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Endocochlear Potential Measures, Local Drug Application and Perilymph Sampling in the Mouse Inner Ear

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Running Head: Inner Ear Methods in the Mouse

Abstract

Functionally and anatomically, the laboratory mouse inner ear is comparable to other mammals, except that it is considerably smaller and operates over a higher acoustic frequency range. Other than miniaturization of methods that might be applied to the inner ears of guinea pigs, gerbils or chinchillas, the major difference lies in fewer points of access, due both to small number of cochlear turns (2¼) and reduced access to cochlear scalae in any single turn. These features do not particularly complicate auditory brainstem, compound action potential, or distortion product emission recording. Due to the close proximity to generators, these responses can be quite large in mice. Instead, they present challenges for endocochlear potential (EP) measurement and for manipulations and measurements of inner ear fluids. Without appropriate modifications, considerable technical challenges and potential pitfalls can render such measurements uninterpretable in mice. This chapter outlines experimental techniques for targeting inner ear fluid manipulations and fluid measures in mice. We specifically consider methods for EP measurement, perilymph sampling, and introduction of chemical agents into the middle or inner ear.

Key words: Endocochlear potential, Auditory bulla, Intratympanic injection, Intralabyrinthine injection, Round window membrane, Posterior semicircular canal

Abbreviations:
EP: endocochlear potential
OHCs: outer hair cells
IHCs: Inner hair cells
IT: intratympanic
TM: tympanic membrane
RWM: Round window membrane
CA: cyanoacrylate adhesive
PSCC: Posterior semicircular canal

1. Introduction

Laboratory mice (*Mus musculus*) have become the dominant experimental model for hearing science (Bowl and Dawson 2015; Ohlemiller et al. 2016a). This reflects their economy, short life span, and especially genetic standardization that facilitates the use of molecular tools to study both the physiology and pathophysiology of hearing. The vast majority of known deafness genes have been discovered and characterized using parallel studies of humans and mice. Instances where mice do not reproduce the phenotype of particular human mutations have typically reflected the use of different protein isoforms or expression by different subsets of cells in humans and mice (e.g., Hosoya et al. 2016a; Hosoya et al. 2016b). As the promise of therapeutics against hearing loss has grown (e.g., Crowson et al. 2017; Wang and Puel 2018; Bielefeld and Kobel 2019; Szeto et al. 2020), local delivery of these to the mouse inner ear has accelerated. This requires mouse protocols specifying where to access inner ear fluids for injection of therapeutic agents, and where and how to sample inner ear fluids to verify the effective dose.

Manipulations and measurements of the mouse inner ear are made challenging by the very small dimensions and volumes involved for the mouse inner ear. Likewise, attempts to extrapolate findings from the mouse ear to their likely outcome in the human ear can be distorted by the large differences

Volume (µL, mm^3)	Human	Guinea Pig	Mouse
Inner Ear Perilymph	196.9	20.0	1.0
Cochlea Endolymph	7.7	2.0	0.3
Scala Tympani	40.5	6.0	0.3
Scala Vestibuli + Vestibule	84.9	10.8	0.5
Length (mm)			
Scala Tympani	28.5	17.1	4.3

Table 1: *Fluid volumes and lengths compared for the human, guinea pig and mouse derived from 3-D reconstructions of the inner ear. Mouse data were reported by Hirose and colleagues (Hirose et al., 2014).*

from the mouse ear to their likely outcome in the human ear can be distorted by the large differences in volume involved. Table 1 compares some fluid volumes and lengths for mice, guinea pigs and humans. The values are derived from 3D reconstructions of thin sheet laser imaging datasets of fixed, intact ears. The total perilymph volume of the mouse is close to 1.0 µL, which is approximately 200-fold less than the perilymph volume of the human ear.

2. Endocochlear Potential Recording

The endocochlear potential (EP) is a positive DC potential recorded from the endolymphatic space of the cochlea. In mice, the normal EP is typically >100 mV, although this is impacted by basal-apical gradients, and can vary by inbred strain. Ion measurements made with glass microelectrodes (EP and ion-specific measures using ion-selective electrodes) are no more difficult in the mouse ear than in other mammals used experimentally, although the number of access points is reduced. As described here, they require a small fenestration of the bony otic capsule 20-30 µm in diameter through which a microelectrode is inserted. Given suitable resources (operating scope, micromanipulators) the small volume of the endolymphatic compartment is not limiting for the measurement.

2.1 Rationale

Sensitive hearing requires a normal middle ear and a full complement of cochlear outer hair cells (OHCs), along with their attending supporting cells in the organ of Corti. The electro-mechanical responses of OHCs in turn help provide the necessary mechanical input to inner hair cells (IHCs), the "coding" sensory cells, to paint a detailed picture of the incoming sound spectrum for processing by the auditory nerve (Dallos et al. 2006; Carney 2012). Both OHCs and IHCs require appropriate electrochemical gradients to drive sound-driven receptor currents. The electrical portion of the

gradient includes the EP, which depends on a normally-functioning stria vascularis and spiral ligament (collectively, the cochlear lateral wall), plus a continuous lining of ion-tight boundaries of endolymph (Jahnke 1975). In supporting EP generation, cells of the lateral wall must regulate a number of ions, including Na^+, K^+, Cl^-, H^+ and Ca^{++} (Wangemann 2006). While K^+ represents the dominant ion in this process, the EP appears more sensitive to fluctuations in pH and Ca^{++} (Gill and Salt 1997; Wangemann 2006). In any event, because of its reliance on multiple ions, a normal EP indicates that a host of metabolic processes are normal, and provides the single most informative metric regarding the micro-environment of hair cells.

The lining of the endolymphatic space is fragile and unforgiving of intrusions (e.g., Suzuki et al. 2017). Numerous technical difficulties, such as drift in DC potentials, preclude chronic EP recording even though long-term monitoring—and even modulation—of the EP have been the goal of some studies (Schmiedt 2010; Mercier et al. 2012). For this reason, we present only an acute (terminal) EP recording procedure using a ventral or lateral approach.

2.2 Materials

The main equipment required includes:

a. Head holder For most procedures accessing the inner ear, it is essential to be able to hold the animal's head rigidly. A head holder suitable for making EP recordings is illustrated in Figure 1. This holder provides two-point support (upper jaw and vertex of the skull) offering stability that is more than sufficient to make EP recordings. Other variants are possible, such as the inclusion of ear bars to limit lateral movements, although these can also restrict access to the inner ear.

b. Electrometer An intracellular amplifier with high input impedance suitable for use with glass microelectrodes is required, with the output routed to a digital display, voltmeter, or data logging system. The amplifier must be capable of making DC potential measurements (no AC-coupling, such

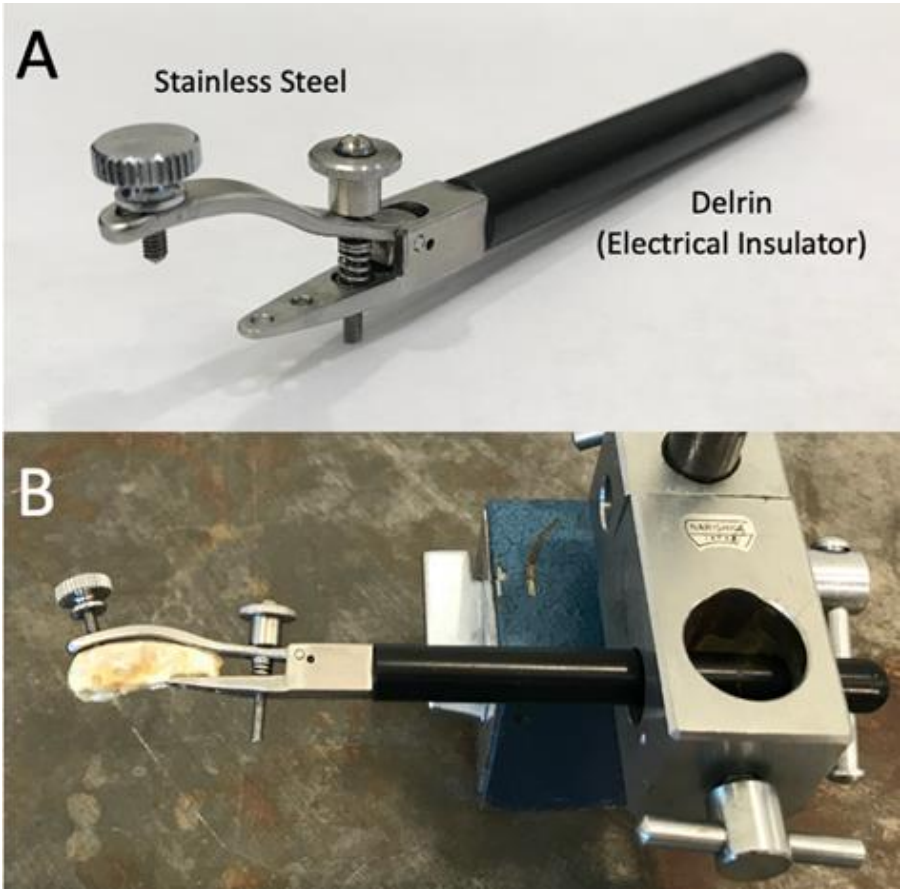

Figure 1: ***A:*** *A custom-made mouse head holder. The lower rigid block has two holes through which the upper incisor teeth are inserted. The two locations are to accommodate different head sizes. The mobile upper bar is tightened to hold the head firmly in place with unimpeded access to both ears. The black shaft of the holder is made from Delrin, which is an electrical insulator.* ***B:*** *The head holder, shown with a mouse skull in place, can be mounted to the table with a magnetic micromanipulator base.*

as the “sound card” input on a laptop). The ability to offset the DC voltage to zero at the amplifier or output device is useful, but not essential.

c. Glass microelectrodes The active measurement electrode is an electrolyte-filled glass pipette, pulled to a fine tip in an electrode puller. The tip size formed by the puller is not critical, but the overall shape of the tip is important. If it is too blunt it will not insert easily, and insertion may be blocked by the bony fenestra. If it is too long and fine, it will bend upon contact with the tissues and will not insert easily. A tip length of about 8 mm works well. The tip of the pipette can either be broken (with fine forceps) to a tip diameter of 2-5 µm or beveled to that diameter on a beveling wheel (Narishige Model EG-40). The advantage of a beveled tip is that it is sharp (like a hypodermic needle) so it penetrates the tissues of the lateral wall more easily. It also allows visual verification of

whether the electrode was inserted successfully without breaking. Filling the electrode with electrolyte is simplified when the electrode glass contains a filling fiber (filament glass) that runs down the inner wall of the tubing. This helps remove bubbles from the tip during the filling process. The pipette is typically filled with 150-500 mM KCl solution, to which electrical contact is made using an Ag/AgCl wire (detailed below). Alternatively, a variety of coupling devices are available that incorporate an Ag/AgCl contact (e.g., WPI model MEH3SF15).

The recording electrode-to-amplifier and reference electrode-to-amplifier junctions are critical for minimizing DC offsets. There are a variety of ways of providing stable fluid/metal junctions (such as using the calomel cells made for pH meters). For EP measurements, we have found that Ag/AgCl wires contacting solutions of fixed Cl^- concentration provide electrically stable junctions. Either commercially available Ag/AgCl pellets or Ag wire, cleaned and after current is passed to give an AgCl coating. This is readily performed by connecting the Ag wire (as the anode) to the positive pole of a 3V battery, and some other wire as the cathode (connected to the negative side of the battery). With both wires immersed in an electrolyte solution containing Cl^-, the silver contact turns gray as AgCl is deposited on the surface. Voltage from an Ag/AgCl wire is stable when the Cl^- concentration of the solution it contacts is constant. If the wire is placed in direct contact with the animal, or near the back of a glass electrode that is “drying up” with time, the Cl^- concentration will change and the voltage generated by the wire will drift with time. In contrast, when the AgCl-coated wire is in contact with solution of stable Cl^- concentration, the voltage remains stable over time. For the active EP electrode, the wire should be inserted down to the shoulder of the glass pipette (where Cl^- concentration will be most stable) or an air-tight coupler with AgCl pellet utilized. For the ground electrode, a AgCl pellet connected to a saline bridge (a length of plastic tubing containing electrolyte) is used to maintain constant Cl^- at the pellet. It should be kept in mind that AgCl pellets are very fragile. They can crack at the stub on which they are mounted, or allow fluid to enter the casing and

contact other metal components, causing instability. Having a supply of new ones available is recommended.

d. Audio monitor An audible baseline monitor (such as the model ABM previously available from World Precision Instruments, Sarasota) converts DC voltage to an audio signal. As the input voltage increases, an audible tone increases in frequency. This provides the operator with valuable feedback about voltage changes while inserting the electrode into endolymph, when attention is focused on the view through the operating microscope.

e. Micromanipulator/microdrive A stereotaxic manipulator, either with magnetic base or bolted in position, is necessary to hold and position the electrode in the animal. A motorized electric or hydraulic microdrive is helpful but not required, as long as the drive allows fine resolution of movement (<5 µm per step) and does not bend or allow other uncontrolled movement when touched. Ideally, there should be some type of read-out of microelectrode depth for verification of when the electrode has been returned to its original position.

f. Surgical instruments Fine surgical scissors and forceps are needed. We have found ocular retractors and small weights hung from bent wire to be useful for gaining access and clearing the view. The pick used to fenestrate the mouse cochlear capsule is a Bauch & Lomb 30 degree stapes pick (Storz N1705 80) which must be sharp and in good condition. The pick should not be used for other purposes. It is advisable to first thin the bone using the sharp edge of an “old” 30 degree pick, turned on its side.

g. Other equipment An adjustable “jack-stand” is required to hold the torso of the animal, ideally with a Plexiglas (or otherwise electrically insulated) bed. Since depressed body temperature will lower the EP, a heating pad is needed to maintain core temperature. A passive or DC current-based heater is preferred over one that relies on circulating water, as the variation in water flow may mechanically destabilize the preparation. While in some cases a ventilator system may aid good

oxygen status, we have found a simple tracheostomy to suffice for this purpose. Care should be taken to prevent any fluids from entering the tracheostomy opening.

2.3 Detailed Methods:

a. Initial surgical approach

Because EP recording is an invasive terminal procedure, the animal should be anesthetized fairly deeply. A number of anesthetics are adequate for this purpose. Light anesthesia risks jerking movements that destabilize the recording. Position the mouse ventral-side up with the upper incisor teeth inserted into the head holder and the tongue moved aside as necessary to ensure a clear airway. A midline incision is then made, as indicated in Figure 2A, after which the underlying fibromuscular tissue is blunt dissected away from the midline to expose the trachea, followed by making a small opening (Fig. 2B). After the connective tissue over the trachea is retracted laterally, the auditory bulla will come into view, surrounded by overlying muscle (Fig. 2C). This muscle can be addressed by a combination of blunt dissection, cutting at the posterior point of attachment on the bulla, and using a retractor or small clip to pull the muscle flap anteriorly. Exposure such as shown in Fig. 2D can be achieved with little bleeding as long as care is taken not to probe too deeply either medially (carotid artery) or posterior to the bulla (jugular vein). The bulla is then opened by drilling a small hole in the most translucent portion with a hand-held needle, then removing small pieces with malleus nippers to fully expose the basal turn of the cochlea and stapedial artery (Fig. 2E). The thin bone is easy to remove. If the bulla is not translucent, or the bone or underlying mucosa appear vascularized, there may be middle ear pathology. Do not use a dental burr to open the bulla or remove large pieces at once, due to the damaging noise that could be transmitted to the cochlea. It is not necessary to remove the bony tympanic ring, although doing so improves the view (assuming no sound to be presented). Avoid touching the stapedial artery, as bleeding from the artery can be

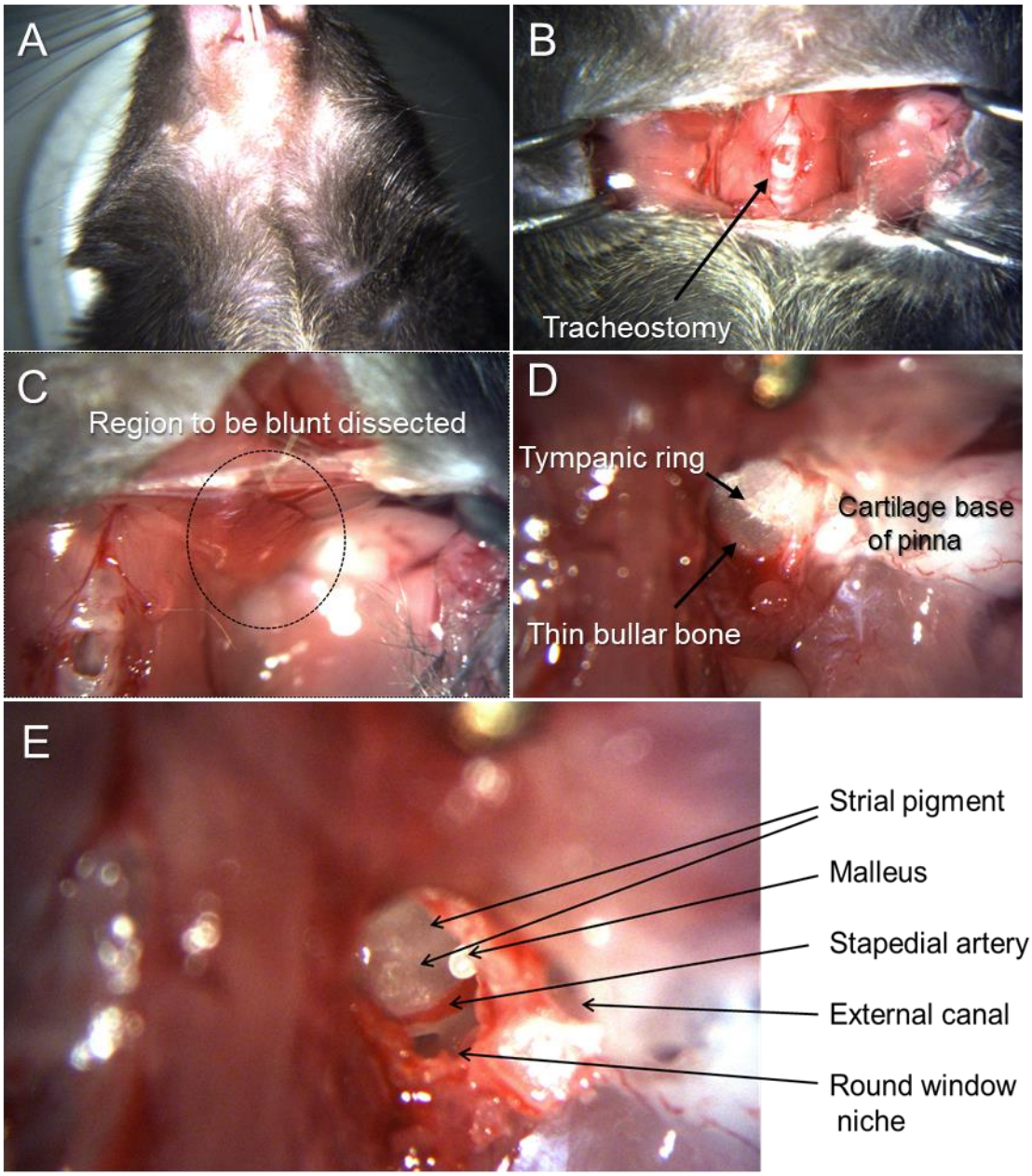

Figure 2. Surgical approach to mouse inner ear for EP recording. A. The location of the initial incision at the midline. B. Occular retractor reveals trachea, from which overlying muscles are blunt dissected and a single hole is made to facilitate breathing. C. Location of musculature overlying the left auditory bulla, which is removed by a combination of blunt dissection, cutting at the posterior attachment, and retracting the anterior flap. D. Exposed bulla, which is nearly transparent in a normal middle ear. Tympanic ring can be seen under the lateral half. E. The bulla has been opened and a portion of the tympanic ring has been removed to expose the cochlear capsule, stapedial artery, and round window niche. In this highly pigmented strain (C57BL/6), the pigmented stripe of the underlying stria of both basal and apical turns can be seen.

difficult to stop. Notably, this artery provides little of the cochlear blood supply, so that if bleeding can be controlled using patient gentle pressure, the procedure may continue.

After the stapedial artery is exposed, cochlear capsule ”targets” for fenestration will appear directly anterior (Fig. 2E). In pigmented mouse strains such as C57BL/6 or CBA/CaJ, the stria of both basal and second turns will often stand out as two medial-lateral-running dark stripes against the white of the capsule bone. For albino mouse strains (e.g., BALB/c) subtle contouring of the capsule and bony septa lines can be helpful. For initial training, it is highly recommended that a pigmented strain be used to learn the anatomic features. The majority of the exposed cochlear capsule borders scala media (providing limited access to other scalae), so that most approach errors are errors of electrode angle. In general, it is best to center the point selected for fenestration along the medial-lateral dimension of the capsule.

b. Fenestration of the cochlear capsule The method for measuring the EP we adapt here was established by Teruzo Konishi (Konishi 1979). Konishi may not have invented the method, but he perfected it and taught it to a considerable number of investigators in the field. The goal is to make a fenestration of the bone with minimal damage to the underlying tissues of the spiral ligament. The main elements of the technique are illustrated in Figure 3, whereby the selected point for fenestration is thinned by shaving the surface. If too little bone shaved, unnecessary effort is needed to make the fenestra through the remaining thick bone. If, however, too much bone is shaved, the remaining bone cracks easily when contacted, and an overly large fenestration is created.

While shaving the surface, the anatomy should repeatedly be evaluated to adjust the site where the fenestration will be located. As the bone is thinned, place the pick gently on the bone at this site and move it *radially* away from the site. Next, gently reposition the pick at the same site and again move it away radially, this time in a different direction. Continue placing the pick on the same site and moving it outwards radially from the site, moving bone (invisibly) with the tip of the pick. This causes the depression to become deeper with time without applying any pressure to the bone. The thinner

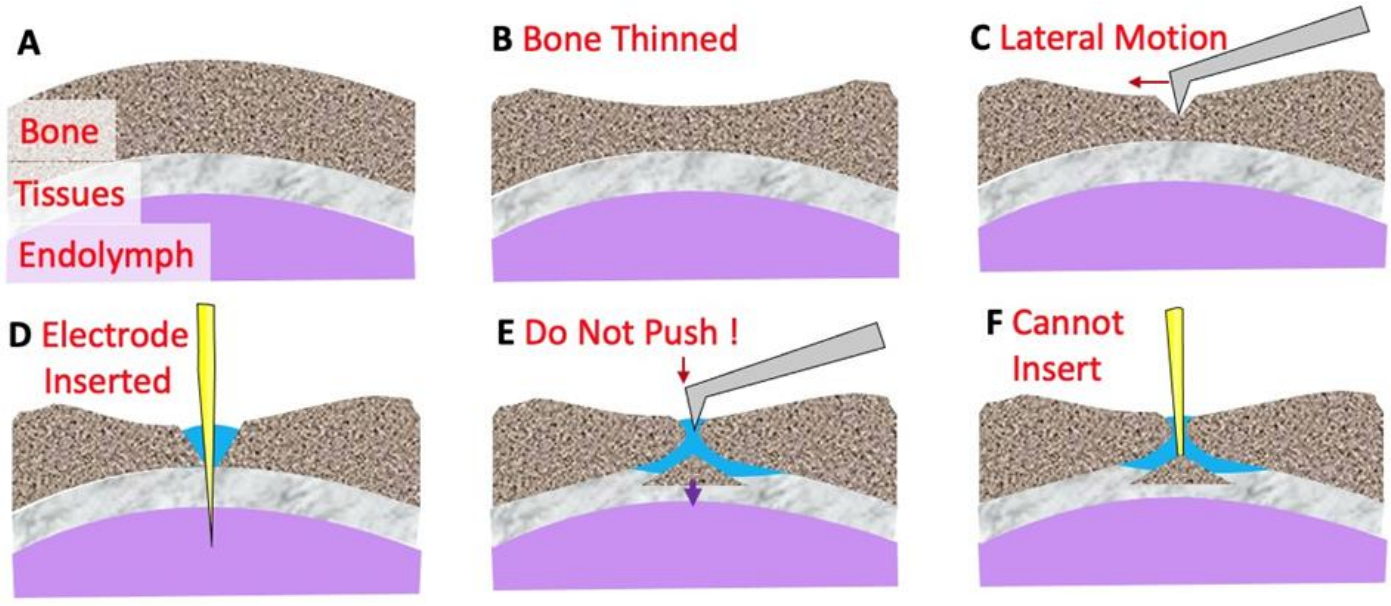

Figure 3: Making a fenestration in the bone for endocochlear potential measurement. From the initial state (A), bone is first thinned by shaving with the sharpened side of an old pick (B). A sharp pick is then gently placed at site for fenestration and moved laterally away from the site to scrape bone away (C). The pick is returned gently to the same site and then moved in a different direction. Performed repeatedly in random radial directions, this creates a slowly enlarging conical depression in the bone. When fluid is seen, the EP measurement can be taken (D). It is important never to push on the pick. This can cause a cone-shaped fragment of bone to be pushed into the tissue/fluid space (E), damaging the underlying tissues and blocking entry of the glass electrode (F).

the bone becomes, the more careful placement must be. Ultimately a point is reached when fluid emerges as the pick is placed. At this point, white edges of the fenestration or just a white dot of bone dust, about 20-30 µm diameter may be visible. During the fenestration process it is important not to force the pick, but to slowly let the tool do the work. It should come as a “surprise” when the appearance of fluid confirms the fenestration is complete. If the bone is thin and the pick is sharp, it may take just 3-5 repetitions for the fenestration to succeed. If many repeats of the pick have been performed without a fenestra appearing, it may be necessary to shave the bone further and start the process again. In older animals the bone is thicker and typically requires more shaving before starting the picking process.

Figure 3 also shows how *not* to make the fenestration. If the pick is pushed prematurely into the thinned bone, it can cause a cone-shaped bone fragment to be pushed into the cochlea (Fig. 3E). The operator will see fluid and through the microscope, and the fenestration may appear successful. However, it can then be extremely difficult to insert the glass electrode into endolymph without breaking the tip (Fig. 3F). It may sometimes be possible to insert the electrode at the edge of the fenestration, to the side of the fragment. Typically, however, the bone fragment will have already detached tissues from the bony wall or damaged them to a degree that the EP will be lower and less stable.

The EP measurement is made by positioning the glass electrode so that the tip makes contact with the fluid emerging from the fenestration. If a drop of fluid has accumulated, it may be necessary to wick it away before positioning the electrode. After fluid contact is made, zero the electrometer to designate this contact potential as “zero voltage” with respect to ground. If the initial voltage varies widely from animal to animal, then something may be wrong with either the ground or electrode metal/fluid interface. There should be minimal potential drift over time. If potential remains stable (<1 mV change over 10 min) then advance the electrode. Be aware that Ag/AgCl wire interfaces require time to stabilize. An electrode that is used immediately after fabrication may show a drift in voltage. If the electrode tip bends as the electrode advances it may be necessary to readjust the manipulator. Commonly, as the tip enters the fenestra, there is a “quiet period” when you know it has passed through the bony aperture but no voltage change is apparent. When the electrode encounters a voltage increase by digital read-out or audio monitor, the electrode should briefly be moved faster to “pop it” through the tissues. As soon as EP reaches a high, stable value, stop advancing.

If there appears no voltage change as the electrode is advanced, the electrode tip may have missed the endolymphatic space, instead entering scala tympani or scala vestibuli. If scala media has not

been breached, it may still be possible to adjust the angle of entry. If the recording is ultimately unsuccessful, it may be worthwhile for future attempts to dissect the preparation to determine how the target was missed.

After a successful EP measurement is made, withdraw the electrode to the “open circuit” reading and reposition it just in contact with the fluid at the fenestration site. Compare the voltage reading with the initial zero voltage reading before the measurement. If it is within a few milliVolts, this gives confidence that the measurement was valid. If the reading differs markedly from zero then either 1) The electrode drifted electrically, 2) The electrode tip was broken during insertion, or 3) The electrode was blunt, and inserted deeply so that a large perforation of the endolymphatic boundary was created, locally polarizing perilymph. Any of these conditions lowers the degree of trust in the validity of the EP measurement.

Repeated or prolonged EP measurements

Some protocols may call for repeat EP measurements in the same preparation. In general, even in the best of circumstances the EP will read a few mV lower than the initial reading. If the electrode is blunt or was broken or too-deeply inserted, repeat EP measures can be substantially lower and less meaningful. Repeated EP measurements are generally unreliable.

Monitoring EP changes over time during a procedure is particularly difficult in the mouse. If the headholder lacks earbars to laterally stabilize the head, there will be a tendency for head position to drift with time, slowly displacing the recording electrode. Variability in the depth of anesthesia, or attempting to maintain anesthesia with an injectable anesthetic, can also mechanically disturb the recording electrode position. Beware also of thermostatically-controlled heating pads that rely on circulating water, as the animals’ head and body may shift between cycles. Finally, the overall

surgical arrangement should not require the experimenter to support or stabilize their hands on the headholder or platform. While continuous EP recording (that is, over the course of a procedure) is possible in mice, the cost and quality of the equipment needed versus brief recordings may be prohibitive.

Choices and significance of EP recording location

The mouse cochlea offers three primary recording sites for the EP: the lower basal turn as described above (~45 kHz frequency place), through the round window membrane (~60 kHz frequency place), and the lower apical turn (~6 kHz frequency place) (Hirose and Liberman 2003). Due in part to the short length of the mouse cochlea (~5.5 mm) (Saunders and Garfinkle 1983) compared to the estimated 2.0 mm length constant of the EP in rodents (Johnstone et al. 1966), there is little frequency specificity to any mouse EP recording. For most research questions, recording from the lower base will suffice to assess strial function in any animal, as most mouse studies have done.

A few papers have described EP recording through the mouse round window membrane (RWM) on the anterior wall of the round window niche (Fig. 2E), which can be accessed using the surgical approach described above. In this approach, the electrode is inserted directly through the RWM, then through a very thin expanse of scala tympani, then through the organ of Corti adjacent to the osseous spiral lamina. Access to the round window requires more aggressive posterior exposure of the cochlea than for other recordings, and may be prohibitively difficult in some inbred strains. Because a rush of perilymph as the RWM is perforated will quickly alter the optics of this approach, and also because of the anatomic disruption the electrode typically causes, the round window approach is especially unsuitable for repeated entry or monitoring the EP over time. EP values reported in mice using this method vary from similar to those obtained from the lateral wall of the lower basal turn (Schrott et al. 1990) to 10-15 mV lower in the same mouse strains (Liu and Zhao 2015; Li et al. 2020),

and it is difficult to say whether this reflects a real spatial gradient or is an artifact of the approach. The round window approach has value for research goals other than EP recording, such as recording saccular and utricular endolymphatic potentials (Liu and Zhao 2015; Li et al. 2020). However, it offers little advantage over the lower basal turn approach described here for EP assessment.

A slight adjustment of location (Fig. 2E) converts the lower basal turn EP recording into a lower apical turn EP recording. The room for error in electrode angle and depth is reduced in the apex, and the extreme thinness of the capsule at this location renders it more susceptible to damage. Typically, thinning of the bone is not needed, and even a light touch with a pick will penetrate the cochlear capsule. Normally the apical turn EP is about 10 mV lower than in the lower base (Fig. 4A). Work in guinea pigs (Gill and Salt 1997) has correlated the EP gradient to endolymphatic Ca^{++} levels. The spatial EP gradient may be smaller in albino animals. Pathological conditions such as aging and broadband noise exposure can both eliminate or even reverse this gradient (Fig. 4B,C). This may reflect preferential effects of most noise exposure protocols and aging on the stria of the basal turn.

2.4 EP changes in pathologic conditions

The EP can be somewhat selectively and temporarily reduced by loop diuretics such as furosemide (Sewell 1984), and may also be affected by genetic mutations (e.g., Steel and Barkway 1989; Ingham et al. 2016; Nishio et al. 2016) and aging (Ohlemiller 2009; Schmiedt 2010). Permanent EP reduction by noise exposure appears uncommon in mice, but may occur as a function of inbred strain, exposure level, and age at exposure (Ohlemiller et al. 2011; Ohlemiller et al. 2018). Nevertheless the universal assertion that a low EP must indicate pathology, and that a higher EP is always better, is false. Acute, mechanically-induced organ of Corti displacements in guinea pigs can increase EP by over 10 mV (Salt et al. 2009), demonstrating there may be conditions when a high EP is “abnormal”. In addition, since hair cells act as a sink to the currents sourced by the stria, ablation of hair cells or

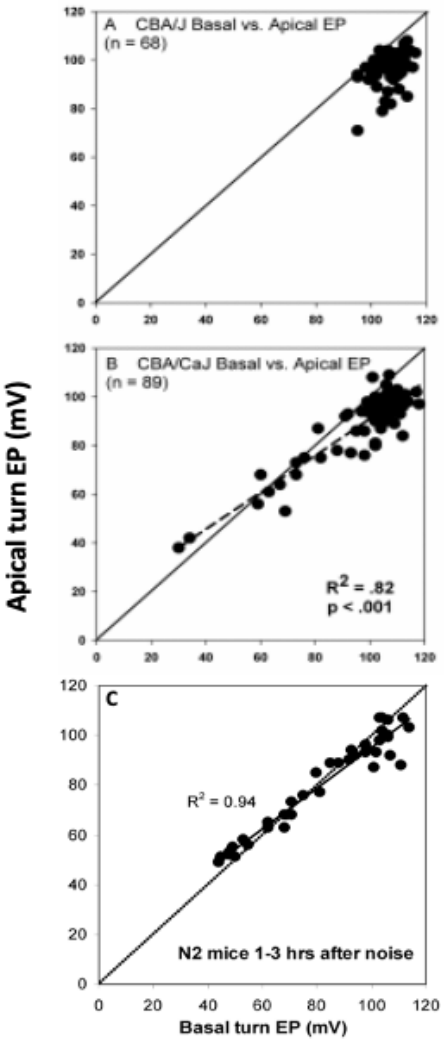

Figure 4. *EP measurements from both basal and apical cochlear turns under three conditions.* ***A.*** *Healthy young CBA/J mice show that the EP in the basal turn averages ~10 mV higher than in the apex.* ***B.*** *Aged CBA/CaJ mice, in which the EP declines with age, show reversal of the normal gradient.* ***C.*** *Similar result for EP measured acutely after noise exposure in N2 backcross mice formed from C57BL/6 and CBA/J. (Adapted from Adapted from Ohlemiller et al. 2010a; Ohlemiller et al. 2010b).*

their stereociliary bundles by ototoxins or noise exposure could yield abnormally high EP values. A comparison of noise-exposed recombinant inbred mouse strains formed from C57BL/6 and BALB/c (Ohlemiller et al. 2016b) identified strains with acutely increased EP and those with an acutely decreased EP. Thus the direction of acute EP change for a given exposure condition appears explicitly genetic, potentially reflecting genes and alleles that impact current-sinking by hair cells.

2.5 Troubleshooting EP recording

A common problem disrupting EP recordings is one of “drift” or instability of the measured electrical potential. Avoidance of drift requires stable electrical interfaces between the metal wires of the amplifier and the aqueous environment of the animal. Correctly grounding the animal is essential for stable EP measurements. The “grounding electrode” (Ag/AgCl pellet and saline bridge or calomel cell) should be the only ground on the animal (Figure 5A). If the voltage reading does not go “open circuit” when the ground electrode is removed from the animal, then something is wrong. If any other metal component of the headholder or animal support (e.g., rectal probe or IV line) effectively grounds the animal, then the circuit shown in Fig. 5B will develop. Moreover, if the voltage of the metal/fluid junction at the second ground differs from that of the initial grounding electrode, the voltage difference will drive a current around the ground circuit “loop”. As this is a low-resistance pathway, the current may be large. Current passing through the ground electrode can cause it to

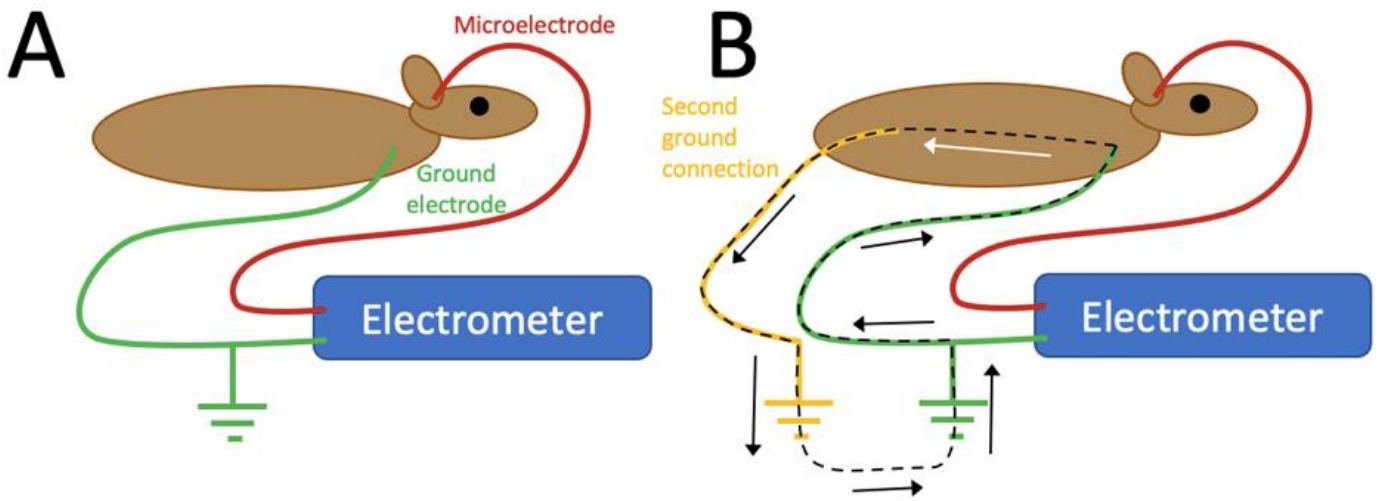

Figure 5: Grounding the preparation. (A) There should only be one electrical pathway to ground on the animal, provided by the “reference” or “grounding” electrode, shown as the green line. (B) In the case where a second ground connection is present, such as through a metal head-holder, IV line or rectal probe (yellow line), large currents can circulate through the low resistance pathway (black dotted lines), causing instability of the reference electrode and drifting potential readings. Left uncorrected the reference electrode can be permanently damaged.

drift, and with time, can damage it permanently. When voltage drift occurs, the first step in diagnosis is to remove the ground electrode to verify the reading goes “open circuit”. If the voltage reading is little changed when the ground electrode is removed, then the second ground connection must be identified and removed.

Other sources of voltage drift can include old or damaged Ag/AgCl cells or inappropriately placed Ag/AgCl pellets or wires. With commercial Ag/AgCl pellet electrodes, instability can arise when fluid seeps down the edge of the pellet and makes contact with the metal wire underneath. Circulating current between the metal and the AgCl will cause the voltage to drift. Similarly, if chloride concentration at the location of the AgCl wire is unstable (e.g., if the wire is simply placed on a cut muscle, or in fluid that is evaporating with time) then voltage will drift. Silver wires and pellets should always be located in some type of fluid bridge containing solution with a stable chloride concentration.

3. Local Drug Application Methods

3.1 Rationale

Local drug application to the inner ear is typically targeted to perilymph since the endolymphatic space is miniscule and does not respond stably to breach. The preferred method will depend on the balance between the permissible degree of invasiveness and the required degree of control of drug levels. Intratympanic applications (IT, through the tympanic membrane) are relatively non-invasive and there is little risk of compromise to the inner ear, as long as the drug itself is not toxic. Because drug levels in perilymph will be governed by the balance between entry from the middle ear and the rate of loss to the vasculature and other compartments, control of perilymph drug levels cannot be precise. Most drugs injected into the middle ear are lost to the vasculature through the middle ear mucosa and potentially the lymphatic system. Mice possess an extremely active mucosa; In our hands, IT solutions that completely fill the middle ear may be entirely absent within 3 hours, and ABR thresholds can recover within the same day. Loss of solution to the pharynx through the Eustachian tube may also contribute to the loss of injected solutions. In mice, moreover, this can lead to quick

aspiration and death, and the inclusion of a “vent” hole is emphasized in our protocol. Middle ear retention may be improved by delivering agents in a gel, including Poloxamer formulations that solidify at normal body temperature (Salt et al. 2011). These may at least temporarily resist flowing back through the injection hole in the tympanic membrane (TM) and may be less inclined to pass through the Eustachian tube. In our hands, however, 17-20% Poloxamer formulations remain in a gel state for less than 30 min in the mouse middle ear. It may be as effective to simply keep animals on their sides (injected ear up) as they recover from anesthesia.

An effective IT injection fills the middle ear, being limited only by the 6.0-8.0 µl volume of the mouse bulla (Saunders and Crumling 2001; Richter et al. 2010). As stated, most of this is lost, yet mice may also offer more effective routes into perilymph than do larger animal models. In the mouse, entry routes into perilymph include not just the RWM, but also the stapes, annular ligament, and even the thin, porous bone of the cochlear apex. In fact, in mice the RWM does not appear to be the major route of drug entry into the cochlea (Salt and Plontke 2009; Salt and Hirose 2018). As one consequence, basal-apical perilymph drug gradients can be much smaller in mice than in other models. Although the importance of the RWM as a drug entry route will depend on the size and polarity of the drug, as well as the existence of transporters that happen to transport the drug (Salt and Plontke 2009; Salt and Hirose 2018), these other routes in mice are more “leaky” and less selective in a way that often works in the investigator’s favor. For these reasons, it may be appropriate to reconsider protocols that call for invasive recovery surgeries to place drugs directly onto the RWM. These come with complications and regulatory headaches, and may hold no advantage since they do not take advantage of the multiple possible routes into the mouse inner ear.

Limitations of intratympanic drug application

After intratympanic application, perilymph drug levels are highly dependent on middle ear retention time, which appears to be shorter in mice than in larger animals. For most drugs, this results in a rapid decline in perilymph concentration. In some cases, it may therefore be preferable to apply drug directly into perilymph. This is more invasive but allows more precise control of the perilymph concentration to be achieved. Any perforation of the otic capsule, however, results in perilymph release, driven by cerebrospinal fluid entering ST through the cochlear aqueduct. If the perilymph leakage cannot be adequately controlled in all animals, then reliable estimates of drug delivery may not be possible. Finally, just because a drug is applied to the perilymph of one ear does not guarantee that its effects will be local to that ear. Due to the small size of the mouse skull, mice may be particularly prone to the “Schreiner effect”, whereby the contents of perilymph on one side of the head may be able to reach CSF via the cochlear aqueduct (Schreiner 1999; Stöver et al. 2000). From there, drugs may reach remote parts of the brain and the opposite cochlea. Although this phenomenon has yet to be explored systematically in mice, the two ears of any mouse subjected to intratympanic or intralabyrinthine injection should not be assumed to be independent.

3.2 Materials

a. Intratympanic injections

In micro-surgical terms, the dimensions of the mouse auditory bulla are forgiving, and one might be tempted to simply hold the mouse and inject through the TM by the unaided eye. The ossicles are easy to dis-articulate, however, and the experiment is compromised if the stapedial artery, which cannot readily be seen through the TM, is breached. We have found that consistent injections can be performed by placing the animals ventral-side-up in a headholder on a stable platform of the type used for EP recording. We inject by mating a 1 cc disposable Luer-lock syringe filled with the experimental compound to a #16 gauge syringe needle, which in turn is inserted into a short length of

1.5 mm ID polyethylene tubing. This size of tubing matches the OD of WPI 1B150F-4 glass filament microcapillary tubing, which is pulled on a forge and then broken to ~50 µm using forceps under a magnifier. When the microcapillary part of the assembly is affixed to a holder and placed on a manual micromanipulator/magnetic stand, it can be maneuvered through the TM under an operating scope. One advantage of this arrangement is that the entire assembly can be moved into place, then operated with one hand, freeing the other hand to re-position the pinna as needed. If the injected media is a gel that may solidify, the microcapillary tip can be placed in or near an ice bath between animals. We do not recommend using a syringe needle of any type for direct IT injections in mice. If the animal and microcapillary tube are mechanically stable, there is little danger of breaking off any part of the tip during the injections. Because only the tip of the microcapillary tube enters the middle ear space, the only danger of infection arises by contamination of the injection media, which should be sterilized by filtration. The variety of mouse strains we have worked with appear extremely resistant to middle ear infections; which are nearly non-existent in our IT-injected animals. For easy visualization of the filling process, we add trace Evans blue to the media.

b. Intralabyrinthine injections

Direct injections into perilymph require a syringe pump capable of injection rates below 1 µl/min, with the syringe connected to a glass or polyimide delivery pipette. As injection rates and volumes are typically very small, it is generally not suitable to use any form of flexible tubing between the syringe and the delivery pipette. Compliance of the tubing results in slow on and off time courses and makes flow rates subject to mechanical disturbance by any movement of the tubing. Instead, a manipulator-mounted syringe pump (e.g., WPI model UMP3 UltraMicroPump) with a plexiglass coupler (WPI MPH6S Microelectrode Holder) allows the pump to be directly connected to the injection pipette. The tip size necessary for injection varies in different applications but can range from 10 µm to 50 µm in diameter. The choice of syringe also varies in different applications, but should be kept as small as

possible, with a volume of around 3x – 10x the total volume to be injected. Suitable gas-tight syringes with volumes of 50 µl and less are available from a number of manufacturers.

Also required is a fenestration pick (detailed earlier) and adhesives and sealants necessary to seal the injection pipette in place in the bony otic capsule, to control fluid leakage or to seal fenestration sites after the injection procedure. There are many brands and types of adhesive suitable.

3.3 Detailed Methods

a. Intratympanic injections

Once an animal is lightly anesthetized and the injection assembly is in place, the entire IT procedure typically requires less than 10 min. For such a short procedure, no heating may be needed and the animals quickly recover. Because of the placement of the ossicles and the angle of the cochlea within the skull, it is not possible to inject compounds directly onto or through the RWM using an IT approach. Our strategy instead has been to select two widely separated points in the TM, one as a vent hole and the other for injection (Fig. 6). If the entry point is below the vent, this encourages the ME to fill from bottom-to-top, with the excess eventually emerging from the vent. Our typical entry point (Fig. 6B,C) offers a safe location for insertion of the microcapillary tube, which is inserted just through the TM. If the media is highly viscous or prone to gelling, it may be necessary to break back the microcapillary tube to a larger size, which may require a more ventral entry hole. The use of a vent hole reduces, but does not eliminate, the danger of media passing through the Eustachian tube and being aspirated. Since the quality of the ME fill depends on a good view, we recommend a slight, bloodless cut at the intratragal notch (Fig. 6A). Placement of additional small weights on hooks as retractors further improves the view. We do not attempt to measure the actual amount of media that is injected, but visual filling of the ME (coloration plus emergence from the vent hole) will mean the delivery of ~6.0-8.0 µl of media.

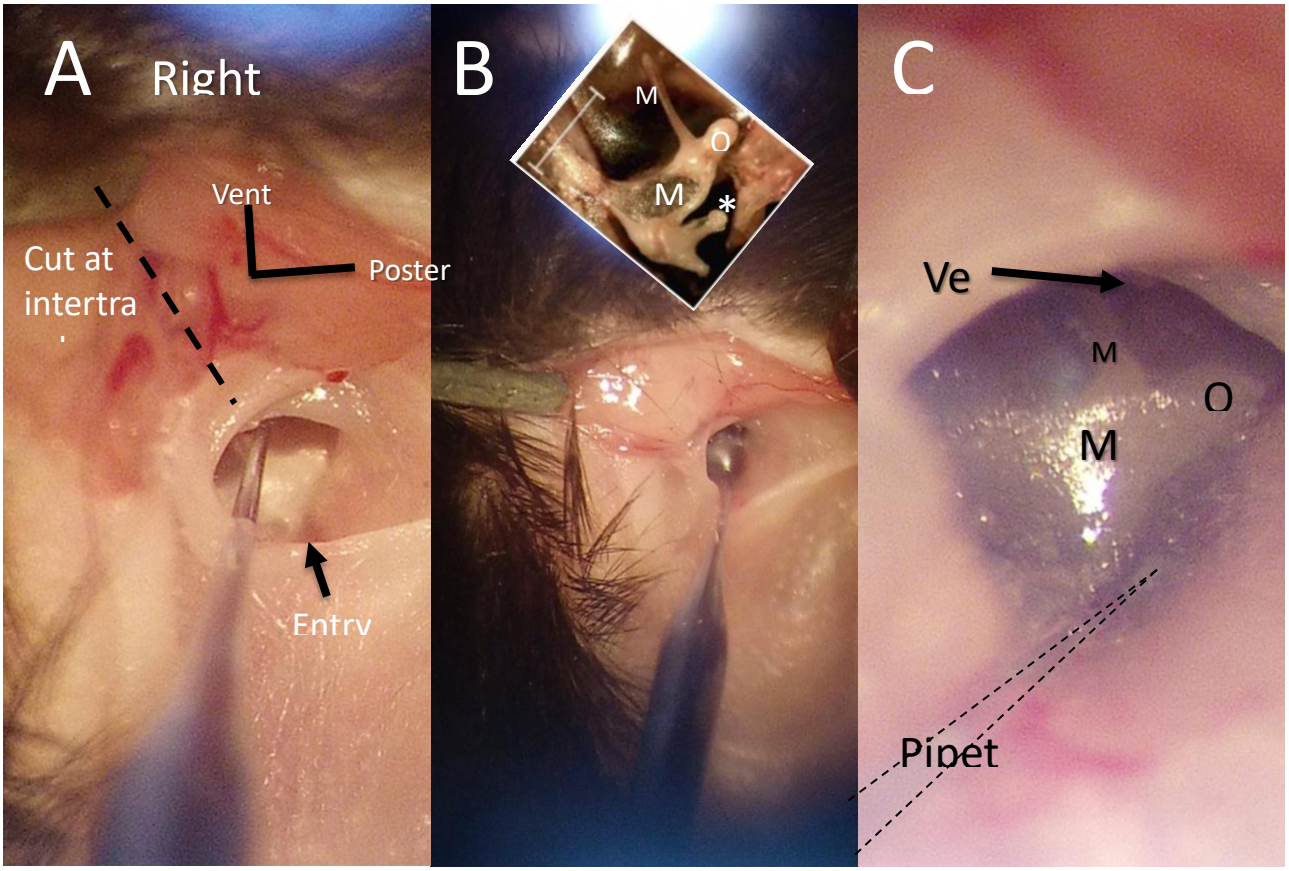

Figure 6. Intratympanic injection into the right ear of a mouse. A. View is optimized by bloodless cut at intratragal notch (dotted line). In this view, the same pipette that is used to make injection is first used to make a vent hole that is ventral (above) and remote from the injection site. B. Injection pipette with Evans blue-labeled Poloxamer 407 is inserted at location indicated by inset (asterisk). C. After fill, blue coloration appears behind the tympanic membrane. Mal: malleus; Oa: orbicular hypophysis; Man: manubrium. (Inset in B modified with permission from Inset in B modified with permission from Mason 2013) Scale bar in inset 1 mm.

b. Intralabyrinthine injections

The two most common sites for intralabyrinthine injections in mice are 1) through the round window membrane and 2) via the posterior semicircular canal (PSCC). Injections through the RWM of the mouse are problematic, due to the close proximity of the cochlear aqueduct. Perforating the RWM with an injection pipette results in immediate CSF efflux at the injection site. The resulting washout of drug has been quantified in guinea pigs (Plontke et al. 2016) but not in mice, where the influence is likely to be larger due to smaller perilymph volumes. This is not to say that some agents (such as adenoviruses) can't be delivered by round window injections, especially in early postnatal specimens

(György et al. 2017). Nevertheless, the method is unlikely to be reliable and effective for smaller drugs in adult mice. Some groups have reported the use of RWM injections combined with canal fenestration for gene therapy in mice using a viral suspension (Yoshimura et al. 2018). Opening the canal relieves the backflow of CSF and passage of CSF through the perilymphatic spaces may help distribute the adenovirus. However, the high degree of washout would also make the technique unsuitable for use with small drug molecules.

Injections into the PSCC obviate most of the problems associated with RWM injections. Injection at this site, which is distant from the cochlear aqueduct, actually causes flow towards the aqueduct, allowing drug loading through almost the entire perilymphatic space. The surgical approach to the

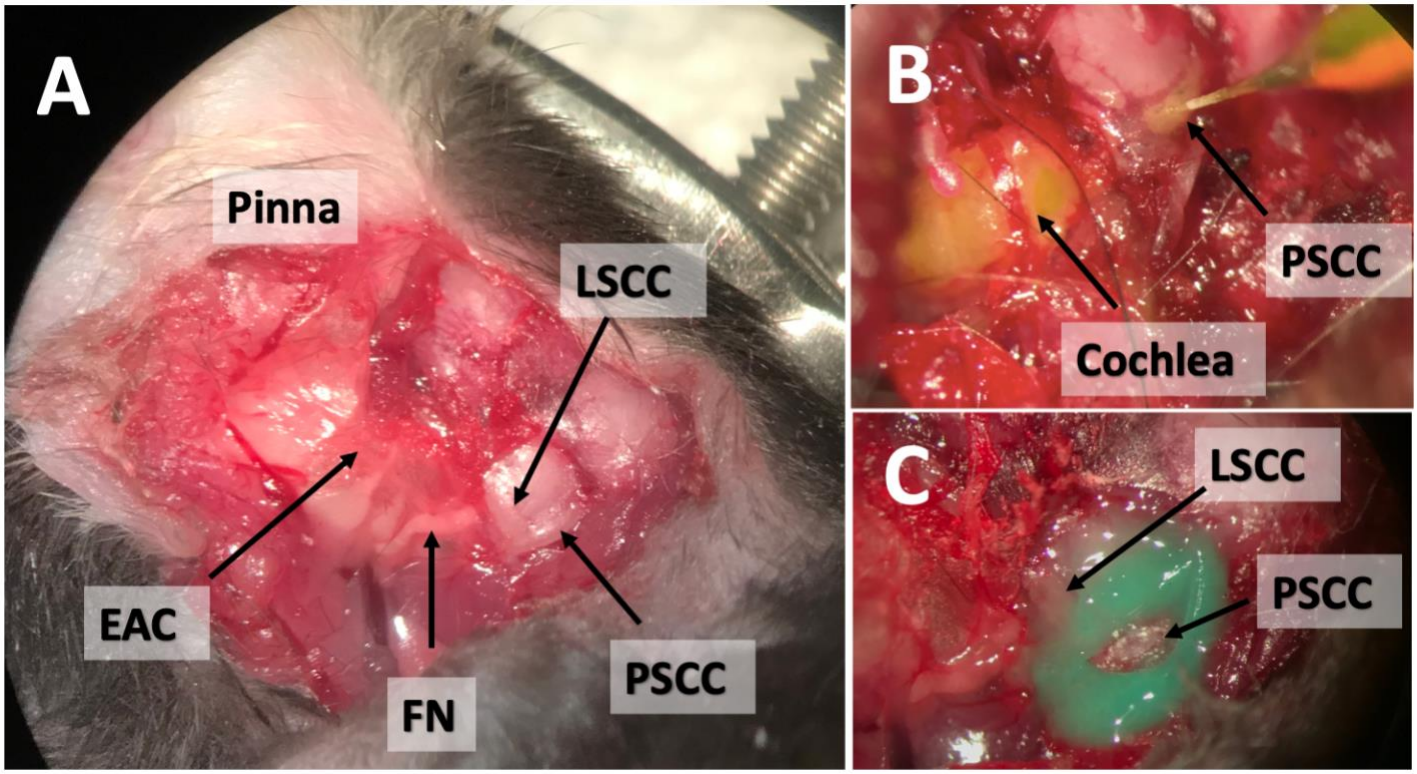

Figure 7: Injection into the posterior semi-circular canal of the mouse. A. Structures revealed after the post-auricular incision. The LSCC and PSCC are seen as a pair of "bumps" orientated at right angles on the surface of the temporal bone. B. Specimen in which fluorescent solution was injected from a pipette sealed into the PSCC. The cochlea is seen to be filled with fluorescent solution. C: The posterior canal prepared for perilymph sampling. The green color is a silicone adhesive that surrounds the site on the PSCC that will be fenestrated. Abbreviations: EAC: external auditory canal, FN: facial nerve, PSCC: posterior semi-circular canal, LSCC: lateral semi-circular canal.

posterior canal is shown in Figure 7. After a post-auricular incision, soft tissues are cleared until the surface of the temporal bone is visible. Following the facial nerve provides a guide to the lateral portion of the temporal bone. The lateral and posterior semi-circular canals are seen as small tube-shaped “bumps” joining at right angles at the edge of the temporal bone.

The procedure for sealing a drug injection pipette into the bony canal is illustrated in Figure 8. We have found it to be extremely difficult to prevent perilymph leaks when adhesives are applied after the injection pipette is inserted, after the bone is fenestrated and wet. At this point, fluid channels between the bone and the adhesive continue to allow small rates of fluid loss. The loss may occur at very low rates, which makes it difficult to visualize, even with concentrated dye in the solution. Instead, we have found it more effective to make the fenestration after a layer of cyanoacrylate (CA) glue (3M Permabond 101) is applied to the dry bone. CA glue is more hydrophobic than bone, allowing it dry more quickly and thoroughly. The bone is first thinned, if necessary, by shaving with a suitably sized blade, such as a flap knife or sharpened edge of a pick (Fig. 8B). The bone is dried

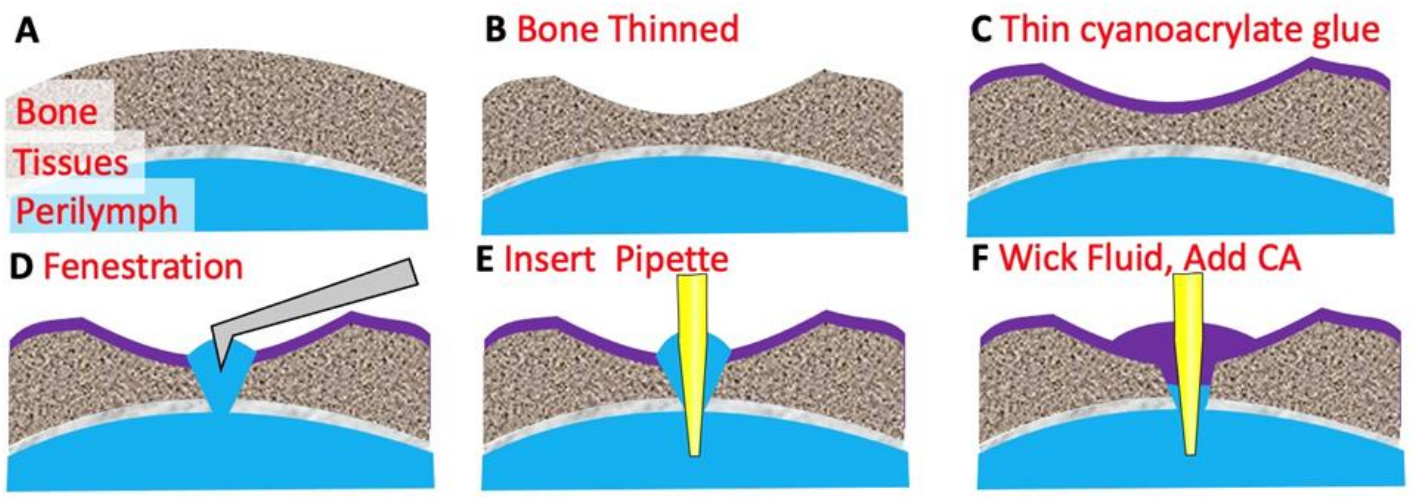

Figure 8: Sealing a pipette into a perilymphatic fenestration made almost anywhere on the otic capsule. From the initial state (A) the bone is first thinned as necessary with a flap knife or other sharp micro blade (B). The bone is carefully dried, and a thin layer of thin, fast setting cyanoacrylate glue is applied to the bone (C). Note that the CA thickness is greatly exaggerated in the figure; the layer should be as thin as possible. A fenestration is made though the glue and bone (D), allowing the pipette to be inserted (E). Perilymph will be leaking at this time. A wick is used to remove the perilymph from the surface and fenestration and a drop of CA glue applied, while the wick is simultaneously removed. CA entering the fenestration sets immediately

and a droplet of thin, fast-setting CA is applied to the bone (Fig. 8C). A small polyethylene scraper, such as a 1 - 2 mm wide piece cut from tubing (PE 240 Becton Dickinson), can aid in spreading the droplet, leaving as thin a layer as possible on the surface of the bone. A fenestration through both the adhesive and bone is made with a sharp pick (Figure 8C), after which the drug injection pipette is inserted (Figure 8E). An injection pipette can be made in a similar fashion to the microelectrodes that were described in the previous section. Non-fiber glass is 1mm outer diameter, pulled by a glass puller to a tip length of 8mm. The tip diameter is broken to 10-50 μm in size, which helps to ensure a successful injection. Perilymph will continue to escape both before, during and after the pipette insertion, accumulating as a droplet at the fenestration site. An absorbent wick is used to remove the accumulating fluid. When the pipette is ready to be sealed, a new dry wick is touched to the pipette/fenestration site, while instantaneously applying a small droplet of CA. At the moment the CA is applied, the wick is removed. Ideally, the wick will transiently remove fluid from the conical fenestration at the instant the CA is applied and CA in the fenestration will set immediately (Figure 8F).

3.4 Troubleshooting

If too much fluid is present at the fenestration site when CA is applied, the CA will float over the fluid and allow a fluid bubble to be formed underneath the CA. A small bubble is acceptable and can be covered with more CA to make it more rigid. Larger fluid bubbles, stretching and thinning the CA, need to be removed with a wick and re-sealed.

The calculated disruptive influence of perilymph leaks on drug distribution and time courses when drug solutions are directly injected into perilymph are illustrated in Figure 9. Calculated spatial distribution (Left column) and time courses (Right column) are shown for a 0.1 μL/min injection of a solution with concentration 1000 (arbitrary units) into the PSCC of the mouse. If the pipette is

completely sealed in place with no fluid leak, this injection rate would displace the 0.8 µL perilymph volume between the injection site and the cochlear aqueduct within about 10 min. The results, as expected, are that the basal part of ST (the region furthest from the injection along the perilymphatic space) becomes filled with drug solution within about 10 minutes. The result of a successful injection

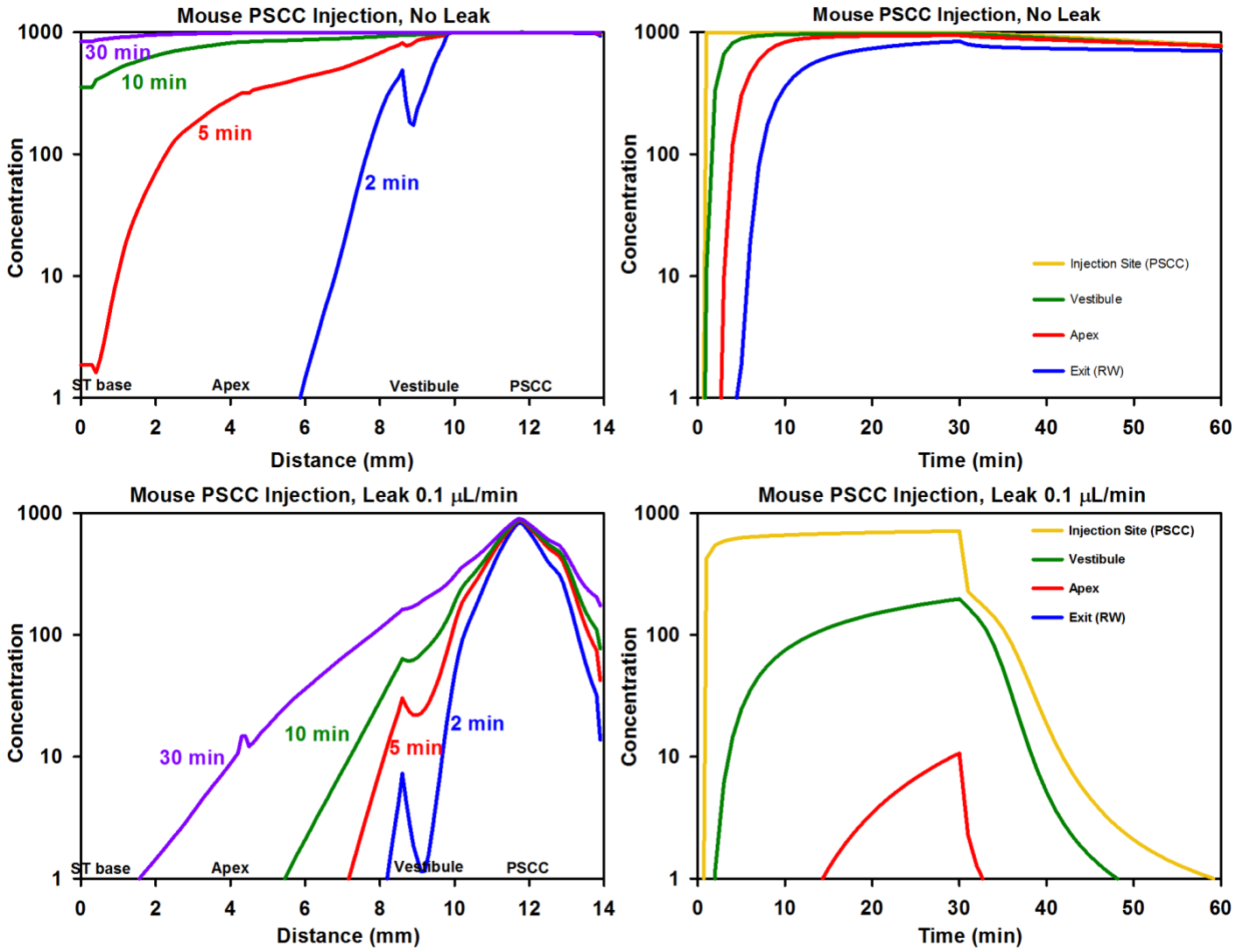

Figure 9: ***Upper Row:*** *Calculated spatial drug distribution (left side) with a 30 min, 0.1 uL/min injection from a pipette sealed into the posterior SCC of the mouse. Time courses at different perilymph locations are also shown (right side). With the pipette sealed, the entire perilymph can be loaded with drug within about 10 min and drug is retained well in the ear after injection ceases.* ***Lower Row:*** *Same calculation for the situation when the pipette is incompletely sealed and is allowing a sustained fluid leak of just 0.1 uL/min. Perilymph concentrations in the cochlea are substantially lower, especially in scala tympani (0 to 4 mm on the plot) and drug is rapidly washed away after the 30 min injection ceases.*

with fluorescein in the injection medium is shown in Figure 7B. The perilymph of the cochlea is visible through the bone and is seen to contain fluorescein at a concentration similar to that in the injection pipette, seen sealed into the PSCC at the right.

If the pipette is not adequately sealed in place, however, a completely different outcome will result. The lower row in Figure 9 shows the calculated results for the same injection when there is a small leak at the injection site. In this instance, the leak is set to 0.1 µL/min, but real leakage rates can be much higher; sometimes greater than 1 µL/min if preventive steps are not taken. With a leakage rate of 0.1 µL/min, drug concentrations in SV are 5–20x lower than the injected concentration, and almost no drug reaches scala tympani. Even more dramatic is the drug washout after the 30 min injection, which occurs in just 5–10 min in the presence of the leak. These calculations show that it is not sufficient simply to inject drug solutions into the ear. Accurate evaluation of drug effects requires the utmost diligence to deal with even the smallest leak to prevent drug washout from occurring. Also of importance is that the volumes and rates of leakage required to wash out drugs are extremely small. For this reason, visual observation of the injection site, even with dye in the solution, may be inadequate to confirm the absence of leakage.

4. Perilymph Sampling

4.1 Rationale

Despite the potential advantages of sampling endolymph for some research questions, endolymph volumes in mice are too small to sample or selectively manipulate without artifacts (Table 1). While the volume of perilymph in mice is also small, it can be accessed and manipulated at multiple locations without compromising cochlear function. For sampling purposes, however, it very much matters where perilymph is accessed. Sampling from the RW results in fluid that is primarily CSF, not perilymph. As the cochlear aqueduct enters next to the RW, fluid taken from the ear is readily replaced by CSF entering at the aqueduct. Thus, pure perilymph samples cannot be collected at the RW of the mouse. Instead, sampling at a distance from the aqueduct using the posterior semi-

circular canal allows relatively pure perilymph to be collected. A fluid sample of 0.8–1.0 µL collected from the PSCC, represents most of the perilymph from one mouse inner ear, including the canals, vestibule, scala vestibuli, and scala tympani.

4.2 Materials

The picks and adhesives required for perilymph sampling are identical to those required for intralabyrinthine injections except that a two-part silicone (Kwik-Cast 2-part silicone adhesive (WPI, Sarasota)) is also required. Perilymph samples are collected into hand-held capillary tubes (VWR catalog # 53432-728 10 µL or Drummond 2-000-005 5 µL) . Samples are diluted and stored in 0.5 mL polyethylene vials with screw top caps incorporating a silicone O-ring. (USA Scientific Cat# 1405-9304)

4.3 Detailed Methods

Accessing the posterior semi-circular canal for sampling begins with the same surgical approach described above for intralabyrinthine injections. Fluid collection must be performed without contamination or loss, so our approach has been to construct a “cup” from silicone adhesive at the perforation site to isolate the emerging fluid. The appearance of the silicone cup over the PSCC during surgery is shown in Fig. 7C. The procedure to make and secure the silicone cup is illustrated in Figure 10. Similar to drug injection, the bone is thinned (Fig. 10B) and coated with a thin layer of cyanoacrylate (Fig. 10C). The silicone is then applied over the CA glue (Fig. 10D). This sequence is necessary because silicone does not readily attach to bone without the CA and is easily detached by mechanical movements during sample collection. Applying the adhesives in order ensures the cup is firmly attached to the bone and is resistant to the moist surrounding tissues. When a fenestration is made through the adhesives, the emerging fluid forms a ball on the silicone surface, allowing it to be readily collected by capillarity into hand-held microcapillary tubes (VWR catalog # 53432-728 10 µL or Drummond 2-000-001 5 µL micropipettes). The latter are demarcated in 1 µl increments. The exact sample volume is measured by measuring sample length under a dissecting microscope with an eyepiece reticule. Sample volume is calculated by comparing sample length with the capillary length

to the calibrated marking. Samples are then expelled into a small volume (25 – 150 µL) of diluent according to the requirements of the measurement assay. The diluent may be aqueous for fluorescence analysis or organic (methanol or acetonitrile) for HPLC/mass spectrometry. Samples are expelled into the diluent using the tubing and capillary adaptor that is included with the micropipettes. The tubing is intended for expelling fluid samples by mouth, but instead we expel them by connecting the tubing to a 1 mL syringe, in which the plunger can be manipulated to drive the sample into the diluent. Diluent is then drawn into the capillary and expelled a few times to rinse the pipette to the maximum degree possible.

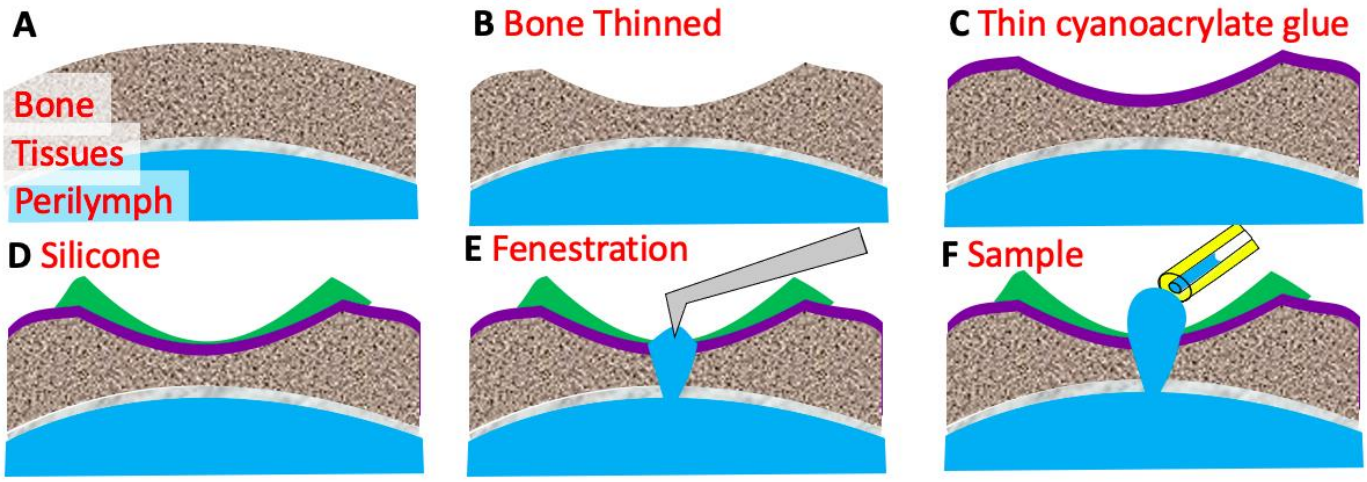

Figure 10. Perilymph sampling from a fenestration anywhere on the otic capsule. From the initial state (A) the bone is first thinned with a flap knife or other sharp micro blade (B). The bone is then dried, and a thin layer of cyanoacrylate glue is applied to the bone (C), followed by a layer of silicone, kept thin in the middle and built up at the edges to form a cup shape (D). A fenestration is then made though both the adhesives and bone (E), allowing perilymph to collect in the silicone cup. A calibrated microcapillary tube is used to draw a precise volume of perilymph as it emerges (F).

Sample handling

It can be challenging to handle samples of less than 1 uL in volume. Even in the capillary, they will evaporate with time, preventing quantitative interpretation. We also avoid the technique of “spraying” the sample into the collection tube by applying a high pressure. The viscosity of water means that in

fine capillaries a substantial portion of the sample may remain on the walls of the capillary tube, or may be lost as mist. We therefore prefer the technique of carefully expelling each sample into a fluid diluent of substantially larger volume. The rinsing of the pipette interior as the diluent is drawn back into the pipette a few times and expelled helps ensure that all the sample is included for analysis.

4.4 Troubleshooting

The musculature over the PSCC is highly vascularized, especially ventral and posterior to the point of fenestration. Bleeding can be minimized by methodical blunt dissection and occasional pressure. Heat cautery should be avoided, as this may heat the fluid in the canal. The ease of fenestration and rate of perilymph flow are greatly impacted by the choice of mouse strain. Some strains (e.g., C57BL/6, BALB/c) feature both very thin bone and relatively large internal diameter, which promote rapid fluid flow. Other strains (e.g., CBA-related strains) possess thicker canal bone plus a smaller internal diameter, which can delay and slow sample flow. Although the PSCC houses both perilymphatic and endolymphatic compartments, the endolymphatic space collapses when the canal is fenestrated, and there is little risk of sample contamination (Hirose et al. 2014).

5. Conclusions

We describe a small set of inner ear research procedures adapted to the laboratory mouse. These procedures—EP recording, local drug delivery, and perilymph sampling—enhance the value of mouse models for the study of stria vascularis pathology, inner ear fluid regulation, and testing of potential therapeutic agents against hearing loss. In mice, these methods leverage genetic standardization and a growing list of molecular tools, imparting unparalleled versatility for hearing research.

Acknowledgement

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