# Deeper Investigation into the Effect of Furosemide on Cochlear Vibratory Responses and Stereocilia Morphology

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## Abstract

The endocochlear potential, EP, the ~+ 80 mV potential in scala media is essential for normal cochlear amplification. In two separate previous studies, we reversibly eliminated the EP by an intravenous (IV) injection of furosemide in gerbil and measured the basal local cochlear microphonic (LCM) (Wang et al., 2019)[1], the vibrations of the OCC (Strimbu et al., 2020)[2], and distortion product otacoustic emissions (DPOAEs). The EP recovered over ~ 40 minutes. Vibrations, cochlear microphonic and DPOAEs recovered later, ~ 2 hours post injection.

Velez-Ortega et al., 2019 have shown that normal transduction currents are necessary to maintain stereocilia morphology[3]. We hypothesized that the loss of EP will thus damage hair cell stereocilia and may, in part, explain the delayed recovery (after EP) of cochlear amplification.

In this study, we further investigate the effect of IV injection of furosemide to the morphology of the OHC bundles and the vibrations of the OCC by 1) ex-vivo imaging of the OHC bundles using scanning electron microscopy (SEM) and 2) In-vivo two dimensional mapping of the axial vibration of the OCC using spectral domain optical coherence tomography (SD-OCT). All experiments in gerbil.

## Results

### *Scanning Electron Microscopy*

To date, results from the SEM images of the OHC bundles after furosemide showed no significant change in the morphology of the OHC stereocilia. In one case, we observed shortening at the first (shortest) row of the OHC stereocilia in the base (Fig. 7 F). However, that result did not persist in later experiments.

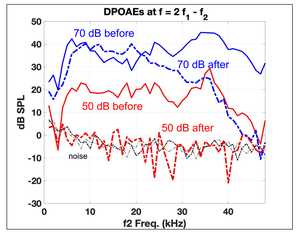



***Figure 6**: DPOAE responses to two equal level tones with 50 dB SPL (in red) and 70 dB SPL (in blue) before (solid lines) and 10 minutes after furosemide injection (dashed lines). g929.*

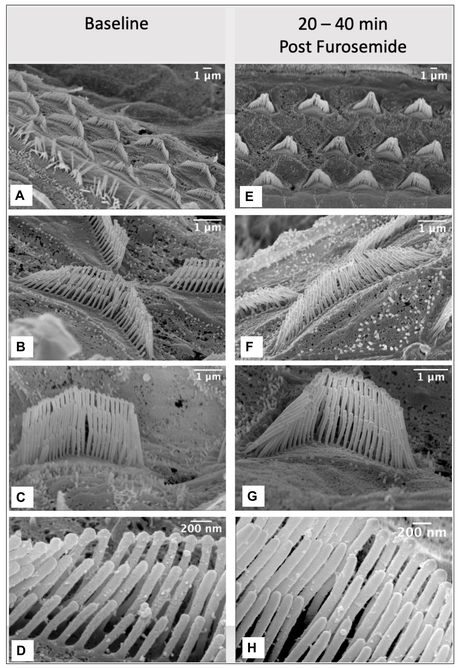



***Figure 7**: scanning electron microscopy (SEM) images of OHC stereocilia bundles in gerbil. A-D) from healthy cochlea (g919-LE). A & B) Basal turn C) Middle turn D) Basal turn (apical end). E – H) SEM images 20 – 40 minutes after IV injection of furosemide (g916-LE, g923-LE). E & F) Basal turn. G & H) Middle turn. EHT = 5 kV.*

### *Displacements in the organ of Corti complex*

Post furosemide, BM vibrations resembled a passive cochlea while those in the OHC-region showed a loss of amplitude but retained the broadband nonlinearity. Vibrations were less tightly focused on the OHC-region. DPOAEs and vibration patterns could recover to baseline conditions over the subsequent 2 – 3 hours.

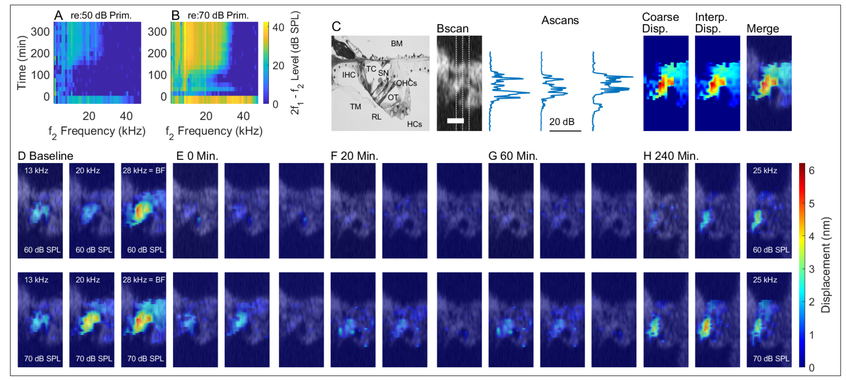



***Figure 8:** A and B. Loss and recovery of DPOAEs at 50 (A) and 70 (B) dB SPL following IV furosemide. Images in (C) explain the method for measuring two-dimensional vibration patterns. The left image shows a photomicrograph of a (guinea pig) cochlea with important structures labeled. BM = Basilar Membrane, RL = Reticular Lamina, IHCs = Inner Hair Cells, OHC = Outer Hair Cell, HCs = Hensen's Cells, TM = Tectorial Membrane, TC = Tunnel of Corti, SN = Space of Nuell, and OT = Outer Tunnel. The second image shows a raw Bscan, 120 µm wide. Scale bar = 50 µm and three representative A-scans spaced 20 µm apart across the radial direction along the dashed lines in the B-scan. The third image shows the axial motion as measured in 10 µm steps in the radial direction and the next image shows the interpolated vibrations. The final image is a merge of the vibration patterns overlaid on the structural Bscan. Panels (D) to (H) show the two dimensional maps of the axial vibrations at selected times evoked at 60 dB (top row) and 70 dB (bottom row). At each time point, vibrations at three frequencies are shown. Experiment #925, 10/13/2021. BF ~ 28 kHz.*

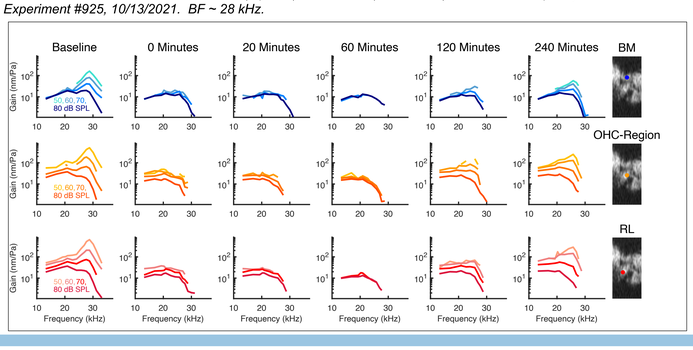



***Figure 9:** Tuning curves measured at the Basilar Membrane (top row in blue), Outer Hair Cell Region (OHC) (middle row in orange), and close to the Reticular Lamina (bottom row in red) at different time points. The Bscans to the right show the locations in the organ of Corti where the data were measured. Same experiment as the previous figure.*

## Background information from our group and Velez-Ortega

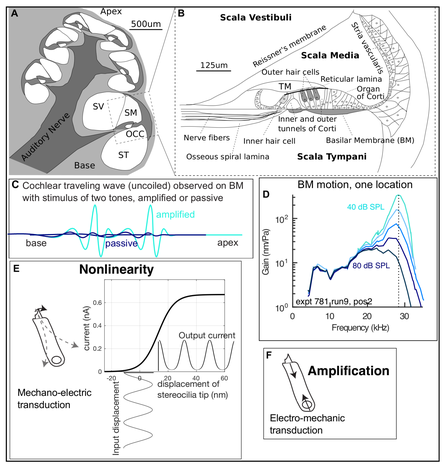



***Figure 1:** Cochlear amplification. A and B: Cross-sectional anatomy of the cochlea. C and D: In vivo, one or more active processes amplify the vibrations in a frequency selective manner. E and F: The nonlinearity in the cochlea likely arises from the mechano-electrical transduction channels in the hair cell bundles while the power delivered to the BM, and other structures, likely comes from the somatic motility of the OHCs.*

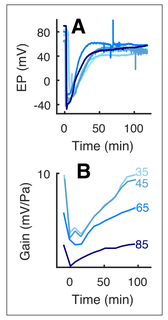



***Figure 2:** A) Endocochlear Potentials measured in five healthy cochleae before and after the treatment with furosemide. The EP is abolished immediately after the drug is injected and stabilizes at a depressed value ~ 30 - 50 minutes later. B) Following furosemide, the LCM gain (or mV per unit pressure) decreases and could recover completely on a slower timescale than in dB SPL. Stimulus levels in dB SPL are indicated by the color coded numbers to the right of the curves [1].*

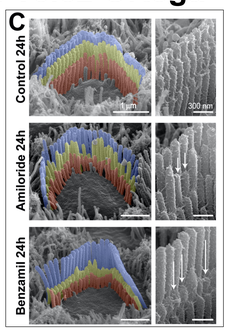
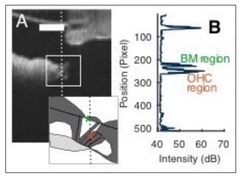



***Figure 4 (copy from paper):** Long-term blockage of the MET channels causes selective shortening of the second and third, but not the first (tallest), rows of stereocilia in mouse outer hair cell (OHC) bundles. C) Representative scanning electron microscopy (SEM) images of OHC stereocilia bundles (false-colored) in mouse organ of Corti explants cultured for 24 hr at 37˚C in vehicle control conditions (top), 100 mM of amiloride (middle), or 30 mM of benzamil (bottom). Right panels show higher magnification images of OHC stereocilia. Arrows point to examples of retracted stereocilia [3].*



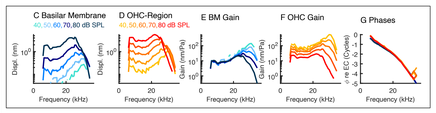




***Figure 3:** SD-OCT imaging and vibrometry. (A) Two dimensional B scan showing the organ of Corti with the BM at the top of the image. Scale bar 200 µm. (B) Vibrations were measured along this Axial or A-scan through the center of the image. Peaks in the A-scan correspond to the strong reflectors. (C) – (F) show the sound-evoked vibrations at the BM and OHC-region from 40 to 80 dB SPL. At the lowest level measured, the OHC-region moved ~ 2× more than the BM [2].*

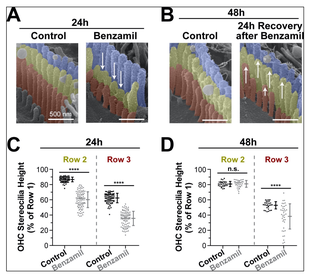



***Figure 5 (copy from paper):** Transducing stereocilia that have retracted due to MET blockage are able to regrow after drug washout. (A and B) Representative false-colored SEM images of mouse OHC stereocilia after (A) 24 hr incubation either in control conditions (left) or with 30 mM of benzamil (right), and after (B) 24 additional hours of recovery after washout. Arrows down point to retracted stereocilia, while arrows up indicate re-growth. (C and D) Heights of second- and third-row stereocilia after (C) 24 hr incubation in control conditions (black, n = 83) or with benzamil (gray, n = 95), and (D) 24 hr after washout (n = 30, control; n = 38, benzamil). Stereocilia heights are shown as a percent relative to the size of tallest (first) row. Data are from 7 to 16 cells per treatment. Error bars indicate mean ± SD. ****p<0.0001; n.s., non-significant (Welch's t tests). Age of the explants: P4 +24–48 hr. All incubations were performed at 37˚C [3].*

## Methods

### *Displacement in the organ of Corti complex*

Cochlear vibrations were measured with a ThorLabs Telesto III OCT system. By taking sequential recordings with a narrow, 10 µm, spacing we construct two-dimensional maps of the axial displacements. Vibrations were measured before, after the furosemide injection (100 mg/kg IV), and in ~20-minute intervals for several hours post injection. DPOAEs were measured at approximately the same time points and used to assess the efficacy of the injection and to provide a real time measurement of cochlear condition.

### *Scanning Electron Microscopy*

For SEM experiments, young gerbils were anesthetized and DPOEAs were measured before and after furosemide injection to confirm the loss of the EP. In order to observe the state of the stereocilia soon after the EP had dropped, the gerbil was euthanized minutes after the confirming DPOAE measurement. Cochleae were extracted, fixed in 2.5% glutaraldehyde, dehydrated in ethanol, critical point dried, sputter coated, and imaged with a ZEISS VP SEM.

## Conclusions

1. The 2D mapping of axial vibration showed that recovery of cochlear amplification occurred along with a return of a vibration pattern that was intense in the OHC region and extended to the BM.
2. The SEM studies did not uncover changes in stereocilia morphology following furosemide. However, the SEM studies are just a single "snapshot", and it is possible that changes occurred or would have occurred before or after this snapshot (as was hinted in one case). While the findings here do not support the hypothesis that the stereocilia are involved in recovery following furosemide, they also do not disprove it. Continuous in-vivo imaging of stereocilia would be useful to probe this hypothesis.

## References:

[1] Y. Wang, E. Fallah, and E. S. Olson. Adaptation of Cochlear Amplification to Low Endocochlear Potential. Biophysical Journal 116. 2019.

[2] C. E. Strimbu, Y. Wang and E. S. Olson. Manipulation of the Endocochlear Potential Reveals Two Distinct Types of Cochlear Nonlinearity. Biophysical Journal, 119(10), 2087-2101. 2020.

[3] A. C. Vélez-Ortega, M. J. Freeman, A. A. Indzhykulian, J. M. Grossheim, and G. I. Frolenkov. Mechanotransduction current is essential for stability of the transducing stereocilia in mammalian auditory hair cells. Elife, 6, e24661. 2017.