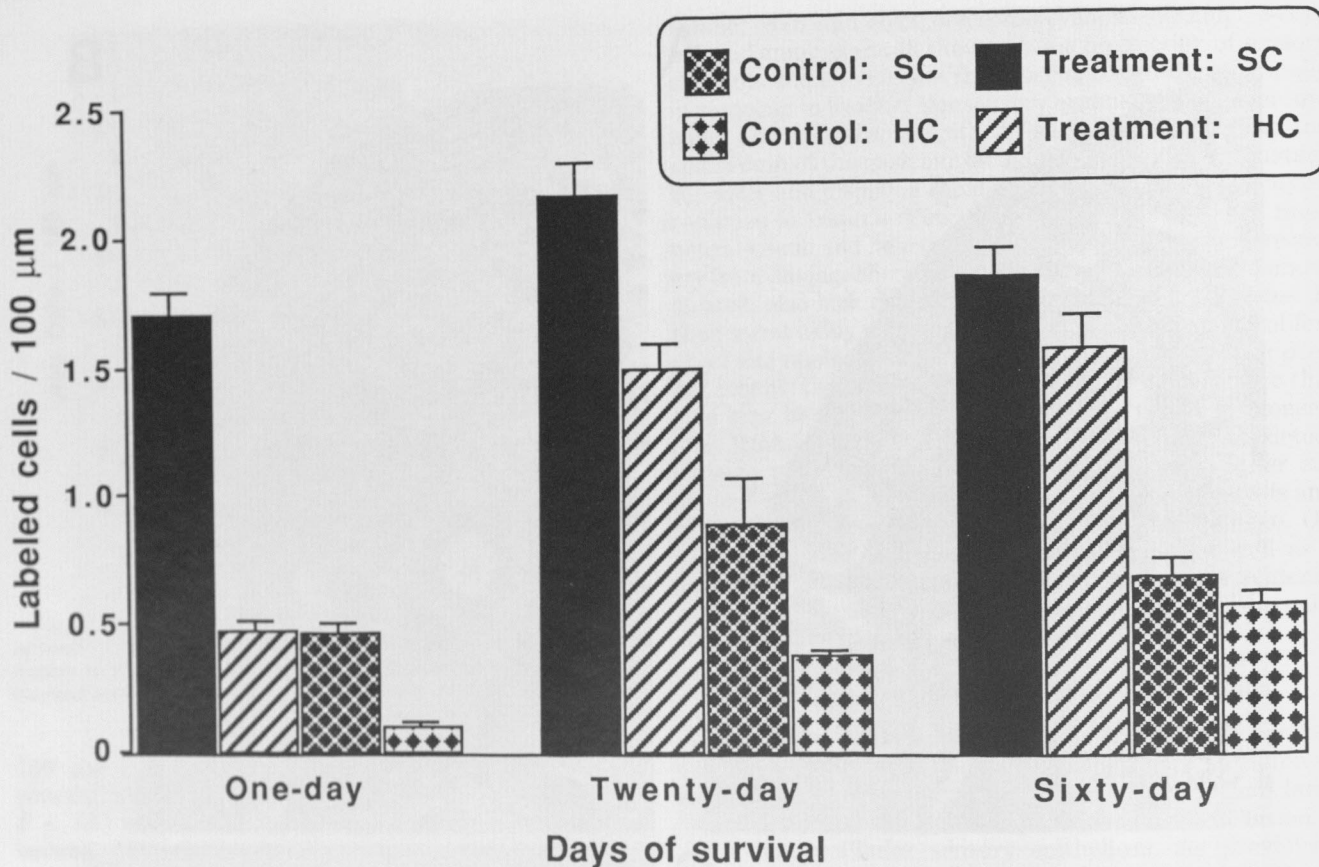
Fig. 9.   Mean number of labeled hair cells (HC) and supporting cells (SC) in superior cristae ampullaris of treatment and control animals at each survival time. Ordinate: number of labeled cells per 100 μm sensory epithelium. The average of three organs for each condition is displayed. Error bars: standard error of the mean.

('91, '92) have recently demonstrated that injections of another aminoglycoside, gentamycin, produce selective lesions to the striolar area, an area where Type I-like hair cells are found. Thus, it appears that the more highly differentiated Type I hair cells (Iurato, '67) have a higher sensitivity to ototoxic drugs. The cellular basis of this differential sensitivity remains unknown.

Hair-cell counts

The stability of hair-cell densities in the epithelia of the four control animals plotted in Figure 5 (two 10 day old and two 70 day old) suggests that a significant number of new hair cells is not added to the avian vestibular sensory epithelium in the first 60–70 days posthatch. Similar results have been found in the murine vestibular system (Ruben, '67), where the normal complement of vestibular hair cells is present in the epithelium at the time of birth. Thus, our results suggest that the normal low level of ongoing proliferation observed in the avian vestibular system may have a reparative function, not an additive function. That is, cell proliferation in the untreated avian vestibular epithelium most likely replaces elements that are lost due to "normal turnover" and as a result of aging, trauma, or disease. This process maintains a stable population of hair cells. These results are in contrast to what Corwin ('81, '83, '85) and Popper and Hoxter ('84) have reported for the vestibular epithelium of fish, where the

number of sensory cells steadily increases throughout the animal's life.

As demonstrated above, the number of hair cells in streptomycin-treated utricles of 60 day survival animals is 20% larger than that in untreated utricles. Girod et al. ('91) have also reported a 12–16% overproduction of hair cells in the apical one-third of cochleae from gentamycin-treated chicks allowed to survive 20 weeks after aminoglycoside injections. One possible reason for this overproduction is that regions of damaged epithelia become exposed to an excessive amount of "trophic signals" which may result in a small overproduction of sensory cells. Alternatively, certain regions of damaged epithelia may be more sensitive to the signals that trigger regeneration, or less sensitive to the signals that limit proliferation.

Cell types identified

An important finding of this research is that all cell types normally present in the vestibular sensory epithelium are replaced after damage. In the two initial studies on the avian vestibular system's regenerative capacity (Jørgenson and Mathiesen, '88; Roberson et al., '92), only supporting cells and Type II hair cells were labeled. In both of these studies untreated animals were killed within 20 days of cell proliferation marker injections. In the present research, we were able to label both Type I and Type II hair cells as well as supporting cells in both untreated and treated animals.

Presumably, the prolonged survival times utilized in this study allowed cell differentiation to take place to the point that labeled Type I hair cells could be observed. This observation is supported by the fact that the majority of labeled Type I hair cells were seen in the tissue from animals allowed to survive 60 days, although some labeled Type I hair cells could be detected in the tissue of treatment animals allowed to survive 20 days.

Evidence of up-regulation

The number of mitotic events which occurred after treatment with vestibulotoxic agents was always greater than that seen in untreated animals. This outcome, together with the results from an experiment in which we studied cell proliferation in the absence of nerve activity (Weisleder and Rubel, '91b), indicates that proliferation in the avian inner ear is a dynamically regulated process. That is, the number of cells that enter the mitotic cycle increases or decreases in response to a variety of external influences. Pardee ('89) and others indicate that external influences prompt quiescent cells to become mitotic. It is conceivable that in response to the streptomycin lesion, the birds either synthesized an abundance of a proliferation-stimulator or, conversely, they ceased production of a proliferation-inhibitor. In recent experiments on electroreceptor organs of weakly electric fish, Fritzsch et al. ('90) have observed the presence of an abundance of microvesicles ($\sim$70 nm diameter) in the afferent terminal of regenerating organs. The authors hypothesize that the microvesicles contain a trophic signal involved in regeneration and cell differentiation of electroreceptors.

Reliably more labeled cells were detected with increasing survival time. This effect was more dramatic between 1 and 20 days than between 20 and 60 days after the treatment (Fig. 9). This analysis suggests that, similar to what is seen during embryonic development (Anniko, '83), there are two phases to the process of vestibular hair cell regeneration. The initial phase is a period of rapid proliferation and slow cell differentiation, during which the number of new, undifferentiated cells increases considerably. The second is a period of rapid cell differentiation and slow proliferation, during which the number of differentiated cells increases significantly. These arbitrarily designated phases coincide, however, with two very interesting observations. First, very few differentiated cells are seen in the tissue during the initial phase. That is, most of the labeled cells in the 1 day survival animals are cells within the supporting cell layer. It is conceivable that these cells undergo several more mitotic cycles during the next 20 days. Since the result of mitosis is doubling of the number of new cells, and since these cells had incorporated the proliferation marker, it is not surprising to encounter many more labeled cells at 20 days than at 1 day. Second, hair cell counts of 1 and 20 day survival tissue also indicate that while the number of supporting cells remains relatively stable, more Type II hair cells can be detected as survival time increases. We are yet to determine whether more progenitor cells are coursing through the cell cycle, whether progenitor cells are cycling faster, or both. Kauffman ('68) reported that during the embryonic period cell cycle times of a given cell type can vary considerably. To address this issue, additional studies involving pulse-fix and pulse-chase paradigms are needed.

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