Effects of Intratympanic Gentamicin on Vestibular Afferents and Hair Cells in the Chinchilla

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Hirvonen, Timo P., Lloyd B. Minor, Timothy E. Hullar, and John P. Carey. Effects of intratympanic gentamicin on vestibular afferents and hair cells in the chinchilla. J Neurophysiol 93: 643–655, 2005. First published September 29, 2004; doi:10.1152/jn.00160.2004. Gentamicin is toxic to vestibular hair cells, but its effects on vestibular afferents have not been defined. We treated anesthetized chinchillas with one injection of gentamicin (26.7 mg/ml) into the middle ear and made extracellular recordings from afferents after 5–25 (early) or 90–115 days (late). The relative proportions of regular, intermediate, and irregular afferents did not change after treatment. The spontaneous firing rate of regular afferents was lower (P < 0.001) on the treated side (early: 44.3 ± 16.3; late: 33.9 ± 13.2 spikes · s−1) than on the untreated side (54.9 ± 16.8 spikes · s−1). Spontaneous rates of irregular and intermediate afferents did not change. The majority of treated afferents did not measurably respond to tilt or rotation (82% in the early group, 76% in the late group). Those that did respond had abnormally low sensitivities (P < 0.001). Treated canal units that responded to rotation had mean sensitivities only 5–7% of the values for untreated canal afferents. Treated otolith afferents had mean sensitivities 23–28% of the values for untreated otolith units. Sensitivity to externally applied galvanic currents was unaffected for all afferents. Intratympanic gentamicin treatment reduced the histological density of all hair cells by 57% (P = 0.04). The density of hair cells with calyx endings was reduced by 99% (P = 0.03); although some remaining hair cells had other features suggestive of type I morphology. Type II hair cell density was not significantly reduced. These findings suggest that a single intratympanic gentamicin injection causes partial damage and loss of vestibular hair cells, particularly type I hair cells or their calyceal afferent endings, does not damage the afferent spike initiation zones, and preserves enough hair cell synaptic activity to drive the spontaneous activity of vestibular afferents.

INTRODUCTION

The aminoglycoside antibiotics are toxic to the sensory hair cells of the inner ear. Loss of hearing or balance function from this ototoxicity frequently limits the use of these agents for treating severe infections. Some aminoglycosides, most notably gentamicin and streptomycin, have selective toxicity for vestibular hair cells, and the loss of bilateral vestibular function from their systemic use can be debilitating (J. C. 1952). Yet this same property has been exploited to treat Ménière’s disease, an idiopathic syndrome marked by attacks of vertigo, hearing loss, tinnitus, and a sense of ear fullness. After intratympanic injection, gentamicin diffuses through the round window membrane and diminishes peripheral vestibular function (Hibi et al. 2001; Hoffer et al. 2001; Smith and Myers 1979). This nonsurgical means of reducing unilateral vestibular function has been studied in small clinical trials and has been found to control vertigo in ~90% of cases of Ménière’s disease (for review, see Blakley 2000). Even a single intratympanic application of gentamicin may suffice to control vertigo attacks (Harner et al. 2001). We have found that a single application of intratympanic gentamicin results in a marked reduction in the function of the labyrinth as measured by the gain of the human angular vestibulo-ocular reflex in response to rapid rotary head thrusts (Carey et al. 2002a,b). The reduction in canal function is not, however, as great after intratympanic gentamicin therapy as that seen after surgical labyrinthectomy, suggesting that the nature of the gentamicin lesion is different from the surgical one.

The precise cellular targets through which aminoglycosides exert their ototoxic effects are not known, but damage may involve binding to plasma membrane phospholipids, inactivation of the enzyme ornithine decarboxylase, or binding to iron and formation of oxygen free radicals (Schacht 1993; Song et al. 1998). These drugs can induce apoptosis and vestibular hair cell loss (Nakagawa and Yamane 1999; Nakagawa et al. 1997). When mammalian cochlear hair cells are lost because of aminoglycoside ototoxicity, the cochlear nerve afferents degenerate rapidly. However, the loss of mammalian vestibular hair cells due to aminoglycoside ototoxicity may not be accompanied by a measurable loss of vestibular afferents, even months after the injury (Dupont et al. 1993).

It is not known what effect aminoglycoside-induced vestibular damage has on the function of mammalian vestibular nerve afferents. Evidence from the chicken shows that repeated systemic injections of the aminoglycoside streptomycin silenced many vestibular afferents and eliminated the responses to vestibular stimulation during the initial period after injection. Recovery of spontaneous activity and some responses to vestibular stimulation were attributed to hair cell regeneration, which is robust in birds but not mammals (Boyle et al. 2002).

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These neurons have characteristically high baseline firing rates that can be modulated up or down by head motion, lending the system its bi-directional sensitivity (Adrian 1943; Goldberg and Fernandez 1971; Lowenstein and Sand 1940). Smith and Goldberg (1986) hypothesized that such resting discharge in vestibular afferents is dependent on transmitter release from hair cells. Consistent with this hypothesis, surgical destruction of the labyrinth (labyrinthectomy) silences most ipsilateral vestibular afferents (Jensen 1983; Sirkin et al. 1984). Labyrinthectomy also results in marked asymmetries in neuronal activity in the central vestibular nuclei, which are ultimately balanced by a number of compensatory changes (for review, see Curthoys and Halmagyi 1995). If aminoglycoside damage did not silence these afferents, central compensatory changes might not be required to the same degree as after labyrinthectomy.

This study was undertaken to determine the physiologic fate of vestibular afferents in chinchillas following a single injection of gentamicin into the middle ear. The treatment was the same as that used for patients with unilateral Ménière’s disease. We performed extracellular recordings from afferents on the treated and untreated sides to determine the effect of intratympanic gentamicin on spontaneous discharge rate, responses to rotational and tilt stimuli, and galvanic sensitivity. Hair cell and support cell densities were calculated using the histological dissector technique. We found that the spontaneous discharge rates of the vestibular nerve afferents decreased only slightly on the gentamicin-treated side. However, the distinct classes of afferents identified on the basis of spontaneous discharge (regular, intermediate, and irregular) were found in normal proportions on the treated side in both the early and late groups. The tilt and rotational sensitivities of the vestibular afferents were greatly reduced on the treated side in both groups, whereas the galvanic sensitivity was not significantly affected. Gentamicin treatment was associated with partial loss of hair cells, especially those that would be significantly affected. Gentamicin treatment was associated with loss of hair cells. Consistent with this hypothesis, surgical destruction of the labyrinth (labyrinthectomy) silences most ipsilateral vestibular afferents recorded in the same laboratory (Hullar and Fernandez 1971). Afferents were classified as regular (CV* > 0.2), or irregular (CV* < 0.2) (Goldberg and Fernandez 1971). Units were tested for tilt and rotational sensitivity.

**Methods**

**Surgical procedures and recording techniques**

Adult chinchillas (500–750 g) were anesthetized with intramuscular ketamine 40 mg/kg (Abbott Laboratories, North Chicago, IL) and xylazine 0.5 mg/kg (Phoenix Scientific, St. Joseph, MO). Gentamicin in buffered solution (26.7 mg/ml, American Pharmaceutical Partners, Los Angeles, CA) was injected through the tympanic membrane or via a small opening made in the bulla in 18 animals. This concentration was chosen as it is widely used clinically to treat Ménière’s disease. Sufficient volume (0.2–0.6 ml) was used to visibly fill the middle ear, and each animal was positioned to keep the round window niche bathed in solution for 30 min. In one of these animals, the buffer alone (without gentamicin) was also injected into the middle ear on the side contralateral to the gentamicin injection to serve as a control for the possibility that the buffer caused changes in afferent physiology.

All chinchillas developed a static roll head tilt (gentamicin-treated ear down) within 4–22 days (median = 8 days) after the gentamicin injection. Head tilt was not precisely quantified, but was graded as mild (≤10°), moderate (11–20°), or severe (>20°) by frontal examination of the animal standing on a level surface. The chinchillas were divided into two groups according to the time interval between gentamicin application and afferent recordings. An early group (n = 10) was tested 5–25 days after the injection. These recordings were made when the head tilt appeared to reach its maximum in each animal. A late group (n = 8) was tested 90–115 days after the injection. By these times, the head tilt had disappeared in one animal, partially recovered in five animals, and persisted in two animals.

For afferent recordings, chinchillas were anesthetized with intraperitoneal 5.5-diallylbarbituric acid, 40 mg/kg (Sigma Chemical, St. Louis, MO). A tracheostomy was performed, and animals were kept at baseline core body temperature with a servo-controlled heating pad (model 40-90-8B, Frederick Haer and Co., Bowdoinham, ME). After placement in a stereotaxic holder, the bullae and internal auditory canals were opened, and the superior vestibular nerves on the treated and untreated sides were exposed. Glass microelectrodes (model M1B100F-4, World Precision Instruments, Sarasota, FL), filled with 3 N sodium chloride and with impedances of 10–30 MΩ, were directed into each superior vestibular nerve using a microdrive (model MO-22, Narishige International). Afferents from the nerve on the gentamicin-treated side were first recorded, and afferents from the nerve on the untreated side were recorded as controls. Most responsive units on either side were derived from the superior canal, horizontal canal, or utricle, as expected given the location of the recording electrode in the superior vestibular nerve. However, occasional posterior canal units were encountered, likely as a result of the electrode passing into the inferior vestibular nerve. Single-unit extra-axonal activity was amplified 500–5,000 times (model 2400A, Dagan, Minneapolis, MN), band-pass filtered from 100 Hz to 3 kHz, digitized at 5 kHz, and stored. A window discriminator was used to trigger on the action potentials of isolated units. Units were considered sufficiently isolated when the action potentials were of uniform amplitude and morphology. Interspike interval histograms were computed for stable periods of baseline discharge before vestibular or galvanic stimulation. The detailed methods for data acquisition and analysis have been previously described (Hullar and Minor 1999).

The stereotaxic apparatus was mounted on a gimbaled structure that allowed any of the semicircular canals to be tilted to rotate, which was parallel to the earth’s surface. The structure was mounted on a servo-controlled rate table (model 130–80/ACT2000, Acutronic USA, Pittsburgh, PA) programmed to provide a variety of earth-horizontal rotations. All procedures were reviewed and approved by the Animal Care and Use Committee of the Johns Hopkins University School of Medicine and conformed to the Guiding Principles for Research Involving Animals and Human Beings of the American Physiological Society.

Each unit was sampled at rest before stimulation, and its CV (CV = SD of interspike intervals/mean of interspike intervals) was calculated. CVs normalized to an interspike interval of 15 ms (CV*) were determined by interpolation with a larger data set from normal chinchilla vestibular afferents recorded in the same laboratory (Hullar and Minor 1999). Afferents were classified as regular (CV* < 0.1), intermediate (0.1 ≤ CV* ≤ 0.2), or irregular (CV* > 0.2) (Goldberg and Fernandez 1971). Units were tested for tilt and rotational sensitivity.

**Tilt and rotational stimulation**

Afferent responses were first determined to a series of static tilts of 10–40° in either direction about the roll and pitch axes. Such a series of tilts served to identify utricular afferents. When tilt responses were obtained, combinations of roll and pitch tilt were used to define the plane in which tilt produced the maximum change in firing rate. Following a tilt of ≤30° in this plane, the firing rate change used to calculate sensitivity was the largest change in rate (averaged over ≥100 spikes) from baseline and did not include the later decrease in that change caused by adaptation. The tilt sensitivity was calculated as the change in firing rate in spikes per second per applied gravitational acceleration (spikes·s⁻¹/g). Applied acceleration was calculated as
g sin (Θ), where g is the gravitational constant, 9.81 m/s², and Θ is the tilt angle relative to the earth-horizontal plane. Rotational sensitivity was evaluated by sinusoidal rotations (0.5–4 Hz, 6–200°/s peak velocity). For each semicircular canal afferent, the canal of origin was brought as close into the plane of rotation as permitted by the stability of the recording. When the canal was not co-planar with the plane of rotation, trigonometric corrections were made to determine the effective velocity acting on the canal, V有效 (Hullar and Minor 1999). Mathematically, this was done by projecting the stimulus velocity vector onto the canal’s axis of maximum sensitivity using dot product multiplication. The axes of the canals were taken from those of the guinea pig (Curthoys et al. 1975).

Galvanic stimulation

Galvanic microcurrents were applied to the bony labyrinth close to the superior vestibular nerve in 12 chinchillas using insulated silver wires (250 μm diam) with 2 mm of exposed, chloride-plated wire at the tips. The active electrode’s tip was located near the round window, and the return electrode with a ball tip was placed in the ventral middle ear. A stimulus isolator (Model 305R-C, World Precision Instruments, New Haven, CT) delivered steps of current (50 μA, 1–10 s). Cathodal currents with respect to the active electrode were excitatory, and anodal currents were inhibitory.

Data analysis

Spontaneous discharge regularity for each afferent was further characterized by creating a normalized histogram of the interspike intervals and fitting that histogram with a lognormal probability density distribution determined by two parameters, μ and σ, using maximum likelihood estimation (MATLAB v. 7, MathWorks, Natick, MA). The values of σ were compared between treated and control afferents for different classes of discharge regularity to determine if intratympanic gentamicin treatment had an effect on the interspike interval distributions. Afferent rotational sensitivity was computed as the change in firing rate per rotational velocity acting on the canal, V有效, expressed as a percentage change. Response phase was computed as the difference (in degrees) between the peak of the sinusoidal firing rate change and the peak of stimulus velocity. A phase lead (positive phase) corresponded to afferent peak response occurring prior to stimulus peak velocity. Average values from 5–10 cycles of the best responses were tabulated. For calculation of rotational sensitivity, cycles in which stimulus velocity was so high as to cause inhibitory cut-off were not used. Tilt and rotational sensitivity were calculated in the same way for afferents on the treated and untreated sides. Afferent galvanic sensitivity in spikes·s⁻¹/μA was calculated by dividing the difference between the firing rates with and without galvanic stimulation by the applied current. Statistical comparisons of these afferent responses were done using Mann Whitney U-test by the Minitab software v. 11.21 (Minitab, State College, PA), the MATLAB software, or Microsoft Excel 2000 (Microsoft, Redmond, WA) on personal computers.

Histological techniques

After completion of the afferent recordings, deeply anesthetized animals were prepared for transcardiac perfusion by opening the thorax, cannulating the left ventricle, opening the right atrium, and perfusing with saline and then fixative. The fixative consisted of 2% paraformaldehyde and 3% glutaraldehyde in 0.1 M phosphate buffer at room temperature. On each side, the bulla was opened, and the perilymphatic space perfused with the same fixative via the oval window and a fenestra made in each semicircular canal. After overnight postfixation, the sensory epithelia were dissected out, washed in PBS, osmicated, dehydrated in graded ethanols, and embedded in Epon (Polysciences, Warrenton, PA) resin. Two-micron-thick cross-sections were taken for quantification of hair cell nuclei and support cell nuclei using the dissector technique (Fernandez et al. 1995; Gundersen 1986). This technique assures that these nuclei, which are larger in diameter than 2 μm, are not overcounted. Two serial sections were photographed, and the images were superimposed. Using one section as a reference section and the other as a lookup section, the nuclei of type I and II hair cells and nuclei of support cells were identified and tabulated only if they appeared in the reference section but not in the lookup section. This process is equivalent to counting the nuclei only if the reference section is the last one in which they appear. Then the same process was repeated for the adjacent section. The tabulated cell counts were averaged between the two sections, and densities of cells were calculated by dividing these averages by the product of the length of the luminal surface of the neuroepithelium and its thickness.

Support cells were identified by their polygonal nuclei that stained darkly and homogeneously except for one to several clumps of chromatins. Support cell nuclei were located low in the epithelium, adjacent to the basement membrane. However, in gentamicin-treated epithelia, support cells can migrate away from the basement membrane and be found higher in the epithelium, likely as part of a repair process (Li et al. 1995). Accordingly, cells found higher in the epithelium in this study were considered support cells if their nuclei had the above features and the cells did not have any associated afferent endings, cuticular plates, or stereocilia. Hair cells were identified by their lighter-staining elliptical nuclei with only one nucleolus per nucleus. Hair cell nuclei were located well above the basement membrane. A cell with such a nucleus was considered a hair cell only if the basal portion of the cell contacted afferent endings or if the apical portion of the cell had a cuticular plate. Type I hair cells had a flask shape, constricted neck, and were surrounded by minimally stained calyceal endings. Their nuclei tended to be round and had several clumps of chromatins. Type II hair cells assumed a cylindrical or barrel shape without a constricted neck; their bouton endings could not be seen with our light microscopic preparation. Their nuclei had a more homogeneous distribution of chromatins than did those of type I hair cells, and type II nuclei tended to be close to the surface of the neuroepithelium because these hair cells were shorter. In gentamicin-treated epithelia, many hair cells were considered to be of indeterminate type because their features were intermediate between these descriptions.

RESULTS

Spontaneous firing rate and discharge regularity

Vestibular afferents continued to fire spontaneously on the gentamicin-treated side in both the early and late groups, and previously defined classes of afferent discharge regularity (Goldberg and Fernandez 1971) were preserved despite gentamicin treatment (Fig. 1). The proportion of irregular versus regular afferents did not differ between the treated and untreated sides in either the early or late group (Fig. 2; P = 0.9 for χ² test), although irregular units tended to be less frequent on the treated versus untreated side (23 vs. 31%). The percentage of intermediate afferents was <10% for either side in both the early and late groups, similar to previous findings in normal chinchillas (Baird et al. 1988). Figure 3 shows that the distribution of CV* values among afferents in the early (B) and late (C) groups after gentamicin treatment were similar to the distribution of CV* values for the control afferents (A).

The average firing rate only changed for regular afferents after intratympanic gentamicin treatment. The spontaneous rate of regular units was 19% lower in the early group and 38%
lower in the late group ($P < 0.001$ for change in rate in both cases). In the early period after intratympanic gentamicin treatment, the average spontaneous firing rate of regular afferents on the treated side was $44.3 \pm 16.3$ spikes $\cdot s^{-1}$ (SD; $n = 117$), which was lower than that of the regular afferents on the untreated side, $54.9 \pm 16.8$ spikes $\cdot s^{-1}$ ($n = 85$; $P < 0.001$). The average spontaneous rate of intermediate units on the treated side was $59.8 \pm 24.2$ spikes $\cdot s^{-1}$ ($n = 10$), which was no different from that on the untreated side of $44.0 \pm 18.0$ spikes $\cdot s^{-1}$ ($n = 12$, $P = 0.09$). The average spontaneous firing rates of irregular afferents also did not significantly decrease on the treated side [$47.3 \pm 19.5$ spikes $\cdot s^{-1}$ ($n = 25$) vs. $51.9 \pm 22.9$ spikes $\cdot s^{-1}$ ($n = 34$) on the untreated side; $P = 0.37$]. At 3 mo after treatment, the average spontaneous firing rate of regular afferents further decreased on the treated side to $33.9 \pm 13.2$ spikes $\cdot s^{-1}$ ($n = 95$; $P < 0.0001$ vs. spontaneous rates on the untreated side). The average spontaneous rate of intermediate units on the treated side was $52.5 \pm 18.4$ spikes $\cdot s^{-1}$ ($n =$
The average spontaneous rate of irregular afferents on the treated side did not significantly change and was 43.5 ± 21.3 spikes·s⁻¹ (n = 25; P = 0.14).

A bivariate regression using a general linear model was performed, considering as variables the individual subject from which the afferent spontaneous discharge was measured and whether or not gentamicin treatment was given. This regression analysis revealed that gentamicin treatment was mainly responsible for the variation in the spontaneous rate (F = 36.8, P < 0.001), although the variation between individual chinchillas, especially in the early group, was also significant (F = 6.2, P < 0.001).

The effect of intratympanic gentamicin on the interspike interval (ISI) distributions of afferents was further explored by fitting a lognormal distribution to the normalized ISI histogram for each afferent. Figure 1A shows the spontaneous discharge of an irregular afferent (G35_21), and Fig. 1B shows the spontaneous discharge of a regular afferent (G35_10), both on the treated side of chinchilla G35 2 wk after intratympanic gentamicin injection. The ISI distribution is broad and skewed to the right for the irregular afferent (Fig. 1C) but narrow for the regular one (Fig. 1D). To compare such distributions, they were normalized and fit with log-normal probability distributions with characteristic parameters μ and σ (Fig. 1, E and F).

The difference in width and skew between these two probability distributions is primarily reflected in the values of σ for the distributions. For the irregular afferent in Fig. 1E, μ = −3.81 and σ = 0.407. For the regular unit in Fig. 1F, the parameters were μ = −3.86 and σ = 0.048.

The value of σ always differed (P < 0.0001, t-test) between regular and irregular afferents. This was true on both the treated and untreated sides and in both the early and late groups. Within classes of afferent discharge, σ values were compared between gentamicin-treated and untreated units to determine whether or not gentamicin treatment had a greater effect on the discharge properties of regular or irregular units.

The only significant effect was found for irregular afferents at 2 wk after treatment, when σ averaged 0.62 ± 0.21 for treated afferents and 0.44 ± 0.14 for untreated afferents (P = 0.002, t-test), suggesting a broadening and/or skewing of the ISI distributions for irregular afferents at this early period after gentamicin treatment.

The increase in the width or skew of the ISI distributions for irregular afferents early after gentamicin treatment might be explained by an increase in injury discharge patterns noted in these units. Rapid bursts of firing with two, three, or more action potentials spaced at unusually short intervals may indicate injury to an afferent’s membrane. Such injury was sometimes seen transiently after passage of the microelectrode in both control and gentamicin-treated units, but bursts of firing ceased in most units after their initial isolation. Among control afferents, exceptions to this were the rare couplets or triplets occurring in <5% of the action potentials in 12 of 131 afferents (9%) recorded on the untreated side early after gentamicin treatment and in 3 of 77 afferents (4%) recorded on the untreated side at 3 mo after gentamicin treatment. However, on the gentamicin-treated side, injury firing was noted on some occasions to persist throughout the length of the recording, sometimes for several minutes, and to involve >10% of the action potentials. Units with such activity were considered to be persistently injured. In the early period after gentamicin treatment, 10 of 152 afferents (7%) on the treated side showed this persistent injury. In the late period, 11 of 131 afferents (8%) on the treated side showed persistent injury. When the 10 gentamicin-treated irregular afferents with persistent injury patterns were excluded from the calculation of a mean value for σ, that value dropped to 0.52 ± 0.13 in the treated irregulars, which was not significantly different from the value for the untreated irregular afferents. Thus intratympanic gentamicin may have increased the ISI breadth and/or skew for irregular afferents 2 wk after treatment, but this effect was not significant when injured afferents were excluded.
Sensitivity to vestibular stimuli

On the treated side, only 18% of the afferents in the early group and 25% in the late group responded to either rotation or tilt. For the 117 afferents recorded on the gentamicin-treated side in the early group, responses adequate to determine the endorgan of origin were recorded in 14 utricular, 3 horizontal canal, and 4 superior canal afferents. For the 95 afferents recorded in the late group, responses adequate to determine the endorgan of origin were recorded in 9 utricular, 8 horizontal canal, 5 superior canal, and 2 posterior canal afferents. There was no difference in the proportion of regular versus irregular afferents that lost vestibular sensitivity (P = 0.6, χ² test). Figure 4A shows the response to a stimulus of 2-Hz sinusoidal head velocity with a peak velocity of 155°/s from a regularly discharging horizontal canal afferent (CV* = 0.03) afferent on the treated side in the late group. The response is barely discernible in the afferent’s firing rate. Fourier analysis of the cycles revealed a rotational sensitivity of 0.01 spikes s⁻¹/deg ⋅ s⁻¹. Figure 4B shows the typical response to a 1-Hz sinusoidal rotational head velocity with peak velocity of 40°/s from a regularly discharging horizontal canal afferent (CV* = 0.03) on the untreated side in the same animal. The rotational sensitivity was 0.27 spikes s⁻¹/deg ⋅ s⁻¹.

Figure 5A shows the response to a 30° static roll tilt in the excitatory direction from a regularly discharging otolith afferent (CV* = 0.02) recorded on the treated side in the early group. The arrow indicates the onset of the roll stimulus. The response sensitivity was 2.8 spikes s⁻¹/g. Figure 5B shows the response to a 30° static roll tilt in the inhibitory direction from a regularly discharging otolith afferent (CV* = 0.03) recorded on the untreated side in the same animal. The response sensitivity was 23.4 spikes s⁻¹/g.

For those few treated afferents that did respond to rotations, the average sensitivity was reduced by nearly 90% in comparison to untreated afferents in both the early and late groups. The rotational sensitivity for irregularly discharging afferents was decreased to 0.12 ± 0.11 spikes s⁻¹/deg ⋅ s⁻¹ (n = 3 with measurable responses) on the treated side compared with 1.01 ± 0.72 spikes s⁻¹/deg ⋅ s⁻¹ (n = 29) on the untreated side (P < 0.007). The rotational sensitivity for regularly discharging afferents was decreased to 0.03 ± 0.03 spikes s⁻¹/deg ⋅ s⁻¹ (n = 19 with measurable responses) on the treated side compared with 0.31 ± 0.28 spikes s⁻¹/deg ⋅ s⁻¹ (n = 29) on the untreated side (P < 0.0001).

For the treated afferents that responded to tilts, the average sensitivity was reduced by 77% in the early group and by 72% in the late group. The tilt sensitivity for irregularly discharging afferents was reduced to 12.1 ± 5.0 spikes s⁻¹/g (n = 9) on the treated side compared with 50.5 ± 22.1 spikes s⁻¹/g (n = 9) on the untreated side (P < 0.0004). The tilt sensitivity for regularly discharging afferents was reduced to 10.3 ± 5.3 spikes s⁻¹/g (n = 13) on the treated side compared with 41.4 ± 27.0 spikes s⁻¹/g (n = 23) on the untreated side (P < 0.0001). The differences in tilt sensitivities between the early and late groups were not significant. None of the intermediate afferents on the treated side was sensitive to tilt or rotation.

To determine if the buffer solution, independent of gentamicin, had an effect on afferent responses, we injected the gentamicin solution into the left ear and the buffer solution (0.6 mM sodium bicarbonate) into the right ear in one chinchilla (B4). The animal developed a tilt of the head to the left as we have seen with all of the chinchillas treated with left intratympanic gentamicin. After 2 wk, we recorded from vestibular nerve afferents. On the side of the buffered vehicle injection, 7 units identified as canal afferents had mean rotational sensitivity of 0.39 ± 0.28 (SD) spikes s⁻¹/deg ⋅ s⁻¹. By comparison, the mean rotational sensitivity of 24 canal afferents from un.injected control ears of chinchillas 2 wk after intratympanic gentamicin treatment was 0.34 ± 0.35 spikes s⁻¹/deg ⋅ s⁻¹ (P = 0.7). Likewise, for seven otolith afferents recorded on the vehicle-injected side of B4, the mean tilt sensitivity was 25.0 ± 12.7 spikes s⁻¹/g, not different from the mean for 19 otolith afferents on the un.injected sides of chinchillas 2 wk after intratympanic gentamicin treatment (44.1 ± 28.4 spikes s⁻¹/g, P = 0.1). These findings indicate that the gentamicin, and not...
the buffer solution itself, was responsible for the changes in afferent responses.

We sought to determine if the control units in this study represented the full morphophysiological range of vestibular afferents. Baird et al. (1988) showed that the relationships between discharge regularity and the sensitivity and phase to rotational stimuli could predict the morphologic features of normal chinchilla semicircular canal afferents. Accordingly, we examined the relationships between discharge regularity and the sensitivity and phase to rotational stimuli for semicircular canal afferents pooled from all of the data on the untreated sides. Figure 6 reveals that our untreated canal afferents behaved similarly to those described by Baird et al. A distinct group of “low-gain irregular afferents,” those with CV* >0.2 and rotational sensitivity <1.5, could be defined in our data (Fig. 6A, ●). These are similar to the low-gain irregulars described by Baird et al. In addition, by excluding these low-gain irregular units, an exponential increase in sensitivity as a function of CV* could be defined for our data (Fig. 6A, solid line). Our rotational gain data were fit by $S = a(CV^*)^b$, where $S$ = rotational sensitivity, $a = 5.38$ spikes·s⁻¹/deg·s⁻¹, and $b = 0.986$. For comparison, the same model fit from the data of Baird et al., where $a = 7.82$ spikes·s⁻¹/deg·s⁻¹, and $b = 0.984$, is plotted (Fig. 6A, dotted line). Likewise, there is a logarithmic relationship (Fig. 6B, solid line) between the phase of the rotational response and CV* given by phase = $a + b \times \log(CV^*)$, where $a = 36.5°$ and $b = 27.1°$. For comparison, the model from Baird et al., where $a = 42.0°$ and $b = 30.2°$, is plotted (Fig. 6B, dotted line).

Galvanic sensitivity

Unlike the responses to vestibular stimuli, the responses to galvanic polarizations did not differ between the treated and untreated sides in either the early or late group. Figure 7 shows typical changes in afferent firing that occurred with applications of 50-µA cathodal (solid bars) and anodal (open bars) currents. Irregular afferents had characteristically large galvanic responses of similar magnitude on treated (Fig. 7A) and untreated (Fig. 7B) sides. Regular afferents had characteristically smaller responses, but again the responses were similar between treated (Fig. 7C) and untreated (Fig. 7D) sides.

For each current polarity, the mean galvanic sensitivity of vestibular afferents was not affected by gentamicin treatment. For regularly discharging afferents, the mean galvanic sensitivity to cathodal currents on the treated side in early and late groups was 0.17 ± 0.16 spikes·s⁻¹/µA ($n = 62$), and it did not differ from that of $0.14 ± 0.09$ spikes·s⁻¹/µA ($n = 21$) on the untreated side ($P = 0.94$). The respective cathodal sensitivities also did not differ between the gentamicin-treated versus untreated sides for intermediate units ($0.49 ± 0.20$, $n = 5$, vs. $0.37 ± 0.12$ spikes·s⁻¹/µA, $n = 7$; $P = 0.37$) or irregular units ($0.47 ± 0.39$, $n = 13$, vs. $0.62 ± 0.28$ spikes·s⁻¹/µA, $n = 8$; $P = 0.10$). Similarly, there were no changes in the sensitivity to anodal currents associated with intratympanic gentamicin treatment. The mean galvanic sensitivity to 50-µA anodal currents for regular units on the treated side in early and late groups was 0.13 ± 0.12 spikes·s⁻¹/µA ($n = 53$), and it did not differ from that of $0.12 ± 0.09$ spikes·s⁻¹/µA ($n = 17$) on the untreated side ($P = 0.97$). The anodal sensitivities also did not differ on the gentamicin-treated versus untreated sides for intermediate units ($0.15 ± 0.05$, $n = 4$, vs. $0.20 ± 0.07$ spikes·s⁻¹/µA, $n = 3$; $P = 0.29$) or irregular units ($0.55 ± 0.40$, $n = 11$, vs. $0.41 ± 0.27$ spikes·s⁻¹/µA, $n = 15$, $P = 0.57$).

Histological results

Figure 8 shows the histological appearance of the midportion of the crista in cross-section from an untreated horizontal canal crista (A) and from a treated horizontal canal crista (B). The untreated control crista appears to have a dense population of hair cells, and type I and II hair cells can clearly be differentiated. In this specimen, there were 2.05 type I hair...
cells per 100 $\mu m^2$ and 1.25 type II hair cells per 100 $\mu m^2$, and there were 3.31 support cells per 100 $\mu m^2$. In contrast, the gentamicin-treated crista (Fig. 8B), which is from an animal 4 wk after intratympanic gentamicin treatment, shows a partial loss of hair cells. The thickness of the hair cell layer of this neuroepithelium appears decreased relative to that of the control (Fig. 8A). Also of note, the gentamicin-treated crista has a number of globular endings (g) beneath the hair cells. These endings are larger than normal boutons (which are not visible at this level) but smaller than calyces, and they may represent swollen boutons or remnants of retracted calyces. In fact, only one intact afferent calyx and type I hair cell could be clearly identified in any of the treated specimens, and it is shown here (I). It yielded a density of 0.06 type I cells per 100 $\mu m^2$. This type I cell and its afferent calyx is shown in detail in Fig. 8C. Type II cells were present at a density of 1.23 cells per 100 $\mu m^2$. The type II cell marked (II) in Fig. 8B is shown in detail in Fig. 8D.

Figure 9 shows hair cells with features intermediate between type I and II cells in a gentamicin-treated crista 15 days after treatment. Hair cells labeled 1 and 2 have cylindrical shapes, with little or no constrictions at their necks. Accordingly, these were considered to be type II hair cells. However, the hair cell labeled 3 has a more flask-shaped appearance with a constriction at the neck. These features are more characteristic of a type I hair cell, but this cell does not have a calyceal afferent ending. Moreover, because of the diminished height of the entire neuroepithelium (see Fig. 8B), the location of the nucleus in the epithelium gives less of a clue to the identity of such a hair cell. Such hair cells were considered indeterminate in this study. These indeterminate cells were present at a density of 0.41 cells per 100 $\mu m^2$ in this example.

Table 1 summarizes the quantitative changes in hair cell and support cell density in the early period after intratympanic gentamicin treatment. Average hair cell and support cell densities in control cristae were similar to those found by Lysakowski and Goldberg (1997). Intratympanic gentamicin treatment resulted in a significant reduction in the total density of hair cells by 57%. The density of type I hair cells was reduced by 99%, as only a single type I hair cell could be identified in the gentamicin-treated specimens (Fig. 8C). The reduction in the density of type II hair cells was not significant. Support cell density was not reduced. After intratympanic gentamicin treatment, 31% of the remaining hair cells were of indeterminate morphology (Fig. 9, 3). Even if all such indeterminate cells were counted as type I cells, there still would have been a significant reduction in type I hair cell density ($P = 0.03$). The density of indeterminate cells was included in the total hair cell density calculation, and the total hair cell density nevertheless was also reduced. These findings indicate that intratympanic gentamicin treatment resulted in a substantial reduction in the total number of hair cells and the density of type I hair cells. The presence of hair cells of indeterminate morphology would not affect the conclusion that total and type I hair cell densities were reduced.

**Discussion**

A single intratympanic application of gentamicin resulted in a severe reduction in the sensitivity of semicircular canal afferents to rotations and of otolith afferents to tilts. The afferents nevertheless continued to fire spontaneously, but regular units had lower resting discharge rates in comparison to the contralateral side. The sensitivity to galvanic stimulation was relatively unchanged. The patterns of discharge regularity were also preserved. The histological findings indicated that total hair cell density was reduced by intratympanic gentamicin treatment. Most of this reduction occurred because of a loss of type I hair cells. While cells of indeterminate morphology appeared after gentamicin treatment, their inclusion in total or type I hair cell counts would still show a reduction in these cells. We conclude that some hair cells—particularly type I cells—are lost after a single intratympanic gentamicin treatment. While the remaining hair cells may provide the critical synaptic input needed to maintain afferent spontaneous activity, they do not provide sufficient information to modulate afferent firing in response to vestibular stimulation.

**Comparison to previous studies**

Two previous reports have examined the effects of intratympanic gentamicin on the structure and function of the chinchilla’s inner ear (Chen et al. 1999; Hilton et al. 2002). In these studies, a single injection of gentamicin at the same concentration that we used was given in two animals. These animals lost vestibular responses to ice water caloric stimulation. In these same animals and in animals receiving multiple injections, scanning electron microscopy showed extensive loss of
hair cell surface features (i.e., stereocilia), especially in the central zones of the cristae and striolar zones of the maculae. While these authors interpreted these findings as indicating hair cell loss, it is also possible that they merely observed the loss of the apical specializations of hair cells, the only features observable with scanning electron microscopy. Because our study of the structural effects of intratympanic gentamicin used cross-sectional light microscopy, we were able to determine the fate of the hair cell bodies within the neuroepithelium. Our findings suggest a partial loss of hair cells in the chinchilla exposed to a single intratympanic gentamicin injection.

Studies in the chicken employing systemic and prolonged exposures to aminoglycosides have given some further information about the fate of vestibular nerve afferents (Duckert and Rubel 1993). Histologically, nearly complete hair cell loss was accompanied by retraction of vestibular afferent nerve endings as far as the basement membrane of the sensory epithelium (Weisleder and Rubel 1993). Boyle et al. (2002) recently used high systemic doses of streptomycin to cause this nearly complete hair cell lesion, and they observed a cessation of spontaneous firing in afferents from the crista in the early period after treatment. Hair cell regeneration was believed responsible for the later return of spontaneous activity in many afferents, but vestibular sensory transduction appeared to recover less completely. Thus this study suggested a dissociation between baseline vestibular hair cell secretory function and the transduction of head acceleration stimuli.

The intratympanic application of gentamicin solution, mimicking that used in the treatment of Ménière's disease, likely results in a very different exposure of hair cells to gentamicin than has been reported in other studies of local aminoglycoside application into perilymph. Lopez and colleagues conducted histological studies on the effects of the direct perilymphatic application of gentamicin to the chinchilla labyrinth (Lopez et al. 1997; Tanyeri et al. 1995). They reported >90% initial loss of both type I and type II vestibular hair cells. Our application, in contrast, was to the middle ear, in which case only a fraction of the drug may cross the round window membrane to exert its effect (Hibi et al. 2001), and the exposure may be brief because of egress via the Eustachian tube. The differences in gentamicin exposure might explain why we see less severe loss of type II hair cells in this study.

Evidence for preservation of synaptic activity but loss of transduction

Physiologically, we observed that spontaneous firing persisted in many afferents after intratympanic gentamicin application. Histologically, we observed that some hair cell bodies remained intact as well. Taken together, these observations suggest that the remaining hair cells may have been capable of...
continued release of neurotransmitter, and that this synaptic activity may have been responsible for the ongoing firing of these afferents. The model proposed by Smith and Goldberg (1986) to explain the spontaneous repetitive discharge in vestibular primary afferents requires baseline synaptic activity to explain the spectrum of discharge regularity and, in particular, the irregular discharge pattern of some afferents. They modeled the spontaneous discharge of vestibular afferents as arising from an outward potassium current activated by the action potential and decreasing with time. This repolarizing current causes theafferent membrane potential to climb back toward threshold for a subsequent action potential. For regularly discharging afferents, the trajectory of the repolarization would bring the membrane potential to threshold in a deterministic fashion, causing a spike to occur at nearly the same interspike interval for each spike. However, superposition of synaptic noise adds some degree of irregularity, giving a range of CV* values among these afferents. For irregularly discharging afferents, the model predicts that the trajectory of the repolarization does not bring theafferent to threshold but just below it, and that baseline synaptic noise, which is stochastic, brings the membrane potential to threshold in an unpredictable fashion.

Figure 3 shows the preservation of CV* values in excess of 0.2, i.e., irregular afferents. If the Smith and Goldberg model is correct, irregular afferents should have ceased firing spontaneously if baseline synaptic noise was entirely removed. In contrast, our data showed that irregular discharge patterns persisted after a single intratympanic gentamicin injection. In fact, the proportion of irregular afferents did not significantly change as a result of intratympanic gentamicin treatment, although there were slightly fewer irregular units after gentamicin treatment (Fig. 2). These findings suggest that the single intratympanic gentamicin injection left intact some hair cell synaptic input to these irregular afferents. The histological findings confirm that some hair cell bodies remained to provide this input. Further ultrastructural analysis will be needed to determine how well the synaptic specializations are preserved in remaining hair cells after intratympanic gentamicin treatment.

How can we reconcile the preservation of transmitter release from hair cells with the observation that rotational and tilt sensitivities were severely reduced by intratympanic gentamicin treatment? The dichotomy would imply a disconnection of the transduction apparatus, which is located in the stereociliary bundle at the apex of the hair cell, and the baseline rate of neurotransmitter release by the hair cell at the basolateral membrane. Early studies of the ototoxic effects of aminoglycosides noted that fusion of stereocilia is among the first signs of hair cell toxicity (Duvall and Wersall 1964). Using cultured rat utricles, Zheng et al. (1999) found that direct exposure to gentamicin caused the loss of hair cell stereocilia and cuticular plates, but not of the cell bodies within the sensory epithelium. The cell body is believed to spontaneously release an excitatory neurotransmitter, which is likely glutamate, and that this release depends on a calcium conductance in the basolateral membrane (Annoni et al. 1984; Cochran and Correia 1995). If our gentamicin treatment damaged stereocilia to a greater extent than it damaged the ion channels and synaptic specializations of the basolateral membrane, then it might be expected that spontaneousafferent activity could be preserved while transduction of vestibular stimulation would be disrupted. Our light microscopic results do show preservation of some hair cell bodies in the epithelium. We are conducting further examinations of these tissues with transmission electron microscopy to determine if the hair cell bodies that we observe after intratympanic gentamicin treatment have lost their apical specializations. However, with the present evidence, an attractive hypothesis is that relatively selective damage to stereocilia may severely reduce transduction in response to vestibular stimuli but preserve spontaneous neurotransmitter release.

Selective toxicity to type I hair cells and/or calyces

The type I hair cell is reported to be more sensitive to the toxic effects of systemic administration of aminoglycosides (Lindeman 1969). Our results appear to confirm this selective toxicity when gentamicin is administered via intratympanic injection. We observed a 99% reduction in hair cells associated with complete calyceal endings. In fact, we could only identify only one example of a calyx in our gentamicin-treated specimens (Fig. 8C). Such findings have previously been interpreted as showing a selective toxicity of gentamicin for the type I hair cell, but another possibility is that gentamicin has a selective toxic effect on the calyceal afferent endings. We observed that 31% of the remaining hair cells after intratympanic gentamicin treatment had features suggestive of type I morphology even though they lacked calyceal endings, features such as constricted necks and flask shapes. Moreover, we observed globular endings in the gentamicin-treated epithelia that might represent remnants of damaged calyces (Fig. 8B). Further studies of the ultrastructural features of these epithelia after gentamicin treatment may help define if some of the remaining hair cells have additional features suggesting that they are type I cells.

Remnants of calyces might retain some ability to transmit synaptic activity to vestibular afferents. Type II hair cells are known to make outer face synapses with normal calyces. It is possible that a calyx could extrude its type I hair cell but still maintain synaptic contacts with surrounding type II hair cells.
via outer face synapses. A relevant observation in this study is that some of the afferents found spontaneously discharging after gentamicin treatment had high values of CV*, suggesting that their input would come only from calyceal endings. This suggestion comes from data that some afferents in the central zone of the normal crista contain only calyceal endings (Fernandez et al. 1988). Baird et al. (1988) characterized such calyx-only units and found that they discharged very irregularly, with CV* > 0.3. Another physiologic signature of these afferents is their low rotational sensitivity at 2 Hz (low-gain irregulars) in comparison with dimorphic units of similar discharge regularity (high-gain irregulars). Figure 6A shows that our extracellular recordings from untreated afferents recapitulated the findings of Baird et al. There was a linear relationship of rotational sensitivity to CV* for most afferents, but a group of very irregular afferents had lower gains. Several of these units have CV* > 0.3. These units in our normal cristae should, therefore, also have been calyx-only afferents. Spontaneously-discharging units with CV* > 0.3 were also found on the gentamicin-treated side in the early and late groups (Fig. 3, B and C). Although these afferents would not appear to have their full calyceal endings based on our histological data, any synaptic input from remnants of these endings might have been sufficient to maintain the spontaneous firing of these units. Since evidence suggests that the discharge regularity of vestibular afferents is a property of the afferent itself and not of its composition of calyx and bouton endings (Goldberg 2000), even altered synaptic input could result in the unit retaining its pretreatment discharge regularity.

Effects on afferent physiology

While spontaneous discharge and patterns of discharge regularity were preserved after treatment with intratympanic gentamicin, there were nevertheless changes in afferent physiology after treatment that suggested both direct and indirect effects on afferents. For afferents that did continue to fire spontaneously in normal patterns, resting rates declined 2 wk after intratympanic gentamicin and further declined 3 mo after treatment. The decline was significant for regular afferents. This decrease in resting discharge rate over time after the single intratympanic injection of gentamicin might indicate either more loss of or damage to hair cells with time after treatment. Delayed hair cell injury from aminoglycosides may occur as the drug accumulates in the lysosomes of hair cells, not exerting a lethal effect until the lysosomes rupture and expose the cytoplasm to the drug (Hashino et al. 1997).

Direct injury to vestibular nerve afferents by gentamicin is also a possible cause of the decreased resting discharge rate seen after injection of gentamicin into the middle ear. Dupont et al. (1993) reported histological evidence of partial damage to vestibular afferents after direct application of an aminoglycoside to the inner ear. They injected sisomicin through the round window membrane of guinea pigs. Near-total destruction of hair cells was noted in the sensory epithelia of the semicircular canals, but vestibular afferents were morphologically intact. Cell bodies in Scarpa’s ganglion were also intact but showed degenerative changes, which included swelling of the myelin sheaths and vacuole formation within the somata.

Our physiological data showed that injury discharge patterns were observed more frequently in gentamicin-treated afferents than in control afferents. After gentamicin treatment, 7 (early group) to 8% (late group) of afferents were found to have firing patterns suggestive of persistent injury on the treated side. In contrast, such a degree of persistent injury was not observed on the untreated side. It is not clear whether or not the vestibular afferents on the gentamicin treated side actually conveyed these injury patterns to the brain stem prior to the encounter with the microelectrode. It is possible that microelectrode penetration initiated the injury discharge pattern and that it persisted longer than in control afferents because gentamicin damaged the neuron’s mechanisms for repairing its membranes or re-establishing its physiological ion gradients after membrane disruption. However, gentamicin might directly damage afferents, so that the observed persistent abnormal discharge would actually be representative of the afferent’s underlying firing and not simply due to an effect of impalement by the microelectrode. If so, the analysis shows that the brain stem would receive an altered static signal from the treated labyrinth in which the ISI distributions would be broadened for irregular afferents in the early period after intratympanic gentamicin treatment. More importantly, our data show that the great majority of afferents on the gentamicin-treated side were still able to generate normal patterns of spontaneous discharge and respond to galvanic stimulation. These findings imply that the spike initiation zones of afferents on the gentamicin-treated side largely remained functional.

Our data do not indicate that unilateral intratympanic gentamicin treatment changed the spontaneous activity, vestibular responses, or galvanic responses of afferents on the contralateral side. Figure 6 shows the similarity in the rotational sensitivities of our untreated canal afferents to those measured by Baird et al. (1988). Likewise, untreated otolith afferents in this study responded to static tilts with sensitivities of 40–47 spikes/s/g, slightly greater but comparable with the mean sensitivities of 33 spikes/s/g for regular afferents and 27 spikes/s/g for irregular afferents in the study of Goldberg et al. (1990). The similarity of our findings from the untreated side with those of others suggests that the peripheral vestibular apparatus on the untreated side is not affected by systemic absorption of gentamicin. It is possible that changes in the physiology of the gentamicin-treated labyrinth could affect the physiology of the contralateral labyrinth via the efferent vestibular system. We cannot exclude this possibility from these experiments because of the use of a barbiturate anesthetic, which would suppress the efferent effects (Plotnik et al. 2002).

Preservation of galvanic sensitivity

Galvanic stimuli act directly on the axons of vestibular nerve afferents, causing depolarization and increased firing (from currents that are cathodal with respect to the perilymphatic electrode) or hyperpolarization and decreased firing (from currents that are anodal with respect to the perilymphatic electrode). Gentamicin treatment did not affect galvanic sensitivity in this study, suggesting that the damage caused by gentamicin is restricted to the hair cells and, perhaps, the distal aspects of the afferent endings, but not including the spike initiation zones. These findings argue that the principal toxic effect of gentamicin as we have used it in this study is on the hair cells and not the afferents themselves.
These findings have considerable significance for work directed toward development of a vestibular prosthesis to encode head acceleration through electrical stimulation of the vestibular nerve. Systemic use of aminoglycosides for treatment of sepsis or severe infections is a common cause of bilateral vestibular hypofunction (Minor 1998). This condition can be severely debilitating, as patients suffer oscillopsia, the illusion of motion of the visual world with everyday head movements (J. C. 1952; Gillespie and Minor 1999). In patients who have suffered disabling loss of peripheral vestibular function as a consequence of the systemic use of these antibiotics, direct galvanic stimulation of the nerve has been proposed as a means of restoring sensorimotor function (Gong and Merfeld 2000). The findings in this study support the feasibility of this approach. Intratympanic gentamicin caused vestibular afferents to lose rotational sensitivity, and this is likely the basis for oscillopsia when it occurs bilaterally in patients who have received the drug systemically. Nevertheless, intratympanic gentamicin treatment did not cause afferents to lose galvanic sensitivity. Direct electrical stimulation of the vestibular nerve may therefore hold promise as a means of restoring sensory input to the vestibular system after aminoglycoside ototoxic damage.

Clinical implications for Ménière’s disease

This study was motivated by a desire to understand the effects of intratympanic gentamicin on the physiology of vestibular nerve afferents. Intratympanic gentamicin is now a commonly used treatment for intractable vertigo due to unilateral Ménière’s disease. Our findings from an animal model show that spontaneous discharge in vestibular afferents is preserved while responses to rotational and tilt stimuli are attenuated. The results provide a basis for understanding changes in the angular vestibulo-ocular reflex for high-acceleration head movements after intratympanic gentamicin treatment. The gain of the vestibulo-ocular reflex attributable to afferents on the treated side after intratympanic gentamicin may therefore hold promise as a means of restoring sensory input to the vestibular system after aminoglycoside ototoxic damage.

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