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Microsurgical access for cell injection into the mammalian cochlea

Sofie Bogaerts [d], Susan Douglas [a], Toby Corlette [b], Henry Pau [d], Darren Saunders [b], Sarah McKay [a], Sharon Oleskevich [a,c,*]

[a] *Neuroscience Research Program, Garvan Institute of Medical Research, Sydney, NSW 2010, Australia*
[b] *Cancer Research Program, Garvan Institute of Medical Research, Sydney, NSW 2010, Australia*
[c] *University of New South Wales, Sydney, NSW 2052, Australia*
[d] *ENT Department, St. Vincent's Hospital, Sydney, NSW 2010, Australia*

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Abstract

The potential use of stem cells to repair hearing loss requires surgical access to the cochlea. Here we describe a microsurgical technique for cell injection into the mouse cochlea. Green fluorescent cells (ZsGreen-MCF10A cells) were successfully injected via a lateral wall cochleostomy into the scala media, scala tympani and scala vestibuli compartments of the cochlea. The effect of surgery on auditory function was investigated with auditory brainstem responses (ABR) to click and tone stimuli. A computerised signal-to-noise ratio detection method was developed to measure ABR thresholds in conjunction with visual inspection. Signal-to-noise ratio detection showed ABR thresholds in control mice were similar for click (33 ± 7 dB) and tone stimuli (33 ± 6 dB), in agreement with visual inspection (click 39 ± 7 dB, tone 35 ± 6 dB). The mean ABR threshold for combined click and tone stimuli was 15–45 dB greater after surgery with minimum hearing loss achieved with a small sized cochleostomy ($\leq$0.4 mm) and by sibling matching to control mice (control 33 ± 4 dB, surgery 48 ± 3 dB). The microsurgical technique will provide a basis for future studies on the use of stem cells in the treatment of hearing loss.

Keywords: Microsurgery; Cochleostomy; Cell injection; Signal-to-noise ratio

1. Introduction

Many forms of deafness result from the degeneration of the sensory cells for hearing in the cochlea (hair cells), which occurs most commonly during ageing and following exposure to excessive noise. Treatments that are currently being explored for hearing loss include gene therapy and cell-replacement therapy. Gene therapy has been applied to protect hair cells (Kawamoto et al., 2001a, 2003a, 2003b, 2004) or to generate new hair cells (Izumikawa et al., 2005; Kawamoto et al., 2003a). Cell-replacement therapy makes use of stem cells to generate new hair cells and auditory neurons. Following injection into normal or deafened cochlea, embryonic and adult neural stem cells survive (Coleman et al., 2006; Hildebrand et al., 2005), differentiate into hair cells or auditory neurons (Hu et al., 2005b; Li et al., 2003a, 2003b; Regala et al., 2005), and provide functional rescue of hearing loss (Hakuba et al., 2005). Recently, Bodmer and colleagues demonstrated that transplanted mouse embryonic neural stem cells migrate close to damaged hair cells in the mouse organ of Corti (Bodmer et al., 2007). *In vitro* data shows that bone marrow mesenchymal stem cells can transform into hair cells (Jeon et al., 2007) while embryonic stem cells can differentiate into neurons (Coleman et al., 2007).

Both gene therapy and cell-replacement therapy require precise delivery of therapeutic agents to the cochlea. Different microsurgical techniques have been reported for accessing the cochlea in guinea pigs (Coleman et al., 2006; Hildebrand et al., 2005; Hu et al., 2005b), gerbils (Hakuba et al., 2005), rats (Ito et al., 2001), and mice (Iguchi et al., 2004; Jero et al., 2001). The round window is accessible, easily identified and provides access to the scala tympani compartment in the basal turn of the cochlea. The round window is routinely used for insertion of a prosthetic hearing device (cochlear implant). Stem cells injected via the round window show survival and partial differentiation in scala media or scala tympani (Coleman et al., 2006; Hakuba et al., 2005; Hildebrand et al., 2005; Ito et al., 2001).

* Corresponding author at: Neuroscience Research Program, Garvan Institute of Medical Research, Darlinghurst, Sydney, NSW 2010, Australia.
Tel.: +61 2 9295 8290; fax: +61 2 9295 8281.
E-mail address: s.oleskevich@garvan.org.au (S. Oleskevich).

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Of particular importance is surgical access to the scala media compartment of the cochlea that contains the sensory epithelium, the organ of Corti. The organ of Corti is composed of the sensory receptors for hearing (inner and outer hair cells), supporting cells (inner and outer phalangeal cells; inner and outer pillar cells; Hensen cells; Claudius cells), the basilar membrane, the tectorial membrane and auditory nerve endings (Raphael and Altschuler, 2003). The inner hair cells are the primary transducers of sound information and there are no endogenous mechanisms in the mammalian cochlea for the replacement of damaged hair cells. This has prompted recent explorations into cell-replacement therapy for sensorineural hearing loss.

The increasing number of available deaf mouse strains makes the mouse an ideal animal model to investigate cell-replacement therapy. Here we describe minimal trauma microsurgery for injection of cells into the mouse cochlea. The microsurgical technique uses well-defined anatomical landmarks to ensure consistent cochleostomy position and improved maintenance of hearing function relative to earlier techniques. The effect of surgery on auditory function is assessed by evoked auditory brainstem responses (ABRs). A computerised threshold detection method was developed to provide more reproducible and objective ABR thresholds than those obtained with the subjective visual inspection method. These techniques will be applied for future transplantation and exploration of stem cells as a potential therapy for hearing loss.

2. Methods

2.1. Ethics

All experiments were performed with the approval of the Garvan Institute and St. Vincent's Hospital Animal Experimentation and Ethics Committee, in accordance with National Health and Medical Research Council (NH&MRC) animal experimentation guidelines and the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes (1990).

2.2. Experimental animals and surgical procedures

CBA/CaH mice aged 3–32 postnatal weeks were used in this study ($n = 30$) for histological analyses ($n = 22$), auditory testing ($n = 6$), and cochlear dissection ($n = 2$). Cochleostomies were performed with minimal trauma surgery on live mice ($n = 9$) or mice sacrificed by cervical dislocation ($n = 6$), ketamine overdose ($n = 3$), or pre-perfusion with 4% paraformaldehyde to improve anatomical preservation ($n = 6$). For live surgery, mice were anaesthetised with 75 mg/kg ketamine (Cenvet Australia, Maryong, NSW, Australia) and 15 mg/kg xylazine (Provet, Castle Hill, NSW, Australia) and placed in a supine position with the neck extended. The level of anaesthesia was monitored with corneal and withdrawal reflexes, and additional anaesthetic was applied at half dose if necessary. A minimally invasive procedure was initiated by micro-drilling through the bulla (Algerbrush II, Design for Vision, NSW, Australia) to access the inner ear and perform a cochleostomy (see Section 3). The surgical procedure was completed in 40 min and performed under an Olympus dissecting microscope with 0.63—4× magnification. Photographs were taken with an Olympus C-7070 wide zoom digital camera attached to the dissecting microscope.

2.3. Cell injection into the cochlea

Green fluorescent cells were used to visualise the spatial distribution of injected cells. MCF10A immortalised human mammary epithelial cells (Soule et al., 1990) expressing the ecotropic retroviral receptor ($MCF10A^{EcoR}$ cells, a kind gift of Drs. Danielle Lynch and Joan Brugge) were infected with pSIREN-retroQ-ZsGreen (Clontech, Mountain View, CA, USA) and cultured as previously described (Debnath et al., 2003). In brief, the ecotropic packing cell line Phoenix-Eco was transfected with 10 μg of plasmid DNA using PolyFect (Qiagen, Doncaster, VIC, Australia). Retroviral supernatants were used to overlay a subconfluent culture of $MCF10A^{EcoR}$ cells. Green fluorescent-positive cells were then sorted to homogeneity using flow cytometry. Prior to injection, cells were harvested with trypsin-ethylenediaminetetraacetic acid (EDTA, Invitrogen, Australia) and resuspended in phosphate buffered saline/EDTA at a concentration of 4×10^6 cells/ml.

Cell injections were made using a Hamilton syringe and a glass capillary needle, broken to a tip diameter of 100 μm and inserted into the cochleostomy. One microliter of fluorescent cells was injected over 1 min. In some cases, the cell suspension was substituted with red paint to visualise the cochlear access provided by the injection site. The red paint was diluted with methyl salicylate to facilitate expulsion from the injection syringe and movement throughout the cochlea. The injected cells or paint remained in the cochlea for approximately 10 min before the cochlea were removed from the animal and prepared for whole mounts or histological analysis in thin sections.

2.4. Preparation of cochlear whole mounts

Following injection, cochleae were prepared as previously described (Pau et al., 2005), fixed in Bodian's fixative (75% ethanol, 5% formalin, 5% glacial acetic acid) for 60–120 min, washed in distilled water for 30 min, and placed in 70% ethanol for 24 h. Specimens were placed in 3% potassium hydroxide for 4 days to remove soft tissues, transferred to a solution containing glycerol, 70% ethanol and benzol (in the ratio of 2:2:1) to render bones transparent, and stored in glycerol and 70% ethanol (in the ratio of 1:1). Whole cochleae were examined with an Olympus dissecting microscope and photographed with an Olympus C-7070 camera.

2.5. Preparation of cochlear thin sections

Cochlea were fixed in 4% paraformaldehyde for 1 day, decalcified in 10% EDTA for 1 day, then placed in 20% sucrose in phosphate buffered saline for 3 days. Cochleae were then transferred to optimal cutting temperature (OCT, Tissue-Tek) medium and placed under gentle vacuum for 10 min prior to freezing and thin sectioning on a cryostat. Adjacent thin sections (25 μm thick) were mounted on slides and viewed under

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normal or fluorescence microscopy. Some sections were stained with haematoxylin and eosin (H&E) for anatomical observation. Images were captured using a Leica DC480 digital camera (Leica, Wetzlar, Germany) attached to a Zeiss Axiophot microscope (Carl Zeiss, North Ryde, NSW, Australia).

2.6. Testing auditory function

Auditory function was assessed by measuring auditory brainstem response (ABRs) thresholds in the left ear of 21–32-week-old mice anaesthetised with ketamine and xylazine ($n = 10$). ABR thresholds were determined in control mice ($n = 7$) and in mice 3–4 months after cochleostomy ($n = 3$), with ($n = 2$) or without saline injection ($n = 1$). Subdermal needle electrodes were positioned at the vertex (active), lateral to the left cheek (reference) and at the base of the back (ground). Electrodes were adjusted to minimize impedance (<5 kΩ). Acoustic stimuli were delivered via an electrostatic insert phone fitted into the animal external ear canal (Tucker Davis Technologies) and held in place with a micromanipulator in a foam-padded, shielded acoustic chamber. The non-tested ear was blocked with a foam earplug in the ear canal. The output was calibrated with a calibration kit (Tucker Davis Technologies), which included a MA3 microphone amplifier, SigCal software and a quarter inch calibrated microphone (AcoPacific). Calibrations were made with reference to the programmed output at 90 dB SPL.

Clicks (1 ms duration) and tone pips (16 kHz; 1 ms rise/fall; 3 ms duration) were delivered and ABRs were obtained by reducing the intensity in 5 dB steps beginning at 90 dB. Threshold detection was determined by two methods: (1) visual detection and (2) signal-to-noise ratio detection. Visual detection was performed by independent observers blind to the experimental conditions. The signal-to-noise ratio was determined by comparing the absolute amplitude of the maximum peak occurring within 1.5–8 ms latency from the onset of stimuli to the standard deviation of the baseline noise over 12–20 ms latency from the onset of stimuli (AxoGraphX Scientific Software). The sound intensity level was deemed threshold when the maximum peak amplitude was three times the standard deviation of the baseline noise (Fig. 4C). Statistics are quoted as mean ± standard error of the mean (S.E.M.). The F statistic was used to confirm equality of variances between populations before applying a two-tailed paired Student's t-test (Statview, SAS Institute, USA).

3. Results

3.1. Microsurgery for mouse cochleostomy

Cochleostomies were performed with minimal trauma surgery on live ($n = 9$) and deceased animals ($n = 15$). A postauricular incision was made and the flap retracted anteriorly. The facial nerve was preserved in its position posterior to the external auditory canal (Fig. 1A). The muscle overlying the bulla was divided with microscissors and electrocautery was applied to prevent bleeding. The minimally invasive technique was aided

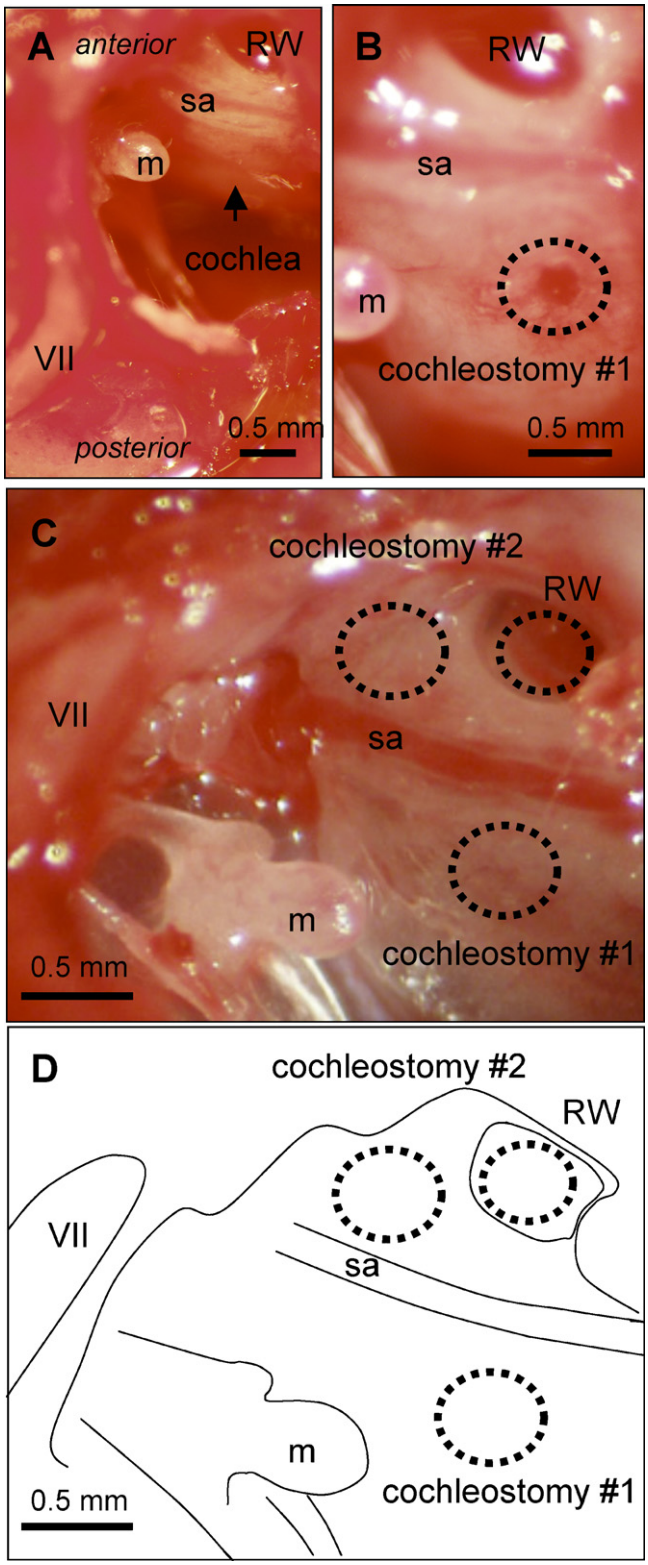

Fig. 1. Surgical procedure for cell injection via mouse cochleostomy. (A) The bulla was opened to expose the lateral wall of the cochlea. Several landmarks are visible including the stapedial artery (sa), the round window (RW) and the facial nerve. The malleus (m) is shown here for demonstration purposes only as malleus exposure indicates damage to the tympanic membrane. (B) Location of injection site via cochleostomy #1 is shown posterior to the stapedial artery (sa) in the lateral wall of the cochlea. (C) Two additional injection sites via cochleostomy #2 and the round window (RW) are shown anterior to the stapedial artery (sa). (D) Schematic diagram of (C).

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by the use of 3/0 silk sutures to retract the adjacent soft tissue and increase the surgical access. The bulla was opened by sharp dissection and/or by drilling with diamond burrs (0.5 and 1.0 mm) to reduce the degree of bony trauma to the bulla and cochlea. Middle ear landmarks including the stapedial artery and round window were identified and served as orientation landmarks for the cochleostomy (Fig. 1A). Cauterisation of the stapedial artery was performed in a limited number of animals ($n = 2$) using a specialised tool for small animal microsurgery that enabled a localized and brief cauterisation (I-Stat, Medtronic).

The cochleostomy was made with a fine gauge needle (27G) on the posterior side of the lateral wall cochlea. A fat and muscle plug was used to seal the cochleostomy site following cell injection. A single dose of analgesic (subcutaneous ketoprofen, 1 mg/kg) was administered prior to surgery and then daily for 3 days post-surgery. Fluid therapy (subcutaneous saline, 10% blood volume) was administered pre- and post-surgery. Animals showed full recovery within 4 h.

3.2. Injection into scala media, scala tympani and scala vestibuli

Cells were injected into the cochlea via three injection sites: cochleostomy #1, cochleostomy #2 and the round window (Fig. 1B and C). Cochleostomy site #1 was posterior to the stapedial artery while cochleostomy site #2 was immediately anterior to the stapedial artery midway between the oval and round windows. Cochleostomy site #2 was chosen after comprehensive drilling on dissected cochlea suggested that cochleostomy site #2 could provide direct access to scala media. However space constraints of cochleostomy #2 caused by the proximity of the stapedial artery and the round window precluded future use of this injection site.

The pipette was advanced through the membranous wall of the cochlea or round window in an anterosuperior direction. In the first instance, red paint was injected to confirm access to the cochlea (Fig. 2A–D). Red labelling was observed in multiple spiral turns of whole cochleae and was present proximal

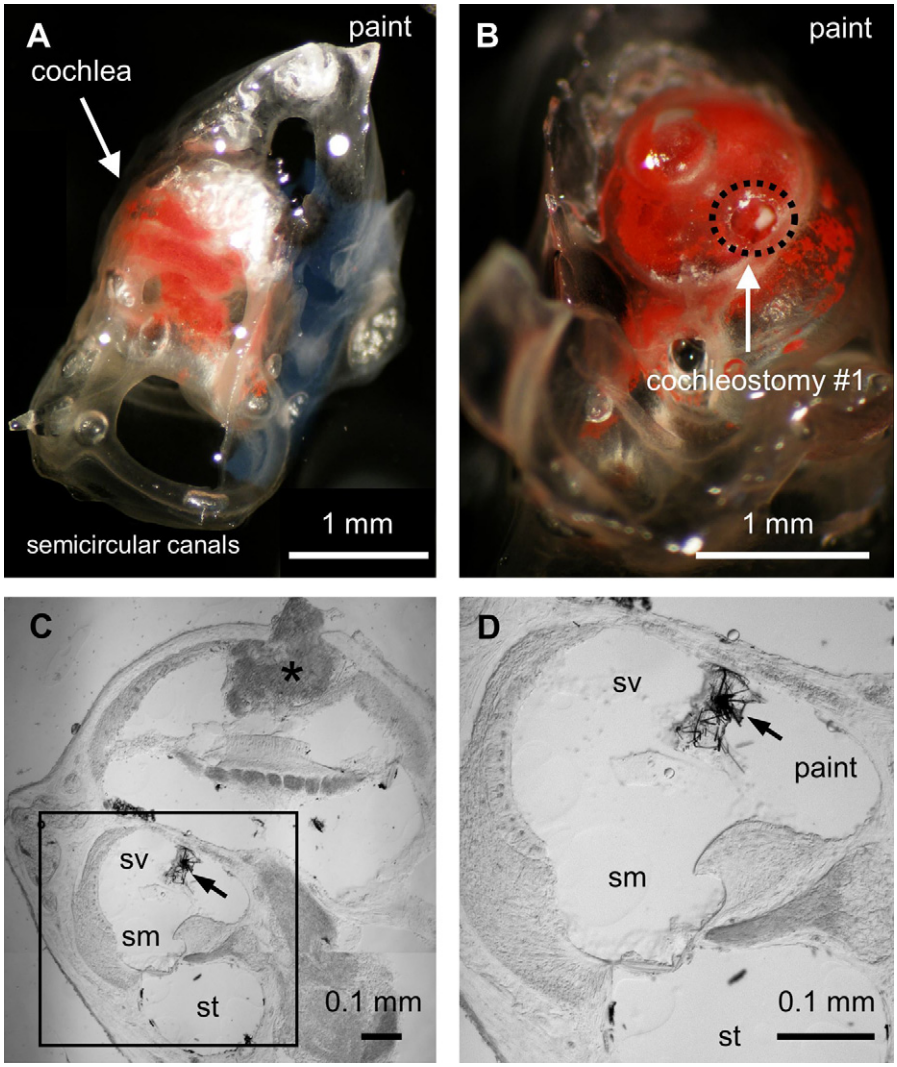

Fig. 2. Paint injection into whole cochleae. (A) Dissected cochlea show red paint in the spiral coils. Bones were rendered transparent with glycerol/benzol/ethanol. (B) Site of cochleostomy and paint injection in lateral wall of cochlea. Paint was evident proximal and distal to the cochleostomy. (C) Thin sections of cochlea showing paint injection into multiple cochlear compartments (arrow) and tissue plug in cochleostomy (asterisk). (D) High magnification image of boxed area in (C).

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and distal to the site of the cochleostomy (Fig. 2A and B). No labelling was observed in the vestibular ducts. Histological analysis of thin-sections of cochleae also revealed paint crystals in the scala vestibuli and the scala tympani (Fig. 2C and D).

Green fluorescent cells (ZsGreen-MCF10A) were injected into the cochlea and their distribution was analysed in thin sections with fluorescence microscopy ($n = 12$; Fig. 3). Control cochleae were sectioned and stained with H&E to establish normal cochlear morphology at successive spirals (Fig. 3A and B). Cochlear tissues were preserved following cell injection with stria vascularis, organ of Corti, and spiral ganglion soma still intact (Fig. 3C). Green fluorescent cells injected at cochleostomy site #1 were observed in the scala vestibuli and the scala tympani ($n = 3$; Fig. 3D). Cells injected at cochleostomy site #2 were observed throughout the cochlea including the scala media, the scala vestibuli and the scala tympani ($n = 2$; Fig. 3E). Cells injected via the round window were observed in the scala tympani ($n = 1$; Fig. 3E). In some cases, fluorescence could not be

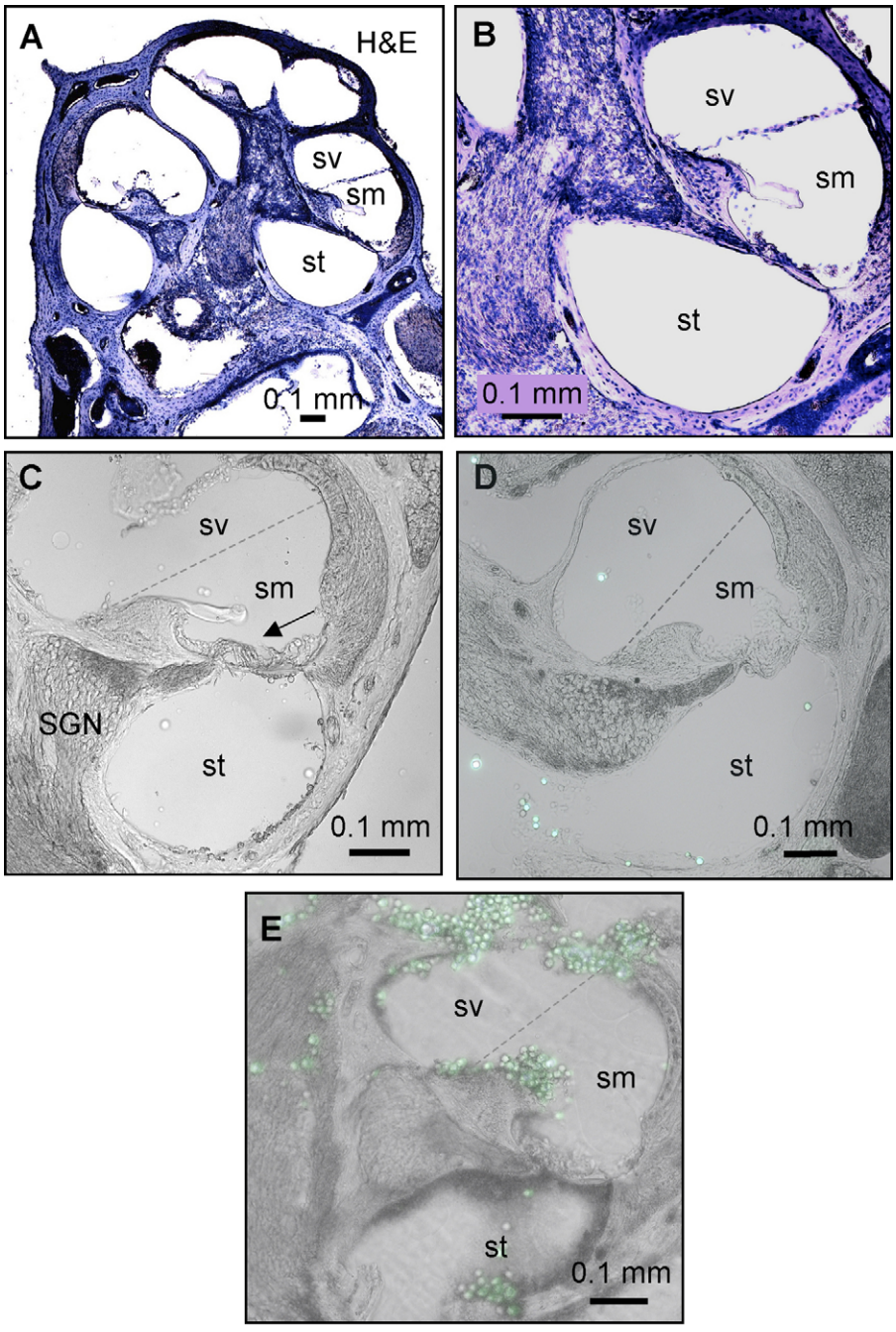

Fig. 3. Cell injection into scala media, scala tympani, scala vestibuli. Thin sections of mouse cochlea stained with haematoxylin and eosin (H&E) showing the anatomical locations of scala vestibuli (sv), scala media (sm) and scala tympani (st) at low (A) and high magnification (B). (C) Unstained thin section of cochlea showing preservation of organ of Corti (arrow) and spiral ganglion soma (SGN). Approximate location of Reissner's membrane is indicated by dashed line. (D) Injection via cochleostomy #1 shows fluorescent cells in scala tympani and scala vestibuli. (E) Injection via cochleostomy #2 shows fluorescent cells in the scala media, the scala tympani and the scala vestibuli.

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detected in the cochlear compartments irrespective of the cell injection site ($n = 6$).

3.3. Effect on auditory function

To determine if the microsurgical technique affected auditory function, the auditory brainstem response was recorded in 21–32-week-old mice ($n = 11$) following ketamine/xylazine anaesthesia. The ABRs were compared between control mice ($n = 7$) and in mice receiving a cochleostomy #1 ($n = 3$; Fig. 4). The ABR was evoked by two types of stimuli (click and 16 kHz tone). ABR thresholds were detected by visual inspection and by computerized signal-to-noise ratio detection (Fig. 4A–C). In control mice, visual inspection yielded a mean ABR threshold that was similar for click (39 ± 7 dB) and tone stimuli (35 ± 6 dB). The signal-to-noise ratio detection method yielded a mean ABR threshold that was similar for the click (33 ± 6 dB) and tone stimuli (33 ± 6 dB). Thus the threshold level was similar for different acoustic stimuli (click or tone) as confirmed by two-independent detection methods.

A comparison of the detection methods revealed that the signal-to-noise ratio detection method estimated similar threshold levels for the tone stimuli and lower threshold levels for the click stimuli ($p < 0.05$; paired t-test; Fig. 4D). In mice receiving a cochleostomy ($n = 6$), animals were deceased due to blood loss during surgery ($n = 1$), anesthetic complications during ABR testing ($n = 1$), and post-operative circling ($n = 1$). The mean ABR threshold for combined click and tone stimuli was 15 dB greater after surgery (control 33 ± 4 dB, surgery 48 ± 3 dB) when the mouse had received a small sized cochleostomy (≤ 0.4 mm) and was sibling matched to control mice. Threshold levels after surgery were equal or lower than threshold

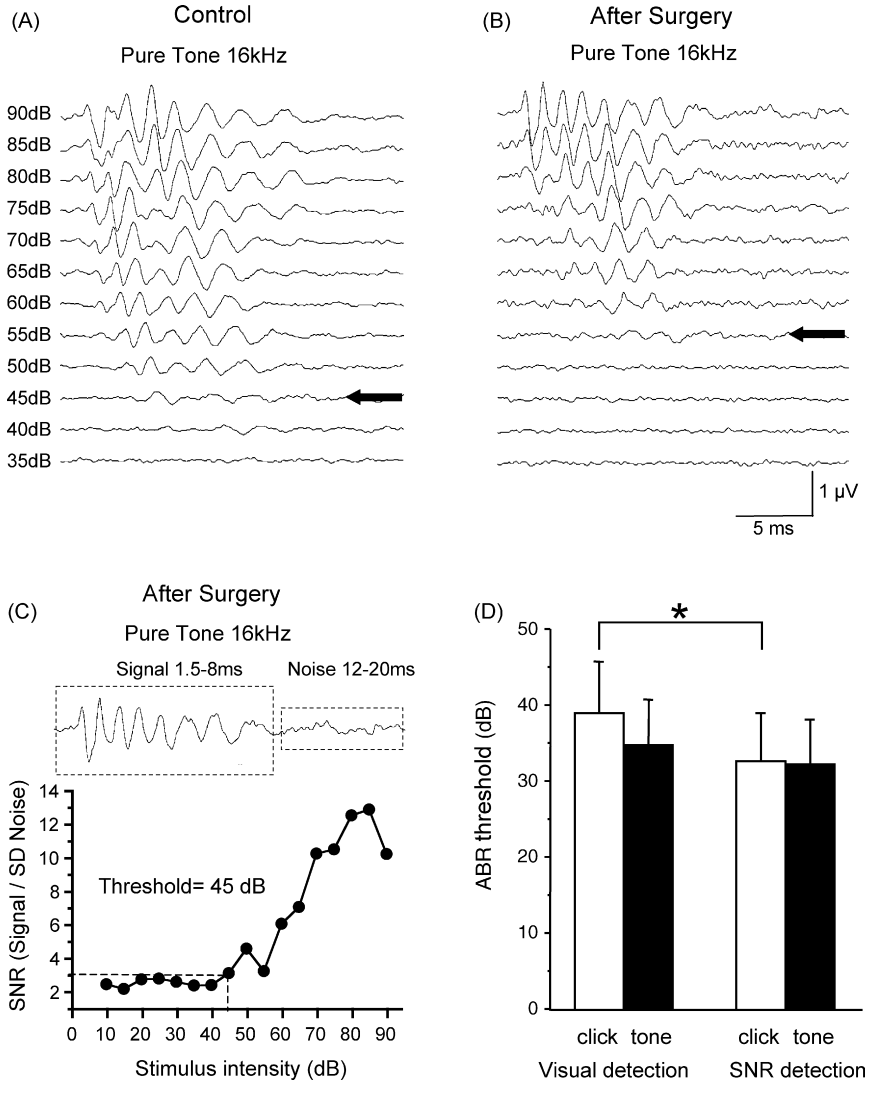

Fig. 4. Surgical treatment minimally affects auditory function. (A and B) Auditory brainstem responses (ABRs) to pure tone stimuli (16 kHz) in control mice and mice after cochleostomy #1. ABR threshold determined by visual detection (arrows). Data for individual animals. (C) ABR threshold determined by signal-to-noise ratio (SNR) analysis (AxoGraphX Scientific). The sound intensity level was deemed threshold when the maximum peak amplitude was three times the standard deviation of the baseline noise. Boxes show measurement regions (inset). (D) Summary data showing that mean threshold was similar for different acoustic stimuli (click vs. 16 kHz tone). The mean threshold was similar for different detection methods (visual vs. SNR) for the tone stimuli and different for the click stimuli (paired t-test, $p < 0.05$). Means ± S.E.M.

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levels in control mice in 23% of auditory tests. In mice that were 10 weeks older than control mice and had received a large sized cochleostomy (0.8–1 mm; $n = 2$), the ABR threshold was 45 dB greater after surgery for combined click and tone stimuli (78 ± 3 dB) compared to control mice.

4. Discussion

Here we describe a robust and reproducible microsurgical technique for cochleostomy and injection of cells into the mouse cochlea. The primary advantages of this surgical technique are access to multiple cochlear compartments, maintenance of cochlear anatomy, and a reduction in the loss of auditory function relative to earlier techniques. Fluorescent cells were observed in the scala media, the scala tympani and the scala vestibuli following cell injection of ZsGreen-MCF10A fluorescent cells. These fluorescent cells were chosen due to their similarity in size to adult olfactory stem cells and were injected in quantities and densities suitable for future injection of stem cells into the cochlea. Histological analysis suggested that gross cochlear anatomy is maintained. Fine cellular detail was beyond the resolution limits of the histological analysis (paraformaldehyde fixation and cryostat sectioning) and loss of fine structures (Reissner's membrane) could be attributable to tissue sectioning and processing. Threshold levels after surgery were equal or lower than threshold levels in control mice in 20% of auditory tests. Surgical procedures resulting in a small cochleostomy correlated with a smaller loss of auditory function compared to a large cochleostomy that resulted in a larger loss of auditory function and post-operative circling.

The computerised signal-to-noise detection method provided an objective estimation of the ABR threshold, with several advantages over the commonly used visual inspection method. Visual inspection yielded significantly different mean threshold values between two experimenters when ABRs were small ($p < 0.05$; data not shown). Computerised detection was consistent between different experimenters, resistant to subjective interpretation, and performed data analysis at a greater resolution than visual inspection of ABR responses. The signal-to-noise ratio detection method estimated lower threshold levels than visual inspection for click stimuli. This could be due to the measurement of threshold levels when the signal amplitude was three times the standard deviation of the baseline noise, a ratio selected to ensure few false positives.

Additional advantages of the surgical technique include the use of distinct anatomical landmarks, the avoidance of major vital structures, and the short duration of the surgery. The short duration minimises the time of anaesthetic application, essential in mouse surgery due to difficulties associated with dose and small body weight. The location of the cochleostomy was refined by identification of cochlear compartments in dissected cochleae and histological analysis of injected green fluorescent cells (ZsGreen-MCF10A) in thin sections of cochlea.

The use of mice in hearing research has increased in popularity due to an increasing number of hearing-impaired mice strains. The advantages of mice as a hearing research model include susceptibility to noise-related hearing loss, natural age-related hearing loss, amenability to genetic engineering, and short life span (Ohlemiller, 2006). Many deaf mice strains mimic various types of hearing loss. These include the Bronx waltzer mouse which demonstrates selective inner hair and pillar cell damage (Inagaki et al., 2006), or mouse strains which carry a Cdh23 but not Myo7a mutation and are more susceptible to noise-induced hearing loss than wildtype mice (Holme and Steel, 2004). Mice have already proven valuable in unravelling some of the mechanisms involved in the origin of different types of auditory pathology (Steel, 1995). The disadvantages of mice hearing models include a different range of hearing compared to humans, and a smaller body and cochlear size compared to other rodents. The mouse cochlea is three times smaller than that of the guinea pig (Jero et al., 2001). This leads to unique challenges when undertaking surgery on the mouse cochlea. The small animal size dictates that anaesthetic, body temperature, hydration and blood loss all be tightly controlled in order to ensure surgical success.

Alternative surgical techniques have been described for accessing the mouse cochlea via the small diameter posterior semicircular canal or via a cochleostomy positioned on the lateral wall of the cochlea, above the stria vascularis (Iguchi et al., 2004; Kawamoto et al., 2001b). In practice, we have found it difficult to locate the stria vascularis in the mouse by visual observation of pigmented melanocytes in the bony lateral wall. The cochleostomy provides good access to cochlear compartments for cell and gene therapy but a loss of hearing function compared to the semicircular canalostomy (Iguchi et al., 2004; Kawamoto et al., 2004). Here we report loss of auditory function 3 months after cochleostomy (15–45 dB shift) in contrast to the decrease in auditory function (40–70 dB shift) reported by others (Iguchi et al., 2004; Kawamoto et al., 2004). Previous reports show that surgery was initiated with the traditional post/retroauricular approach in agreement with our technique (Iguchi et al., 2004), while other reports describe a ventral approach that necessitates bypassing numerous vital structures such as the trachea, large facial veins and branches of the carotid artery as well as retraction of the submandibular gland (Chen et al., 2006; Jero et al., 2001). Although this technique preserves hearing function (Chen et al., 2006), any inadvertent injury to these structures may result in the demise of the animal.

Surgical techniques have been described to access the cochlear compartments for subsequent injection of stem cells (Coleman et al., 2006; Hildebrand et al., 2005; Hu et al., 2005a, 2005b). Access was achieved via the round window or a cochleostomy in the basal turn, and embryonic stem cells were injected into the scala media or the scala tympani. Histological analyses demonstrated cell survival and migration into the scala media, the scala tympani and the scala vestibuli. Although these techniques show relatively low cell survival and migration to scala media, they provide promising results for the application of stem cells to repair cochlear damage.

This study describes a robust and reproducible method for injecting cells into the three compartments of the mouse cochlea. The microsurgical technique uses well-defined anatomical landmarks to ensure consistent cochleostomy position and improved maintenance of auditory function relative to earlier techniques.

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A computerised threshold detection method provides consistent and objective estimates of ABR threshold levels between different observers. Minimum hearing loss was achieved with a small sized cochleostomy and by sibling matching to control mice. These techniques provide a basis for future injections of stem cells into the cochlea of deaf mice and the exploration of cell-replacement therapy as a treatment for hearing loss.

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