Research Article

Rhesus Cochlear and Vestibular Functions Are Preserved After Inner Ear Injection of Saline Volume Sufficient for Gene Therapy Delivery

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ABSTRACT

Sensorineural losses of hearing and vestibular sensation due to hair cell dysfunction are among the most common disabilities. Recent preclinical research demonstrates that treatment of the inner ear with a variety of compounds, including gene therapy agents, may elicit regeneration and/or repair of hair cells in animals exposed to ototoxic medications or other insults to the inner ear. Delivery of gene therapy may also offer a means for treatment of hereditary hearing loss. However, injection of a fluid volume sufficient to deliver an adequate dose of a pharmacologic agent could, in theory, cause inner ear trauma that compromises

functional outcome. The primary goal of the present study was to assess that risk in rhesus monkeys, which closely approximates humans with regard to middle and inner ear anatomy. Secondary goals were to identify the best delivery route into the primate ear from among two common surgical approaches (i.e., via an oval window stapedotomy and via the round window) and to determine the relative volumes of rhesus, rodent, and human labyrinths for extrapolation of results to other species. We measured hearing and vestibular functions before and 2, 4, and 8 weeks after unilateral injection of phosphate-buffered saline vehicle (PBSV) into the perilymphatic space of normal rhesus monkeys at volumes sufficient to deliver an *atoh1* gene therapy vector. To isolate effects of injection, PBSV without vector was used. Assays included behavioral observation, auditory brainstem responses, distortion product otoacoustic

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emissions, and scleral coil measurement of vestibulo-ocular reflexes during whole-body rotation in darkness. Three groups ($N = 3$ each) were studied. Group A received a 10 μL transmastoid/trans-stapes injection via a laser stapedotomy. Group B received a 10 μL transmastoid/trans-round window injection. Group C received a 30 μL transmastoid/trans-round window injection. We also measured inner ear fluid space volume via 3D reconstruction of computed tomography (CT) images of adult C57BL6 mouse, rat, rhesus macaque, and human temporal bones ($N = 3$ each). Injection was well tolerated by all animals, with eight of nine exhibiting no signs of disequilibrium and one animal exhibiting transient disequilibrium that resolved spontaneously by 24 h after surgery. Physiologic results at the final, 8-week post-injection measurement showed that injection was well tolerated. Compared to its pretreatment values, no treated ear's ABR threshold had worsened by more than 5 dB at any stimulus frequency; distortion product otoacoustic emissions remained detectable above the noise floor for every treated ear (mean, SD and maximum deviation from baseline: -1.3 , 9.0 , and -18 dB, respectively); and no animal exhibited a reduction of more than 3 % in vestibulo-ocular reflex gain during high-acceleration, whole-body, passive yaw rotations in darkness toward the treated side. All control ears and all operated ears with definite histologic evidence of injection through the intended site showed similar findings, with intact hair cells in all five inner ear sensory epithelia and intact auditory/vestibular neurons. The relative volumes of mouse, rat, rhesus, and human inner ears as measured by CT were (mean $\pm$ SD) 2.5 ± 0.1 , 5.5 ± 0.4 , 59.4 ± 4.7 and 191.1 ± 4.7 μL . These results indicate that injection of PBSV at volumes sufficient for gene therapy delivery can be accomplished without destruction of inner ear structures required for hearing and vestibular sensation.

Keywords: inner ear, volume, safety, auditory brainstem response, otoacoustic emission, vestibular ocular reflex, gene therapy

INTRODUCTION

Sensorineural losses of hearing and/or vestibular sensation due to hair cell dysfunction are common, disabling, and costly to individuals and to society (Sun et al. 2014). While cochlear implants can partially restore auditory sensation and vestibular implants may someday restore an artificial sensation of head move-

ment, neither of these interventions is likely to achieve function that is as good as might in theory be achieved through interventions that repair, regenerate, or replace hair cells. Recent preclinical research demonstrates that treatment of the inner ear with a variety of compounds can elicit regeneration or repair of hair cells in animals exposed to ototoxic medications (Jung et al. 2013; Mizutani et al. 2013; McCall et al. 2010; Staecker et al. 2011, 2014; Staecker and Rodgers 2013; Kraft et al. 2013; Praetorius et al. 2009; Klickstein 2013; Borenstein 2011). Delivery of gene therapy may also offer a means for treatment of hereditary hearing loss and vestibular dysfunction (Akil et al. 2015). However, uncertainty exists regarding the potential for inner ear trauma due to injection of fluid volumes sufficient to carry an adequate dose of a pharmacologic or gene delivery agent.

Clinical evidence from cochlear implantation surgeries performed with the intent to preserve residual natural hearing in the implanted ear demonstrates that displacement of a ~ 17 μL volume of perilymph by a cochlear implant stimulating electrode array inserted via the round window or a cochleostomy at ~ 100 $\mu\text{L}/\text{min}$ can be performed without degradation of natural hearing and vestibular sensation from preoperative levels (e.g., Helbig et al. 2013; Havenith et al. 2013; Erixon et al. 2012; Bruce et al. 2011; Brown et al. 2010). Moreover, insertion of a cochlear implant array through a narrow slit cut in the round window typically does not cause visible efflux of perilymph, so it seems likely that the human inner ear can tolerate volume displacement of that amount and rate without necessarily sustaining barotrauma that causes detectable post-implantation changes in hearing thresholds. However, hearing preservation after cochlear implant array insertion is an unpredictable outcome, and insertion of a silicone and metal device may not accurately model the risk of barotrauma during fluid injection.

The primary goal of the present study was to investigate this risk in a primate inner ear that closely models human anatomy, using an experimental design that is independent of effects caused by any particular pharmacologic agent or gene delivery vector. We accomplished this by assaying hearing and vestibular function before and after inner ear injection of fluid into the inner ears of preoperatively normal rhesus monkeys. To avoid confounding effects due to a particular active ingredient, only phosphate-buffered saline vehicle (PBSV) was injected—no investigational drugs were administered.

A secondary goal was to optimize the injection site, technique, and volume of administration into the inner ear perilymphatic space in this animal model, using techniques closely modeled on those under consideration for use in a planned human inner ear

gene therapy trial. Although rhesus and human temporal bone anatomy are very similar, the rhesus ear canal is only about half the caliber of a human ear canal, and this makes endaural (i.e., trans-ear canal) injection difficult in rhesus without a break in sterile technique. Moreover, the endaural approach results in at least temporary dislocation of the tympanic membrane and deliberate placement or post-operative accumulation of material in the ear canal, both of which can cause conductive hearing losses and confound measurements of cochlear function that depend on normal ear canal and middle ear function. We therefore performed injections via a transmastoid surgical approach, which provides sterile access to both the round window and the oval window in rhesus monkeys without entry into the ear canal or disruption of the ear drum, allowing for hearing measurements shortly after injection.

A final goal was to facilitate generalization of these physiologic results from rhesus macaques to humans and to other species that may be used for study of inner ear pharmacology. Toward that end, we measured total inner ear fluid space volume in temporal bone specimens from rhesus monkeys, humans, mice, and rats. Although other factors contribute to an inner ear's ability to undergo fluid injection without sustaining significant damage (including dimensions relative to surgical instruments and the amplitude of a surgeon's hand tremor; compliance of the stapes footplate and round window membrane, relative size of the endolymphatic and perilymphatic compartments; and relative patency of the cochlear and endolymphatic aqueducts), total inner ear volume is a readily measurable single quantity that provides a useful metric for the relative size of the compartment into which one is injecting. We therefore measured total inner ear volume for rhesus, human, mouse, and rat temporal bones using 3D reconstructions of micro-computed tomography (microCT) images.

METHODS

Overview

Three groups of rhesus macaque monkeys (*Macaca mulatta*, 4–7 kg, 4 years old, source: Johns Hopkins University Research Farm) were studied. Each group comprised three previously untreated animals without history of ear infection, ear surgery, meningitis, exposure to ototoxic agents, radiation, abnormally loud sound environment, signs of imbalance, or other risk factors for loss of inner ear or middle ear function. Group A (animals Rh24, Rh66 and Rh73) received right ear transmastoid/trans-oval window/stapes (TM/OW/S) injection of 10 μ L PBSV via a stapedotomy created using a clinical-grade fiber-

optically coupled carbon dioxide/infrared otologic laser. Group B (animals Rh12, Rh32 and Rh43) received right ear transmastoid/trans-round window (TM/RW) injection of 10 μ L PBSV. Group C (animals Rh36, Rh37 and Rh49) received right ear transmastoid/trans-round window (TM/RW) injection of 30 μ L PBSV.

We measured hearing and vestibular function before and 2, 4, and 8 weeks after unilateral injection of phosphate-buffered saline vehicle (PBSV) into the perilymphatic space of normal monkeys at volumes chosen because they would be sufficient to deliver an *atoh1* gene therapy vector (Klickstein 2013). To isolate effects of injection as opposed to agents used to create or treat hair cell injury, the study was performed in normal animals and involved PBSV without administration of potentially toxic or therapeutic agents. Hearing was assessed using auditory brainstem responses (ABR) and distortion product otoacoustic emissions (DPOAE). Vestibular sensation was assessed using vestibulo-ocular reflex (VOR) responses to whole-body passive yaw rotations in darkness. Behavioral assessments were also performed. Animals were then euthanized, transcardially perfused, and dissected for harvest and preservation of temporal bones, to allow for subsequent histologic examination in the event that hearing or vestibular assessments identified a deficit.

We also measured inner ear fluid space volume via 3D reconstruction of microCT images of adult C57BL6 mouse, Wistar rat, rhesus macaque, and human temporal bones ($N = 3$ each).

All experiments were performed in accordance with a protocol approved by the Johns Hopkins Animal Care and Use Committee, which is accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care (AAALAC) International and consistent with European Community Directive 86/609/EEC.

Surgical Procedures

Perioperative Anesthesia, Analgesia, and Antibiotic Administration. No agents known to be ototoxic were used in care of study animals, and no agents known to suppress vestibular reflexes were administered except as part of perioperative anesthetic and analgesic management. For procedures under general endotracheal anesthesia, a monkey was pre-treated with glycopyrrolate (0.005 mg/kg intramuscular injection, IM) or atropine (0.02–0.04 mg/kg IM or SC) to control secretions, then injected 15 min later with ketamine (10–12 mg/kg IM) prior to intubation. Dexamethasone 4 mg was given once intravenously (IV) with the intent of reducing fibrosis and inflammatory responses associated with inner ear surgical

procedures and for its anti-nausea effect. Local anesthesia for skin incision was achieved using subcutaneous injection field block with lidocaine 1–5 mg/kg as 1 % lidocaine in 1:100 K epinephrine solution prior to incision. Local anesthesia for the conjunctiva was achieved by administration of proparacaine hydrochloride ophthalmic solution 5 mg (5 %), 2–3 drops placed on the conjunctiva 1 min before procedure. Warm lactated Ringer's solution was administered via an IV placed in a limb vein. Enrofloxacin (10 mg/kg, IM) and cefazolin (25 mg/kg IV) were given prior to incision, with cefazolin dosing repeated every 2 h during surgery. After administration of the preanesthetic regimen, the animal was intubated and connected to a ventilator/gas vaporizer. Isoflurane in oxygen was delivered for induction (typically 5 %) and used for maintenance of anesthesia (typically 0.8–2 %). Oxygen flow was set at 700 mL/min (for non-rebreathing machines) or 300 mL/min (rebreathing machines).

Upon completion of surgery, animals were treated with buprenorphine (0.01 mg/Kg) IM BID and meloxicam (0.2 mg/kg) IM/PO QD or carprofen (4.4 mg/kg IM) just prior to cessation of isoflurane and then observed for postural imbalance, nystagmus and other signs of vestibular dysfunction as they recovered from anesthesia. Buprenorphine was repeated at 12 and 24 h post-operatively, and additional buprenorphine, meloxicam, and/or carprofen were provided (typically over the subsequent 3–7 days) until the animal appeared to be pain-free without medication. Animals also received enrofloxacin 10 mg/kg IM or PO for 5 days post-operatively.

Implantation of Head Cap/Bolt and Eye Coils for Vestibular Assessments. In preparation for vestibular function assessments, a head cap and post were placed to stabilize the animal in an experimental apparatus during vestibulo-ocular reflex testing. Our methods for surgical implantation of a head-fixation mechanism and scleral coils for magnetic search coil recording of 3D eye movements in primates have been described previously (Minor et al. 1999; Dai et al. 2011c). With the animal under general endotracheal anesthesia, a titanium head post was affixed to the skull under sterile conditions using blunt-tipped titanium or steel bone screws and poly-methyl methacrylate. During this portion of the surgery, a stereotactic frame was used to ensure that the post was placed in the midsagittal plane at a known angle with respect to the mean axis of the horizontal semicircular canals.

For subsequent use in measuring eye movement responses to head rotation, two wire coils were fashioned from polytetrafluoroethylene-coated, multistranded stainless steel wire (Cooner Wire, Chatsworth, CA) and sutured to the sclera of one eye, with one around the iris and the

other approximately orthogonal to the first. Wires were tightly twisted to reduce inductive artifacts and then tunneled subcutaneously to connectors within a plastic housing embedded in the head cap.

Inner Ear Injection. With the animal under general inhalational endotracheal anesthesia and using sterile technique, an incision was made behind the pinna and the mastoid bone was exposed. A cortical mastoidectomy was performed using a surgical drill. The facial recess was opened to provide access to the middle ear. For transmastoid/round window (TM/RW) injection, a small slit was made in the round window for access to the inner ear using the tip of a 25-gauge needle under direct visualization via a binocular operating microscope. For transmastoid/oval window/stapes (TM/OW/S) injection, a small hole was made in the oval window (i.e., the stapes footplate) using an otologic CO₂ laser (OmniGuide Surgical, Cambridge MA; OTO-S fiber, typically 3.5 W, 250 mS/pulse) for access to the inner ear, as is typically performed for stapedotomy in a clinical setting.

The phosphate-buffered saline vehicle used in this study was BSS® Balanced Salt Solution (Alcon Laboratories, Inc., Fort Worth, TX; http://ecatalog.alcon.com/PI/BSSbag500_us_en.pdf). Its composition is as follows: sodium chloride (NaCl) 0.64 %, potassium chloride (KCl) 0.075 %, calcium chloride dihydrate (CaCl₂·2H₂O) 0.048 %, magnesium chloride hexahydrate (MgCl₂·6H₂O) 0.03 %, sodium acetate trihydrate (C₂H₃NaO₂·3H₂O) 0.39 %, sodium citrate dihydrate (C₆H₅Na₃O₇·2H₂O) 0.17 %, sodium hydroxide, and/or hydrochloric acid (to adjust pH), and sterile water for injection. The pH is approximately 7.5. The osmolality is approximately 300 mOsm/kg. This solution was chosen because (1) it is FDA-approved for use in the human body (and is commonly used during ophthalmologic surgery), (2) it is readily available, and (3) it approximates the ionic composition of mammalian perilymph. The molarities of Na, K, and Cl are, respectively, 155, 10, and 129 in BSS and 152, 3.5, and 134 in an artificial perilymph commonly used in inner ear pharmacology experiments in mammals (e.g., Salt et al. 2006). A previously unopened, sterile BSS® vial was used for each animal.

Injection of PBSV was performed using a volume-controlled syringe pump (Unilife Corp., York, PA) connected via a thin, relatively noncompliant silicone tube to a 30-gauge, ~2.5-cm-long, blunt-end steel injection catheter. During injection, the catheter was held in place such that its tip was visibly inserted to a depth of ~0.2 mm through the OW or RW hole. Injection rate and volume settings are listed in Table 1. This procedure should be considered an “intent to treat” intervention, because (1) some reflux of PBSV and/or perilymph may occur around the

Table 1

Study design and animals randomized to each treatment group

Treatment group	Injection site	Flow rate ($\mu\text{L}/\text{min}$)	Injection volume (μL)	Number of animals	Animal ID numbers
A	Stapes/oval window	20	10	3	Rh24x, Rh66x, Rh73x
B	Round window	20	10	3	Rh12x, Rh32x, Rh43x
C	Round window	20	30	3	Rh36x, Rh37x, Rh49x

catheter at the injection site, making it impossible to precisely control the volume that is delivered to the inner ear and (2) despite direct microscopic observation of catheter insertion through surgically-created holes in the OW and RW, three specimens did not exhibit clear evidence of a healed injection-point injury on histologic examination (see “Results”).

After completion of the injection, a piece of fascia was harvested and placed over the injection point to occlude it. To avoid inner ear injury, fascia was not inserted into the perilymphatic space, but rather was stabilized in place over the entry to the inner ear by tucking it under the stapes arch or against bone of the posterior mesotympanum. The wound was sutured closed in layers, followed by application of skin glue.

Vestibulo-ocular Reflex Assessment

The vestibulo-ocular reflex (VOR) drives eye movements opposite the direction of head movement to maintain stable vision during head rotation. When the oculomotor system is intact (as it was in the animals used in this study) and the labyrinths are normal, VOR gain (the ratio of eye response angular velocity to head angular velocity) is close to 1 during rapid head rotations in darkness. When one labyrinth’s semicircular canal function is deficient, responses during rapid, passive whole-body rotation toward that side are slower or nonexistent, and VOR gain is decreased (Sadeghi et al. 2007; Dai et al. 2011b). VOR responses to rapid, high-acceleration, passive whole-body rotation to the right of an animal sitting upright in darkness are primarily driven by excitation of the right horizontal semicircular canal (SCC). Inhibition of the mirror-image left horizontal SCC also contributes to the response, but to a lesser degree, particularly for high-acceleration ($> \sim 1000 \text{ }^\circ/\text{s}^2$) head rotation stimuli. Therefore, normal VOR responses to rightward high-acceleration whole-body rotation in darkness indicate normal function of the right horizontal semicircular canal. (For a review of relevant VOR physiology, see Carey and Della Santina 2009.)

The eye coil system used to measure angular eye position has been described in detail previously (Robinson 1963; Migliaccio et al. 2004). The monkey was seated in a plastic chair with its head restrained atraumatically by the head post. Animals did not exhibit signs of distress or discomfort during testing.

The chair was mounted atop the Earth-vertical-axis rotator. Counterclockwise motor rotation as viewed from above (i.e., nose toward the left ear) was considered a positive sense rotation and excited the left horizontal SCC while inhibiting the right horizontal SCC. Three pairs of field-generating coils were rigidly attached to the chair and moved with the animal, generating three fields orthogonal to each other and aligned with the X (naso-occipital, +nasal), Y (interaural, +left), and Z (superoinferior, +superior) head coordinate axes. The X, Y, and Z fields oscillated at 79.4, 52.6, and 40.0 kHz, respectively. Signals induced across each scleral coil were demodulated to produce three voltages proportional to the angles between the coil and each magnetic field. They were then analyzed using 3D rotational kinematic methods detailed by Straumann et al. (1995). All signals transducing motion of the head or the eye were passed through eight-pole Butterworth anti-aliasing filters with a corner frequency of 100 Hz prior to sampling at 200 Hz. Coil misalignment was corrected using an algorithm that calculated the instantaneous rotation of the coil-pair with reference to its orientation when the eye was in a reference position (Tweed et al. 1990). Angular position resolution of the coil system was 0.2° (tested over the angular range of $\pm 25^\circ$ combined yaw, pitch, and roll positions), and angular velocity noise was $\sim 2.5^\circ/\text{s}$ peak.

During sinusoidal 0.05/0.1/0.2/0.5/1/2/5 Hz, $50^\circ/\text{s}$ peak velocity; passive, whole-body rotations in darkness about the mean horizontal semicircular canal axis (with the animal oriented to align that axis with the rotator’s Earth-vertical axis); eye movement angular position; and velocity data were acquired for the horizontal component of 3D eye movements, then separately averaged for ≥ 10 cycles free of saccades and blinks. For each response and for each stimulus trace, we used a single-frequency Fourier analysis to compute the magnitude of the single, symmetric best fit sinusoid, under the constraint that the response frequency was the same as the known stimulus frequency.

For responses to steps of acceleration, we computed the “acceleration gain” ratio ($G_A = \text{mean eye acceleration}/\text{mean head acceleration}$) during the constant-head-acceleration segment of the record, prior to the onset of the first nystagmus quick phase. This method is described in detail elsewhere (Migliaccio et al. 2004). Only the

horizontal component of the 3D VOR was used to compute G_A during responses to yaw rotations. Data from 10–20 stimulus trials in each direction were averaged to obtain a representation of the response.

Auditory Brainstem Response

Measurement of auditory brainstem responses (ABR) allows objective measurement of the threshold of auditory sensation as a function of stimulus frequency. The presence of normal ABR responses to air-conducted sound at normal thresholds indicates normal function of the ear canal, tympanic membrane, middle ear, inner ear, and auditory brainstem. The ABR recording technique used is similar to that we have previously described (Dai et al. 2011a); except that ABRs were acquired using a clinical-grade auditory evoked potential system (Auditory Evoked Potential System, AEP, Biologic-Natus) with sampling rate 100 kHz. ABR measurements were recorded with solid gel skin electrodes (GN Otometrics) attached in the midline above the supraorbital ridge, on the right and left ear lobes, and on the cheek ipsilateral to the test ear (ground). Individual electrode impedances were below 3 k Ω . Before any measurements, a sound level meter and calibrated microphone (Nicolet) were used to calibrate the ABR speakers in the sound-isolating experimental chamber.

During ABR measurements, animals were anesthetized with ketamine (5–10 mg/kg IM), seated on a chair inside the chamber and presented with clicks (duration of 100 μ sec) and tone pips at 1, 2, 4, and 8 kHz via ear-canal-insert speakers with alternating polarity and a Blackman window with 2-ms rise-fall and 1-ms plateau times (Etymotic ER-2, calibrated and flat to 16 kHz). An average ABR waveform was measured during 200 stimulus presentations at a rate of 30 per second. At each frequency, stimulus level was presented in 10 dBHL (human A-weighted per standard IEC 61672:2003) descending increments until the ABR waveform was no longer visible above the noise floor, then in alternating 5 dBHL ascending and descending increments until a clear threshold was identified. Because the system employed is designed and maintained for clinical use, it measures and reports sound intensities using dBHL as per clinical audiometric standards, rather than dB SPL. This choice of normalization does not affect comparisons between treated and untreated ears or for within-ear changes over time.

Distortion Product Otoacoustic Emissions

Distortion product otoacoustic emissions (DPOAE) are generated in the cochlea in response to two tones of a given frequency and sound pressure level

presented in the ear canal. Presence of normal DPOAEs (i.e., responses detectable above the measurement system's noise floor) are an objective indicator of normally functioning cochlear outer hair cells, and also indicate normal or near-normal sound transmission through the ear canal, ear drum and middle ear. DPOAEs were measured using a clinical-grade system (Biologic, Model 580-NAVPR2), two Etymotic ER-2 earphones, a probe unit containing an Etymotic ER-10B microphone, and an Etymotic amplifier providing 40 dB of amplification. Silicone tubing (0.95 mm OD, 0.5 mm ID) transmitted the sound generated by the two earphones through the probe housing and into the ear canal. Microphones were recalibrated before each DPOAE measurement.

The cochlea was stimulated with two primary frequencies (f_1 and f_2 , where $f_2 > f_1$), and the distortion product ($2f_1 - f_2$) emitted by the cochlea was measured. These emissions were measured using an f_2/f_1 ratio = 1.22 while f_2 was varied over 1, 2, 3, and 8 kHz. Tone levels of primary frequencies (L_1 and L_2) were constant at 65 and 55 dB SPL, respectively. Absolute DPOAE level and noise floor level were measured, and DPOAE/noise floor ratio (DP-NF) was determined by subtracting the noise floor in dB from the DPOAE level in dB.

Histology

Histologic processing of temporal bone blocks harvested after transcardiac paraformaldehyde perfusion was optimized for examination of both middle and inner ear structure, with a particular focus placed on examination of the oval and round windows (to determine presence or absence of evidence of injection) while maintaining some ability to examine hair cells, the membranous labyrinth, and other inner ear structures. Neither the stapes footplate nor the round window was deliberately disrupted as would typically be done to allow intracochlear perfusion with fixative when only inner ear tissues are of interest. Tissues from the inner ear were retained in neutral buffered 10 % formalin for at least 48 h, after which time they were transferred to an ethylenediaminetetraacetic acid (EDTA) solution for decalcification. Tissues were transferred to fresh EDTA periodically for several months until fully decalcified, then embedded in paraffin blocks, step-sectioned (10 μ m sequential sections), mounted on glass slides, and stained with hematoxylin and eosin (H&E) for microscopic exam.

Histopathologic evaluation was performed by three examiners, two of whom were initially blinded to animal ID, ear and surgical history for a given set of specimens until after reporting analysis of histopathologic features. Blinding was limited by the fact that the mastoidectomy in each operated (i.e., right) ear

resulted in an obvious absence of bone septae in the mastoid sinus, providing an obvious clue to a history of surgery but not to the site of injection. The fixation, blocking, embedding, sectioning, staining, and microscopy techniques we employed were sufficient to examine specimens for the presence of intact neurons, but we did not perform rigorous stereologic counting of all neurons in each specimen.

Specimen Processing, Imaging, Reconstruction, and Analysis for Volumetric Measurements

Three postmortem temporal bone specimens of each species were used to measure total inner ear volume. Adult C57BL6 mouse, Wistar rat, and human specimens were denuded of soft tissue and dried; monkey specimens were imaged without soft tissue removal, so that they would be available for subsequent histologic study. Mouse specimens were imaged in a microCT imaging unit (Scanco, Bassersdorf, Switzerland) using isotropic voxels measuring 16 μm per side, as has been previously described (Calabrese and Hullar 2006). Rat, monkey, and human specimens were imaged in the Johns Hopkins Small Animal Imaging Resource Center MicroCT using isotropic voxels measuring 70 μm per side for rat and monkey and 100 μm per side for human. Image stacks were reconstructed by semi-automatic segmentation, smoothed in 3D and translated into volume using Amira software (Visualization Sciences Group). During segmentation of CT images, we did not attempt to separately segment the cochlear or vestibular aqueducts or membranous labyrinth; instead, we segmented the osseous labyrinth (i.e., the space enclosed by the endosteal lining of the semicircular canals, vestibule, and cochlea). The volume was determined by multiplying the number of voxels by the volume per voxel.

Statistical Analysis

Statistical analysis was performed using R and SAS® to evaluate two predictions/hypotheses for each dependent variable: left and right ear data do not significantly differ for normal monkeys prior to treatment (hypothesis H1); and compared to pre-treatment control data, there are no significant, persistent changes in right/treated ear data after inner injection (hypothesis H2). Due in part to the necessarily small sample size per treatment group in this study of nine nonhuman primates divided into three groups of three animals per treatment subjected to repeated measures over time, data did not always meet normality criteria required for application of standard analysis of variance (ANOVA), general linear models, and post hoc *t* tests. In those cases, we employed

nonparametric analyses, including the two-way Friedman ANOVA with repeated measures and post hoc Wilcoxon signed-rank tests, which do not require normally distributed data and do incorporate corrections for small sample size and multiple comparisons. (Hill and Lewicki 2007)

Comparison of paired left vs. right ear ABR data for normal/pretreatment monkeys was performed to test H1 using a Friedman two-way repeated measures analysis of variance [ANOVA] by ranks (i.e., the nonparametric alternative to ANOVA with repeated measures) followed by post hoc left-right comparisons for each ABR stimulus frequency using the Wilcoxon signed-rank test with adjustment of α for each comparison from 0.05 to $1-0.95^{(1/5)} = 0.01$ to account for the five individual tests representing data for click, 1-, 2-, 4-, and 8-kHz stimuli. H2 was evaluated for ABR data using only data from right ears, with a Linear Mixed Model and corresponding *F* test used to test whether there was any significant persistent change in right ear ABR threshold after inner injection. H1 and H2 were analogously tested for OAE, sinusoidal VOR gain, and G_A data, with the following exceptions.

For OAE data, we anticipated that presence of a post-operative middle ear effusion that would interfere with conductive hearing to such an extent that OAEs might not be detectable above the noise floor. We therefore tested H1 as defined above, and (hypothesis H3) compared to pre-treatment controls, DP-NF is detectable significantly above zero at every time point. For those data, H1 was tested using Friedman and Wilcoxon signed-rank tests, and H3 was tested using a Linear Mixed Model. For sinusoidal VOR gain testing, we did not attempt to extract half-cycle gains, because we anticipated that head acceleration was not high enough during those stimuli to selectively test the function of only one ear by effectively silencing input from the other. We therefore did not attempt to test H1 for that case.

RESULTS

Confirmation of Intended Intervention

All nine injections were completed under direct binocular microscopic view, with the surgeon (CCDS) confirming insertion of the injection cannula through the intended site in each case. However, subsequent histologic exam (described in greater detail below) did not always identify signs that an injection catheter had been inserted all the way through the round window. Evidence of catheter penetration through the oval or round window was clearly apparent in six of nine operated ears; however, the right temporal bones of Rh43X (group B), Rh12X (group B), and Rh49X (group C) did not exhibit

Fig. 1. Auditory brainstem response (ABR) thresholds in response to clicks and pure tones at 1, 2, 4 and 8 kHz of three monkeys/group in three treatment groups. *Top row:* group A (right ear transmastoid/trans-oval window/stapes [TM/OW/Stapes] injection of 10 µL phosphate-buffered saline vehicle [PBSV] via a stapedotomy). *Middle row:* group B (right ear transmastoid/trans-round window [TM/RW] injection of 10 µL PBSV). *Bottom row:* group C (right ear

TM/RW injection of 30 µL PBSV). *N* and *black triangles/error bars:* mean $\pm$ SD thresholds measured in 18 normal rhesus ears before any manipulation. *L* left/untreated; *R* right/treated; *0* before injection; 2, 4, 8 number of weeks after right inner ear PBSV injection. Threshold units are dB HL as reported by a clinical ABR system using the human A-weighted scale. Monkey 24X=●, 73X=●, 66X=●, 32X=◆, 43X=◆, 12X=◆, 36X=■, 37X=■, 49X=■.

obvious histologic evidence of round window disruption. That does not necessarily mean that no injection

was performed, because artifacts due to temporal bone harvesting and processing can obscure indica-

Fig. 2. DP-NF (i.e., DPOAE amplitudes relative to noise floor amplitude, in dB) at $f_2 = 1, 2, 4$, and 8 kHz for the same treatment conditions, animals, ears, and time points shown in Fig. 1. Monkey 24X=●, 73X=●, 66X=●, 32X=◆, 43X=◆, 12X=◆, 36X=■, 37X=■, 49X=■. A DP-NF value >0 indicates presence of functional outer hair cells in cochlea.

tions of surgical trauma, especially if that trauma is remote in time and minimal. In fact, one control/left ear was read as having damage to the round window, and two right ears in group C were read as having displacement of the stapes, despite direct in-life confirmation of stapes integrity by microscopic exam immediately after round window injection during surgery and again during temporal bone harvesting. These findings suggest that identification of presence or absence of surgical trauma to the oval or round window via blinded histopathologic exam can be confounded by histologic processing artifacts. Physiologic and histologic data presented below should be considered in light of the lack of histologic corroboration of catheter penetration for right temporal bones of Rh43X, Rh12X, and Rh49X.

Behavioral Observation

All animals resumed normal activity by 48 h post-injection, and all but one behaved normally in the first post-operative day. Animal Rh66X (group A) exhibited postural imbalance signs suggesting an acute but mild vestibular weakness in the first 48 h after trans-stapes injection. In this animal, surgical drilling required to obtain access for the trans-stapes

approach from the mastoid cavity led to “blue-lining” (exposure but not violation) of the right horizontal semicircular canal endosteum. The animal’s behavior was otherwise normal, and its posture, ambulation, and activity were indistinguishable from normal by post-op day 3.

Auditory Brainstem Responses

ABR thresholds were tested in the 18 ears of the 9 normal rhesus monkeys before any ear surgical procedure. Mean $\pm$ SD normal/pre-injection ABR hearing thresholds for clicks and for 1-, 2-, 4-, and 8-kHz tone pips were 32.2 ± 3.1 , 35 ± 2.4 , 30.6 ± 3.4 , 27.8 ± 2.6 , and 21.9 ± 3.5 dB HL (A-weighted), respectively. These thresholds are similar to previously reported results for this species (Dai et al. 2011a; Torre et al. 2004).

Figure 1 shows all ABR threshold data for every treatment group, animal, ear, stimulus (clicks and 1-, 2-, 4- or 8-kHz tone pips) and time point. The pre- to post-operative change in ABR hearing threshold for the *non-injected* (left) ear was less than 5 dB for every measurement at every time point except for a single instance (monkey Rh24X at 8 kHz on week 4) out of 45 measurements. This provides an estimate of test-

Fig. 3. VOR Gain [eye velocity/head velocity] during eye movement responses to passive sinusoidal whole-body rotation in darkness at frequencies of 0.05 to 5 Hz. *N* normal, mean $\pm$ SD VOR gain measured in all nine monkeys before inner ear injection; *B* VOR gain data from (Dai et al. 2012) for four rhesus monkeys with bilateral

vestibular deficiency after bilateral ototoxic injury; *0* before injection; 2, 4, 8 number of weeks after inner ear PBSV injection. Monkey 24X= $\circ$, 73X= $\bullet$, 66X= $\circ$, 32X= $\diamond$, 43X= $\diamond$, 12X= $\diamond$, 36X= $\square$, 37X= $\square$, 49X= $\square$. SD < 0.03 when SD bars are not visible for *N* and *B* data.

retest variation in ABR threshold over time independent of inner ear injection and is similar to the variability reported by Torre et al. (2004) for normal rhesus monkeys. The fact that rhesus pretreatment threshold levels cluster around ~30 dB HL and apparently trend toward reduced ABR threshold (i.e., improved hearing) with increasing frequency represents interspecies difference between rhesus and humans, for whom the audiologic equipment used was calibrated. Therefore, only comparisons across time and left/right comparisons should be used as assays of change in hearing.

Left vs right comparisons of normal monkeys for a given stimulus frequency revealed no significant differences (Friedman two-way ANOVA: $\chi^2 = 3$, $P = 0.083$). Assuming the click ABR threshold mean and variance for all 18 normal ears are accurate estimates of the true population, and assuming equal variances for pre- and post-injection measurements, a paired *t* test comparing three post-injection ears vs. 18 normal ears should have statistical power of 90 % to detect a true click ABR threshold increase of 8 dB.

Compared to pre-treatment controls, there was no significant, persistent change in right ear ABR thresholds after inner injection (Linear Mixed Model: $F(1138) = 1.27$, $P = 0.2609$). At the 8-week, post-treatment measurement, the mean change in ABR threshold from baseline (for a given ear and stimulus frequency) was 0.34 dB (SD 3.2 dB), and no treated ear's ABR threshold for any stimulus frequency was more than 5 dB worse (greater) than that ear's pre-treatment ABR threshold. The greatest pre- to post-injection change in ABR threshold in any individual injected ear over all time points was a single instance of a +15-dB change (monkey Rh37X, right ear response to 8 kHz tones 2 weeks after injection). Notably, the ABR threshold shift in this individual was accompanied by a change in middle ear compliance suggesting middle ear effusion (which is commonly observed after mastoid surgery), wax in the ear canal, or both. Presence of wax impaction was confirmed on otoscopic examination at the time of the post-op week 4 measurement, and the wax was removed prior to that test. ABR threshold returned to normal and was still normal at the 8-week, measurement.

Distortion Product Otoacoustic Emissions

We tested DPOAE responses in the 18 ears of the 9 normal rhesus monkeys before any ear surgical procedure. Mean $\pm$ SD pre-injection/normal DPOAE amplitudes for $f_2 = 1, 2, 4$, and 8 kHz were 12.1 ± 4.7 , 14.5 ± 4.5 , 15.1 ± 5.7 , and 19.6 ± 5.8 dB SPL, respectively. Mean $\pm$ SD pre-injection DP-NF values (DPOAE amplitude relative to the noise floor amplitude) for $f_2 = 1, 2, 4$, and 8 kHz were 12.6 ± 5.8 ,

21.8 ± 8.5 , 25.6 ± 6.5 , and 30.1 ± 9.6 dB, respectively. These results are within the range previously reported for the same species (Dai et al. 2011a; Lasky et al. 1995; Martin et al. 1988; Park et al. 1995). Because absolute DPOAE and relative DP-NF values are equal except for correction due to variation in the noise floor, further analysis focused on DP-NF, which yields a more meaningful comparison across test sessions. Figure 2 shows all DP-NF data for every animal, ear, stimulus, and time point.

Pre- to post-intervention mean change in DP-NF relative amplitudes for non-injected (left) ear was less than 10 dB for every group at 8-week, post-injection. This provides an estimate of test-retest variation in DP-NF results over time independent of inner ear injection, and is similar to the variability previously reported (Dai et al. 2011a).

Left vs right comparisons of normal monkeys for a given stimulus frequency revealed no significant differences in DP-NF (Friedman Two Way ANOVA: $\chi^2(1) = 0$, $P = 0.92$). Assuming the DP-NF mean and variance for 18 normal ears at $f_2 = 4$ kHz are accurate estimates of the true population distribution, and assuming equal variances for pre- and post-injection measurements, a paired *t* test comparing three ears before and after surgery should have statistical power of 90 % to detect a true 4 kHz DP-NF change of 16 dB and 80 % power to detect a change of 14 dB.

The variability of these OAE measurements is such that they are most conservatively interpreted as categorical, with the presence of a DPOAE above the noise floor indicating detectable cochlear outer hair cell function and a middle ear capable of transmitting acoustic vibrations both inward and outward. Compared to pre-treatment measurements, DP-NF values did vary over time; however, no treated ear's DP-NF at 8 weeks post-treatment had worsened by more than 18 dB at any stimulus frequency (mean, SD, and maximum deviation from baseline: -1.3 , 9.0 , and -18 dB, respectively), and DP-NF values were detectable significantly above zero for every ear at every time point (ANOVA for comparison of each group and each time point vs pretreatment control: $F(1251) = 18.2$, $P = 0.000081$).

The group A 8-kHz data for weeks 2 to 8 exhibited a right ear post-injection DP-NF drop and apparent recovery over time (compare left and right ear data), but the time 0 data were obtained just *before* injection, so they do not indicate a change in hearing related to injection.

Vestibular Assessment (Horizontal Vestibulo-ocular Reflex)

The sinusoidal steady-state VOR gain measured during 10–20 cycles of 50 °/s peak passive, whole-body

rotations in darkness about the mean horizontal semicircular canal axes, aggregated over all nine monkeys prior to injection, was (mean $\pm$ SD) 0.85 ± 0.04 , 0.89 ± 0.02 , 0.93 ± 0.01 , 0.96 ± 0.02 , 1.01 ± 0.01 , 1.01 ± 0.02 , and 1.01 ± 0.02 at 0.05, 0.1, 0.2, 0.5, 1, 2, and 5 Hz, respectively. These results are similar to data previously reported for the rhesus VOR during yaw rotations about an Earth-vertical axis (Dai et al. 2011b; Fetter and Zee 1988; Sadeghi et al. 2006).

Fig. 4. Mean $\pm$ SD VOR "acceleration gain" G_A [eye velocity/head velocity, averaged over the constant-acceleration segment of the head movement] for whole-body, passive $1000^\circ/\text{s}^2$ impulse head rotations in darkness before (0) and at 2, 4, and 8 weeks after right inner ear PBSV injection. *N* Normal, *B* bilateral vestibular deficiency due to intratympanic administration of gentamicin (Dai et al. 2011b). *Left* panels show data for leftward head turns, predominantly reflecting left (non-injected ear) horizontal semicircular canal function. *Right* panels show rightward rotation data, predominantly reflecting right (treated ear) horizontal semicircular canal function. Monkey 24X=○, 73X=●, 66X=○, 32X=◇, 43X=◇, 12X=◇, 36X=□, 37X=□, 49X=□. SD <0.03 when SD bars are not visible beyond markers for *N* and *B* data. RW injected via round window, OW injected via oval window (stapes).

Figure 3 shows mean sinusoidal VOR gain data for every animal, ear, stimulus frequency, and time point as measured using $50^\circ/\text{s}$ peak velocity stimuli. For comparison, Figure 3 also shows data from Dai et al. (2011b) for monkeys with bilateral loss of vestibular sensation after ototoxic injury due to middle ear application of gentamicin. Compared to pre-treatment measurements, sinusoidal VOR gain values varied little over time: excluding one apparent outlier

Fig. 5. Representative hematoxylin and eosin stained light microscopy images of treated right ears. **a** In an animal that underwent oval window injection (group A monkey 24X right ear), the mastoid exhibits fibrous tissue and minimal inflammation after surgical mastoidectomy, with preservation of the horizontal SCC (*H*), facial nerve (*FN*), incus (*I*), and tympanic membrane (*TM*). **b** In the same ear, a part of the stapes footplate has been replaced by a thin fibrous scar band (arrow), indicating the site of catheter penetration and injection. Blood products are visible with occasional mononuclear cells. **c** In an animal that underwent round window injection (group B monkey 12X right ear), the round window (arrow head) appears to be intact without evidence of fibrosis or fenestration. Blood products are visible in the round window niche.

(an 11 % drop for Rh24X at 0.1 Hz), no treated ear's sinusoidal VOR gain at 8 weeks post-treatment had worsened by more than 6 % from its pretreatment value, and the mean, SD, and maximum deviation from baseline were -0.1 , 2.5 , and -5.4 %, respectively.

Transient, high-acceleration, passive head or whole-body rotations are more sensitive than sinusoids as probe stimuli for detection of unilateral loss of semicircular canal sensation; however, we found no significant changes in VOR gain under that more stringent test condition. Prior to ear injections, responses of all nine monkeys during acceleration segment VOR gains measured during 20 cycles of $1 \text{ k } ^\circ/\text{s}^2$ (to $150 \text{ } ^\circ/\text{s}$ plateau) passive, whole-body transient rotations in darkness about the horizontal semicircular canal axes were similar for leftward (Mean $\pm$ SD G_A 1.01 ± 0.01) and rightward (1.02 ± 0.02) rotations and therefore pooled (1.01 ± 0.02). These results are similar to data previously reported for the same species (Dai et al. 2011b, c).

Figure 4 shows transient VOR gain data for every animal, ear, stimulus frequency, and time point. For comparison, Figure 4 also shows data from Dai et al. (2011b) for monkeys with bilateral loss of vestibular sensation after ototoxic injury due to middle ear application of gentamicin. Compared to pretreatment measurements, G_A varied little over time: no treated ear's G_A at 8 weeks post-treatment had worsened by more than 2 % from its pretreatment value, and the mean, SD, and maximum deviation from baseline were -0.3 , 1.2 , and -2 %, respectively.

Histology

All 18 temporal bones were harvested and processed, but the cochlea of the left (control) ear of Rh36X (group C) was transected during temporal bone harvesting. The vestibule and semicircular canals and middle ear structures were assessed histologically for that specimen, but no histologic data were acquired for its cochlea. As noted above (see “Confirmation of Intended Intervention”), all surgically treated temporal bones exhibited clear evidence of entry into the inner ear via the intended injection route except for those of Rh43X (group B), Rh12X (group B), and Rh49X (group C).

All 18 temporal bones, including all control ears and all operated ears, exhibited similar findings, including presence of intact hair cells in all inner ear sensory epithelia, intact auditory/vestibular neurons, signs of post-mortem trauma due to bone harvesting/trimming, presence of products of blood breakdown in the area of the injection, absence of bacteria or inflammation that would suggest active or recent infection, and absence of fibrosis or osteoneogenesis within perilymphatic spaces of the inner ear. In both treated and untreated ears, Reissner's membrane was intact in some sections while being displaced in adjacent sections, more consistent with post-mortem sectioning artifacts in this thin structure than injection trauma.

Figure 5a shows representative H&E stained light microscopy images of the middle ear and the oval and round windows in treated right ears. In every operated ear, the mastoid exhibited removal of

Fig. 6. Representative hematoxylin and eosin stained light microscopy images of the cochleae in a normal ear (**a**, **e** monkey 37X left ear) and the treated right ears from animals in group A (**b**, **f** monkey 24X), group B (**c** **g** monkey 12X), and group C (**d**, **h** monkey 36X). Mid-modiolar sections (**a–d**, 2.5X) show intact basal, mid, and apical

turns in normal ear and all treatment groups. High magnification images of the organ of Corti for the same specimens (**e–h** 20X) show variable disruption of Reissner's membrane (RM) but the presence of intact inner (arrow head) and outer hair cells (arrow) and cochlear nerve fibers. ST scala tympani, SM scala media, SV scala vestibuli.

bone septae and presence of fibrous tissue and minimal inflammation in the mastoid cavity (typical after surgical mastoidectomy) with preservation of the horizontal semicircular canal, facial nerve and tympanic membrane. Figure 5b shows that in an ear that underwent oval window injection (Rh24X right ear, group A), part of the stapes footplate has been replaced by a thin fibrous scar band (arrow), indicating the site of catheter penetration and injection. Blood products are visible, as are occasional mononuclear cells. Figure 5c shows a representative section for the right ear of an animal that underwent attempted/intended round window injection (Rh12X right ear, group B) in which the round window (arrow head) appears to be intact without evidence of significant fibrosis or fenestration. Products of blood breakdown are evident in the round window niche.

Figure 6 shows representative light microscopy images of the cochleae of study ears. Mid-modiolar

sections (a–d) reveal intact basal, mid, and apical turns across treatment groups, without evidence of infection, scar formation, or osteoneogenesis. High magnification images of the organ of Corti (e–h) reveal the presence of intact hair cells, outer hair cells, and cochlear nerve fibers.

Figure 7 shows representative light microscopy images of cristae, utricles, and saccules in three study ears, one from each group. Although histologic artifacts and differences in the plane of section result in variation across specimens, all specimens revealed intact hair cells in vestibular neuroepithelia, intact neurons, and absence of intraluminal fibrosis or signs of infection.

MRI or CT Assessment of Inner Ear Fluid Volume

Figure 8 shows examples of 3D reconstruction surface model views for one specimen of each species, and Table 2 summarizes volumetric results for all speci-

Fig. 7. Representative light microscopy images of cristae (a, d, g, j), utricles (b, e, h, k), and saccules (c, f, i, l) in three study ears, one from each group. a–c Monkey 73X left ear, normal. d–f Monkey 73X right ear, group A. g–i Monkey 32X right ear, group B. j–l Monkey

36X right ear, group C. Although histologic artifacts and differences in the plane of section are apparent, all specimens exhibit intact hair cells in the neuroepithelia, intact neurons, and absence of intraluminal fibrosis or signs of infection.

mens. According to these data, the 191 ± 16 μL total fluid space volume of the human inner ear is approximately 76, 35, and 3.2 times that of C57BL/6J mice, Wistar rats, and rhesus monkeys, respectively. Conversely, mice, rats and monkeys had mean inner ear volumes that are 1.3, 2.9, and 31 % of the human volume, respectively. It is interesting to note that the 10 and 30 μL volumes injected in this study represent ~16 and 50 %, respectively, of the ~60 μL total rhesus inner ear volume. Combined with the observation during surgery that reflux of fluid back out around the injection catheter during injection was not prominent, these data suggest that some perilymph or injected fluid left the inner ear via another path, such as the cochlear aqueduct and/or through expansion of the perilymphatic compartment allowed by movement of endolymph out of the membranous labyrinth and into the endolymphatic sac.

DISCUSSION AND CONCLUSIONS

These data reveal that neither injection of 10 μL PBSV via the oval window (i.e., through the stapes footplate) after laser stapedotomy nor injection of 10–30 μL via the round window causes significant physiologic signs of injury to structures necessary for normal auditory and vestibular function. If these findings can be directly extrapolated from rhesus to human inner ear volumes, then the human inner ear should be able to tolerate trans-stapes or trans-round window injection of ~30–90 μL of PBSV.

This study should be considered an “intent to treat” study, because reflux of fluid around the injection catheter and out of the inner ear prevents one from knowing with certainty whether the entire volume intended for the inner ear actually entered and remained in the inner ear. The uncertainty is

Fig. 8. Inner ear fluid space volume surface reconstructions from micro-computed tomography scans of **a** C57BL/6 J mouse, **b** Wistar rat, **c** rhesus monkey, and **d** human.

TABLE 2

Total inner ear volumes (μL) in mouse, rat, rhesus, and human

Specimen	C57BL6 mouse	Wistar rat	Rhesus monkey	Human
	Voxel length 16 μm	70 μm	70 μm	100 μm
1	2.5	5.5	54.9	185.5
2	2.4	5.9	64.3	179.0
3	2.6	5.1	59.1	208.9
Mean	2.5	5.5	59.4	191.1
SD	0.1	0.4	4.7	15.7

increased by the lack of histologic evidence of catheter penetration in three specimens. However, even if the three temporal bones without clear evidence of injection trauma at the stapes or round window did not receive the same treatment as the other surgically treated ears (which seems unlikely given the direct microscopic view during surgery), results observed for the six temporal bones with certain histologic evidence of injection still provide strong evidence in support of this conclusion.

Although changes over time were noted in ABR threshold, DPOAE amplitude, DP-NF relative amplitude, sinusoidal VOR gain, and transient VOR gain, and although individual measurements occasionally fell outside of narrow $\pm 2\text{SD}$ normative data ranges established by measurements made prior to injection, we observed no loss of hearing >5 dB by ABR threshold or loss of vestibular function greater than 11 % of pre-treatment G_A at 8 weeks in the injected ear of any animal. Results for related tests in clinical settings help provide context for interpreting the clinical significance of the changes we observe. Studying test-retest reliability of standard clinical audiometric equipment, Stuart et al. (1991) found that the critical difference for auditory thresholds over the 1–8-kHz range in young adults without known or suspected hearing loss ranged from 8.8–13.0 dB. Studying the head thrust test in 19 normal human subjects undergoing passive, transient, high-acceleration yaw head rotations similar to the motion stimuli we used in the present study, Schubert et al. (2006) found that the VOR gain G_A was 0.94 ± 0.06 . Based on that study, a decrement in G_A of up to $2 \times \text{SD} = 0.12$ or 13 % would be considered within normal range in a clinical setting. In the current study, no treated ear's G_A at 8 weeks post-treatment had worsened by more than 2 % from its pretreatment value.

One group A animal (Rh66X), in which the endosteum of the horizontal semicircular canal was inadvertently exposed during surgery, exhibited mild postural signs of imbalance during the first two post-operative days, and then fully recovered to normal activity. This was likely unrelated to the trans-stapes injection and instead reflects the added surgical

drilling required to access the stapes from the mastoid cavity approach (as opposed to the endaural approach employed in human application (Klickstein 2013)) and the comparatively small size of the rhesus temporal bone. One group C animal (Rh37X) exhibited a self-limited worsening of ABR threshold at the 2-week measurement associated with a wax impaction seen on otoscopic exam. Both animals exhibited normal ABR, DPOAE, and VOR function at 8 weeks post-op. No other animal exhibited any sign suggestive of deficits in hearing or vestibular function.

In clinical otologic practice, middle ear and mastoid surgery almost invariably causes at least a transient accumulation of fluid in the middle ear and, consequently, a transient conductive hearing loss that resolves as the middle ear is cleared via the Eustachian tube over the initial 2–4 weeks after surgery. Small changes observed in rhesus ABR thresholds and DPOAE amplitudes in the early post-operative period therefore reflect the expected post-operative course after middle ear and mastoid surgery. In the present study, data obtained at 8 weeks post-operatively are the best indicator of long-term cochlear and vestibular function. These data indicate that administration of PBSV directly into the perilymphatic space of the inner ear either via the oval window and stapes footplate or via the round window does not cause a persistent, functionally significant change in hearing or vestibular sensation as measured using ABR thresholds, presence of otoacoustic emissions, and vestibulo-ocular reflex gains. More subtle changes might have been identified using other assays beyond these tests, which we chose to emulate the test battery required to assess safety in the CGF166 first-in-human clinical trial of inner ear gene therapy (Klickstein 2013). For example, ABR growth function analysis might provide additional detail regarding changes in hair cell and spiral ganglion neuron health and survival. Nonetheless, the comparatively coarse methods we employed provide meaningful data that are directly comparable to standard clinical metrics.

Several previous studies investigated perilymph and endolymph volumes across species (Shinomori et al. 2001; Buckingham and Valvassori 2001; Zou et al. 2010; Ekdale 2013). Our volumetric data agree approximately with a recently published study by Ekdale (2013) describing inner ear volume in multiple mammalian species. Ekdale reported inner ear volumes (combined cochlea + vestibular labyrinth volume) of 2.3, 63 and 237 μL for individual specimens of mouse, rhesus monkey and human temporal bones, respectively, with mouse and rhesus monkey having 0.9 % and 26.5 % the inner ear volume of human respectively. In that study, Ekdale noted a strong linear correlation between inner ear volume and body mass ($r = 0.95$ and $p < 0.01$ when

data for 36 species ranging in size from mice to whales are logarithmically scaled). It is therefore tempting to simply extrapolate between animals and humans for any inner ear injection safety study; however, the extent to which an inner ear's delicate contents can withstand injection of fluid likely depends not only on the ear's fluid volume but also on the relative patency of the cochlear/perilymphatic and vestibular/endolymphatic aqueduct systems. Therefore, the ultimate determination of whether the human inner ear can tolerate injection of fluid volumes large enough to carry gene therapy agents in sufficient dose can only come from studies in humans, such as the CGF166 trial, which is currently underway.

Although the surgical approaches and injection methods we employed were limited to those that were under consideration for a planned human inner ear gene therapy trial (Klickstein 2013), microinjection of gene therapy or other agents directly into the scala media or elsewhere in the endolymphatic compartment is another option worthy of consideration, particularly because it may ultimately prove more effective at delivering agents to hair cells and supporting cells. Wise et al. 2010 found, in guinea pigs, that injection into scala media resulted in more localized gene expression, neuron survival, and peripheral projections of nerve fibers than did injection into the guinea pig scala tympani. However, our focus in the current work was on assessing the risk of injecting PBSV into the primate inner ear using techniques closely modeled on stapedotomy and cochlear implantation, two common and well-established techniques that are routine in clinical otologic surgical practice. Direct injection into the scala media in humans is not a standard surgical technique, and the techniques typically used for performing it in research animals (e.g., using a motorized microdrive to advance a glass micropipette being used to monitor endocochlear potentials (Wise et al. 2010)) are not easily translated into a clinical setting. Injection into the endolymphatic sac might offer a clinically tractable alternate route for entry, given that most ear surgeons have experience with endolymphatic sac surgery; however, that approach requires a mastoidectomy, whereas the oval and round windows can be accessed in humans via a more limited endaural (i.e., through the ear canal) approach

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