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An Investigation into Adult Mammalian Hair Cell Regeneration

Abstract

The mammalian inner ear includes the sensory organ, the organ of Corti, in which there are mechanoreceptors called hair cells. These hair cells are the units that respond to sound vibrations and transmit the signal to the central nervous system for recognition. If these hair cells are damaged by either prolonged noise exposure or age-related causes, hearing loss occurs and can greatly deteriorate someone's standard of living. In chickens and zebrafish, hair cells can be regenerated after damage, however adult mammals do not have that same ability. In mammals, there are cell proliferation pathways, Wnt/ß-catenin and Notch, that can be harnessed for hair cell regeneration. Although there is promising research for these pathways, there is still something lacking in the adult mammalian system preventing successful regeneration. Epigenetic manipulation through histone demethylation and acetylation may provide the missing link for these cell proliferation pathways by allowing transcription of genes that are normally inactive in adult mammalian cochlea, which houses the organ of Corti. This hypothesis is multi-faceted through three approaches. A comparison between chicken, zebrafish, and mice, will be made to outline the reasons for that lack of hair cell regeneration in adult mammals. An exploration of mouse variants will shed light on the effects of DNA manipulation on hair cell structure. Then, through the administration of azacitidine (DNA methylation inhibitor) and panobinostat (histone deacetylase inhibitor), 205 female C57BL/6J mice will recover full hair cell numbers after prolonged noise exposure at 114 dB and 118 dB, for 16 kHz. The methods include dissection of the cochlea and immunostaining for hair cells counts. To broaden the effects of this project on the scientific community, a summer course for high school students, summer camps for kids ages eight to fourteen, and a mural about hearing loss awareness will all be designed. We present this project proposal with the request for a $1,000,000 budget to conduct everything previously mentioned.

Introduction

The ear is a sensory organ that allows organisms to hear sounds and process them into electrochemical signals to the brain. The ear also maintains one's balance. The function of the mammalian ear and its auditory system is dependent on the structure of the outer, middle, and inner ear. The outer ear includes the auricle, or pinna, and the external auditory meatus, or ear canal (Alvord and Farmer, 1997). The auricle is made up of a top layer of skin covering a yellow elastic fibrocartilage network and the entire structure is separated into different regions: helix, antihelix, lobe, tragus, triangular fossa, auricular tubercle, scaphoid fossa, concha, antitragus, crus of helix, and crura of antihelix. The function of the auricle is to funnel sounds from the environment into the external auditory meatus, moving further inward (Ekdale, 2016). Of the external auditory meatus, there is an outer cartilaginous portion and an inner bony portion. This structure is a tube that leads into the cranium to the tympanic membrane. The cartilaginous portion is an extension of the concha from the outer ear and, located more medial to the tympanic membrane, there is a narrowing of the canal called the "isthmus." The middle ear consists of the tympanic membrane, the tympanic cavity, and three tiny bones. The tympanic membrane, also called the eardrum, is the smooth, translucent membrane that separates the outer ear structures from the other regions. This membrane is elliptical in shape and concave, jutting inwards towards the inner ear. When soundwaves pass through the ear canal and meet the membrane, they cause the membrane to vibrate and this vibration gets transferred to the tiny bones (Volandri et al., 2011). The tiny bones, inside of the tympanic cavity, form a "chain" connecting the tympanic membrane to the oval window, however the last bone touches the petrosal bone, which surrounds the inner ear cavities. The three bones are named, in order of laterally to medially, the malleus, the incus, and the stapes. They transmit the soundwaves from the tympanic membrane to the fluid filled chambers of the inner ear by articulating with the oval window, which is one of two openings to the inner ear.

The inner ear includes interconnected spaces within the petrosal bone, which is named the bony labyrinth (Ekdale, 2016). Inside of this bony labyrinth, there is a series of interconnected soft tissue ducts, making up the membranous labyrinth, which is separated into inferior and superior divisions. The inferior portion contains the cochlea and the saccule of the membranous vestibule. The superior portion contains the utricle of the vestibule and the three semicircular ducts. Inside the cochlea, the organ of hearing, called the organ of Corti, resides.

The membranous labyrinth is filled with lymphatic fluid, but, more specifically, perilymph fills the bony labyrinth and endolymph fills the cochlear duct and membranous vestibular apparatus. Auditory receptors in the cochlea and motion receptors in the vestibular system are stimulated by vibrations transferred through the endolymph. Previously stated, the ear can maintain balance as well as act as a sensory organ. The vestibule is the portion that maintains the balance; however, endolymph can affect both the cochlea and the vestibule (Swartz et al., 1996; Ekdale, 2016). The location of the organ of Corti inside the cochlear duct is upon the vestibular surface of the basilar membrane. The structures inside the cochlea that detect the auditory sensations are called hair cells. There are two distinctions of hair cell: inner hair cells and outer hair cells. The inner hair cells are located above the edge of the osseous spiral lamina, while the outer cells are located on the basilar membrane (Lim, 1986). The basilar membrane lines the entirety of the interior of the organ of Corti. To distinguish the two types, outer hair cells are more cylindrical and inner hair cells are more flask shaped. Outer hair cells comprise the majority of the hair cell types. The reason for their sensory function is that the hair cell structure is highly specialized with the presence of stereocilia. The stereocilia are microvilli that are linked together, which causes them to be referred to as bundles. These microvilli are sensitive to any pressure or movement changes in the endolymph and the stereocilia displace when provoked by these changes. Additionally, there is another type of cell that surrounds the hair cells: the supporting cell. Each hair cell is separated from another in their respective bundles by these supporting cells (McPherson, 2018).

To be more precise, the soundwaves that transfer through the endolymph have different frequencies, which are measured in hertz (Hz). The organization of the hair cells in the mammalian cochlea is tonotopically organized, with the apical end responding to low frequencies and the basal end responding to high frequencies (Kros and Evans, 2006). The stereocilia "respond" to these frequencies through their displacement. The audible frequency range for humans is 20 Hz to 20 kHz. However, for some mammals, the range can reach up to 100 kHz.

In order for the sound to be transmitted to the brain, the hair cells need to be connected to the nervous system. Through the innervation of the cochlea, the mechanical impulse is recognized by the hair cells and turned into an electrical impulse. Both afferent and efferent fibers innervate the organ of Corti and the ganglion cells in both types of nerve terminate at inner and outer hair cells (Engström and Sjöstrand, 1954). However, specifically outer hair cells

receive 90% innervation of efferent nerves. Afferent nerves carry stimuli to the central nervous system, while efferent nerves are motor nerves that carry impulses away from the central nervous system. No mature hair cells produce action potentials, so all transmissions are graded receptor potentials that are produced due to the hair bundle displacements (McPherson, 2018). At the base of each hair cell contains a membrane with several pre-synaptic active zones, and this area is where chemical neurotransmitters are released into the synapse. The displacement of the hair cell bundle causes the release due to membrane depolarization. Once the neurotransmitters are released, they bind to receptors in the membrane of the afferent neuron and causes an influx of positive ions, which results in depolarization of the neuron. This depolarization travels further down the neuron and, eventually, the nerve transfers the electrical impulse all the way to the central nervous system, where the sound is processed. This is the simplified process of hearing.

Hearing loss includes the destruction of the hair cell bundles, and, even potentially, the destruction of the nerves that allow for signal transmission to the brain. The damage of the hair cells is due to mechanical stress and there can be many types of injury to the hair cell with some being more serious than others (Wagner and Shin, 2019). There can be tip link breakage, stereocilia core damage and loss of synapse. Tip links that connect stereocilia can be repaired within 24 hours of damage, while synapse loss and stereocilia core damage may be harder to fix. Damaged hair cells can also affect balance. If hair cells that recognize a certain frequency become damaged, it is possible that the individual won't be able to hear the specific frequency any longer. This is where hearing loss starts to become significant, especially for mammals. In mammals, mature hair cells can't be regenerated and repaired, which causes permanent hearing loss. Non-mammalian vertebrates, however, do have mature hair cells with regenerative abilities (Cox et al., 2014; Groves et al., 2013). Hearing loss is a common health defect, the prevalence coming close to heart disease and arthritis, and mainly affects human individuals over 70 (Wagner and Shin, 2019; Goman, et al., 2017). In the U.S., about two thirds of adults over 70 years of age have serious hearing loss (Goman, et al., 2017). However, the population of those aged 20 or older in the U.S. with hearing loss is predicted to increase from about 44 million as of 2020 to 73.5 million by 2060. Additionally, the number of people with hearing loss in the U.S. is supposed to double in the next 40 years and this will surpass population growth. With the forthcoming increase of hearing loss in adults, there is an increased need for intervention. Hearing impairment can be genetic in humans and progress into old age. While many cases of

hearing loss are due to changes to the ear structure during old age, there is also noise-induced hearing loss.

Since the invention of electronic devices, the human ear has received more focused auditory stimuli with the transmission of the sound directly into the ear canal. Younger generations have started using earbuds or headphones at a young age. There have been studies that have shown that the increased usage of personal music players and some type of headphone can lead to higher risk of noise-induced hearing impairment (Widén et al., 2017; Kim et al., 2009; Peng et al., 2007). One study showed that longer lifetime exposure and increased listening frequency in adolescents were associated with poorer hearing thresholds, which means that the level at which an individual can perceive sound becomes higher and their hearing sensitivity decreases (Widén et al., 2017). It's not the overall act of listening to music that is the cause of the hearing loss, but it is the volume and amount of exposure when listening to music that causes issues. In groups who listened to music between 90 and 100 dB for long periods, there was a greater risk for developing hearing impairment. Even activities that do not include personal music devices cause noise-induced hearing loss: attending night clubs or rock concerts, using firearms or power tools (Carter and Black, 2017). There is evidence that loud noise exposure and listening to music with headphones is an escalating trend among younger people (Swierniak et al., 2020).

Any level of hearing loss, regardless of the how or when is permanent in humans and the only current way to fix it is to prevent it. People can use personal hearing protectors, like ear plugs, etc., when taking part in activities that can cause hearing loss. However, there is evidence that adolescents, even when they are aware of these forms of protection, use little to none and even participate in more dangerous habits, like standing close to speakers and doing loud activities for long periods of time (Vogel et al., 2010). However, hearing aids do exist to help those with hearing loss because hearing loss greatly affects an individual's mental and physical health. Hearing aids can increase the quality of life for an individual, although, adherence to the usage of hearing aids can be low for people who have them (Bainbridge and Wallhagen, 2014). Therefore, it would be incredible if people were able to regain their hearing loss by regenerating the damaged or destroyed hair cells. As previously stated, non-mammalian vertebrates can regenerate their hair cells, yet mammals can't. This includes humans, but there have already been many studies trying to figure out the best strategy towards this regeneration in mammals. To be

more specific, the regeneration of hair cells includes the ability to create new hair cells to replace the old ones. Birds, fish, reptiles and amphibians are able to spontaneously regrow hair cells, even if there is no damage to them.

Popular model systems for birds, fish, and mammals are chickens, zebrafish, and mice. Chickens and zebrafish can regenerate hair cells, therefore there must be a different mechanism in these animals compared to mammals. There are many ways that hair cells could potentially regenerate and restore their numbers, including migration of cells to the damaged region, differentiation of cells into hair cells, and mitotic production of new hair cells. Focusing on the avian auditory system, the structure is quite similar to the mammalian auditory system. In fact, due to their similarities, the avian inner ear is one of the best understood biological models of hair cell recovery. The regeneration occurs in the basilar papilla, which is analogous to the organ of Corti with an organization of hair and supporting cells similar to that of the mammal (Warchol, 2011). Supporting cells are the progenitors to new hair cells during avian regeneration because they enter the cell cycle after damage in the avian vestibular epithelium (Stone and Rubel, 2000). In the chicken cochlea, direct transdifferentiation of supporting cells into hair cells and mitotic asymmetric division of supporting cells are both processes for hair cell regeneration. However, it has also been found that symmetric divisions of supporting cells occur (Scheibinger et al., 2018).

Although the avian inner ear is well studied, it is actually the lateral line in fish that first indicated hair cell regeneration. The lateral line system is a sensory organ in fish and amphibians, where the detection of water motion and pressure occurs. This helps the fish identify any organisms nearby, which aids in social behaviors and predator/prey detection. The lateral line is made of series of neuromast cell clusters, which contain mechanosensory hair cells surrounded by support cells. These hair cells share multiple similarities with the mammalian inner ear hair cells (Ma and Raible, 2009). The hair cells are also innervated by afferent and efferent neurons. In the lateral line system, there is an anterior and posterior branch, differentiated by their location in the organism. The structure of the posterior lateral line is quite different than the avian and mammalian inner ear.

Finally, one of the most common model systems for mammals is mice. Genetically modified mice are commonly used in many diverse experiments. While adult mice can’t regenerate damaged hair cells like most mammals, prenatal and neonatal mice, however,

maintain that ability or at least some portions of it. Many types of therapies for hair cell regeneration are being developed and investigated for the potential of adapting them for human usage. Key signaling pathways include the canonical Wnt and Notch signaling pathways. The co-regulation of these pathways can potentially influence regeneration of hair cells. The Wnt pathway is characterized by a family of secreted glycoproteins called Wnts; they bind to frizzled receptors and low-density lipoprotein receptor related protein 5/6 (LRP5/6) to form Wnt-Frizzled-LRP5/6 complexes. These complexes lead to the downregulation of glycogen synthase kinase 3ß (GSK3ß). This kinase normally phosphorylates cytoplasmic ß-catenin. So, with the downregulation of GSK3ß, ß-catenin translocates to the nucleus and interacts with T cell factor/lymphoid enhancer factor (TCF/Lef) transcription factors, which then initiates gene expression.

The Notch pathway is characterized by the Notch family of receptors that interact with membrane-bound Notch ligands: Jagged (Jag) 1-2, Delta-like (Dll) 1, 3, 4. These ligands are in adjacent cells to the Notch receptors, which are transmembrane proteins. The interactions between ligand and receptor trigger cleavage of the Notch receptor by the ADAM family of metalloproteases and y-secretase. This forms the Notch receptor intracellular domain (NCID) that then interacts with the recombination signal binding protein for the immunoglobulin kappa J region (RBPJk) transcription factor in the nucleus. This triggers gene expression. It's important to mention Atoh1, a transcription factor also necessary for hair cell differentiation, and Sox2, the regulator of Atoh1. Sox2 interacts with Atoh1 3' enhancer, which activates Atoh1. After, Sox2 is downregulated, which is required for Atoh1 expression. This mechanism is affected by both Wnt and Notch signaling. Specifically, if Notch signaling is downregulated, Atoh1 expression is upregulated, although Sox2 is also downregulated. If Notch signaling is upregulated, then the activation stimulates expression of Hes/Hey transcription factors and they inhibit Atoh1 expression by recruiting a co-repressor (Samarajeewa et al., 2019). Therefore, the pathways of Notch and Atoh1 are intertwined.

When both Wnt and Notch pathways trigger targeted gene expression, their effectors influence and regulate cell proliferations, differentiation, and fate determination during embryonic development. However, the Wnt pathway also regulates stem cell control and homeostasis in adult tissues, while the Notch pathway promotes maintenance of adult tissues.

The combined regulation of these pathways has led to some form of hair cell regeneration in previous mice studies.

Regardless of these regenerative successes, there still seems to be limitations in the adult cochlea, which may be the result of age-related epigenetic modifications (Samarajeewa et al., 2019). This means that some of the important genes in these pathways are methylated or deacetylated and their subsequent transcription/translation are prevented from happening. Most of the time, when a gene is methylated, it basically becomes inactive. However, genes can also become active depending on the side chain that is methylated. The methylation could either be histone methylation, which is constantly being manipulated by the cell, and DNA methylation, which is permanently methylated unless the methyl group is removed. Histone methylation is the process of proteins attaching methyl groups onto lysine and arginine residues in histone proteins. These histone proteins are the reason for DNA fitting inside nuclei, where the DNA is wrapped around the histone proteins in order to condense the DNA into chromatin (Jambhekar et al., 2019). The DNA being wrapped around the histone proteins form nucleosomes and further wrapping of nucleosomes eventually leads to chromosome formation, which are formed during prophase of mitosis for cell division. The process of histone wrapping is a form of gene transcription regulation. The place of methylation on the histones are the nitrogen atoms in amino acid side chains and/or the amino termini at the end of histone tails. Histone H3 is the primary site of histone methylation, being one of the four histone proteins that pair to form an octamer in a nucleosome. However, if a gene promoter is located in the site that is methylated, then this usually acts to repress gene transcription.

DNA methylation is the direct addition of methyl groups to the DNA molecule, specifically addition to the carbon 5 position of cytosine. The repression of transcription due to DNA methylation is thought to be caused by direct interference of the recognition sequence of transcription factor complexes (Johnson et al., 2012). Age-related methylation of DNA is characterized by genome-wide hypomethylation and promoter-specific hypermethylation (Johnson et al., 2012). There is also increased DNA damage and high levels of DNA breaks in aging genomes, which may have affected histone modifications (Oh et al., 2013). Deacetylation can also inactivate genes through DNA condensation, which means that acetylation does the opposite and leaves the chromatin in a more relaxed state allowing transcription. The specific mechanism that causes tightening or relaxation of DNA is a change in charges. DNA is

negatively charged, while the histone tails are positively charged. The addition of an acetyl group to the tail neutralizes the charge, which prevents the histone tail from being attracted to the DNA and the DNA stays more relaxed (less coiled). The addition of a methyl group to the tail maintains the positive charge, which usually makes DNA more coiled. However, as previously stated, sometimes methylation causes relaxation of DNA. Both acetylation/deacetylation and methylation/demethylation are regulated as part of gene expression regulation.

With increasing hearing loss in younger generations due to the technological age, there is a need for innovative research surrounding hearing regeneration. There is evidence for triggering regeneration of damaged hair cells through the harnessing of pathways already present in adult mammals, most notably the Wnt and Notch pathways. One aspect that has been widely explored in other fields of study but has yet to be in regard to hair cell proliferation is epigenetics. Mice cochleas can be removed and analyzed to determine the effects of this epigenetic manipulation. Adult mice are the best model because their genome is similar to the human genome and their hearing organ is same structurally to humans. Additionally, the small size and accessibility of mice makes them perfect candidates for invasive experiments.

A possible reason for the absence of hair cell regeneration in adult mammals is that target genes for Wnt and Notch pathways in the ear are inactive and unable to be transcribed due to methylation and deacetylation of DNA and histones. Through administration of histone deacetylase (HDAC) inhibitors and demethylation drugs, the Wnt and Notch pathways of adult mice could be activated leading to subsequent regeneration of hair cells damaged by noise exposure. A budget of $1,000,000 will be necessary to explore the three approaches of this multi-faceted hypothesis: the comparison of chicken, zebrafish, and mice hair cell development and regeneration; the observation of mutant and transgenic mice with mutations causing difference in number and structure of stereocilia of hair cells; the experimental approach through administration of HDAC inhibitors and demethylation drugs in adult mice after loud noise exposure. The comparison of vertebrates will determine what makes the mammalian auditory organ an outlier in regard to hair cell regeneration. Observing mice with mutations in hair cell and stereocilia structure will help understand effects of genetic manipulation on the mechanosensory receptors. Finally, experimenting directly with the mice model will hopefully lead to results that show epigenetics can be the answer to hair cell regeneration in the cochlea of an adult mammal. Therefore, our predicted results will show that mice who were administered

the epigenetic drug combination, azacitidine (DNA methylation inhibitor) and panobinostat (histone deacetylase inhibitor), will yield almost total recovery of noise damaged cochlear hair cells.

Chicken Hair Cell Regeneration

Both the avian and fish inner ear are able to regenerate their hearing when hair cells are damaged, by sound, age, or toxins. The peripheral auditory system of birds includes the ear canal, the tympanic membrane, and the middle ear ossicle. These structures are different to that in the mammal. The avian ear canal is short, yet still leads to the tympanic membrane that still functions as transmitter of sound waves into vibrations. There is a single ossicle, instead of three in the mammal, which is called the columella. This ossicle transmits vibrations from the tympanic membrane to the inner ear fluids in the avian labyrinth (Saunders, 2010). In the avian labyrinth, the chambers and the two types of lymph remain the same as the mammal.

The chicken inner ear structure is very similar to that of other vertebrates, including humans. The chicken inner ear can be divided into dorsal vestibular and ventral auditory components, where the cochlear duct act as the auditory component. Instead of the cochlear duct being coiled like in humans, the structure is that of a straight tube to extends medially to laterally. The proximal and distal ends of the cochlear duct form an arc shape, with both ends pointed posteriorly. The vestibular component of the ear includes the utricle and saccule, semicircular canals and their corresponding ampullae. The ampullae contain the cristae. Each canal is located in different planes, which is most likely due to the differential growth of the otic epithelium. The otic epithelium is what the inner ear arises from during development. In the chicken inner ear, there are eight sensory organs: one auditory and seven vestibular. These organs are organized into anterior and posterior groups, with four organs in each. The anterior group contains the superior and lateral cristae, and the maculae utriculi and sacculi. The posterior group contains the posterior crista, the basilar papilla, the lagena, and the macula neglecta (Wu et al., 1998). The basilar papilla is the sensory organ of the cochlear duct, which contains endolymph as part of the scala media. Of the hair cells in the papilla, there are two types called tall and short hair cells with tall hair cells on the superior half of the papilla and short hair cells on the inferior half (Sanders, 2010). The organization of the hair cells into bundles of stereocilia, made of actin filaments, is similar to that of mammals. Tall hair cells are innervated by mainly

afferent neurons and short hair cells are innervated mainly by efferent neurons. Short hair cells lie over the basilar membrane creating a gradient of mechanical stiffness along the papilla with the changing thickness of the basilar membrane. This gradient gives rise to a traveling wave, which produces a frequency-to-place translation where there is maximum basilar membrane movement. The tall hair cells lie in an epithelial layer of the basilar papilla above a fibrocartilaginous plate, although the epithelium and plate do not move with sound stimulation. Tall hair cells and short hair cells are likened to mammalian inner and outer hair cells. The organization of the hair cells includes a W-shaped pattern in the superior and posterior cristae, while there is a V-shaped pattern in the lateral crista (Wu et al., 1998). Hair cell depolarization is caused by hair bundle displacement, like in the mammal. With louder stimuli, the greater the hair bundle displacement is. Supporting hair cells surround hair cells, with each hair cell surrounded by six or seven supporting cells (Goodyear and Richardson, 1997). Hair cells are regenerated through cell division of supporting cells.

Even though mitotic activity ceases during embryonic development, when hair cells are damaged, supporting cells re-enter the cell cycle and differentiate into either supporting or hair cells. Membrane proteins expressed by hair cells prevent surrounding supporting cells from reentering the cell cycle, so the absence of hair cells triggers cell proliferation (Sanders, 2010). Additionally, supporting cells can spontaneously differentiate into hair cells through direct transdifferentiation. This process doesn't require the re-entry of supporting cells into the cell cycle; however, it is considered a significant source of new hair cells during avian hair cell regeneration. Also, the likelihood of supporting cells undergoing either process depends on their location along the basilar papilla, which is also called the auditory epithelium. Re-innervation is rapid during regeneration, which leads to a successful, functional recovery (Stone and Cotanche, 2007). In the mature basilar papilla, a basic helix loop helix (bHLH) proneural transcription factor Atoh1 is reactivated after hair cell loss, which induces mitosis and transdifferentiation in supporting cells. The regulation of Atoh1 transcription is influenced by Wnts, epidermal growth factors, fibroblasts growth factors, and Notch molecules. Atoh1 directs the supporting cell towards the hair cell fate during regeneration. The Notch pathway is the most well studied of the vertebrate inner ear.

In the avian inner ear, the Notch ligand, Notch1 negatively regulates Atoh1 through mediation of Hes factors. Hes, Hairy/Enhancer of Split, factors are bHLH transcription factors

and they enact repressor activity when the Notch receptor is bound by Notch1. If the Notch pathway is activated, Atoh1 activity is repressed. When the basilar papilla is undamaged, supporting cells transcribe Notch1and Notch receptor, which prevents expression of Atoh1 and this inhibits progenitor/precursor cells from acquiring the hair cell fate (Stone and Cotanche, 2007). When the basilar papilla is damaged, Notch activity decreases and increased Atoh1 transcription leads to transdifferentiation toward hair cells (Żak et al., 2015). Inhibition of supporting cell activity is important in, not only allowing differentiation, but also in regulating the correct number of cells. Additionally, there are many different types of signals that trigger supporting cells to enter the cell cycle. For example, adenylate cyclase increases division of supporting cells and other second messengers in signaling cascades can cause supporting cell division (Stone and Cotanche, 2007). Also, multiple signaling pathways interact to regulate the progression of supporting cells in their cell cycle.

Zebrafish Hair Cell Regeneration

Since zebrafish have been a popular model for hair cell regeneration research, they act as convenient models for comparison to the mammalian auditory system. The two branches of the lateral line system are the anterior lateral line and posterior lateral line. The anterior portion is located around the head, while the posterior portion runs down the trunk and tail. The lateral line placode gives rise to sensory structures in the head, like the ear, and it also gives rise to the primordium. The anterior cells of the posterior lateral line placode give rise to posterior lateral line ganglion, which innervate lateral line hair cells. The posterior cells give rise to the primordium and this mass of cells migrates to the tip of the tail, depositing neuromasts in intervals. The neuromasts form the posterior lateral line with there being primary and terminal neuromasts, while the lateral line ganglion innervates the neuromasts. The trailing region cells are organized into rosette patterns, due to maturation of the apical junction belt, and the remaining cells remain disorganized. Chemokine receptors produced in the cells of the primordium interact with their ligand, which is expressed along the path of migration. Along the path of migration, rosettes, each corresponding to a protoneuromast, mature gradually and are deposited sequentially from the leading end. Once two or three rosettes are formed, migration occurs and the deposition of a neuromast begins the formation of a fourth rosette at the leading edge of the primordium. This process continues throughout migration with cells at the leading

edge being the progenitor zone for protoneuromast formation. Interestingly, Wnt/ß-catenin and another signaling pathway, the FGF pathway, regulate organization and migration of the primordium (Ma and Raible, 2009). The FGF pathway regulates the primordium into rosettes and Wnt/ß-catenin pathway regulates patterning by modulating FGF signaling.

The primordium forms about a day after fertilization and neuromasts mature with hair cells by the third day. At five days after fertilization, the neuromasts vary in amount of hair cells, which are surrounded by support cells with projections that intercalate between the hair cells. The support cells are surrounded by mantle cells and they form a ring around the neuromast. Division of a hair cell progenitor forms pairs of hair cells with opposite hair-bundle polarity with two mirrored sets of hair cells. Some hair cell bundles of neuromast are oriented parallel to the body axis and some are oriented perpendicular. Hair cells are innervated with afferent neurons specific for their orientation. New hair cells are regenerated when old ones die, and this regeneration is rapid. These new hair cells arise from mitotically dividing supporting cells, which also proliferate (Ma et al., 2008). The new number of hair cells is quite similar to the original number of hair cells and the new hair cells have the same polarity as the original with newly developed innervations. FGF signaling is also required for proper specification of hair cell precursors, specifically through activation of the bHLH transcription factor Atoh1a. Notch signaling plays a role in hair cell regeneration through the limitation of support cell proliferation in order to control the number of hair cells regenerated (Ma and Raible, 2009). The specific proteins with elevated expression during regeneration are Notch3, delta, Atoh1a. This feedback inhibition regulates the size of regenerating neuromasts.

Mice Hair Cell Regeneration

The anatomy of the mouse ear is identical to the mammalian description provided earlier. To recap, the sensory organ inside of the inner ear is the organ of Corti, where the hair cells are tonotopically organized along the spiraled structure. There are both supporting cells and hair cells with stereocilia organized into hair bundles, which displace when the endolymph vibrates through sound stimulation. Adult mammals, including mice, can’t regenerate hair cells when they are damaged. Neonatal (newborn) mice, however, still have the ability to regenerate for a brief period through the interaction of multiple pathways. Specifically, the hair cells in the neonatal mouse vestibular utricle, which is located in the inferior portion of the membranous

labyrinth, can mitotically regenerate (Groves et al., 2013). The supporting cells in the neonatal mouse cochlea are capable of re-entering the cell cycle and undergoing mitosis. In this immature cochlea, *Lgr5*-positive supporting cells have shown they have Wnt-responsive progenitor cell characteristic and have limited ability to generate new hair cells. However, as the cochlea matures, the Wnt response changes in these cells, losing the ability to regenerate hair cells after damage (Jansson et al., 2015). Neonatal cochlear supporting cells can regenerate hair cells upon inhibition of the Notch pathway, overexpression of β-catenin, or ectopic Atoh1. Overall, there is a limited time window for hair cell regeneration in the neonatal mouse cochlea (Cox et al., 2014). The Wnt/ß-catenin and Notch pathways, details described previously, are the most impactful pathways regarding hair cell regeneration. Therefore, the analysis on the similarities and differences between chicken, zebrafish, and mice hair cell regeneration mechanism will be focused on these pathways.

Comparison

While the chicken, zebrafish, and mice auditory/sensory systems have differences, there are similarities that make them all great models for comparison. Every anatomical model system has hair cells and non-sensory supporting cells. When regeneration occurs in all systems, new hair cells originate from the supporting cells through mitosis, but avian supporting cells can regenerate hair cells through direct transdifferentiation as well. The similar components of this hair cell regeneration include the signaling pathways: the Wnt/ß-catenin pathway, Notch pathway, and the basic helix loop helix (bHLH) proneural transcription factor gene, Atoh1. Each model includes the Notch pathway, which acts functionally the same. The function of the Notch pathway is to inhibit expression of Atoh1 analogs through lateral inhibition. This acts mainly as a way to prevent regeneration of hair cells when it is not needed, so we only see decreased Notch activation after damage to the hair cells. Therefore, we see that Atoh1, and Atoh1a in zebrafish, are increasingly expressed to induce mitosis, or transdifferentiation in chickens, of supporting cells. In each model, the Notch pathway interacts with Hes/Her/Hey factors, which are the transcription factors that directly inhibit Atoh1 expression through repressor or co-repressor activity. The Notch pathway becomes activated through ligands: Jagged (Jag) 1-2, Delta-like (Dll) 1, 3, 4 in mammals; DSL (Delta, Serrate/Jagged, LAG-2) family in zebrafish; and Notch1 in chickens. So, both the function of the Notch pathway and Atoh1 genes are common

throughout each model. The Wnt/ß-catenin pathway is found in each model; however, it directly affects hair cell regeneration in the chicken and mice. The biggest difference in signaling pathways is the presence of the FGF pathway in zebrafish. The Wnt/ß-catenin pathway regulates the formation of rosettes by modulating FGF signaling. Coincidentally, FGF signaling is required for proper specification of hair cell precursors by activating Atoh1a.

The biggest differences in each model system are their anatomical components, especially due to the lateral line system of zebrafish. This is wholly different than the organ of Corti and basilar papilla, which are located in the respective cochlea of the mammal and chicken. Since the anatomy is entirely different besides the innervation and the presence of supporting and hair cells, the focus will be on the chicken basilar papilla and the organ of Corti. In the basilar papilla, there are tall and short hair cells, which are assembled in the superior or inferior portion depending on their cell type. Short hair cells lie over the basilar membrane with changing stiffness following the changing stiffness of the membrane. Tall hair cells lie in the epithelial layer of the basilar papilla, however this epithelium doesn't move with sound stimulation. The basilar membrane does create a gradient with its thickness causing it to give rise to a wave that travels to the tall hair cells. The endolymph is the fluid that carries the vibrations in the basilar papilla as well, and the ionic gradient between the perilymph and endolymph is necessary for hair cell transduction. In the mammal, there is also an ionic relationship between endolymph and perilymph. In the organ of Corti, there are inner and outer hair cells, which are located at the edge of the osseous spiral lamina (inner hair cells) and on the basilar membrane (outer hair cells). The difference in the cell types, despite their locations in the different anatomical sensory structures, is their characteristics. The tall and short hair cells are named for the height of their stereocilia, but the outer and inner hair cells are named for their locations. Additionally, the inner and outer hair cells have different shapes. Both in the chicken and mouse, the hair cells have stereocilia linked together as bundles with supporting cells surrounding the hair cell bundles.

Despite all of these anatomical differences, the biggest difference is the ability of supporting cells in the chicken to re-enter the cell cycle to regenerate hair cells. The reason for this could be that there are multiple pathways that take part in this regeneration by assisting the progression of supporting cells through their cycle. Also, different factors including Wnts, epidermal growth factors, fibroblasts growth factors, and Notch molecules, influence the regulation of Atoh1 in the chicken basilar papilla. However, the main pathways being studied for

mammalian hair cell regeneration are the Wnt/ß-catenin and Notch pathways. In the mammalian auditory organ of Corti, all cells are terminally mitotic by embryonic day 14, while this is embryonic day nine for the avian basilar papilla. However, the cells in the avian basilar papilla still re-enter the cell cycle and proliferate after cochlear maturation (Stone and Rubel, 2000). Since adult mammals can't regenerate and adult birds can, it is understandable to expect that there is a discrepancy in the pathways in charge of inducing mitosis in the supporting cells. Regardless of whether a specific pathway is absent in the adult mammalian organ of Corti, it's possible that DNA won't allow for the transcription of genes involved in these signaling pathways. Further analysis of the different animal model systems will be conducted.

Mouse Variant Observation

While hair cell regeneration is not natural in adult mice, there are mouse variants that have been generated in order to develop different hair cell numbers/structure. Most mice that have been produced in research are those that have lost their hearing due to damage or destruction of hair cells. There aren't really any cases of mice with natural or artificial mutations that increase the number of hair cells. The reason for this observation of mouse variants is to understand the effects of genetic manipulation on the sensory hair cell. This will also show how hearing loss is caused by both damage and loss of hair cells.

Using the ethylnitrosourea (ENU) mutagen, the *samba* mutation was introduced into the Loxhd1 gene of a mouse, developing a *samba* mouse line. This specific mutation is a missense mutation, which means that a change in the DNA causes a change in the amino acid in the protein amino acid chain. The missense mutation in this Loxhd1 gene leads to congenital deafness. However, the mutation is homozygous recessive. Traditionally, the LOXHD1 protein has been evolutionarily conserved with at least 15 PLAT domains making up the entirety of the protein. A PLAT domain stands for polycystin/lipoxygenase/α-toxin and these domains are present in proteins with diverse functions. Mutations in these domains are common causes of human disease. We know that Loxhd1is expressed in cochlear hair cells and vestibular hair cells, along the stereociliary membrane. Due to it being expressed along the membrane, one function of the LOXHD1 protein may be the action of coupling the plasma membrane to the F-actin cytoskeleton of the hair cell. Since the LOXHD1 protein acts as a stabilizing protein for the stereociliary bundle after stereocilia maturation, then it's understandable to assume that the

missense mutation destabilized the structure of the protein and therefore destabilized the stereocilia (Grillet et al., 2009). While the mutation didn't affect the development of the hair cell, it did cause deafness shortly after birth due to the degeneration of the stereocilia.

One mouse that is used frequently in hearing research is the DBA/2J mouse. It's enticing qualities are that it has early onset and progressive hearing loss due to the *ahl* mutation of cadherin 23 ($Cdh23^{ahl}$) and the mutation of the ahl8 locus. Both, acting in combination, cause early onset hearing loss, but the mutation of cadherin 23 increases the susceptibility of age-related hearing loss in inbred mice. The specific gene that harbors the mutation of the ahl8 locus is fascin-2 and this encodes the FSCN2 protein, which links actin in the hair cell stereocilia. Also, the expression of FSCN2 in hair cell bundles temporarily lengthens the tallest row of the stereocilia. There is proof that FSCN2 also influences the growth of stereocilia by regulating actin treadmilling or elongation. Cadherin 23 is known for being a part of stereocilia tip links. If mice are homozygous for $Cdh23^{ahl}$ and ahl8, then hearing loss progresses more rapidly than if they were heterozygotic mice (Shin et al., 2010). Hair bundle loss occurs near the cochlear base and then moves apically, which follows the pattern of age-related hearing loss. There is eventual degeneration of stereocilia within hair bundles as well.

Thirdly, there is a spontaneous mutation called the jerker mutation, which is present in homozygous jerker mice. This mutation features a frameshift mutation in the espin gene on chromosome 4 in mice. This mutation causes the mice to lack espin proteins, while heterozygosity only produces loss of half of the espin. A frameshift mutation is either insertion or deletion of DNA bases, which shifts the DNA sequence so that the wrong amino acid gets put into the protein sequence during translation. Espin proteins bundle actin by binding to and cross-linking actin filaments into bundles in stereocilia of hair cells. They also produce a concentration-dependent elongation of actin bundles in cells. An inbred mouse strain, CBA/CaJ.JE/LeJ-$Espn^{je}$, has been produced to analyze the role of espins in jerker mice because CBA/CaJ mice normally exhibit minimal age-related hearing loss. In homozygous jerker mice, cochlear stereocilia shorten and disappear, which shows that espins allow for resistance to degeneration. The espin protein cross-links stabilize the parallel actin bundle against fragmentation and depolymerization (Sekerková et al., 2011). Based off of observations of the CBA/CaJ congenic jerker mouse line, the primary job of espins is morphogenesis and stabilization of hair cell stereocilia.

In a recessive mouse mutant, the whirler mutation causes mouse deafness. In homozygous whirler mice, inner hair cell stereocilia are half the length of heterozygotic mice and outer hair cell stereocilia are arranged in a ‘U’ rather than the normal ‘V’ or ’W’ shape. This sensory deficit in humans is caused by mutations in at least 100 genes. One novel autosomal recessive deafness locus (DFNB21) is homologous to the region where the whirler deafness gene is located in mice. Both short C-terminal and long isoforms of the whirlin protein in the cochlea have a role in stereocilia development and function. They also both contain PDZ domains, which interact with many proteins at semimembranal sites; harmonin and cadherin 23 interact with harmonin PDZ domains and PDZ-binding interfaces on cadherin 23 in stereocilia. The harmonin isoform is an F-actin bundling protein and provides a link between cadherin and actin (Mburu et al., 2003). It also interacts with Myosin VIIa, which act as a functional unit for cohesion of the stereocilia bundle. Overall, the key molecular component of stereocilia membrane and F-actin growth is a PDZ protein, whirlin. A defect in the whirlin protein causes the deafness in whirler mice.

These different mouse variants demonstrate that changes in the DNA sequence can lead to disastrous effects in stereociliary function and hair cell loss of the cochlea. If mutations like these can fully change the functional component of these mechanosensory cells, then there is a possibility that artificial mutations can be used to create the opposite outcome. We want to be able to regenerate any hearing loss due to damage to these hair cells, so the answer could lie in our own genetic code. If neonatal mice can regenerate their hair cells for a short time window, then there may be hope that the same pathways can be activated in the adult mouse and, therefore, in adult humans. In our experiment, we want to epigenetically change the DNA sequence of adult mice in order to re-activate pathways for hair cell regeneration. Although epigenetic mutation is different than direct mutations of the DNA code, the premise is the same, being that manipulating our own genetic code can lead to the answers of our most major weaknesses. Further analysis of mouse hearing loss variants will be conducted.

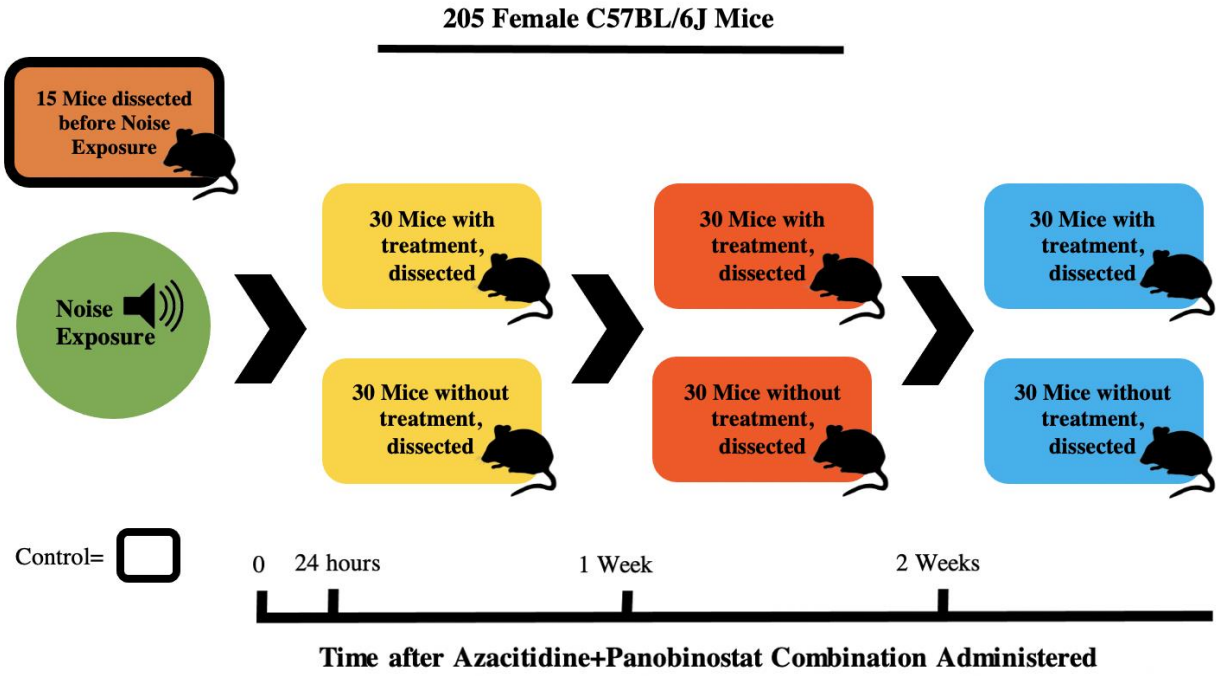

Figure 1. Schematic Representation of the Noise Exposure Experimental Process. A total of 205 female C57BL/6J mice will be the experimental group. Data values will be taken from 15 mice before the noise exposure at 16k Hz. The rest of the mice will undergo noise exposure. Then, after 24 hours of administration of the drug combination, 60 mice will be euthanized for analysis, 30 with drugs (15 at 114 dB and 15 at 118 dB), 30 who went without (15 at 114 dB and 15 at 118 dB). Then two more groups of 60 will be euthanized after one week and then after two weeks.

Methods for Mouse Model Noise Exposure Experiment

Animals

About 205 female C57BL/6J mice around 50-55 weeks old, each weighing around 30g, will be used in this study. There will be seven separate groups to take data from (Figure 1). Before any experimenting is conducted, 15 mice will be euthanized, and their cochlea dissected. The rest of the mice will undergo the noise exposure experiment. The next 30 mice will be euthanized 24 hours after the drugs are administered depending on the intensity of the sound they were exposed to (15 at 114 dB and 15 at 118 dB, for 16 kHz), and another 30 mice will also be euthanized at the same time, however they will not be given any drugs. Then 60 mice will be euthanized after one week of administration of the drug combination, with the same grouping as before. Finally,

another 60 mice will be euthanized after two weeks of administration of the drug combination with the same grouping. There will be 10 extra mice leaving room for any error.

Noise exposure

The 190 Mice will be anesthetized for three hours using a mixture of ketamine (50 mg/kg), atropine (0.05 mg/kg), and xylazine (10 mg/kg). Then each, in groups of six with 10 of the 30 chambers having groups of seven, will be placed into a sound attenuated chamber (AccuScan Sac/ss) for one hour of noise exposure. 190 mice will be exposed to one of two 16 kHz pure tones (114 dB or 118 dB for 2 hours each) from a Logitech S120 wired speaker (Figure 1).

Administration of drugs

The drug combination will include azacytidine and panobinostat. Azacitidine is a hypomethylating agent, which inhibits DNA methylation. Panobinostat is a histone deacetylase inhibitor, which inhibits the acetylation of histone proteins. The dose will be a combination of 2 mg/kg of azacitidine and 2 mg/kg panobinostat, which will be injected into the tail veins of the mice. Once administration of the drugs is finished for each mouse, a stopwatch will start to record the time after dosage.

Dissection

When the appropriate time marker is reached, then the mice in that group will be euthanized by carbon dioxide asphyxiation using a method consistent with the euthanasia guidelines of the American Veterinary Medical Association. The skin will be removed above the temporal bone with standard fine forceps and the temporal bone will be dissected away with the forceps and 10.5 cm fine scissors. The cochlea will be dissected from the otic capsule (inner ear) by pressing on the otic capsule and using the same scissors to assist in the release of the cochlea. Then the temporal bones will be fixed (placed in 2 ml microcentrifuge tubes containing 250 - 500 µl 4% paraformaldehyde diluted in 10 mM phosphate buffered saline pH 7.4) and decalcified (paraformaldehyde replaced with 120 mM ethylenediaminetetraacetic acid) in order to make it easier to cut into the petrosal portion of the temporal bone. Then, using #4 straight jeweler's forceps and 2.5mm Vannas spring scissors, we will cut into the oval window and make cuts along the spiral ligament of the cochlear basal turn. Once the basal turn is fully dissected, the

2.5mm Vannas spring scissors will be used again to separate the middle turn from the apical turn by cutting into the scala media and along the spiral ligament of the middle turn. The middle turn of the cochlea will be mounted on a slide for each mouse to make a whole-mount slide with ~50 µl of mounting media and a coverslip. When the whole mounts of the cochlear middle turn are finished, they will be sealed and stored in a slide box at room temperature until imaged.

Immunostaining

After dissection, each cochlea will be stained with antibodies for fluorescent imaging. During immunostaining, each cochlear turn will be submerged in ~500 µl of 10 mM PBS pH 7.4 of a well-plate and a stereo dissection scope will be used to make sure the turn won't be sucked into the 200 µl pipette. PBS will be removed from each well and replaced with ~200-300 µl per well of blocking/permeabilization solution (1% Triton X-100, 1% bovine serum albumin (BSA), and 10% normal goat serum (NGS) diluted in 10 mM PBS pH 7.4). Then the wells will be incubated for an hour at room temperature on a 3D rotator. The blocking/permeabilization solution will be removed using a pipette and will be replaced with ~100 µl per well of primary antibody solution (0.1% Triton X-100, 1% BSA, and 5% NGS diluted in 10 mM PBS pH 7.4). The wells will be incubated overnight at four degrees Celsius on a 3D rotator. Using a micropipette, the primary antibody solution will be removed, and the wells will be washed three times with 10 mM PBS pH 7.4 at ~500 µl per well. Each wash will be incubated for a minimum of five minutes at room temperature on a 3D rotator. The last PBS wash should be removed and replaced with ~100 µl per well of secondary antibody solution (0.1% Triton X-100, 1% BSA, and 5% NGS diluted in 10 mM PBS pH 7.4).

Each antibody will be diluted by a certain factor: rabbit anti-myosin VIIa primary antibody (1:200 dilution), goat anti-Sox2 primary antibody (1:500 dilution), donkey anti-goat Alexa 568-conjugated secondary antibody (1:1,000 dilution), donkey anti-rabbit Alexa 488-conjugated secondary antibody (1:1,000 dilution). To protect the fluorescent tags from the light, the well plate will be placed inside a black box, followed by incubation for two to three hours at room temperature on a 3D rotator. Anytime the well plate is resting without being used, it will be inside of the black box for protection. The secondary antibody solution will be removed and then three washes of 10 mM PBS pH 7.4 at ~500 µl per well will be performed. Each wash will be incubated for a minimum of five minutes at room temperature on a 3D rotator. The last PBS

wash will be removed and then replaced with ~100 µl per well of Hoechst 33342 (diluted 1:2,000 in 10 mM PBS pH 7.4) to label nuclei. Then the wells will be incubated for 15 - 20 minutes at room temperature on a 3D rotator. The Hoechst solution will be removed and then another three washes of PBS will be performed. Then the well plate will be incubated for a minimum of five minutes at room temperature on the 3D rotator.

Cellular imaging and analysis

Whole mount images of immunofluorescence will be taken using a Zeiss LSM 700 confocal microscope with 405, 488, and 555 wavelengths, at x20 objective. Additionally, images at the x60 oil objective can be used to get higher magnification of individual areas. The combined rabbit anti-myosin VIIa primary antibody and donkey anti-rabbit Alexa 488-conjugated secondary antibody will label hair cells as magenta. The combined goat anti-Sox2 primary antibody and donkey anti-goat Alexa 568-conjugated secondary antibody will label the supporting cells as green. The Hoechst stains the nuclei blue. The images can be overlaid to create a more complete picture of the cochlear turn.

Hair cell and supporting cell counts

Using the immunofluorescence images at the x20 objective, cell counts can be performed. ImageJ 1.44 software and the cell counter plug-in will be utilized to count from the images. Both hair cells and supporting cells will be counted. Regions are randomly chosen, and a 12x12 grid will be overlaid on the image. Only regions that totally encapsulate the cochlear epithelium will be counted and 15 regions will be counted. Then averages will be taken across all 15 regions per cochlear turn. Using ImageJ, the area will be computed to figure out the percentage of epithelial area that was sampled. The x60 objective images will be used to make hair cell and supporting cell densities (cells/μm^2) in the area that the image entails. Then cell counts per turn are estimated by multiplying average cell density by average cochlear turn area measured in the slide. Only cells that are fully magenta will be counted as hair cells and only cells that are fully green will be counted as supporting cells.

Graphs and statistical analyses

There will be at least five graphs produced to showcase different comparisons of data: average cell counts between supporting and hair cell counts from mice who had drugs administered, average cell counts between hair cells before noise exposure and after drug administration, average cell counts between supporting cells before noise exposure and after drug administration, average cell densities of the cochlear middle turn from all time points with drugs, average cell densities of the cochlear middle turn of mice with drugs and without drugs. Statistical analyses of the data will be conducted to determine significance in hair and supporting cell count before noise exposure and after drug administration. Additionally, comparisons between mice with drugs and without drugs will be analyzed to see how much of an effect the drugs have on the regeneration, if any. The data from mice who were exposed to the different noise intensities will be analyzed to observe how noise intensity affects amount of depletion regeneration of cells.

Budget

To carry out this project, all of the materials mentioned in the methods will be bought. There are several materials needed for the noise exposure portion of the experiment: 205 female C57BL/6J mice around 50-55 weeks old, 30 AccuScan Sac/ss sound attenuating chambers, 30 Logitech S120 Wired Speakers, two carbon dioxide tanks, stop watches, ketamine, atropine, and xylazine. Azacitidne and Panobinostat will be bought for administration in the tail veins of the mice. There are materials needed for the dissection of the mice to extract the cochlea: five standard fine forceps, five 10.5 cm fine scissors, slides, coverslips, mounting media, five boxes of 200µl micropipettes and tips, five #4 straight jeweler's forceps, five 2.5 Vannas spring scissors, a slide box, a box of 2ml microcentrifuge tubes, 10 mM PBS pH 7.4, 4% paraformaldehyde, and 120 mM ethylenediaminetetraacetic acid. The final materials needed are for immunostaining of the hair cell mounts: 1% Trion X-100, a 3D rotator, 1% bovine serum albumin, 10% normal goat serum (NGS), a black cardboard box, a well-plate, Hoechst 33342, a Zeiss LSM 700 confocal microscope, and a stereo dissection scope. In total, the materials needed for the methods will be about $103,047.16 (Table 1). Additionally, three undergraduate students, five graduate students, and one postdoctoral researcher will be paid each year as staff. Undergraduates will be paid $15/hour, graduates will be paid $45,000/year salary, and the postdoctoral researcher will be paid

$70,000/year salary (Table 1). Meanwhile, I will only be paid two months out of the year, each year. We will need theoretically $896,187.16, however, a budget of $1,000,000 will provide us with the most optimal results.

Table 1. Summarized Layout of Experiment Budget. In year one, the materials for the experiment will be bought, but there will be the same number of staff each of the two years. Materials from the first year will be used in the second year as well.

Year	**Budget**	
1	Materials	$103,047.16
	3 Undergraduates	$84,870
	5 Graduates	$225,000
	1 Postdoc	$70,000
	Casie Grogan	$16,700
Total		**$499,617.16**
2	3 Undergraduates	$84,870
	5 Graduates	$225,000
	1 Postdoc	$70,000
	Casie Grogan	$16,700
Total		**$396,570.00**
Total budget:		$896,187.16

Broader Impacts Statement

On top of furthering the research towards a treatment for noise induced hearing loss, this project will broaden its effects through a summer course for high school students focused on developing lab techniques and skills. In this program, students will learn how to use stereo dissecting and confocal microscopes, micropipettes, centrifuges, and how to prep slides. There will also be an introduction to cell counting and immunostaining with imaging using a ZOE fluorescent cell imager. This course will hopefully encourage more students to pursue careers in microbiology/biology and become the future of scientific research. Additionally, there will be a

public outreach campaign to fund summer camp programs, specifically designed for kids ages eight to fourteen to explore biology and nature. This will be a science camp to spark an interest in life science in young kids, while also teaching kids the basics of biology, including cells and ecosystems. There would also be a portion of the program focusing on human anatomy, where kids will learn the functions of the organ systems and how they interact with each other. For the older kids, there will be dissection days, but kids of all ages will be able to go on nature hikes and field trips to develop an appreciation for the environment.

To collaborate with the arts and to create community engagement, this project will host multiple events to create a mural focusing on awareness for the hard of hearing community and hearing loss, in general. The mural will bring together people of all ages to brainstorm creative ideas and paint the mural on the side of a community building. The mural will display the beauty in human nature and the perseverance of those with hearing loss. This will bridge multiple sections of the community and bring hearing loss to light in a way that builds a passion for funding time and money into ways to fix onset hearing loss, age-related or noise induced.

Overall, this project isn't just about finding ways to fix hearing loss, but it is about furthering scientific progress through research and the future generations. High schoolers will find that learning useful lab skills in the summer can give them the edge in their college career. Young kids may find their passion for life through nature and the human body, while also creating lasting memories in summer camp. Communities will come together for a greater cause and unite the arts and science with the painting of a mural that will last for years to come. Ultimately, we want to create an impact in multiple ways, but our project goal is to find a treatment for onset hearing loss to aid future generations.

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