Posterior Semicircular Canal Gene Delivery in the Adult Mammalian Inner Ear

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Abstract

Hearing loss is a common disability affecting the world's population today. While several studies have shown that inner ear gene therapy can be successfully applied to mouse models of hereditary hearing loss to improve hearing, most of these studies rely on inner ear gene delivery in the neonatal age, when mouse inner ear has not fully developed. However, the human inner ear is fully developed at birth. Therefore, in order for inner ear gene therapy to be successfully applied in patients with hearing loss, one must demonstrate that gene delivery can be safely and reliably performed in the mature mammalian inner ear. The posterior semicircular canal approach has been shown to be an effective gene delivery method in the neonatal mouse inner ear. In this study, we examine the steps involved in posterior semicircular canal gene delivery in the adult mouse inner ear. We observe that the adult mouse inner ear is more susceptible to surgical trauma. We also find that the duration of perilymphatic leakage and injection rate have a significant effect on the post-surgical hearing outcome. Our results show that AAV2.7m8 is capable of transducing the adult mouse inner and outer hair cells with high efficiency.

Introduction

Hearing loss is a common disease process affecting the world's population today. Approximately 3 in every 1000 newborns are affected by hearing loss every year[1]. Over the past few years, several studies have shown that inner ear gene therapy is effective at improving the auditory function in mouse models of hereditary hearing loss[2]. In most of these studies, gene delivery is done in the neonatal age (< P5). One major difference between human and mouse ears is the fact that the auditory system is fully mature at birth in humans, whereas the onset of hearing is not until ~ P12 in mice[3]. The mouse inner ear is immature at birth and continues to undergo development after birth[3,4]. Therefore, in order for inner ear gene therapy to be successfully translated to patients with hereditary hearing loss, one needs to demonstrate that gene therapy can be effective when delivered to the mature mammalian inner ear. In addition, one also needs to identify viral vectors which can successfully transduce target cells in the mature mammalian inner ear.

Various gene delivery methods have been examined for delivering gene therapy to the inner ear in animal models of hearing loss[5]. Three delivery methods (cochleostomy, round window injection, and canalostomy) are commonly used in mice[6]. Cochleostomy allows for transgene delivery directly into the scala media, where the mechanosensory hair cells in the cochlea are located. Even though one study showed no hearing loss in adult mice with this approach[7], other studies have shown that this surgical approach causes significant hearing loss, likely due to the trauma incited by drilling through the lateral wall of the cochlea[8–10]. Round window injection is another method for administering gene therapy into the inner ear. The round window is a membranous structure at the base of the cochlea which separates the middle ear and the cochlea. It can be accessed via the middle ear after the opening of the tympanic bulla. Despite being less invasive than cochleostomy, it can lead to middle ear effusion, which negatively affects hearing temporarily[11]. In addition, the transduction efficiency of round window injection is not

evenly distributed throughout the cochlear turns, with a lower transduction rate in the apical turn, which is further away from the injection site (round window)[12,13].

The canalostomy approach involves gene delivery through one of the semicircular canals located superficially in the temporal bone. It does not require opening the tympanic bulla, which minimized the chances for surgical trauma and middle ear effusion[14–16]. In rodents, the posterior semicircular canal (PSC) is the most prominent and easily accessible out of the three semicircular canals. Therefore, PSC approach is a commonly used surgical method for inner ear gene therapy studies in mice[17,18]. In adult mice, additional surgical aspects need to be considered when using this method, since the mouse otic capsule is initially cartilaginous and becomes ossified by postnatal week 2[4,19], which potentially makes PSC gene delivery in adult mouse inner ear more challenging technically.

In this study, we examine the surgical steps involved in the PSC approach in order to refine this surgical technique for safe and reliable gene delivery in the adult mouse inner ear. We find that the adult mouse inner ear is more susceptible to surgical trauma compared to the neonatal mouse inner ear. In addition, we find that the duration of perilymphatic leakage and injection rate have significant effect on hearing in the adult mouse inner ear. We also show that the synthetic AAV2.7m8 is capable of transducing the adult mouse inner and outer hair cells with high efficiency.

Results

Adult mouse inner ear is more susceptible to hearing loss than neonatal inner ear for inner ear gene delivery

Posterior semicircular canal approach (PSC) has been shown to be a safe and effective surgical approach for inner ear gene delivery in the neonatal mouse inner ear[17,20]. In addition, some studies have also shown that it can be safely implemented in the adult mouse inner ear[15,21]. However, our initial attempts at using the PSC approach for gene delivery in the adult mouse inner ear showed significant ABR threshold elevation in many mice (Fig. 1a). Therefore, we decided to investigate the implementation of PSC approach in adult mice more carefully. The three main surgical steps in the PSC approach are, 1) fenestration of PSC, 2) insertion of injection tubing into the PSC, and 3) injection of fluid into the PSC (Fig. 1b). We decided to examine each of these surgical steps involved with PSC gene delivery to see if we could refine this surgical technique to minimize trauma to the adult mouse inner ear.

The duration of perilymphatic leakage after PSC fenestration negatively affects hearing in adult mouse inner ear

We first examined the effect of PSC fenestration on the adult mouse inner ear. The fenestration of PSC is performed using a small 27-gauge needle to expose the canal lumen. Observation of perilymphatic leakage is used as confirmation for successful access to the PSC lumen. In this experiment, we performed PSC fenestration on adult CBA/J mice. The PSC fenestration was left open for several minutes to allow for perilymphatic leakage to occur, and then sealed off using a muscle plug. We found that some mice developed significant hearing loss after PSC fenestration while others didn't (Fig. 2a). We decided to examine the PSC fenestration more closely by timing the duration of PSC opening and perilymphatic leakage. We separated our animals into three groups based on various durations of PSC opening and perilymphatic leakage: 2, 5, and 10 minutes. We found that mice with 2-minute and 5-minute PSC opening had minimal ABR threshold elevation compared with non-surgery control mice. However, mice in the 10-minute PSC opening group had significant ABR threshold elevation compared to the non-surgery control mice (Fig. 2b; p=0.0153 for 4kHz, p=0.0006 for 8kHz, p=0.0082 for 16kHz, and p=0.0010 for 32kHz, t-test). This indicates that prolonged PSC opening time and perilymphatic leakage can adversely affect the hearing outcome in adult mouse inner ear.

Tube insertion into the PSC had no effect on hearing in the adult mouse inner ear

Next, we compared ABR thresholds in mice with or without insertion of injection tubing into the PSC to determine whether this surgical step would adversely affect auditory function. It is important to remember that mice undergoing tube insertion will have to undergo PSC fenestration. Therefore, this surgical step cannot be evaluated on its own, but must be evaluated after the PSC fenestration has been created. The PSC opening time was kept below 5 minutes to minimize perilymphatic leakage. We found that there was no significant difference in ABR thresholds between mice that underwent tube insertion and non-surgery control mice when the PSC opening was kept below 5 minutes (Fig. 3).

The rate of injection has significant effect on hearing in the adult mouse inner ear

Lastly, we evaluated the effect of injection rate in the adult mouse inner ear via the PSC. Again, it is important to remember that in order for mice to be injected with gene therapy, the PSC must be fenestrated first, and then the injection tubing must be inserted into the PSC lumen in order for the injection to take place. In neonatal mice, we have shown previously that we could deliver approximately 1 µl of fluid volume within a span of 30 seconds into the inner ear without any ABR threshold elevation compared to non-surgery control mice[18]. However, when we used the same injection rate in adult mice, significant ABR threshold elevation was observed (Fig. 4a). Substantial IHC and OHC loss was found throughout the cochlear turns (Fig. 4b). In the basal turn of the cochlea, all IHCs and OHCs were damaged, suggesting the hearing loss observed resulted from hair cell damage after injection (Fig. 4c).

Therefore, we decided to examine the effect of injection rate in the adult mouse inner ear more carefully. The micro-injector that we use allows us to set the fluid volume per injection (e.g. 13.8 nl, 27.6 nl, 46 nl, etc.), and the injection interval can be spaced out as determined by the investigators. We assessed the following three different injection regimens: 72 injections of 13.8 nl per injection every 10 sec (for a total volume of 993.6 nl), 36 injections of 27.6 nl per injection every 10 sec (for a total volume of 993.6 nl), and 20 injections of 46 nl per injection every 10 sec (for a total volume of 920 nl). We found that there was no significant difference in the average ABR thresholds between mice in the 13.8 nl per injection and 27.6 nl per injection groups compared to non-surgery control mice (Fig. 4d). However, mice in the 46 nl per injection group exhibited significantly higher ABR thresholds compared to non-surgery control mice (Fig. 4d). The differences in ABR threshold were significant at all tested frequencies except 4kHz (p=0.0641 for 4kHz, p=0.0027 for 8kHz, p=0.0258 for 16kHz, and p=0.0318 for 32kHz, t-test).

AAV2.7m8 transduced adult cochlear hair cells with high efficiency

We previously showed that AAV2.7m8 is a powerful viral vector for gene delivery in the neonatal mouse inner ear[18]. However, it has been shown that AAV transduction efficiency can be different between neonatal and adult mouse inner ears[16,21,22]. Therefore, we assessed the transduction pattern and efficiency of AAV2.7m8 in the adult mouse inner ear using our newly refined PSC approach. When 1 µl of AAV2.7m8 was delivered via PSC approach by 72 injections of 13.8 nl per injection every 10 sec, IHC and OHC transduction rates were 65.3±10.1 and 37.9±7.4% in the apical turn, 69.2±10.8 and 35.2±7.7% in the middle turn, and 40.3±7.8 and 10.8±6.3% in the basal turn of the cochlea (Fig. 5a). Even though the overall transduction rate is lower compared to our previous study in neonatal mouse inner ears, there were some adult mice that had very high rates of IHC and OHC transduction, comparable to neonatal ears. The reduction in overall IHC and OHC transduction rates in the adult mouse inner ear is likely due to the increased technical challenge with adult mouse inner ear gene delivery compared to neonatal ears.

To determine whether overall injection volume affects transduction efficiency, we injected some mice with ~2 µl of AAV2.7m8-GFP using 72 injections of 27.6 nl per injection every 10 sec (Fig. 5b). IHC and OHC transduction efficiencies with 2 µl were 91.2±0.9% and 86.3±4.3% in the apical turn, 91.0±2.5% and 56.9±12.7% in the middle turn, and 83.7±6.3% and 21.6±9.8% in the basal turn of the cochlea (Fig. 5c). When compared with 1 µl injection, the overall transduction rate was higher across the cochlear turns, and the difference in transduction rate was significant in the basal turn of the cochlea for IHCs, and the apical turn of the cochlea for OHCs. This indicates that AAV2.7m8 is capable of transducing cochlear IHCs and OHCs at high levels, and the transduction efficiency in adult mice increases in a dose-dependent manner.

Hearing is preserved in adult mouse inner ear after 2 µl injection using the PSC approach

Even though AAV2.7m8 is capable of transducing cochlear IHCs and OHCs at high levels in the adult mouse inner ear, we had to increase the total injection volume to 2 µl in order to match the transduction efficiency seen in the neonatal mice. Since the adult mouse inner ear is more vulnerable to surgical manipulation and injection volume, we assessed whether a 2 µl injection would have any effect on auditory function in these animals. We found that neither 1 µl nor 2 µl injection volume caused significant ABR threshold shift compared to non-surgery control mice, as long as the injection parameters were kept below 27.6 nl every 10 seconds. We also found that the average ABR threshold was not significantly different between mice injected with 1 µl and 2 µl (Fig. 6). These results demonstrate that up to 2 µl of fluid volume can be safely injected into the adult mouse inner ear using the PSC approach without causing any significant ABR threshold elevation.

Discussion

Inner ear gene therapy has been shown to be effective at improving the auditory function of several mouse models of hereditary hearing loss[2,23–28]. While these proof-of-concept studies are very promising, most of these studies require gene delivery to be performed at the neonatal age (before P5), when the mouse inner ear is still not fully mature. In contrast, the human inner ear begins to have auditory perception by ~ 19-week gestation and is fully developed at birth[29]. Therefore, in order to increase the probability of success in translating inner ear gene therapy from mouse models of hearing loss to patients with hearing loss, one must try to demonstrate successful gene delivery in the mature mammalian inner ear. While PSC approach is a well-established surgical approach for gene delivery in neonatal mouse inner ear, few studies have examined the impact of PSC approach on hearing outcome in the adult mouse inner ear[14,16,21]. Even though the anatomy between the neonatal and adult mouse inner ear is similar, one major difference is the fact that the adult otic capsule is completely ossified, whereas the neonatal otic capsule is still cartilaginous[4,19]. This difference makes accessing the adult inner ear much more challenging, and potentially more traumatic. Indeed, we found that adult mouse inner ear is more susceptible to hearing loss after PSC gene delivery compared to neonatal ears[18]. Therefore, we decided to see if we could refine the surgical techniques of PSC gene delivery in the adult mouse inner ear to minimize hearing loss. We examined the three main surgical steps involved with PSC gene delivery: 1) fenestration of PSC, 2) insertion of injection tubing into the PSC, and 3) injection of fluid into the PSC. We found that prolonged perilymphatic leakage and injection rate have significant effects on hearing in the adult mouse inner ear.

Fluid leakage from the fenestra is a good indicator of having obtained access to the perilymphatic space during canalostomy approach. In order to decrease the fluid volume and pressure in the perilymphatic space to accommodate for gene therapy injection, Suzuki et al. recommended waiting for 5 minutes after the fenestra of the PSC wall is opened to allow perilymph to leak out[21]. Similarly, in a study by Yoshimura et al., they recommended fenestrating the PSC when performing round window gene delivery to allow perilymphatic leakage in order to decrease the pressure in the inner ear[13]. In the present study, we found that a major factor for hearing loss in adult mice undergoing PSC gene delivery is perilymphatic leakage.

We found that mice with a fenestra opening time of 10 minutes prior to securing the injection tubing exhibited significantly higher ABR thresholds compared to non-surgery control mice (Fig. 2b). The concept of minimizing the duration of time for perilymphatic leakage is well known in the otologic surgery literature. In cholesteatoma surgery, when the cholesteatoma has eroded through the otic capsule (most commonly involving the horizontal semicircular canal), it is generally recommended to leave the cholesteatoma matrix on the perilymphatic fistula to avoid perilymphatic leakage and exposure of the inner ear[30,31]. In cases where a decision is made to open the eroded horizontal semicircular canal wall, it is usually recommended to seal the fistula immediately in order to minimize perilymphatic leakage and preserve inner ear function[32,33]. Therefore, our data suggest that it is important to minimize the leakage of perilymph after the fenestra on the posterior semicircular canal is opened in order to minimize hearing loss. We recommend trying to insert and secure the injection tubing as soon as the fenestra on the posterior semicircular canal is opened.

The injection fluid into the inner ear could potentially cause barotrauma and mechanical trauma to the inner ear. In mice injected with 1 µl of viral vectors, a larger volume of fluid per injection was significantly associated with ABR threshold shift (Fig. 4c). In addition, mice that received fast injection rate (20 injections in 30 seconds) exhibited significant hair cell loss and ABR threshold elevation (Fig. 4a). Both large fluid volume per injection and shortened duration between injections can induce large and fast displacement of perilymph, which may lead to increased pressure within the cochlea, causing damage. Clinical experience with hearing preservation cochlear implant surgery supports our findings. Thick diameter of cochlear implant array is analogous to large volume per injection in this study. It has been shown that larger diameter cochlear implant array leads to higher insertion force and results in increased risk of loss of residual hearing during cochlear implant surgery[34]. Similarly, an increase in implant insertion speed is analogous to injection speed (the time interval between injections) in the present study. It has also been shown that increased cochlear implant insertion speed can cause adverse effects on residual hearing during cochlear implant surgery[35,36]. Therefore, our data suggest that a smaller volume of fluid per injection with slower injection speed (longer time interval between injections) offers the best chance for hearing preservation in the adult mouse inner ear with PSC gene delivery.

In our previous study, we showed that AAV2.7m8 is capable of transducing neonatal cochlear IHCs and OHCs at high levels[18]. In this study, we tested the transduction efficiency of AAV2.7m8 in the adult mouse cochlea. We found that AAV2.7m8 was also capable of transducing the adult cochlear IHCs and OHCs, but the overall transduction rate was lower than what we observed in the neonatal inner ears[18]. The decrease in transduction efficiency in the adult mouse inner ear has been reported in other studies[9,37,38]. In a study comparing the transduction efficiency of several AAV serotypes between neonatal and adult mouse inner ears, Shu et al. found that the viral transduction efficiency in the adult mouse inner ear was significantly lower than the neonatal mouse inner ear[9]. In another study, when exogenous *Tmc1* gene was delivered into the inner ear of *Tmc1* deficient mice using Anc80L65, infected hair cell rates decreased as a function of injection age from 93% at P1 to 3% at P14[38]. A recent study in which AAV9-PHP.B was used as the viral vector for inner ear gene delivery in a mouse model of Usher syndrome type 3A, the authors

observed that AAV9-PHP.B was able to transduce both OHCs and IHCs in neonate mice (P0-P1), while adult mice (P28) exhibited transduction in only IHCs[37]. Although this decrease may be due to the maturation of cellular architecture of the inner ear that prevents the diffusion of AAV to infect hair cell[9], the exact mechanism involved is still unclear. Therefore, further study is needed to better understand the mechanism behind this phenomenon.

We observed that the transduction rates of IHCs and OHCs increased in a dose-dependent manner with AAV2.7m8, with no significant ABR threshold shift up to a total injection volume of 2 µl. The average volume of perilymphatic space in mice is 0.62–1.72 µl[39,40]. Therefore, it is interesting that an injection volume of 2 µl did not negatively impact auditory function. In addition to our results, other studies have also shown that up to 2 µl of fluid volume can be delivered into the mouse inner ear without any significant effect on the auditory function[14,41–43]. The ability of the mouse inner ear to accommodate a large fluid volume may be explained by the presence of a relatively large and patent cochlear aqueduct, which communicates between the perilymphatic and subarachnoid spaces[18,44]. The cochlear aqueduct essentially acts as an outflow valve which allows excess fluid volume to escape into the subarachnoid space. The fact that up to 2 µl of fluid volume can be safely delivered to the adult mouse inner ear is particularly useful in gene therapy studies requiring the use of multiple viral vectors (e.g. dual-AAV approaches for delivering large cDNA), since these studies often require larger injection volume.

In conclusion, our study shows that although the adult mouse inner ear is more susceptible to surgical manipulation, the auditory function can be preserved by reducing the PSC opening time to minimize perilymphatic leakage, as well as utilizing a slower fluid injection rate. In addition, the synthetic AAV2.7m8 is capable of transducing adult mouse IHCs and OHCs with high efficiency. It is our hope that the detailed methods described in this study can be utilized for safe and efficient gene delivery in the adult mouse inner ear.

Methods

AAV vector construction

The AAV2.7m8-CAG-EGFP (9.75 x 10^{12} GC/mL) was produced by the Research Vector Core at the Center for Advanced Retinal and Ocular Therapeutics (University of Pennsylvania). The production method for these viruses have been previously described[45].

Animal surgery

Animal surgery was approved by the Animal Care and Use Committee at the National Institute on Deafness and Other Communication Disorders (NIDCD ASP1378-18). All animal procedures were done in compliance with the ethical guidelines and regulations set forth by the Animal Care and Use Committee at NIDCD. The study was performed in compliance with the ARRIVE guidelines for animals. Adult (P30-

90) CBA/J mice were used in this study. Anesthesia was induced using isoflurane gas (Baxter, Deerfield, IL) through a nose cone at a flow rate of 0.5 L/min. Gene delivery was done using the PSC approach. A post-auricular incision was made using small scissors. The soft tissues were bluntly dissected to expose the PSC. To expose lumen of the ossified canal, a 27-guage hypodermic needle was used. A small hole was created by rotating the needle with gentle pressure. After creating a hole, perilymph leakage from the PSC was identified and the hole was kept open for 2, 5 or 10 minutes to determine whether the opening time affects post-injection hearing results. A Nanoliter Microinjection System (Nanoliter2000, World Precision Instruments, Sarasota, FL) was used in conjunction with a polyethylene tube attached with glass micropipette to load viral vector. AAV2.7m8-CAG-EGFP (9.75×10^{12} GC/mL) was injected according to following different volume and rates to define best option for fluid injection: 13.8 nl × 72 injections every 10 seconds, 27.6 nl × 36 injections every 10 seconds, 46 nl × 20 injections every 10 seconds, 46 nl × 20 injections in 30 seconds total, and 27.6 nl × 72 injections every 10 seconds. Incision was closed with 5-0 vicryl sutures.

Auditory brainstem response

Auditory brainstem response (ABR) testing was used to evaluate hearing sensitivity at ~P30. Animals were anesthetized with ketamine (100 mg/kg) and dexmedetomidine (0.375 mg/kg) via intraperitoneal injections and placed on a warming pad inside a sound booth (ETS-Lindgren Acoustic Systems, Cedar Park, TX). The animal's temperature was maintained using a closed feedback loop and monitored using a rectal probe (CWE Incorporated, TC-1000, Ardmore, PN). Sub-dermal needle electrodes were inserted at the vertex (+) and test-ear mastoid (-) with a ground electrode under the contralateral ear. Stimulus generation and ABR recordings were completed using Tucker Davis Technologies hardware (RZ6 Multi I/O Processor, Tucker-Davis Technologies, Gainesville, FL, USA) and software (BioSigRx, v.5.1). Click and tone-burst ABR thresholds were measured at 4, 8, 16, and 32 kHz using 3-ms, Blackman-gated tone pips presented at 29.9/sec with alternating stimulus polarity. At each stimulus level, 512-1024 responses were averaged. Thresholds were determined by visual inspection of the waveforms and were defined as the lowest stimulus level at which any wave could be reliably detected. A minimum of two waveforms was obtained at the threshold level to ensure repeatability of the response. Physiological results were analyzed for individual frequencies, and then averaged for each of these frequencies from 4 to 32 kHz.

Immunohistochemistry and quantification

After completion of functional testing, mice were euthanized by CO2 asphyxiation followed by decapitation. Temporal bones were harvested and fixed overnight with 4% paraformaldehyde followed by decalcification in 120mM EDTA for 4 days. The vestibular organs and cochlear sensory epithelia were micro-dissected, blocked, and labeled with rabbit anti-myosin 7a antibody to label hair cells (1:200, product # 25-6790, Proteus BioSciences, Ramona, CA), and chicken anti-GFP antibody to label GFP (1:1000, product # ab13970, abcam, Cambridge, MA), and Hoechst stain (1:500, product # 62249, Life

Technologies, Carlsbad, CA) to label nuclei. Primary and secondary antibodies were diluted in PBS. Images were obtained using a Zeiss LSM780 confocal microscope at 10x and 40x using z-stacks.

For quantification of cochlear hair cell and supporting cell infection efficiency, two 40x images were taken at the apex, middle turn, and base of cochlea. The number of hair cells and supporting with GFP expression was counted and averaged at each location along the cochlea. Each 40x image contains ~30 IHCs and ~90 OHCs. The overall transduction rate was calculated by averaging the transduction rates obtained from the entire cochlea. For quantification of utricular hair cell infection efficiency, two 40x images (each containing ~300 vestibular hair cells) were taken per utricle specimen and the number of hair cells with GFP expression was counted and averaged.

Statistics

Student's t-test (two-tailed) was used to assess differences in transfection efficiency. It has been shown that different AAV serotypes can have different transfection efficiencies in different regions of the cochlea[43]. Therefore, transfection efficiencies from each region of the cochlea (apex, middle turn, and cochlear base) were treated as separate measurements in the calculation of mean, standard error, and statistical significance. For ABR threshold, Student's t-test was used to assess differences in the thresholds. The p-value of <0.05 indicates statistical significance.

Declarations

Data availability

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

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Author Contributions

W.C. and J.B. were involved with the conception of the study and final approval of manuscript. J.Z., J.W.C., K.I., Y.I., M.G., and W.C. were involved with data collection, data analysis, data interpretation, and drafting of manuscript.

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Figures

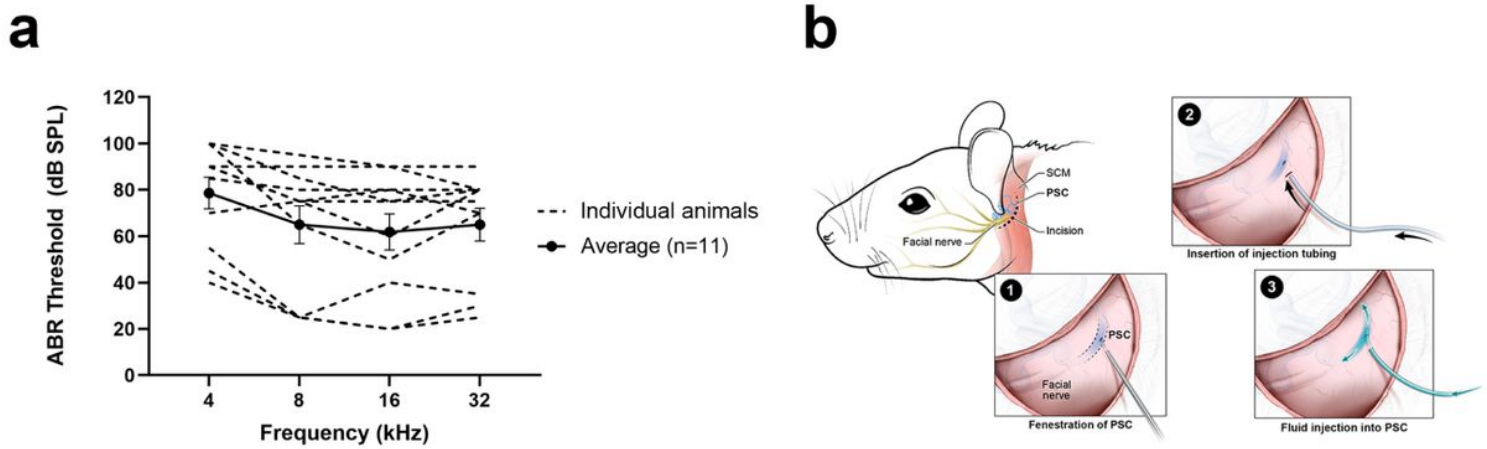

Figure 1

Adult mouse inner ear is more susceptible to surgical trauma with inner ear gene delivery. (a) ABR thresholds in adult mice that underwent PSC gene delivery. Many mice exhibited significant ABR threshold elevation, while others did not. (b) Schematic drawing of PSC approach used in this study for inner ear gene delivery in adult mouse.

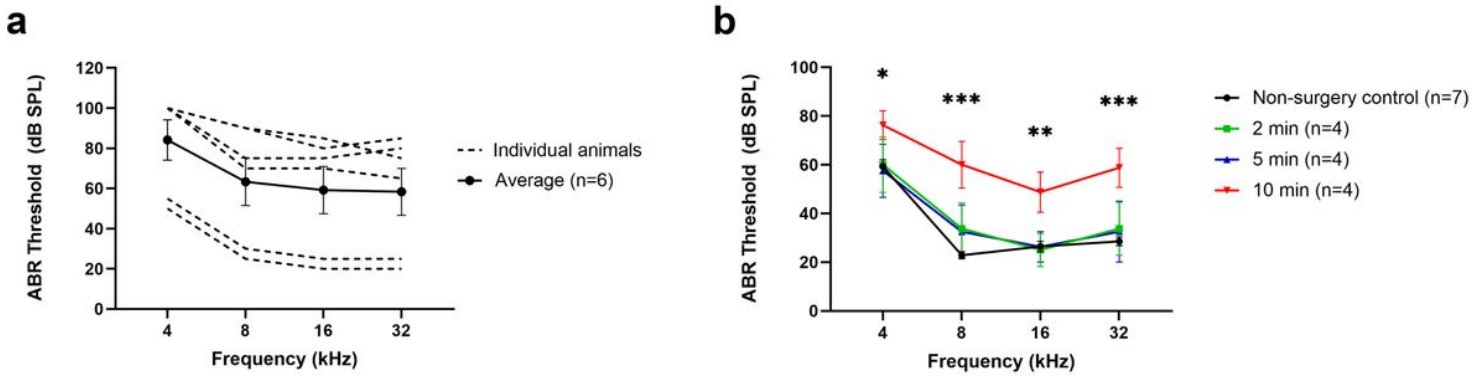

Figure 2

Prolonged perilymphatic leakage causes hearing loss. (a) ABR thresholds of adult mice undergoing PSC fenestration. Some mice developed hearing loss after PSC opening while others did not. (b) ABR thresholds of adult mice undergoing PSC fenestration, controlling for perilymphatic leakage time. Mice with 10-minute PSC opening time exhibited significant ABR threshold elevations across all frequencies compared with non-surgery control mice (t-test). Data are represented as mean ± SEM. n represents the number of animals tested. Statistical significance between groups is shown above error bars (* represents $p < 0.05$, ** represents $p < 0.005$, *** represents $p < 0.0005$).

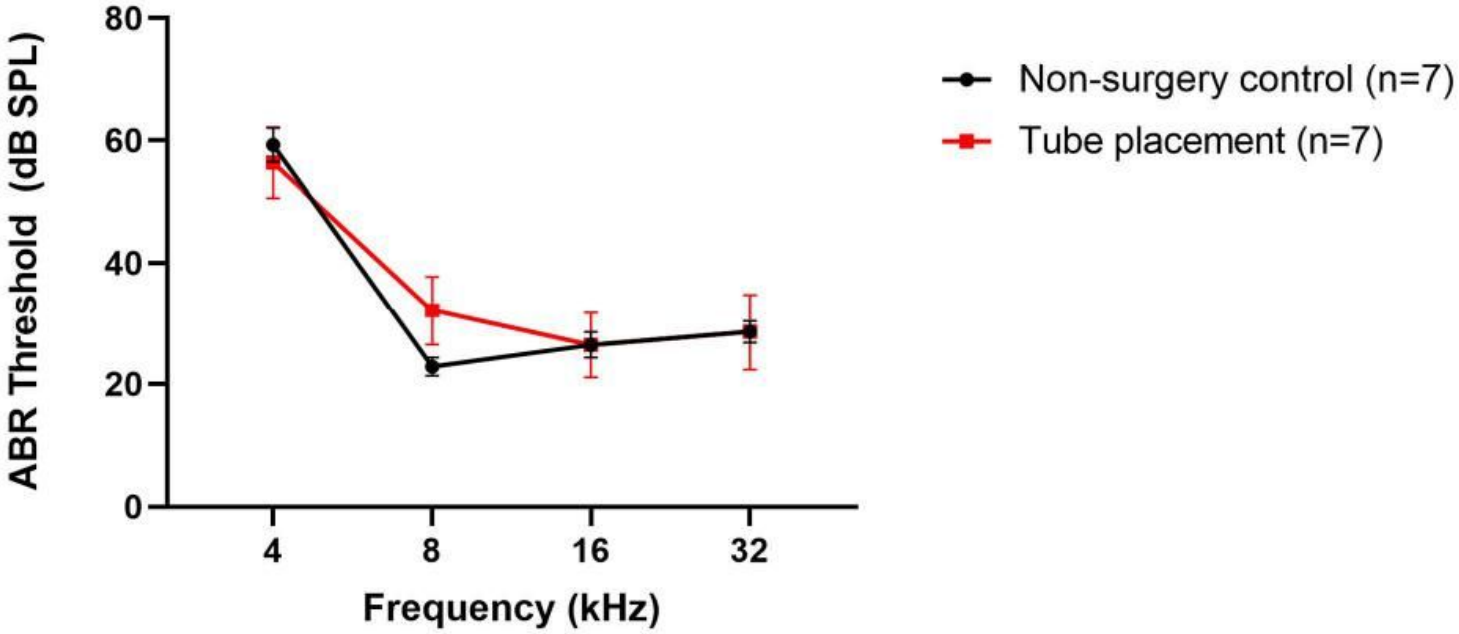

Figure 3

Injection tube insertion into the PSC lumen does not cause hearing loss. ABR thresholds in mice that underwent injection tube insertion are comparable to non-surgery control mice ($p > 0.05$, t-test). Data are represented as mean ± SEM. n represents the number of animals tested.

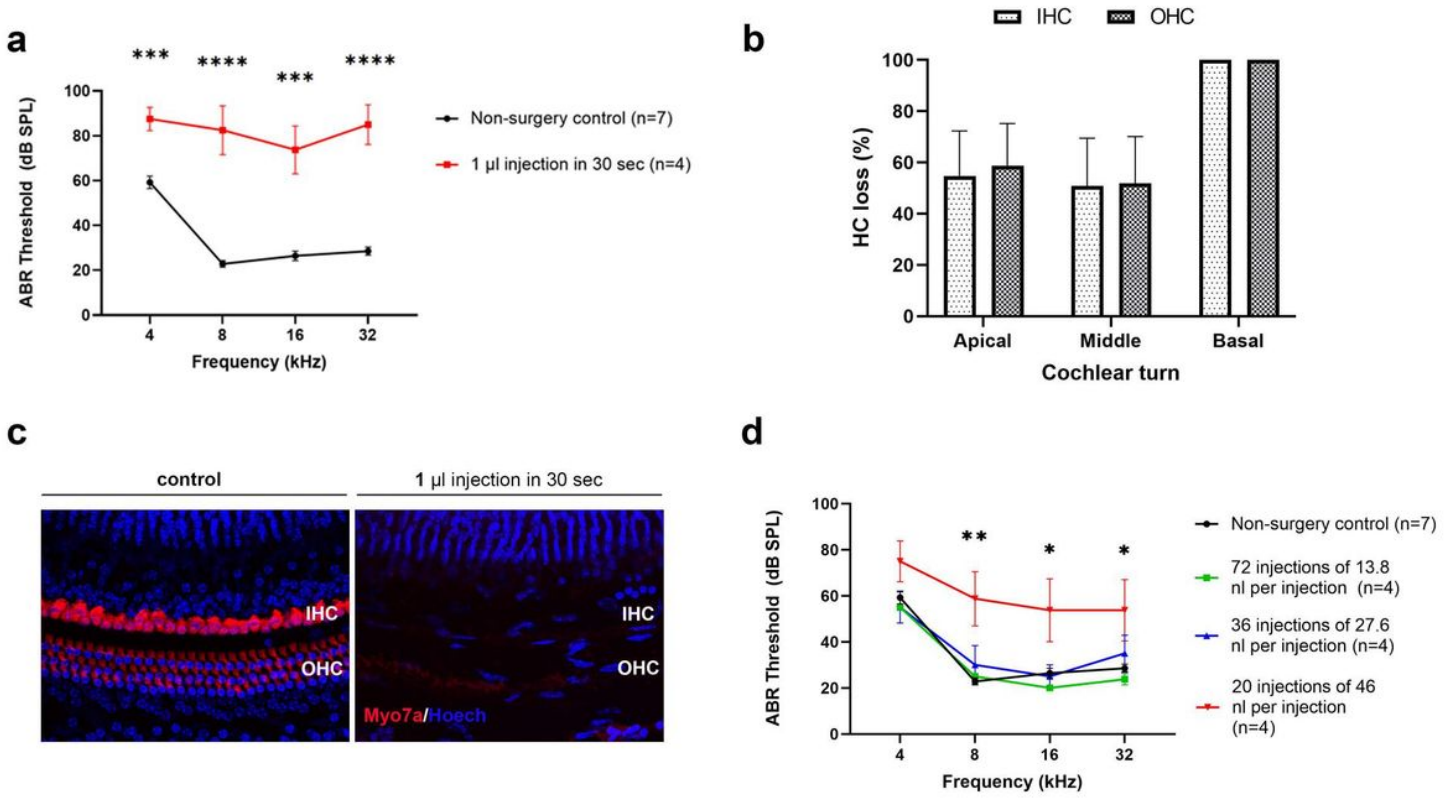

Figure 4

Injection rate has significant effect on hearing in adult mouse inner ear. (a) Adult mice that received 1 µl gene delivery within 30 seconds had significant ABR threshold elevation at all frequencies tested in comparison with non-injection control mice ($p<0.05$, t-test). (b) Substantial IHC and OHC losses were observed throughout the cochlear turns in mice that received 1 µl gene delivery within 30 seconds. (c) Confocal images of the basal turn of the cochlea in a non-surgery control mouse (labeled “control”) and a mouse that underwent 1 µl gene delivery within 30 seconds (labeled “1 µl gene delivery in 30 seconds”). The cochlear specimen from the animal that underwent 1 µl gene delivery within 30 seconds had complete loss of IHCs and OHCs. (d) Adult mice that underwent 20 injections of 46 nl per injection every 10 seconds exhibited significant ABR threshold elevation, whereas mice that received 72 injections of 13.8 nl per injection every 10 seconds, and 36 injections of 27.6 nl per injection every 10 seconds did not, compared with non-surgery control mice. Data are represented as mean ± SEM. n represents the number of animals tested. Scale bar represents 20µm. Statistical significance between groups is shown above error bars (* represents $p < 0.05$, *** represents $p < 0.0005$, **** represents $p < 0.0001$).

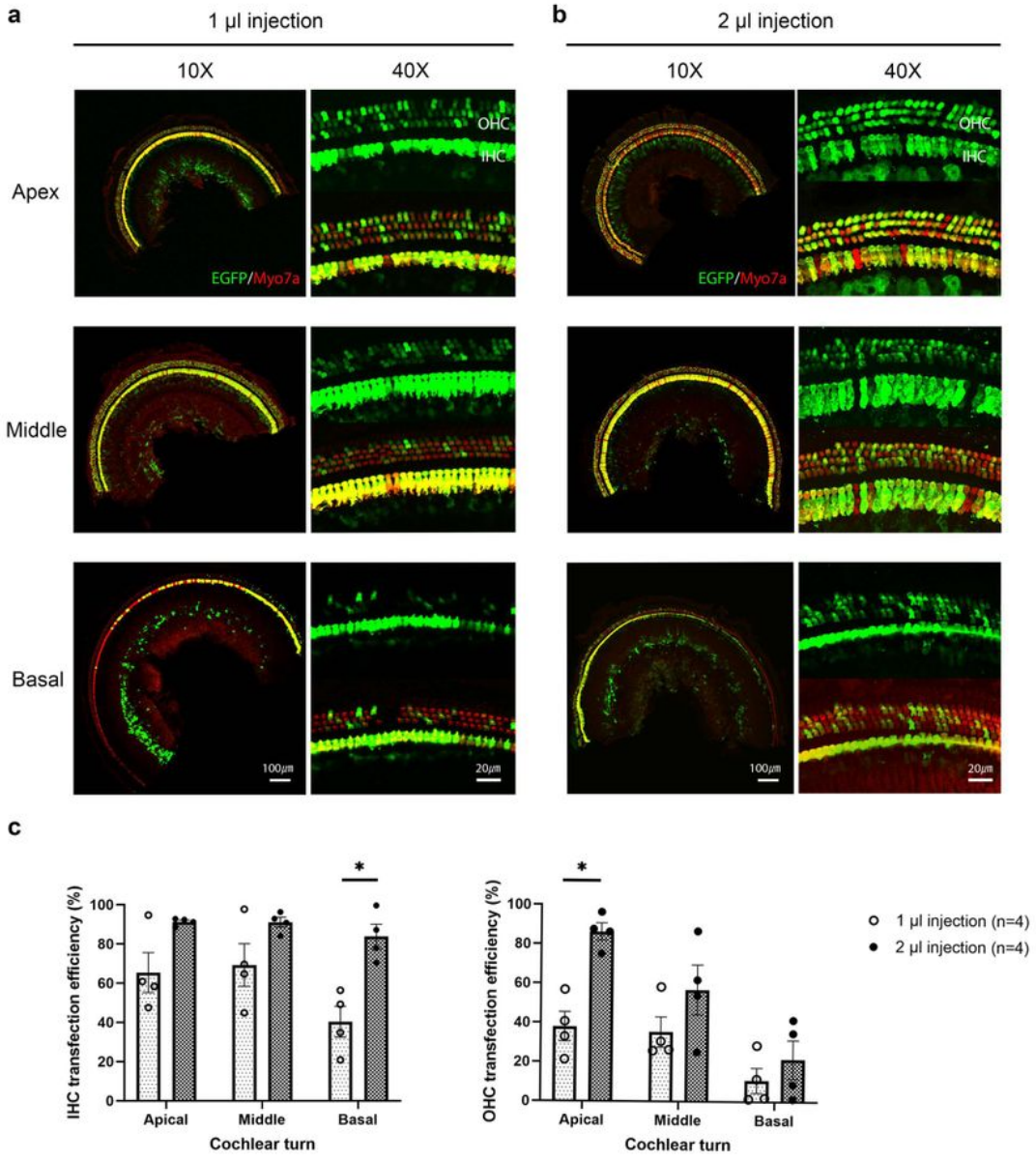

Figure 5

AAV2.7m8 is capable of transducing IHCs and OHCs in the adult mouse cochlea, and the transduction efficiency increased in a dose-dependent manner. (a & b) Representative 10X and 40X images from apical, middle, and basal turns of the cochlea of mice injected with 1 µl (a) and 2 µl (b) of AAV2.7m8-GFP via the PSC approach. The IHC and OHC transduction rates are better in the animal that received 2 µl of AAV2.7m8-GFP gene delivery, as evidenced by higher number of IHCs and OHCs with GFP expression. (c)

Quantitative comparison of transduction efficiency of IHCs and OHCs in adult mice that received 1 µl and 2 µl injections revealed a dose-dependent increase in hair cell transduction efficiency, especially in IHCs of the basal turn and OHCs of the apical turn ($p < 0.05$; t-test). GFP expression is shown in green and Myo7a expression is shown in red. Scale bar represents 100µm and 20µm in 10X images and 40X images, respectively. For each animal, hair cell infection was quantified at six different locations along the cochlea: two in the apical turn, two in the middle turn, and two in the basal turn. Data are represented as mean ± SEM). Statistical significance between groups is shown above error bars (* represents $p < 0.05$; t-test).

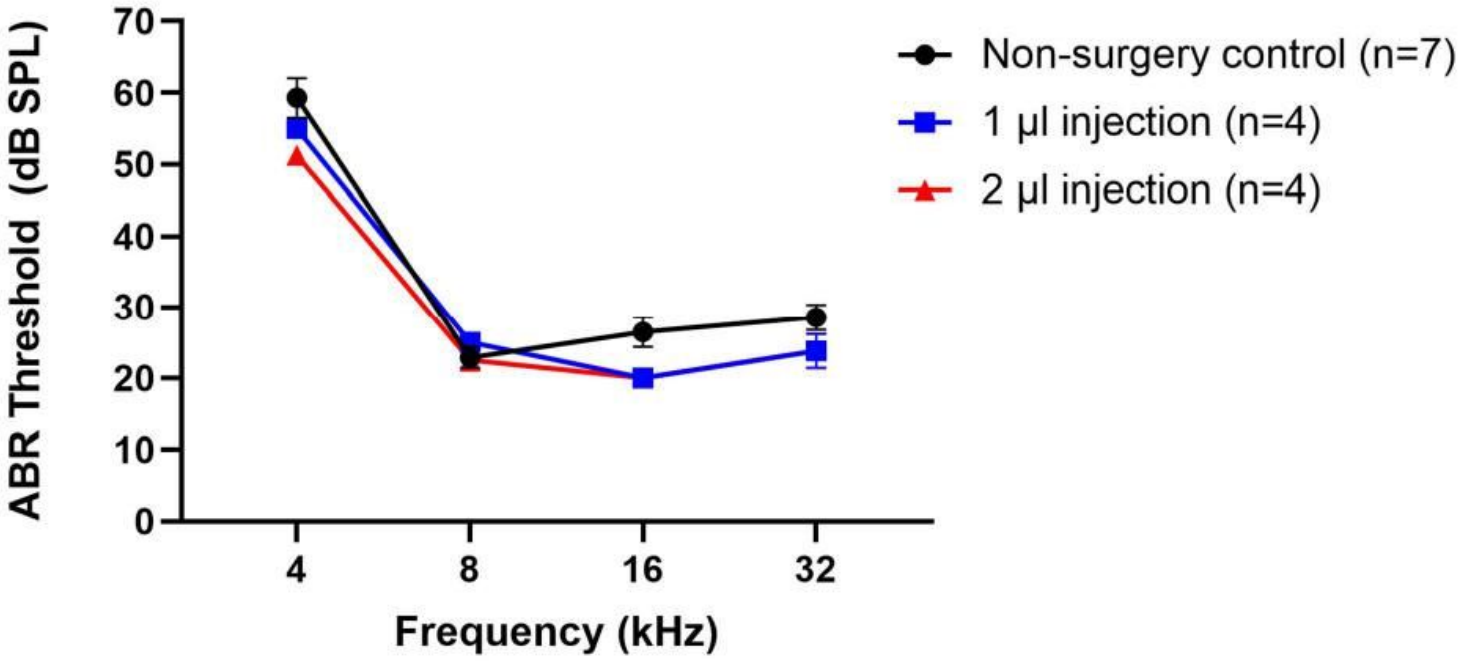

Figure 6

Adult mice injected with 2 µl of AAV2.7m8-GFP did not have hearing loss. ABR threshold elevation was not observed in adult mice at one week following 1 µl and 2 µl of AAV2.7m8-GFP injection, compared with non-surgery control mice. Data are represented as mean ± SEM. n represents the number of animals tested.