Discussion of the Animal Care Protocol: Mouse *In Utero* Gene Transfer

General Considerations

Gene transfer to the developing mouse inner ear requires major surgery that compromises the abdominal cavity. Consequently, Institutional Animal Care and Use Committees (IACUC) will focus particular attention on the animal care and use issues associated with the protocol such as anesthesia, analgesia, and medial asepsis. The best practice is to develop the animal care protocol under the guidance of the home institution's IACUC and veterinary staff. The following information does not represent a complete animal care protocol. Rather, the document provides language that may be useful in developing an approvable protocol.

This discussion of animal protocol development is organized according to main topics that will likely need to be addressed by any investigator in a university setting. Obtain the current animal care protocol form from your home institution and identify the consonance of main topics. Complete a draft protocol and arrange to meet with a member of the IACUC to further refine the protocol in a manner that reflects the sponsoring institution's animal care and use perspectives. There are undoubtedly institution-specific focus areas in the protocol that will require special treatment. For example, the Oregon Health & Science University IACUC is particularly focused on preemptive analgesia and environmental enrichment for post-surgical rodents.

Finally, be aware that the IACUC will require an approved recombinant DNA protocol that covers the expression plasmids that are electroporated into the mouse otocyst. Consult with your institutional biosafety committee staff to learn if there are unique requirements for recombinant DNA delivered *in vivo*. We sterilize our expression plasmids by 0.22μm filtration (any low retention, centrifuge format, microfuge compatible filter is acceptable) prior to administration *in vivo*.

Protocol Title

It is likely that this protocol will be incorporated into a main protocol that governs the existing animal research conducted in the lab. As such, it will likely be submitted as an amendment to the existing protocol that is already titled. The amendment will then be evaluated by (almost certainly) full committee review. We refer to this element of our protocol as "Mouse *In Utero* Gene Transfer."

Alternatives

The mouse is the vertebrate species that most closely models human inner ear anatomy, physiology, and development. In addition, mice are the most developmentally accessible mammalian species available with defined natural and engineered mutations in genes affecting inner ear development and function. No appropriate cold-blooded vertebrates, invertebrates, or tissue/cell culture lines model the complexity and sophistication of human vestibular and auditory function as completely as the mouse. In addition, computer simulations that may contribute to our understanding of the genetic interactions responsible for normal and abnormal development and function of the inner ear are in their infancy and will only be

practically useful if further informed by carefully controlled experiments in warm-blooded, higher vertebrates.

Medical Asepsis

Surgical instruments are sterilized in a steam autoclave. The instrument tray is double-wrapped and contains a sterility indicator. A separate set of sterile instruments is used for each subject, or the surgical instruments are sterilized between subjects by glass bead sterilization. The surgeon wears sterile gloves, scrubs covered by a sterile, disposable gown, face mask, and hair bonnet.

Preoperative Animal Care/Husbandry/Transport

Timed-pregnant mice will be subjected to ventral laparotomy and transuterine microinjection as described in detail below. On the morning of surgery, the dam's cage will be fitted with a water bottle-compatible cage top, a water bottle, food, and a microisolator bonnet prior to transport from the animal facility in a closed, sterilized paper bag. We use each bag once, autoclave, and then redeploy the sterile bag.

Anesthesia

Anesthetic sodium pentobarbitol (Nembutal)

Dose: 65 µg sodium pentobarbitol per gram body weight

Route: Intraperitoneal injection

Frequency: one injection per dam

Assessment of Anesthetic Efficacy

Five minutes after intraperitoneal injection of 65 µg/gram body weight sodium pentobarbitol, the dam is evaluated for response to noxious stimuli (i.e., tail and toe pinches) and for the presence of the ocular reflex (i.e., vibrissae and cheek touches) to ascertain the initial depth of anesthesia. Disinfection of the surgical site begins after no responses to noxious stimuli are observed and a negative ocular reflex is validated. Negative tests for noxious stimuli are recorded with other operative data on the Individual Mouse Survival Surgery Data Sheet (appended to this protocol as supplementary material). The data sheet is also used to record other pre-surgical, surgical, and post-surgical parameters *for each surgical subject*.

To ensure that adequate anesthesia is maintained throughout the procedure, tail and toe pinches are administered 1) just before the abdominal wall is incised; 2) after the uterus is exposed; and 3) prior to stitching the abdominal wall and integument.

Ventral Laparotomy

The abdominal fur is shaved from the suprapubic region to just below the rib cage. Disinfection of the skin is accomplished by alternating 70% ethanol with Betadine. The sequence is 70% ethanol, Betadine, and finish with 70% ethanol. We prefer ethanol-Betadine-ethanol sequence because the final ethanol pass removes residual Betadine, allowing us to see the *linea alba* in the abdominal wall beneath the skin. The location of the *linea alba* predicates where the skin incision is made since they should be aligned. The two disinfection passes with ethanol can

lower body temperature, so the disinfected dam is placed on a sterile drape resting on a 37°C surface for ~2-5 minutes prior to beginning surgery.

A ventral midline incision (10-14 mm length) through the skin and then the *linea alba* of the abdominal wall is made with sterile, ball-tipped dissection scissors. The abdominal cavity is flushed with 1-2 mL of sterile, pre-warmed (37ºCelsius), lactated Ringer's solution to facilitate uterine horn extrusion through the incision site. Rarely, venous bleeding is observed at the edges of the incised skin and the abdominal wall which, if encountered, is controlled by direct pressure for 2 minutes using a vascular clamp.

Transuterine Microinjection

The uterus is fed through the incision in the abdominal wall with ring forceps and transilluminated with a fiber optic light (2850 K) that transfers little heat at its output end. The uterus and fiber optic light housing are kept moist by irrigation with 37°C lactated Ringer's solution every 1-2 minutes. Each embryo is localized and positioned for injection using gentle palpation of the uterus (i.e., finger pressure). A 12-16µm outer diameter, beveled glass capillary pipette is inserted through the uterus and into the otocyst of the mouse embryo. Approximately 0.05-0.2µl of solution will be injected per otocyst with the Picospritzer pressure injector (source gas >99.9% purity) depending on the embryonic stage. The estimated time to inject 4-6 embryos by this method is approximately 25 minutes and the entire surgical procedure with injections takes ~1-1.5 hours per dam.

In Vivo Electroporation

The uterus is freshly irrigated with prewarmed lactated Ringer's solution. The otocyst that was filled with expression plasmid is centered in the path of a tweezer-style electrode that consists of a 5mm outer diameter tungsten cathode and anode. The uterus is gently compressed to couple the electrode to the uterus and a single square wave pulse train with the following characteristics is initiated: five, 43volt pulses at 50msec per pulse and a 950msec interpulse delay. The uterus is immediately irrigated with prewarmed lactated Ringer's solution after completion of the electroporation cycle. Each embryo experiences only one, 5 pulse, direct current stimulus train. Six to eight embryos are electroporated per dam. An injection of 0.2-0.4µl of Alexa Fluor-conjugated dextran (10mg/mL aqueous) into the nascent 4th ventricle is performed after successful electroporation in those embryos that must be identified and phenotypically evaluated after birth.

Closing Up

The freshly irrigated uterine horns are reinserted into the abdominal cavity with ring style forceps. The abdominal cavity is flushed with 2-4 mLs of prewarmed lactated Ringer's solution. The abdominal wall and skin are sutured with a 6-0 (0.7 metric) Polysorb Violet Braided Lactomer 9-1 suture with CV-11 needle. A running stitch is used for both the abdominal wall and skin in which a conventional throw is followed by a locking stitch. Bupivicaine (4mg/kg maximum), a short duration topical anesthetic, is applied to the freshly sutured skin.

Analgesia

The nonsteroidal antiflammatory Meloxicam is administered subcutaneously at 5mg/kg body weight after suturing and before the dam is placed in the heated recovery cage. The first

subcutaneous injection is therefore administered while the dam is under sodium pentobarbital anesthesia. Though prophylactic administration of Meloxicam after anesthesia induction and before the start of surgery is ideal, many analgesics can affect uterine tone which could alter our ability to efficiently conduct transuterine microinjection at embryonic days 11.5-12.5. Dams are assessed 24 hours after the first Meloxicam dose to determine if a follow up dose is required. Typically, dams display normal eating, grooming, and social behavior shortly after surgery and are unlikely to require additional dosing. Meloxicam will be re-administered at 48 and 72hrs postoperatively if the dam shows signs of pain or discomfort. Veterinary staff will be consulted if assistance is required to assess postoperative pain status.

Post-Operative Care

Heating the recovery cage facilitates maintenance of normal body temperature during recovery and significantly reduces physiological stress. Heating is achieved by 1) placing the recovery cage on a Hallowell Hard Pad connected to a GayMar T-Pump with thermostatic control; and 2) a 660Watt ceramic heat lamp permanently mounted 35cm above the recovery cage. Temperature is monitored at the surface of the hard pad with a liquid crystal thermometer placed directly on the pad surface. The Hallowell Hard Pad thermostat and heat lamp are adjusted to achieve a constant 25°C *at the surface of the bedding* where the recovering dam is placed.

The recovery cage is enriched with a transparent red tube that provides a suitable hiding place without interfering with postoperative monitoring. Moistened lab chow is placed in a dish at the bottom of the cage to supplement the overhead water bottle and food. A standard mouse nesting pad is mixed with about 12-13grams of sterile Enviro-dri (Shepherd Specialty Papers; www.ssponline.com) to encourage nesting behavior.

Ambulation, eating, drinking, defecation, and species-specific grooming and social behaviors are monitored to determine if the mice are experiencing post-surgical pain. The data for each dam is recorded on her own Mouse Survival Surgery Data Sheet (appended to this protocol). Unmitigated vaginal bleeding indicates vascular compromise and this is detected while the dam is still anesthetized in the recovery cage. Should vascular compromise be detected, the dam will be euthanized by Nembutal overdose (100mg/kg intraperitoneally) followed by prophylactic cervical dislocation. All postoperative care that will be recorded on the Individual Mouse Survival Surgery Data Sheet (appended to this protocol).

Reagents Administered to Mice (not for anesthesia or analgesia)

Sterile ophthalmic ointment, lactated Ringer's solution, fast green (trace quantities), Alexa Fluor conjugated dextran (0.2-0.4µl of 10 mg/mL aqueous stock solution), phosphate buffered saline (pH=7.2-7.4), and expression plasmid (250-500ng recombinant DNA per otocyst) are reagents administered to the mice not for anesthesia or analgesia.