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Hair cell regeneration in the European starling (*Sturnus vulgaris*): Recovery of pure-tone detection thresholds [1]

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Behavioral detection thresholds were obtained from four starlings before, during, and after 11 days of subcutaneous injections of kanamycin. Birds were operantly conditioned to respond to pure-tones ranging in frequency from 0.25 kHz to 7 kHz using the method of constant stimuli and were tested daily for 141 days after the first injection of aminoglycoside. All four birds sustained hearing losses greater than 60 dB at frequencies from 4 kHz to 7 kHz by the end of the 11 day injection schedule. Two birds had a slight shift in threshold at 3 kHz. No change in threshold occurred for any of the birds at lower frequencies. Recovery of detection thresholds began soon after the injections ceased and continued for approximately 50 days. In all four birds there was some degree of permanent hearing loss: 5 dB to 15 dB at frequencies between 4 kHz and 6 kHz, and approximately 25 dB at 7 kHz. Scanning electron microscopy (SEM) was performed at 0 and 5 days post-injection in a separate group of starlings given the same injection schedule. Hair cell loss and damage was observed across the basal 34% to 36% of the basilar papilla. SEM in two behaviorally tested birds sacrificed 142 days after the first injection showed that there was regeneration of hair cells to populate the previously damaged region, but that disorientation of stereocilia bundles in the basal third of the basilar papilla was common. The other two behaviorally tested birds were treated with kanamycin again for 16 days beginning at 142 days after the first injection. Thresholds shifted again, but less than during the first dosing period. SEM of these birds' basilar papillae showed less hair cell loss than observed in the birds given only a single, 11 day dosing of kanamycin. This result suggests that birds may be less susceptible to the ototoxic effects of kanamycin in repeated treatments. In all four birds, the degree and position of damage observed with SEM corresponded with the extent and frequency of hearing loss.

Regeneration; Function; Behavior; Thresholds; Avian; Starling

Introduction

The first evidence of hair cell regeneration in the avian cochlea was reported by Cruz et al. (1987) and Cotanche (1987) using light microscopy and scanning electron microscopy (SEM). Corwin and Cotanche (1988), Ryals and Rubel (1988), and Lippe et al. (1991) documented that post-embryonic cellular mitosis contributed to the production of new hair cells after noise or aminoglycoside ototoxicity. Anatomical descriptions of the recovery process suggest that regenerative processes proceed rapidly after ototoxic drug treatment or noise exposure, and that by 30 days post treatment, many hair cells look mature (Cotanche, 1987; Girod, Duckert and Rubel, 1989; Duckert and Rubel, 1990; Saunders et al., 1992; Hashino et al., 1992). These data suggest that if synaptic connections are made between auditory nerve fibers and hair cells, some recovery of function might be possible, and that this recovery could begin rapidly after drug treatments are terminated. There is, however, residual damage, such as disorientation of stereociliary bundles, and scars or empty spaces on the basilar papilla (e.g., Duckert and Rubel, 1990; Hashino et al., 1992; Raphael, 1992; Raphael and Altschuler, 1992; Duckert and Rubel, 1993; Raphael, 1993). Therefore, despite significant regeneration of hair cells within 30 days, the basilar papilla is not normal and residual, functional deficits could remain. Little is known, however, about the relationship between hair cell regeneration and auditory perception.

Functional recovery following aminoglycoside ototoxicity has been assessed in a gallinacious bird (chicken) and in psittacines (budgerigars). Hashino and Sokabe (1989) tested adult budgerigars, obtaining behavioral detection thresholds for pure-tones before and after kanamycin injections. Budgerigars sustained transient threshold shifts at all frequencies tested and residual deficits primarily at low frequencies. Tucci and Rubel (1990) tested 16 to 20 week old chicks with frequency specific auditory evoked potentials following administration of gentamicin. They found that chicks also sustained transient threshold shifts at all frequen-

[1] A preliminary report of these data was presented at the Sixteenth Midwinter Research Meeting of the Association for Research in Otolaryngology, St. Petersburg, FL, February, 1993.

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cies tested, but residual hearing loss was greatest at high frequencies. These observations suggest that some degree of functional recovery can be expected across orders of birds in both developing and mature animals.

Certain auditory functions in the budgerigar may represent special cases among birds and vertebrates in general (Okanoya and Dooling, 1987). Studies of functional recovery during and following hair cell regeneration in a passerine songbird (oscines) would most likely generalize to more birds than budgerigars or chickens, because oscines are a suborder of birds containing almost 50% of all avian species. The psychoacoustic abilities of different species of songbirds are remarkably similar (see Fay, 1988). Moreover, hearing in songbirds has garnered considerable interest because of the inter-relationship among hearing, vocal behavior and development (Konishi, 1985). It might be possible to exploit this relationship to explore the limits of functional recovery if auditory thresholds recover in songbirds following hair cell death and subsequent regeneration.

In the current study we describe the effects of kanamycin on hearing in the European starling (*Sturnus vulgaris*), a passerine songbird. Starlings were chosen because they learn to perform auditory tasks quickly, and like the budgerigar, their auditory system and capabilities have been well characterized (e.g., Fay, 1988). Additionally, a cochlear frequency map has been obtained in this species (reported in Manley, 1990), thus allowing for the correspondence between structural damage and functional consequences to be investigated.

Methods

Subjects

Nine adult European starlings were used as subjects. Four birds were used for behavioral testing. Three birds were sacrificed and processed for SEM immediately following the injection series to determine the initial hair cell loss following kanamycin treatments. Two birds were given the same injection series and were allowed to survive for 5 days. They were then processed for SEM an examined for evidence of hair cell regeneration. All birds were captured locally by baiting to a cage equipped with a trap door. Birds were released who appeared juvenile or senescent.

During behavioral testing, birds were food rationed so as to keep them at the highest possible weight which maintained their motivation to perform the task, which for most birds was about 90% of their free-feeding weight in the laboratory, but within the range of normal weights we commonly measure in the field. Weights during testing ranged from 65 g to 75 g.

Stimulus and apparatus

The birds that were tested behaviorally after aminoglycoside treatments were a subset of birds from an investigation of auditory thresholds in starlings. In order to obtain audibility curves, birds were tested at ten frequencies (0.25 kHz, 0.5 kHz, 1 kHz, 2 kHz, 3 kHz, 4 kHz, 5 kHz, 6 kHz, 7 kHz, and 10 kHz) prior to dosing with kanamycin. It was not possible to test a bird on all ten frequencies every day. Thus, during and immediately following aminoglycoside treatments only six frequencies were studied in detail (0.25 kHz, 1 kHz, 3 kHz, 4 kHz, 5 kHz, and 7 kHz). Limiting the number of test frequencies allowed for more accurate tracking of daily changes in threshold. There were 12 intensity levels per frequency separated by 6 dB increments. Tone bursts were 200 ms in duration and had linearly ramped 20 ms rise-fall times. Maximum intensity outputs of our acoustic stimuli ranged from 67 dB SPL at 0.25 kHz to 82 dB SPL at 4 kHz.

Stimuli were generated digitally with a 44 kHz sampling rate and stored on an NEC 386SX PC. During presentation, they were sent through a Data Translation DT2821 AD/DA board. Stimuli were amplified by a Technics SA-303 amplifier and then fed to a Daven T-693 manual attenuator. The loudspeaker (Realistic MC-600) was situated approximately 2 feet from the bird's left ear.

The birds were trained and tested in an IAC 1.21 m $\times$ 1.21 m $\times$ 2.14 m sound attenuating booth. Ambient noise measurements and calibration of test stimuli were made with a Bruel and Kjaer sound level meter with a Bruel and Kjaer 1/2 inch microphone placed in the same location as the bird's head and pointed towards the loudspeaker. The signal was then fed in to a Hewlet Packard spectrum analyzer for analysis. Harmonic distortion from the loudspeaker was at least 30 dB down from the stimulus frequency. Ambient noise levels ranged from 40 dB SPL to 50 dB SPL in the soundbooth. Spectral analysis of the ambient noise measured in 1/3 octave bands showed that most of the noise was in the low frequencies (see Fig. 1).

Training and testing procedure

Birds were trained and tested in their home cages (49 cm $\times$ 41 cm $\times$ 41 cm) within the soundbooth. A panel containing two piezo-electric peck keys and a solenoid-activated feeder was inserted in the cage door opening. Stimulus presentation was under computer control.

Birds were trained to peck the 'observation key' repeatedly until a signal trial was detected. Trials were stopped and restarted if the bird failed to peck the observation key for more than 1 s. The amount of time from when a bird initiated a trial to when the stimulus was presented was varied randomly from 2 to 5 s so that the birds could not use time cues to improve

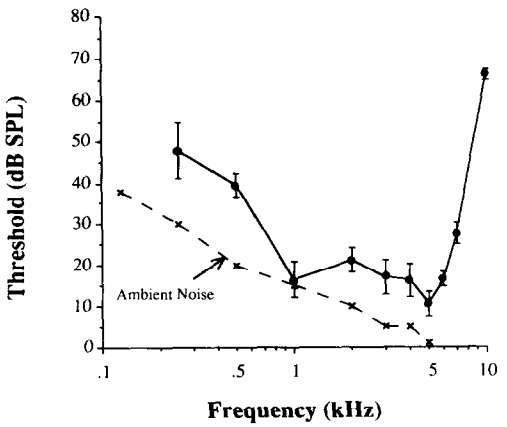

Fig. 1. Average audibility curve of 4 birds used in the present study prior to dosing with kanamycin. Error bars indicate ±1 standard error of the mean. Total power of ambient noise levels measured in 1/3 octave bands are also shown. Center frequencies of octave bands denoted by 'x's.

performance. Stimuli were only presented immediately following a peck on the observation key to ensure that the bird's head was in a known position when sounds were presented. A peck on the 'response key' within 1 s after the end of the tone burst was reinforced with a food reward (dog food kibble). A peck on the response key during a no-signal trial (a false alarm) was punished by darkening the sound booth for 4 s. Food rationed birds were tested daily in sessions consisting of approximately 172 trials. Once a trial had been initiated, the probability of a signal trial was 0.8.

Data for threshold estimation at each frequency was gathered by the method of constant stimuli in which intensity and frequency were randomly varied from trial to trial. A range of 30 dB (five stimuli in 6 dB steps at each frequency) was used. For each frequency, a psychometric function was derived by plotting the proportion of 'Yes' responses as a function of intensity. Threshold was taken as the 70% point on this function based upon linear interpolation. Each bird's normal threshold at any given frequency was computed from the average of at least ten individual daily thresholds prior to the dose period. Only thresholds from sessions with false alarm rates below 0.40 were counted. Table I shows average false alarm rate for each individual bird across the entire experiment. It should be noted that false alarm rates between 0.25 and 0.40 were rare, comprising only 6.9% of the total sessions used (n = 376) and were almost exclusively due to the performance of a single bird (92–1). Examination of the data revealed no tendency for an increased hit rate in these sessions, thus, they were not excluded from the analysis.

Prior to kanamycin dosing, stimulus sets were alternated on a daily basis because it was not possible to obtain thresholds at every frequency on every day. One stimulus set contained 0.25 kHz, 1 kHz, 3 kHz, 5 kHz, and 7 kHz. On the alternate day, birds were tested at 0.5 kHz, 2 kHz, 4 kHz, 6 kHz, and 10 kHz. However, to track changes in threshold more precisely during and immediately after the drug treatment, the stimulus set was fixed when dosing with kanamycin began (Day 1). The 4 kHz stimuli were added to the first stimulus set, and that set was used until approximately Day 50 after the first injection, when the practice of alternating sets was reinstituted. Additional sessions of separate frequency-trial blocks after the main session were run approximately every 2 days with 2 kHz, 6 kHz and 10 kHz stimuli to 'spot check' performance at those frequencies between Day 1 and Day 50.

Kanamycin injections and testing schedule

Once thresholds had been determined, each bird was given subcutaneous injections of 100 mg/kg/day for 2 days and 200 mg/kg/day kanamycin for 9 days. * Birds were tested several hours after their daily injection. Food rations were provided immediately after behavioral testing.

The five birds which were not tested behaviorally but used for SEM were given the same dosage of kanamycin. Three birds were sacrificed the day after the last injection (Day 12), and two birds were sacrificed 5 days later (Day 17). Two of the four behaviorally tested birds were sacrificed on Day 142. The other two behaviorally tested birds were redosed with kanamycin beginning on Day 142 for 16 days and sacrificed on Day 158. These birds were redosed in order to demonstrate that hearing had recovered due to the regeneration of hair cells. It was presumed that loss of hair cells in the same position as the regenerated hair cells and concomitant hearing loss would provide further evidence that the hearing recovery was

TABLE I

Average false alarm rate observed for each bird during the experiment computed from the daily sessions included in the final sample

Bird	92-1	92-3	92-7	92-8	ALL
Average f.a. rate	0.19 [1]	0.07	0.02	0.07	0.09
SD	0.12	0.07	0.03	0.07	0.10

[1] False alarm rate is the proportion of 'yes' responses during no-signal trials to the total number of no-signal trials.

* Initial attempts to cause hair cell damage in the starling with gentamicin injections were unsuccessful. Gentamicin did not cause hair cell damage in the starling, but frequently caused death from presumed renal failure. Kanamycin, on the other hand, evoked no outward signs of distress in the starling and produced highly reproducible damage to the basal third of the basilar papilla (see results). An 11 day dosing period was used instead of the standard 10 day dosing period so that the first day's dose could be spread over 2 days to reduce the shock of these high dosages of aminoglycoside.

mediated at least in part by the new hair cells. It should be noted that this reasoning is parallel to the common assumption that hearing loss due to the initial exposure to noise or aminoglycosides is mediated primarily by the loss of hair cells. The dose of kanamycin was to be administered until the birds had approximately the same audiometric configuration observed following the first dosing period (see results).

Scanning electron microscopy

For SEM, birds were decapitated following administration of an overdose of pentobarbitol sodium. Both cochleas from each bird were perfused via the round window with 2.5% gluteraldehyde and 2% paraformaldehyde in 0.1 molar phosphate buffered saline (PBS). The temporal bones were removed and the basilar papilla exposed using standard microdissection techniques. The specimens were then placed in 1% osmium tetroxide for one hour followed by several washes in PBS. Specimens were then dehydrated in a graded ethanol series, followed by final dissection. The tectorial membrane was removed under 70% ethanol. After critical point drying, specimens were mounted on aluminium stubs and coated with gold palladium prior to examination with the JEOL 63005 electron microscope.

The SEM observations were used to document hair cell loss following aminoglycoside treatment and subsequent repopulation of hair cells in the damaged portion of the basilar papilla. Our goal was not to provide detailed correlations between structural and functional properties as a function of frequency and place. Thus, detailed quantitative analyses of hair cell parameters such as number, size, and bundle orientation were not undertaken.

Results

Pretreatment thresholds: Audibility curve

Fig. 1 shows the average audibility curve of the four birds prior to dosing with kanamycin. The frequency of best hearing observed in the present study was slightly higher than previously reported (Kuhn et al., 1982; Dooling et al., 1986). Thresholds from an additional 8 starlings tested behaviorally but not given aminoglycosides fell within the error bars of thresholds shown here.

There was no obvious change in performance parameters such as false alarm rate, session length, or latency of response from sessions run before, during, or after injections of aminoglycosides. For example, average false alarm rate for the 12 days prior to the dose was 0.05 for all birds and average false alarm rate during the dosing period was 0.07.

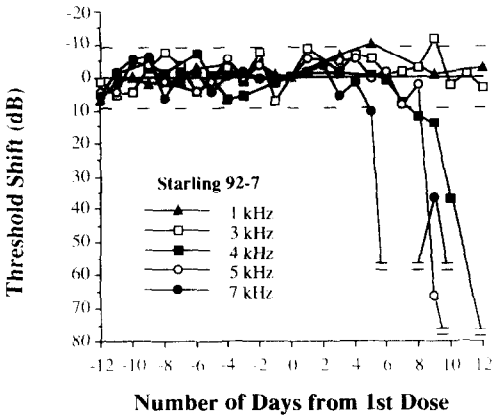

Fig. 2. Individual daily threshold shift (Starling 92–7) at 5 frequencies from 12 days before to 12 days after the first injection of kanamycin (dosing period from Day 1 to Day 11). Dashed lines indicate ±2 standard deviations of threshold shifts prior to the dosing period for 4 birds at the frequencies displayed. Limits of test equipment indicated by broken lines.

General pattern of threshold shift during and following kanamycin dosing period

During the 11 day dosing period, threshold shifts greater than 60 dB were observed in all four birds at all frequencies above 3 kHz. An example of the threshold shifts observed in one bird from 12 days prior to the first dose to 12 days after the first dose of kanamycin is shown in Fig. 2. The dashed lines indicate 2 standard deviations of the mean of thresholds obtained prior to dosing with kanamycin (Day −12 to Day −1). Threshold shifts of less than 2 standard deviations were not considered significant changes. On Day 5, this bird's threshold at 7 kHz was just beyond the 2 standard deviation criterion. On Day 9, both 4 kHz and 5 kHz were below this point. Note that no significant change in threshold occurred for frequencies below 4 kHz in this bird during the dosing period.

Threshold shifts occur initially at high frequencies and then at progressively lower frequencies. This result is evident in the mean threshold shifts of all four birds, shown in Fig. 3. The horizontal dashed lines indicate two standard errors of the mean based upon thresholds obtained prior to dosing with kanamycin. When the threshold shifts from all four birds are considered, significant threshold shift first occurred at 7 kHz on Day 4; at 5 kHz on Day 7, and at 4 kHz on Day 9. No shifts in threshold were observed at lower frequencies for the birds during the dosing schedule. No responses could be obtained from any of the birds at 7 kHz or 5 kHz by Day 10, and at 4 kHz, by Day 12. The improvement in performance seen in the average data at 7 kHz on Day 9 is largely due to the performance of a single starling (92–7), the data for which are shown in Fig. 2. The average value of some the thresholds obtained at 7 kHz was beyond the limit of the test equipment; when responses could not be obtained at a given frequency, a

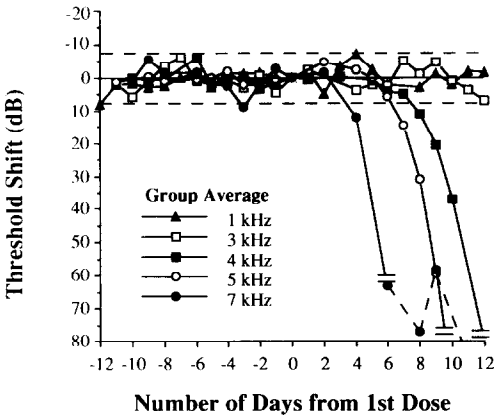

Fig. 3. Group daily threshold shifts at five frequencies from 12 days before to 12 days after the first injection of kanamycin. Horizontal dashed lines indicate ±2 standard errors of the mean of threshold shifts prior to the dosing period for all birds at the frequencies displayed. Dashed lines are used between data points which are beyond the limits of the test equipment. This occurs because a value of 90 dB threshold shift is assigned to sessions where no response could be obtained from a bird.

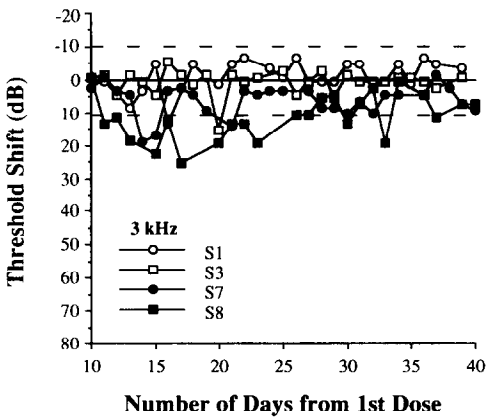

Fig. 4. Individual daily threshold shifts of all birds at 3 kHz from 10 days after the first dose to 40 days after the first dose. Dashed lines indicate ±2 standard deviations of threshold shifts prior to the dosing period for all birds at 3 kHz.

threshold shift of 90 dB was assigned for the purposes of averaging.

Because thresholds shifted beyond the limit of the test equipment above 3 kHz, it was not possible to measure the total amount of hearing loss at these frequencies. For frequencies below 4 kHz, significant threshold shifts occurred in two birds at 3 kHz. Individual data for all birds at 3 kHz are plotted in Fig. 4. One bird (92–8) had a significant threshold shift on Day 11 which continued for approximately 12 days, while another bird (92–7) had a significant threshold shift at 3 kHz on Day 14. This bird's threshold shift at 3 kHz did not stay below the 2 standard deviation criterion for more than a few days. The other two birds showed no reliable shifts at 3 kHz. No threshold shifts were observed in any bird at 1 kHz or 0.25 kHz during or following the dosing period.

At Day 50, when the practice of alternating stimulus sets was reinstituted such that every two days all ten frequencies were tested, responses to 10 kHz could not be obtained in any bird. In fact, thresholds at 10 kHz could not be obtained in any bird on any day for the remainder of the experiment. It should be noted, how-

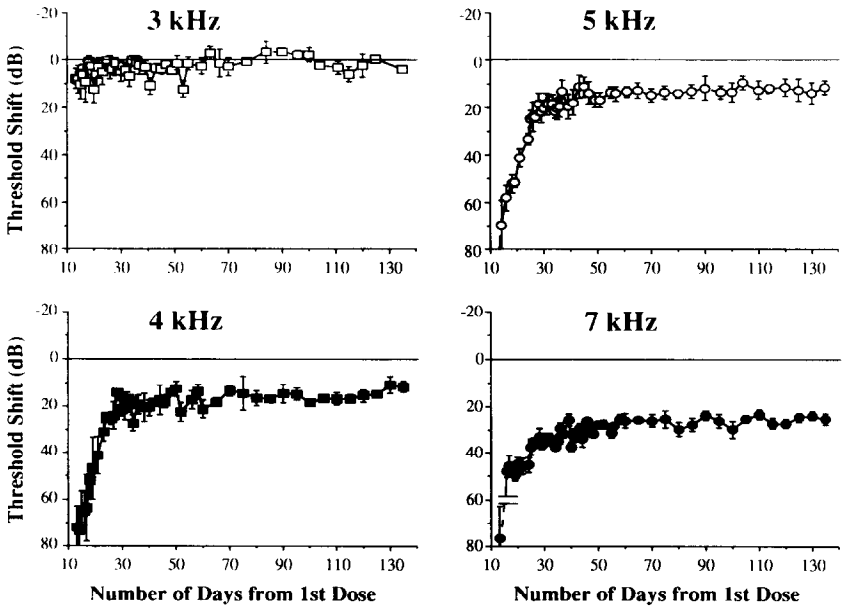

Fig. 5. Group daily threshold shifts at 4 frequencies from 10 to 141 days after the first dose of kanamycin. Error bars represent ±1 standard error of the mean. Dashed lines are used between data points which are beyond the limits of the test equipment. This occurs because a value of 90 dB threshold shift is assigned to sessions where no response could be obtained from a bird.

ever, that the maximum output of our system at 10 kHz was 79 dB SPL. Thus, we can only be confident of a 12 dB hearing loss at 10 kHz, since thresholds at this frequency prior to drug administration were typically about 67 dB. Thresholds at 0.5 kHz and 2 kHz at Day 50 were not different from those obtained prior to the first dosing period. 'Spot check' thresholds at these frequencies made between the end of the first dosing period and Day 50 (see Methods) also showed no significant change from thresholds obtained prior to the first dosing period. The threshold shift on Day 50 and in previous 'spot checks' at 6 kHz was similar to 5 kHz (see below).

General pattern of recovery

Recovery toward normal hearing threshold began within a few days of the end of the 11 day dosing period. Average threshold shifts at 4 frequencies from Day 10 to Day 141 are plotted in Fig. 5. At 3 kHz, day-to-day threshold shift varied considerably which was not surprising given that two birds had significant threshold shifts and two did not. In addition, the thresholds of birds with significant shifts at 3 kHz appeared to vary more day-to-day than the birds without threshold shifts (see Fig. 3). At 4 kHz and 5 kHz, recovery of threshold proceeded rapidly for approximately 20 days after the end of kanamycin treatments, then more gradually for another 20 days. The group average threshold shift at 4 kHz and 5 kHz by Day 141 was approximately 15 dB. At 7 kHz, recovery was more gradual than at other frequencies and resulted in a residual threshold shift of 25 dB. Thresholds at 3 kHz recovered completely by the time thresholds stabilized at 4 kHz, 5 kHz, and 7 kHz (e.g., by Day 60).

Other than the individual differences described in the transient threshold shifts at 3 kHz, there was only one notable difference between birds in the recovery of behavioral thresholds. Starling 92–8 showed complete recovery at 5 kHz, although not at 4 kHz, 6 kHz or 7 kHz. This result was not observed in any of the other birds.

The effect of redosing with kanamycin

Two of the birds were redosed with kanamycin beginning at Day 142. Their average threshold shifts at 4 kHz, 5 kHz, and 7 kHz across the entire experiment are plotted in Fig. 6. Redosing produced threshold shifts similar to those seen following the first dose. There was, however, one notable difference. Despite 5 additional days of exposure to high dosages of kanamycin during the second dosing period, threshold shifts were not as pronounced as those seen during the initial kanamycin treatment.

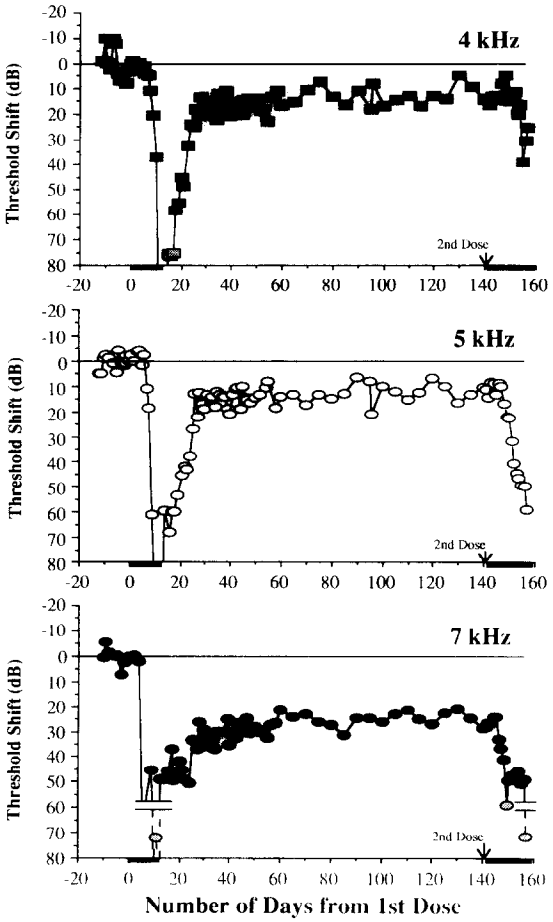

Fig. 6. Average daily threshold shifts of 2 birds (Starlings 92–7 and 92–8) at three frequencies from 12 days before to 158 days after the first dose of kanamycin. Days during dosing periods are indicated by a thickening of the x-axis. Data points which fall below the limits of the test equipment (denoted by broken lines) are shaded and connected by dashed lines.

Scanning electron microscopy

Fig. 7A shows a surface view of the basal half of the basilar papilla from a normal starling. Panels B and C are higher magnification micrographs from the same regions to be shown in Figs. 8, 9, and 11 from treated animals. The approximate positions are indicated by arrows and are from just below the midway point between neural and abneural edge. The apparent clumping of stereocilia which can be seen along the neural (superior) edge is due to incomplete fixation in this region and was commonly observed in both normal and damaged papillae.

The basilar papillae of five starlings given 11 daily injections of kanamycin were examined at Day 12 or Day 17 (corresponding to 0 days and 5 days survival, respectively). Representative photomicrographs taken from a starling sacrificed at Day 12 are shown in Fig. 8. The damaged region extended approximately 727 μm or 34% of the length of the papilla from the base towards the apex (measurements of distance from the

basal tip were made along the superior edge of the papilla). The region from the base to approximately 582 μm, or 0% to 28%, appears to be devoid of any surviving mature hair cells (Fig. 8B). There appeared to be a 'transition zone' from 30% to 34% distance from base where some hair cells remained, but were fewer in number. A higher magnification micrograph from this region (Fig. 8C) shows that the remaining hair cells in this transition zone were not normal. There was considerable damage to the apical surface and stereociliary bundles of the surviving cells. Some of these cells had lost all or most stereocilia. In others, the apical surface appeared grossly enlarged and the stereocilia bundles appeared to be splayed or fragmented. In addition, some hair cells appeared to be in the process of being extruded from the epithelium. It is possible that these hair cells are in the process of dying. It is also evident that newly regenerated hair cells are emerging, especially in the most basal regions (Fig. 8B). These are identified by smaller stereocilia which have not yet attained their adult lattice structure, by numerous microvilli emerging from the cell's luminal surface outside the area of the stereocilia bundle, and by a prominent kinocilium (Cotanche, 1987; Henry et al., 1988; Duckert and Rubel, 1990). The position of damage resulting from kanamycin dosing in the two other birds sacrificed at Day 12 covered the basal 29% to 36% of the papilla; all birds had a 'transition zone' approximately 200 μm in length. Birds sacrificed at Day 17 (5 days survival) showed the same position of damage except that there appeared to be a greater number of regenerating hair cells in the most basal regions of the papillae from these birds compared with the birds sacrificed on Day 12. In as much as SEM of the luminal surface of the basilar papilla could reveal the extent of damage caused by aminoglycoside ototoxicity, there was no obvious apical spread of damage during the 5 days after the dosing period. Thus, in adult starlings, it appears that an 11 day treatment of kanamycin results in a reproducible area of damage to the basal 34% of the basilar papilla, with little continuing damage beyond the dosing period.

The basilar papillae from two of the behaviorally tested birds were processed for SEM on Day 142. Micrographs of one papilla are shown in Fig. 9A. The basal 34% of the papilla appeared fully repopulated by

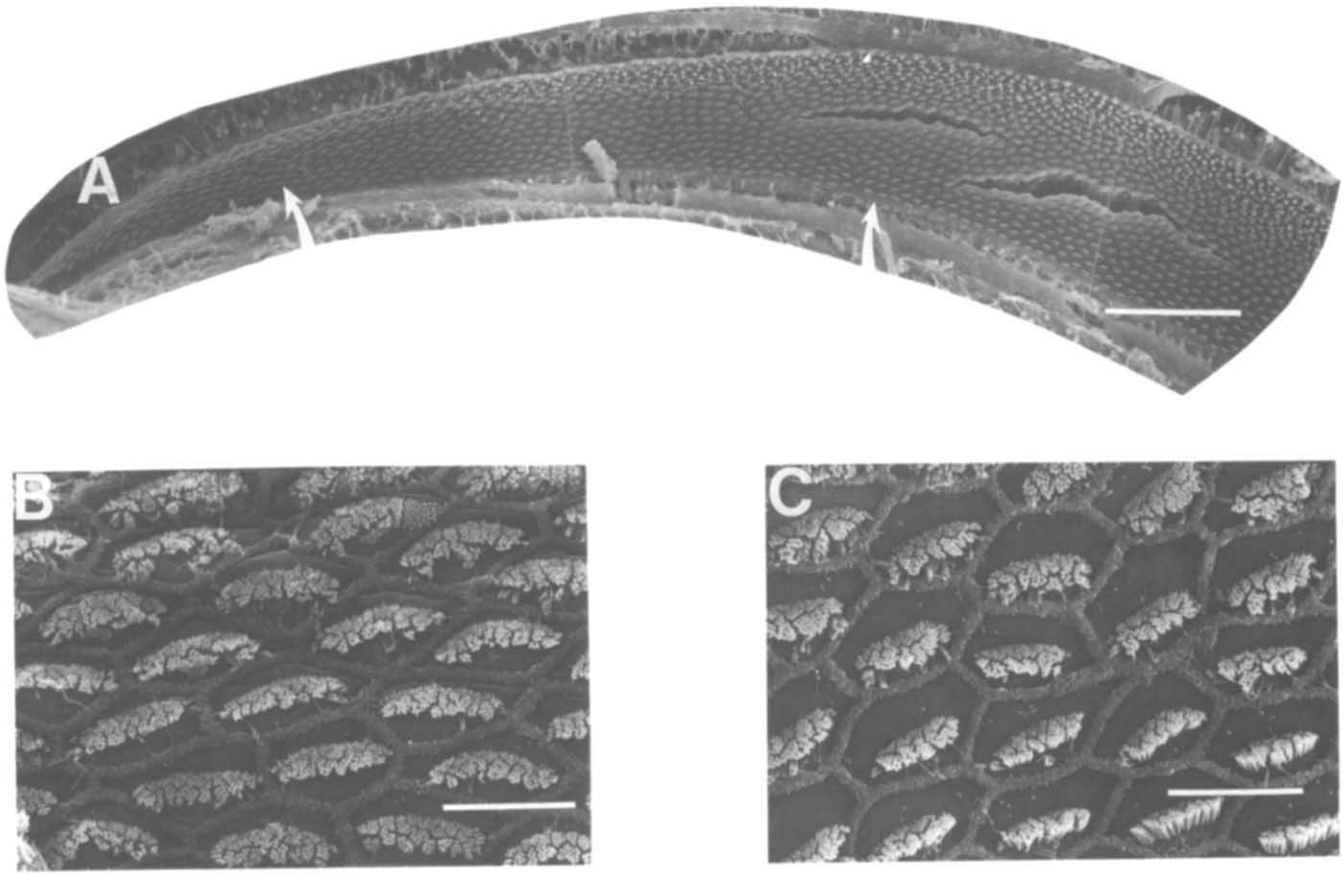

Fig. 7. A. Scanning electron micrographs of basal half of a basilar papilla of an untreated, control Starling. Arrows indicate positions of higher magnification micrographs seen in B and C. Scale bar = 100 μm. B. Scanning electron micrograph taken from an area approximately 200 μm from basal tip. C. Scanning electron micrograph taken from an area approximately 30% of the length from the basal tip. Scale bar for B and C = 10 μm.

hair cells, but the orientation of stereociliary bundles was often not normal. It is possible that the transition between disorganized stereociliary organization and organized orientation corresponded to the extent of damage from the first injection series, because the position at which we observed disorganization was similar to the position of damage found in birds sacrificed on Day 12. This observation further suggests that little to no spread of damage occurred beyond the dosing period. In the most basal regions, some of the presumed regenerated hair cells were not only disorganized in terms of their orientation, but also had abnormal hair cell bundle morphology (e.g., Fig. 9B). These abnormalities included multiple, oddly shaped ciliary bundles, distorted lattice structure, and visually observable variation in the number of cilia on adjacent hair cells. This was also true for many cells in the region of the presumed transitional zone (Fig. 9C).

The stereociliary bundle disorientation seen in aminoglycoside treated birds is further exemplified in Fig. 10A, taken from the other behaviorally tested bird sacrificed at Day 142. The location of these hair cells is approximately 300 μm from the basal tip. In this case, the apparent loss of appropriate signals for stereociliary bundle orientation resulted an interesting pattern, resembling the petals of a flower. This pattern is either an unlikely accidental occurrence or suggests that the signals influencing bundle orientation are from adjacent hair cells. Fig. 10B was taken from a control bird at the same location as Fig. 10A. Patterns of stereocilia orientation such as those shown in Figs. 10A and 9B have never been observed in the 10 ears we've processed from normal starlings.

The two birds subjected to a second dosing period of kanamycin at Day 142 were also processed for SEM on Day 158 (Fig. 11). There was a considerable difference in the extent of hair cell loss observed comparing birds sacrificed after a single dosing period to those subjected to a second dosing period (see Figs. 8A–C for comparison). With the exception of a region between 0% and 10% from the base, the basal third of the papilla was still populated by hair cells after the sixteen day dosing period which began on Day 142. Furthermore, these hair cells appeared too mature to have regenerated during this 2nd dosing period. Because this region had been devoid of surviving hair

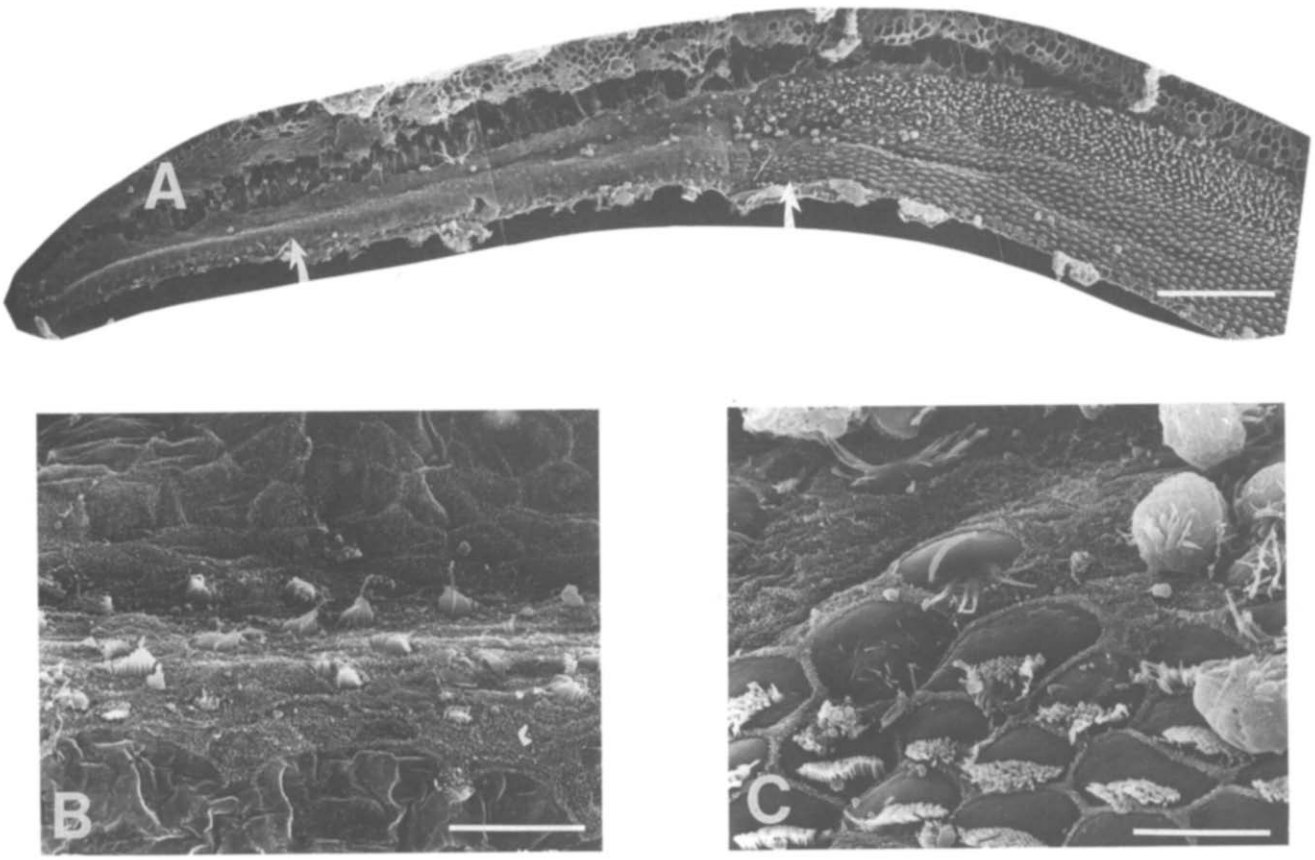

Fig. 8. A. Scanning electron micrographs of basal half of a basilar papilla of Starling 92-WB11, a bird dosed at 200 mg/kg/day kanamycin for 11 days (see Footnote 1) and sacrificed on Day 12. Arrows indicate positions of higher magnification micrographs seen in B and C. Scale bar = 100 μm. B. Scanning electron micrograph taken from an area approximately 200 μm from basal tip. C. Scanning electron micrograph taken from an area approximately 30% of the length from the basal tip. Scale bar for B and C = 10 μm.

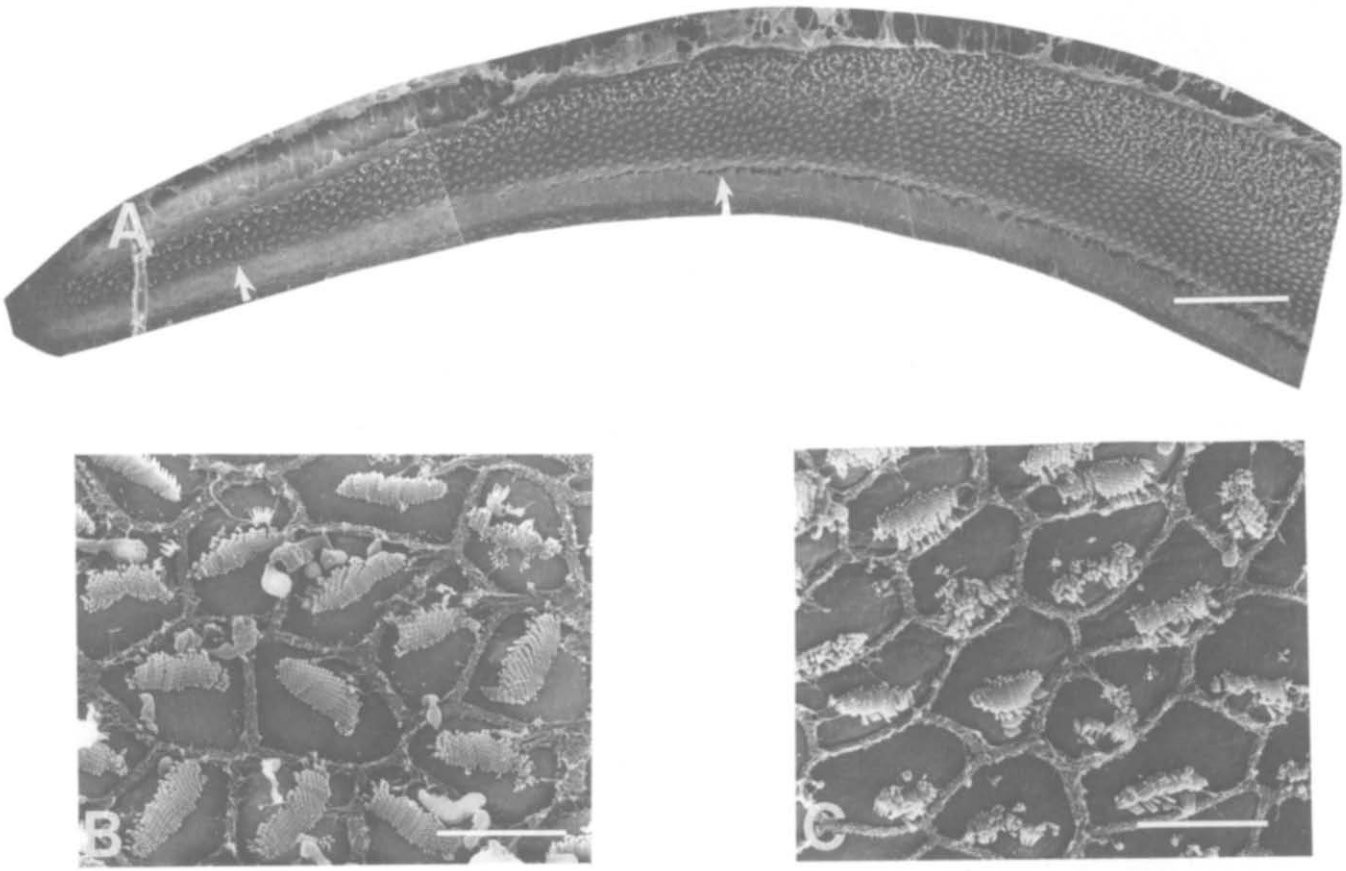

Fig. 9. A. Scanning electron micrographs of basal half of a basilar papilla of Starling 92–1, a bird dosed at 200 mg/kg/day kanamycin for 11 days (see Footnote 1) and sacrificed on Day 142. Arrows indicate positions of higher magnification micrographs seen in B and C. Scale bar = 100 μm. B. Scanning electron micrograph taken from an area approximately 200 μm from basal tip. C. Scanning electron micrograph taken from an area approximately 30% of the length from the basal tip. Scale bar for B and C = 10 μm.

cells after the first dosing period of 11 days (see Fig. 8), it appears that the papillae of birds with regenerated hair cells are more resistant to the ototoxic effects of kanamycin than are the inner ears populated by normal, embryonically generated hair cells.

Discussion

The present results show that auditory behavioral detection thresholds recover following aminoglycoside ototoxicity in the starling. The pattern of loss and recovery was similar across the four birds tested. Large initial threshold shifts (> 60 dB) are associated with residual threshold shifts, 5 dB to 15 dB from 4 kHz to 6 kHz, and approximately 25 dB at 7 kHz. While we presume these residual threshold shifts to be permanent, it is possible they are not. Mild threshold shifts (< 30 dB) such as those which occurred for two birds at 3 kHz are not permanent.

Unlike the budgerigar (Hashino and Sokabe, 1989), starlings do not show a low frequency hearing loss following kanamycin treatments of the same dose and duration. Rather, low frequency thresholds remain unchanged. The differences between starling and budgerigar in behavioral thresholds are likely the result of an increased spread of damage from the base towards the apex seen in the budgerigar (Hashino et al., 1992). Budgerigars given 200 mg/kg/day for 10 days sustained damage to the basal 55% to 75% of the basilar papilla. In the present study, starlings sustained damage to only the basal 30% to 34% of the papilla, similar to the extent of damage observed by Hashino et al. (1991) in neonatal chicks. It is still an open question as to whether or not starlings would demonstrate transient or permanent threshold shifts in the low frequencies if the dosing period was extended until the position of damage in each species was equivalent.

In the chick, Tucci and Rubel (1990) found that thresholds continued to get worse beyond the dosing period, and that both high and low frequencies were affected. These results were not observed in the present study. Starling thresholds appeared to improve immediately upon termination of drug exposure, and low frequency thresholds (< 3 kHz) did not change. Differences in the age and species of the bird as well as

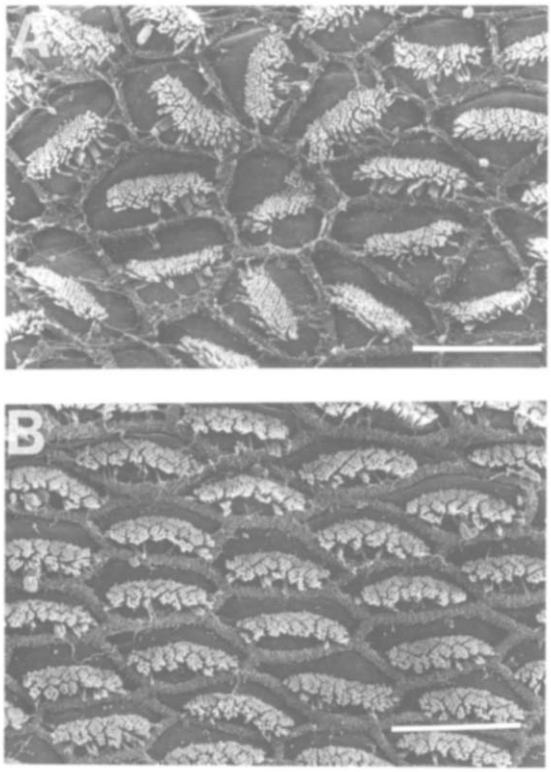

Fig. 10. A. Scanning electron micrograph of a group of hair cells approximately 300 μm from the basal tip of the left papilla of Starling 92–3, a bird dosed at 200 mg/kg/day kanamycin for 11 days (see Footnote) and sacrificed on Day 142. B. The same area in an untreated, control Starling. Both scale bars = 10 μm.

the drug used most likely resulted in a different pattern of hair cell loss and recovery in these two studies and contributed to the differences observed in auditory thresholds.

In the present study, there appeared to be a close correspondence between the overall amount and position of damage as indicated by SEM and behavior. Starlings sustained observable hair cell damage to the basal 34% of the papilla. Using the frequency map of Gleich (reported in Manley, 1990), the basal 34% of the papilla corresponds to coding sites for frequencies greater than 3 kHz. These were, in fact, the frequencies at which a significant threshold shift was reliably observed. Two birds had slight shifts at 3 kHz and all four birds had normal hearing at frequencies below 3 kHz.

Residual threshold shifts remained at long survival times for starlings (present study), chicks (Tucci and Rubel, 1990) and Budgerigars (Hashino and Sokabe, 1989). There are a number of factors that may be responsible for these residual, potentially permanent threshold shifts. Systematic hair cell counts were not made in the present study. Thus, it is possible that fewer hair cells occupied the regenerated portions of the epithelium than normal. The innervation of new hair cells and their sub-luminal conditions were not examined; Ryals et al. (1989) report degeneration of ganglion cells following hair cell loss in young adult quail. Finally, transduction efficiency may be reduced or cochlear mechanics may be altered when stereociliary bundles are not normally aligned. It is not known what size of an effect (in dB threshold shift) might be expected from such abnormalities. Future studies using otoacoustic emissions, such as the measurements obtained by Norton et al. (1990) in the chick during regeneration might be helpful in addressing these issues.

The residual hair cell disorientation of regenerated hair cells found in the present study was also observed by Hashino et al. (1992) in adult budgerigars. Studies with chicks (e.g., Cotanche and Corwin, 1991; Cotanche et al., 1991) have observed changes in stereocilia orientation during development and regeneration. It would be instructive to obtain precise measurements of changes in orientation and correlate these changes with functional recovery.

The finding that repeating the injection series resulted in reduced hair cell damage has not been previously reported, although similar phenomena have been observed following low to moderate levels of noise exposures (e.g., Subramaniam et al., 1991). The effect has been termed 'toughening' because hair cells appear to become resistant to the damaging effects of noise after long term exposure to moderate noise levels. This phenomenon is thought to explain the reduction in the amount of temporary threshold shift observed with behavioral measures after repeated sound exposures. The possibility that the ototoxic effects of kanamycin are reduced following exposure to high dosages needs to be systematically investigated since the present data were from only two birds (four ears). Moreover, there may be any number of mechanisms contributing to reduced ototoxicity which may not be due to altered properties of regenerated hair cells. For example, it is possible that there are changes in the characteristics of how aminoglycosides are metabolized following exposure to high dosages. In any case, behavioral data showing relatively less total threshold elevation corresponded to the reduced loss of hair cells observed after the second dosing period in both birds. Finally, regenerating hair cells were found in birds exposed to aminoglycosides for a second time. Similar observations have been made in neonatal chicks following repeated exposure to intense noise (Adler et al., 1992).

The relationship between basic auditory capabilities following hair cell regeneration and the perception of complex, species-specific communication signals could be addressed in songbirds. Because the songs of birds share several important acoustic similarities with human speech and are a critical component of their social behavior, changes in the perception or production of

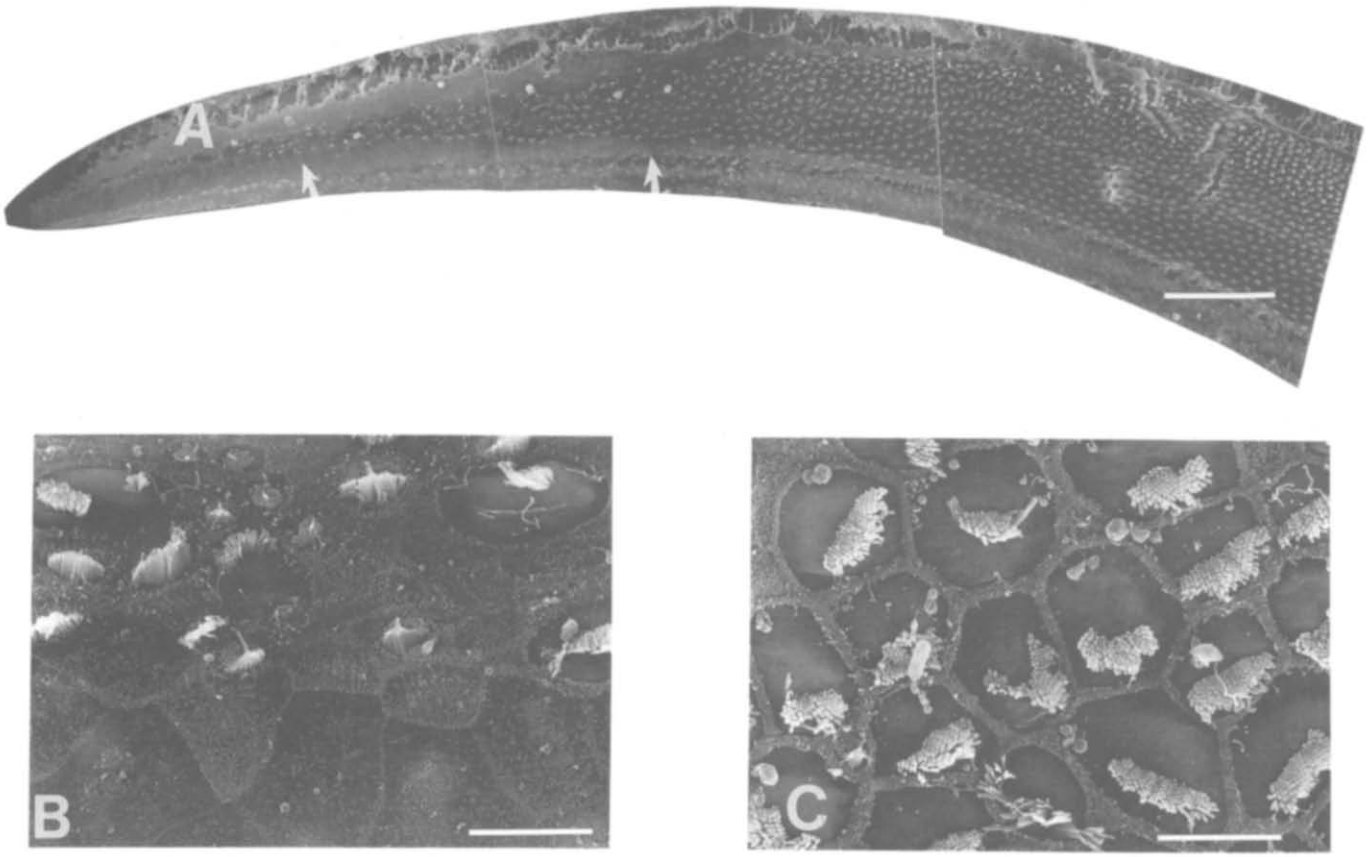

Fig. 11. A. Scanning electron micrographs of basal half of a basilar papilla of Starling 92–7, a bird dosed at 200 mg/kg/day for 11 days (see Footnote 1) with kanamycin; the bird was allowed to recover until Day 142 when it was dosed again with 200 mg/kg/day kanamycin for 16 days and sacrificed on Day 158. Arrows indicate positions of higher magnification micrographs seen in B and C. Scale bar = 100 μm. B. Scanning electron micrograph taken from an area approximately 200 μm from basal tip. C. Scanning electron micrograph taken from an area approximately 30% of the length from the basal tip. Scale bar for B and C = 10 μm.

song following hair cell regeneration may provide insights regarding the relationships between measures of auditory performance and perception.

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