Video Article

Surgical Method for Virally Mediated Gene Delivery to the Mouse Inner Ear through the Round Window Membrane

Omar Akil[1], Stephanie L. Rouse[1], Dylan K. Chan[1], Lawrence R. Lustig[1]

[1]Department of Otolaryngology-Head and Neck Surgery, University of California, San Francisco

Correspondence to: Omar Akil at oakil@ohns.ucsf.edu

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Abstract

Gene therapy, used to achieve functional recovery from sensorineural deafness, promises to grant better understanding of the underlying molecular and genetic mechanisms that contribute to hearing loss. Introduction of vectors into the inner ear must be done in a way that widely distributes the agent throughout the cochlea while minimizing injury to the existing structures. This manuscript describes a post-auricular surgical approach that can be used for mouse cochlear therapy using molecular, pharmacologic, and viral delivery to mice postnatal day 10 and older via the round window membrane (RWM). This surgical approach enables rapid and direct delivery into the scala tympani while minimizing blood loss and avoiding animal mortality. This technique involves negligible or no damage to essential structures of the inner and middle ear as well as neck muscles while wholly preserving hearing. To demonstrate the efficacy of this surgical technique, the vesicular glutamate transporter 3 knockout (VGLUT3 KO) mice will be used as an example of a mouse model of congenital deafness that recovers hearing after delivery of VGLUT3 to the inner ear using an adeno-associated virus (AAV-1).

Video Link

The video component of this article can be found at http://www.jove.com/video/52187/

Introduction

Gene therapy has long been suggested as a potential treatment for genetic hearing loss, but success in this area has remained elusive[1]. To date, virally mediated methodologies have predominated due the theoretic ability to target specific cell types within the relatively inaccessible cochlea. Both adenovirus (AV) and adeno-associated virus (AAV) have been used for cochlear gene delivery. AAVs are advantageous in the cochlea for a number of reasons. They are replication-deficient viruses and can efficiently transfer transgenic molecules to different cell types including neurons, an important target for a number of causes of hearing loss. AAV entry into the cell is mediated by specific receptors[2]; thus, the choice of a particular serotype must be compatible with the cell types to be transduced. AAVs can effectively transfect hair cells[3] and incorporate into the host genome, resulting in stable, long-term expression of the transgenic protein and phenotypic change in the cell[4]. While not necessarily advantageous for short-term applications such as hair-cell regeneration, long-term expression is very important for stable rescue of genetic defects. Because AAVs are not associated with any human disease or infection and demonstrate no ototoxicity[5,6,7], they are an ideal candidate for use in gene therapy for inherited forms of hearing loss[8].

Transfer of exogenous genetic material into the mammalian inner ear using viral vectors has been studied over the last decade and is emerging as a promising technique for treating both genetic and acquired forms of hearing loss[9]. The cochlea is potentially an ideal target for gene therapy for several reasons: 1) its small volume necessitates a limited amount of the virus needed; 2) its relative isolation from other organ systems limits side effects; and 3) its fluid-filled chambers facilitate viral delivery throughout the labyrinth[10,11,12,13,14,15].

Mouse models of congenital deafness allow for use of many methods of study to monitor development of the inner ear in a systematic, replicable way. While the small size of mouse cochleae does present some surgical difficulty, the mouse serves as an extremely important model in the study of genetic hearing loss, with several experimental advantages over other species[16]. Mouse models allow assessment of a range of characteristics through genetic linkage analysis, collection of detailed morphological observations, and simulating pathogenic scenarios; as such, they are good candidates for virally mediated gene therapy. Extensive genetic studies in mice combined with technological advances have made it possible to generate genetically modified mice in a reproducible way across laboratories[17,18,19,20,21]. Furthermore, there exist numerous models for both acquired and inherited hearing loss phenotypes in mice, allowing rigorous testing in this animal model[22,23,24]. Thus, correcting hearing using virally mediated gene therapy in a mouse model is an appropriate first step in the search for a cure for human disease.

We have previously shown that transgenic mice lacking vesicular glutamate transporter 3 (VGLUT3) are born deaf due to lack of glutamate release at the IHC ribbon synapse[25]. Because this mutation does not lead to a primary degeneration of the sensory hair cells, these mutant mice are potentially an excellent model in which to test cochlear gene therapy for congenital hearing loss.

To date, a number of viral delivery techniques for cochlear gene therapy have been described, including round window membrane diffusion, round window membrane injection, and delivery via a cochleostomy. There are potential advantages and disadvantages of each of these approaches[9].

Here we report a surgical method for virally mediated gene delivery to the VGLUT3 KO mouse inner ear through the round window membrane (RWM). The post-auricular RWM injection method is minimally invasive with excellent hearing preservation, and is relatively fast. As we have previously published, in an effort to restore hearing in this mouse model, an AAV1 vector carrying the VGLUT3 gene (AAV1-VGLUT3) was introduced into the cochlea of these deaf mice at postnatal day 12(P!@), resulting in the restoration of hearing[26]. Hearing in the VGLUT3 KO mice was verified by auditory brainstem response (ABR), while transgene protein expression was verified using immunofluorescence (IF). This methodology thus demonstrates that virally-mediated gene therapy can correct a genetic defect that would otherwise results in deafness.

Protocol

NOTE: All procedures and animal handling complied with NIH ethics guidelines and approved protocol requirements of the Institutional Animal Care and Use Committee of the University of California, San Francisco.

1. Preparing the Animal for Surgery

1. Carry out surgical procedures in a clean, dedicated space. Autoclave all surgical instruments, sterilize with a glass-bead sterilizer prior to surgery.
   NOTE: In this protocol, use postnatal day 10-12 (P10-12) FVB mice. Different ages and strains of mice can be used to meet the needs of a specific project. Mice older than P40 can be challenging because the bulla bone gets harder.
2. Anesthetize the mice with intraperitoneal injection of a mixture of ketamine hydrochloride (100 mg/kg), xylazine hydrochloride (10 mg/kg), and acepromazine (2 mg/kg). Begin surgical preparation only after the animal no longer responds to painful stimuli, such as toe pinch. If necessary, administer a booster dose (one-fifth the original dose) of the anesthetic cocktail to restore the original anesthetic plane.
   NOTE: Take care at this step because most of the mortality observed at this age is caused by anesthetic overdose.
3. Cover the animal's eyes with a protective ophthalmic ointment to keep the eyes moist during anesthesia, suppressing the animal's blink reflex.
4. Position the mouse with neck extended on a heating pad throughout the procedure until the mouse is totally awake, to prevent post-anesthesia hypothermia.
5. Shave the left post-auricular region with a clipper and disinfect with 70% ethanol and povidone-iodine before surgical manipulation.

2. The Surgical Procedure and Vector Injection

1. Use a post-auricular approach to expose the tympanic bulla. Incise the subcutaneous tissue with small scissors to expose the post-auricular muscle. After retracting the adipose tissue to the posterior side of the incision, separate the muscles to the right and left side perpendicular to the incision to expose the temporal bone. Ensure that this incision is long enough to work comfortably with adequate visualization.
2. Perforate the tympanic bulla with a 25 G needle and expand the hole as necessary with forceps by peeling the bone back to allow access to the basal turn of the cochlea, and then widen sufficiently to visualize the stapedial artery and the round window membrane (RWM).
3. Puncture the RWM gently in the center with a borosilicate capillary pipette. Observe fluid efflux through the RWM at this point; this is normal. Wait until the efflux has stabilized (the effluxed fluid from the cochlea is dried with a sterile filter paper).
   NOTE: Take care when holding and advancing the glass needle so that the RWM perforation is as small as possible. Depending on the microscope used, hold the pipettes using a micromanipulator or by hand using a pipette holder.
4. Prepare the pipettes for injection using a pipette puller the same way patch clamp pipettes are prepared. Ensure that the tip diameter is large (about 15 µm in diameter) so that pipetting the virus in and out is done easily.
5. Draw up 1 to 2 µl of AAV1-VGLUT3, AAV1-GFP or AAV2-GFP into an injection pipette. After the efflux is stabilized (5 to 10 min), microinject this fixed volume into the scala tympani through the same hole previously made in RWM.
6. Seal the RW niche quickly after pulling out the pipette with a small plug of muscle and secure it with a small drop of tissue adhesive placed on the muscle to avoid leakage from the round window.
   NOTE : Seal this hole very well after the needle is removed with a small plug of muscle to prevent any perilymph leakage from the cochlea- this step is critical to preserve hearing. Failure to seal completely the RWM will result in hearing loss over time.
7. After injection, cover the hole in the auditory bulla with adipose tissue, return the post-auricular muscles and adipose tissue to their normal position and suture the wound in layers with a 6-0 or smaller absorbable chromic suture.
8. Disinfect the wound with povidone-iodine.

3. Postoperative Care

1. Place mice in a warm cage and do not leave them unattended until they are fully recovered then move them back with the mother. It is recommended to remove the male from the cage before putting the pups back with the mother.
2. Administer subcutaneous Carprofen (2 mg/kg) for analgesia postoperatively and every 24 hr thereafter for 3 days, to manage inflammation and pain. Monitor the animals daily for signs of distress, abnormal weight loss, pain, or infection. Generally by the 3rd day after the surgery all mice should be acting normally. If any signs of distress or disease appear in a mouse after the 3rd day, consider euthanizing the mouse as per institutional guidelines.

4. Assessment of Cochlear Function Following Viral Delivery Using Auditory Brainstem Response (ABR) Recordings

NOTE: The auditory brainstem response (ABR) is an auditory evoked potential extracted from ongoing electrical activity in the brain and recorded via electrodes placed under the scalp. The animal is stimulated with sound. The resulting recording consists of five waves that reflect the electrical activity of successive points in the auditory pathway in the first 10 msec after onset of an auditory stimulus.

1. Anesthetize mice by intraperitoneal injection of a mixture of ketamine hydrochloride and xylazine hydrochloride as described in section 1.2 of this protocol, except without acepromazine.
2. Place the mouse on a heating pad throughout the hearing test until the mouse is totally awake, to prevent post-anesthesia hypothermia.
   NOTE: Use the following sound stimuli in this experiment: click (5 msec duration; 31 Hz presentation rate).
3. Place subdermal needle electrodes at the vertex, below the pinna of the left ear (reference), and below the contralateral ear (ground) and start recording ABRs as previously described in a sound-proof chamber[27,28]. Record ABR waveforms in 5 dB sound pressure level intervals down from maximum amplitude.
4. Determine ABR thresholds postoperatively as early as 4 days after viral delivery
   NOTE: Threshold is defined as the lowest stimulus level at which response peaks for waves I–V were clearly and repeatedly present on visual inspection.
5. Measure Wave I to analyze the activity from the cochlear nerve. The lowest stimulus level that yields a detectable ABR waveform is defined as the threshold.

5. Cochlear Transgene Protein Expression Using Immunofluorescence

1. Anesthetize mice by an overdose of intraperitoneal injection of a mixture of ketamine hydrochloride and xylazine hydrochloride as described in section 1.2 of this protocol, except without acepromazine.
2. Decapitate the head only after the animal no longer responds to painful stimuli, such as toe pinch.
3. Dissect the cochleae out and process for whole-mount immunofluorescence as described[26]. A detailed protocol of cochlear whole-mount immunofluorescence using anti-VGLUT3 and anti-Myosin 7a antibodies is described in Akil *et al.*, 2013[29].
4. For GFP labeling, incubate the cochlear whole-mounts overnight at 4 °C with a rabbit anti-GFP antibody at 1:250 dilution.
5. Rinse the cochleae twice for 10 min with PBS and then incubate for 2 hours in goat anti-rabbit IgG conjugated to Cy2 diluted 1:4,000 in PBS.
6. Rinse the cochleae with PBS twice for 10 minutes and incubate them with DAPI for 15 min.
7. Mount the cochleae on glass slides and observe under a microscope with confocal immunofluorescence.

Representative Results

To verify the technical features and utility of the post-auricular approach for cochlear molecular therapy, AAV1-VGLUT3, AAV1-GFP and AAV2-GFP were delivered into P10-12 mice inner ear via the RWM. This approach demonstrates successful transgene expression within inner hair cells (IHC) (VGLUT3 **Figure 1** and GFP **Figure 2** and GFP **Figure 3A**), outer hair cells (OHC) (GFP **Figure 2**) and supporting cells (GFP **Figure 2** and **Figure 3A**)[26] without significant organ of Corti injury. The observation of different cell types expressing GFP seen in the cochlear whole-mount immunofluorescence images (**Figure 2** and **Figure 3A**) demonstrates that the choice of AAV serotype must be compatible with the cell types to be transduced. **Figure 1**, **Figure 2** and **Figure 3A**[26] show very good transfection in both IHCs and OHCs, as well as distribution of the transgene protein (GFP/VGLUT3) throughout the cochlea after AAV1 or AAV2-GFP or AAV1-VGLUT3 transfection. The distribution of IHCs expressing VGLUT3 after AAV1-VGLUT3 transfection (**Figure 3B**)[26] suggests that transfection via the RWM is more effective and uniform through the whole cochlea than other methods that have reported higher transfection rates closer to the site of viral delivery. We have found that transfection rates can be made more efficient by increasing the volume of the virus injected into the inner ear (**Figure 1**)[26].

Our observation of successful VGLUT3 expression in VGLUT3 KO mouse IHCs with no cochlear damage led us to test hearing by measuring ABRs in the rescued KO mice. **Figure 4A** and **Figure 4B**[26] document the ability of post-auricular RWM delivery of AAV1-VGLUT3 into the inner ear of the VGLUT3 KO mice to produce hearing rescue in this mouse model of congenital hearing loss. Auditory brainstem response (ABR) traces were restored in the VGLUT3 rescued KO (**Figure 4A**), and the ABR thresholds were measurable and comparable to those seen in wild-type mice (**Figure 4B**).

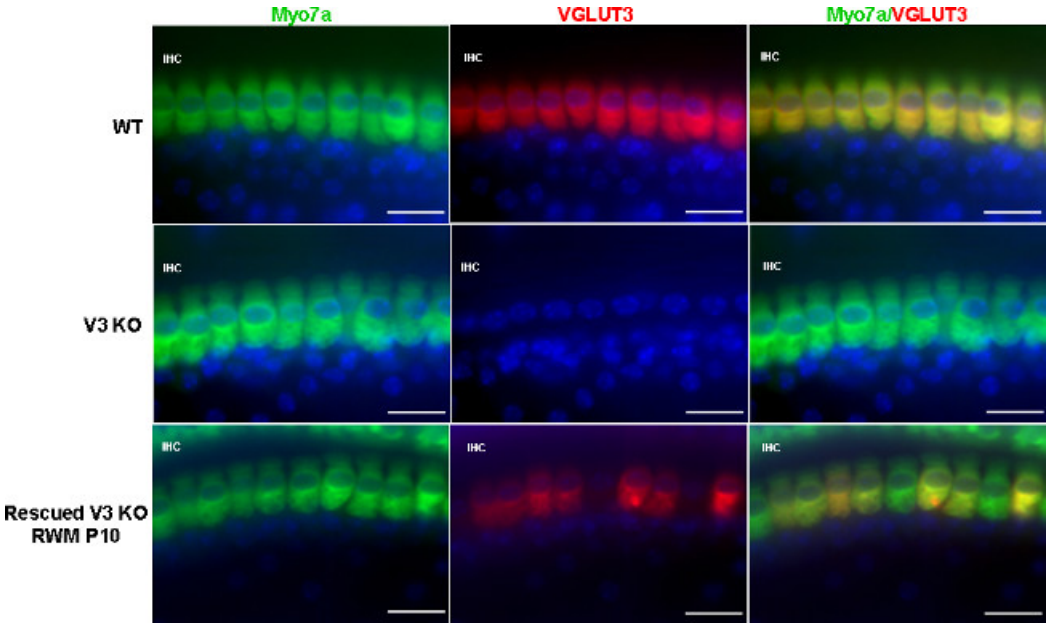

Figure 1: VGLUT3 IHC transfection after RWM viral delivery to P10–P12 KO mice. The transfected cochleae were examined at P30 by immunofluorescence using anti-Myo7a antibody, a hair-cell marker, and an antibody against VGLUT3. Myo7a and VGLUT3 were expressed in all IHCs only in the WT, while IHCs from KO mice expressed only Myo7a. The rescued mouse cochleae showed some IHCs expressing VGLUT3, and all IHCs expressed Myo7a (row 3). **IHC**: inner hair cells. Reprint with permission from [26]. Please click here to view a larger version of this figure.

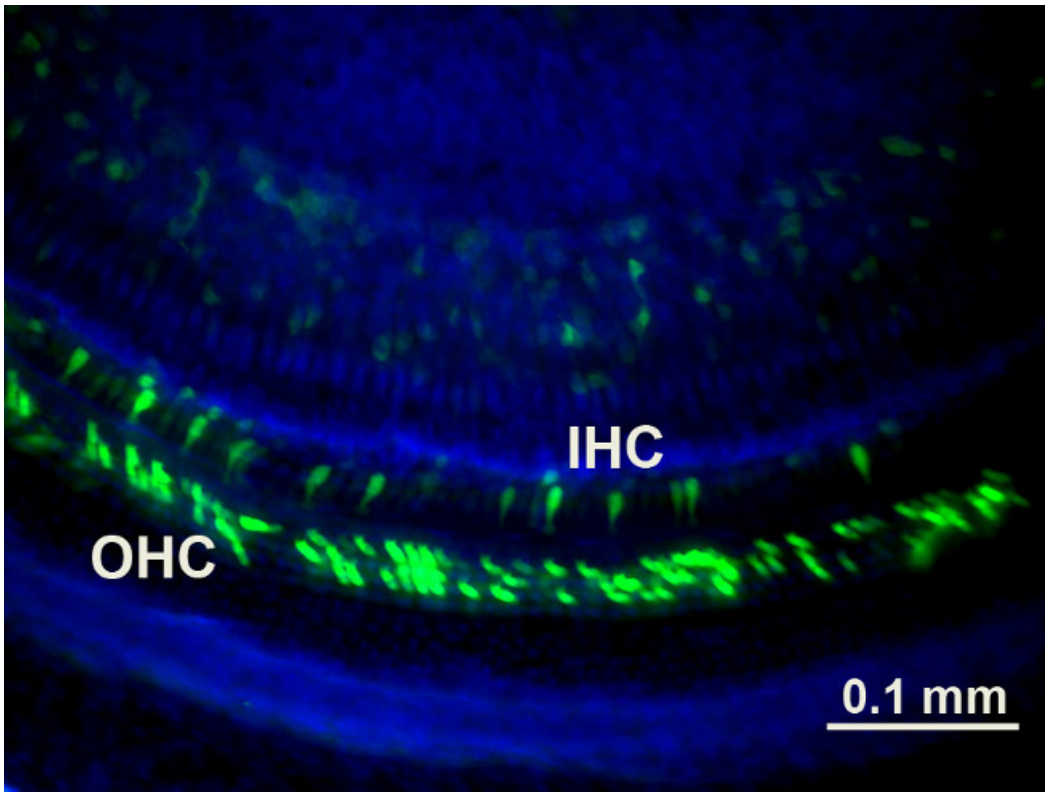

Figure 2: Mouse cochlear whole-mount immunofluorescence after RWM AAV2-GFP delivery to wild-type P10-P12 mice. AAV2-GFP was used to assess viral delivery using the post-auricular approach to the mouse cochlea. AAV2-GFP delivery was done at P10-12 and transgenic GFP expression was examined in the cochlea at P21 using an anti-GFP antibody. GFP expression (green) in WT mice cochlear whole-mount show stronger GFP expression in outer hair cells (OHC) than inner hair cells (IHC).

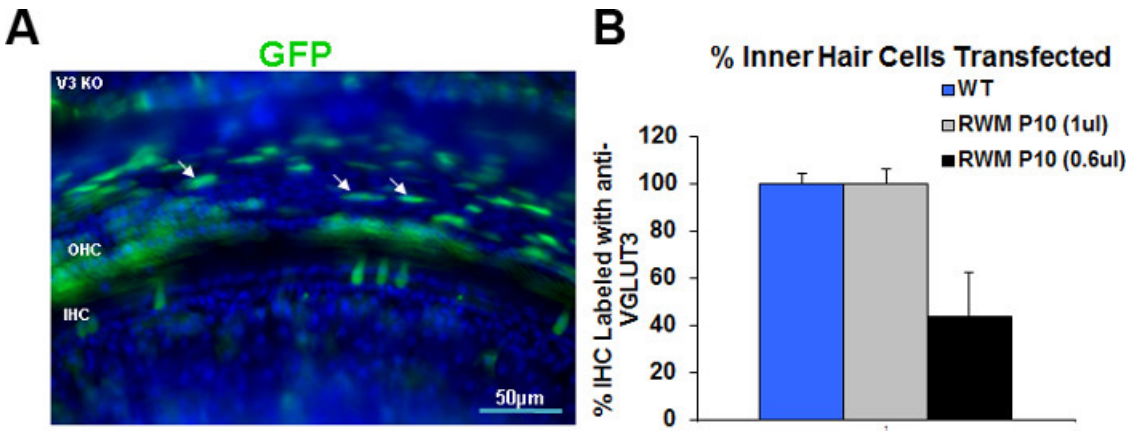

Figure 3: Mouse cochlear whole-mount immunofluorescence after RWM AAV1-GFP delivery to wild-type P10-P12 mice. After AAV1-GFP delivery to the cochlea expression of GFP is seen in inner hair cells (IHC) and supporting cells **(A)**, but not OHCs, whereas AAV1-VGLUT3 delivery to the cochlea of the VGLUT3 KO mice **(B)** resulted in VGLUT3 expression only in IHCs. The number of IHCs expressing VGLUT3 in the VGLUT3 KO mice cochlea after AAV1-VGLUT3 delivery depends of the amount of virus delivered to the inner ear; for example, 40% of IHCs expressed VGLUT3 when 0.6 µl of the virus was injected, whereas 100% of IHCs expressed VGLUT3 after 1 µl of the virus was delivered. There was no difference in VGLUT3 expression between the apex, mid-turn, or base when 0.6 µl of virus was injected. Reprint with permission from [26].

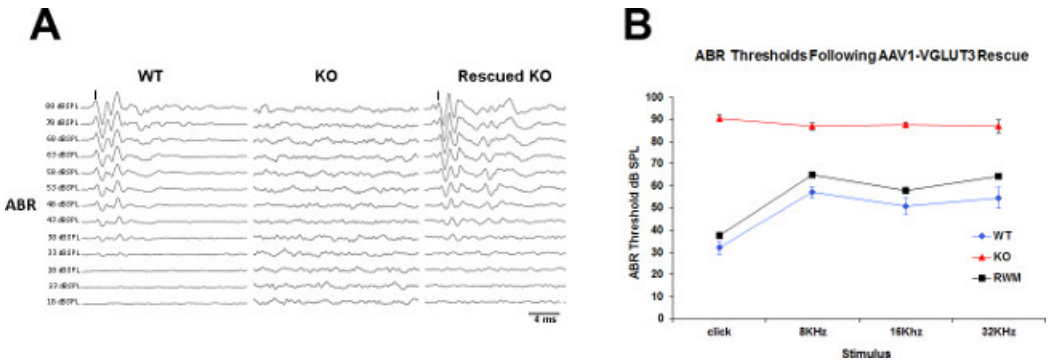

Figure 4: Auditory brainstem response (ABR) assessment. Representative ABR waveforms after AAV1-VGLUT3 delivery were similar between rescued and wild-type mice; in contrast, no ABR waveforms were seen in KO mice **(A)**. Rescued KO mice also showed measurable ABR thresholds. These ABR thresholds were comparable to those seen in WT mice for all frequencies measured **(B)**. I: ABR wave I. Reprint with permission from [26]. Please click here to view a larger version of this figure.

Discussion

In this work, we describe in detail a technique that can be used for cochlear gene therapy, with the goal of restoring or rescuing normal auditory function that is compromised by a genetic defect. As it is typically atraumatic, this approach is safe for cochlear gene transfer or other potential molecular therapies[30]. Other approaches for cochlear therapy have been described, including a ventral approach[24], cochleostomy[31,32] and endolymphatic sac delivery[33] in mouse and guinea pig. In our experience, the post-auricular approach is faster, simpler, and associated with reduced injury and animal mortality. Further, the post-auricular approach offers improved visualization of the RWM with less chance of trauma and associated loss of hearing[34,35,36]. This report documents the technical aspects and utility of the post-auricular approach for cochlear molecular therapy by demonstrating delivery of AAV1-VGLUT3 into the inner ear via the RWM, resulting in transgenic expression of both VGLUT3 and GFP (**Figure 1** and **Figure 2** and **Figure 3A**) and hearing rescue in this mouse model of congenital hearing loss. Prior studies describing methods for gene delivery showed only limited transgenic expression in critical areas of the organ of Corti[12,37,38,39,40,41]. These differences may be due to the quality of the viral preparation, the titer of the viral vector, serotype used, or simply insufficient viral delivery into the cochlea. In contrast, using this post-auricular approach we were able to obtain very good transfection rates to both inner and outer hair cells and distribution of a reporter gene (GFP) after AAV2-GFP transfection (**Figure 2** and **Figure 3A**) throughout the cochlea. Based on the percentage of IHCs transfected with AAV1-VGLUT3 (**Figure 3B**), we suggest that transfection via RWM is more effective and uniform through the whole cochlea than other methods, including that described in the guinea pig, which reported higher transfection rates in the basal turn, close to the site of viral application[42]. The long-term expression of the GFP/ VGLUT3 within the cochlea, for at least a year and a half, is consistent with data obtained from other animal models and different organ systems[38,43].

Additionally, histologic analysis of the transfected cochlea demonstrated no evidence of an inflammatory response, with excellent preservation of the cytoarchitecture of the organ of Corti, including the stria vascularis and spiral ganglion neurons[26]. Further, in contrast to methods that employed a cochleostomy, the RWM transfection used in the present study did not cause cochlear damage, and higher volumes could be injected [31,32]. Lack of trauma using this technique was verified by demonstrating equivalent ABR thresholds between operated and control ears, a finding similar to that seen by Xia *et al.*[4] Finally, AAV-VGLUT3 delivery through the RWM resulted in ABR thresholds comparable to those seen in WT mice (**Figure 4A** and **Figure 4B**)[26]. We have used this technique in all ages of mice, but it is technically challenging in mice older than P40

because the bulla bone becomes harder to perforate and the broken bone from the bulla is sharper in older than in younger mice. At older ages, if caution is not taken bleeding can occur if a piece of the bulla bone lacerates the neck tissues.

In conclusion, this report documents successful gene transfer into several types of cochlear cells in the mouse using an AAV-based vector as was previously shown. This technique of gene delivery minimizes trauma, does not lead to hearing loss, and can result in widespread transfection of a variety of cell types within the cochlea, including hair cells and spiral ganglion neurons. It has great potential application for other types of congenital and acquired forms of hearing loss, and could also be applied to a variety of molecular therapies in mouse models of hearing loss.

Disclosures

No conflicts of interest declared.

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