Published in final edited form as:

Hear Res. 2017 November ; 355: 33–41. doi:10.1016/j.heares.2017.09.004.

Severe streptomycin ototoxicity in the mouse utricle leads to a flat epithelium but the peripheral neural degeneration is delayed

Guo-Peng Wang^{1,2}, Ishani Basu², Lisa A. Beyer², Hiu Tung Wong², Donald L. Swiderski², Shu-Sheng Gong¹, and Yehoash Raphael^{2,*}

¹Department of Otolaryngology – Head and Neck Surgery, Beijing Friendship Hospital, Capital Medical University, Beijing 100050, China

²Kresge Hearing Research Institute, Department of Otolaryngology – Head and Neck Surgery, University of Michigan, Ann Arbor, MI 48109, USA

Abstract

The damaged vestibular sensory epithelium of mammals has a limited capacity for spontaneous hair cell regeneration, which largely depends on the transdifferentiation of surviving supporting cells. Little is known about the response of vestibular supporting cells to a severe insult. In the present study, we evaluated the impact of a severe ototoxic insult on the histology of utricular supporting cells and the changes in innervation that ensued. We infused a high dose of streptomycin into the mouse posterior semicircular canal to induce a severe lesion in the utricle. Both scanning electron microscopy and light microscopy of plastic sections showed replacement of the normal cytoarchitecture of the epithelial layer with a flat layer of cells in most of the samples. Immunofluorescence staining showed numerous cells in the severely damaged epithelial layer that were negative for hair cell and supporting cell markers. Nerve fibers under the flat epithelium had high density at the 1 month time point but very low density by 3 months. Similarly, the number of vestibular ganglion neurons was unchanged at 1 month after the lesion, but was significantly lower at 3 months. We therefore determined that the mouse utricular epithelium turns into a flat epithelium after a severe lesion, but the degeneration of neural components is slow, suggesting that treatments to restore balance by hair cell regeneration, stem cell therapy or vestibular prosthesis implantation will likely benefit from the short term preservation of the neural substrate.

Keywords

Hair cell; innervation; vestibular; mouse; utricle; streptomycin; vestibular prosthesis

*Corresponding author: Address: MSRB-3, Rm. 9301, 1150 W. Medical Center Drive, Ann Arbor, MI 48109-5648, Telephone: (734) 763-4444, yoash@umich.edu.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

1. Introduction

The mammalian vestibular sensory epithelium undergoes a limited amount of spontaneous hair cell (HC) regeneration after damage (Burns et al., 2012; Forge et al., 1998; Forge et al., 1993; Golub et al., 2012; Kawamoto et al., 2009; Slowik et al., 2013; Tanyeri et al., 1995; Wang et al., 2015; Warchol et al., 1993). The regenerated HCs are largely derived by transdifferentiation of surviving supporting cells (SCs) (Lin et al., 2011; Slowik et al., 2013; Wang et al., 2015). Therefore, the surviving SCs are potential targets for treatments to enhance the spontaneous HC regeneration, which is otherwise incomplete.

The models initially used for studying HC regeneration in the mammalian vestibular epithelium were mostly based on aminoglycoside ototoxicity which eliminated all or most HCs but spared SCs (Forge et al., 1998; Forge et al., 1993; Kawamoto et al., 2009). More recently, the *Pou4f3^{DTR}* mouse has become a useful model for HC elimination in the cochlea and the vestibular epithelium (Cox et al., 2014; Golub et al., 2012). After the administration of diphtheria toxin (DT) in *Pou4f3^{DTR}* mice, HCs are lost in a cell autonomous fashion, sparing the SCs, in both auditory and vestibular systems (Golub et al., 2012; Tong et al., 2015). A severe ototoxic insult may damage both HCs and SCs, leading to a flat epithelium (FE) as seen in the cochlea (Kim et al., 2007) and in some cases of severe degeneration in human ears (McCall et al., 2009). Although the effects of severe HC lesions on the SCs are well characterized in the auditory epithelium (Kim et al., 2007; Morais et al., 1988; Nadol et al., 2006; Oesterle, 2013; Pawlowski et al., 2006; Raphael et al., 2007; Shibata et al., 2010; Taylor et al., 2012), this is not the case in the utricle.

It is unclear whether a severe ototoxic insult to the vestibular epithelia can lead to a FE and how such severe lesions affect the neural substrates of these organs. Because the remaining SCs are the substrate for regenerative approaches and the survival of neurons is a prerequisite for recovery of function, the effects of severe injury on vestibular SCs and on the neural substrates of the vestibular end organs need to be better understood. In this study, we induced a severe lesion in the mouse utricle by local perfusion of a high dose of streptomycin. We found that the normal cytoarchitecture of the vestibular sensory epithelium was replaced by a monolayer of very thin flat cells of irregular contour as previously shown in the severely deafened cochlea. We found that despite the loss of HCs and appearance of a FE, many nerve fibers remained in the epithelium and the superior vestibular ganglion neurons (SVGN, the superior division of vestibular ganglion neurons) survived for at least 3 months.

2. Materials and Methods

2.1 Animals and surgery

All animal experiments in this work were approved by the University of Michigan Institutional Animal Care and Use Committee (IACUC) and were performed using acceptable veterinary standards. Female CD-1 mice at the age of 3-4 weeks were purchased from Charles River Laboratories International Inc. (Wilmington, MA, USA) and were housed at the animal care facility at the University of Michigan. The animals were 4-5 weeks old at the beginning of the experiment. We administered a high dose of streptomycin

(400 µg) into their left ears via the posterior semicircular canal to induce a severe lesion in the vestibular epithelium. Streptomycin powder was purchased from X-gen Pharmaceuticals Inc. (Northport, NY, USA) and was dissolved in normal saline. The surgical procedure was described previously (Wang et al., 2010), except that here, 400 µg streptomycin (2 µl at 200 g/l or 1 µl at 400 g/l) was used. Mice were sacrificed at 1 month or 3 months after surgery. Surgeries were only performed on left ears; right ears were used as controls.

2.2 Immunofluorescence staining

Once deeply anesthetized with an intraperitoneal injection of xylazine (7 mg/kg) and ketamine HCl (120 mg/kg), animals were decapitated and the temporal bones removed and fixed in 4% paraformaldehyde in phosphate buffered saline (PBS) for 2 hours. Utricles were dissected out and treated with 0.3% Triton X-100 in PBS for 15 minutes. For F-actin labeling, samples were incubated with Alexa Fluor 488-conjugated phalloidin (diluted 1:300, Invitrogen, A12379) for 30 minutes. For staining with primary antibodies, samples were blocked using 10% normal donkey serum (Jackson ImmunoResearch, West Grove, PA, USA) and 0.1% bovine serum albumin in PBS for 1 hour at room temperature. Tissues were then incubated overnight at 4 °C in a primary antibody. The following primary antibodies were used: rabbit anti-Myosin VIIa antibody (diluted 1:300, Proteus BioSciences Inc., 25-6790), goat anti-Sox2 antibody (diluted 1:300, Santa Cruz, SC-17320) and rabbit anti-neurofilament 200 (diluted 1:400, Sigma-Aldrich, N4142). After rinsing in PBS, samples were incubated in fluorescence-labeled secondary antibodies (Alexa Fluor 488 or 594, Invitrogen) at a dilution of 1:300 for 45 minutes at room temperature. When visualization of the nucleus was required, the DNA-binding fluorescent stain 4',6-diamidino-2-phenylindole (DAPI) was used. After staining, specimens were mounted on glass slides with Fluoro-Gel mounting media (Electron Microscopy Sciences, Hatfield, PA, USA), and examined with a Zeiss LSM 510-META Laser Scanning Confocal Microscope. Confocal images were cropped, labeled, and spaced with Zeiss LSM Image Browser software and Adobe Photoshop software.

2.3 Scanning electron microscopy (SEM)

Mice were anesthetized as described above and fixed by intracardiac perfusion of 2% glutaraldehyde (Electron Microscopy Sciences, Hatfield, PA, USA) in 0.15M cacodylate buffer. Temporal bones were removed and utricles were dissected out and incubated in the same fixative for 2 hours. The OTOTO method was used for tissue proceeding for SEM (Davies et al., 1987; Kawamoto et al., 2009; Osborne et al., 1991). Then, samples were dehydrated with graded series of ethanol solutions, critical point dried, mounted on stubs using silver paste, studied and photographed using an Amray FE 1910 SEM.

2.4 Plastic sections

Animals were deeply anesthetized and perfused as described above. The temporal bones were removed and decalcified in 3% EDTA solution with 0.25% glutaraldehyde for 1 week. Subsequently, the samples were embedded in JB-4 (Electron Microscopy Sciences) and serially sectioned in planes parallel to the axis of the cochlear modiolus from the level of SVGN to that of the utricle. Sections were 3 µm thick, and every sixth section, at an interval of 18 µm, was collected on a glass slide and stained with 1% Toluidine Blue in 1% sodium

borate. Sections of utricles and SVGN were studied and photographed using a Leica DMRB light microscope (Leica, Eaton, PA, USA) with SPOT Imaging™ software.

2.5 Quantification and statistical analysis

We calculated the density of SVGN nuclei based on plastic sections in each of the following groups: left ears at 1 month, right ears at 1 month, left ears at 3 months and right ears at 3 months. Four ears were analyzed for each group. For each ear, 6 to 8 sections spanning approximately 100 to 130 μm of SVGN were collected. Four of the slides were randomly chosen for calculation of SVGN density in each ear. We only counted ganglion cells which exhibited a well-defined nucleus and nucleoli. We used the tpsDig software (F. James Rohlf, Ecology & Evolution, SUNY at Stony Brook, Stony Brook, NY, USA) to count the number of SVGN and to measure the area of the profile of the superior vestibular ganglion by tracing the outer edge of each region of SVGN. The density of SVGN was calculated by dividing SVGN number by the area of the profile using Microsoft Excel. Statistical differences were examined using one-way ANOVA (Prism 6, GraphPad Software, Inc., La Jolla, CA, USA), and p value of <0.05 after Bonferroni adjustment for multiple tests was considered significant.

3. Results

3.1 Mortality of mice and gross manifestations of dysfunction after surgery

Approximately 50% (32/67) of mice given 2 μl of streptomycin solution (200 g/l) died just after the infusion. When the same dose was given in a lower volume and higher concentration (1 μl at 400 g/l), the mortality rate decreased to less than 20% (9/47). Severe vestibular dysfunction was detected after the surgery in all surviving mice, manifested by head tilt, circling, and longitudinal body-axis rolling.

3.2 Morphological changes of the severely damaged utricular epithelium

In control utricles labeled with phalloidin to visualize the actin-rich areas in the sensory epithelium (primarily the HC stereocilia and adherens-type intercellular junctions), HC stereocilia were seen throughout the vestibular sensory epithelium (Fig. 1A). The bundles of stereocilia and the actin in the intercellular junctions were better visualized at higher magnification (Fig. 1A inset). Following the streptomycin injection (400 μg), two types of lesions were found: a moderate lesion with surviving SCs and a few remaining HCs and a severe lesion with a FE and nearly no HCs. Summed over all animals in the study whether examined by immunofluorescence staining, SEM or plastic sections (described below), 18% (13/73) had a moderate lesion and 82% (60/73) had a severe lesion. In the cases with the moderate lesion (Fig. 1B, and in higher magnification in the inset), we observed well patterned SCs and a few HCs with short stereocilia randomly scattered throughout the utricle. This finding was similar to that found in utricles damaged by a lower dose of streptomycin (Wang et al., 2014; Wang et al., 2010). In contrast, animals with a severe lesion displayed an epithelium consisting of large cells with irregular contours (Fig. 1C). The differences between the striolar and extrastriolar regions of the utricle were erased and indiscernible with the flattening of the epithelium. When viewed on the microscope, the FE of these utricles had an extremely low thickness (distance in the z-axis between the top of

the cell and the basement membrane beneath it, not shown). This damaged tissue resembled the FE previously shown in severely deafened auditory epithelia (Izumikawa et al., 2008; Kim et al., 2007; Shibata et al., 2010; Taylor et al., 2012). At 3 months after the severe lesion (Fig. 1D), the epithelium was still flat and similar to that seen in the 1 month samples.

Ultrastructure of the epithelial surface of control utricles visualized by SEM showed normal organization of HC stereocilia and kinocilia evenly covering the epithelial surface (Fig. 2A). In the minority of lesioned ears, the utricle had SCs that were organized and densely covered with microvilli and a few HCs with short stereocilia and a prominent kinocilium (Fig. 2B), similar to utricles that received a moderate insult (Kawamoto et al., 2009). The majority of ears exhibited a severe lesion at 1 month (Fig. 2C): no stereocilia bundles were found and the cells of the vestibular epithelium appeared large and irregularly shaped, resembling the FE seen in the phalloidin-stained whole-mounts. Cells had either a dense coat of microvilli protruding from the cell surface (cell depicted by arrowhead in Fig. 2C and shown in higher magnification in Fig. 2D) or very sparse microvilli (arrow in Fig. 2C points the cell shown in Fig. 2E). At 3 months (Fig. 2F), the epithelium still consisted of large and flat epithelial cells with irregular shapes. No stereocilia were seen among the large and irregular flat cells.

Analysis of plastic sections of a normal utricle showed a full complement of HCs and SCs covered by an otoconial membrane (Fig. 3A). At 1 month after a severe lesion (Fig. 3B), HCs and SCs were absent, replaced by a FE, and the otoconial membrane was absent. The FE was composed of a monolayer of very thin flat cells with slender nuclei (arrow in Fig. 3B). At 3 months after the lesion (Fig. 3C), the utricular epithelium was still flat, similar to that of the 1 month group animals, and the otoconial membrane was still absent (arrow in Fig. 3C).

3.3 Identification of FE cells based on Myosin VIIa and Sox2

Myosin VIIa is a specific HC marker present in both type I and type II utricular HCs (Hasson et al., 1997) delineating the cytoplasm of all HCs throughout the utricle (Fig. 4A). At 1 month after the severe streptomycin trauma, double staining with phalloidin and anti-Myosin VIIa antibody revealed a few Myosin VIIa-positive cells in the FE (Fig. 4B). Most of these HCs had no stereocilia on the surface; but in some cases we observed small stereocilia-like structures (Fig. 4C) that were thinner than those of new HC bundles in the utricles lesioned by a moderate trauma (Kawamoto et al., 2009; Wang et al., 2010). At 3 months after the severe insult (Fig. 4D), the apical contour the FE included some areas with SCs that resembled normal SC apical contour. Myosin VIIa-positive cells were also detected at 3 months. Immature stereocilia-like structures were found on the surface of a few HCs in the FE (Fig. 4E).

We used anti-Sox2 antibody to label SCs and type II HCs (Oesterle et al., 2008). In the HC layer of a control utricle, Sox2 was present in the nuclei of a subset of HCs, likely the type II HCs (Fig. 5A), and the nuclei of SCs at the lower focal plane where they reside (Fig. 5B), as previously described (Oesterle et al., 2008). In utricles that were moderately traumatized (Fig. 5C), Sox2 pattern of expression remained similar to the normal tissue in the SC layer. In severely traumatized utricles with flat epithelia, several configurations of staining for Myosin VIIa and Sox2 were found (Fig. 5D-F''). Sox2-positive cells were sparsely

distributed and their nuclei were larger than those of normal Sox2-positive HCs or SCs (Fig. 5D-D'). Some cells had Sox2-positive nuclei and Myosin VIIa-negative cytoplasm (Fig. 5E). Of the few Myosin VIIa-positive cells (presumed to be HCs), most were Sox2-positive and a minority were Sox2-negative (Fig. 5E). Cells in some areas of the FE were negative for both Sox2 and Myosin VIIa (Fig. 5F-F''), which is different from the normal tissue.

3.4 Alterations of nerve terminals and vestibular ganglion neuron density

The baso-lateral portion of type I HCs is enclosed in a nerve calyx, which can be identified by neurofilament staining. Nerve calyces and nerve fibers were distributed throughout the normal epithelium of the mouse utricle (Fig. 6A-B). In the FE 1 month after the treatment of streptomycin (Fig. 6C-D), most calyces were absent, but nerve fibers remained. At 3 months (Fig. 6E-F), the FE was completely devoid of nerve calyces. The population of nerve fibers associated with the utricle also appeared to be reduced at 3 months compared to 1 month, although a few fibers were still visible at 3 months. Fibers were seen in the same focal plane as the adherens junctions (Fig. 6F) as well as beneath the epithelium (as revealed by confocal optical sections, not shown).

The vestibular ganglion neurons are composed of two populations, SVGN and inferior vestibular ganglion neurons (IVGN, inferior division of vestibular ganglion neurons). Because the afferent fibers in the utricle mainly project to SVGN, we assessed the changes of SVGN in plastic sections. In ears that had FE, revealed by analysis of plastic sections, we performed quantification of surviving neurons. At 1 month after the insult (Fig. 7A-B), there was no difference of SVGN density between streptomycin-injected (left) ears and non-injected (right) ears, but by 3 months (Fig. 7C-D), injected ears had a lower number of surviving SVGN than non-injected ears. In addition, lesioned ears exhibited larger stromal intercellular spaces. In the surviving SVGN cells, the cytoplasm and nuclei were well demarcated and normally shaped (Fig. 7C). The mean density of SVGN in injected ears of 3 month group was significantly lower ($p < 0.05$) than non-injected ears of 3 month group and lower than injected or non-injected ears of the 1 month group (Fig. 7E).

4. Discussion

4.1 The mouse model

In this study, we present a mouse model of severe impairment in the utricular epithelium. We determine that a high dose of streptomycin induced degeneration of both HCs and SCs, resulting in a FE. This lesion is different from lesions induced by a lower dose of aminoglycosides (Forge et al., 1993; Kawamoto et al., 2009; Lopez et al., 1998; Meiteles et al., 1994; Wang et al., 2010), and from the lesion seen in the *Pou4f3^{DTR}* mouse (Golub et al., 2012). In these studies, SCs survive the lesion and are able to transdifferentiate to new HCs. The present study is different in that the loss of utricular HCs is accompanied by formation of a FE instead of the survival of differentiated SCs. A negligible number of Myosin VIIa-positive cells is found in the samples with FE. Despite the severity of the lesion, nerve fibers and SVGN survive for months after the insults. Only partial degeneration of fibers is noted at 3 months. The relatively robust survival of the neuronal elements despite the severe loss of HCs and the appearance of the FE suggests that this model may be useful

for investigating neuronal responses to regenerative therapies and prostheses targeting vestibular dysfunction.

The outcome of the ototoxic insult we observe includes a FE in most mice, but in some animals the lesion is moderate and differentiated SCs appear to remain in the epithelium. It is unclear why a fifth of the mice have a moderate lesion that does not involve a FE. We speculate that the variability is due to fluid leakage from the injection site or to variability in sensitivity to ototoxic drugs among animals.

In the study, approximately half of the animals die after the infusion of 2 μ l of streptomycin solution at 200 g/l. We then change the drug regimen by lowering the volume to 1 μ l and increasing the concentration to 400 g/l, thus retaining the total amount of drug delivered, and determine that the mortality decreased to less than 20%. The cause of death of mice may be related to activity of streptomycin in the brain, which is connected to the inner ear via the cochlear aqueduct and cerebrospinal fluid.

4.2 Vestibular HCs and innervation in the FE

The vestibular epithelium of mammals in vivo has spontaneous HC regeneration after HCs are lost (Forge et al., 1998; Forge et al., 1993; Golub et al., 2012; Kawamoto et al., 2009; Lopez et al., 1998; Rubel et al., 1995; Slowik et al., 2013). Both in vivo and in cultured explants, the extent of regeneration in the vestibular epithelium is limited and depends to a large extent on trans-differentiation of SCs (Forge et al., 1998; Forge et al., 1993; Golub et al., 2012; Kawamoto et al., 2009; Slowik et al., 2013; Tanyeri et al., 1995; Wang et al., 2010; Wang et al., 2015; Warchol et al., 1993). In the current study, a small number of Myosin VIIa-positive cells are sparsely detected in the vestibular FE. We cannot determine whether these are HCs that survived the extremely severe ototoxic insult, or are newly regenerated. The majority of those HCs resemble immature or type II-like HCs (positive for both Myosin VIIa and Sox2, Fig. 5), similar to new HCs in the utricle after a moderate injury (Forge et al., 1998; Kawamoto et al., 2009; Wang et al., 2010), but we cannot rule out these are HCs that survived the injury. It is unclear whether a subtype of cells within the FE is capable of undergoing transdifferentiation to new HCs, or, some original SCs remain in the epithelium and contribute to the regeneration. Lineage tracing studies of vestibular HCs or SCs are needed to identify the origin of new HCs in the vestibular FE.

We observe immature stereocilia-like structures on the surface of a few cells that are positive for Myosin VIIa (Fig. 4). These structures are thinner and different from new stereocilia on HC in the moderately lesioned epithelium (Kawamoto et al., 2009; Wang et al., 2014; Wang et al., 2010). Unequivocal identification is impossible at this stage, but even if they develop into mature functional HCs, their small number makes it unlikely they would meaningfully improve vestibular function.

In the auditory epithelium damaged by aminoglycosides, nerve fibers and neurons die within a few days such that the FE becomes devoid of nerve fibers (Shibata et al., 2010). In contrast, nerve fibers and SVGNs remain for several months after utricular HCs and SCs are replaced by FE. It is currently unclear what cells, if any, provide neurotrophic support for maintaining vestibular neurons in the absence of HCs and SCs. The presence of SVGNs and

fibers in the FE provides a window of opportunity for innervating new HCs when better interventions become available for enhancing their formation.

Surprisingly, the density of SVGNs in non-lesioned ears of the 3 month group shows a trend to decrease compared to both the lesioned and non-lesioned ear of the 1 month group. This decline may have been caused by streptomycin that spread into the non-lesioned ear via the cerebral spinal fluid and resulted in a chronic toxicity of SVGN.

4.3 The origin of flat cells

It is unknown whether the cells of the FE are dedifferentiated SCs or other types of cells that migrated into the sensory area from the flanking regions of the epithelium. In the chick auditory system damaged by severe noise exposure, flat cells are derived from the hyaline cells located along the inferior edge of the basilar papilla, which migrate and invade the sensory area (Cotanche et al., 1995). In the FE of mammalian cochlea, flat cells are morphologically quite different from the original SCs, and some of their molecular components indicate they have migrated from the flanks (Elizabeth Oesterle, Personal Communication). The newly formed FE in the guinea pig auditory epithelium contains cells that can undergo a phase of proliferation that repopulates the epithelial sheet (Kim et al., 2007), further suggesting that the identity of these cells may be different from the original cells.

At the gross-morphology level, the current data on the degradation of the vestibular epithelium to the flat stage concurs with findings in the cochlea. Closer examination of the vestibular FE reveals that the cells are not completely homogenous. We observe four kinds of cells that exist in the FE after triple staining by anti-Myosin VIIa antibody, anti-Sox2 antibody, and DAPI (Fig. 5): Myosin VIIa-positive / Sox2-positive cells, Myosin VIIa positive / Sox2-negative cells, Myosin VIIa-negative / Sox2-positive cells, and Myosin VIIa-negative / Sox2-negative cells. The two groups of Myosin VIIa-positive cells are readily identified as HCs. With the current methods, we cannot distinguish between HCs surviving the lesion and regenerating HCs. The Myosin VIIa-negative/Sox2-positive cells also are readily identifiable, as SCs. The nuclei of these cells are larger than in the original SCs, possibly because they may be preparing to divide to cover the confluent layer of the epithelium as seen in the FE of the organ of Corti (Kim et al., 2007). The identity of the Myosin VIIa-negative /Sox2-negative cells remains to be determined. These cells may be transformed from SCs that have lost some features of SCs (de-differentiation) or they may have migrated from the flanking region. SEM data show that some cells have abundant microvilli cover and others do not (Fig. 2), but presence or absence of dense microvilli is not enough to determine their origin unequivocally.

4.4 Future therapies for the vestibular FE

Inhibition of Notch signaling and *Atoh1* over-expression generate new HCs by transdifferentiation of SCs (Kawamoto et al., 2003; Lin et al., 2011; Mizutani et al., 2013; Staecker et al., 2007). SCs with severely altered morphology are less likely to produce a large amount of new HCs by the above techniques, as was shown in the cochlear FE (Izumikawa et al., 2008). Instead, stem cell transplantation might be a more promising

strategy for placing new HCs in the FE. The slow degeneration of neural components may benefit the re-innervation of new HCs. Feasibility for implanting exogenous cells has been demonstrated for the auditory FE (Lee et al., 2017; Park et al., 2014) and might also be developed for the vestibular organs. It may still be necessary to follow stem cell transplantation with protocols for inducing the transdifferentiation of exogenous cells into vestibular HCs and SCs.

A vestibular prosthesis is under development to treat patients with severe vestibular dysfunction (Hageman et al., 2016; Merfeld et al., 2007; Phillips et al., 2015; Wall et al., 2002). Similar to the cochlear implant, surviving primary ganglion neurons are the targets of stimulation by the proposed vestibular prosthesis. We now show that nerve fibers and SVGN survive for a while in the severely traumatized mouse vestibular sensory epithelium, which presents a therapeutic opportunity for a vestibular prosthesis. Although nerve fibers and SVGN do degenerate as time advances after the lesion, it may be possible to preserve nerve terminals and neurons in the FE by stimulating them with a prosthesis, as in the auditory system (Pfungst et al., 2015; Ramekers et al., 2012; Roche et al., 2015). Implanted stem cells and over-expression of neurotrophins (Shibata et al., 2010; Wise et al., 2010) might also be used as adjunct therapies to maintain neural tissue in the vestibular organs.

5. Conclusions

In this study, a severe lesion in the mouse utricle is induced by a local perfusion of a high dose of streptomycin. In most ears examined, both vestibular HCs and SCs degenerate, leaving behind a FE. A negligible number of Myosin VIIa-positive cells are found in the FE. However, nerve fibers and primary utricular neurons do not undergo a rapid degeneration, thereby presenting a time window for therapies to restore balance by HC regeneration, stem cell therapy or vestibular prosthesis implantation.

Acknowledgments

This work was supported by the R. Jamison and Betty Williams Professorship, the A. Alfred Taubman Medical Research Institute, National Natural Science Foundation of China (grants 81100717 and 81570912), the China Scholarship Council (Grant number 2008616087) and NIH-NIDCD Grants R01-DC01634, R01-DC014832, and P30-DC05188.

References

- Burns JC, Cox BC, Thiede BR, Zuo J, Corwin JT. In vivo proliferative regeneration of balance hair cells in newborn mice. *J Neurosci.* 2012; 32:6570–7. [PubMed: 22573679]
- Cotanche DA, Messana EP, Ofsie MS. Migration of hyaline cells into the chick basilar papilla during severe noise damage. *Hear Res.* 1995; 91:148–59. [PubMed: 8647716]
- Cox BC, Chai R, Lenoir A, Liu Z, Zhang L, Nguyen DH, Chalasani K, Steigelman KA, Fang J, Rubel EW, Cheng AG, Zuo J. Spontaneous hair cell regeneration in the neonatal mouse cochlea in vivo. *Development.* 2014; 141:816–29. [PubMed: 24496619]
- Davies S, Forge A. Preparation of the mammalian organ of Corti for scanning electron microscopy. *J Microsc.* 1987; 147:89–101. [PubMed: 3305958]
- Forge A, Li L, Nevill G. Hair cell recovery in the vestibular sensory epithelia of mature guinea pigs. *J Comp Neurol.* 1998; 397:69–88. [PubMed: 9671280]
- Forge A, Li L, Corwin JT, Nevill G. Ultrastructural evidence for hair cell regeneration in the mammalian inner ear. *Science.* 1993; 259:1616–9. [PubMed: 8456284]

- Golub JS, Tong L, Ngyuen TB, Hume CR, Palmiter RD, Rubel EW, Stone JS. Hair cell replacement in adult mouse utricles after targeted ablation of hair cells with diphtheria toxin. *J Neurosci.* 2012; 32:15093–105. [PubMed: 23100430]
- Hageman KN, Kalayjian ZK, Tejada F, Chiang B, Rahman MA, Fridman GY, Dai C, Pouliquen PO, Georgiou J, Della Santina CC, Andreou AG. A CMOS Neural Interface for a Multichannel Vestibular Prosthesis. *IEEE transactions on biomedical circuits and systems.* 2016; 10:269–79. [PubMed: 25974945]
- Hasson T, Gillespie PG, Garcia JA, MacDonald RB, Zhao Y, Yee AG, Mooseker MS, Corey DP. Unconventional myosins in inner-ear sensory epithelia. *J Cell Biol.* 1997; 137:1287–307. [PubMed: 9182663]
- Izumikawa M, Batts SA, Miyazawa T, Swiderski DL, Raphael Y. Response of the flat cochlear epithelium to forced expression of Atoh1. *Hear Res.* 2008; 240:52–6. [PubMed: 18430530]
- Kawamoto K, Ishimoto S, Minoda R, Brough DE, Raphael Y. Math1 gene transfer generates new cochlear hair cells in mature guinea pigs in vivo. *J Neurosci.* 2003; 23:4395–400. [PubMed: 12805278]
- Kawamoto K, Izumikawa M, Beyer LA, Atkin GM, Raphael Y. Spontaneous hair cell regeneration in the mouse utricle following gentamicin ototoxicity. *Hear Res.* 2009; 247:17–26. [PubMed: 18809482]
- Kim YH, Raphael Y. Cell division and maintenance of epithelial integrity in the deafened auditory epithelium. *Cell Cycle.* 2007; 6:612–9. [PubMed: 17351334]
- Lee MY, Hackelberg S, Green KL, Lunghamer KG, Kurioka T, Loomis BR, Swiderski DL, Duncan RK, Raphael Y. Survival of human embryonic stem cells implanted in the guinea pig auditory epithelium. *Sci Rep.* 2017; 7:46058. [PubMed: 28387239]
- Lin V, Golub JS, Nguyen TB, Hume CR, Oesterle EC, Stone JS. Inhibition of Notch activity promotes nonmitotic regeneration of hair cells in the adult mouse utricles. *J Neurosci.* 2011; 31:15329–39. [PubMed: 22031879]
- Lopez I, Honrubia V, Lee SC, Li G, Beykirch K. Hair cell recovery in the chinchilla crista ampullaris after gentamicin treatment: a quantitative approach. *Otolaryngol Head Neck Surg.* 1998; 119:255–62. [PubMed: 9743081]
- McCall AA, Ishiyama GP, Lopez IA, Bhuta S, Vetter S, Ishiyama A. Histopathological and ultrastructural analysis of vestibular endorgans in Meniere's disease reveals basement membrane pathology. *BMC Ear Nose Throat Disord.* 2009; 9:4. [PubMed: 19493357]
- Meiteles LZ, Raphael Y. Scar formation in the vestibular sensory epithelium after aminoglycoside toxicity. *Hear Res.* 1994; 79:26–38. [PubMed: 7528737]
- Merfeld DM, Haburcakova C, Gong W, Lewis RF. Chronic vestibulo-ocular reflexes evoked by a vestibular prosthesis. *IEEE Trans Biomed Eng.* 2007; 54:1005–15. [PubMed: 17554820]
- Mizutani K, Fujioka M, Hosoya M, Bramhall N, Okano HJ, Okano H, Edge AS. Notch inhibition induces cochlear hair cell regeneration and recovery of hearing after acoustic trauma. *Neuron.* 2013; 77:58–69. [PubMed: 23312516]
- Morais D, Gonzalez M, del Villar R, Gayoso MJ. Long-term ototoxic effects of neomycin applied topically in the middle ear: a morphological study in the guinea pig. *J Laryngol Otol.* 1988; 102:304–7. [PubMed: 3385319]
- Nadol JB Jr, Eddington DK. Histopathology of the inner ear relevant to cochlear implantation. *Adv Otorhinolaryngol.* 2006; 64:31–49. [PubMed: 16891835]
- Oesterle EC. Changes in the adult vertebrate auditory sensory epithelium after trauma. *Hear Res.* 2013; 297:91–8. [PubMed: 23178236]
- Oesterle EC, Campbell S, Taylor RR, Forge A, Hume CR. Sox2 and JAGGED1 expression in normal and drug-damaged adult mouse inner ear. *J Assoc Res Otolaryngol.* 2008; 9:65–89. [PubMed: 18157569]
- Osborne MP, Comis SD. Preparation of inner ear sensory hair bundles for high resolution scanning electron microscopy. *Scanning Microsc.* 1991; 5:555–64. [PubMed: 1947938]
- Park YH, Wilson KF, Ueda Y, Tung Wong H, Beyer LA, Swiderski DL, Dolan DF, Raphael Y. Conditioning the cochlea to facilitate survival and integration of exogenous cells into the auditory epithelium. *Mol Ther.* 2014; 22:873–80. [PubMed: 24394296]

- Pawlowski KS, Kikkawa YS, Wright CG, Alagramam KN. Progression of Inner Ear Pathology in Ames Waltzer Mice and the Role of Protocadherin 15 in Hair Cell Development. *J Assoc Res Otolaryngol*. 2006;1–12.
- Pfingst BE, Zhou N, Colesa DJ, Watts MM, Strahl SB, Garadat SN, Schwartz-Leyzac KC, Budenz CL, Raphael Y, Zwolan TA. Importance of cochlear health for implant function. *Hear Res*. 2015; 322:77–88. [PubMed: 25261772]
- Phillips JO, Ling L, Nie K, Jameyson E, Phillips CM, Nowack AL, Golub JS, Rubinstein JT. Vestibular implantation and longitudinal electrical stimulation of the semicircular canal afferents in human subjects. *J Neurophysiol*. 2015; 113:3866–92. [PubMed: 25652917]
- Ramekers D, Versnel H, Grolman W, Klis SF. Neurotrophins and their role in the cochlea. *Hear Res*. 2012; 288:19–33. [PubMed: 22465680]
- Raphael Y, Kim YH, Osumi Y, Izumikawa M. Non-sensory cells in the deafened organ of Corti: approaches for repair. *Int J Dev Biol*. 2007; 51:649–54. [PubMed: 17891723]
- Roche JP, Hansen MR. On the Horizon: Cochlear Implant Technology. *Otolaryngol Clin North Am*. 2015; 48:1097–116. [PubMed: 26443490]
- Rubel EW, Dew LA, Roberson DW. Mammalian vestibular hair cell regeneration. *Science*. 1995; 267:701–7. [PubMed: 7839150]
- Shibata SB, Cortez SR, Beyer LA, Wiler JA, Di Polo A, Pfingst BE, Raphael Y. Transgenic BDNF induces nerve fiber regrowth into the auditory epithelium in deaf cochleae. *Exp Neurol*. 2010; 223:464–72. [PubMed: 20109446]
- Slowik AD, Bermingham-McDonogh O. Hair cell generation by notch inhibition in the adult mammalian cristae. *J Assoc Res Otolaryngol*. 2013; 14:813–28. [PubMed: 23989618]
- Staecker H, Praetorius M, Baker K, Brough DE. Vestibular hair cell regeneration and restoration of balance function induced by math1 gene transfer. *Otol Neurotol*. 2007; 28:223–31. [PubMed: 17255891]
- Tanyeri H, Lopez I, Honrubia V. Histological evidence for hair cell regeneration after ototoxic cell destruction with local application of gentamicin in the chinchilla crista ampullaris. *Hear Res*. 1995; 89:194–202. [PubMed: 8600126]
- Taylor RR, Jagger DJ, Forge A. Defining the cellular environment in the organ of Corti following extensive hair cell loss: a basis for future sensory cell replacement in the Cochlea. *PLoS One*. 2012; 7:e30577. [PubMed: 22299045]
- Tong L, Strong MK, Kaur T, Juiz JM, Oesterle EC, Hume C, Warchol ME, Palmiter RD, Rubel EW. Selective deletion of cochlear hair cells causes rapid age-dependent changes in spiral ganglion and cochlear nucleus neurons. *J Neurosci*. 2015; 35:7878–91. [PubMed: 25995473]
- Wall C 3rd, Merfeld DM, Rauch SD, Black FO. Vestibular prostheses: the engineering and biomedical issues. *J Vestib Res*. 2002; 12:95–113. [PubMed: 12867668]
- Wang GP, Guo JY, Peng Z, Liu YY, Xie J, Gong SS. Adeno-associated virus-mediated gene transfer targeting normal and traumatized mouse utricle. *Gene Ther*. 2014; 21:958–66. [PubMed: 25119376]
- Wang GP, Chatterjee I, Batts SA, Wong HT, Gong TW, Gong SS, Raphael Y. Notch signaling and Atoh1 expression during hair cell regeneration in the mouse utricle. *Hear Res*. 2010; 267:61–70. [PubMed: 20433915]
- Wang T, Chai R, Kim GS, Pham N, Jansson L, Nguyen DH, Kuo B, May LA, Zuo J, Cunningham LL, Cheng AG. Lgr5+ cells regenerate hair cells via proliferation and direct transdifferentiation in damaged neonatal mouse utricle. *Nature communications*. 2015; 6:6613.
- Warchol ME, Lambert PR, Goldstein BJ, Forge A, Corwin JT. Regenerative proliferation in inner ear sensory epithelia from adult guinea pigs and humans. *Science*. 1993; 259:1619–22. [PubMed: 8456285]
- Wise AK, Hume CR, Flynn BO, Jeelall YS, Suhr CL, Sgro BE, O'Leary SJ, Shepherd RK, Richardson RT. Effects of localized neurotrophin gene expression on spiral ganglion neuron resprouting in the deafened cochlea. *Mol Ther*. 2010; 18:1111–22. [PubMed: 20216530]

Abbreviations

DAPI	4', 6-diamidino-2-phenylindole
DT	diphtheria toxin
FE	flat epithelium
HC	hair cell
IVGN	inferior vestibular ganglion neurons
PBS	phosphate buffered saline
SC	supporting cell
SEM	scanning electron microscopy
SVGN	superior vestibular ganglion neurons

Highlights

A severe ototoxic insult results in a flat epithelium (FE) in the mouse utricle

Nerve fibers and vestibular ganglion neurons undergo a slow degeneration

The survival of neurons under the FE provides a window of opportunity for therapy

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Fig. 1.

Confocal images of whole mounts of utricles stained by phalloidin. (A) A control utricule with a normal morphology of HC bundles. Inset: higher magnification image of stereocilia and adherens junctions. (B) In animals that had a moderate lesion, surviving SCs are well patterned and HCs with immature hair bundles (arrow and inset) are found. (C) In utricles with a severe lesion at 1 month after streptomycin injection, the epithelium consists of expanded cells of irregular contour. (D) At 3 months after severe trauma, the epithelium is still poorly organized. Scale bars (A for A - D, and in each of the insets) represent 20 μm.

Fig. 2.

SEM images of utricles in a normal control (A), 1 months after the lesion (B-E) and 3 months after the lesion (F). (A) A normal density and appearance of HC stereocilia and kinocilia in a control utricle. (B) A utricle moderately traumatized, with a few HCs exhibiting round stereocilia bundles (arrow) or short stereocilia bundles with a kinocilium (arrowheads). (C) In a utricle displaying a severe insult, the epithelium contains no HC bundles and the surface of SCs is expanded. Those cells can be classified into two classes: cells with plentiful microvilli (arrowhead) and cells with sparse microvilli (arrow). (D) and (E) are high magnification images of the area indicated by the arrowhead and the arrow in (C), respectively. (F) At 3 months after a severe insult, the epithelium is flat and stereocilia bundles are absent. Scale bars represent 20 μm .

Fig. 3.

Light microscope images of plastic sections from a normal (A) and flat utricular epithelia (B-C). (A) The cytoarchitecture of the normal vestibular utricular epithelium from a control ear. O, otoconia; B, HC bundles; H, HCs; S, SC nuclei. At 1 month (B) and 3 months (C) after the insult, the epithelia are composed of a monolayer of very thin flat cells with slender nuclei, and no stereocilia or kinocilia are found (arrows). The scale bar, for all images, represents 20 μm .

Fig. 4.

Confocal images of whole mounts of normal (A) and flat utricular epithelia obtained at 1 month (B-C) and 3 months (D-E) following the lesion, stained with anti-Myosin VIIa antibody (red), and phalloidin (green, B-E). (A) HCs of a normal utricle are evenly distributed along the entire surface. Myosin VIIa is positive in the cytoplasm and negative in the nuclei. (B) A FE in which a HC (red) without stereocilia is present. (C) A FE containing a HC with an abnormal stereocilia-like structure (arrow). (D) In some regions of the FE, the apical contour of some cells resemble regular SC organization and a few cells were positive for Myosin VIIa. (E) A FE containing a cell positive for Myosin VIIa displaying an immature stereocilia-like structure (arrow). Scale bars are 20 μ m (B applies to B, D and E).

Fig. 5.

Confocal images of whole mounts of utricles showing the distribution of Sox2 in normal (A-B) and traumatized tissues 1 month after the lesion (C-F''). (A) In a normal utricle, Myosin VIIa (red) stains all HCs. Sox2 (green) is expressed in nuclei of type II HCs. (B) Sox2 localizes in nuclei of SCs in the normal utricle. (C) In a moderately damaged utricle, Sox2 is expressed in nuclei of SCs which are negative for Myosin VIIa. (D) In a severely damaged utricle, the density of Sox2 positive cells is low and spaces appear between them. (D'') Double staining of the image in (D) by DAPI (blue) showing that Sox2 is localized to nuclei. (E) Myosin VIIa-positive cells are either Sox2 positive or Sox2-negative (arrows) in nuclei. (F-F'') Some nuclei in severely traumatized utricles are negative for both Sox2 and Myosin VIIa (arrows). Scale bar, 20 μ m, for all images.

Fig. 6.

Confocal images of whole mounts of a normal utricle (A-B) and utricles obtained at 1 month (C-D) or 3 months (E-F) following a severe lesion, stained with anti-NF200 antibody (red) and/or phalloidin (green). (A) The focal plane just beneath the luminal surface reveals nerve calyces (arrows). (B) A lower focal plane than that of (A) reveals the course of nerve fibers. (C) A low magnification view of a utricle 1 month after the lesion, showing the innervation in the whole sensory area of the utricle. (D) The high magnification view of (C) shows that almost all nerve calyces are lost but numerous nerve fibers survive in the FE. (E-F) The density of nerve fibers is reduced at the 3 months group. Scale bars (A for A, B, D and F; C for C and E) represent 20 μ m.

Fig. 7.

Light microscopy images of plastic sections of superior vestibular ganglion neurons (SVGN) of mice after a unilateral severe lesion (A-D) and the quantification analysis of SVGN (E).

(A-B) In the 1 month group, no significant difference in the density of SVGN is detected between the lesioned left side (A) and the non-lesioned right side (B). (C-D) In the 3 months group, the lesioned left side (C) has a lower density of surviving SVGN than the non-lesioned right side (D). The scale bars (A for A-C) represent 50 μm. (E) Quantitative analysis of the density of SVGN shown in (A-D). Asterisks label significant differences ($p < 0.05$).