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Research paper

Intra-tympanic delivery of short interfering RNA into the adult mouse cochlea

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ABSTRACT

Trans-tympanic injection into the middle ear has long been the standard for local delivery of compounds in experimental studies. Here we demonstrate the advantages of the novel method of intra-tympanic injection through the otic bone for the delivery of compounds or siRNA into the adult mouse cochlea. First, a fluorescently-conjugated scrambled siRNA probe was applied via intra-tympanic injection into the middle ear cavity and was detected in sensory hair cells and nerve fibers as early as 6 h after the injection. The fluorescent probe was also detected in other cells of the organ of Corti, the lateral wall, and in spiral ganglion cells 48 h after the injection. Furthermore, intra-tympanic delivery of Nox3 siRNA successfully reduced immunofluorescence associated with Nox3 in outer hair cells 72 h after injection by 20%. Drug or siRNA delivery via intra-tympanic injection does not compromise the tympanic membrane or interfere with noise-induced hearing loss, while trans-tympanic injections significantly altered the cochlear response to noise exposure. In summary, intra-tympanic injection through the otic bone into the middle ear cavity provides a promising approach for delivery of compounds or siRNA to cochlear hair cells of adult mice, relevant for the study of mechanisms underlying inner ear insults and, specifically, noise-induced hearing loss.

1. Introduction

Local application of drugs to the inner ear has the potential to increase their efficacy in specific cell types and reduce side effects in comparison to systemic administration. For instance, local application of medication has been used clinically to treat inner ear disorders, such Meniere's disease with gentamicin or of idiopathic sensorineural hearing loss with corticosteroids (Assimakopoulos and Patrikakos, 2003; Seggas et al., 2011). Similarly, compounds have been delivered to the inner ear of guinea pigs, rats, chinchillas, and mice for laboratory research (Mikulec et al., 2009; Plontke et al., 2007; Roehm et al., 2007; Wagner et al., 2005; Xu et al., 2010). In addition, biodegradable gels loaded with therapeutic compounds and placed on the round window membrane reduce noise-induced hearing loss (NIHL) in guinea pigs, chinchillas, and rats (Coleman et al., 2007; Iwai et al., 2006; Lee et al., 2007). Among the types of local administration, the simplest and most frequently used is injection through the tympanic membrane (Borkholder, 2008), often using the mouse as a model as it offers an advantage in the availability of molecular data and genetic information. The kinetics of drug distribution in the inner ear after this procedure have been documented; gentamicin can be detected in the perilymphatic fluids of the mouse 1 day after intra-tympanic injection and its concentration drastically decreases after 3 days (Xu et al., 2010).

Short interfering RNA (siRNA) is a powerful tool for the study of molecular pathways and pathologies. In inner ear research, local application is required, as systemic administration would lead to dilution or degradation before significant concentrations in target cells can be achieved. Indeed, local delivery of siRNA into the rat cochlea has been successfully used for investigation of the mechanisms of cisplatin-induced hearing loss (Kaur et al., 2011; Mukherjea et al., 2010, 2008). However, it remains to be established which method of siRNA application would be most suitable for the study of NIHL, which cells take up siRNA after the local application, and how long after delivery of siRNA the compounds are effective in the targeted cells.

To define these parameters, adult CBA/J mice at 3 months of age were used for investigation. We first characterized the effects of local injection through the tympanic membrane (trans-tympanic

Abbreviations: ABR, auditory brain stem response; BBN, broadband noise; NIHL, noise-induced hearing loss; PTS, noise-induced permanent threshold shifts; siRNA, short interfering RNA.

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administration) or through the otic bone into the middle ear cavity (intra-tympanic administration) on subsequent auditory function as measured by auditory brain stem response (ABR) and on the response to noise exposure with broadband noise (BBN) from 2 to 20 kHz at 108 dB for 2 h. Next, the localization of a fluorescently-tagged scrambled siRNA probe delivered into the middle ear via the intra-tympanic route was determined at several time points after the delivery. Finally, the ability of Nox3 siRNA treatment to suppress Nox3-associated immunofluorescence in outer hair cells was assessed.

2. Materials and methods

2.1. Materials

Alexa Fluor 546-conjugated Nox3 siRNA (target sequence: 5′-AAGGTGGTGAGTCACCCATCT-3′), scrambled siRNA, Phalloidin, Hoechst 33342 and Alexa Fluor 488 secondary fluorescent antibody were purchased from Invitrogen (Carlsbad, CA). Polyclonal rabbit anti-Nox3 (# SC67005) was from Santa Cruz Biotechnology. Tissue adhesive was purchased from 3M Vetbond (#1469SB, St. Paul, MN). All other reagents were purchased from Sigma-Aldrich Co. (St. Louis, MO).

2.2. Animals

Male CBA/J mice at 12 weeks of age (Harlan Sprague Dawley, Indianapolis, IN) were allowed free access to water and a regular mouse diet (Purina 5025, St. Louis, MO) and were kept at 22 ± 1 °C under a standard 12 h:12 h light–dark cycle for one week of acclimation before the experiments. All research protocols were approved by either the University of Michigan (UM) Committee on Use and Care of Animals or by the Institutional Animal Care & Use Committee at the Medical University of South Carolina (MUSC). Animal care was under the supervision of either the Unit for Laboratory Animal Medicine at UM or the Division of Laboratory Animal Resources at MUSC.

2.3. Auditory brain stem response

Animals were anesthetized via intra-peritoneal injections of xylazine (7 mg/kg), ketamine (65 mg/kg), and acepromazine (2 mg/kg) and then placed in a sound-isolated and electrically shielded booth (Acoustic Systems, Austin, TX). Body temperature was monitored and maintained with a heating pad. Acoustic stimuli were delivered monaurally to a Beyer earphone attached to a customized plastic speculum inserted into the ear canal. Subdermal electrodes were inserted at the vertex of the skull, under the left ear, and under the right ear (ground). Tucker Davis Technology (TDT) System III hardware and SigGen/Biosig software were used to present the stimuli (15 ms duration tone bursts with 1 ms rise-fall time) and record the response. Up to 1024 responses were averaged for each stimulus level. ABR responses were determined for each frequency by reducing the intensity in 10 dB increments and then in 5 dB steps near threshold until no organized responses were detected. Thresholds were estimated at the lowest stimulus level at which a response was observed, identified by the presence of ABR waves I and II. All ABR measurements were evaluated by an expert blinded to the treatment conditions.

2.4. Noise exposure

Mice were exposed to a BBN from 2 to 20 kHz at 108 dB SPL for 2 h. Four mice were exposed at the same time in separate stainless steel wire cages (approximately 9 cm × 9 cm × 9 cm). The sound exposure chamber was fitted with a loudspeaker (model 2450H; JBL, Northridge, CA) driven by a power amplifier (model XLS 202D; Crown Audio, Elkhart, IN) fed from a CD player (model CD-200; Tascam TEAC American, Montebello, CA). Audio CD sound files were created and equalized with audio editing software (Audition 3; Adobe System, Inc., San Jose, CA). Sound levels were calibrated with a sound level meter (model 1200; Quest Technologies, Oconomowoc, WI) at multiple locations within the sound chamber to ensure uniformity of the sound field, and were measured before and after exposure to monitor stability.

2.5. Surgical procedure for intra-tympanic application

Catheter tubes 29.5 cm in length were prepared from two types of tubing. Twenty-eight cm of micro medical polyethylene tubing (#BB31695, Scientific Commodities Inc., Lake Havasu City, AZ) and 2 cm of medical grade polyimide tubing (#823400, A-M Systems Inc., Sequim, WA) were fitted with an overlap of 0.5 cm under a dissection microscope and bonded with one drop of Quick Bond (#72588, Electron Microscopy Sciences, Hatfield, PA). After bonding overnight, the catheter tubes were sterilized with ethylene oxide gas. Animals were anesthetized with an intra-peritoneal injection of xylazine (20 mg/kg) and ketamine (100 mg/kg). Body temperature was maintained with a heating pad during the surgical procedure. A retroauricular incision was made for the approach to the temporal bone (Fig. 1). The facial nerve was identified deeply along the wall of the external auditory canal. The otic bulla was identified ventrally to the facial nerve and a small hole was made in the thin part of the otic bulla with a 30 G needle, located ventrally to the facial nerve (Fig. 1 arrowhead). Injury of the stapedial artery can be avoided at this location. The catheter tube described above was fed through the hole for about 0.5 cm and 10 μL of the experimental solution was slowly injected. After delivery, the hole was covered by the surrounding muscles. No seal was necessary to prevent leakage. The skin incision was closed with tissue adhesive.

2.6. Immunocytochemistry for surface preparations or cryosections

Following decalcification with 4% EDTA for 72 h at 4 °C, cochleae were processed for immunocytochemistry on surface preparations or frozen sections. For surface preparations, the decalcified cochleae were dissected under a microscope by removing the softened otic capsule, stria vascularis, Reissner's membrane, and tectorial membrane. The remaining tissue, including the modiolus and cochlear sensory epithelium, was permeabilized in 3% Triton X-100 solution for 30 min at room temperature. For cryosection analysis, sections of 8 μm thickness were incubated in 0.3% Triton X-100 in PBS for 30 min at room temperature. The specimens for either cryosections or surface preparations were washed three times with PBS, and incubated with phalloidin at a concentration of 1:100 for 1 h at room temperature. After the final wash with PBS, the tissue for surface preparations was dissected by removing the modiolus. The epithelia were divided into three segments (apex, middle, and base). Specimens were mounted on slides with anti-fade mounting media. Immunolabeled surface preparations were imaged with an Olympus laser confocal microscope. Immunolabeled cryosections were imaged with a Zeiss laser confocal microscope. For each group a sample size of 3 was used.

2.7. Quantification of the immunofluorescence signals in outer hair cells from surface preparations

Immunofluorescence of Nox3 in outer hair cells on surface preparations was quantified from original confocal images, each taken with a 63× magnification lens under identical conditions and