ABSTRACT

Molecular Correlates Of Hearing Loss: Oncomodulin And Prestin

Preston L. Simpson

Director: Dwayne D. Simmons, Ph.D.

The exquisite hearing sensitivity of mammalian hearing is due to cochlear outer hair cells (OHCs) that amplify sound signals through changes in cell membrane potential and cell length. The OHC motor protein, prestin, is responsible for this phenomenon. Oncomodulin (OCM) is the dominant calcium-binding protein in OHCs and therefore plays a major role in calcium homeostasis. Adult Ocm knockout (KO) mice are deaf and prestin expression is abnormally distributed. Since prestin and OCM are localized in the periphery of OHCs, we hypothesized that they may interact. We performed high-resolution confocal imaging in adult wildtype (WT) mice at various ages. Measures of labeling intensity found OCM and prestin labeling in OHCs colocalized. Since prestin is expressed in Ocm-KO mice, we hypothesized that increasing prestin expression might delay hearing loss in Ocm-KO mice. Previous studies show that long-term administration of nonsteroidal anti-inflammatory drugs increases prestin protein expression, OHC electromotility and improved hearing. We performed salicylate injections in *Ocm*-KO mice to potentially delay hearing loss. Although we found that salicylate had an effect in WT mice, there was no or little effect in Ocm-KO mice. Taken together, our results suggest that the loss of OCM may have deleterious effects on prestin function in OHCs.

APPROVED BY DIRECTOR OF HONORS THESIS:

_____

Dr. Dwayne Simmons, Ph.D., Department of Biology

APPROVED BY THE HONORS PROGRAM

_____

Dr. Elizabeth Corey, Director

DATE:

MOLECULAR CORRELATES OF HEARING LOSS: ONCOMODULIN AND PRESTIN

A Thesis Submitted to the Faculty of

Baylor University

In Partial Fulfillment of the Requirements for the

Honors Program

By

Preston Simpson

Waco, Texas

May, 2020

TABLE OF CONTENTS

TABLE OF FIGURES AND TABLES

CHAPTER ONE

Introduction

Hearing loss

Hearing loss affects 38.2 million American adults. (Clason, 2019). Understanding the mechanism of hearing loss is crucial to discovering a cure. Two categories of hearing loss are age-related hearing loss (ARHL) and noise-induced hearing loss (NIHL). Although both ARHL and NIHL result from different causes, the underlying mechanism remains the same: cochlear outer hair cells (OHCs) lose their function. This can occur for a variety of reasons and it is important to understand the physiology of the ear and how hearing occurs.

Physiology of the ear

The process of hearing begins when auditory sound waves come into contact with the outer ear. The pinna, or outside of the ear, helps funnel incoming sound waves travel further into the ear canal. Towards the end of the ear canal, sound waves come into contact with the tympanic membrane, otherwise known as the eardrum. As a result, the eardrum vibrates and causes movement of an ossicular bony chain inside of the middle ear. This bony chain is composed of three segments: the maleus, incus, and stapes. Sound waves vibrate the bony chain, causing it to push on the oval window (Brownell, 2017). An important event occurs at this stage where sound vibrations are transformed into fluid vibrations. This occurs because vibrations of the bony chain cause perilymphatic fluid of the inner ear to vibrate. The middle ear also interacts with the inner ear through the round

window, an opening that connects to the ear drum and is used to relieve pressure. The organ inside the inner ear that allows for hearing to occur is the cochlea. The cochlea is a snail-shaped organ which interacts with the middle ear using the oval and round window. The cochlea is located in the temporal bone and consists of three canals: the scala tympani, scala vestibuli, and scala media. These canals are wrapped around a bony axis called the modiolus. The Organ of Corti, located between the scala media and scala tympani, contains outer and inner hair cells that play a role in sensorineural transduction. Two different fluids fill the cochlea and are located in different canals. The scala tympani and scala vestibule are filled with perilymph and the scala media is filled with another type of fluid, endolymph. Vibration of the perilymph caused by the ossicular chain causes the basilar membrane (located in the Organ of Corti) to vibrate as well. Basilar membrane vibration triggers tectorial membrane vibration, which causes the tectorial membrane to rub against hair cells in the cochlea specific for certain frequencies. In turn, this bends OHCs and depolarizes them via an influx of potassium ions. Potassium ions allow for calcium ions to flow into the cell and initiate depolarization that sends electrical signals to the brain (Dallos, 2008). Depolarization of OHCs leads to OHC contraction, amplifying the original sound stimulus. OHCs are important because without them, sounds would not be amplified to a magnitude with which we could hear them. Our hearing range would be much narrower without them (Ashmore, 1970). Not having OHCs would be like swimming to the bottom of a pool and then trying to listen to a conversation outside the pool. OHCs can amplify sound because of their ability to lengthen and contract upon mechanical stimulation (Ciganović, 2018). OHC excitations lead to IHC excitation and activates the synapse between IHCs and auditory nerve fibers,

which sends the information to the brain. OHC contraction stimulates sound amplification whereas elongation inhibits sound amplification. There are two important types of hair cells in the inner ear: OHCs and IHCs. OHCs amplify incoming sound vibrations; in turn, increasing the sensitivity and frequency selectivity of the sound. On the other hand, inner hair cells (IHCs) are responsible for releasing glutamate from ribbon synapses. This is important for exciting auditory never fibers which then transmit sound information to the brain, where the information is interpreted.

Electromotility

Electromotility allows OHCs to be mobile and change shape when mechanically stimulated. Electromotility is the process of sound vibrations being transformed into electrical signals that the brain can understand. Without electromotility, hearing would not be possible. This process is able to occur because of voltage-gated conformational changes in motor proteins of OHCs (Zheng et al., 2000). Cochlear OHCs amplify sound signals through changes in cell membrane potential and cell length that are a function of prestin. The primary protein responsible for OHC amplification is prestin (SLC26A), a transmembrane protein found in OHCs. Without prestin, the OHCs lose their sensitivity and frequency selectivity. If prestin is removed from OHCs, there is a significant decrease in hearing threshold (Liberman et al., 2002). This is likely because the removal of prestin causes the loss of electromotility. Thus, OHCs are responsible for the exquisite hearing sensitivity in mammals. When OHCs become damaged, either due to aging, noise exposure, or other factors, the result is hearing loss.

Calcium-binding proteins in the inner ear

Calcium acts as a buffer, aids in neurotransmitter release, and plays a role in sensory adaptation in the inner ear. Calcium is important in the inner ear because it acts as a signal that causes modification of cell shape (Pack and Slepecky, 1995). At rest, cells have low levels of calcium. Under normal circumstances, low levels of calcium are maintained by calcium-buffering proteins. However, when stimulated, these calcium-buffering proteins release calcium that changes the cytoskeleton and shape of hair cells (Lewit-Bentley and Stéphane Réty, 2000). Because of calcium's importance to cochlear hair cells, it is necessary to have a method of modulating and controlling calcium levels. This is performed by mobile EF-Hand Calcium-binding proteins (CaBPs) that have a sequence of approximately 30 residues that cause a conformational change when they are bound to calcium; therefore, allowing them to affect downstream proteins (Fischer, 2019).CaBPs are important for OHC function, but when they are removed, no phenotype is observed. This rule holds true for every CaBP except for one—oncomodulin (Climer et. al., 2019).

Oncomodulin

Although there are multiple calcium-binding proteins in the inner ear, oncomodulin (Ocm) is unique in that it is a member of the parvalbumin family, is subcellularly compartmentalized, is found preferentially in OHCs (relative to other EF-hand Calcium buffers) and its role in the regulation of calcium may be essential to proper OHC function (Hackney et al., 2005; Simmons et al., 2010). OCM is expressed in outer hair cells (OHCs) where mechanoelectric transduction and electromotility occur. OCM is also unique from other CaBPs because when removed in *Ocm* KO mice, they exhibit a phenotype of elevated hearing thresholds. In other words, *Ocm* KO mice do not hear as well as WT mice. Interestingly, even though the mice have difficulty hearing or cannot hear at all due to the loss of *Ocm*, they still have intact OHCs that express prestin. Another interesting phenomenon seen was that *Ocm* KO OHC's appeared to modify prestin protein expression compared to WT OHC's (Tong et al., 2016). Therefore, it appears the loss of *Ocm may* affect prestin expression. *Ocm* KO mice were created by cloning 17 kilobases of the *Ocm* gene (found on exons 2-4). A LoxP site was engineered on the 5' end of exon 2 and an Flp-neo-Flp-LoxP cassette eas engineered on the 3' end of exon 4 (Tong et al., 2016). The KO mouse construct was first created in C57/Bl6 mice and then transferred to CBA mice.

Colocalization of OCM and prestin

Because both prestin and OCM are localized in the periphery of OHCs, and a deletion of Ocm gives rise to accelerated hearing loss as well as OHC loss, it is possibile that prestin and Ocm may be interacting. To test this hypothesis, first we take high-

resolution confocal images of OHCs labeled for prestin and Ocm in wild-type (WT) and Ocm-knockout (KO) mice (mice that lack the Ocm protein). Second, we calculate colocalization coefficients and observe the morphology of prestin expression to assess the degree to which Ocm and prestin interact with one another. Because both prestin and Ocm play a monumental role in OHC function, it is paramount to study their potential interaction. Additionally, if prestin expression is modified/ by loss of *Ocm*, discovering a way to rescue prestin expression is crucial.

Salicylate

Previous studies have shown that short-term salicylate injections negatively affect hearing thresholds but are beneficial if given as long-term injections. The same studies have also shown that prestin mRNA and protein levels are up-regulated in response to chronic (i.e. long-term) salicylate injections (Yu et al., 2008). Assuming that this phenomenon occurs, we hypothesize that salicylate injections could potentially restore hearing to *Ocm* KO mice. This is because we hypothesize that OCM and prestin are interacting. A method that increases prestin expression could potentially be long-term salicylate injections. They could restore the function of prestin that remains in non-functioning *Ocm* KO OHCs. Theoretically, prestin up-regulation could serve as a compensatory mechanism for the loss of *Ocm*.

CHAPTER TWO

Materials and Methods

Animal use and preparation

All mice (Mus musculus) used in these studies were bred in-house. Transgenic mice (Ocm<-/->) strains were either on a mixed C57/Bl6 and 129/svJ genetic background or a CBA/CaJ background. Animals were euthanized with lethal intraperitoneal injections of sodium pentobarbital (Nembutal, 100 mg/kg). The day of birth (E19.5 – E20.5 for mice) represented postnatal day 0 (P0). All experimental procedures were approved by the Institutional Animal Care and Use Committee (IACUC) and conducted according to the guidelines for Animal Research at Baylor University, Waco, TX.

Embedding Medium for Tissue Sectioning

An agarose-gelatin mixture served as our tissue embedding medium. To make this medium, 0.5 g of Agarose and 10 mL of DI water are mixed in a 50 mL conical. In another 50 mL conical, 0.15 g Gelatin and 10 mL of saline are mixed. Both tubes are placed into a beaker of boiling water heated by a hot plate. The gelatin in the tube with saline will dissolve first. Once it does, vortex and centrifuge the tube. Now pour the saline-gelatin mixture into the other tube containing agarose and DI water. Heat the tube until the contents are dissolved. This usually takes around ten minutes. After the contents have dissolved, remove the tube from boiling water and let it cool for approximately 8-10 minutes. The agarose-gelatin embedding medium is necessary for preparing tissue before it is sectioned.

Immunocytochemistry protocol

Cochlear spiral tissue samples were collected via microdissection. The samples were stored in 1x Phosphate Buffered Saline (PBS) until they were ready to be used. The first step of the immunocytochemistry assay is to place the microdissected pieces into 30% sucrose (diluted with DI water or 1x PBS). They are stored in 30% sucrose for a minimum of 30 minutes. Usually, it took around 1 hour for the samples to fully become fully submerged. Soaking the samples in sucrose allowed the membrane of cells to become more permeable. This makes it easier for antibodies to penetrate and label the cells of interest. Sucrose shaking occurred on a Benchmark ORBI-Shaker™ JR. shaker at room temperature. After the tissue soaked in sucrose, it froze on dry ice for 15 minutes and then were thawed in a 37°C oven for 15 minutes. This process also made the cells more permeable for future antibody labeling. After the tissue froze and was thawed, it was washed with 1x PBS for a total of 10 minutes. The thawing process served as an additional step to enhance cell permeability to future antibody labeling. This procedure was performed in one 4 minute interval followed by two 3 minute intervals. PBS washing removed the sucrose used in a previous step. Washes were performed at room temperature and on a Benchmark ORBI-Shaker™ JR. shaker. The samples were blocked in a solution of 5% Normal Horse Serum and 0.3%Triton X-100 (5% NHS-T). The blocking step prevented nonspecific binding. In other words, the NHS-T blocked antibodies from binding to proteins that were not of interest. The tissues were blocked for a one hour period at room temperature on a Benchmark ORBI-Shaker™ JR. shaker. After blocking, the tissues were washed again with 1x PBS. They were washed in the same manner as the first PBS washing step with one 4 minute interval followed by two 3

minute intervals. This washing step removed the 5% NHS-T blocking solution added during the previous step. The primary antibodies were added after the second 1x PBS washing step. The specific antibodies used are listed below in the "Immunocytochemistry protocol for colocalization experiments section." Primary antibodies were diluted with 1% NHS-T solution. The specific dilutions used can be seen below in the "Immunocytochemistry protocol for colocalization experiments section." Tissues containing primary antibodies incubated overnight in a 37°C oven. After overnight incubation, samples were washed in 1x PBS for three 10 minute intervals on a Benchmark ORBI-Shaker™ JR. shaker at room temperature. Next, secondary antibodies were added (see below in the "Immunocytochemistry protocol for colocalization experiments section" for specifics). Secondary antibodies were diluted with 1% NHS-T. Tissues were incubated with secondary antibodies for one hour on a Benchmark ORBI-Shaker™ JR. shaker for 1 hour at room temperature. After secondary antibody incubation, the samples were washed with 1x PBS for three 10 minute intervals on a Benchmark ORBI-Shaker™ JR. shaker at room temperature. This is the final necessary PBS rinsing step. After this, the samples were mounted on 25x75x1.0 mm Fisherbrand© Precleaned Frosted Microscope Slides. This was accomplished by inserting the tissue samples into a small drop of VECTASHIELD Mounting Medium with DAPI (4′,6-diamidino-2-phenylindole). Fisherbrand© Microscope Cover Glasses (22x40-1.5 mm) were placed on top of the tissue samples submerged in mounting medium. The microscope slides dried with help from a Fisher Slide Warmer. Slides were warmed for 15 minutes or until excess mounting medium was dry. L.A. COLORS® Electron Microscopy Sciences Nail Polish was used to adhere the microscope cover slips to the microscope slides. The slides were

placed again on the slide warmer for 15 minutes or until the nail polish dried. Once mounted, the microscope slides were ready for imaging. For colocalization assays, there must be cochlear tissue pieces that act as a control. This means that the control cochlear samples are stained with either Ocm or prestin, while the other experimental pieces are colabled with Ocm and prestin. Additionally, other pieces are colabeled with Ocm and prestin. For control pieces designated for only Ocm labeling, the SWANT OMG 4 Goat (Gt) anti-Ocm primary antibody (1:2000 dilution). For pieces that are only to be labeled with prestin, they are labeled with Rabbit (Rb) anti-prestin (1:2000 dilution). Pieces designated for colabeling are treated with both antibodies. Dilutions of primary antibodies are made using a 1% NHS-T solution. To facilitate the antibodies in adhering to the tissue, they are left in a 37°C oven overnight. After washing with 1x PBS to remove the primary antibodies the next day, the secondary antibodies Northern Lights Donkey (Dk) anti-Gt 493 for Ocm (1:200 dilution) and Dk anti-Rb Biotin (1:200 dilution) for prestin can be applied. They will incubate in 37°C for one hour. Wash with 1x PBS to remove secondary antibody. To complete the immunocytochemistry assay, streptavidin 647 (1:200) needs to be applied to pieces that are designated for prestin labeling. Pieces are mounted with mounting-medium containing DAPI.

Perfusion

Animals were perfused/fixed with 4% Paraformaldehyde (PFA) prior to cochlear morphological experiments. PFA fixes the animal's tissue and makes it optimal for physiological studies. Prior to perfusion, animals were anesthetized and euthanized with lethal intraperitoneal injections of sodium pentobarbital (at a concentration of at least 100-150 mg/kg). After mice no longer had respiratory or tactile reflexes (confirmed by toe pinch) the mice underwent transcardial perfusion followed by decapitation. The chest cavity is not open until all vital signs have ceased.

Microdissection procedure

Prior to dissection, tissues were fixed in 4% paraformaldehyde overnight at room temperature and decalcified in 0.1 M Ethylenediaminetetraacetic acid (EDTA) for 2 days at room temperature. The following tools were used for microdissections: Fine Science Tools Dumont Forceps, Fine Science Tools Fine Forceps, Fine Science Tools Vannas Spring Scissors, Fine Science Tools Blade Holder and Breaker – Flat Jaws, and Fine Science Tools Scalpel Blades – Breakable. Microdissections were performed in a 35x10 mm tissue culture dish. The medium used for dissection was 1x PBS. Before bisection of the cochlea, the brain-side bulge was removed by standing the temporal bone on its vestibular end. The bulge was removed using the blade breaker and scalpel blade. Cochleae were bisected through the oval window. Bisection of the cochlea results in two cochlear halves: an apical half and a basal half. Either half was chosen and non-cochlear tissue was removed using spring scissors. Next, the cochlear capsule was removed using spring scissors. Cut modiolar connections between cochlear turns using spring scissors.

When working on the apical half, the tectorial membrane must be removed at this point. Not doing so blocks your view of some outer hair cells (OHCs). The tectorial membrane can be removed using fine forceps. After removal of the tectorial membrane, peripheral tissue was cut off to further expose cochlear turns. Peripheral tissue was removed with both the blade breaker and scalpel blade and spring scissors. Microdissection of the first cochlear half is almost complete. The final step is to trim off extra-cochlear tissue above the OHCs and below the limbus. If done with extreme caution, extra-cochlear tissue that could obscure view of OHCs will be removed. The same procedure was performed on the remaining cochlear half.

Immunocytochemistry procedure for microdissected samples

The same steps are used for immunocytochemistry assays. The difference is that the procedures are performed in a 1.5 mL Eppendorf tube apparatus instead of in a 32 well plate. The apparatus was created by removing the top off of an Eppendorf tube. The next step is to tape the tube cap to a petri dish lid. Samples, including the medium they are submersed in (e.g., 30% sucrose, 1x PBS, or antibody solutions). To seal the sample and medium from the outside environment, the tube is pushed back onto the cap. The maximum volume of fluid the Eppendorf tube caps can hold is approximately 100 μL.

Imaging and colocalization

One method of measuring the interaction between two proteins is through colocalization assays. Simply put, colocalization occurs when two molecules associate with the same structure (McDonald et al., 2011). Colocalization coefficents were

calculated to assess the degree to which prestin and Ocm interact. A ZEISS confocal microscope (LSM800 with Airyscan) was used to capture images (63x Plan Apo objective) of prestin and Ocm labeling. The same settings were used to capture prestin and Ocm co-labeling, Ocm-only labeling, and prestin-only labeling. Once the images were acquired, colocalization coefficents were determined representing the interaction between Ocm and prestin signals. Image analysis was done with Zeiss Zen software. We decided to use Pearson's Colocalization Coefficient because its measurements are independent of signal background (the same cannot be said for Mander's Overlap Coefficient values). Pearson Colocalization coefficients were used to gauge the degree to which prestin and Ocm interact.

$$MOC = \frac{\sum_i (R_i \times G_i)}{\sqrt{\sum_i R_i^2 \times \sum_i G_i^2}}$$

$$PCC = \frac{\sum_i (R_i - \bar{R}) \times (G_i - \bar{G})}{\sqrt{\sum_i (R_i - \bar{R})^2 \times \sum_i (G_i - \bar{G})^2}}$$

ZEN software calculates two colocalization coefficents: the Pearson Colocalization Coefficent (PCC) and Manders Overlap Coefficent (MOC). PCC can have values ranging from -1 to +1 and measures the pixel-by-pixel covariance in the signal levels of two images. MOC can have values ranging from 0 to 1 and measures the degree to which pixels of two molecules overlap with one another (McDonald et al., 2011). Since PCC is independent of signal background, we chose to use PCC as a measure of colocalization.

Quantifying prestin circulation

We used ImageJ FIJI Analysis software to quantify prestin circularity. 63x confocal images labeled only for prestin were subject to analysis. Only outer hair cells (OHCs) that contained nuclei (indicated by DAPI staining) were measured for prestin circularity. Circles were drawn around the periphery of OHCs (labeled by prestin) using the "freehand selections" tool on ImageJ. After the circles were drawn, the software measured a circularity value; circularity values range from 0-1. The closer the circularity value is to 1, the more circular the prestin expression, and vice versa. The more circular the prestin expression, the closer the OHC is to being perfectly circular. Around 30 regions of interest (ROIs) were drawn per sample (i.e., for one cochlea, around 30 OHCs had their circularity measured). The OHCs chosen to be measured were ones where it was possible to draw a complete circle around them. This indicated prestin must have been seen around the entire periphery of the OHC. This was done to eliminate the possibility of error caused by drawing an ROI around parts of an OHC where prestin is not visible.

Another way of quantifying prestin expression was by measuring how much OHCs deviated from a perfect circle. We used ImageJ Fiji software to do this. First, we drew a perfect circle around each individual OHC. Next, we used the "freehand line" tool to measure how much each OHC deviated from a perfect circle. We did this by tracing the part of the OHCs that deviated with the freehand line tool. FIJI gave us a length with this measurement. The longer the length measured, the more the OHC deviated from a perfect circle; and thus, the more irregularly shaped it was. We measured OHC/prestin

circularity for CBA/CaJ WT and KO mice and for C57 mice. These measurements were performed in the 32 kHz region of the cochlea.

Counting Irregular OHCs

Another method used to quantify the prevalence of irregular OHCs was calculated by the number of collapsed OHCs within the 8 kHz and 32 kHz region for both CBA/CaJ WT and KO mice. OHCs were counted using prestin labeling. An OHC was considered collapsed if it had prestin labeling that indicated that the OHC was folding in on itself or appeared to have a concave or convex shape.

Frequency Mapping

To validate the frequency regions of our cochlea samples, we created frequency maps using ImageJ. The measure line plugin allows users to trace cochlear sections where OHCs are (or should be). The plugin then takes the traced distance and determines the approximate frequency region of the cochlea. 10x confocal microscopy images were used to make frequency maps. Before the frequency map was made, all images of microdissected pieces must be merged into a single montage. This can be accomplished by turning microdissected piece images into a stack and then turning that into a montage. To do this, image > stacks > images to stack; then, image > stacks > make montage. OHCs from each microdissected piece were traced using the segmented measure line tool. OHCs were traced from the base of the cochlea (higher frequency region) to the apex of the cochlea (lower frequency region). Frequency maps were created to show

observers the location of the 8 and 32 kHz regions. This allowed us to know where to image.

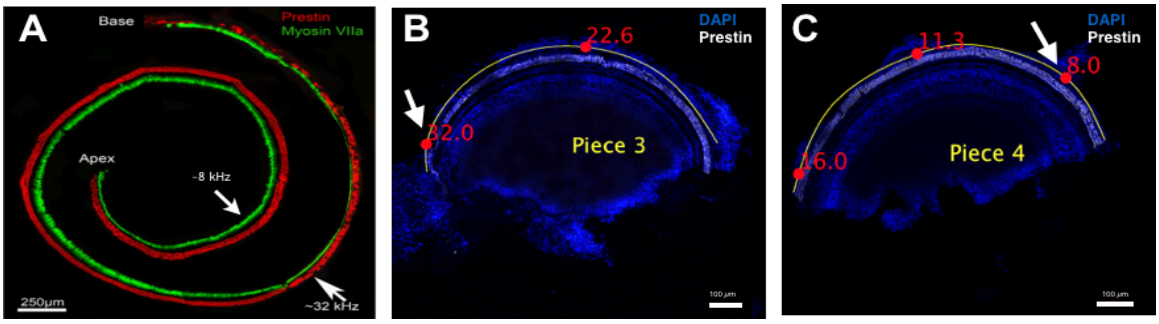

Figure 1. The full spiral of a mouse cochlea is shown (*A, from Zheng et al., 2010*). In this study, we focused our work on 32-kHz and 8-kHz regions as show by arrows (1A). The cochlear spiral was micro dissected and the 32-kHz (1B) and 8-kHz (1C) were separately processed and viewed.

Vibratome sectioning

The vibratome 3000 was used to section tissue. Obtained sections were 100 microns thick. Prior to sectioning, cochlear tissue is embedded in an agarose-gelatin mixture (instructions to make are written above). The cochlear tissue is embedded to where the cochlear spiral is facing upward.

Distortion Product Otoacoustic Emission Thresholds (DPOAE)

DPOAE tests evaluated animal hearing. A DPOAE is the only in-vivo measurement of OHC function. Prior to DPOAE testing, animals are anesthetized with a 80 mg/kg ketamine, 15 mg/kg xylazine mixture via intraperitoneal injection. ¼ supplementary doses are given, if needed. To measure DPOAE's, the ear canal must be visualized. This is done by making a 1 cm incision inferiorly between the cartilage of the tragus and anti-tragus. If bleeding occurs, it can be stopped by applying pressure with a

cotton applicator. After the surgery, the wound is swabbed with a topical analgesic, such as lidocaine, and alcohol. Before testing begins, an antibiotic ointment is applied to the eyes of the anesthetized animal to prevent them from drying out. Ear tubes containing mini speakers and a microphone are inserted into the ear canal of the anesthetized animal. Measurements are obtained by presenting two primary frequencies (f1 and f2) with an f1:f2 ratio of approximately 1.22. Typically, sound levels of the tones are varied by 5 dB steps from 55 to 85 dB SPL where f2 is 10 dB below the level of f1. Test frequencies range between 5 and 45 kHz. Input/output curves are obtained and compared for all subjects.

Salicylate protocol

C57BL/6 mice on an alpha parvalbumin (aPV) background aged 8-12 weeks were used for this experiment. First, mice had their DPOAE thresholds measured before salicylate injections. After the pre-injection DPOAE, mice were intraperitoneally injected with 200 mg/ml sodium salicylate saline solution (Sigma-Aldrich Sodium Salicylate, CAS: 54-21-7). One hour after the injection, the mice had their DPOAE's tested again and were allowed to recover. Recovery consisted of housing the mice in cages inside of a heated sound chamber. 24 hours later, mice had their DPOAE's measured again. This procedure was done on WT and Ocm KO mice. Saline-only injections served as a vehicle control for salicylate injections. Five aPV WT mice were tested; of those five, 2 were given a saline-only injection and the other 3 received salicylate injections. Seven aPV WT mice were tested; of those seven, 2 were given a saline-only injection and the other 5 received salicylate injections.

Salicylate analysis

The effect of salicylate injections on hearing was investigated by measuring DPOAE thresholds and magnitude. The EPL Cochlear Function Test Suite application was used to measure DPOAE data. We graphed DPOAE data comparing WT and Ocm KO mice and vehicle and salicylate injections. Frequency (kHz) served as our independent variable and DPOAE threshold (dB SPL) and magnitude (dB SPL) served as our dependent variables. DPOAE threshold data was categorized based on if an Ocm WT mouse or Ocm KO mouse was injected. Next, the data was further categorized based on the type of injection received (vehicle or salicylate). Scatterplots were made to see if DPOAE thresholds changed pre-injection, 1-hour post-injection, and after a 24-hour recovery period. Error bars represent standard error mean (SEM).

CHAPTER THREE

Results

The loss of Ocm contributes to progressive hearing loss

DPOAEs are the only in-vivo measurement of OHC function. Because OCM is found exclusively in OHCs, DPOAEs are ideal for measuring the effect an absence of *Ocm* has on OHC function. The function of OHCs can be measured with DPOAE threshold shifts. A distortion product (DP) threshold is the minimum sound stimulus needed to elicit a hearing response. Therefore, if a mouse has a low DP threshold, then it does not take an intense sound stimulus for the mouse to hear, suggesting that they can hear well. On the other hand, if a mouse has a high DP threshold, it takes a more intense sound stimulus for them to hear, suggesting that something could be wrong with their hearing. Figure 2 shows typical DPOAE threshold measurements for CBA/CaJ *Ocm* KO mice at ages of 5-8 weeks, 10-13 weeks and 16-31 weeks (2A) and WT mice at ages of 7-8 weeks, 9-10 weeks and 18-31 weeks (2B). *Ocm* KO mice ages 5-8 and 10-13 weeks have nearly identical DP thresholds in response to low frequency sound stimuli (because the blue and red lines are overlapping). 10-13 week-old *Ocm* KO mice have thresholds around 10 dB higher than 5-8 week old *Ocm* KO mice in high frequency regions. *Ocm* KO mice aged 16-31 weeks have much higher DP thresholds than the 5-8 and 10-13 week old mice (2A). This suggests that the absence of *Ocm* causes progressive hearing loss with onset at around 16 weeks (Tong et al., 2016). At ages ranging from 7-31 weeks old, *Ocm* WT mice had similar DP thresholds to *Ocm* KO mice of roughly the same age. The large

increase in hearing threshold does not occur with WT mice when they age beyond 16 weeks as was seen in the *Ocm* KO mice (2B).

High-Resolution Imaging Reveals Overlap between Prestin and Ocm in OHCs

To begin our investigation of whether OCM and prestin interact, we took high-resolution airyscan confocal images of OHCs immunostained for both proteins. When prestin protein expression (Fig. 3B) is overlayed with OCM protein expression (Fig. 3C), both proteins appear to be localized in the OHC periphery (Fig. 3D). This figure shows that OCM and prestin protein are expressed in similar OHC regions. Prestin is localized in the periphery of OHCs and OCM is in close proximity. Because the two proteins appear to be localized in similar areas of OHCs, it suggests that they could be interacting with one another. High-resolution images were taken in the 8 and 32 kHz cochlear regions. These particular frequency regions were chosen because we feel that they are the best representation of low frequency and high frequency regions, respectively. It is important to study OHCs in both high and low frequency regions because they are uniquely damaged. OHCs in the basilar region of the cochlea, where high frequency regions are located, are more susceptible to damage. This explains why as people age, they usually lose their ability to hear high frequency sounds before low frequency sounds.

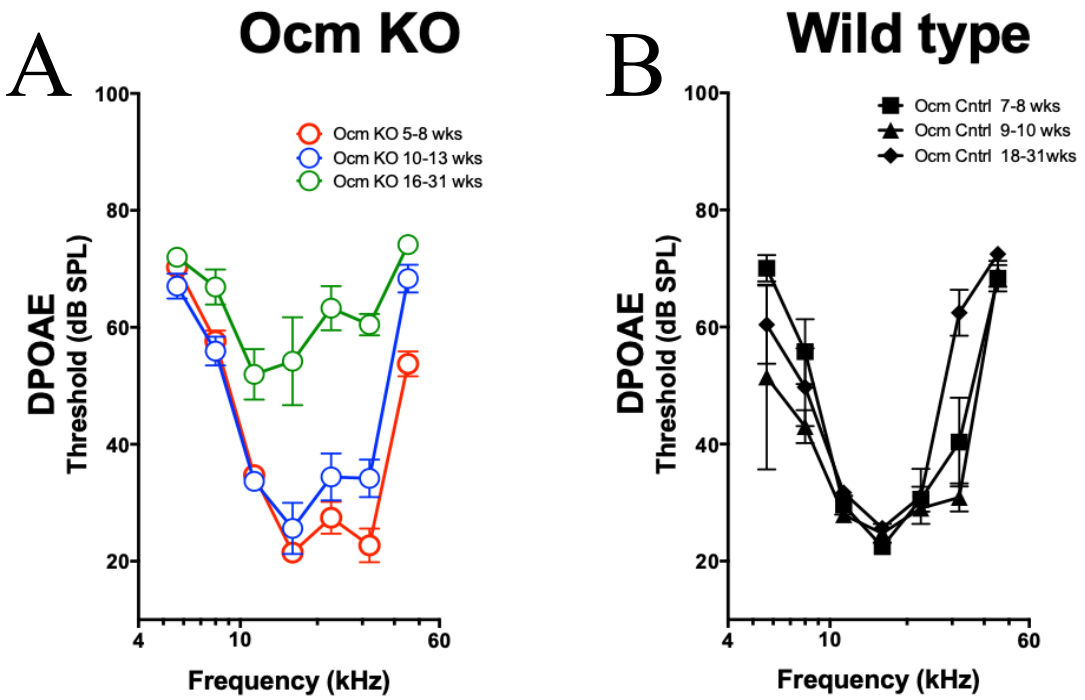

Figure 2. Typical DPOAE threshold measurements for *Ocm* KO mice at ages of 5-8 weeks, 10-13 weeks and 16-31 weeks (2A). Typical DPOAE threshold measurements for WT mice at ages of 7-8 weeks, 9-10 weeks and 18-31 weeks (2B). DP thresholds are shown from a stimulus range of approximately 4 kHz to 60 kHz. The frequencies of sound stimuli given were 6, 8, 11, 16, 23, 32, 36, and 45 kHz. This is a good reference to compare DPOAE's in response to salicylate injections that are shown in Figure 10.

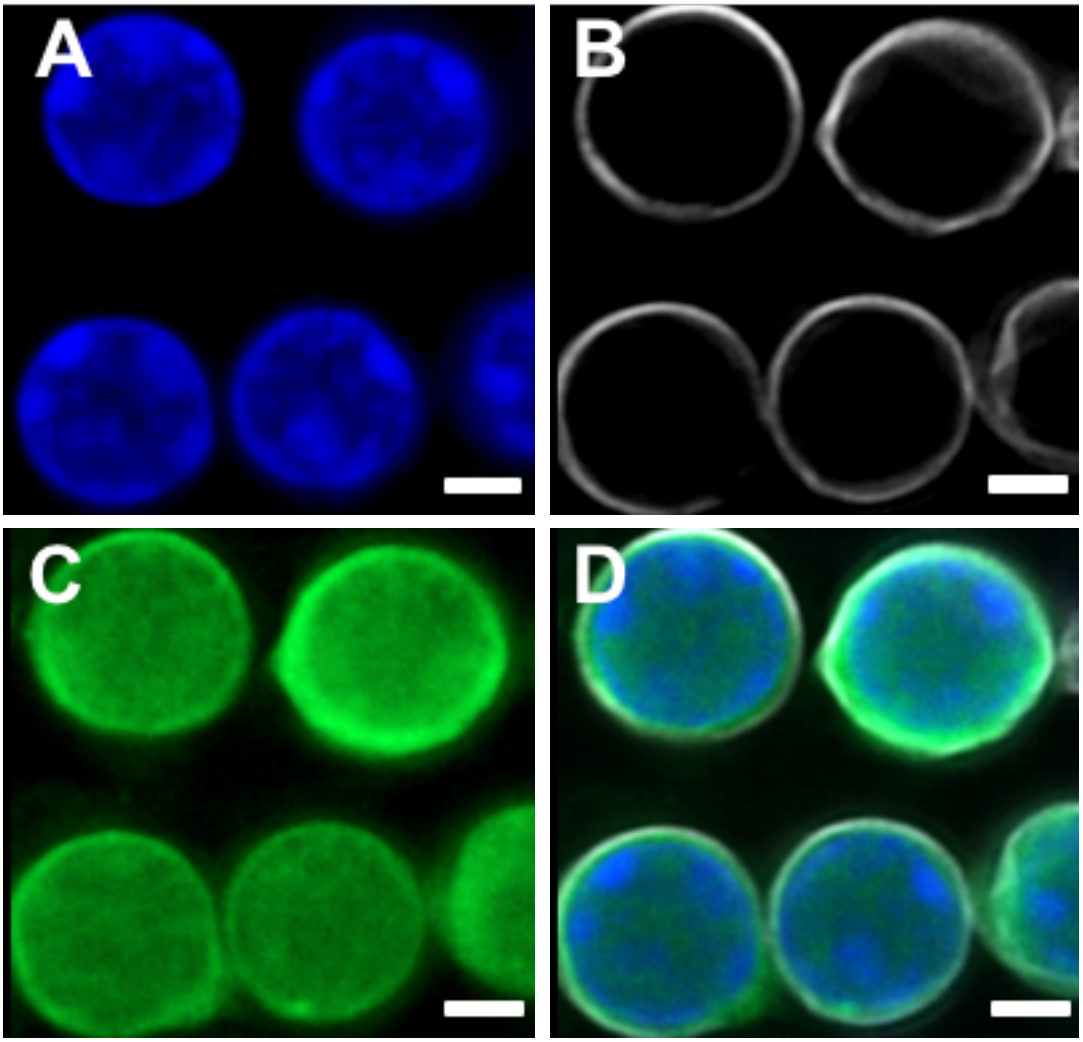

Figure 3. High resolution confocal Airyscan images of cochlear OHCs in 8 kHz region. Airyscan images are near super resolution (140 nm resolution) and serve as a useful tool to visualize molecular interactions. DAPI-stained nuclei (A), prestin-labeled OHC membranes (B), Ocm labeling within OHC cytoplasm and membrane (C), and a composite image (D) are shown. Due to the apparent overlap of prestin (gray) and Ocm (green), we hypothesize that the two proteins have overlapping distributions in OHCs. The scale bar represents 2 μm.

Ocm and Prestin Appear to Colocalize in Cochlear OHCs

To further test whether OCM and prestin protein are interacting, we used ZEN software to test for colocalization between the two proteins.We calculated the Pearson Colocalization Coefficent (PCC) for CBA/CaJ *Ocm* WT mice (Fig. 4A), C57BL/6 OCM WT mice (Fig. 4B) and CBA/CaJ *Ocm* KO mice (Fig. 4C). PCC values give an indication to the amount of interaction occurring between two proteins. Therefore, the higher the value, the more two proteins are interacting, theoretically. CBA *Ocm* WT mice had a significantly higher PCC value than both C57 WT mice and CBA *Ocm* mice (Fig. 4D).

Morphology of Prestin Expression is not Uniform in OHCs

While testing for colocalization between OCM and prestin, we noticed that some CBA WT OHCs had abnormal prestin expression (Fig. 5A, 5C). Normal prestin protein expression is characterized by circular cellular profiles that resemble a circle around the periphery of OHCs. In other words, prestin appears as an outline of each OHC (Fig. 5B). Therefore, abnormal prestin protein expression is characterized by anything that deviates from this pattern. Some OHCs had prestin expression where it looked like the OHC was collapsing in on itself.

Differences in Prestin Expression between CBA, C57 and Ocm KO at 3 months in 32-kHz Region

Because we saw some OHCs with abnormal prestin expression in CBA WT mice, we wondered if this phenotype occurred more frequently in C57 WT and CBA *Ocm* KO mice. We hypothesized that C57 WT OHCs would have more OHCs with abnormal

prestin expression and that CBA *Ocm* KO OHCs would have that greatest amount of OHCs with abnormal prestin expression. Upon further investigation, we observed that CBA WT OHCs typically had circular prestin expression patterns that you would expect (Fig. 6A). C57 WT mice had slightly more irregular prestin expression compared to CBA WT mice (Fig. 6B). CBA *Ocm* KO mice had the least regular prestin protein expression of the genotypes tested. OHCs with prestin expression suggesting that the cell is collapsing in on itself are indicated by arrows (Fig. 6C).

Circularity of Prestin expression Deviates among Mouse Strains and Genotype

After observing that C57 WT mice and CBA *Ocm* KO mice appear to have less circular prestin expression, we wanted to quantify this observation. We did this by calculating the average "circularity deviation index" for each mouse genotype. This was done by capturing 63x confocal images of OHCs immunostained for prestin protein. Next, ImageJ software was used to determine how much the prestin expression deviated from a perfect circular. Theoretically, the more prestin protein expression deviated from a perfect circular, the more irregular the protein expression was. We hypothesized that CBA *Ocm* KO mice would have the highest circularity deviation index because of the loss of *Ocm.* If OCM and prestin are interacting as we hypothesize, then the loss of *Ocm* should make prestin expression more irregular and thus increase its circularity deviation index value. CBA *Ocm* KO and C57 WT mice had a significantly higher circularity deviation index than CBA WT mice.

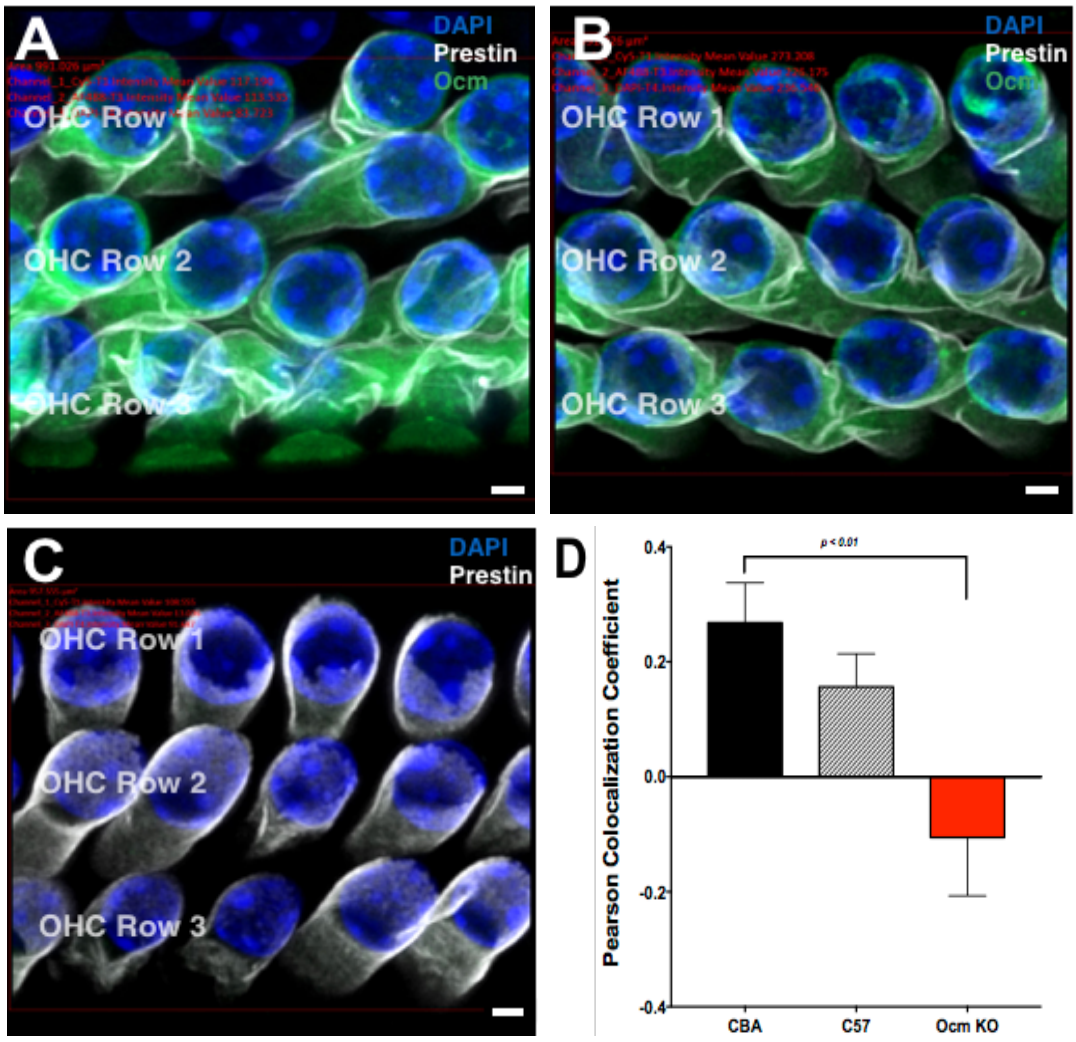

Figure 4. High resolution Airyscan images of OHCs are shown for 3 month old CBA (A), C57 (B), and Ocm KO (C). All images were taken in the 32 kHz region of the cochlea. PCC values were calculated for similar size ROIs containing OHCs. The bar chart (D) shows the mean (±SEM) PCC values for CBA and Ocm KO (n=3) and C57 (n=2) at 3 months. The scale bar represents 2 µm.

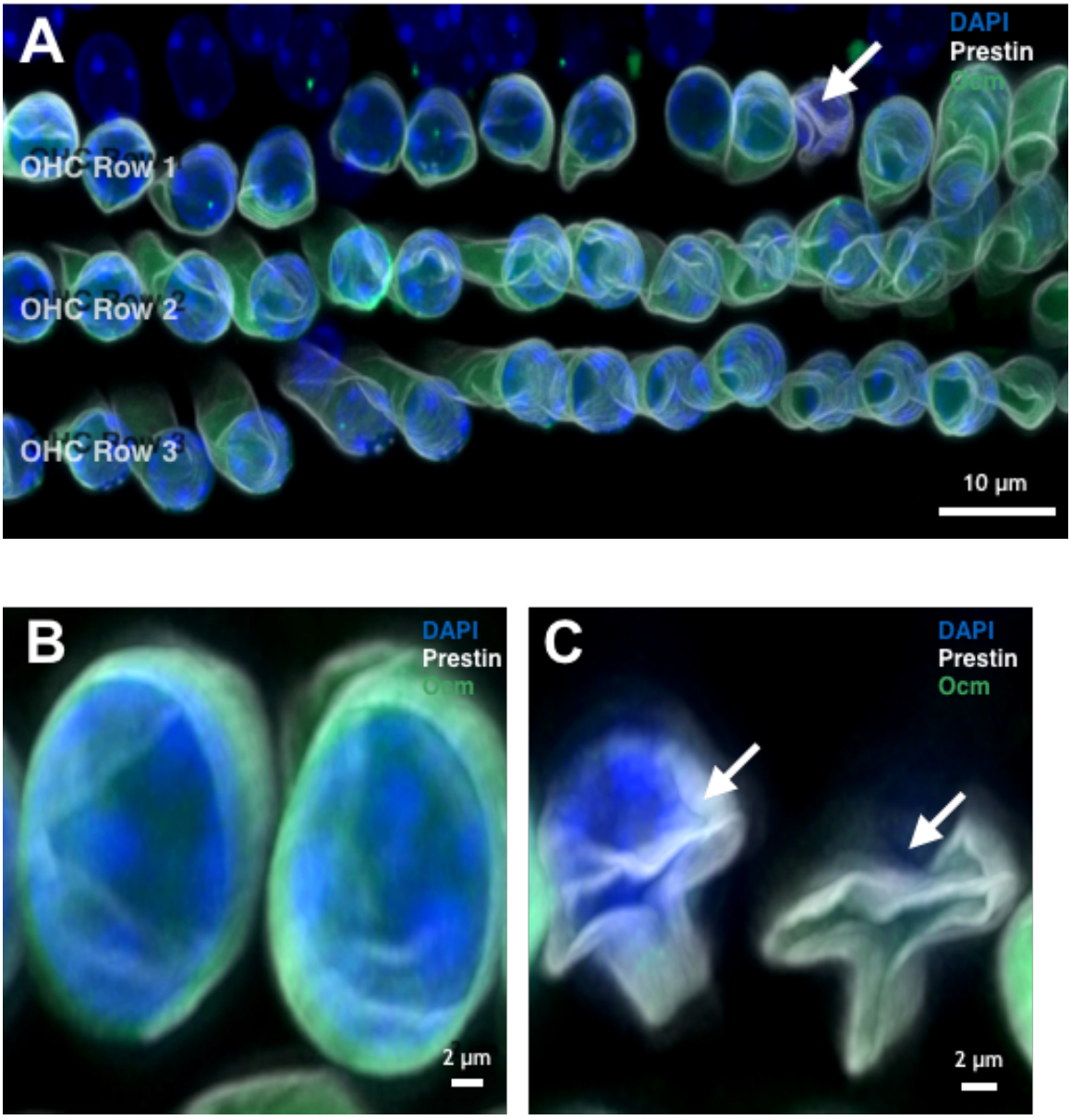

Figure 5. High magnification images of cochlear OHCs from a 3-month-old CBA WT mouse. All OHCs appear to express prestin normally except for the one emphasized by the arrow (A). An Airyscan image of two OHCs that express normal prestin and Ocm (B). An Airyscan image of two OHCs that show abnormal prestin morphology and diminished Ocm expression is shown in (C). This occurrence in normal CBA raise several questions about the potential interaction between prestin and Ocm. These images were captured in the 8-kHz region.

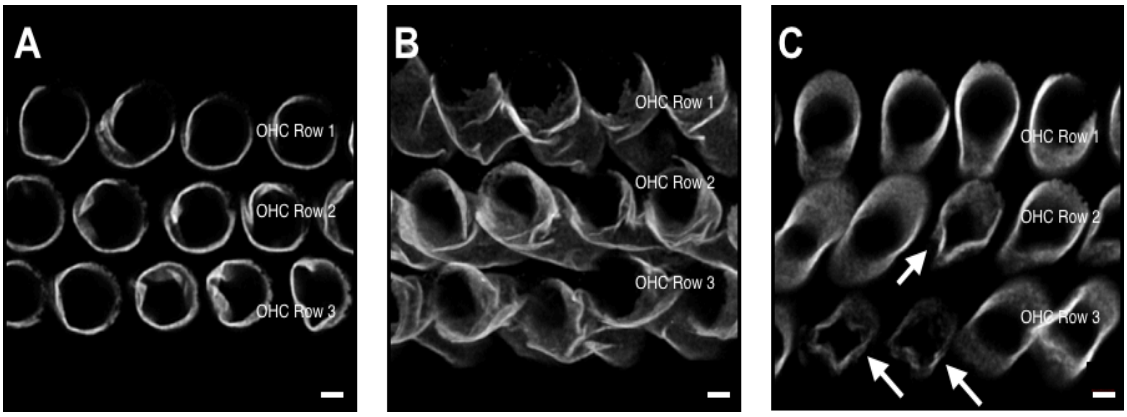

Figure 6. Airyscan images of 3-month-old cochlear OHCs expressing prestin from CBA (A), C57 (B), and Ocm KO (C) mice are shown. Prestin morphology changes across strains and genotype. CBA mice have mostly circular prestin profiles whereas Ocm KO mice have the least circular. Arrows indicate OHCs with irregular prestin shapes. All images were taken in the 32 kHz region. Scale bar represents 2 µm.

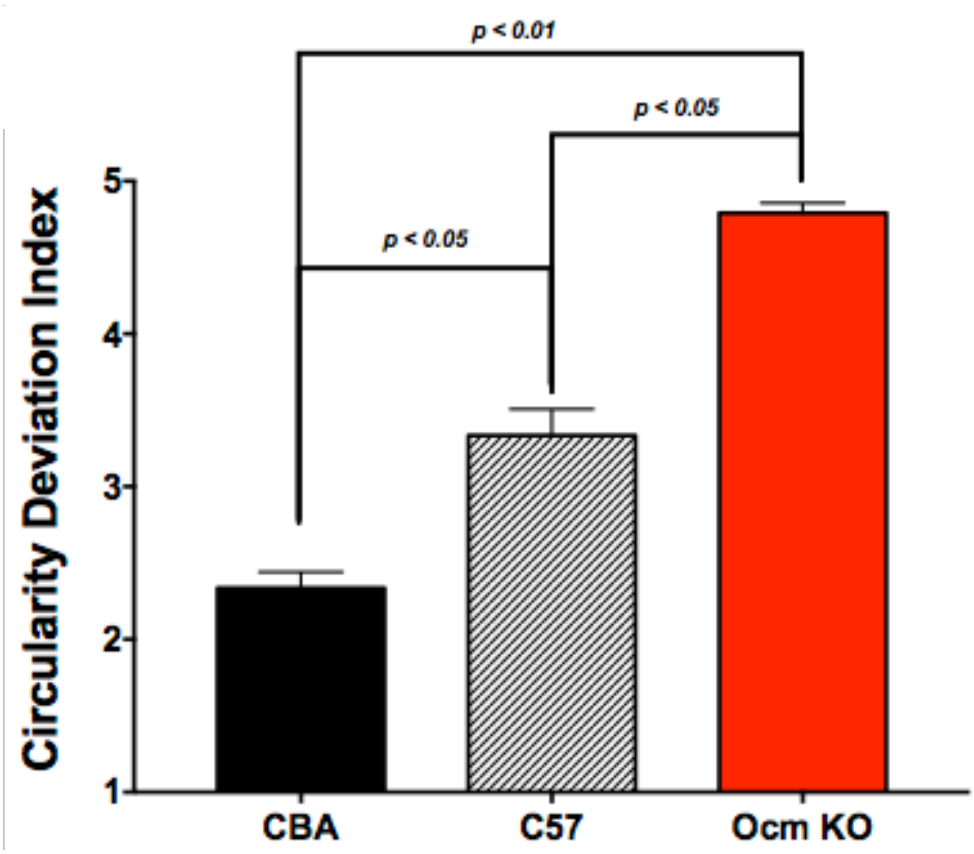

Figure 7. Bar plot quantifies the circularity of OHCs among CBA, C57 and Ocm KO mice at 32-kHz. Irregular OHC morphology deviates from a perfect circle. Deviation from circularity among OHCs was measured using ImageJ. OHCs in Ocm KO mice (red bar) were least circular, on average. n=2 for each independent variable. Error bar represents 1 SEM.

Collapsed OHCs increase with Frequency in Ocm KO mice

Another way to test how the loss of *Ocm* affected prestin expression was by measuring the number of collapsed OHCs in mice. We investigated this phenotype because of our previous observations of seeing OHCs that appeared to be collapsing in on themselves. We hypothesized that the loss of *Ocm* altered prestin protein expression in a way that compromised the cytoskeletal structure of the affected OHCs; thus, allowing them to collapse structurally. Collapsed OHCs were defined as ones with abnormal prestin expression such as the ones seen in Fig. 5C. At 3 months of age, CBA *Ocm* KO mice had a significantly higher percentage of collapsed OHCs than CBA WT mice (Fig. 8). This held true in both the apex (8 kHz region) and base (32 kHz region) of the cochlea. This suggests that the damage of *Ocm* absence is severe enough to impact the whole cochlea instead of only the basilar region.

The Effect of Salicylate on Prestin Expression in the Absence of Ocm

To investigate whether salicylate could act as a compensatory mechanism for the loss of *Ocm,* we immunostained cochleae of mice prior to salicylate injections. Saline was used as a vehicle (Veh) control because it was used as the medium to make salicylate solutions. C57BL/6 WT mice were either injected with saline or salicylate. Also, the same was done for C57BL/6 *Ocm* KO mice. In addition to immunostaining, we also used DPOAEs to assess how hearing was affected by salicylate injections.

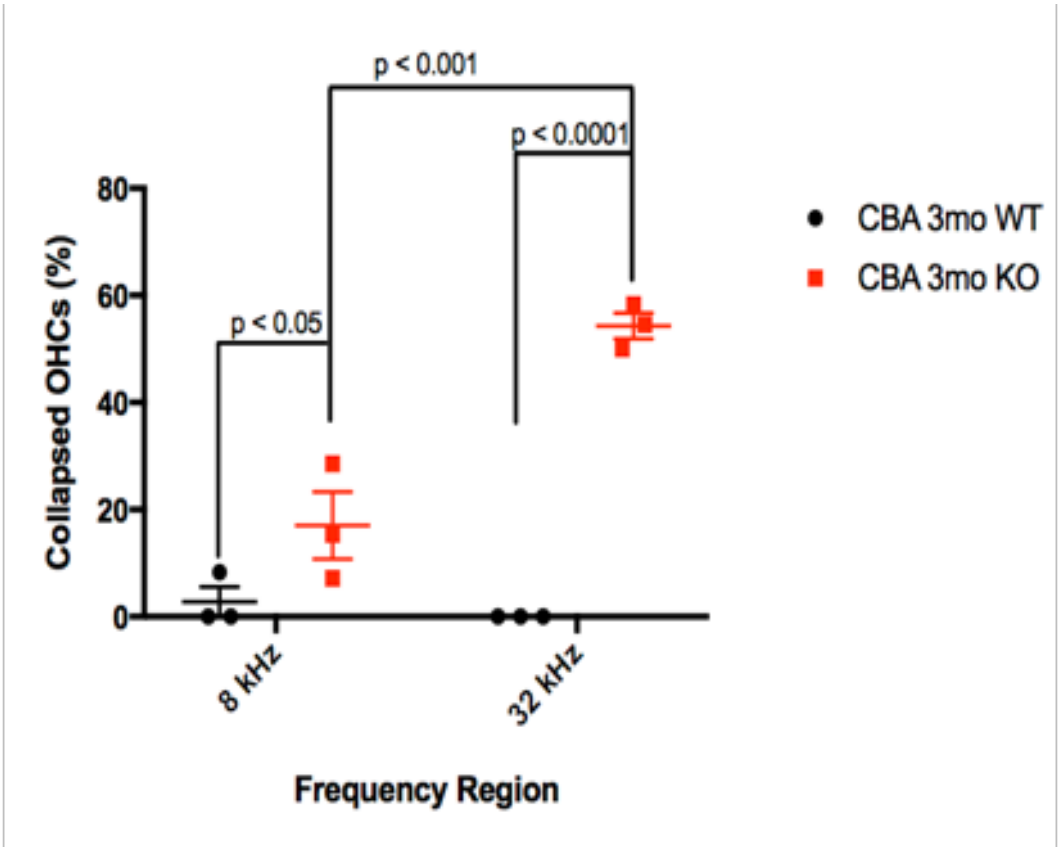

Figure 8. Collapsed OHCs were counted using prestin staining. Dot plot shows percentage of collapsed OHCs (±SEM) in the 8 and 32-kHz region for WT (n=3) and KO mice (n=3) (A). High frequency regions in KO mice have more significantly collapsed OHCs than low frequency regions. However, both frequency regions of KO are significantly different than the WT (high frequency region has more significant difference).

Mice had their DPOAE thresholds recorded prior to injection, after injection and after a 24 hour recovery period. This procedure was performed for both C57 BL/6 WT and *Ocm* KO mice. Once again, Saline was used as the vehicle control for salicylate injections.

A previous study showed that prestin protein levels increased after chronic salicylate injections and decreased following acute salicylate injections (Yu et al., 2008). We used immunohistochemistry and confocal microscopy to see if this same phenomenon occurred in *Ocm KO* mice. Images were taken with the same optical parameters. This enables us to compare prestin shape and intensity fairly among different variables. Normal prestin expression is characterized by a round circle surrounding the membrane of outer hair cells. Abnormal prestin expression is shown by cells with irregular shapes instead of round circles. For example, an outer hair cell with regular prestin expression might look like a four leafed clover or a dinosaur footprint. Abnormal prestin expression such as this suggests that the hair cell is collapsing in on itself. There does not appear to be a difference in the prestin expression and intensity in WT mice injected with salicylate or WT mice injected with a saline (Veh) control. Prestin expression after saline injection is shown in panel A and prestin expression after salicylate injection is shown in panel B. Both rows of hair cells appear to have regular prestin expression around the membrane of the hair cells and the prestin protein intensity appears to be similar between the two as well. *Ocm* knockout mice appear to have increased prestin expression intensity after saline injection compared to the WT mice injected with saline (9C). *Ocm KO* mice were missing hair cells and the hair cells that are present have irregular prestin expression (9D). For example, the bottom row of outer hair cells in 9D look like rectangles instead of round circles. It is interesting that salicylate injected *Ocm* KO have more OHCs

missing compared to Saline control *Ocm* KO mice, suggesting that salicylate is more effective in killing OHCs in *Ocm* KO mice. The intensity of prestin protein expression appears greater in saline-injected *Ocm* KO mice (9C) than in WT saline-injected mice (9A). OHC shape in *Ocm* KO mice appears most irregular after salicylate injection, as indicated by the irregular shaped OHCs in 9D. This is interesting because the salicylate injections might have caused the outer hair cell prestin shape to become more irregular. Our data offer different conclusions than previous studies (Yu et al., 2008). Previously, it was shown that single, acute salicylate injections decreased prestin protein expression and intensity but our data suggest that it did not have a major effect on the prestin expression intensity. We made this conclusion based on the observation that prestin intensity did not appear to change when mice were injected with salicylate. This held true for both WT and *Ocm* KO mice (i.e. prestin intensity does not change from 9A to 9B or from 9C to 9D). Using confocal images is not a fool-proof method for observing whether certain conditions change protein expression. For example, the protein expression might appear more intense as indicated by a brighter color but this does not necessarily mean that the protein expression is actually increasing. Further tests need to be done such as western blots to determine if the amount of protein is actually changing after salicylate injections. Due to time constraints, we did not have the opportunity to do these tests or investigate the effect of chronic salicylate injections on prestin protein expression in *Ocm* knockout mice. This would be interesting to look at in future studies.

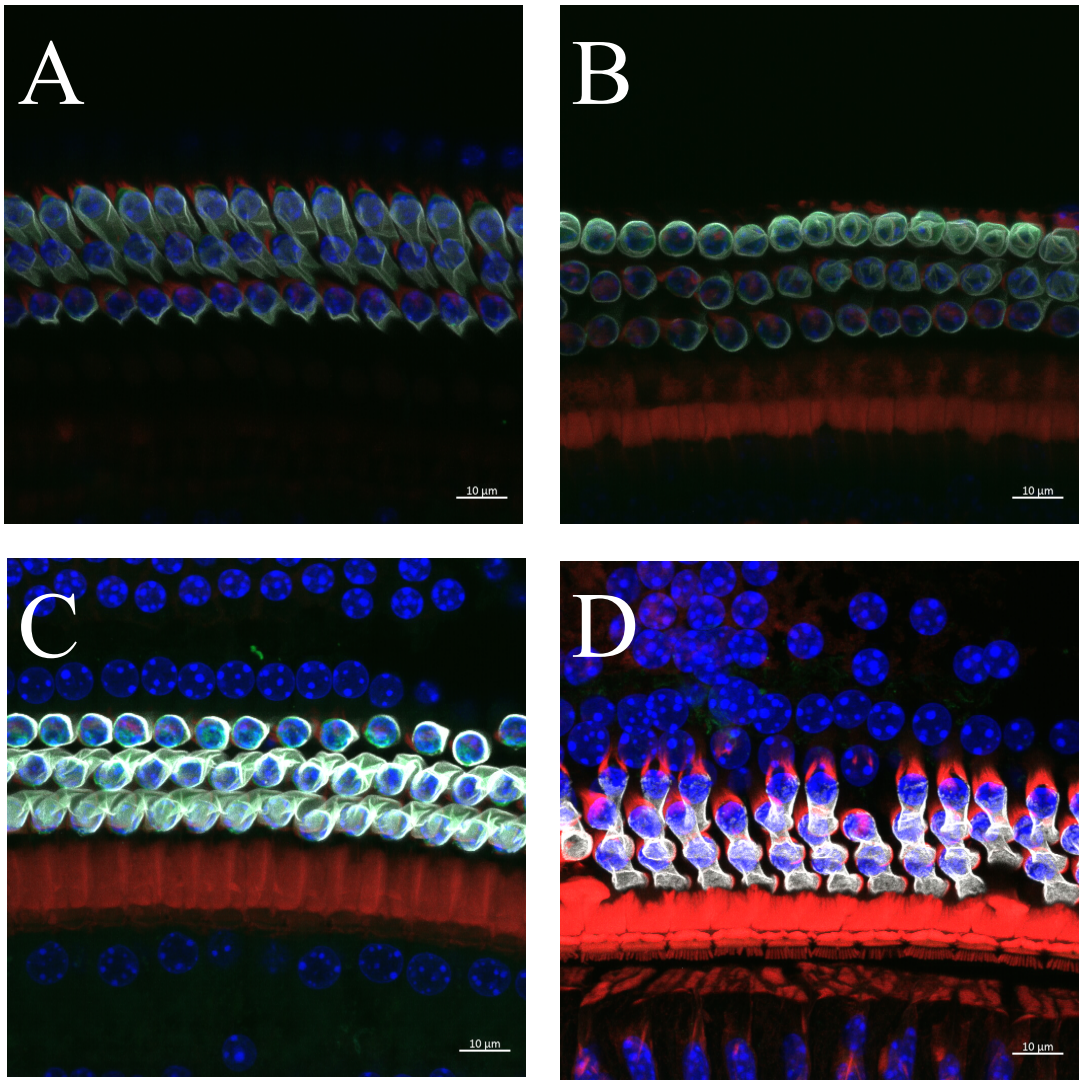

Figure 9. A) WT Vehicle (Veh, only saline injection) 3 month old mouse. Green fluorescence is OCM, blue is DAPI, and far red is prestin. B) WT Salicylate (Sal) injection 3 month old mouse. C) Ocm KO Veh injection 3 month old mouse. D) Ocm KO mouse Sal injection 4 month old mouse. Images were taken with the same optical parameters.

The Effect of Salicylate DPOAE's in the Absence of Ocm

Figure 10 shows the typical DPOAE threshold curve for a WT C57 BL/6 mouse at 9 weeks (triangle) and 15 weeks of age (circle). The y-axis is the hearing threshold, or the minimum sound intensity needed to elicit a cochlear response. The x-axis (labeled "F2") is the frequency of the sound stimulus given to the mouse. The frequencies of sound stimuli given were 6, 8, 11, 16, 23, 32, 36, and 45 kHz. This figure is important because it shows the natural increase in hearing thresholds as C57 BL/6 mice age. From 9 to 15 weeks of age, C57 BL/6 mice have an increased hearing threshold of approximately 20 dB across all frequency regions, a large detriment to their hearing. It shows that aging can be a major factor in the quality of hearing in this strain of mice.

Prestin Expression Changes with Age

The absence of *Ocm* does not appear to be the only factor affecting prestin protein shape. Aging seems to have the same effect on prestin protein shape that removing *Ocm* does. Figure 11A shows OHCs in the 32 kHz region from a 3 month old C57 BL/6 mouse. At this age, prestin expression still appears circular, suggesting that it is functioning normally. Figure 11B also shows OHCs from the 32 kHz region, but from a 6 month old C57 BL/6 mouse. Notice that prestin expression is no longer circular. Instead, some OHCs have rectangular-shaped prestin and others look shriveled and/or collapsed. This change is not only seen in the 32 kHz region, but also the 8 kHz region. Figure 11 C shows normal circular prestin protein expression from a 3 month old C57 BL/6 mouse in the 8 kHz region. Figure 11 D shows OHCs from a 6 month old C57 BL/6 mouse in the 8 kHz region. As seen in the 32 kHz region, the OHCs are not as circular as the ones from

the 3 month old mice; they appear oblong and oval. Just as how age appeared to modify prestin protein expression in the 32 kHz region, it seemed to have the same effect in the 8 kHz region as well.

A previous study showed that the hearing of WT mice (increased hearing threshold) is worsened for a 1-2 hour period when given a single "acute" injection of salicylate. This effect was reversible because the hearing returns to normal after approximately 7 hours (Yu et al., 2008). However, when mice were given daily salicylate injections (two injections per day for a two-week period), their hearing threshold was decreased compared to their hearing thresholds prior to salicylate injection. For wild type saline control (Veh) injected mice, there was not a noticeable difference in the hearing thresholds prior to injection and after a 24-hour recovery period except for in the 16 kHz (12A). In the low frequency regions (from 6 kHz to 16 kHz), the wild type salicylate-injected mice had similar DP thresholds prior to injection and after a 24-hour recovery period (12B). However, there was a noticeable difference in the DP thresholds in high frequency cochlear regions (from 16 kHz to 45 kHz). In the 23, 32, 36 and 45 kHz regions, the difference in DP threshold prior to injection and after a 24-hour recovery period was approximately 20, 15, 20, and 20 decibels, respectively. The saline-injected *Ocm* KO control DP thresholds showed a similar pattern to the wild type vehicle control DP thresholds. The recovery DP thresholds are similar to the pre-injection measurements except for in the 16 kHz region (12C).

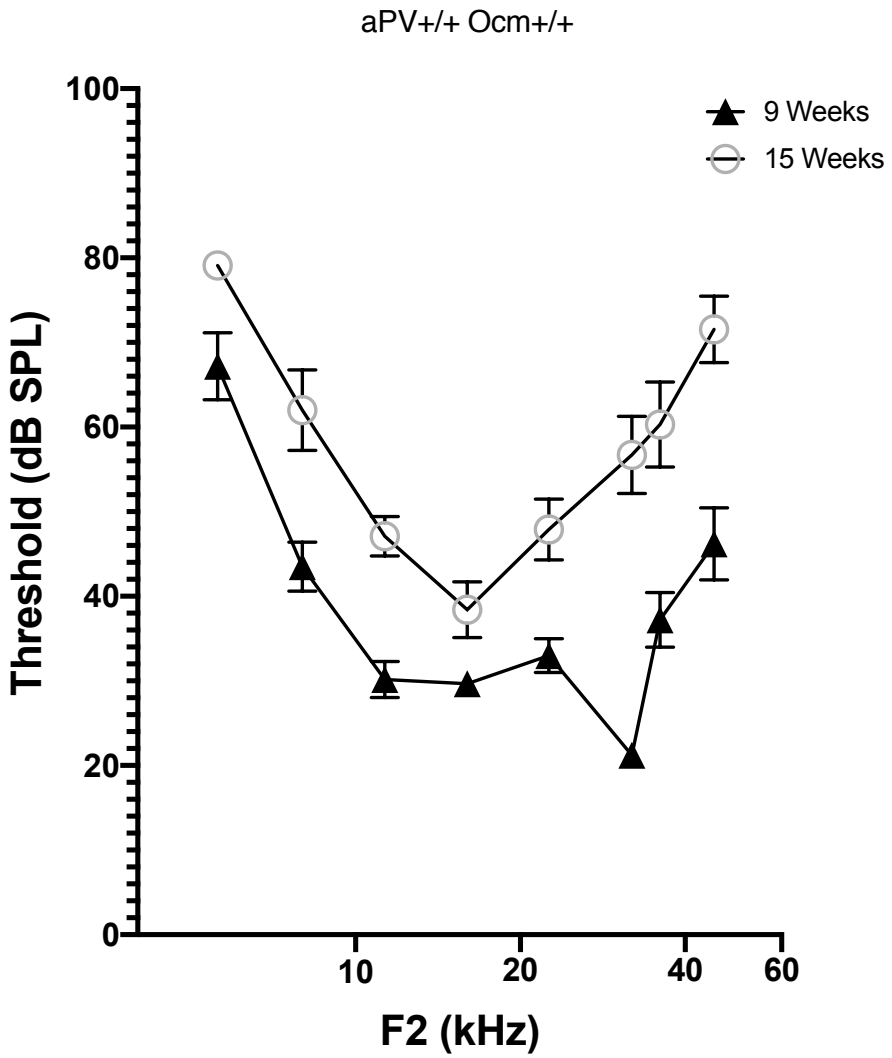

Figure 10. DPOAE thresholds for 9 wk and 15 wk old C57 BL/6 WT mice. The frequencies of sound stimuli given (F2) were 6, 8, 11, 16, 23, 32, 36, and 45 kHz.

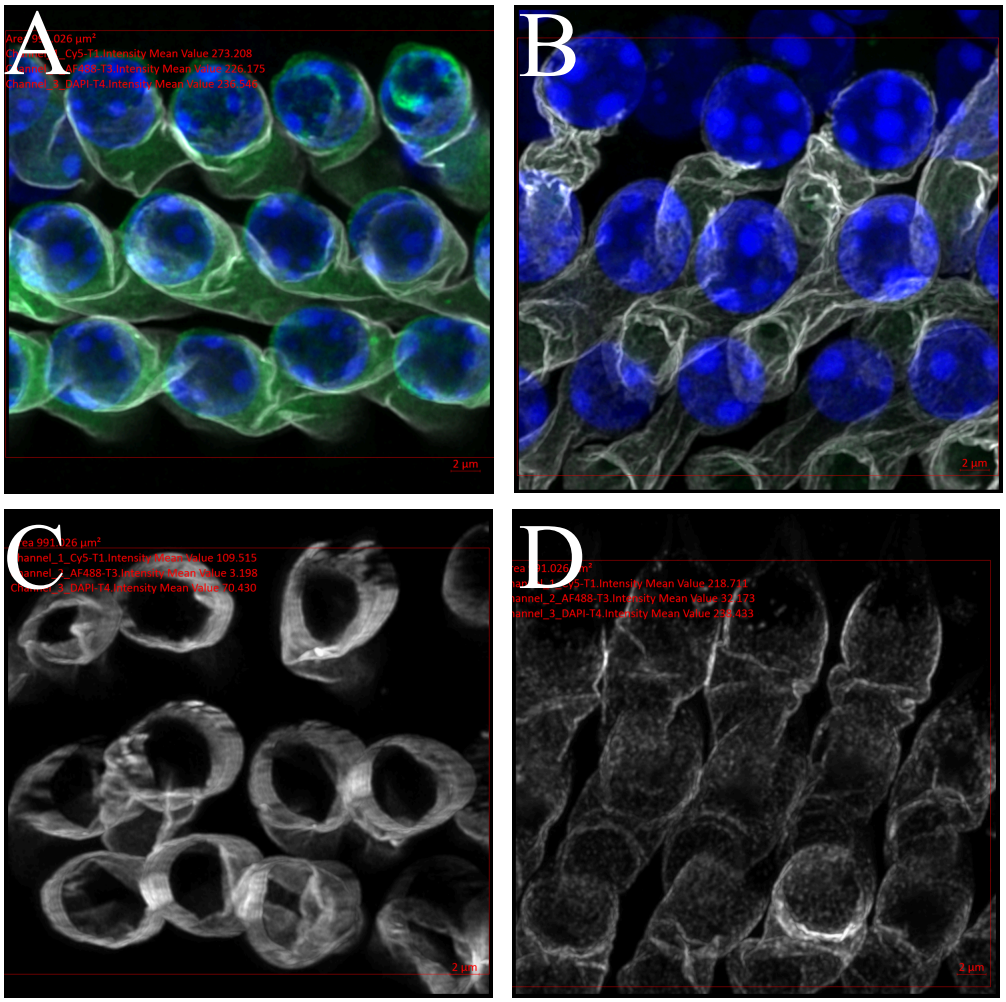

Figure 11. 63x Airyscan confocal images of 3 month old WT C57 BL/6 prestin expression in the 32 kHz region (A). 6 month old WT C57 BL/6 prestin expression in the 32 kHz region (B). 3 month old WT C57 BL/6 prestin expression in the 8 kHz region (C). 6 month old WT C57 BL/6 prestin expression in the 8 kHz region (D). OCM is green, DAPI is blue, and prestin is grey. All images taken with the same optical parameters.For the *Ocm* KO salicylate-injected mice, there was not a noticeable difference in low frequency regions (from 6 kHz to 16 kHz) just like the wild type salicylate-injected mice. Again, there was a noticeable difference in the DP thresholds prior to salicylate injection and after a 24-hour recovery period. The difference here is that the change in DP threshold for the *Ocm* KO mice was not as great as it was for the wild type mice (12D). This suggests that salicylate injections have less of an effect on DP threshold in the absence of *Ocm*. In other words, the absence of *Ocm* reduces the effect that salicylate has on prestin. There was not a noticeable difference in DP thresholds prior to or after salicylate injection.

DP Threshold Shift	5.6 kHz	8.0 kHz	11.2 kHz	16.0 kHz	22.5 kHz	32.0 kHz	36.0 kHz	45.2 kHz
9 -15 wk	16 dB	18 dB	18 dB	8 dB	16 dB	36 dB	22 dB	24dB
WT Control	4 dB	6 dB	2 dB	12 dB	6 dB	2 dB	8 dB	-2 dB
WT Salicylate	0 dB	-4 dB	-6 dB	0 dB	-16 dB	-14 dB	-20 dB	-20 dB
KO Control	0 dB	6 dB	6 dB	22 dB	6 dB	-2 dB	6 dB	0 dB
KO Salicylate	0 dB	6 dB	0 dB	10 dB	2 dB	-10 dB	-6 dB	-12 dB

Table 1.This table shows the threshold shifts that occurred in Figures 10 and 12 at various frequencies. A negative value indicates a decreased threshold shift and a positive value indicates an increased threshold shift. Thus, a negative value represents an improvement in OHC function and vice versa. This suggests that salicylate improves OHC function in WT mice and *Ocm* KO mice in high frequency regions. *Ocm* KO mice appear less susceptible the effects of salicylate injection. 15 wk old mice have decreased OHC function compared to 9 wk mice.

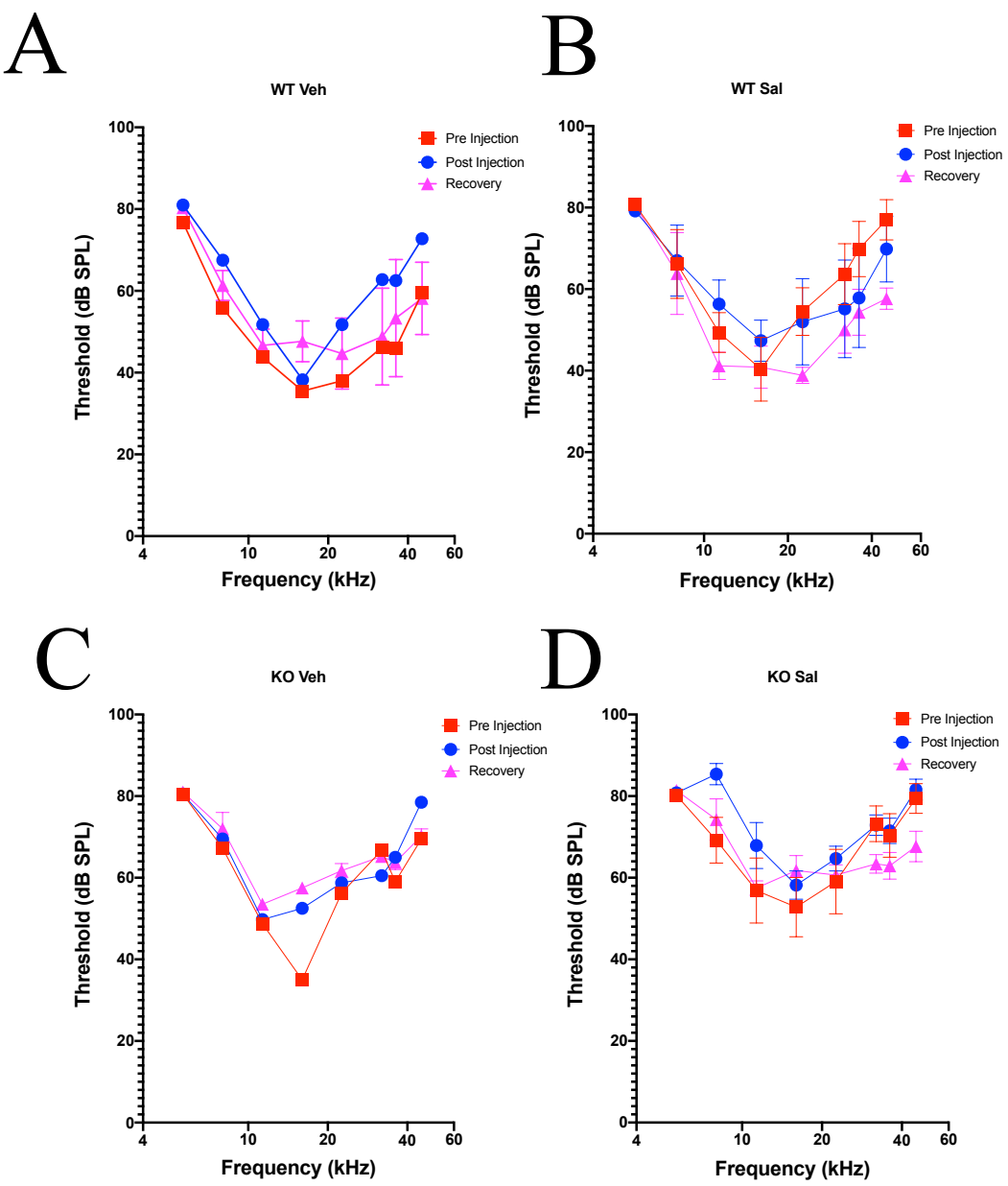

Figure 12. DP thresholds of APV C57BL/6 WT mice prior to saline (Veh) injections, after injections, and 24 hours after injection (A). DP thresholds of APV C57BL/6 WT mice prior to salicylate (Sal) injection, after injection, and 24 hours after injection (B). DP thresholds of APV C57BL/6 *Ocm KO* mice prior to saline (Veh) injection, after injection, and 24 hours after injection (C). DP thresholds of APV C57BL/6 *Ocm KO* mice prior to salicylate (Sal) injection, after injection, and 24 hours after injection (D). The sound stimuli given were 6, 8, 11, 16, 23, 32, 36, and 45 kHz.

CHAPTER FOUR

Discussion

The Interaction of prestin and OCM

Ocm KO mice are deaf and have irregularly distributed prestin expression. Based on this observation, the first main objective of this thesis was to investigate whether OCM and prestin colocalize in cochlear OHCs. To answer this question, we used immunocytochemistry as our qualitative measurement and circularity deviation index, number of collapsed OHCs, and Pearson Colocalization Coefficients as our quantitative measurements. We first used immunocytochemistry to discover that OCM and prestin protein appear to both localize in the periphery of OHCs (Fig. 3D). To further test our observations, we used PCC values to test the extent to which the two proteins were interacting. CBA WT mice, C57BL/6 WT mice and CBA *Ocm* KO mice had average PCC values of approximately 0.25, 0.17, and -0.10 respectively. Interestingly, the data suggest OCM and prestin have a more intimate interaction in CBA WT mice compared to C57BL/6 WT mice. This is noteworthy because C57BL/6 WT mice have an *ahl* mutation (cadherin 23) that causes them to have progressive hearing loss. C57 start having elevated thresholds in high frequencies as early as 4 – 5 months (Fig. 10) and are deaf by 12 – 14 months. This is in contrast to CBA mice that do not have elevated DP thresholds until 24 months. With Ocm mutation, C57Bl/6 start progressive hearing loss as early as 2 months and are completely deaf by 6 months. An Ocm mutation in CBA mice causes progressive hearing loss to begin at 3-4 months and they are deaf by 12–14 months.

This suggests that a mutation of *Ocm* or another type of alteration to *Ocm* affects its interaction with prestin, potentially affecting OHC function. We also observed a unique phenomenon where WT OHCs had abnormal prestin expression and were irregularly-shaped. Thus, we hypothesized that if this phenotype occurs in CBA WT mice, then it might occur more frequently in C57BL/6 WT and CBA *Ocm* KO mice. We used circularity deviation index (Fig. 7) and counting the number of collapsed OHCs (Fig. 8) as measurements to test this hypothesis. The measurements confirmed our hypothesis and increased our evidence that OCM and prestin are interacting. To further confirm that OCM and prestin are colocalized, more experimental techniques need to performed. Co-immunoprecipitation with OCM and prestin is a potential experiment we could perform in the future. We also investigate how prestin changes with age using immunohistochemistry. The disadvantage of this is that it is purely a qualitative measurement. Its not reliable to make conclusions based off only qualitative measurements. Thus, future quantitative measurements should be done, such as measuring circularity deviation, to ensure this phenomenon is actually occurring.

Investigating a Potential Therapeutic Intervention to Delay Hearing Loss

Although Ocm-KO mice are deaf, their OHCs still express prestin. Using this observation, we hypothesized that increasing prestin protein might delay hearing loss in *Ocm*-KO mice. Previous studies show that long-term administration of nonsteroidal anti-inflammatory drugs increases prestin protein expression, OHC electromotility and improved hearing. We hypothesized that using salicylate (aspirin), a nonsteroidal anti-inflammatory drug, could increase prestin in *Ocm-KO* mice. Thus, we made the second

main objective of our thesis to test salicylate's ability to rescue OHC function, by increasing prestin expression, in the absence of *Ocm*. To do so, we used immunohistochemistry to observe if prestin expression changed in response to salicylate injections (Fig. 9). We also measured DPOAE's of mice to observe if their hearing was affected by salicylate. The data show salicylate injections had an effect on C57/Bl6 mice but had little to no effect on *Ocm* KO C57/Bl6 mice (Fig. 12). As a whole, my thesis project serves as a preliminary experiment to test the feasibility of using a substance, such as salicylate, to rescue non-functioning OHCs. Also, it offers a potential mechanism or pathway for why OHCs lose function in an aging model. If increasing the amount of prestin compensates for the loss of OCM, then drugs like salicylate that potentially increase the amount of prestin could be a simple therapeutic intervention to slow or even prevent age-related hearing loss. When we started the salicylate experiment, we investigated two variables at the same time—saline vs salicylate injection and WT vs *Ocm*-KO mice. This was done because I was not able to do summer research at Baylor, preventing me from having enough time to test each variable separately. If the Simmons lab wants to continue this project, it would be ideal to test the effect of salicylate only on WT mice before adding the additional variable of *Ocm* KO mice. This would ensure whether salicylate is actually having an effect on its own and is not being affected by confounding variables. The paper that inspired us to see the effects of salicylate of prestin in *Ocm* KO mice looked at acute and chronic outcomes (Yu, 2008). Future experiments should focus on the chronic effects of salicylate on prestin expression in *Ocm* KO mice as we did not have time to investigate it. Also, the injection protocol might need revision. We injected mice via intraperitoneal injections. This might not be the best method of

injection. Other methods, such as intravenous injections (tail injections) might be more efficient at ensuring the injection is successful. Additional experiments should be done to investigate salicylate's long-term effects on prestin expression. If salicylate does increase prestin expression after long-term exposure, this could be a potential method of restoring OHC function in *Ocm* KO mice. We believe it is worth the effort to continue studying the relationship between OCM and prestin for its potential clinical implications.

Bibliography

Ashmore, Jonathan. "Outer Hair Cells and Electromotility." *Cold Spring Harbor Perspectives in Medicine*, Cold Spring Harbor Press, 1 Jan. 1970, perspectivesinmedicine.cshlp.org/content/9/7/a033522.long.

Brownell WE. What Is Electromotility? -The History of Its Discovery and Its Relevance to Acoustics. *Acoust Today*. 2017;13(1):20–27.

Ciganović, Nikola, et al. "Static Length Changes of Cochlear Outer Hair Cells Can Tune Low-Frequency Hearing." *PLoS Computational Biology*, Public Library of Science, 19 Jan. 2018, www.ncbi.nlm.nih.gov/pmc/articles/PMC5792030/.

Clason, D. (2019, November 30). Hearing loss statistics at a glance. Retrieved from https://www.healthyhearing.com/report/52814-Hearing-loss-statistics-at-a-glance.

Climer, Leslie K, et al. "Oncomodulin: The Enigmatic Parvalbumin Protein." *Frontiers in Molecular Neuroscience*, Frontiers Media S.A., 9 Oct. 2019, www.ncbi.nlm.nih.gov/pmc/articles/PMC6794386/.

Dallos, P, and Fakler B. Prestin, a new type of motor protein. Nature. 2002. doi: 10.1038/nrm730.

Dallos, P. Cochlear amplification, outer hair cells and prestin. *Curr Opin Nuerobiol.* 2008. doi:10.1016/j.conb.2008.08.016.

Fischer N, Johnson Chacko L, Majerus A, Potrusil T, Riechelmann H, Schmutzhard J, Schrott-Fischer A, Glueckert R: Age-Dependent Calcium-Binding Protein Expression in the Spiral Ganglion and Hearing Performance of C57BL/6J and 129/SvJ Mice. ORL 2019;81:138-154. doi: 10.1159/000499472.

Hackney CM, Mahendrasingam S, Penn A, Fettiplace R (2005) The concentrations of calcium buffering proteins in mammalian cochlear hair cells. J Neurosci 25:7867–7875. CrossRef Medline.

Homma, K, Miller, KK, Anderson, CT, Sengupta, S, Guo-Guang, D, Aguiñaga, S, Cheatham, M, Dallos, P, and Zheng, J. Interaction between CFTR and prestin (SLC26A5). Biochimica et Biophyscia Acta (BBA) – Biomembranes. 2010. doi.org10.1016/j.bbamem.2010.02.001.

Lewit-Bentley, A. and Réty, S. EF-hand calcium-binding proteins. Current Opinion in Structural Biology. 2000. doi: 10.1016/s0959-440x(00)00142-1.

Liberman, M. C., Gao, J., He, D. Z. Z., Wu, X., Jia, S., & Zuo, J. (2002). Prestin is required for electromotility of the outer hair cell and for the cochlear amplifier. *Nature*, *419*(6904), 300–304. doi: 10.1038/nature01059.

Tong, B, Hornak, AJ, Maison, SF, Ohlemiller, KK, Liberman, MC, and Simmons, DD. Oncomodulin, an EF-Hand Ca^{2+} Buffer, Is Critical for Maintaining Cochlear Function in Mice. The Journal of Neuroscience. 2016. doi: 10.1523/JNEUROSCI.3311-15.2016.

Xia, A. Song, Y. Wang, R, Gao, SS, Clifton, W, Raphael, P, Chao, S, Pereira, FA, Groves, AK, and Oghalai, JS. Prestin Regulation and Function in Residual Outer Hair Cells after Noise-Induced Hearing Loss. PLoS One. 2013. doi: 10.1371/journal.pone.0082602.

Yu, N. Zhu, ML. Johnson, B. Liu, YP. Jones, RO and Zhao HB. Prestin up-regulation in chronic salicylate (aspirin) administration: An implication of functional dependence of prestin expression. Cellular and Molecular Life Sciences. 2008. doi: http://dx.doi.org/10.1007/s00018-008-8195-y.

Zheng, Jing, et al. “Prestin Is the Motor Protein of Cochlear Outer Hair Cells.” *Nature News*, Nature Publishing Group, www.nature.com/articles/35012009.