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# Hair cell regeneration and recovery of auditory thresholds following aminoglycoside ototoxicity in Bengalese finches

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**Abstract**

Birds regenerate auditory hair cells when original hair cells are lost. Regenerated hair cells become innervated and restore hearing function. Functional recovery during hair cell regeneration is particularly interesting in animals that depend on hearing for vocal communication. Bengalese finches are songbirds that depend on auditory feedback for normal song learning and maintenance. We examined the structural and functional recovery of the Bengalese finch basilar papilla after aminoglycoside ototoxicity. Birds were treated with the ototoxic aminoglycoside, amikacin, daily for 1 week. Treatment resulted in hair cell loss across the basal half of the basilar papilla and corresponding high frequency hearing loss. Hair cell regeneration and recovery of auditory brainstem responses were compared in the same animals. Survival times following treatment were between 1 day and 12 weeks. Analysis of structural recovery at weekly intervals indicated that hair cells in the Bengalese finch papilla require a maximum of 1 week to regenerate and appear with immature morphology at the epithelial surface. An additional 6 days are required for adult-like morphology to develop. Repopulation of the damaged region was complete by 8 weeks. Recovery of auditory thresholds began 1 week after treatment and reached asymptote by 4 weeks. Slight residual threshold shifts at 2.0 kHz and above were observed up to 12 weeks after treatment. Direct comparison of structural and functional recovery indicates that auditory thresholds recover maximally before a full complement of hair cells has regenerated. 

*Key words:* Amikacin; Hearing; Bird; Basilar papilla; Cochlea; Stereocilia

## 1. Introduction

The sensory epithelium of the avian hearing organ (basilar papilla) is a curvilinear sheet of hair cells. Hair cells are the sensory receptor cells responsible for hearing function in vertebrates. In birds and mammals, these specialized cells are tonotopically organized along the auditory epithelium such that high frequency sounds are encoded at the basal end and lower frequencies are encoded by cells located in progressively more apical positions. Two types of hair cells populate the basilar papilla, and are located in distinct but continuous regions of the epithelium (Manley et al., 1987; Gleich, 1989; Fischer, 1992, Fischer, 1994; Smolders et al., 1995). Short hair cells are located in the inferior, predominantly basal-most regions, and, like mammalian outer hair cells, are largely innervated by efferent fibers. Tall hair cells are located along the superior edge of the papilla and resemble mammalian inner hair cells in that they are innervated by afferent fibers.

Unlike mammals, birds regenerate auditory hair cells in the basilar papilla following loss of original hair cells due to treatment with ototoxic aminoglycosides (Cruz et al., 1987; Lippe et al., 1991) or acoustic overstimulation (Cotanche, 1987; Corwin and Cotanche, 1988; Ryals and Rubel, 1988). Hair cells in the basal (high frequency) end of the papilla are the most vulnerable to aminoglycoside ototoxicity; they are the first cells to die and be extruded from the epithelial surface during or after drug treatment (see Cotanche et al., 1994; Cotanche, 1999 for review). Both tall and short hair cells are vulnerable to aminoglycosides. Hair cell loss occurs

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across the entire papilla width, but the basal to apical extent of hair cell loss is dose dependent (Cotanche, 1999). The extent of hair cell loss sweeps apically with increased dose or duration of drug treatment.

Regenerated hair cells become innervated by both afferent and efferent fibers (Duckert and Rubel, 1990, 1993; Wang and Raphael, 1996) and restore hearing (Tucci and Rubel, 1990; Marean et al., 1993, 1998; Niemiec et al., 1994, see Smolders, 1999 for review). The recovery of auditory function during hair cell regeneration has now been studied in a variety of species including chicks (Tucci and Rubel, 1990), quail (Niemiec et al., 1994; Ryals et al., 1999), starlings (Marean et al., 1993, 1998), budgerigars (Dooling et al., 1997; Ryals et al., 1999), pigeons (Müller et al., 1996; Müller and Smolders, 1998), canaries and zebra finches (Ryals et al., 1999). In all species examined, hair cell regeneration results in the recovery of auditory thresholds to normal or near-normal levels. Studies report that some permanent hearing losses occur at high frequencies; elevated thresholds persist after hair cells encoding high frequencies have fully regenerated (Tucci and Rubel, 1990; Marean et al., 1993, 1998; Dooling et al., 1997). Most studies that have examined the relationship between structural and functional recovery from hair cell damage have used focal lesions created by acoustic overstimulation (Saunders et al., 1992, 1995; Adler et al., 1993; Umemoto et al., 1993; Niemiec et al., 1994; Saunders and Salvi, 1995; Müller et al., 1996; Ryals et al., 1999).

Songbirds are particularly interesting birds in which to study the recovery of auditory function after hair cell loss because they depend on vocalizations to communicate, and several species have been shown to depend on hearing to vocalize normally. For example, adult Bengalese finches normally sing stereotyped songs that are stable over time (Immelmann, 1969; Dietrich, 1980; Clayton, 1987; Woolley and Rubel, 1997). However, their songs deteriorate following surgical deafening or extensive hair cell loss (Woolley and Rubel, 1997, 1999a; Okanoya and Yamaguchi, 1997). Thus, the relationship between hearing recovery and vocal recovery during hair cell regeneration can be addressed in these animals. Additionally, the effectiveness of regenerated hair cells in supporting natural behaviors that depend on auditory input such as song in birds can be examined.

We were interested in examining the relationship between hair cell regeneration along the basilar papilla and recovery of auditory function across a range of frequencies by examining both processes in the same animals. We damaged hair cells in adult Bengalese finches by treating them with daily aminoglycoside injections for 1 week. Hair cells populating the basal half of the basilar papilla were killed by treatment. This species was chosen because recovery of song behavior has been shown to occur over 8 weeks following hair cell loss in these birds (Woolley and Rubel, 1999b). Auditory thresholds were measured electrophysiologically to determine the time course of sensitivity loss as hair cells died and recovery as regenerated hair cells repopulated the basilar papilla. Each bird was tested only once, either 0, 1, 2, 4, 8, or 12 weeks after treatment. Immediately following measurement of thresholds, birds' papillae were fixed and processed for anatomical analysis using scanning electron microscopy (SEM). With this design, we could directly compare the morphology of regenerated hair cells along the basilar papilla and the recovery of auditory function across a range of frequencies.

## 2. Materials and methods

### 2.1. Animals

We used 34 adult Bengalese finches (22 females and 12 males) that were aviary-raised (Magnolia Bird Farm, Anaheim, CA, USA) and purchased by us as young adults. Birds were housed in groups of 5–10 individuals of both sexes and maintained on a 14:10 light/dark cycle. Each bird was between 4 and 12 months of age at the beginning of this study.

### 2.2. Experimental design

All birds were treated with daily injections of the ototoxic aminoglycoside, amikacin, for 1 week (details below). Pilot studies indicated that this treatment results in hair cell loss across roughly one half (basal end) of the basilar papilla in this species. After treatment to cause hair cell loss, birds were allowed to recover for 0 ($n = 5$), 1 ($n = 5$), 2 ($n = 4$), 4 ($n = 6$), 8 ($n = 5$) or 12 ($n = 4$) weeks. To document auditory sensitivity loss resulting from treatment and auditory recovery following the regeneration of hair cells, thresholds were determined by recording auditory brainstem responses (ABRs) to pure tone stimuli. Thresholds for recovering birds were compared to normal thresholds determined by recording ABRs from untreated control birds ($n = 5$). To assess the location and extent of hair cell loss and the morphology of regenerated hair cells, basilar papillae were processed for SEM immediately following ABR recording. Both papillae in three birds from each recovery group and three control birds (a total of 42 papillae) were analyzed.

### 2.3. Aminoglycoside treatment

We used the ototoxic aminoglycoside, amikacin, to

induce hair cell damage in the basal (high frequency) half of the basilar papilla. Experimental birds were given daily subcutaneous injections of amikacin (Faulding Puerto Rico Inc.) in alternating doses of 150 mg/kg/day and 300 mg/kg/day for 1 week. In birds, systemic administration of ototoxic aminoglycosides results in dose dependent destruction of hair cells located in the basal (high frequency) region of the basilar papilla, and sparing of hair cells in the apex (Tucci and Rubel, 1990; Hashino et al., 1992; Marean et al., 1993; Salvi et al., 1994, Woolley and Rubel, 1999a). Amikacin was chosen for this study because it is highly toxic to auditory hair cells and less toxic to renal function than other aminoglycosides (Lenoir and Puel, 1987; Kitasato et al., 1990; Beaubien et al., 1995; Vago et al., 1998).

### 2.4. Electrophysiological recordings from auditory brainstem

Evoked potential thresholds were determined by recording from auditory brainstem nuclei during presentation of sound stimuli at the following test frequencies: 0.25, 0.5, 1.0, 1.5, 2.0, 3.0, 4.0, 5.0, and 6.0 kHz. Tone bursts with a 1 ms rise/fall time and 10 ms total duration were delivered at a rate of 9 per s. Stimuli were delivered with a free field speaker (Realistic Minimus-7) placed at a 90° angle to the mid-line of the head and at a distance of 7 cm from the head. The stimulus delivery system was calibrated at the beginning of each experiment using an ER-10 microphone (Etymotic Research) placed at the ear. Custom software was used to acquire and analyze evoked potential traces. All threshold data were recorded in decibels (dB) sound pressure level (SPL).

Animals were anesthetized with urethane ($\sim$2000.0 mg/kg). Body temperature was maintained at 39°C. Birds were placed on a flat platform and their heads were stabilized in a custom-designed holder. Two pin electrodes (Grass Instruments Co., Quincy, MA, USA) were implanted bilaterally through the cranium into the telencephalon and into the cerebellum just above the auditory brainstem nuclei (active electrodes) and one electrode was inserted into leg muscle (ground electrode). Responses were amplified, filtered (0.03–3.0 kHz band pass), and digitized at a rate of 200 kHz. Responses were averaged over 200 presentations at stimulus levels that produced a clear suprathreshold response. Responses were averaged over 500 presentations at and around threshold. Stimulus presentations for each frequency were begun at 90 dB and decreased or increased in intensity by 10 dB steps above and below threshold. Stimulus presentations were decreased or increased by 5 dB steps around threshold. Threshold was defined as the intensity at which the averaged response was at least twice the amplitude of baseline variation. If no response could be detected at the highest intensities our system delivers, a default value of 120 dB was used for calculation of threshold averages and threshold shifts.

### 2.5. SEM

Immediately following auditory brainstem recordings, birds were euthanized by an intramuscular injection of sodium pentobarbital (Anpro Pharmaceuticals) and decapitated. Under a dissecting microscope, the external auditory meatus, tympanic membrane and columella were removed, exposing the basilar papilla through the oval window. The opposite end of the cochlear duct was exposed by creating a small hole in the bone overlying the lagena with a scalpel tip. Papillae were then perfused via the oval window with 2.5% glutaraldehyde and 2.0% paraformaldehyde in 0.1 M phosphate-buffered saline (PBS). Heads were post-fixed in the same fixative overnight. Temporal bones were then dissected out of the head and papillae were completely exposed by removing the roofs of their bony encasements. Specimens were placed in 1% osmium tetroxide for 1 h and washed in PBS. Specimens were then dehydrated in a graded ethanol series to 70% ethanol, the tectorial membrane was removed in a final dissection, and specimens were dehydrated to 100% ethanol. After critical point drying, specimens were mounted on aluminum stubs with graphite glue and sputter coated with gold palladium. SEM was performed with a JEOL 6300F electron microscope (accelerating voltage of 15 kV) to document the extent and location of hair cell damage, loss and regeneration in each animal.

### 2.6. Quantification of hair cell damage and regeneration

Quantification of hair cell damage was done by digitally acquiring SEM images of papilla segments at $\times$250 onto a PowerMac 8100/80 and montaging images to compose a complete digital image of each papilla using image analysis software (NIH Image v.1.61b7). Montages were coded and randomized so that, during quantification, the experimenter was blind as to the group to which each papilla belonged. The following measures were made for each basilar papilla: (1) total papilla area; (2) area of damaged papilla; and (3) area of the papilla populated by mature-looking regenerated hair cells. Additionally, each papilla was given a score between 0 and 5 of overall quality, with 0 being the lowest quality and 5 being the highest quality. Quality in this case was defined in terms of the most 'normal-like' appearance. For example, a normal undamaged papilla would be of the highest quality and receive a score of 5, and a papilla showing hair cell loss with no

evidence of regeneration would be of the lowest quality and receive a score of 0. This score was meant to provide an overall measure of papilla recovery after hair cell loss. For quality scoring, analysis was limited to the damaged area of each papilla. The damaged area was defined as the region showing hair cells with expanded surfaces that were missing stereocilia, showing no hair cells, or showing regenerated hair cells. Regenerated hair cells and original hair cells were distinguished by their morphological differences. Immature regenerated hair cells were identified by their smaller luminal surfaces, smaller stereocilia, the presence of microvilli next to stereocilia on their luminal surfaces and by the presence of kinocilia (see Duckert and Rubel, 1990, 1993; Marean et al., 1993). Regenerated hair cells were considered mature-looking if they had no kinocilia, the same surface size as normal adult hair cells and stereocilia bundles that appeared to be of adult-like morphology. At all times, regenerated hair cells could be distinguished from original hair cells by the disorientation of their stereocilia bundles with respect to the bundles on neighboring cells.

### 2.7. Statistical analyses

Auditory thresholds, percentages of the papilla area missing original hair cells, percentages of papilla area containing mature-looking regenerated hair cells, and

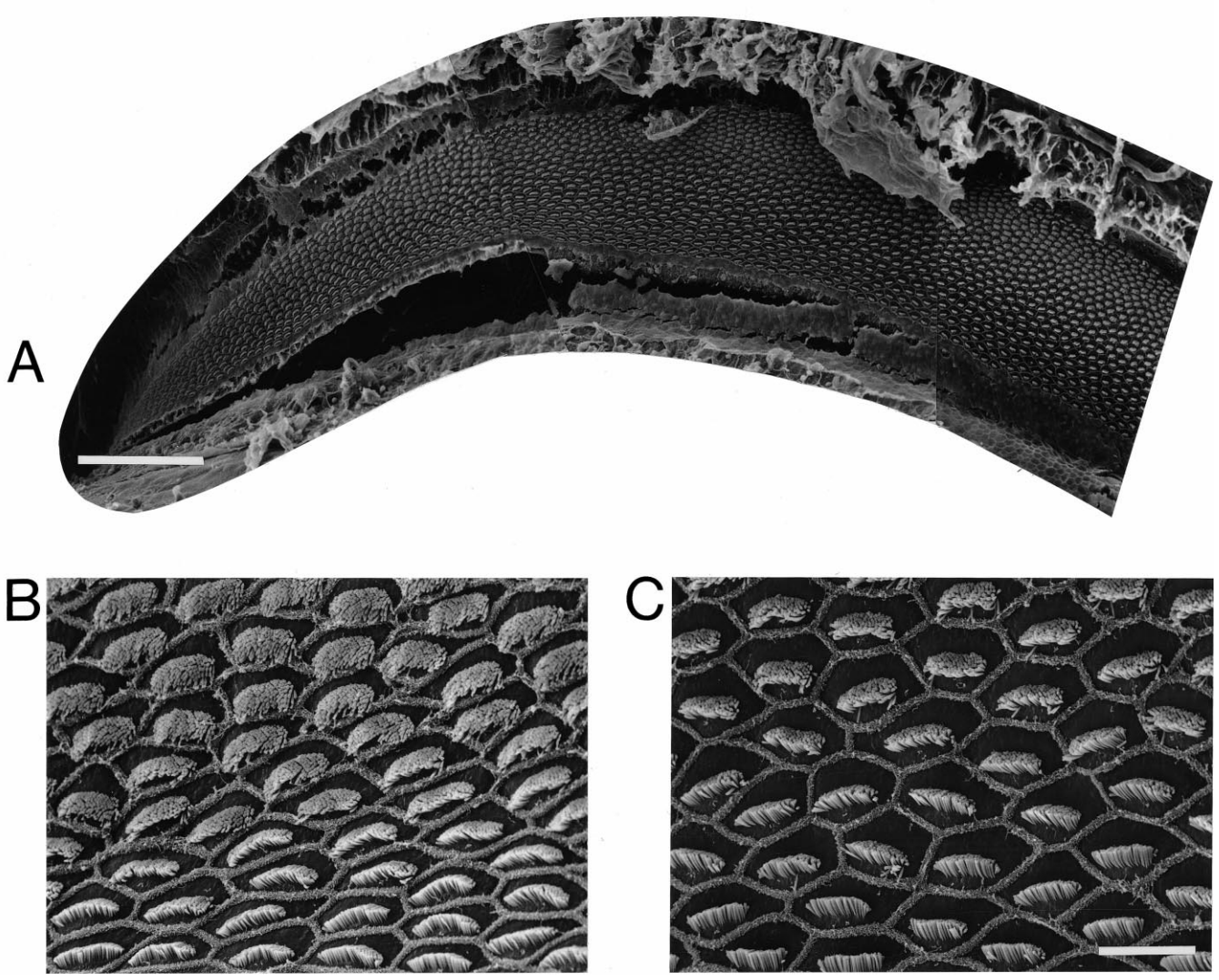



Fig. 1. Scanning electron photomicrographs of a normal Bengalese finch basilar papilla. A: Low magnification montage of the basal half of the papilla showing the highly organized array of normal hair cells. Basal is to the left. Apical is to the right. The superior edge is at the top and the inferior edge is at the bottom. Scale bar indicates 100 μm. B: High magnification view of hair cells in the basal end, approximately 100 μm from the basal tip. Each hair cell has one stereocilia bundle showing the characteristic staircase structure projecting from its luminal surface and is surrounded by the microvilous surfaces of supporting cells. C: High magnification view of hair cells in the mid-region of the papilla, approximately 700 μm from the basal tip. Scale bar indicates 10 μm and applies to B and C.

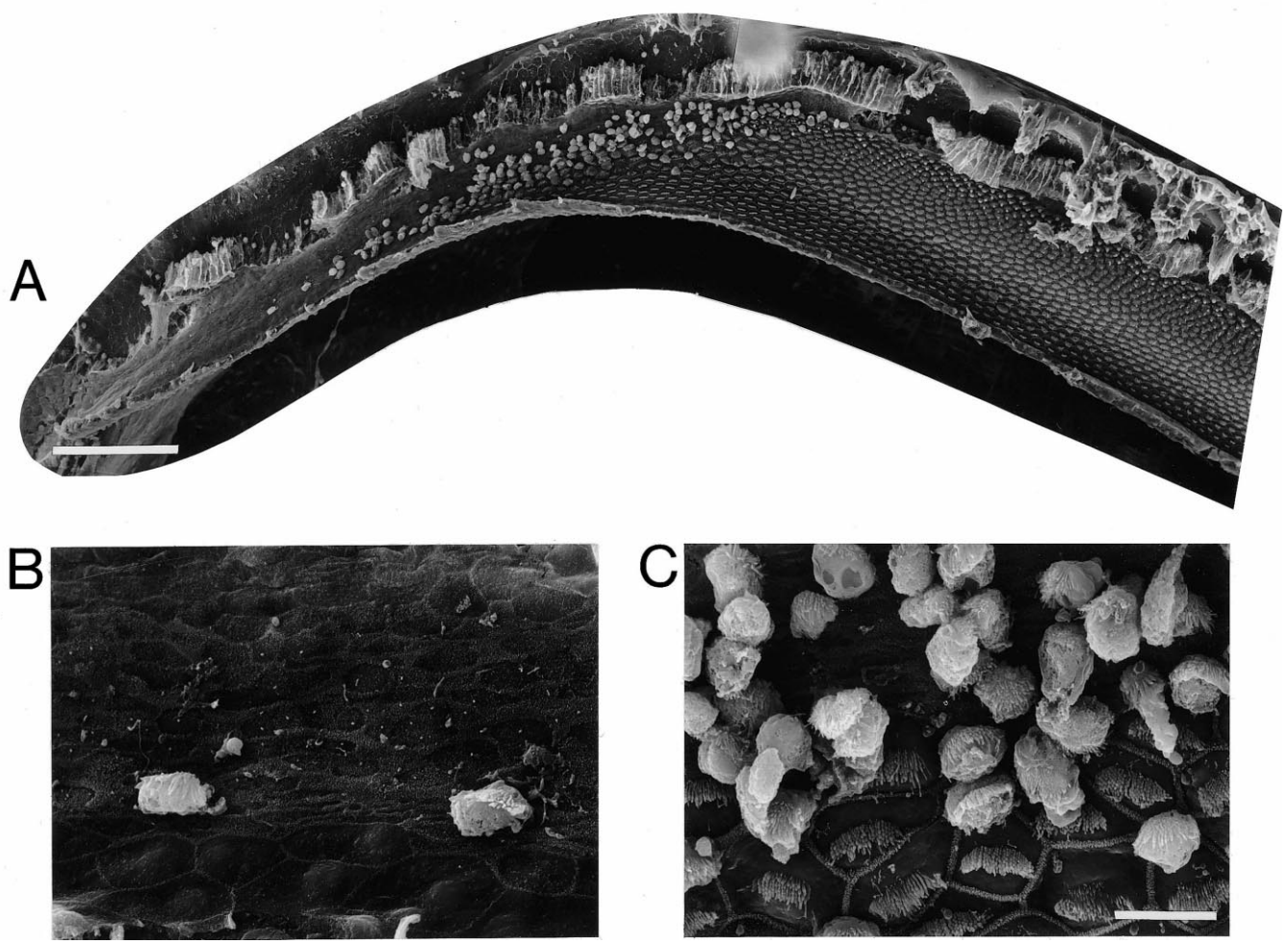



Fig. 2. Scanning electron photomicrographs of the right basilar papilla from a bird treated with amikacin for 1 week and allowed to recover for 1 day (0 weeks). A: Low magnification montage of the basal half of the papilla shows hair cell loss in the basal end. Extruded hair cells are visible along the transition zone. Scale bar indicates 100 μm. B: High magnification view of the epithelial surface approximately 100 μm from the basal tip. Hair cells have been extruded from the epithelium and supporting cells have expanded to cover the surface. Two extruded hair cells are visible. C: High magnification view of hair cells that have been extruded from the epithelium along the transition zone, approximately 400 μm from the basal tip. Normal-looking original hair cells are visible next to extruded hair cells. Scale bar indicates 10 μm and applies to B and C.

papilla quality scores were tested for statistical differences across groups using factorial design, one-factor ANOVAs. Post-hoc comparisons were made with the Sheffé *F*-test.

All animal husbandry procedures and animal use protocols were approved by the University of Washington Animal Care Committee.

## 3. Results

### 3.1. The normal basilar papilla

The Bengalese finch basilar papilla has been briefly described previously (Woolley and Rubel, 1999a). The sensory epithelium is a curvilinear sheet of hair cells that is ~1450 μm in length and of the typical avian shape (Fig. 1). The epithelial surface is a well organized array of hair cells most similar in appearance to that of the canary and zebra finch (Gleich et al., 1994; Ryals et al., 1999). The basal tip is the narrowest portion of the epithelium. There are ~9 hair cells across the basal end at 100 μm from the very tip. The epithelium progressively widens toward the apex (~25 hair cells across at 400 μm from the apex) and then narrows further toward the apex (~15 hair cells across at 100 μm from the apical tip). Hair cells are hexagonal in surface shape and are ~10 μm in diameter (Fig. 1). One stereocilia bundle projects from the inferior luminal surface of each hair cell.

### 3.2. Hair cell loss following aminoglycoside treatment

SEM analysis of basilar papillae 1 day (0 weeks) after

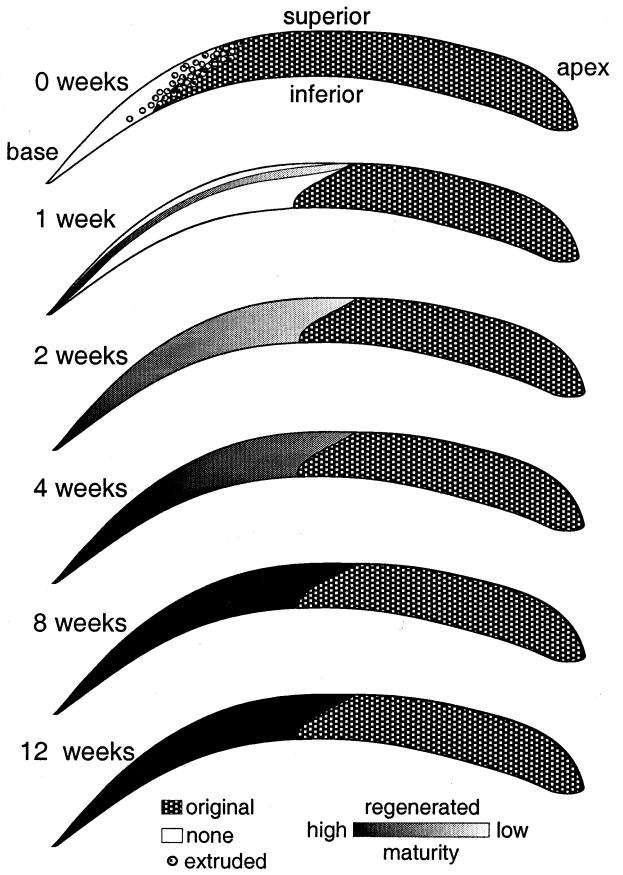



Fig. 3. Schematic drawings of basilar papillae from each recovery time following treatment. The extent and patterns of hair cell damage and regeneration are shown.

1 week of daily amikacin injections showed that 22.81 ± 3.0% (mean ± S.E.M.) of the total papilla surface area was damaged by treatment. An example is shown in Fig. 2A and schematized in Fig. 3. The basal end of the basilar papilla was devoid of hair cells and expanded support cells covered the epithelial surface (Fig. 2B). Hair cell loss along the superior edge extended more toward the apex than did hair cell loss along the inferior edge. Extruded hair cells were visible along the transition zone, which is the border between epithelium denuded of hair cells and epithelium containing original hair cells (Fig. 2C). No regenerating hair cells could be seen in the basal tip or elsewhere on the epithelial surface (Figs. 2B, 3 and 4A). Papilla quality scores for all papillae that were examined 1 day following treatment were 0 on a scale of 0–5, with 0 being the lowest quality and 5 being a 'normal-looking' papilla (Fig. 4B).

By 1 week after treatment, hair cell lesions were greater in size than at 1 day after treatment (Figs. 3 and 4A). Original hair cells were missing from an average of 42.41 ± 2.80% of the total epithelial surface, nearly twice that seen at 1 day. There was no evidence of ongoing hair cell extrusion from the epithelial surface by 1 week after treatment. Hair cell lesions did not increase in size after 1 week (Figs. 3 and 4A); a mean of 43.54 ± 4.25% and 37.80 ± 6.33% of the epithelial surface was missing original hair cells at 4 weeks and 12 weeks after treatment, respectively.

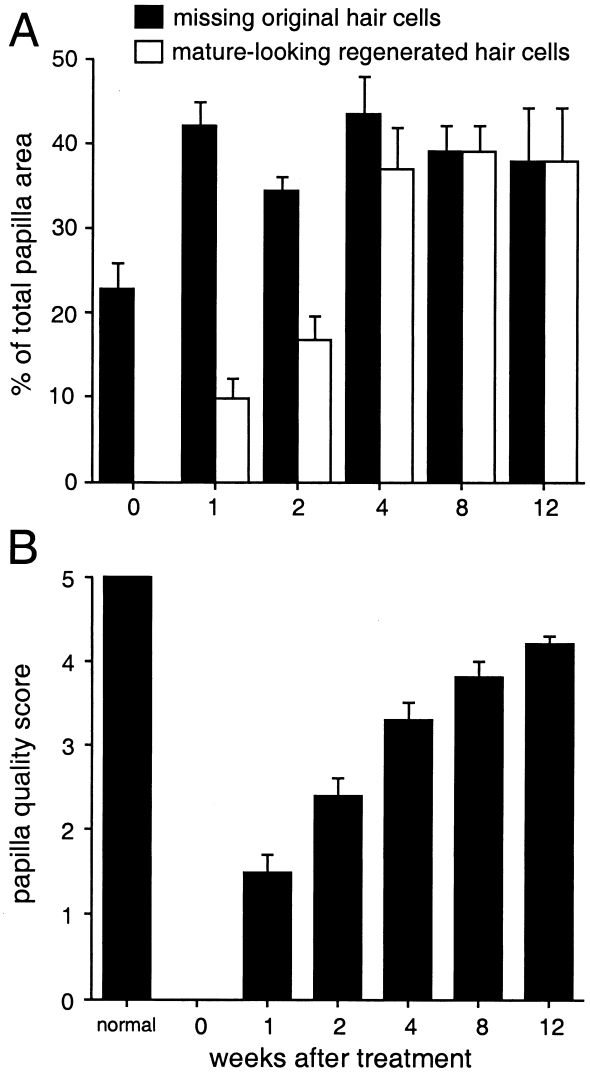



Fig. 4. Percent of total papilla area damaged by treatment, percent of total papilla area showing mature regenerated hair cells, and papilla quality scores are plotted for each recovery time. A: The percent of total papilla area damaged by treatment (black bars) was significantly ($P < 0.01$) smaller 1 day after treatment than 1 week after treatment. The percent of total papilla area showing mature regenerated hair cells (white bars) increased gradually between 1 day and 4 weeks after treatment. Percent of papilla area damaged by treatment was the same as the percent of papilla area covered by mature regenerated hair cells by 8 and 12 weeks after treatment. B: Papilla quality scores increased significantly ($P < 0.001$) with recovery time, but were still significantly lower ($P < 0.05$) than scores for control papillae 12 weeks after treatment. Error bars indicate S.E.M.

### 3.3. Hair cell regeneration

By 1 week after the end of treatment, regenerated hair cells were visible across the entire length but not the entire width of the damaged region (Figs. 3 and 5A,B). A stripe of regenerated hair cells extended from the basal tip through a portion of the superior half of the papilla width and merged with the superior edge of the papilla at the apical border of the damaged region. This pattern is schematized in Fig. 3. Mature-looking hair cells were visible in the basal tip of the papilla, covering $9.88 \pm 2.19\%$ of the total epithelial surface (Fig. 4A). These regenerated hair cells had no kinocilia and were nearly mature in surface size and morphology (Fig. 3). Regenerated hair cells in the apical extent of the damaged region were more sparsely distributed and were smaller in size, indicating that they were less mature than those in the basal end (Fig. 5B). Papilla quality scores increased significantly ($P < 0.001$) between 1 day (0 weeks) and 1 week after treatment (Fig. 4B). The average quality score for papillae 1 week after treatment was $1.5 \pm 0.2$ (mean ± S.E.M.).

By 2 weeks after treatment, regenerated hair cells were visible across the length and width of the damaged epithelium. Cells across the entire damaged region appeared more mature than at 1 week after treatment (Figs. 3 and 5), and little indication of the stripe pattern evident at 1 week could be seen. Mature-looking regenerated hair cells covered $16.80 \pm 2.72\%$ of the total papilla area (Fig. 4A). Across the entire damaged region, immature hair cells, presumably still in the process of erupting at the epithelial surface, were seen commingled with mature-looking hair cells (Fig. 5C,D). The mean score for papilla quality at 2 weeks after treatment was $2.4 \pm 0.2$ (Fig. 4B).

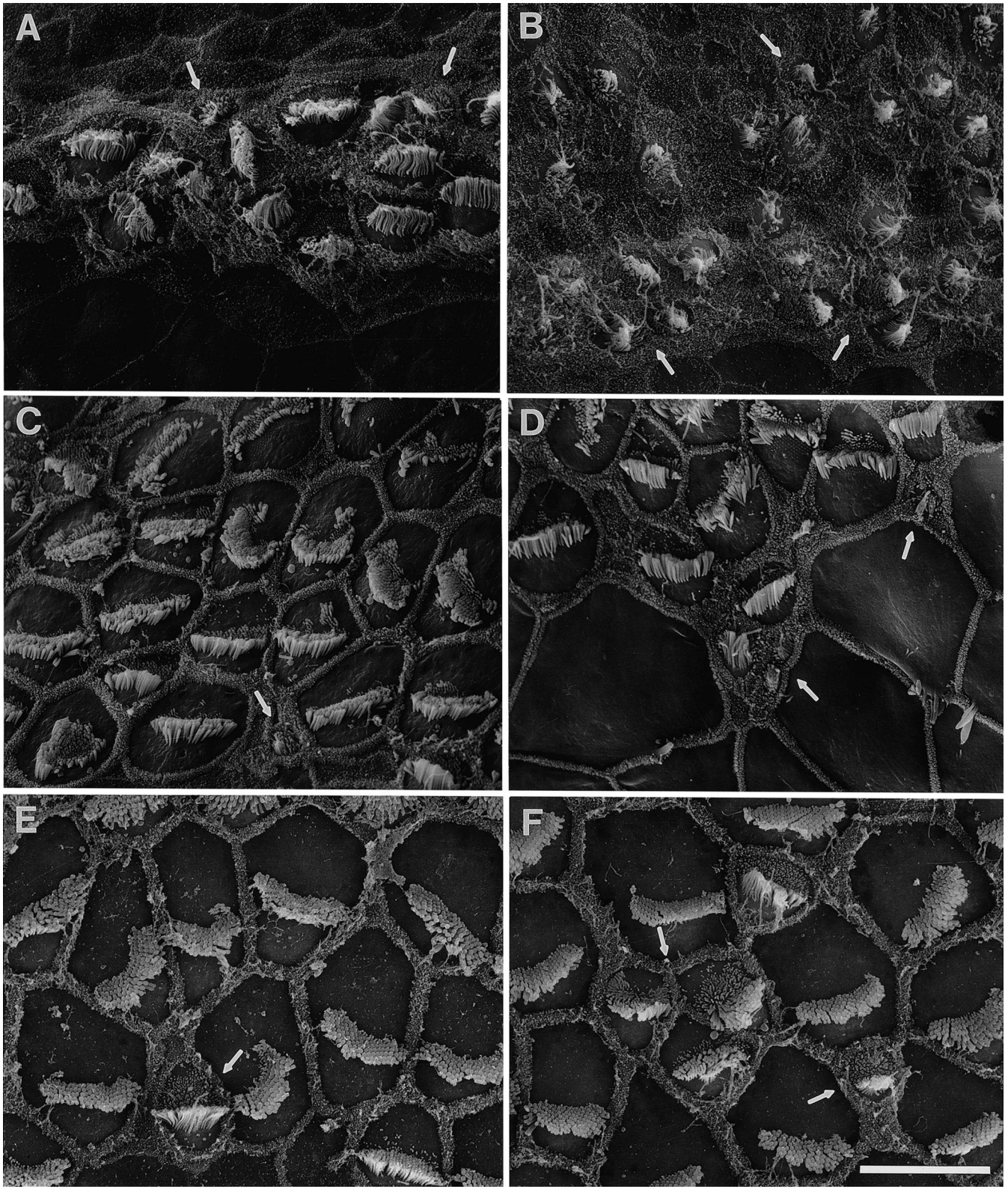
Four weeks after treatment, nearly the entire epithelial surface contained mature-looking hair cells. Most new hair cells looked fully regenerated as judged by their surface morphology. The total papilla area populated with mature-looking regenerated hair cells was $37.30 \pm 4.81\%$, and was only slightly smaller than the total area missing original hair cells at 4 weeks ($43.54 \pm 4.25\%$; Fig. 4A). A small portion of the damaged region contained what looked to be injured hair cells with expanded surfaces located at the edge of the damaged area next to the undamaged epithelium. In the basal tip, no immature-looking hair cells were visible. However, the maturity of hair cells apical to the basal tip was not uniform; surface size was highly variable among regenerated cells. Immature hair cells with small surfaces were still observed at the junctions between mature-looking hair cells across the damaged region except for the basal tip. Immature-looking cells were more sparsely distributed in the basal end than in the apical end of the damaged region, however (Fig. 5E,F). Thus, 4 weeks after treatment, mature-looking hair cells had repopulated the damaged region but some new cells were still erupting at the epithelial surface. Papilla quality scores were a mean of $3.3 \pm 0.2$ (Fig. 4B).

By 8 and 12 weeks of recovery, no immature hair cells were visible. The epithelial surface was completely covered with mature-looking cells as characterized by normal adult-like morphology (Fig. 6). Papilla quality scores were significantly increased ($P < 0.05$) compared to scores at 4 weeks (Fig. 4B). Scores were $3.8 \pm 0.2$ and $4.2 \pm 0.1$ for 8 and 12 weeks, respectively. Some hair cells in the transition zone had multiple, small tufts of stereocilia, rather than one larger stereocilia bundle (Fig. 6C). Stereocilia bundles across the entire damaged region were misaligned with respect to those on neighboring cells at both 8 and 12 weeks of recovery. This type of disorientation is atypical of normal hair cells (compare Figs. 1 and 6) and typical of an array of regenerated hair cells (Girod et al., 1991; Hashino et al., 1992; Marean et al., 1993; Woolley and Rubel, 1999a). This abnormality appeared to be slightly more severe at 8 weeks than at 12 weeks, suggesting that basilar papillae were still undergoing repair up to 3 months after treatment. The improvement in stereocilia bundle orientation over long-term survival times has been reported previously (Duckert and Rubel, 1993).

### 3.4. Loss of auditory sensitivity following aminoglycoside treatment

Immediately following treatment, auditory thresholds at test frequencies of 0.5 kHz and above were affected by aminoglycoside treatment. Thresholds for frequencies between 0.5 and 2.0 kHz were consistently 3–13 dB higher than in control birds (Fig. 7A, Table 1), but differences did not reach significance ($P > 0.1$). These slight threshold shifts were likely the result of aminoglycoside toxicity that did not kill but temporarily affected the functioning of hair cells in the mid- and apical (low frequency) regions of the basilar papilla. This effect of aminoglycoside toxicity on hair cells that are not killed by treatment has previously been reported (Tucci and Rubel, 1990).

Thresholds between 2.0 and 6.0 kHz were significantly elevated compared to controls ($P < 0.05$ at 2.0 and 3.0 kHz; $P < 0.001$ at 4.0, 5.0 and 6.0 kHz) and maximally shifted 1 day following the end of treatment (Fig. 7, Table 1). No responses were detected at 6 kHz and, in two out of five birds, no responses were detected at 5 kHz. In these cases, default values of 120 dB were used for threshold calculations. Threshold shifts resulting from treatment were between 24 and 43 dB at frequencies above 2.0 kHz. For example, thresholds at 4.0, 5.0 and 6.0 kHz were greater than 100 dB.




### 3.5. Auditory recovery

By 1 week after treatment, auditory thresholds at 5.0 and 6.0 kHz were slightly but not significantly ($P > 0.5$) decreased compared to thresholds measured 1 day (0 weeks) after treatment (Fig. 7A). For example, thresholds at 6 kHz decreased from $120 \pm 0.0$ dB at 1 day after treatment to $111 \pm 5.6$ dB by 1 week after treat-

Fig. 5. High magnification photomicrographs of hair cells in the basal and mid-regions of recovering basilar papillae 1, 2, and 4 weeks after treatment. A: Hair cells 100 μm from the basal tip in a basilar papilla at 1 week after treatment. B: Hair cells from the same papilla as in A but 700 μm from the basal tip. Hair cells in A are more mature than hair cells in B. C: Hair cells 100 μm from the basal tip in a basilar papilla at 2 weeks after treatment. D: Hair cells from the same papilla as in C but 700 μm from the basal tip. More immature hair cells are present in D than in C. Hair cells with expanded surfaces and missing stereocilia are present along the border between regenerated hair cells and original hair cells. E: Hair cells 100 μm from the basal tip in a basilar papilla at 4 week after treatment. F: Hair cells from the same papilla as in E but 700 μm from the basal tip. More immature hair cells are present in F than in E. Arrows point to immature regenerated hair cells. Scale bar indicates 10 μm and applies to all panels.
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ment. Thresholds at these frequencies were still elevated compared to controls ($P < 0.05$). In contrast to the slight decrease in thresholds at higher frequencies, thresholds at 3 kHz significantly increased ($P < 0.05$) between 1 day and 1 week after treatment. The average threshold increased from $67 \pm 5.4$ dB 1 day after treatment to $85 \pm 4.2$ dB by 1 week. This increase in threshold at 3 kHz may reflect the apical progression of hair cell loss seen between 1 day and 1 week after treatment. Thresholds at 2.0 kHz and below were not different ($P > 0.05$) between 1 day and 1 week of recovery.

By 2 weeks after treatment, thresholds at 2.0 kHz and below were not different ($P > 0.5$) from control thresholds (Fig. 7A, Table 1). Thresholds at 3.0, 4.0 and 5.0 kHz were still elevated compared to controls ($P < 0.05$ for 3.0 kHz; $P < 0.01$ for 4.0 and 5.0 kHz) but sig-

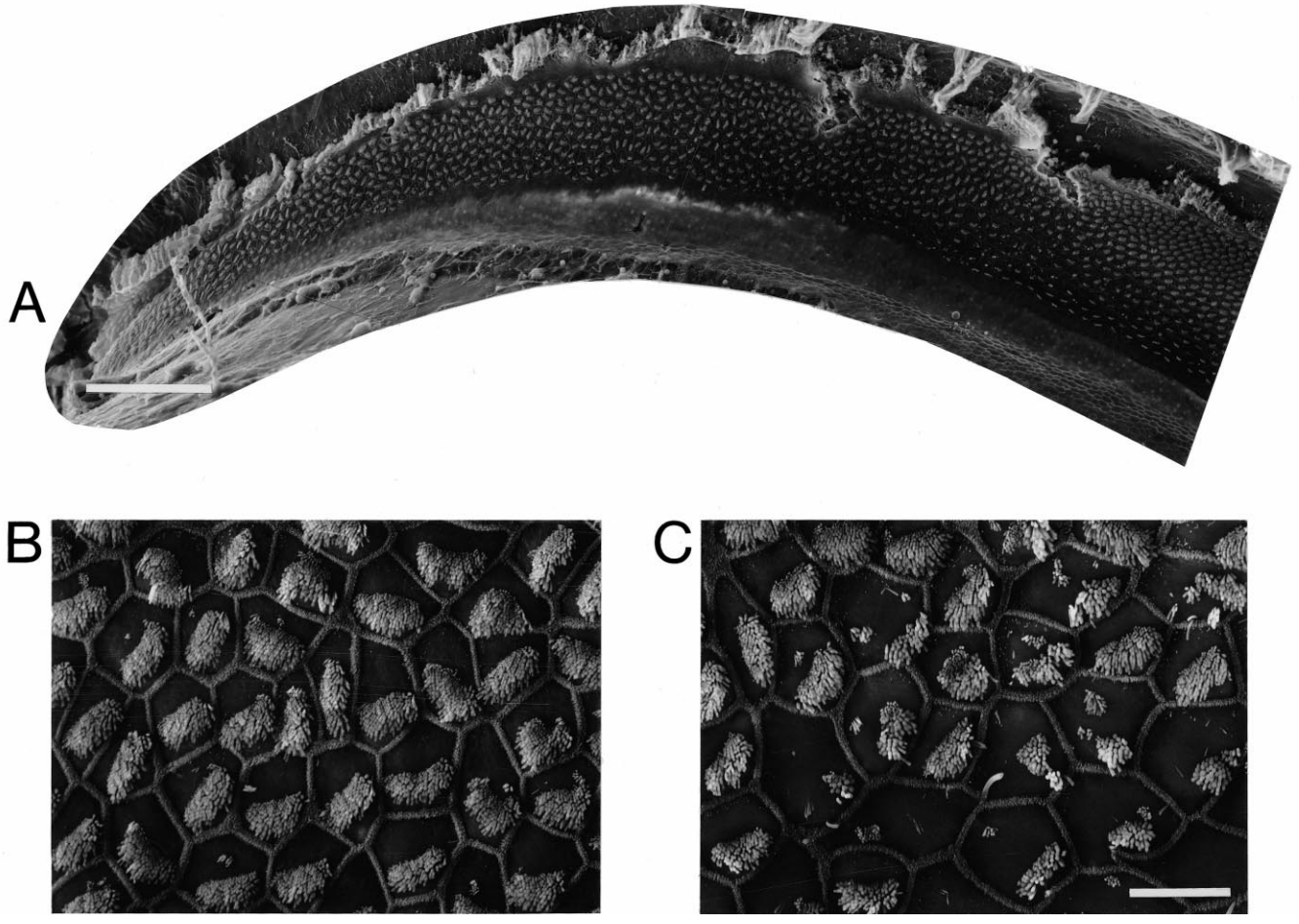


Fig. 6. Scanning electron photomicrographs of the right basilar papilla from a bird treated with amikacin for 1 week and allowed to recover for 12 weeks. A: Low magnification montage of the basal half of the papilla showing a full complement of regenerated hair cells. The hair cell array is less well organized than in control papillae. Scale bar indicates 100 μm. B: High magnification view of the epithelial surface approximately 100 μm from the basal tip. Each regenerated hair cell has an adult-like stereocilia bundle and is normal in surface size. Stereocilia bundles are disoriented with respect to bundles on neighboring cells. C: High magnification view of hair cells approximately 700 μm from the basal tip, showing abnormal stereocilia bundles on regenerated hair cells. Some cells had numerous small tufts of stereocilia rather than the one large bundle characteristic of normal hair cells. Scale bar in C indicates 10 μm and applies to B and C.

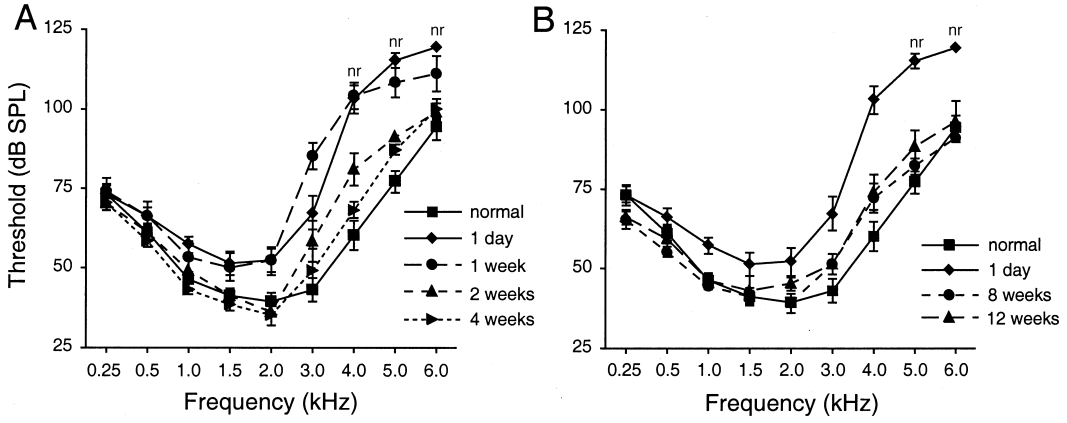


Fig. 7. Auditory threshold plots for control birds and treated birds from each recovery time. Thresholds were determined by recording ABRs to pure tone stimuli. A: Hearing thresholds were significantly elevated at 2 kHz and above by 1 day after treatment. Thresholds decreased at the highest frequencies by 1 week after treatment, and at all frequencies by 2 weeks. Recovery of thresholds reached asymptote by 4 weeks after treatment. B: Hearing thresholds did not improve between 4 and 12 weeks after treatment, and remained slightly but not significantly elevated compared to control thresholds at 12 weeks after treatment. nr indicates averages that include default values of 120 dB.

nificantly decreased compared to thresholds measured 1 week after treatment ($P < 0.01$). Thresholds at 6.0 kHz were not different from controls by 2 weeks of recovery ($P > 0.1$).

By 4 weeks of recovery, when immature hair cells were still erupting at the epithelial surface (Fig. 5E,F), auditory thresholds had recovered maximally (Fig. 7, Table 1). Thresholds were not significantly different from controls at any test frequency ($P > 0.1$). However, they were slightly elevated. For example, average thresholds were $8.3 \pm 8.2$ dB (mean $\pm$ S.D.) higher than controls at 4 kHz and $9.7 \pm 8.2$ dB higher than controls at 5 kHz (Table 1). At 8 and 12 weeks after treatment, hearing thresholds at all frequencies (except for 5.0 kHz at 12 weeks, see Table 1) were not significantly different from controls or from thresholds at 4 weeks after treatment ($P > 0.1$; Fig. 7B). Slight threshold shifts at high frequencies persisted after 12 weeks of recovery.

## 4. Discussion

SEM analysis of basilar papillae showed that daily injections of the ototoxic aminoglycoside, amikacin, for 1 week resulted in hair cell loss across the basal half of the basilar papilla. Recordings of ABRs to pure tone stimuli indicated that thresholds were significantly elevated at 2.0 kHz and above after treatment. Hair cell regeneration and auditory recovery were first evident 1 week after treatment. Hair cell regeneration was ongoing at 2 and 4 weeks of recovery and appeared to be complete by 8 weeks. At 8 and 12 weeks after treatment, papillae appeared fully recovered except that stereocilia bundles were disoriented with respect to those on neighboring cells. Thus, some disorganization of the epithelial surface in the regenerated region remained at 12 weeks of recovery. Recovery of auditory thresholds was evident by 1 week after treatment and reached asymptote by 4 weeks. Slight elevations in high

Table 1
Mean $\pm$ S.D. threshold shifts compared to controls for each recovery time after treatment

| Frequency (kHz) | 0 weeks recovery | 1 week recovery | 2 weeks recovery | 4 weeks recovery | 8 weeks recovery | 12 weeks recovery |
|---|---|---|---|---|---|---|
| 0.25 | $0.0 \pm 5.5$ (ns)[a] | $1.0 \pm 12.0$ (ns) | $-3.0 \pm 6.8$ (ns) | $-3.0 \pm 9.3$ (ns) | $-8.0 \pm 10.3$ (ns) | $-6.8 \pm 7.5$ (ns) |
| 0.5 | $5.0 \pm 5.5$ (ns) | $5.0 \pm 11.8$ (ns) | $0.2 \pm 5.5$ (ns) | $-3.5 \pm 6.9$ (ns) | $-6.0 \pm 5.8$ (ns) | $-2.2 \pm 6.7$ (ns) |
| 1.0 | $9.8 \pm 3.5$ ($P < 0.01$) | $7.0 \pm 11.7$ (ns) | $2.8 \pm 9.1$ (ns) | $-3.5 \pm 6.9$ (ns) | $-2.0 \pm 5.1$ (ns) | $0.2 \pm 6.5$ (ns) |
| 1.5 | $10.0 \pm 7.1$ (ns) | $9.0 \pm 10.2$ (ns) | $0.2 \pm 6.3$ (ns) | $-2.7 \pm 5.2$ (ns) | $0.0 \pm 8.4$ (ns) | $1.7 \pm 9.1$ (ns) |
| 2.0 | $13.0 \pm 10.8$ ($P < 0.05$) | $13.0 \pm 10.8$ ($P < 0.05$) | $-2.8 \pm 6.7$ (ns) | $-4.0 \pm 12.4$ (ns) | $0.0 \pm 8.4$ (ns) | $6.0 \pm 6.6$ (ns) |
| 3.0 | $24.0 \pm 14.3$ ($P < 0.05$) | $42.0 \pm 8.1$ ($P < 0.001$) | $14.5 \pm 16.8$ ($P < 0.05$) | $6.2 \pm 12.9$ (ns) | $8.0 \pm 12.5$ (ns) | $8.2 \pm 8.9$ (ns) |
| 4.0 | $43.0 \pm 13.3$ ($P < 0.001$) | $44.0 \pm 14.3$[b] ($P < 0.001$) | $20.5 \pm 12.4$ ($P < 0.01$) | $8.3 \pm 8.2$ (ns) | $12.0 \pm 15.7$ (ns) | $13.8 \pm 6.3$ (ns) |
| 5.0 | $38.0 \pm 7.5$[b] ($P < 0.001$) | $31.0 \pm 8.6$ ($P < 0.01$) | $14.2 \pm 7.5$ ($P < 0.01$) | $9.7 \pm 8.2$ (ns) | $5.0 \pm 11.4$ (ns) | $11.8 \pm 11.9$ ($P < 0.05$) |
| 6.0 | $25.0 \pm 7.1$[b] ($P < 0.001$) | $17.0 \pm 13.3$ ($P < 0.05$) | $4.8 \pm 6.8$ (ns) | $5.2 \pm 5.3$ (ns) | $-3.0 \pm 6.8$ (ns) | $2.2 \pm 7.0$ (ns) |

[a] All statistical comparisons are from post-hoc tests (Sheffé) following ANOVAs at each recovery time.
[b] Threshold shift averages include default values of 120 dB SPL. Default values were used when no responses were detected at the highest intensities tested.

frequency thresholds persisted at 12 weeks of recovery. Analysis of structural and functional recovery after hair cell damage in the same animals indicates that maximal recovery of auditory thresholds occurs before hair cell regeneration is complete in the Bengalese finch basilar papilla.

### 4.1. Correspondence between anatomical and functional recovery

Immediately following treatment, the basal 22.81% of the total papilla area was missing original hair cells, and cells in the process of extrusion from the epithelial surface were visible. Recordings of ABRs showed that aminoglycoside treatment resulted in significant threshold elevation at 2.0, 3.0, 4.0, 5.0 and 6.0 kHz. No responses were detected at 6.0 kHz and, in some animals, no responses were detected at 5.0 kHz. This lack of responses at the highest hearing frequencies likely reflects the loss of hair cells in the basal tip of the epithelium. At 1 day after treatment, thresholds for frequencies below 2.0 kHz were slightly but not significantly elevated compared to controls.

Regenerated hair cells in the Bengalese finch papilla were clearly evident 1 week after aminoglycoside treatment. Initial repopulation of the epithelial surface progressed apically from the basal tip, following the same pattern as hair cell loss. At 1 week of recovery, thresholds at the highest test frequencies, 5.0 and 6.0 kHz, encoded by the hair cells in the basal tip had decreased slightly but not significantly toward normal levels. Sensitivity losses at 3.0 kHz were more severe 1 week after treatment than at 1 day after treatment; thresholds increased significantly between 1 day and 1 week after treatment. This threshold shift at 3.0 kHz was likely due to the apical progression of hair cell loss to encompass 42.41% of the papilla surface during the first week after treatment. At this time, no evidence of ongoing hair cell extrusion was still evident at the epithelial surface and the hair cell lesion had reached its maximum size.

High magnification analysis of papillae 2 and 4 weeks after treatment showed immature hair cells at the junctions between mature-looking hair cells. These results agree with earlier studies showing that hair cells are not uniform in maturity during regeneration (Hashino et al., 1992; Duckert and Rubel, 1993) and suggest that proliferation of regenerating hair cells does not occur synchronously for all hair cells that will eventually repopulate the basilar papilla. Auditory threshold measurements indicated that a significant amount of functional recovery occurred by 2 weeks following treatment. Hearing thresholds at 6.0 kHz were significantly different from those at 1 day of recovery and not significantly different from controls. The time frame of this recovery corresponds well with the maturation of regenerated hair cells in the basal tips of the basilar papillae. Thresholds at 5.0, 4.0 and 3.0 kHz were still elevated compared to controls at 2 weeks but were significantly improved compared to thresholds at 1 week of recovery. At this time, immature hair cells were present in large numbers across the damaged region apical to the basal tip, while regeneration in the basal tip appeared to be complete.

Recovery of evoked response thresholds reached asymptote with only slight high frequency threshold shifts by 4 weeks after treatment. Thresholds were not statistically different from normal thresholds at any frequency. Immature hair cells were still repopulating the damaged region at this time. This direct comparison of structural and functional recovery indicates that the completion of functional recovery precedes the completion of hair cell regeneration, suggesting that, in the Bengalese finch, a full complement of regenerated hair cells is not required for thresholds to maximally recover. Duckert and Rubel (1990) observed synaptogenesis and innervation of still unerupted regenerated hair cells in the chick basilar papilla. The innervation of unerupted hair cells suggests that immature cells could be functional. However, it is unlikely that the immature hair cells observed in this study could have been functioning as mature hair cells. Their stereocilia bundles were small tufts lacking the staircase structure characteristic of mature hair cells. Therefore, their contacts with the tectorial membrane (which are required for hair cell excitation) were not typical of normally functioning hair cells. Other more complex auditory capabilities such as frequency resolution may require complete hair cell regeneration in order to recover maximally. For example, Marean et al. (1998) reported that the recovery of frequency resolution was slower than the recovery of detection thresholds in aminoglycoside-treated starlings.

By 8 and 12 weeks after treatment, all regenerated hair cells appeared fully mature based on the morphology of their stereocilia bundles and luminal surface size. The major pathology remaining at these long-term recovery times was the disorientation of stereocilia bundles. Thresholds at high frequencies remained slightly elevated at both 8 and 12 weeks. These thresholds, however, were not different from thresholds at 4 weeks after treatment, when immature hair cells were still present. The disorientation of stereocilia bundles has been reported previously (Cotanche, 1987; Cotanche and Corwin, 1991; Hashino et al., 1992; Marean et al., 1993; Duckert and Rubel, 1993; Müller and Smolders, 1998) and has been suggested to be responsible for the residual hearing losses that persist past the completion of hair cell regeneration. In this study, the fact that thresholds did not improve between 4 and 12 weeks and the

orientation of stereocilia bundles did improve over that time suggests that the two pathologies may not be causally linked.

### 4.2. Comparative assessment of hair cell regeneration

Structural recovery of the auditory sensory epithelium after hair cell damage has been examined in a wide variety of avian species, and with several hair cell damage protocols. The time course of regeneration and the pattern with which new hair cells repopulate the papilla appear to be different among different species, ages and treatment protocols. Studies that can be compared to this work are those that have used aminoglycosides to damage a large region of the papilla, affecting a range of hearing frequencies.

Our treatment resulted in ongoing extrusion of original hair cells beyond the treatment period. The maximal extent of damage was not achieved until 1 week after the end of treatment. Loss of hair cells continuing beyond the treatment period has been demonstrated previously in studies administering gentamicin over several days (Duckert and Rubel, 1990; Girod et al., 1991). This ongoing increase in hair cell loss after treatment does not occur in studies using kanamycin to induce hair cell damage (Hashino et al., 1991; Hashino et al., 1992). Thus, gentamicin and amikacin appear to have progressive and prolonged toxicity characteristics that are not typical of kanamycin.

The exact timing of hair cell regeneration is impossible to determine in this type of study. Damage to original cells progresses apically from the basal tip over time and the birth dates of new cells are not known. However, some reasonable estimates of the time required for new hair cells to regenerate and mature can be made. We first observed new hair cells in the damaged region 1 week after the end of treatment. New hair cells in the basal tip were not visible 1 day after treatment but appeared to be nearly mature by 1 week after treatment. Also at 1 week after treatment, immature hair cells were present in the apical end of the damaged region, where original hair cells were still present 1 day after treatment. These results indicate that hair cells in the Bengalese finch papilla require a maximum of 1 week to regenerate and appear with immature morphology at the epithelial surface, and an additional approximately 6 days to develop a mature-appearing surface morphology. The timing of hair cell regeneration in the recovering Bengalese finch papilla appears to correspond well with the timing of initial hair cell regeneration in budgerigars, chicks and pigeons. Chicks and budgerigars treated with kanamycin for 10 days show the small stereocilia bundles of immature hair cells in the basal tip immediately after treatment (Hashino et al., 1991; Hashino et al., 1992). These results indicate that the maximum amount of time that is required for hair cells to regenerate and erupt at the papilla surface in aminoglycoside-treated chicks and budgerigars is 10 days. New hair cells have been observed in the chick basilar papilla as early as 3–5 days after the first day of gentamicin treatment (Duckert and Rubel, 1990; Girod et al., 1991; Janas et al., 1995). Müller and Smolders (1998) found that immature hair cells were present in the pigeon basilar papilla within 11 days of local gentamicin application. Even though the birth dates of new hair cells cannot be pinpointed in these studies, the timing of initial regeneration after aminoglycoside toxicity appears to be similar in chicks, budgerigars, pigeons and Bengalese finches.

In this study, a base to apex gradient in new hair cell maturity was evident for the first 4 weeks after treatment. At any one time, the maturity of new hair cells decreased toward the apical extent of the damaged region. At 1 week following treatment, the recovery appeared in a stripe pattern extending the entire length of the damaged region but only covering a portion of the width. The stripe of regenerated hair cells decreased in maturity toward the apex and ran in close proximity to the superior edge of the sensory epithelium, suggesting that tall hair cells regenerated before short hair cells. Duckert and Rubel (1993) reported that the opposite might be true in gentamicin-treated chicks. They observed that the superior portions of damaged papillae exhibited more immature cells (based on surface morphology) than the inferior portions of the same regions. The stripe pattern evident in the Bengalese finch papilla has not been reported before. A base to apex pattern of new hair cell proliferation and maturation has been observed in other studies administering aminoglycosides over several days (Duckert and Rubel, 1990; Girod et al., 1991; Hashino et al., 1991, 1992, 1995).

Between 2 and 8 weeks after treatment, papillae exhibited heterogeneous populations of new hair cells, showing a high degree of variability in surface size and maturity. The variability in maturity and the presence of new hair cells over a protracted period of recovery indicate that new hair cells were generated over several weeks, rather than synchronously. This corresponds well with reports of similar asynchronies in regeneration over several weeks following aminoglycoside treatment (Girod et al., 1991; Hashino et al., 1992; Duckert and Rubel, 1993). In this study, the completion of hair cell regeneration as judged by mature hair cell surface morphology occurred between 4 and 8 weeks following the end of treatment. This is in contrast to the longer period of regeneration seen in gentamicin-treated chicks. Girod et al. (1991) and Duckert and Rubel (1993) reported that immature hair cells were still present in the recovering chick papilla up to 20 weeks following treatment. In Bengalese finches, the orienta-

tion of stereocilia bundles on regenerated hair cells did improve toward normal between 8 and 12 weeks. These findings agree with studies reporting that stereocilia bundle orientation among regenerated hair cells in the chick basilar papilla improves over time (Cotanche, 1987; Cotanche and Corwin, 1991; Girod et al., 1991; Duckert and Rubel, 1993).

### 4.3. Comparative assessment of hearing recovery

The recovery of hearing or auditory thresholds after aminoglycoside ototoxicity has been examined previously in chicks, starlings and budgerigars (Hashino and Sokabe, 1989; Tucci and Rubel, 1990; Marean et al., 1993, 1998; Dooling et al., 1997). These studies have used electrophysiological methods similar to those used in this study and behavioral methods that examine hearing detection thresholds in the same birds over several weeks. We report here that recovery of auditory thresholds in Bengalese finches occurred between 1 day and 4 weeks after aminoglycoside administration. By 4 weeks, thresholds had decreased such that they were only slightly elevated compared to normal, and did not improve further by 12 weeks following treatment. Residual high frequency hearing losses similar to those reported here have been observed in most studies examining this topic (Tucci and Rubel, 1990; Marean et al., 1993, 1998; Dooling et al., 1997).

Comparing the recovery of auditory function during hair cell regeneration among different species indicates that the timing of auditory recovery may be more similar among closely related species. Near normal auditory thresholds in Bengalese finches recover with a similar time course to that in starlings and more rapidly than in chicks and budgerigars. Marean et al. (1993) reported that most recovery of behavioral detection thresholds in kanamycin-treated starlings occurs over 30 days following treatment. Detection thresholds in budgerigars require 8 weeks to recover maximally (Dooling et al., 1997). Thresholds in gentamicin-treated chicks improve some time between 5 and 16 weeks after treatment (Tucci and Rubel, 1990). Additionally, the formation of synapses onto new hair cells is still ongoing 8 weeks after gentamicin treatment (Hennig and Cotanche, 1998). Starlings and Bengalese finches are both oscine songbirds in the order Passeriformes. These two species are more closely related to each other than budgerigars (order Psittaciformes) and chicks (order Galliformes) are to each other or to songbirds. Comparison of functional recovery patterns in these four species suggests that more closely related species may show stronger similarities in the timing of auditory recovery. To this end, Ryals et al. (1999) recently showed that canaries and zebra finches (songbirds) recover hearing thresholds more rapidly and completely than do budgerigars (non-songbirds) after the identical acoustic trauma.

### 4.4. Hearing and vocal recovery

Dooling et al. (1997) examined both hearing and vocal recovery after hair cell damage in budgerigars. Birds were treated with kanamycin to cause hair cell loss, and their abilities to produce calls that matched externally generated acoustic templates were measured. One bird lost the ability to produce matching calls after kanamycin treatment. That bird regained the accuracy of its vocal production within 15 days following treatment. Behavioral detection thresholds reached maximal recovery 8 weeks following treatment. Vocal production in that case recovered before detection thresholds reached maximal recovery.

Bengalese finches were chosen for this study because vocal recovery during hair cell regeneration has been examined in this species. Adult Bengalese finches normally sing stereotyped and stable songs over time (Immelmann, 1969; Dietrich, 1980; Clayton, 1987; Woolley and Rubel, 1997). Normal song behavior depends on auditory feedback, however. When hearing is disrupted by surgical deafening or inducing hair cell damage, song behavior deteriorates (Woolley and Rubel, 1997; Okanoya and Yamaguchi, 1997; Woolley and Rubel, 1999a).

Woolley and Rubel (1999b) examined the recovery of song behavior after inducing degradation of normal song by destroying nearly all auditory hair cells in adult male Bengalese finches. Birds with degraded songs gradually recovered the ability to sing their original songs during hair cell regeneration. The ordering of syllables within songs recovered to normal between 1 and 4 weeks after treatment. The time course for recovery of normal syllable ordering corresponds well with the timing of threshold recovery in this study. Here, thresholds in birds with hair cell damage recovered between 1 and 4 weeks following treatment. Since hair cell loss in that study was greater than the hair cell loss in the present study, we cannot assume that the recovery of hearing in birds in which vocal recovery was studied was exactly the same as the auditory recovery reported here. However, given that normal singing depends on hearing, the fact that vocal recovery occurred between 1 and 4 weeks after hair cell loss suggests that hearing recovery in that study followed a similar time course to the threshold recovery reported here.

Woolley and Rubel (1999b) found that the structure of song syllables recovered more gradually than syllable order. Eight weeks were required for post-treatment syllables to match acoustically with syllables of the original song. Marean et al. (1998) showed that recovery of detection thresholds precedes recovery of more

complex measures of auditory perception such as frequency discrimination in starlings with regenerated hair cells. The more gradual and delayed return of syllable structure reported by Woolley and Rubel (1999b) suggests that refinement of acoustically complex vocalizations may require hearing capabilities that are more complex than detection thresholds alone.

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