Recovery of the Vestibulocolic Reflex After Aminoglycoside Ototoxicity in Domestic Chickens

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Goode, Christopher T., John P. Carey, Albert F. Fuchs, and Edwin W Rubel. Recovery of the vestibulocolic reflex after aminoglycoside ototoxicity in domestic chickens. *J. Neurophysiol.* 81: 1025–1035, 1999. Avian auditory and vestibular hair cells regenerate after damage by ototoxic drugs, but until recently there was little evidence that regenerated vestibular hair cells function normally. In an earlier study we showed that the vestibuloocular reflex (VOR) is eliminated with aminoglycoside antibiotic treatment and recovers as hair cells regenerate. The VOR, which stabilizes the eye in the head, is an open-loop system that is thought to depend largely on regularly firing afferents. Recovery of the VOR is highly correlated with the regeneration of type I hair cells. In contrast, the vestibulocolic reflex (VCR), which stabilizes the head in space, is a closed-loop, negative-feedback system that seems to depend more on irregularly firing afferent input and is thought to be subserved by different circuitry than the VOR. We examined whether this different reflex also of vestibular origin would show similar recovery after hair cell regeneration. Lesions of the vestibular hair cells of 10-day-old chicks were created by a 5-day course of streptomycin sulfate. One day after completion of streptomycin treatment there was no measurable VCR gain, and total hair cell density was ~35% of that in untreated, age-matched controls. At 2 wk postlesion there was significant recovery of the VCR; at this time two subjects showed VCR gains within the range of control chicks. At 3 wk postlesion all subjects showed VCR gains and phase shifts within the normal range. These data show that the VCR recovers before the VOR. Unlike VOR gain, recovering VCR gain correlates equally well with the density of regenerating type I and type II vestibular hair cells, except at high frequencies. Several factors other than hair cell regeneration, such as length of stereocilia, reafferentation of hair cells, and compensation involving central neural pathways, may be involved in behavioral recovery. Our data suggest that one or more of these factors differentially affect the recovery of these two vestibular reflexes.

INTRODUCTION

Birds are able to regenerate auditory hair cells, the sensory transducers of the inner ear, after they are destroyed by ototoxic aminoglycoside antibiotics (Cruz et al. 1987; Lippe et al. 1991). Auditory hair cell regeneration is also evident after severe noise damage (Cotanche 1987; Corwin and Cotanche 1988; Ryals and Rubel 1988). Anatomic data have shown that afferent connections are reestablished on these regenerated hair cells (Duckert and Rubel 1993).

Several studies at different levels of the auditory system have shown that the regenerated cells and their afferents are functional. In chickens, compound action potentials, recorded at the round window, return after kanamycin damage (Chen et al. 1993). Auditory evoked potentials, measured at the brain stem, recover after damage caused by noise (McFadden and Saunders 1989) and gentamycin (Girod et al. 1991; Tucci and Rubel 1990). In budgerigars, behavioral detection thresholds for pure tones return after kanamycin damage (Hashino and Sokabe 1989). In the European starling, pure tone detection thresholds recover after similar damage (Marean et al. 1993). Another measure of auditory function, evoked otoacoustic emissions, shows the same pattern of recovery after kanamycin damage (Norton and Rubel 1990).

Avian vestibular hair cells also are susceptible to ototoxic aminoglycoside damage and show a similar pattern of regeneration and recovery, as assessed by their anatomic appearance and afferent connections (Weisleder and Rubel 1993). Only two studies have tested whether recovered vestibular hair cells are functional. In the first, evoked potentials recorded at the skull and elicited by linear acceleration recovered after streptomycin treatment (Jones and Nelson 1992). Thresholds for these potentials returned to normal by 2 wk after streptomycin treatment, but the amplitudes and latencies of the responses remained below normal for 8 wk after aminoglycoside treatment.

Recently, we found that the vestibuloocular reflex (VOR) in chickens also recovers after streptomycin damage (Carey et al. 1996). The VOR produces eye movements that compensate for angular acceleration of the head, thereby helping to stabilize visual images on the retina. Because the VOR originates at the hair cells, the viability of the vestibular epithelium can be estimated on the basis of the relative size of compensatory eye movements during head rotation. One may gauge the efficacy of the VOR by calculating its gain, the ratio of eye velocity to head velocity. VOR gain becomes zero when hair cells are at a minimum and returns to normal values as they regenerate (Carey et al. 1996).

Just as the VOR stabilizes the eye during head movements, the vestibulocolic reflex (VCR) stabilizes the head in space. Therefore the VCR aids the VOR in stabilizing images on the retina. The VCR is particularly prominent in birds, as can be appreciated by watching a sparrow perched on a wire. The sparrow's body movement is canceled by the VCR, such that the head remains stable in space. The VCR is a closed-loop system, i.e., the output of the reflex, head movement, is fed back to reduce the reflex input from the vestibular hair cells.

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It has been suggested that in mammals the VOR originates from signals delivered by regularly discharging vestibular afferents, whereas the VCR is thought to originate from irregularly discharging afferents (Goldberg et al. 1987; Highstein et al. 1987; Minor and Goldberg 1991). In chickens, type I hair cells are contacted by regularly firing afferents and type II hair cells are contacted by irregularly firing afferents (Yamashita and Ohmori 1990). Our earlier study showed that the recovering VOR correlates better with the reappearance of type I than type II hair cells (Carey et al. 1996). We wondered whether the VCR also would recover and whether the recovery would correlate better with the regeneration of type I or type II hair cells. We tested this in chickens whose hair cells were destroyed by aminoglycoside antibiotics.

METHODS

Experimental subjects

Subjects were 40 white Leghorn chicks (*Gallus domesticus*), ~10 days posthatch, and 19 age-matched controls. The chicks were housed in brooders that allowed light (16-h days) and free access to food and water. Animal care and experimental procedures conformed to standards of the Institutional Animal Care and Use Committee at the University of Washington.

Lesions of the vestibular hair cells in the 40 experimental chicks were created by a 5-day course of streptomycin sulfate (Sigma) in normal saline (1,200 mg $\cdot$ kg^{-1} $\cdot$ day^{-1} im). Each injection resulted in unconsciousness in all subjects, and 40% of the chicks ($n = 16$) died over the 5-day course of injections. Surviving chicks regained consciousness 3–6 h after each injection. They exhibited a slight tremor, most evident in the head, but showed no other motor abnormality. This tremor was evident in head position traces only on day 1 postlesion. Postlesion time points, e.g.,"1 wk," refer to time after the last day of streptomycin injection, not age. Because treatment began at 10 days posthatch and lasted 5 days, a "1-wk" bird was actually 3 wk old at the time of testing. The VCR was assessed at 1 day ($n = 5$), 1 wk ($n = 5$), 2 wk ($n = 5$), 3 wk ($n = 3$), 4 wk ($n = 3$), and 8 wk ($n = 3$) postlesion. Some subjects were killed at each time point for histological analysis.

Rotational stimulation

Alert chicks were placed on a perch coupled to a rotating turntable, which was set to oscillate sinusoidally at each of the following frequencies: 0.1, 0.3, 0.5, 0.8, 1.0 and 1.4 Hz. The angle of excursion was fixed at ±10°. Larger chicks, which tended to leap from the perch, were secured to it with a leg leash or double-sided tape affixed to the perch. All subjects were tested in a dark chamber. To ensure that they were properly positioned on the perch, a light was turned on periodically, and subjects were observed with a video camera and monitor. Data were collected only during periods of darkness.

Head movement measurement

Head movements were measured by means of an electromagnetic method in which an alternating magnetic field induced an electromotive force in a small coil of wire fixed to the subject's head. A similar method is generally used to measure eye movements with a small coil of wire affixed to the eye (Robinson 1963). The coil, which consisted of four turns of wire, was square and measured 1.5 cm on a side. To attach it to the subject's head, we clipped feathers in a crescent-shaped area, 1–2 cm long, immediately posterior to the external auditory canal on the right side of the head, and secured a patch of Velcro (hook side out) to the skin with cyanoacrylate adhesive. The coil was sewn to a complementary piece of Velcro (fuzzy side) and attached to the subject's head before VCR testing. This allowed us to use the same coil on a number of subjects. The lightweight coil and Velcro did not cause the head to sag or list, even in the smallest chicks.

Two pairs of 14-in. magnetic coils, coupled to the turntable, provided alternating magnetic fields (35 kHz) centered at the subject's head. Signals proportional to horizontal and vertical angular head position were extracted from the head coil signal by phase-sensitive detectors, sent to analog amplifiers, filtered at 500 Hz, and recorded on tape with a Vetter 5000A PCM recorder (sampling rate = 5 kHz/channel). Turntable position was measured simultaneously with a potentiometer. All signals were digitized later with a Macintosh IIfx computer (sampling rate = 1 kHz) and a MIO16 Digitizer (National Instruments). The digitizer sampling rate was dependent on the turntable oscillation frequency so that 600 samples/cycle occurred at all frequencies.

In each test session, before attaching the coil, we calibrated it by positioning it at the location of the chicken's head and rotating it through precise angular displacements in the pitch and yaw planes. Measurements of angular displacement were linearly related to turntable angle within ±0.5° over a range of ±30°. We also calibrated the entire system by fixing the coil in space and rotating the turntable sinusoidally at a frequency of 0.5 Hz about the fixed coil, simulating a perfectly compensatory VCR. We then made minor calibration changes to the phase detector amplifiers so that our analysis program (see VCR DATA ANALYSIS) returned a gain and phase (head velocity relative to perch velocity) of 1.00 ± 0.02 and 180.0 ± 0.5°, respectively. Body movement was monitored via a second coil attached to the subject's body. Any stimulus cycle in which body movement exceeded 10% of head movement was not included in the analysis. For very large subjects, at the later testing periods (4–8 wk postlesion) it is possible that the body coil was outside the homogenous region of the magnetic field, thus underestimating the actual body movement. However, in addition, each subject was observed from time to time during testing. On the basis of these observations and the body coil signal, we conclude that our recorded head movements largely could not be attributed to body motion.

VCR data analysis

An interactive analysis program for Macintosh computers simultaneously displayed single cycles of sinusoidal turntable position and head and body movements (Fig. 1, *top two traces*). The program provided digital derivatives of head and perch position and fit each with a best-fit sine wave via a discrete Fourier transform. Rapid head "saccades," which are characterized by fast oscillations, were easily identified on the head velocity trace and deleted by an observer (Fig. 1, *bottom two traces*). The program then calculated VCR gain as the ratio of the amplitude of the best-fitted horizontal head velocity sinusoid to the amplitude of the fitted perch velocity sinusoid. Thus a perfectly compensatory VCR would have a gain of 1.00. The phase shift of the VCR was calculated as the difference in phase between horizontal head and turntable velocity. A perfectly compensatory VCR would have a head velocity that was 180° out of phase with the turntable. Phase differences between 0 and 180° indicate that the peak head velocity preceded peak perch velocity. Phase differences between 180 and 360° indicate that head velocity lagged perch velocity. In the example of Fig. 1, VCR gain was 0.74 and phase was 184°.

At all frequencies >0.1 Hz, ≥10 and ≤ 30 cycles were analyzed at each frequency for each subject. Average gains and phases were calculated from data in cycles considered acceptable on the basis of two criteria. *1*) After head saccades were removed, ≥60% of the data points in the cycle remained; and *2*) ≥60% of the variance of head velocity was sinusoidal. When VCR gain was ≤0.1, we could not measure head velocity reliably. Thus measured gains ≤0.1 should be considered indistinguishable from 0.0.

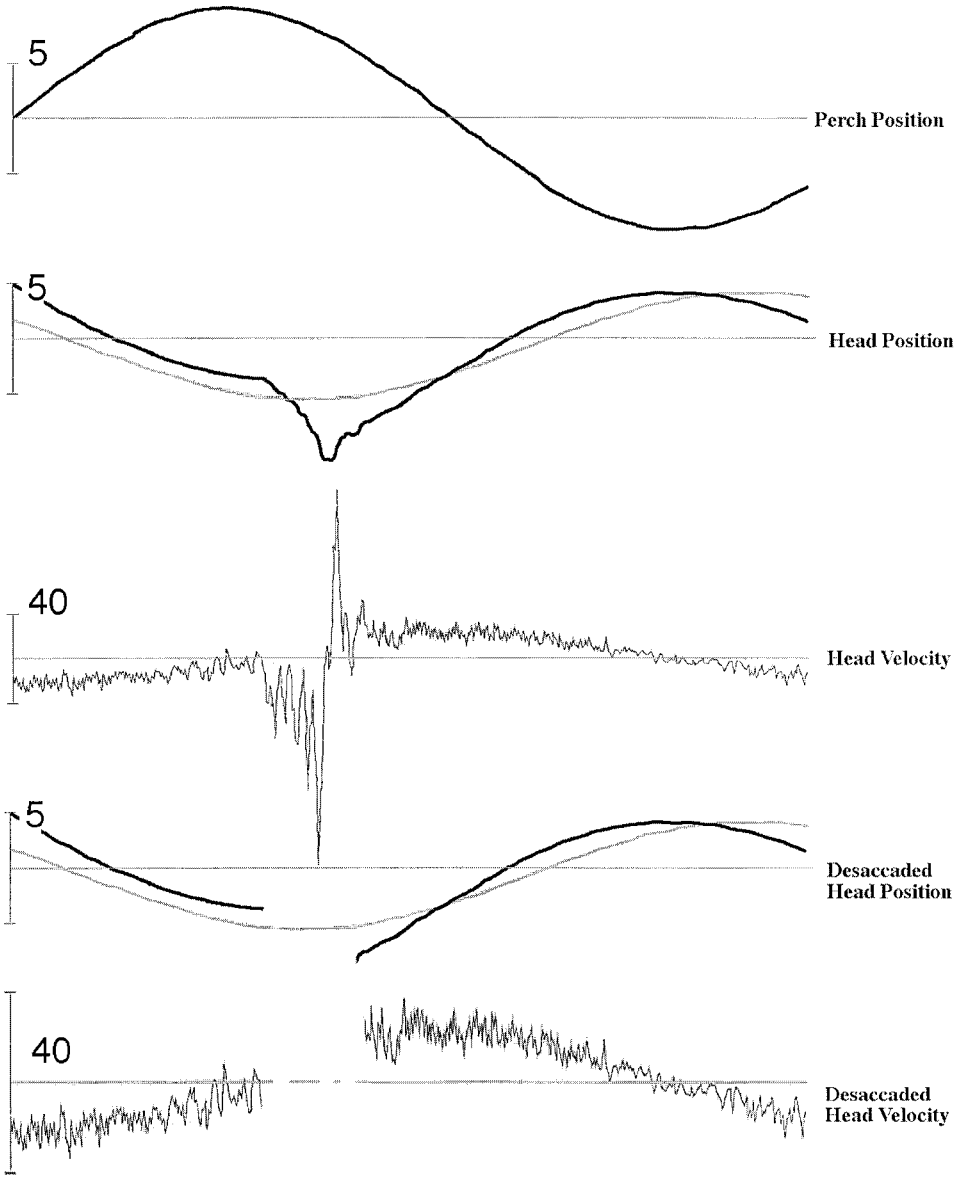

FIG. 1. Representative vestibulocolic reflex (VCR) data of a chick at 0.5 Hz to illustrate the analysis procedure. Calibration bars are in degrees for position traces and degrees/second for velocity traces. "Desaccaded" head position and velocity traces reflect the deletion of a nonperiodic, rapid head movement or "saccade." The shaded trace is the best fit curve imposed by the computer program.

Histological preparation and analysis

Within 12 h after final VCR testing, subjects were killed with an overdose of pentobarbitol (ip). Labyrinthine tissues were fixed by transcardiac perfusion of heparinized saline followed by fixative (4% paraformaldehyde, 2.5% glutaraldehyde, 0.1 M sucrose, and 0.1 M phosphate buffer, pH 7.4). The labyrinths were exposed and perfused directly with the same fixative. The entire head was placed in fixative for 1–4 days at 4°C. The canal cristae were then dissected out, washed several times with PBS (pH 7.4), and postfixed in 1% osmium tetroxide for 30 min. After three washes in PBS, the specimens were dehydrated in graded ethanol solutions.

Vestibular organs from the left side of each chick were embedded in Spurr's resin for light microscopy (LM). The entire length of each canal crista was then cut in 2-μm transverse serial sections with diamond or glass knives. Three of every 25 sections were mounted, stained with Richardson's stain, coverslipped, and examined under a light microscope at $\times$400 total magnification.

Organs from the right side of each chick were prepared for scanning electron microscopy (SEM). The roof of the ampulla and the cupula were removed from the crista before critical point drying and gold–palladium sputter coating. Specimens were viewed with a JEOL 63005 electron microscope at 15-kV accelerating voltage.

In LM sections, hair cells were identified on the basis of the following criteria: *1*) a nucleus with visible nucleolus above the row of supporting cell nuclei at the basement membrane, *2*) a cell body extending to the luminal surface of the sensory epithelium; and *3*) contact with underlying neural elements. Hair cells were typed according to the morphology of their afferent endings. Type I hair cells have an afferent calyx that completely surrounds the cell, which appears clear in our Richardson's stained sections, whereas type II hair cells have afferent endings that do not completely surround the cell. The morphology of the unstained calyces was consistent with previous anatomic analyses of cristae (Lysakowski 1996). Supporting cells were identified on the basis of three criteria: *1*) a nucleus with a visible nucleolus, *2*) a lack of afferent endings, and *3*) a cell body that did not have a broad projection to the sensory epithelium.

The entire length of the crista (transverse to the flow of endolymph) was sectioned in 2-μm slices. Three of every 25 sections were mounted (a sample of 3 2-μm sections every 50 μm). Hair cells were counted in one of three sections in each sample. The width of the basement membrane in each section was measured with the use of

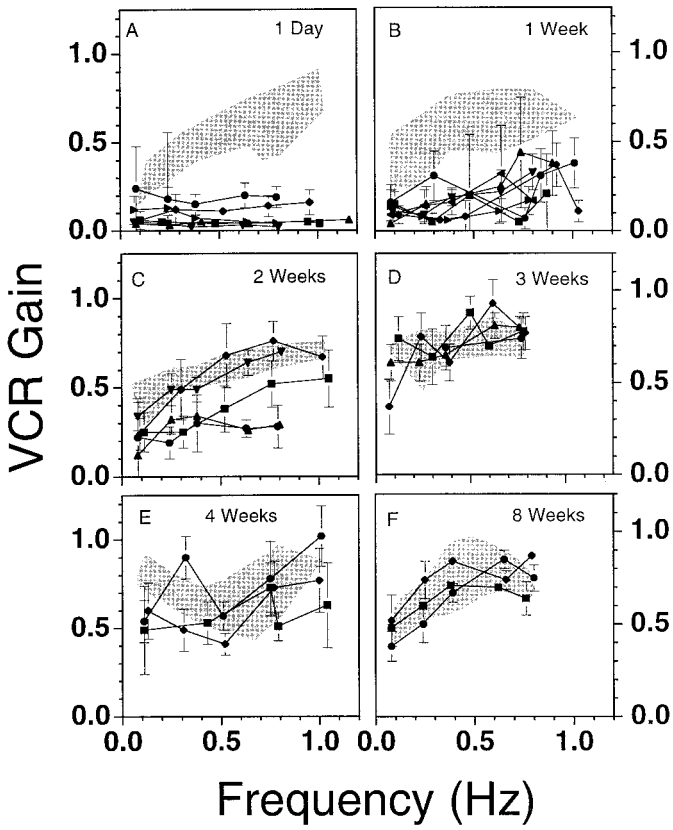

FIG. 2. VCR gain (head velocity/perch velocity) as a function of the frequency of horizontal rotation for 31 treated chicks at 6 recovery times after streptomycin treatment. Shaded areas represent the range of VCR gains for untreated control chicks ($n = 19$). Each symbol represents the mean gain of an individual chick over 10–20 sinusoidal rotations at a given frequency. Brackets are SDs. At the low gain levels shown in *A*, the signal-to-noise ratio was very low, the phase shifts were extremely variable, and the sinusoidal variance of most cycles was below our acceptance criteria. Thus data for some experimental chicks in *A* are means of <10 cycles. The majority of the cycles analyzed from these chicks showed gains that were essentially 0, so that if these were included the population average would be even lower.

NIH Image (version 1.57) on a Macintosh Power PC computer. Hair cell density was calculated as the number of hair cells per 100 μm width of basement membrane (see Weisleder and Rubel 1993). The densities of type I and type II hair cells were averaged from six or seven regularly spaced sections along the complete length of the crista.

RESULTS

Functional recovery

VCR GAIN. VCR gain in control chicks tended to increase with frequency between 0.1 and 1.4 Hz at all tested ages. The range of VCR gains for control chicks of each age is shown by the shaded areas in Fig. 2, *A–F*. These data are comparable with previous measurements of the pigeon VCR (Outerbridge 1969). The VCR gains from control chicks also tended to increase with age through ~3 wk postlesion, especially at lower frequencies. Recall that these time points refer to time after the last streptomycin injection (these "3-wk" age-matched, untreated controls were ~5 wk old). In other words, the chronological age of the subject is offset by ~2 wk from the postlesion time point indicated for every subject (0 wk postlesion = 2 wk old). We adopted this nomenclature to be consistent with our previous experiment (Carey et al. 1996). A repeated measures analysis of variance (ANOVA) for control VCR gain revealed a significant main effect of oscillation frequency ($P < 0.001$) and a significant interaction between oscillation frequency and age ($P < 0.002$).

There was no measurable VCR in treated chicks 1 day postlesion, so the responses could not be fit by the data analysis program. The data for experimental chicks in Fig. 2*A* are means of fewer than the requisite number of cycles for each frequency and are presented only for reference. A repeated-measures ANOVA at 0 wk showed a significant main effect of treatment (streptomycin vs. control), a significant main effect of frequency, and a significant treatment × frequency interaction ($P < 0.001$ for each effect). As seen in Fig. 2*B* (solid lines), some recovery in VCR gain occurred by 1 wk postlesion. Most of the chicks had measurable VCRs, especially at higher frequencies, but responses were highly variable. At 2 wk postlesion, three of five treated chicks showed VCR gains less than control values, but the other two recovered to within the range of normal values (Fig. 2*C*). By 3 wk postlesion, all subjects had VCR gains within the range of normal values (Fig. 2*D*). At all time periods after 3 wk, treated subjects generally had VCR gains within the normal range at all frequencies >0.3 Hz (Fig. 2, *E* and *F*). A repeated-measures ANOVA showed no significant main effect of treatment at 3 wk postlesion ($P > 0.10$) nor were there any significant effects of treatment at any time period after 3 wk. At 4 wk postlesion gains were more variable (Fig. 2*E*), even among control chicks. VCR gains were quite high for one subject at 0.3 Hz and low for another subject at 0.8 and 1.0 Hz (Fig. 2*E*). We have no explanation for this variability. There was no significant main effect of treatment at 8 wk ($P > 0.10$).

VCR PHASE SHIFTS. Figure 3 shows the phase shifts of the recovering VCR over a range of frequencies at several time points after streptomycin treatment. A perfectly compensatory VCR has a phase of 180° relative to perch velocity. All control chicks exhibited a fairly constant phase ~180° at frequencies >0.3 Hz. There was a tendency for phase to lead at 0.1 Hz in controls at all ages (Fig. 3, shaded areas). Experimental chicks exhibited an extremely variable phase both within and between individuals at 1 wk postlesion (Fig. 3*B*); error bars represent SDs for each individual tested at a particular frequency. By 2 wk, when gain had shown considerable recovery, phase became less variable and approached control values (Fig. 3*C*). By 3 wk, all treated chicks showed phase shifts within the normal range (Fig. 3*D*). A repeated-measures ANOVA of phase data showed no significant main effects of treatment (streptomycin vs. control) on VCR phase. This is probably due to the insensitivity of the ANOVA to the huge variability within subjects at each frequency tested (values for subjects in the analysis were means of >10 trials for each frequency). Although we acknowledge no statistical effect, the huge variability within subjects in terms of VCR phase is an important observation.

SUMMARY. Figure 4 shows the time course of average VCR gain and phase shifts at a frequency of 0.5 Hz for control and streptomycin-treated chicks. Gain was quite depressed immediately after streptomycin treatment and was not significantly different from normal by 3 wk ($P > 0.10$, Student's *t*-test). Average phase shifts tended to lead normal values and were extremely variable for ≥1 wk postlesion. Both the magnitude

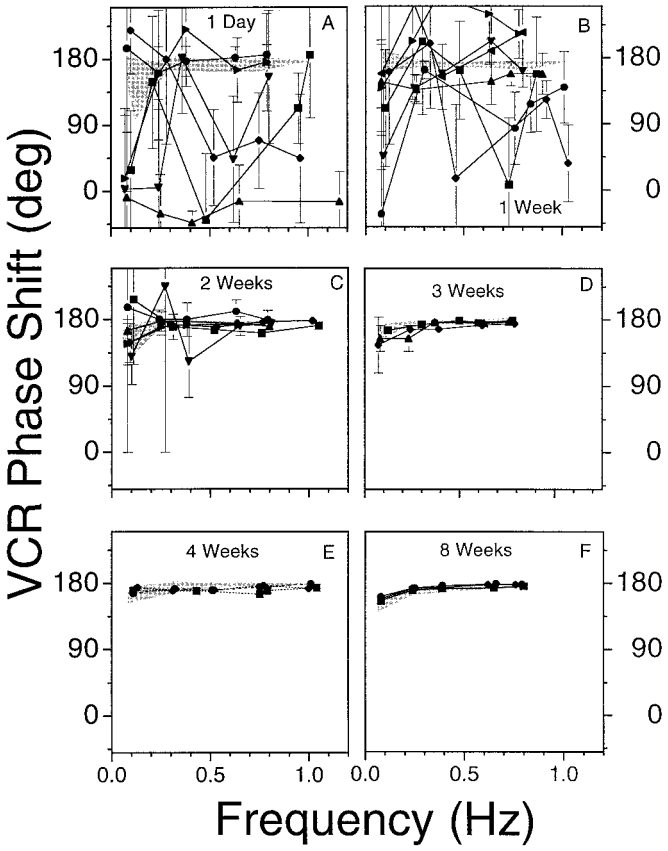

FIG. 3. VCR phase shift (head velocity sinusoid relative to perch velocity sinusoid) as a function of the frequency of horizontal rotation for 31 treated chicks at 6 recovery times. Shaded areas indicate the range of control values ($n = 19$). Each symbol indicates mean phase of an individual treated chick over 10–20 sinusoidal rotations at a given frequency. Brackets indicate SDs.

of average phase lead and its SD returned nearly to normal by 2 wk postlesion.

Anatomic recovery

SEM ANALYSIS. Figure 5 shows SEMs of the horizontal semicircular canal ampulla with the cupula removed. Control chicks at all ages showed dense, mature stereocilia across the entire length of each crista ampullaris (Fig. 5*A*). In contrast, 1 day after streptomycin treatment all treated chicks showed a consistent pattern of damage with mature stereocilia surviving only along the edge of the sensory epithelium (Fig. 5*B*). A high-magnification SEM (Fig. 5*C*) shows small white tufts at the top of the crista, which appear to be either dying cells that are being extruded from the cuticular plate (arrow) or immature hair cells characterized by their kinocilium and short tufts of stereocilia (arrowhead) (Weisleder and Rubel 1993). In the subject from which this tissue was taken, VCR gain was measured as <0.04 at 0.5 Hz and therefore was taken to be zero.

At 3 wk postlesion, the density of stereocilia bundles increased considerably, and mature stereocilia could be seen over the entire crista (Fig. 5*D*). However, the stereocilia appeared less dense at this time than those of the control epithelium (Fig. 5*A*). Reflex gain for this subject was 0.88 at 0.5 Hz versus the mean control gain of 0.68 at this time point (Fig. 2). At 8 wk postlesion, cristae from treated chicks were indistinguishable from those of control chicks (Fig. 5*E*). The stereocilia were long and dense over the entire length of the crista. VCR gain for the chick whose tissue is shown in Fig. 5*E* was 0.67 at 0.5 Hz (mean control gain at this time was 0.82).

LM ANALYSIS. Figure 6 shows an example of the population of hair cells in transverse sections through the horizontal crista ampullaris (perpendicular to its long axis) from a control chick and from a streptomycin-treated chick 1 day postlesion. In the untreated control chick (Fig. 6*A*), hair cell bodies were visible all along the middle third of the sensory epithelium. At this distance along the crista, type I hair cells were most numerous at the top, whereas type II hair cells predominated more laterally. Type I hair cells, characterized by their large calyx afferents (which, with Richardson's stain, appear as white spaces surrounding the hair cell), were visible near the top of the crista (arrowhead). Several type I hair cells may be contacted by a single afferent calyx as seen at the top of this image. A higher magnification of this region in the untreated control (Fig. 6*B*) shows a typical type II hair cell with a cylindrical body projecting to the top of the crista (arrow) next to a type I hair cell (arrowhead). The stereocilia bundles attached to these hair cells are visible. In a higher magnification of the lateral edge of the crista of Fig. 6*A*, there are several type I hair cells visible at the right edge of the image (Fig. 6*C*, underlined). The edge of the crista, which is to the left in this image, contains the nuclei of several type II hair cells (arrowheads).

In the streptomycin-treated chick, there were no visible stereocilia bundles in this particular section 1 day postlesion (although the SEM photos show a band of stereocilia remain-

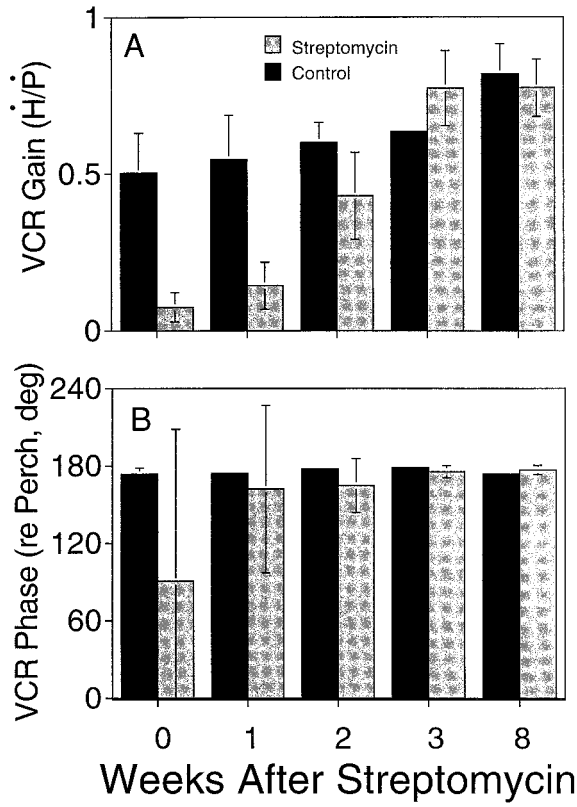

FIG. 4. Mean VCR gains (*A*) and phase shifts (*B*) at 0.5 Hz for streptomycin-treated chicks (shaded bars) and controls (solid bars) as a function of recovery time postlesion. Each bar on the histogram is the mean of data from ≥4 chicks. Brackets indicate SDs.

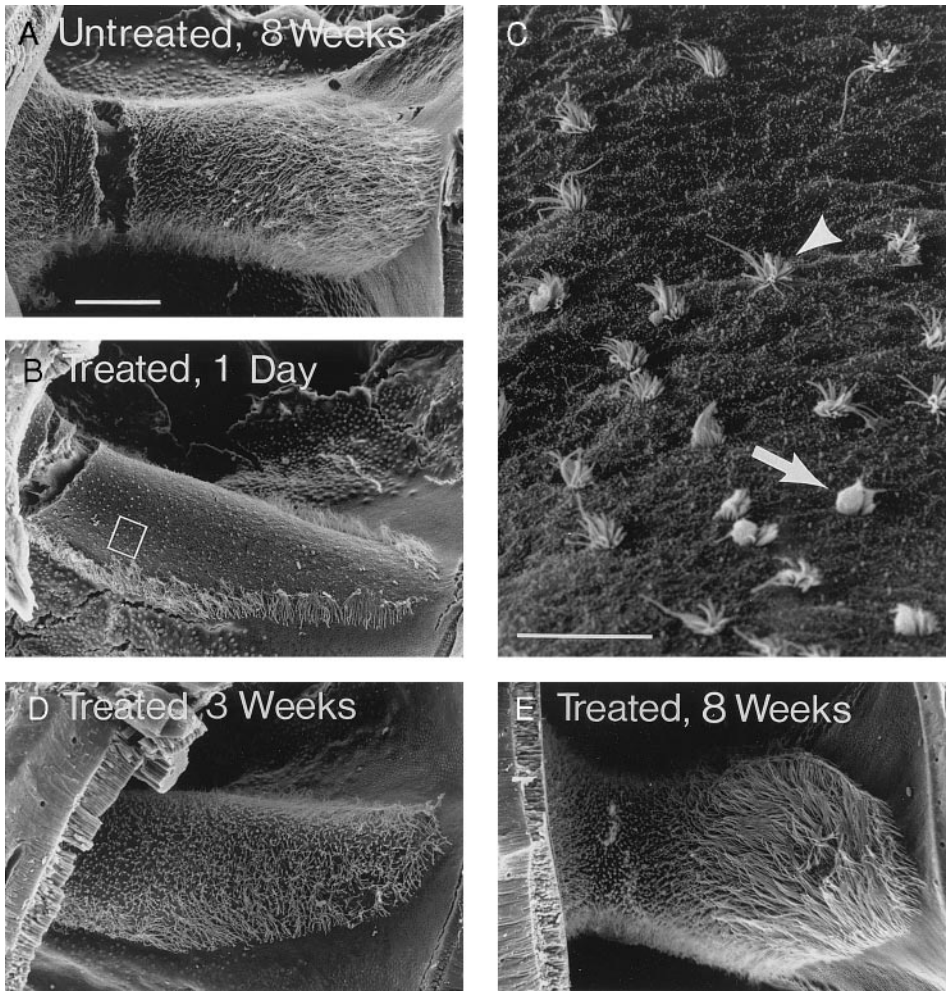

FIG. 5. Scanning electron micrographs of the crista ampullaris of the horizontal semicircular canals of untreated and treated chicks. *A*: 8-wk untreated chick. *B*: 1-day treated chick. A narrow band of surviving hair cells is seen along the edge of the sensory epithelium. *C*: high-magnification view of the boxed area in *B*. Note the long kinocilium and short tufts of stereocilia typical of immature vestibular hair cells (white arrowhead). Cells that are blebbing out of the cuticular plate (white arrow) are thought to be dead hair cells being extruded from the sensory epithelium. *D*: 3-wk treated chick. *E*: 8-wk treated chick. Calibration bar in *A* is 100 μm; in *C*, 10 μm. For scale in *B*, *D*, and *E*, refer to calibration bar in *A*.

ing along the edge of the crista) and few identifiable hair cell nuclei (Fig. 6*D*). Two type II hair cell nuclei were visible near the lateral edge of the crista (arrowheads). Under higher magnification, the top of the same crista was completely lacking in type I hair cells (Fig. 6*E*). Two type II hair cell nuclei are indicated by black arrowheads. No afferent calyces are visible anywhere along the top of the crista.

Figure 7 shows representative LM sections of cristae from streptomycin-treated subjects at 3 wk (*A–C*) and 8 wk (*D–F*) postlesion. At 3 wk some type I hair cells were visible near the top of the crista (Fig. 7*A*, arrowheads), and all afferent calyces appeared to innervate only single type I hair cells (Fig. 7*B*, arrowheads). Figure 7*C* shows type II hair cells (above line) near the edge of the crista and two type I hair cells at the left of the image. At 8 wk postlesion, the crista resembled those of untreated chicks (compare Fig. 7, *D–F*, with Fig. 6, *A–C*). Bundles of stereocilia were visible all over the crista (Fig. 7*D*). Along the top edge of the crista (Fig. 7*E*) it was again common to see several type I hair cells enveloped by a single afferent calyx (arrows). Type II hair cells were numerous toward the edge of the crista (Fig. 7*F*).

Quantitative analysis

All subjects tested behaviorally were subject to LM measurement of hair cell density. Analyses of hair cell density were conducted on 2-μm sections spaced at 50-μm intervals through the entire length of each horizontal semicircular canal crista, yielding six to eight sections/crista. Our objective was not to estimate the absolute number of hair cells but rather to determine relative hair cell densities for control and experimental chicks in each group. Thus stereological analyses were not undertaken. Furthermore, thick sections, which are advantageous for stereological analyses or for applying various corrections for "double counting," obscure the morphology and therefore the identification of hair cell phenotypes.

Figure 8 shows the mean densities of type I, type II, and total hair cells at various survival times from at least four chicks at each time period. Total hair cell density 1 day postlesion (0 wk) was ~35% of mean control hair cell density (Fig. 8*A*). This percentage is comparable with that reported in our previous publication on streptomycin damage (Carey et al. 1996). At this time, most of the remaining hair cells in streptomycin-treated chicks were type II (Fig. 8*C*); type I hair cell density was near zero (Fig. 8*B*). By 1 wk postlesion, type II hair cell density averaged 70% of normal, and type I hair cell density increased to ~35% of normal.

Total hair cell density was significantly higher in control chicks than in streptomycin-treated chicks at 0, 1 and 2 wk postlesion ($P < 0.002$, $P < 0.05$, and $P < 0.001$, respectively, factorial ANOVA). At 3 wk postlesion, there was no significant difference between control and streptomycin-treated sub-

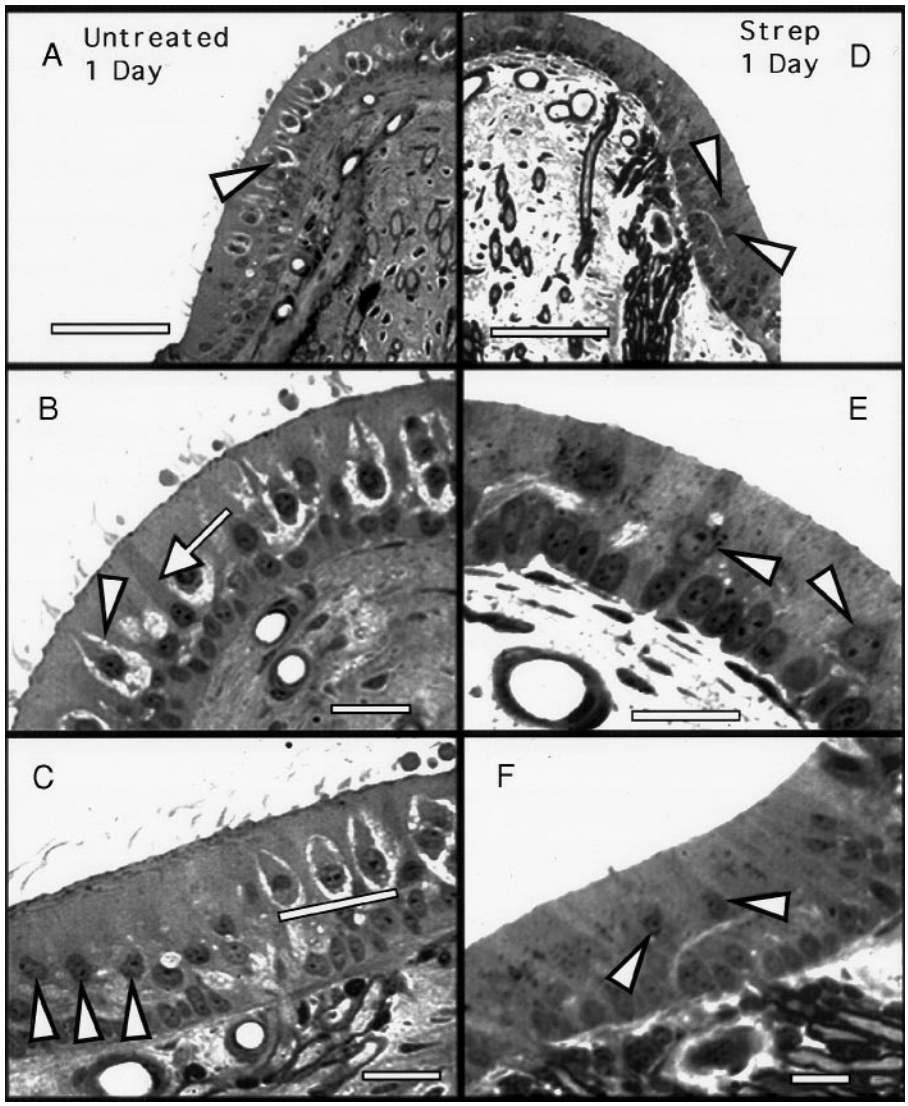

FIG. 6. Light microscopy (LM) sections through the horizontal crista ampullaris cut perpendicular to the long axis of the crista. *A*: canal side of crista from an untreated control age-matched to 1 day postlesion. *B*: top of crista shown in *A* under higher magnification. *C*: lateral edge of crista shown in *A* under higher magnification. *D*: canal side of crista from a streptomycin-treated subject 1 day postlesion. *E*: top of crista shown in *D* under higher magnification. *F*: lateral edge of crista shown in *D* under higher magnification. Calibration bars in *A* and *D*, 100 μm; in *B*, *C*, *E* and *F*, 25 μm.

jects in terms of total, type I, or type II hair cell density ($P > 0.10$ for each test, factorial ANOVA). At 4 wk postlesion, type II hair cell density was not significantly different from normal ($P > 0.10$, factorial ANOVA), and type I density averaged ~65% of normal. At 8 wk, type I and total hair cell density in treated chicks was not significantly different from control hair cell density ($P > 0.10$). Carey et al. (1996) showed a similar pattern of recovery, i.e., streptomycin-treated chickens' type I hair cell density was not significantly different from controls at later time points, even though the mean hair cell densities (type I, type II, and total) were always lower in treated subjects compared with controls.

For several frequencies of rotation, we considered the relation between VCR gain recovery and hair cell regeneration. By using data from treated chickens, we plotted VCR gain at several different frequencies against type I and type II hair cell density and against total hair cell density (Fig. 9). At low frequencies, VCR gain correlated at least as well with total hair cell density as with hair cell densities of either type alone. At higher frequencies VCR gain correlated better with type I and total hair cell density than with type II hair cell density (Fig. 9*A*, ■). Figure 9, *B* and *C*, illustrates the linear regressions of hair cell density with gain at 0.5 Hz.

DISCUSSION

As in our previous study on the VOR in streptomycin-treated chicks (Carey et al. 1996), we have shown that regeneration of hair cells in the vestibular epithelium is correlated with functional recovery. We see the return of a reflex that depends on the integrity of hair cells and their central connections. Streptomycin treatment resulted in the loss of 65% of vestibular hair cells. This loss eliminated the VCR completely, as it did the VOR. When the vestibular epithelium returned to near normal numbers of hair cells, VCR gain also returned to normal.

However, we found two major differences in the recovery of the VOR and VCR. First, by the third week of recovery, VOR gain was only one-half normal on average, whereas VCR gain consistently was near control values (Figs. 2 and 4). Thus it appears that the VCR recovers before the VOR. Second, the gain of the VOR over the course of recovery is best correlated with the density of type I hair cells, whereas recovering VCR gain correlates slightly better with total hair cell density over

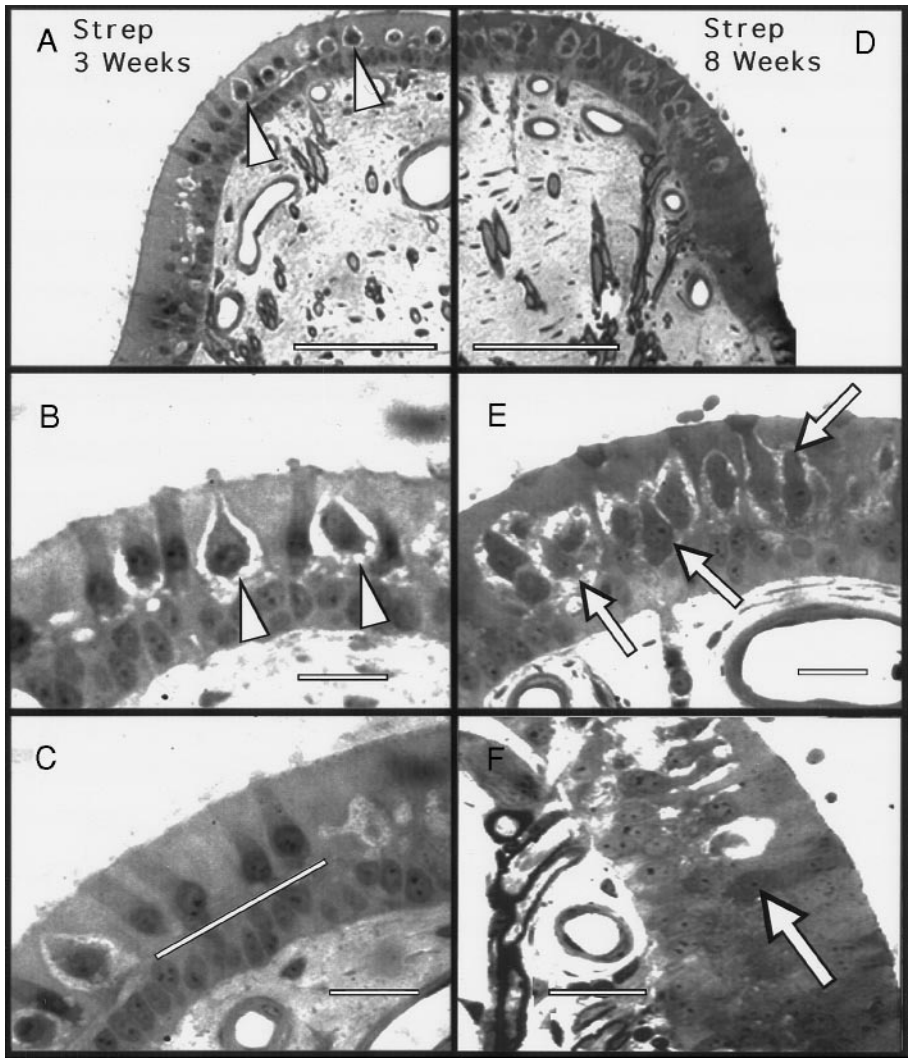

FIG. 7. LM sections through the horizontal crista ampullaris cut perpendicular to the long axis of the crista. *A*: canal side of crista 3 wk postlesion. *B*: top of crista shown in *A* under higher magnification. *C*: lateral edge of crista shown in *A* under higher magnification. *D*: canal side of crista 8 wk postlesion. *E*: top of crista shown in *D* under higher magnification. *F*: lateral edge of crista shown in *D* under higher magnification. Calibration bars in *A* and *D*, 100 μm; in *B*, *C*, *E* and *F*, 25 μm.

all frequencies (Fig. 9) and correlates poorly with type I hair cell density at higher frequencies.

Because little is known about the VCR in birds, we will first review our behavioral data from control subjects, and then we will comment on the relation between hair cells of different type and the VCR and VOR. Finally, we will examine the differences between the VCR and VOR in terms of the time courses of recovery and discuss the limitations of our methodology.

VCR in normal chickens

The gain of the VCR in untreated chickens depends on the frequency of stimulation and on age (Fig. 2, shaded areas). At the earliest ages tested, VCR gains ranged from ~0.3 to 0.8, increasing with the frequency of oscillation (Fig. 2*A*, shaded area) and with age (Fig. 4, filled bars). Statistical analysis indicates that VCR gain at the lower frequencies increases significantly more with age than VCR gain at higher frequencies (Fig. 2, shaded areas). It is important to note, however, that our test apparatus did not allow us to deliver a constant peak velocity at different frequencies. It may be therefore that the increase in gain with age is apparent only at lower peak velocities and may be unrelated to the frequency of oscillation.

VCR gains generally are higher than VOR gains. Mean VOR gain from untreated chicks that were age matched to 1 day postlesion (<10 days old) ranged from 0.2 to 0.3 and also increased with frequency. Mean VOR gain also increased with age to ~0.5 at most frequencies tested at 10 wk of age (Carey et al. 1996). Although it may seem counterintuitive for VCR gain to be greater than VOR gain, especially at higher rotation frequencies, bear in mind that the eyes of birds are quite massive with respect to the head and together outweigh the brain in many species (Pearson 1972). Furthermore, the disk shape of the galliform eye limits eye movement such that larger gaze changes must be accomplished mainly by head movement (Pearson 1972). VCR gains comparable with those reported here were also demonstrated in pigeons (Outerbridge 1969), where they also increase with oscillation frequency. Gioanni (1988) found higher pigeon VCR gains that also increased with oscillation frequency, ranging from 0.7 at 0.1 Hz to 0.9 at 1 Hz. However, these birds were treated with amphetamines to maintain alertness (Gioanni 1988).

VCR gain and hair cell type

There is some evidence in mammals that the VCR and VOR are subserved by separate pathways and that these pathways

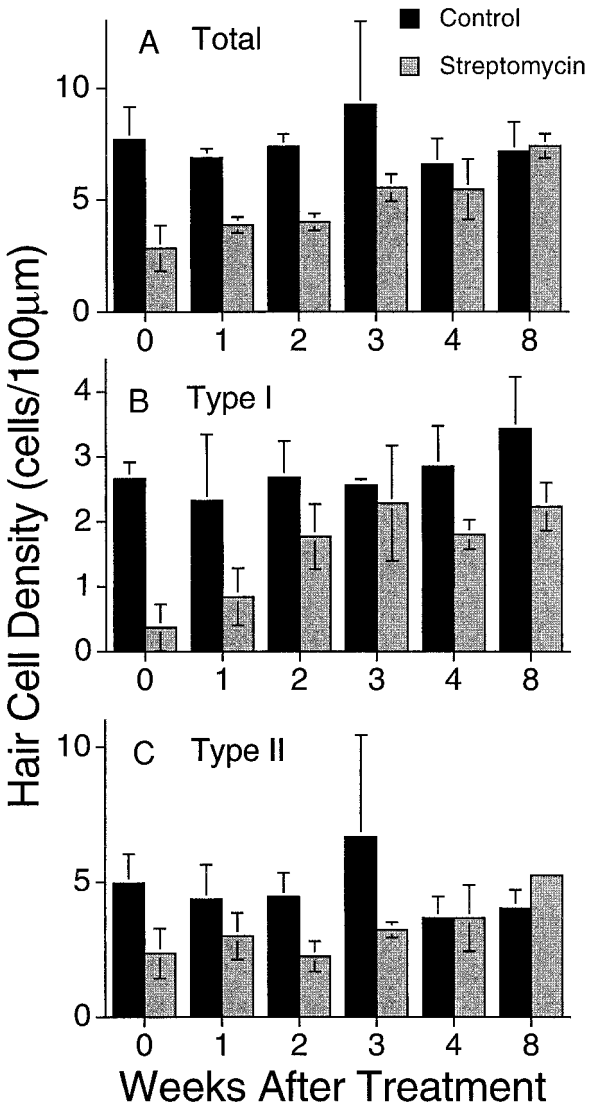

FIG. 8. Mean hair cell densities vs. weeks after streptomycin treatment. Data at 0 wk are from 1 day postlesion. Each bar represents the mean (±1 SD) of ≥4 chicks. *A*: total hair cell density. *B*: type I hair cell density. *C*: type II hair cell density.

may originate at different types of vestibular afferents. The squirrel monkey VOR is thought to originate in vestibular nerve fibers with regular discharge patterns (Goldberg et al. 1987), which generally receive inputs from type II hair cells in mammals. In contrast, the VCR in the cat is thought to originate in afferents that discharge at irregular rates and are driven mostly by type I hair cells (Bilotto et al. 1982).

It was suggested that in chickens this pattern of hair cell innervation is backward from mammals; type I hair cells contact regularly firing afferents, and type II hair cells contact irregularly firing afferents (Yamashita and Ohmori 1990). We previously showed that recovering VOR gain correlates well ($r^2 = 0.79$) with the regeneration of type I hair cells (Carey et al. 1996). If the chicken VCR is primarily driven by irregularly firing afferents as it is thought to be in mammals, we might expect to see a strong correlation between VCR gain and type II hair cell density. However, at higher frequencies (0.8–1.0 Hz) gain correlates poorly with type II hair cell density ($r^2 = 0.25$–0.35, Fig. 9). At lower frequencies, gain correlates equally well with type I, type II, and total hair cell density. For example, at 0.5 Hz $r^2 = 0.51$, 0.49, and 0.59 for gain × type I, gain × type II, and gain × total hair cell density, respectively (Fig. 9, *B* and *C*). This does not support the hypothesis that the VCR is driven solely by one type of hair cell or the other.

Mechanisms for VCR recovery

Two hypotheses could account for both the weaker correlation between VCR gain and hair cell density and the differential time courses of recovery between the VCR and VOR. The first and simplest hypothesis is that the VCR does not need as many hair cells as the VOR to operate normally. A second hypothesis involves the process of central compensation.

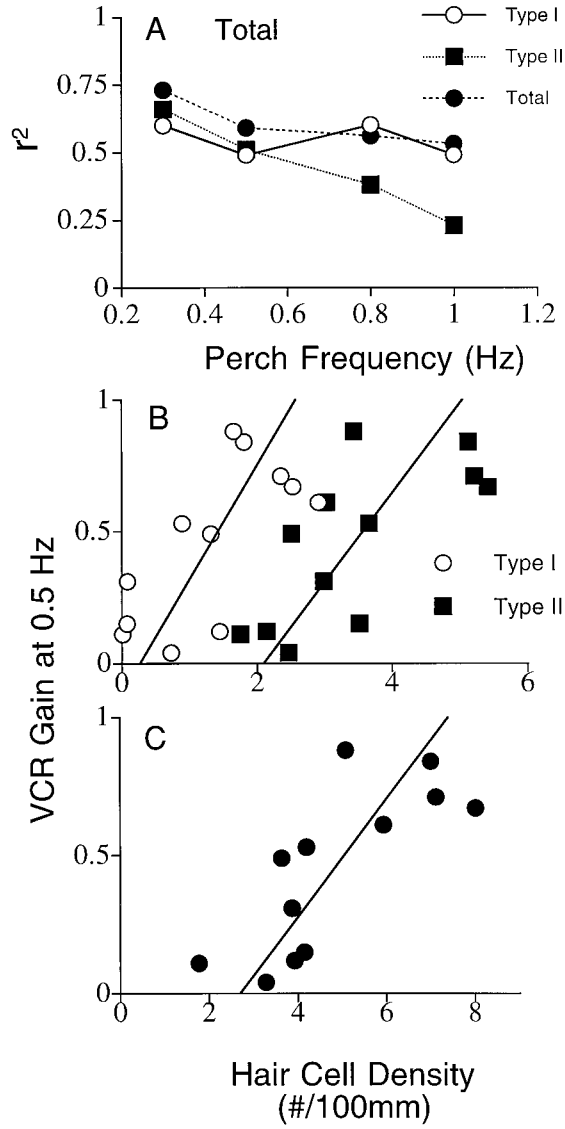

FIG. 9. *A*: square of correlation coefficients (r^2) of treated subjects' hair cell density vs. VCR gain as a function of perch oscillation frequency (Hz). ○: type I relation; ■: type II relation; ●: total hair cell correlation ($n = 12$). *B:* VCR gain at 0.5 Hz vs. type I and type II hair cell density. Linear fit to type I data: $x = 2.309y + 0.267$ ($r^2 = 0.51$) and to type II data: $x = 2.987y + 2.113$ ($r^2 = 0.49$). *C:* VCR gain vs. total hair cell density. Data fit by linear regression: $x = 4.706y + 2.695$ ($r^2 = 0.59$).

Because the VCR is functioning at a time when hair cell regeneration is incomplete (3 wk postlesion) it is not unreasonable to assume that this reflex can function normally with input from a limited number of hair cells. The VCR differs from the VOR in that it is a negative-feedback system. The goal of the VCR is to reduce its input signal, head rotation, to zero. Therefore, as soon as any head rotation signal exists, the VCR control system would act to stabilize the head in space, i.e., produce a substantial VCR gain. The VOR, on the other hand, is an open-loop system; it does not "know" whether the eye movement it creates is appropriate until the visual scene is stable. Thus, although VCR gain might return to normal values rapidly in the presence of any useful head movement signal (as our current data show), the VOR might depend on the regeneration of a larger population of hair cells to provide a more complete head movement input signal.

It is also possible that the lack of a strong correlation between the density of hair cells and VCR gain and the differential recovery rates of the VCR and VOR can be attributed to differential plasticity in central pathways. Reversing goggles, for example, reduce the gain and can even reverse the direction of the human and feline VOR (Gonshor and Melvill Jones 1976). This compensation is driven by a retinal slip signal. If gaze (eye position in space) is not maintained during head and/or body movements, the visual world will "slip" across the retina in a direction opposite that of the movement. Slip must have been apparent during our experiment, where hair cell loss reduced VCR gain to zero at a time when VOR gain was also near zero. Therefore a slip signal may also cause central compensation in the VCR. If so, part of the recovery of the VCR after hair cell loss could be due to changes in the CNS. If central compensation affects the VCR pathway and is more rapid than in the VOR pathway, it could account for differential recovery times. Furthermore, if central processes also are relatively more important to VCR recovery than to VOR recovery, we might see a weaker relation between the density of viable vestibular hair cells and VCR gain, as we did. Central compensation could certainly explain the increase in VCR gain between 2 and 3 wk, when hair cell density did not change appreciably.

We must also consider the implications of the fundamentally different control systems for the VOR and the VCR. The VOR is an open-loop system, whereas the VCR is closed loop. The closed-loop nature of the VCR may allow it to recover more rapidly than the VOR. Restoration of the VCR reduces both retinal slip and the acceleration acting on the semicircular canals. Thus, as the VCR recovers, retinal slip, the error signal presumably driving VOR compensation, is also diminished, a situation that might cause VOR recovery to be relatively delayed.

Finally, it is possible that over the course of recovery the streptomycin-treated subjects learn to use proprioceptive cues from the neck to direct head movements. We tested this possibility by measuring the VCR of two chickens that had undergone bilateral labyrinthectomy. We were not able to detect VCR gains above 0.3 in either chicken $\leq$8 wk after surgery. Thus the compensatory head movements that develop in our streptomycin-treated subjects must be driven primarily by the regenerating vestibular system.

Technical considerations

Using morphological criteria to classify vestibular hair cells may lead to an inaccurate estimate of the ratio of type I to type II regenerated hair cells. We distinguished a type I from a type II hair cell only by the morphology of its afferent connection. It is thought that hair cells that eventually become type I have a type II hair cell morphology during regeneration (Weisleder and Rubel 1993). At this time there is no accepted method of identifying which cells would go on to become type I hair cells. Therefore we may have incorrectly identified type I hair cells as type II because they had not yet developed an afferent calyx.

In trying to link the recovery of the VCR with the regeneration of hair cells we assumed that the hair cells we counted were functional. Although streptomycin treatment resulted in the loss of ~65% of hair cells in our LM counts (Fig. 8, *top*), our SEM data suggest that only a narrow band of stereocilia survived 1 day after treatment (Fig. 5*B*). At this time some hair cell regeneration already was evident (Fig. 5*C*, arrowhead). However, the stereocilia were immature and may not have been functional. The band of surviving hair cells eventually disappeared, leaving the ampulla stripped of long stereocilia. Thus it is likely that >65% of hair cells were lost after streptomycin treatment. Our estimate is highly conservative because we cannot distinguish early regenerated hair cells from surviving hair cells. It is possible that VCR (as well as VOR) recovery was at least partially due to the repair of hair cells that survived the streptomycin treatment (also mentioned in Baird et al. 1993) and remained viable in the subluminal environment despite loss of their stereocilia (Sobkowicz et al. 1992). Thus the repair of surviving hair cells that lost their stereocilia but not their afferent connections may contribute along with the development of new hair cells to the recovery of the VCR.

In conclusion, the recovery of the VCR in streptomycin-damaged chicks has broad parallels with VOR recovery under similar circumstances. However, the VCR recovers before the VOR, suggesting that the force driving recovery of the vestibular reflexes operates differently on the VOR and VCR. Our data from both VCR and VOR experiments suggest that, although the chicken VOR appears to be driven primarily by type I hair cells, the VCR is not produced by type II hair cells alone, especially at higher frequencies. It seems that factors in addition to hair cell regeneration drive the recovery of the VCR because this reflex has normal characteristics even before all hair cells have been reconstituted.

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