ABSTRACT

Endoplasmic Reticulum Stress and Noise-Induced Hearing Loss

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Hearing loss is influenced by many environmental factors, and it greatly affects the quality of life. A specific sensorineural form of hearing loss is noise-induced hearing loss (NIHL). Excessive noise exposure causes damage to sensitive structures within the inner ear resulting in NIHL. The cochlea of the inner ear is extremely vulnerable to stressful conditions. The noise-induced damage is most apparent in the outer hair cells (OHCs) of the cochlea. Noise exposure results in hair cell injury and death, which is most notable in the OHCs due to their crucial role in the amplification of sound for hearing. Noise exposure can induce endoplasmic reticulum stress (ER stress) in cells by oversaturating cells with misfolded and unfolded proteins in the endoplasmic reticulum. Here we identify that elevated noise exposure induces ER stress in the OHCs leading to the onset of the unfolded protein response (UPR), which upregulates certain molecular components that activate apoptosis and inflammatory pathways.

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ENDOPLASMIC RETICULUM STRESS AND NOISE-INDUCED HEARING LOSS

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PREFACE

My education has led me to develop unique habits of thought manifested in every dimension and modality of learning. Here, I absorbed knowledge through thinking and reasoning which has allowed me to become more confident in my thoughts. My desire to learn did not end in the classroom as it expanded to include opportunities that have enhanced my knowledge. Seeking to further my understanding of biology, I became an undergraduate research assistant in Dr. Simmons' lab at Baylor University. The lab gave me a place to grow my intellectual abilities.

The desire for this research work has grown from my interest in biological research. The Simmons Lab at Baylor University has offered me the time and space to grow intellectually, feed my curiosity, and delve deeper into my study of biology beyond the classroom setting. The lengthy process involved tremendous work and dedication, which would have been impossible without my support system of family, friends, and mentors.

Thank you to all the members of the Simmons Lab at Baylor University for the stimulating discussions and encouragement. Thank you to my family and friends for their support.

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Finally, I would like to thank my parents and brothers for their unconditional love and support throughout the completion of my thesis. Thank you for always being there for me.

CHAPTER ONE

Introduction

Noise-induced hearing loss (NIHL) is a result of persistent or prolonged exposure to elevated noise levels causing damage to sensitive structures within the inner ear. The damage to the inner ear affects sensorineural capabilities (Wu et al., 2020). This is due to the increased sensitivity of the sensory hair cells of the cochlea to noise exposure. Of the two types of cochlear hair cells, the outer hair cells (OHCs) are extremely selective and sensitive to stress. Noise exposure can induce stress, specifically endoplasmic reticulum stress (ER stress) in the OHCs. Stressful conditions can lead to hair cell injury and the death of predominantly OHCs.

ER stress can lead to the onset of the unfolded protein response (UPR) in stressed cells to assist in the proper folding of proteins. The UPR plays a role in increasing the presence of certain inflammatory, repair, and pro-apoptotic components. Our experiments focus on only a few of the many components involved in apoptosis, inflammation, and repair pathways. The components we studied are CHOP, Manf, and macrophages. The increased expression and abundance of these components activate two distinct pathways, which are apoptosis and inflammatory pathways. The activation of inflammatory responses leads to an infiltration of immune cells, such as macrophages, to address cell injury in stressed tissues. Likewise, the increase in pro-apoptotic components such as CHOP activates apoptosis in damaged hair cells. Apoptosis is programmed cell death. The two distinct pathways activated by ER stress can lead to the increased presence of components associated with inflammatory pathways and apoptosis. When studying ER

stress, Manf appeared to play a significant role in attenuating apoptosis. This may be justified by the increased presence of Manf seen in OHCs of noise-exposed mice. As these OHCs are undergoing ER stress, the presence of Manf increases to address the hair cell injury and activate autophagy. Autophagy is the natural degradation of cells, which involves the breakdown of various other cytoplasmic components (Glick et al., 2010). By serving a housekeeping role, autophagy removes misfolded or aggregated proteins, pathogens, damaged mitochondria, and damaged endoplasmic reticulum (Glick et al., 2010). Though autophagy entails the natural degradation of intracellular components, it serves an important role in repair pathways by ridding the cells of debris from stress-induced injury. There is crosstalk between apoptosis and autophagy, which is determined in response to specific stress. Further research would be needed to address the crosstalk between the two cellular pathways in response to stress from noise exposure. However, ER stress can be induced by noise exposure and lead to the activation of two distinct pathways of apoptosis and inflammation.

Considering the sensitivity of OHCs to noise, we hypothesized that noise exposure will induce endoplasmic reticulum stress (ER stress) within the hair cells leading to the onset of unfolded protein response (UPR) and eventually apoptosis resulting in hair cell death. Previous studies have shown that noise exposure can induce inflammatory pathways and stress conditions injuring hair cells which may result in hair cell death. We exposed adult Oncomodulin wildtype (Ocm WT) and adult Oncomodulin knock-out (Ocm KO) mice, aged 2 to 7 weeks, to broadband noise at an elevated noise level to induce stress effects of noise. Our experiments were focused specifically on ER stress. We performed immunofluorescent experiments, confocal imaging, and Fiji/Image

J analysis of adult Ocm WT and adult Ocm KO mice cochlea post-noise exposure. Our results suggest that noise exposure induces ER stress in the OHCs of the cochlea due to the upregulation of inflammatory and repair pathways such as UPR and pro-apoptotic components.

CHAPTER TWO

Background

Auditory System & Physiology of the Ear

The auditory system allows organisms to hear and understand sounds within the environment. Through constructing a perceptual space that takes information from objects and groups, segregating sounds, and providing meaning and access to communication tools, the auditory system serves as the sensory system for hearing (Litovsky, 2015). It is comprised of the central auditory nervous system and peripheral structures which consist of the outer, middle, and inner ears. The outer ear consists of the auricle and auditory canal. The middle ear is made up of the tympanic membrane known as the eardrum, and three small bones called the ossicles. The ossicles are the malleus, incus, and stapes. The inner ear consists of the vestibular organs and the cochlea, which is the central focus of this thesis.

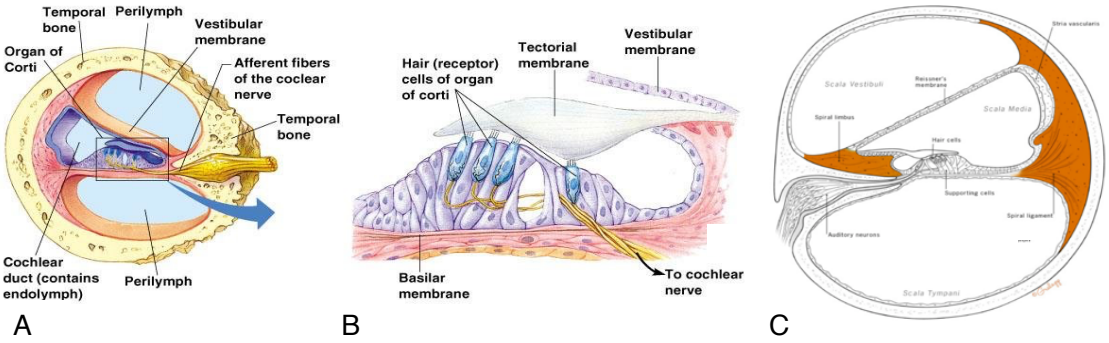

Figure 1. Important Structures of the Cochlea. The structures surrounding the organ of Corti are depicted in (A). The inner hair cells (IHCs) and outer hair cells (OHCs) of the organ of Corti are illustrated in (B), along with the tectorial membrane and innervation from the cochlear nerve. The important structure of the cochlea that will be focused on in our results are presented in (C), such as the spiral limbus, stria vascularis, and spiral ganglion (labeled as auditory neurons in the image). The images of (A) and (B) are adopted from Ilik's 2018 Master's thesis "MEMS THIN FILM PIEZOELECTRIC ACOUSTIC TRANSDUCER FOR COCHLEAR IMPLANT APPLICATIONS." The image in (C) is adopted from Verdoodt et al.'s 2021 paper "On the pathophysiology of DFNA9: Effect of pathogenic variants in the COCH gene on inner ear functioning in human and transgenic mice."

Energy waves produce sound. The sound waves reach the auditory canal and travel down the external acoustic meatus to the tympanic membrane. These sound waves create pressure which moves the tympanic membrane and initiates the vibration of the middle ear ossicles. The malleus is the first ossicle to receive vibrational pressure. Next, the vibration is passed onto the incus. Then, the incus passes on the vibration to the stapes. During this chain of vibrational pressure among the ossicles, each small bone plays a role in amplifying the sound waves. The amplification of sound is achieved by the ossicles working together as a lever system to generate a mechanical advantage. The lever action of the middle ear ossicles is necessary to generate forces that amplify sound so humans can hear. Once the ossicles complete their role, the sound energy is in a mechanical force of waves that passes through the oval window into the cochlea.

The oval window serves as a passage between the middle ear and inner ear. In the fluid-filled cochlea, the mechanical energy of the sound wave is converted to electrical energy by sensory hair cells. The cochlear hair cells transduce the fluid pressure waves (Henkel, 2018). This action activates the auditory components of the vestibulocochlear nerve, known as cranial nerve VIII (Henkel, 2018). The cochlea consists of three sections, which are the scala tympani, scala vestibuli, and scala media. The organ of Corti resides on the basilar membrane located inside the scala media. The sensory hair cells are housed in the organ of Corti (Figure 1). The stereocilia of the hair cells are connected to the tectorial membrane within the scala media. As oscillations occur in the scala of the cochlea, it shifts the stereocilia of the hair cells which open or closes mechanically gated channels. These channels play a role in activating or deactivating the hair cells via cell depolarization. This converts the sound waves from mechanical energy to electrical energy in the cochlea.

Hearing Loss

Hearing loss is a burden to many across the world, as it is the fourth leading cause of disability. Affecting approximately 5.3% of the world's population, it is the most common sensory defect in humans (Gonzalez-Gonzalez, S., 2017). In the United States, hearing loss doubles with every 10 years of aging (Cunningham and Tucci, 2017). Humans typically hear between a frequency range of 20-20,000 Hz (Peterson et al., 2021). The two categories of hearing loss are conductive hearing loss and sensorineural hearing loss. Conductive hearing loss is caused by impairment of the outer and middle ear. Sensorineural hearing loss is a result of dysfunction in the cochlea or spiral ganglion.

There are various types of acquired hearing loss such as ototoxic drug-induced hearing loss (ODIHL), age-related hearing loss (ARHL), and noise-induced hearing loss (NIHL). Each type of hearing loss has its own pathology in the ear. ODIHL is hearing loss due to exposure to certain drugs that induce conditions such as ototoxic stress. It is driven by cochlear sensory hair cell death (Wu et al., 2020). ARHL is the loss of hearing with age. This type of hearing loss results in the loss of hair cells, spiral ganglion, and vascular striated cells (Wu et al., 2020). Noise-Induced Hearing Loss (NIHL) is due to excessive or overexposure to noise (Wu et al., 2020), which can lead to cochlear hair cell injury and death.

Noise exposure can create many concerns regarding the function of hearing. Overexposure to noise can be a major cause of noise-induced hearing loss and perceptual difficulties such as speech-in-noise difficulties, tinnitus, and hyperacusis (Boero et al., 2018). In terms of high-frequency energy, it is defined as 5.7 to 22 kHz in human speech and vocal communication (Monson et al., 2014). Humans begin to lose hearing in the higher frequency regions, which are associated with the basal regions of the cochlea (Cunningham et al., 2017). This implies that speech and hearing are affected by high frequencies leading to speech-in-noise difficulties. Tinnitus is a high-pitched ringing experienced in one or both ears; this ringing may be due to exposure to elevated noise levels. Hyperacusis is defined as a disorder of loudness perception (Baguley, 2003). The dynamic of hyperacusis presents serious challenges to individuals as they have an unusual tolerance to ordinary sounds in the environment. These ordinary sounds relay exaggerated or inappropriate responses that would not be seen in an individual without hyperacusis. Thus, hyperacusis is a type of hearing hypersensitivity that creates

difficulties for individuals in an environment with elevated noise exposure. This may be explained by loudness recruitment, which is described as experiences when humans perceive loudness faster than normal with increasing sound due to cochlear outer hair cell injury and loss.

NIHL is a sensorineural hearing loss that stems from high vulnerability to noise. Several studies allude to the idea that inflammatory pathways may be upregulated with noise. Mice of C57BL/6 and BALB/c backgrounds are quite easily susceptible to permanent NIHL with a minimum of 10 minutes of exposure to broadband noise at 110dB SPL (Herranan et al, 2020).

The Functions of the Inner Ear

The inner ear is responsible for detecting and processing electrical signals. It serves two main functions in sound detection and body balance (Sundar et al., 2021). The two functional parts of the inner ear are the cochlear and vestibular systems (Sundar et al., 2021). The vestibular system is responsible for the function of balance. The cochlea is responsible for the function of hearing.

There are special sensory cells in the cochlea that play a significant role in hearing (Figure 1). Hair cells are specialized sensory cells of the cochlea that serve in converting mechanical signals produced by sound waves into electrochemical signals through fluid waves (Zhao and Müller, 2015). There are two types of hair cells, outer hair cells (OHCs) and inner hair cells (IHCs), involved in sound transduction into electrical responses (Tong et al., 2016). The sensory hair cells reside in the organ of Corti (Peterson et al., 2021). OHCs are sensory hair cells in the organ of Corti which are responsible for

amplifying the sound-induced vibrations (Climer et al., 2019) of the cochlear partition which directly enhance sensitivity and frequency selectivity (Tong et al., 2016). The OHCs are targets of noise and aging, leading to elevated hearing thresholds and loss of cochlear frequency tuning when damaged (Tong et al., 2016). There are three rows of OHCs in the organ of Corti (Peterson et al., 2021). Inner hair cells (IHCs) are the second type of sensory hair cells in the organ of Corti, which are responsible for neurotransmission of sound stimuli directly to cochlear nerve fibers (Climer et al., 2019). There is a single row of IHCs in the organ of Corti (Peterson et al., 2021).

The level of inner hair cells (IHCs) and auditory nerve neurons demonstrates the adaptation mechanism used by sensory systems to enhance a range of responses to incoming stimuli (Goutman, 2017). The adaptation site is represented by the synapses between the inner hair cells and the dendrites of afferent auditory neurons. This is responsible for converting graded acoustic signals into trains of action potentials. The depletion of releasing vesicles and desensitization of postsynaptic receptors can induce an early depression of synaptic response and lead to synaptic decay (Goutman, 2017). The presynaptic and postsynaptic mechanisms contribute to this depression which may impart features of auditory nerve function.

IHCs are present in the cochlea and act as mammalian phono-receptors as they transduce sound energy into graded changes in membrane potentials. There are ribbon synapses that exist between IHCs and afferent neurons that are responsible for this role (Goutman, 2017). The desensitization of postsynaptic receptors has a twofold effect as it abbreviates signaling between IHCs and afferent auditory nerves but also balances differences in decay kinetics between responses to different stimulation strengths

(Goutman, 2017). There are presynaptic determinants of synaptic depression that influence these effects.

The presence and active roles of IHCs and OHCs are crucial to maintaining the functional capabilities of the cochlea. The sensory hair cells of the cochlea influence the inner ear's role in hearing.

Calcium Signaling and Oncomodulin

Calcium plays a major role in the mechanisms of the ear. It is a key component in mechanoelectrical transduction, cochlear amplification, and synaptic function (Tong et. al, 2016). The levels of Ca^{2+} in the hair cells are controlled by a multitude of proteins that serve as buffers, sensors, and pumps for calcium. Ca^{2+} homeostasis is linked with hearing loss in addition to an increased free Ca^{2+} concentration in OHCs following acoustic overstimulation (Tong et. al, 2016). The hair cells contain mobile EF-hand Ca^{2+}-binding proteins that act as buffers in Ca^{2+} signaling and homeostasis. Such proteins have a common primary structure of 30 residues in their sequence. The residues form a helix-loop-helix motif that undergoes a conformational change when bound to Ca^{2+} and relays interactions with downstream proteins.

Oncomodulin (Ocm) is a small EF-hand, specifically the helix-loop-helix structural domain of the protein, Ca^{2+} binding enigmatic parvalbumin protein (Climer et al., 2019). There are other EF-hand calcium-binding proteins present in addition to Ocm such as calmodulin, calbindins, calretinin, and sorcin (Climer et al., 2019). The presence and role of oncomodulin (Ocm) are significant in the study of acquired hearing loss due to changes in cochlear function. In mammals, it is present in the beta isoform with a

restrictive post-embryonic expression pattern that limits its presence to sensory hair cells in the inner ear and certain immune cell subtypes.

The presence of Ocm is limited to the cochlear OHCs, vestibular hair cells, and macrophages (Tong et. al, 2016). The expression of Ocm is predominantly restricted to other hair cells (OHCs) in the adult mammalian cochlea. The proposed models for Ocm function are in hearing through calcium signaling pathways present in OHC function and nerve regeneration. It is suggested that Ocm expression decreases with age because the presence of calcium-binding proteins has diminished within vestibular tissues. The expression of Ocm begins postnatal day two but becomes significantly reduced in vestibular tissues as the animals age (Simmons et al., 2010). The research examines the Ocm labeling pattern in calcium-dependent processes that affect the motor function of the outer hair cells.

Stress Pathways: ER Stress and Programmed Cell Death

Endoplasmic reticulum stress, or ER stress, can induce various pathways such as the unfolded protein response (UPR), inflammatory responses, repair pathways, and more. Cells undergo ER stress when the amount of misfolded or unfolded proteins saturates and exceeds the capacity of the endoplasmic reticulum. This places the cells under stressful conditions that lead to the onset of several different pathways. ER stress can be caused by many factors that impair protein glycosylation or disulfide bond formation, or by overexpression of or mutations in proteins entering the secretory pathway. These factors can be detected by measuring levels of specific UPR factors, as seen in Figure 1. Endoplasmic reticulum machinery is a major regulator of intracellular calcium homeostasis (Herranen 2020). ER stress is associated with elevated levels of

cytoplasmic calcium. Thus, outer hair cells in high-frequency regions of the basal part of the cochlea are more sensitive to Manf inactivation (Herranen et al., 2020).

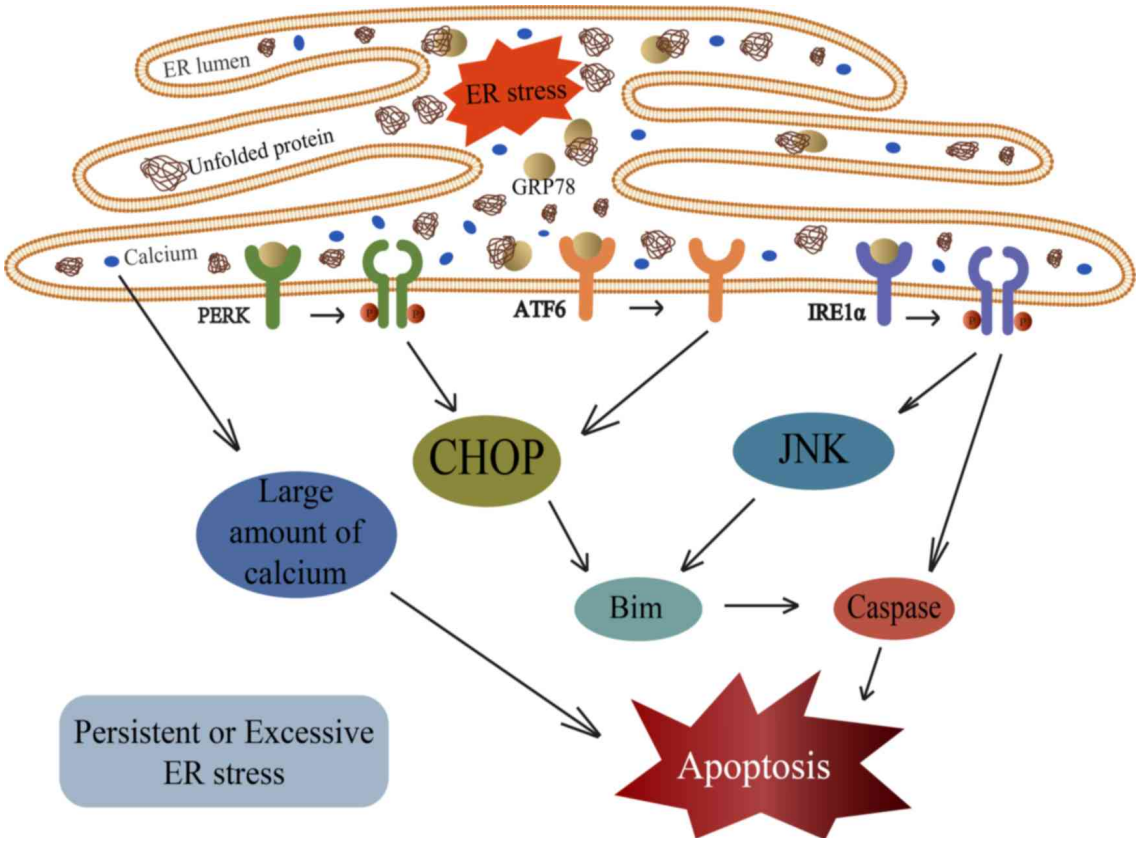

Figure 2. Schematic of unfolded protein response (UPR) inducing apoptosis. Cells undergo ER stress when the protein folding process is disrupted as it results in the accumulation of unfolded and misfolded proteins in the ER. During ER stress, the UPR is activated to help minimize unfolded protein overload. The three branches of UPR illustrated in this schematic are PERK, ATF6, and IRE1. If UPR is unsuccessful under ER stress conditions, it may lead to the upregulation of pro-apoptotic components such as CHOP and excess calcium which can induce apoptosis. This figure is adopted from Mao et al.'s 2019 paper "Role of endoplasmic reticulum stress in depression.

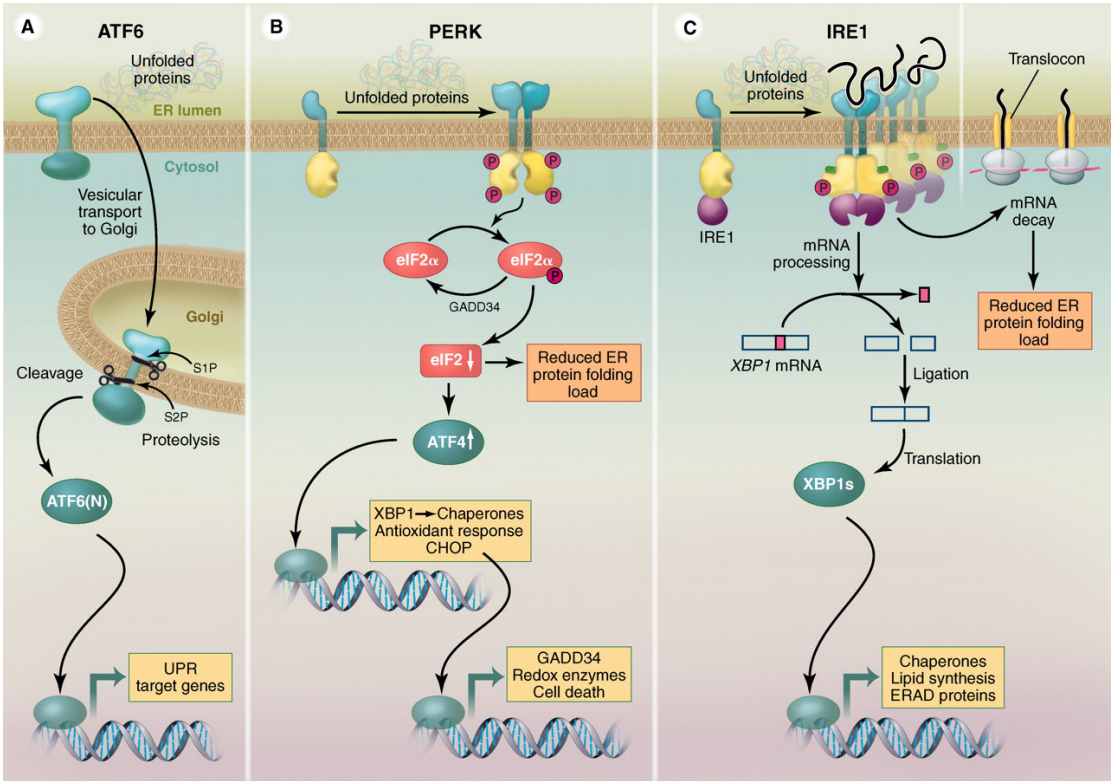

Figure 3. Schematic of unfolded protein response (UPR) under stressful conditions. There are three branches of the UPR. Each branch has its own family of signal transducers, ATF6, PERK, and IRE1, which sense protein-fold-conditions in the ER. The signal transducers transmit information, which results in the production of bZIP transcription regulators. The bZIP transcription regulators enter the nucleus and drive the transcription of the UPR target genes. (A) depicts the UPR pathway via the signal transduction mechanism of ATF6 in the regulation of proteolysis. (B) displays the signal transduction mechanism of PERK via translational control in the UPR pathway. PERK reduces the ER protein folding load by down-turning translation. (C) portrays nonconventional mRNA slicing via the signal transduction pathway of IRE1 in the UPR pathway. IRE1 reduces the ER folding load by degrading the ER-bound mRNAs, which can help attenuate prolonged ER stress. These are examples of transcriptional responses that increase the protein-folding capacity in the ER. (A), (B), and (C) have been adopted from Walter and Ron's 2011 paper "The Unfolded Protein Response: From Stress Pathway to Homeostatic Regulation."

ER stress is characterized by the accumulation of misfolded and unfolded proteins, as well as the disruption of calcium and redox balances (Wu et al., 2020). The misfolded and unfolded proteins trigger the unfolded protein response, known as UPR. UPR is a cellular stress response related to ER stress. As seen in Figure 3, it is like an

adaptive reaction that reduced the unfolded protein load to maintain cell viability. Cells can cope with ER stress by increasing their expression of chaperones such as GRP78 and PDI and enhancing ER-associated degradation of misfolded proteins via UPR (Figures 2 and 3). By increasing the expression of proteins involved in ER function such as ER chaperones like GRP78 and PDI, they can assist with properly folding proteins (Figures 2 and 3). Calcium and redox balances affect hearing loss as calcium signaling plays a predominant role in the function of outer hair cells which serve to amplify sound in the cochlea for hearing (Figure 4).

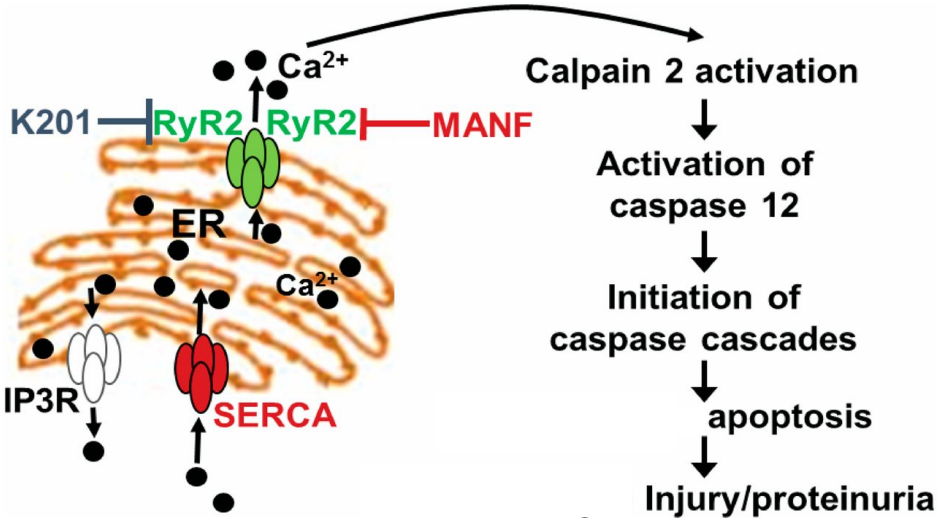

Figure 4. Schematic of Manf and apoptosis. This figure depicts how calcium depletion in the ER can induce apoptosis and injury but may be inhibited by Manf. RyR2 is a specific ryanodine receptor that serves as a cytoplasmic scaffold for regulatory proteins. Cells undergoing ER stress experience hyperphosphorylation of RyR2 channels which releases aberrant ER calcium that can induce apoptosis and injury to the cells. As shown in the schematic, Manf blocks ryanodine receptors from releasing excess calcium which can aid in the inactivation of caspases and other pro-apoptotic components that lead to cellular apoptosis and injury. This figure is adopted from Park et al.'s 2019 paper "Discovery of endoplasmic reticulum calcium stabilizers to rescue ER-stressed podocytes in nephrotic syndrome."

However, if stress pathways activated by ER stress prolong, then this may induce pathways of programmed cell death. This includes mechanisms involved in apoptosis, autophagy, and programmed necrosis (Figures 2 and 4). Programmed cell death plays a critical role in the development and diseases of the cochlea (Wu et al., 2020). Apoptosis is an ATP-driven, highly ordered death of cells. More specifically, apoptosis is defined as programmed cell death which is highly active and regulated by genes including Bcl-2, p53, and c-Jun, as well as enzymes such as caspases and endonuclease G (Wu et al., 2019). The process of apoptosis occurs through intrinsic, extrinsic, and endoplasmic reticulum pathways. The intrinsic pathway of apoptosis occurs through the mitochondria and the extrinsic pathway occurs using a death receptor. The ER pathway involves the endoplasmic reticulum and calcium release alongside caspase activity.

Important Proteins: Antibodies

msCHOP

CHOP, C/EBP homologous protein, is an ER stress marker. More specifically, CHOP is a pro-apoptotic component of unfolded protein response (UPR). Outer hair cell death has revealed upregulation of CHOP alongside increased UPR. CHOP is a downstream transcription factor in UPR. Severe ER stress can induce the expression of CHOP. This can lead to ER stress-related apoptosis based on the downregulation of the expression of anti-apoptotic proteins, like Bcl-xL (Wu et al., 2020) and Manf (Xu 2019). CHOP has shown to have an increased presence preceding OHC death as they are upregulated in the OHCs of conditional knock-out (KO) mice cochlea (Herranen et al.,

2020). Thus, prolonged ER stress can activate a pro-apoptotic branch of UPR involving CHOP as a downstream component of the pathway (Herranen et al., 2020).

msCoxIV

Cytochrome c oxidase subunit 4, known as CoxIV, is a mitochondrial protein that is essential for mitochondrial maintenance and viability. It serves as a mitochondrial stress marker. CoxIV is the last electron transport chain protein in the mitochondria (Guan et al., 2019). This protein is involved in oxidative phosphorylation. The release of CoxIV is initiated by apoptosis and caspase release. The levels of CoxIV can be disrupted by exposure to toxic agents or elevated noise levels, which impairs the functioning of mitochondria (Guan et al., 2019). A disruption in CoxIV levels impairs mitochondria function by creating mitochondrial stress. Exposure to elevated noise levels may induce oxidative stress that originates from the mitochondria (Yuan et al., 2015). Since CoxIV is involved in oxidative phosphorylation, the levels of CoxIV can be impacted by noise exposure inducing mitochondrial stress.

rbIBA

Ionized calcium-binding adaptor molecule 1, known as IBA1, is a cytoplasmic calcium-binding protein that serves as a microglial marker (O'Malley et al., 2016). It labels resident macrophages of the central nervous system. The expression of IBA1 can be used to label the presence of macrophages. More specifically, IBA1 is a key participant in membrane processes associated with phagocytosis in macrophages and microglia (O'Malley et al., 2016). IBA1 engages in actin-cross-linking activity that plays a role in membrane motility and phagocytosis (O'Malley et al., 2016). Thus, IBA1 can

serve as a marker for resident macrophages in the cochlea (Liu et al., 2021). IBA1-positive cells have been associated with the spiral ligament, spiral limbus, spiral lamina, and stria vascularis (O'Malley et al., 2016). As a macrophage indicator, IBA1 can be crucial in assessing inflammation in cochlear tissue.

rbManf

The mesencephalic astrocyte-derived neurotrophic factor, Manf, is a 20 kDa nonconventional neurotropic factor. Neurotrophic factors (NTFs) regulate the life and death of neuronal and non-neuronal cells. Breaking down the name of the protein: mesencephalic is the midbrain, astrocytes are star-shaped glial cells in the central nervous system which form tight junctions and are responsible for the blood-brain barrier, and neurotropic refers to the growth of nervous tissue. It is an endoplasmic reticulum resident protein that is produced in the ER itself. Manf promotes ER homeostasis and may serve to protect against hearing loss induced by ER machinery stressors (Herranen et al., 2020). It serves a local function by promoting protein folding homeostasis; thus, it interacts physically with major ER chaperones like GRP78, which is a glucose-regulated protein also known as BiP.

In the cochlea, Manf plays a local function. It is expressed in sensory hair cells, neurons, and selected non-sensory cells of the cochlea such as Dieter cells/pillar cells. Manf is more heavily expressed in the inner hair cells and supporting cells, such as Dieter cells than compared to its expression in outer hair cells which is confined to the borders of the cells as that is where most of the ER congregates. Manf promotes outer hair cell survival and normal hearing function. Thus, the inactivation of Manf can trigger the upregulation of ER chaperones, such as GPR78, that assist in proper protein folding. The

major targets of Manf inactivation are outer hair cells. A study on Manf's significance in the cochlea has shown that Manf conditional knock-out (KO) mice have predominant outer hair cell loss from their previously complete hair cell population at postnatal day 12 (P12) to about 25% outer hair cell loss by the age of 5 to 11 weeks (Herranen et al., 2020). This is evidenced by consistently elevated auditory brainstem response thresholds, which measure the nerve signals stimulated by noise exposure recognition. Manf has been shown to promote cell survival through regulating the unfolded protein response that will thereby relieve ER stress (Danilova et al., 2019). The inactivation of Manf can trigger an upregulation of ER chaperones, which assist in proper protein folding. Ultimately, Manf inactivation can result in outer hair cell death.

CHAPTER THREE

Materials and Methods

Animal Use and Preparation

All mice selected for these studies were from the APV colony with a C57/mixed background (aged 2-7 weeks old). There was a total of 12 mice selected for these studies. This included six male Ocm WT mice and six male Ocm KO mice selected for these studies. Three Ocm WT mice and three Ocm KO mice were selected for noise exposure and the other mice remained as control subjects. Four conditions were considered in these studies. The four conditions are noise-exposure, non-noise exposure, biological sex, and genotype (Ocm WT or Ocm KO). All experimental procedures were approved by the Institutional Animal Care and Use Committee (IACUC). They were conducted according to the guidelines for Animal Research at Baylor University, Waco, TX.

Auditory Testing with Distortion Product Otoacoustic Emission Thresholds

To assess the effect of noise exposure, hearing levels were measured via Distortion Product Otoacoustic Emission Thresholds (DPOAE) pre-and post-noise exposure. DPOAEs is a standard test for evaluating animal hearing. A DPOAE serves as only an in-vivo measurement of OHC function. The animals are anesthetized with an intraperitoneal injection of a mixture of 80mg/kg ketamine and 15 mg/kg xylazine. If needed, one-fourth of the booster doses were given. Once the mouse was anesthetized, a 1 cm incision was made inferiorly near each ear between the cartilage of the tragus and anti-tragus. This is necessary to make the ear canal visible for measuring DPOAEs. A

cotton applicator was used to apply pressure if bleeding occurs during the incision. Following the incision, a topical analgesic such as lidocaine was applied to the wound with a cotton application, followed by alcohol. An antibiotic ointment was applied to the anesthetized mouse's eyes to prevent any dryness during the DPOAE. To begin the DPOAE, a probe containing mini speakers and a microphone was inserted into the ear canal. DPOAE measurements were taken for both ears at the following test frequencies: 5, 8, 11, 16, 22, 32, and 45 kHz.

Noise Exposure

The noise exposure was conducted in a dark soundproof noise box. The parameters of the noise box were set to a voltage of 0.5V for a duration of 120 minutes. The mice were exposed to 2 hours of broadband noise at a 110 dB sound pressure level (SPL). The current outside decibel measurement was 61dB SPL. The broadband noise allowed for a frequency range that was not just limited to 5 to 45 kHz, which includes the exact frequencies at which the DPOAE measurements were taken for each mouse. As broadband noise stems from an array of frequencies, it was more representative of the natural environment of humans.

Perfusion and Tissue Fixation

For tissue fixation, the animals were perfused with 4% paraformaldehyde (PFA). PFA is used to fix animal tissues and make them optimal for experimentation. First, the mice were anesthetized and euthanized with lethal intraperitoneal injections of sodium pentobarbital. The set concentration for these injections was 100-150 mg/kg of the mouse's weight. The mice's toes were pinched to confirm that the mice had no respiratory

or tactile reflexes. After this, the mice underwent transcardial perfusions with phosphate-buffered saline (PBS) and PFA. This was followed by decapitation and dissection of the right and left ears' cochlea.

Cochlear Whole Embedding

To embed the fixed cochlea, an agarose-gelatin embedding medium is necessary to prepare for tissue sectioning. An agarose-gelatin mixture was prepared to serve as the tissue embedding medium. First, a 50 mL conical vial was filled with 10 mL of water and 0.5 g of agarose. Another 50 mL conical vial was filled with 10 mL of water and 0.15 g of gelatin powder. Another 50 mL conical vial was filled with 4-5 mL of 1x Phosphate Buffered Saline (PBS). All three conical vials were loosely capped, to prevent the buildup of pressure, and placed into a beaker of boiling water heated by a hot plate. After five minutes, the conical vials were removed and tightly capped to be vortexed. Following the vortex step, the conical vials were loosed and placed back in the beaker of boiling water. Once the gelatin in the vial dissolved in the saline, the vial was vortexed again. Then, the saline-gelatin mixture was poured into the vial containing agarose and deionized water and placed back into the beaker of boiling water. After ten minutes, all the contents in the conical vial were dissolved. After the contents of the vial dissolved, the beaker of boiling water was removed from the hot plate. The beaker was set aside to cool with the conical vial still in the beaker of water. During this cooling period, 4-5 mL of the agarose-gelatin mixture was poured into the vial containing PBS until it reached the 10 mL mark line. The cooling process took approximately 5-8 minutes.

For tissue embedding, the fixed cochlea was placed in a concave orientation with the oval window visible, on a petri dish. First, 1 ml of the mixture from the conical vial

containing 50% 1x PBS and 50% agarose gelatin was perfused through the oval window of the cochlea. Immediately, the 50% perfused whole fixed cochleae were individually placed into a plastic embedding mold in the same orientation. Then, 3-5 ml of the agarose-gelatin embedding medium was pipetted in a circular motion on top of the cochlea laying on the bottom of the mold in the same concave orientation. Afterward, the mold was set aside for the embedding medium to set. This setting process took approximately 20-25 minutes. After the mold was set, the mold was placed into a 50 ml conical vial of 1x PBS and placed at 4ºC overnight.

Tissue Sectioning

The cochlear tissue samples were collected via tissue sectioning. The samples were stored in 1x PBS as the embedded molds until they were ready to be used. To prepare the embedded cochlea for sectioning, the mold was cut into a house shape with a large base using a sharp-edge blade. The Vibratome 3000 (Technical Products International, St. Louis, MO) was used for cochlear tissue sectioning; it serves as a standard laboratory resource for sectioning fresh and fixed tissue. In this case, the Vibrotome was used to cut fixed cochlear tissue. The tissue sections were achieved using a vibrating razor blade set at a 15º angle, with a high vibration amplitude of 7, and a very low blade movement speed of almost zero to move extremely slowly. The section thickness was set to cut 100 microns thick sections of the fixed cochlea. This instrument requires an element of temperature control; thus, the fixed embedded cochlea was chilled before sectioning (in a 50 ml conical vial filled with 1x PBS in a bucket of ice) and ice was used to surround the stage. Both measures of temperature control are essential for obtaining high-quality cochlear sections. The stage of the Vibrotome was filled with ice-

cold 1x PBS, which created an ice-cold sterile PBS Vibrotome bath for the tissue. Ice was placed surrounding the stage to maintain a cool temperature for the embedded cochlea. This helps with smooth sectioning. A half of a small double edge razor blade was inserted into the blade holder. The blade angle was adjusted to a 15º angle. The house-shaped embedded cochlea was superglued to the chuck or metal mounting block using Loctite® 404® Quick SetTM Instant Adhesive (Henkel). The chuck was submerged in the 1x PBS bath on the stage. Individual sections of 100 µm thickness were collected with a fine-tip paintbrush and transferred into a 24-well tray for immunostaining. Each of the 24 wells contained approximately 0.5 mL of ice-cold 1x PBS and 1 cochlear section. The samples were stored at 4ºC until the immunostaining process began.

Immunostaining and Immunofluorescence

Immunostaining is a standard technique used for detecting the distribution and localization of specific proteins within cells or tissues. It is defined using specific antibodies. The detection of specific proteins in tissues is referred to as immunohistochemistry. The process began with placing cochlear sections into 30% sucrose diluted with 1x PBS (0.3 g sucrose in 1 mL 1x PBS). The 24-well tray was placed on the Benchmark ORBI-Shakerä JR at 80 rpm for 30 minutes. Next, the 24-well tray was frozen at -80ºC for 20 minutes or overnight. Then, the 24-well tray was covered with a cover film and thawed in the 37ºC oven for 30 minutes. Once thawed, 2 quick rinses with 1x PBS then 3 rounds of 10-minute washes with 1x PBS were completed on the Benchmark ORBI-Shakerä JR. During the washes, a 5% NHS block was made with Normal Horse Serum (NHS) and 0.3% Phosphate Buffer Saline with Triton X-100 (PBST). Approximately 5% NHS block was applied, and the tissues were blocked for 1

hour on the Benchmark ORBI-Shakerä JR. Then, the blocking solution was removed, and the prepared primary antibody aliquots were added to the cochlear tissue sections. The 24-well tray was covered with a coverslip film and incubated overnight for 24 hours in the 37ºC oven. The following primary antibodies were used for immunostaining: rabbit polyclonal MANF (Icosagen AS Catalog# 310-100, RRID: AB_11135308, 1:200), mouse monoclonal C/EBP homologous protein (CHOP; Novus Catalog# N860093555l; 1:100), goat polyclonal Oncomodulin protein (gtOcm; Santa Cruz Biotechnology Catalog# SC-7446; 1:1000), and goat polyclonal anti-choline acetyltransferase (gtChAT; Millipore Catalog# AB114D; 1:200). After the overnight incubation period, the samples were washed with 2 quick rinses of 1x PBS and 6 rounds of 10-minute intervals with 1x PBS on the Benchmark ORBI-Shakerä JR.

Following immunostaining, the immunofluorescence step occurs which involves staining primary antibodies with a secondary antibody containing a fluorescent that will label the protein of interest. After the primary antibody washes, the secondary antibodies were applied to each sample to better detect the primary antibody labeling. Before applying the secondary antibodies to the samples, the secondary antibodies were diluted with the 1% NHS block. The secondary antibodies have a fluorophore attached to the antibody to allow for the protein to fluoresce under the confocal microscope. The secondary antibodies selected for these immunofluorescent experiments were Alexa Fluor 488/557/647-conjugated goat anti-rabbit/mouse IgG secondary antibodies (Invitrogen). Also, the nuclei were stained with Hoechst at this time. The tissue sections were incubated with secondary antibodies for two hours in the 37ºC oven. Following the incubation, the samples were washed with 2 quick rinses of 1x PBS and 6 rounds of 10-

minute intervals with 1x PBS on the Benchmark ORBI-Shakerä JR. All the 1x PBS washing steps were completed.

The tissue samples are mounted on 25 x 75 x 1.0 mm Fisherbrand® Precleaned Frosted Microscope Slides. A clean microscope slide was taken and one drop of 1x PBS was added per section mounted on the slide. Four cochlear tissue sections were mounted on each microscope slide. A drop of 1x PBS was added to the slide, then a cochlear tissue section was transferred from the 24-well tray and rolled onto the microscope slide using a large-tip paintbrush. Next, a small drop of VECTASHIELD Mounting Medium was added on top of each cochlear tissue section on the microscope slide. A Fisherbrand® Microscope Cover Glass (22 x 40 x 1.5 mm) was placed on top of the cochlear tissue samples submerged in the drop of 1x PBS and VECTASHIELD Mounting Medium. Then, a Kimwipe was used to remove any excess liquid around the coverslip. Afterward, L.A. COLORS® Electron Microscopy Sciences Nail Polish was used to seal the microscope coverslips onto the microscope slides. The slides were placed in a flat slide tray. The slide tray was placed in a closed drawer away from direct light to maintain the immunofluorescence ability and allow the microscope slides to dry for confocal microscopy imaging.

Confocal Microscopy Imaging

Confocal microscopy was used to capture images of the cochlear tissue samples. A ZEISS confocal microscope was used to acquire images of CHOP and MANF immunofluorescence labeling with a 10x air object and 63x Plan Apo oil objective. The same laser setting was used to capture the CHOP labeling, MANF labeling, gtOcm

labeling for Ocm WT samples, and gtChAT labeling for Ocm KO samples. The image analysis was completed with Zeiss Zen software and Imaris imaging software.

Statistical Analyses

ImageJ or Fiji was used to count the number of macrophages and calculate the level of Manf expression in the area of interest. The integrated density and mean gray value were calculated for the entire OHC and its nuclei in the noise-exposed and control mice. Integrated density accumulated the value of each pixel in the captured confocal images of the cochlear samples in the area of interest. It is equivalent to the product of the area and the mean gray value. The higher integrated density values correlated with greater fluorescence. The mean gray value summed the gray values of all the pixels of the cochlear samples in the area of interest and divided that number by the number of pixels.

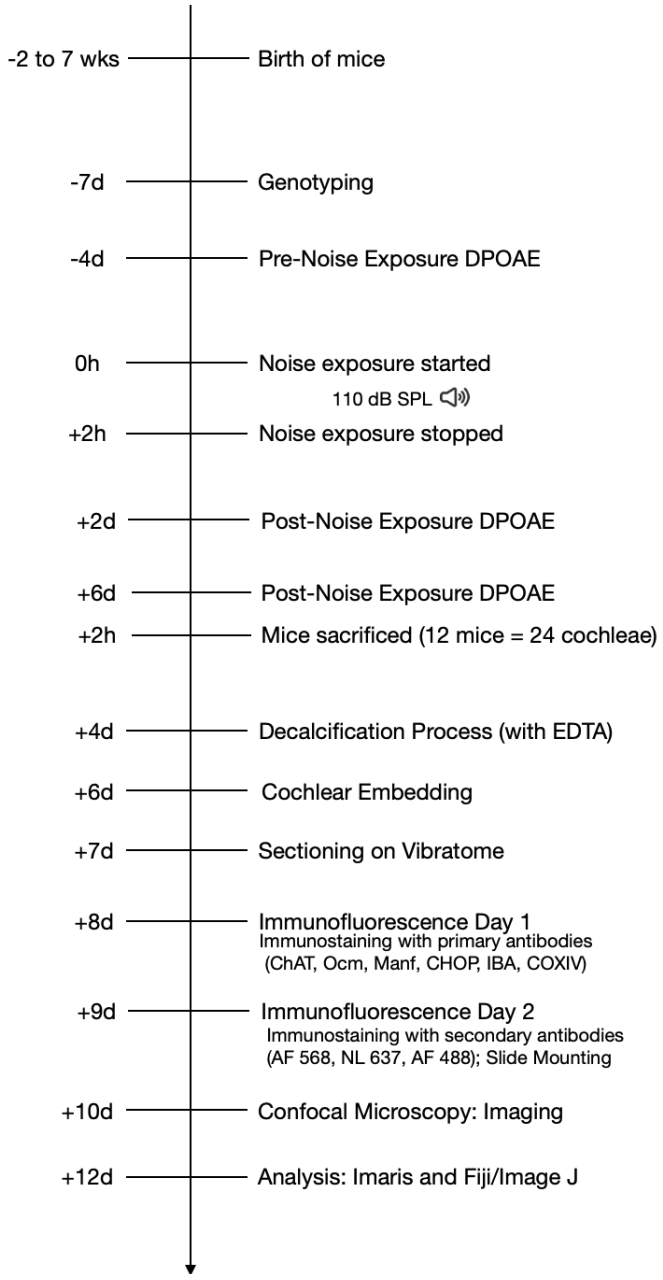

Figure 5. Experimental Timeline. The animal samples were mice (aged 2-7 weeks). One week (7d) before the noise exposure, they were genotypes to determine whether they were Oncomodulin wildtype (Ocm WT) or Oncomodulin knock-out (Ocm KO). The baseline hearing function of the mice was measured via DPOAE testing 4 days (4d) before the noise exposure. The mice were exposed to noise at 110 dB SPL for 2 hours (2h). The control mice were not exposed to any elevated noise levels; however, they were still in the same room just adjacent to the soundbox. The following day, post-noise exposure DPOAE testing for performed on the mice. There were three types of primary antibodies used for the immunofluorescence, which served as the ER stress markers, mitochondrial stress markers, and inflammatory markers in these experiments.

CHAPTER FOUR

Results

Assessment of Hearing and Stress

Before noise exposure, all nine mice had a DPOAE performed to assess their hearing at Day 0, which served as the baseline standard hearing measurements for each mouse. Following pre-noise exposure DPOAEs for all the mice, the control Ocm WT and Ocm KO mice were set aside. The Ocm WT and Ocm KO mice selected for noise exposure were exposed to 110 dB SLP of broadband noise for 2 hours. Broadband noise entails a variety of frequencies; thus, it is more representative of the human environment and allows for an assessment of the entire cochlear frequency range. Final DPOAEs were taken 48 hours or 2 days and 6 days post-noise exposure. The DPOAEs were performed on day 2 to assess the immediate effects of noise exposure. The measurements taken on Day 2 account for the temporary threshold shift, which describes objective changes in hearing acuity immediately after an episode of noise exposure. On day 6, DPOAEs were repeated to assess the aftermath of noise exposure. The measurements taken on Day 6 account for a more permanent threshold shift. The time, between Day 2 and Day 6, granted the injured cochlear hair cells of the noise-exposed mice to activate any inflammatory and repair pathways for potential recovery. The Ocm WT mice had a greater difference in thresholds than compared to that of Ocm KO mice post-noise exposure (Figures 6 and 7). This was more apparent between 8 to 32 kHz (Figures 6 and 7). Noise exposure may have increased the loss of OHCs towards the apical regions near the apex of the cochlea, which is associated with low-frequency regions. Even without

noise exposure, Ocm KO mice have naturally greater thresholds than Ocm WT mice because they lack Oncomodulin (Simmons et al., 2010). There was an increase in thresholds for both Ocm WT and Ocm KO post noise exposure, which indicated that there was some hearing loss experienced due to the elevated noise levels. However, when considering noise as the primary variable, the Ocm WT mice displayed a greater difference in thresholds than the Ocm KO mice post-noise exposure.

To assess ER stress post-noise exposure, certain antibodies were selected that identify key components of the ER stress pathways. Manf was used to identify ER-resident proteins, which are upregulated in correspondence with ER stress (Figure 5). The other ER stress marker used was CHOP labeling a pro-apoptotic component of unfolded protein response (UPR), which is a key process regarding ER stress (Figure 5). ER stress has the potential to induce inflammatory responses which involve the activation of macrophages. Specifically, microglia were identified using the antibody IBA (Figure 5). The CoxIV antibody was used to analyze mitochondrial stress in the cochlea as it can induce mitochondrial insufficiency (Bravo et al., 2011), which can be a contributing factor to ER stress (Figure 5). The interaction between the mitochondria and ER plays a role in cellular adaptation, which in this case may be OHCs' adaptation post-noise exposure.

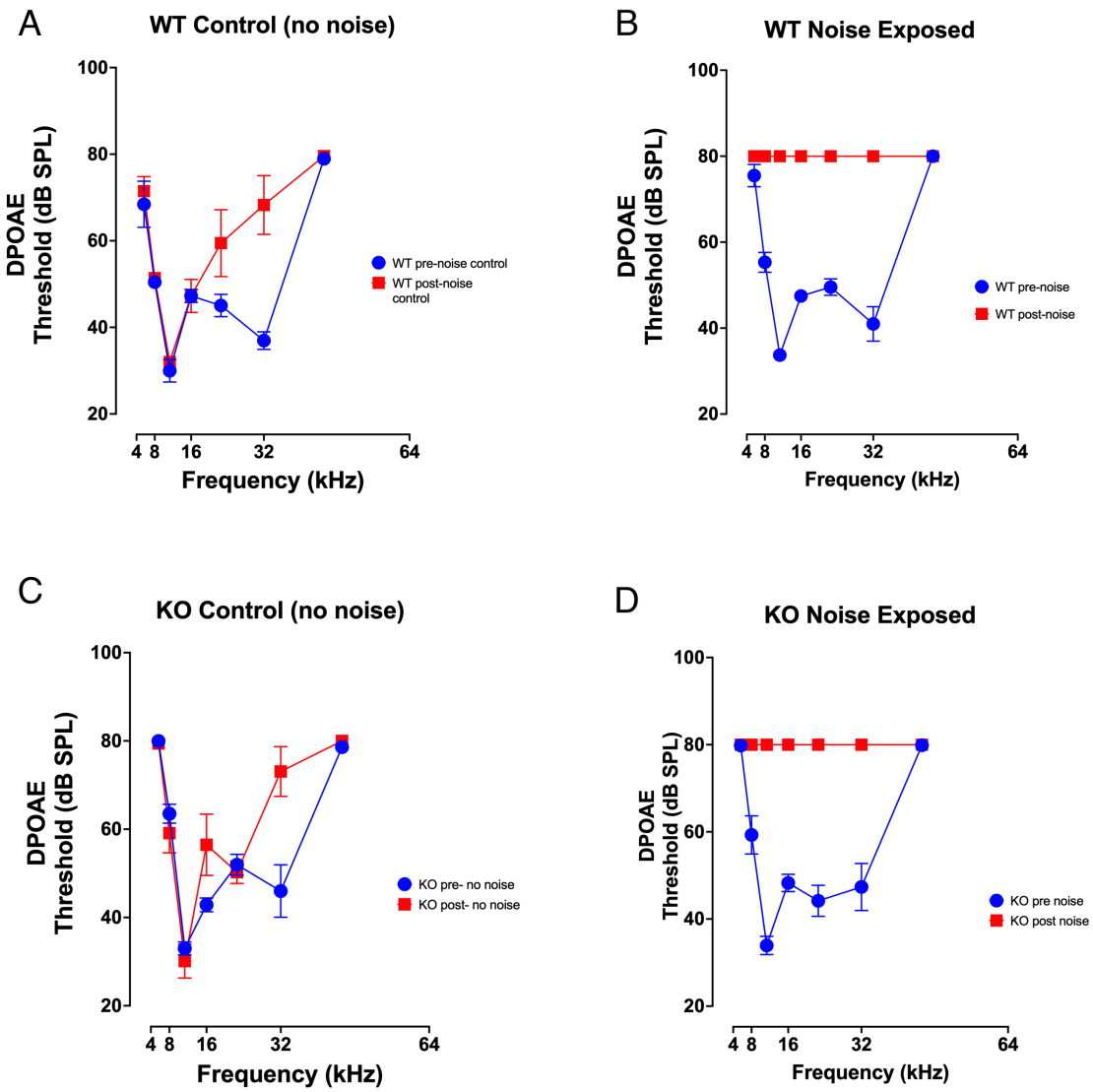

Figure 6. Representative distortion product otoacoustic emission (DPOAE) threshold measurements (n = 12). Post-noise-exposure, Ocm WT mice (aged 2-7 weeks) showed greater differences in DPOAE thresholds. The frequencies of sound stimuli given to the mice were the following: 5.66, 8, 11.32, 16, 22.56, 32, 36, and 45.2 kHz. The blue color illustrates the pre-noise exposure DPOAE thresholds, and the red color illustrates the post-noise exposure DPOAE thresholds for (A-D). (A) is the pre-and post-noise exposure DPOAE of the Ocm WT control mouse. (B) is the pre-and post-noise exposure DPOAE of the Ocm WT mouse exposed to noise. (C) is the pre-and post-noise exposure DPOAE of the Ocm KO control mouse. (D) is the pre-and post-noise exposure DPOAE of the Ocm KO mouse exposed to noise. This is a good reference to compare DPOAEs in response to noise exposure.

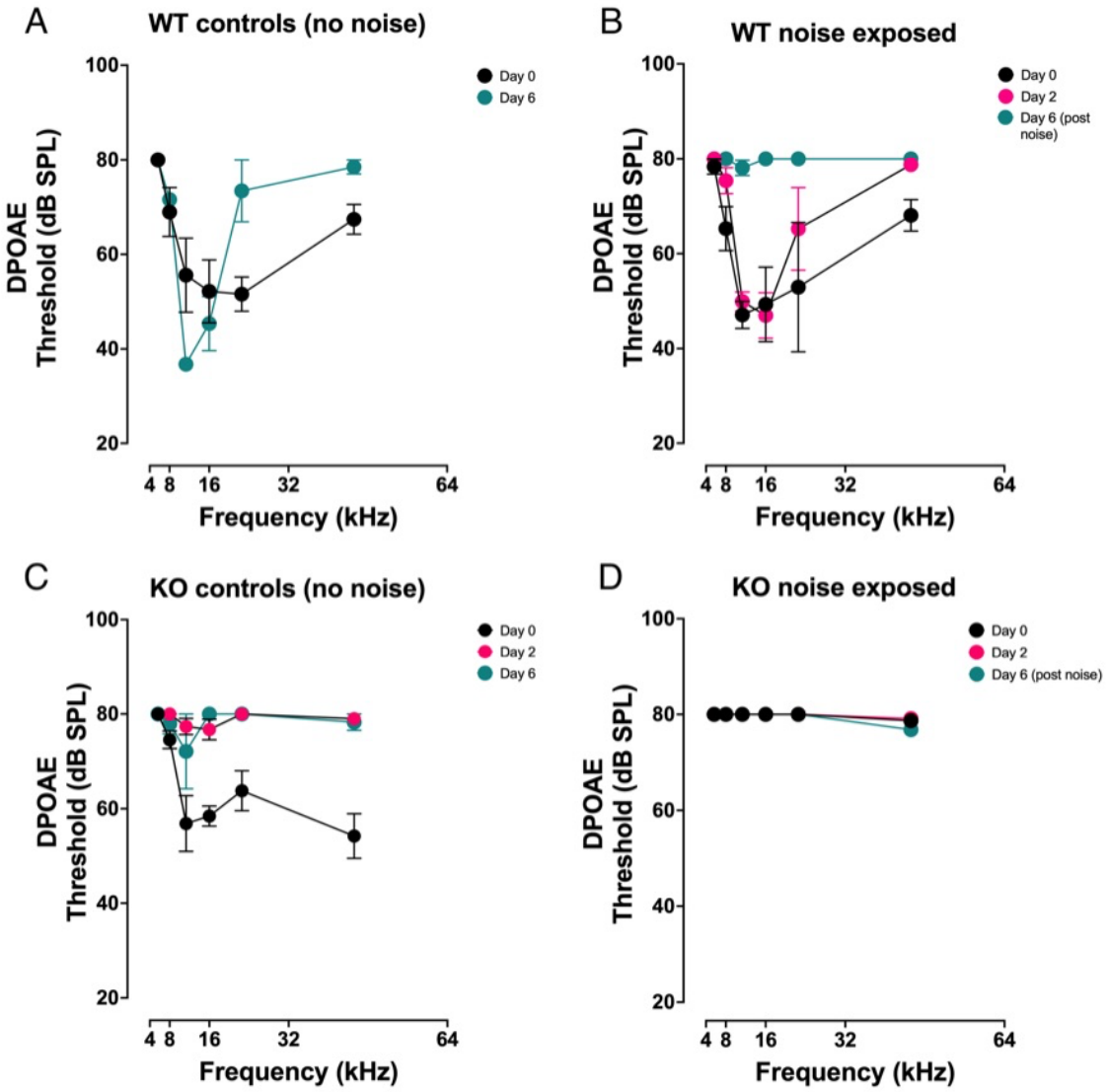

Figure 7. Representative distortion product otoacoustic emission (DPOAE) threshold measurements at baseline and several days post- noise exposure (n = 12). Post-noise-exposure, Ocm WT mice at ages of 2-7 weeks showed greater differences in DPOAE thresholds, even after recovery time (Day 6). The stimuli given were at the following frequencies of sound: 5.66, 8, 11.32, 16, 22.56, 32, 36, and 45.2 kHz. The black color illustrates the baseline DPOAE thresholds (Day 0), the pink color illustrates the DPOAE thresholds 2 days after noise exposure (Day 2), and the teal color illustrates the DPOAE threshold measurements 6 days post-noise exposure (Day 6) for (A-D). (A) is the pre-and post-noise exposure DPOAE of the Ocm WT control mouse. (B) is the pre-and post-noise exposure DPOAE of the Ocm WT mouse exposed to noise. (C) is the pre-and post-noise exposure DPOAE of the Ocm KO control mouse. (D) is the pre-and post-noise exposure DPOAE of the Ocm KO mouse exposed to noise. This is a good reference to compare DPOAEs in response to noise exposure and its long-term effects on hearing.

Presence of Macrophages Varies among Mice Depending on Noise Exposure

The location and number of macrophages vary depending on the presence of stress and inflammatory responses occurring in the tissue at the time of observation. The resident macrophages reside in the cochlea and vestibular organs. They accumulate within damaged regions of the inner ear and engulf hair cell debris (Hirose et al.) IBA-1 served as a macrophage marker for mouse cochlea (red in Figures 8 and 9).

The abundance of macrophages in major areas of the noise-exposed mice's cochlea such as the spiral limbus (SL), stria vascularis (SV), and spiral ganglion (SG) suggest an inflammatory response induced by noise (Figure 9). The SL consists of fibrocytes, and it is located on the osseous spiral lamina. The fibrocytes of the SL are vulnerable to the stress that can be induced by infection and trauma, such as elevated noise exposure (Maynard and Downes, 2019). Located in the lateral wall of the cochlear duct, the SV maintains the ion composition of the endolymph, which entails a high ratio of potassium ions to sodium ions. This is crucial in producing the endocochlear potential, which serves as the driving force for hair cell mechanotransduction (Liu et al., 2016). The SG is a collection of cell bodies of cochlear neurons. Located at the conical central axis of the cochlea, the SG contains two types of bipolar sensory neurons which are Type I cells and Type II cells (Henkel, 2018). The Type I cells of the SG synapse with inner hair cells of the cochlea, whereas type II cells of the SG synapse with outer hair cells of the cochlea (Henkel, 2018), providing afferent and efferent innervation respectively. The bipolar neuron cells of the SG serve as the origin of the cochlear modular auditory nerve (CN). The CN is responsible for the transmission of electrical impulses generated for hearing and localized sound.

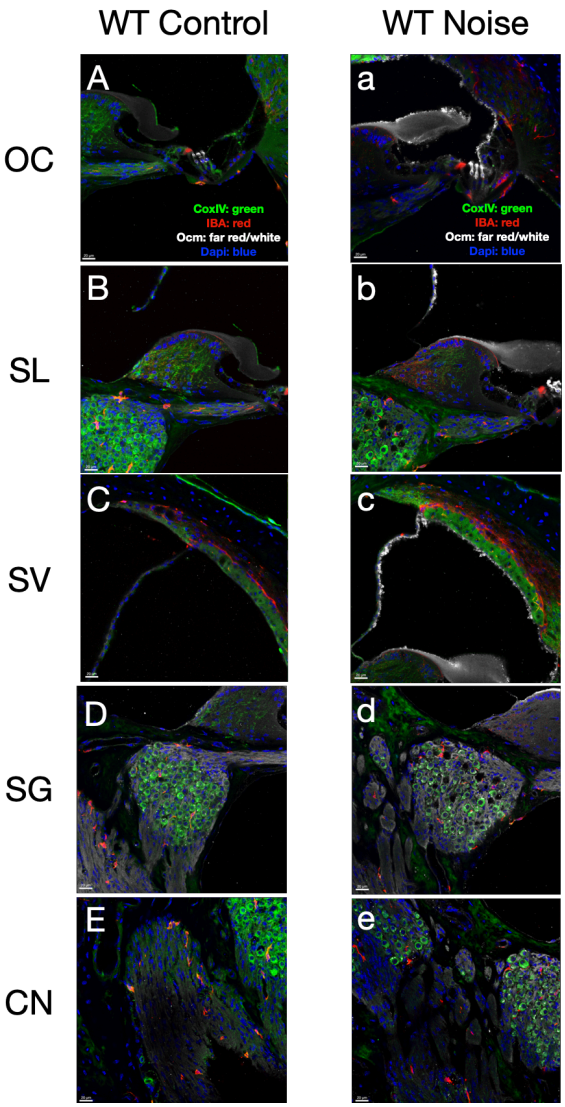

Figure 8. Confocal images comparing noise exposure with mitochondrial and ER stress markers' expression on Day 6. All images were taken at a magnification of 63x with oil immersion. The images were all captured with the same confocal settings. These images were captured from region II of the left cochlea, which is representative of the base or basal regions of the cochlea. The expression of mitochondrial stress and macrophages in no noise control mice are shown in the first column and noise-exposed mice are shown in the second column. The following areas are shown in this figure: organ of Corti (OC), spiral limbus (SL), stria vascularis (SV), spiral ganglion (SG), and cochlear modular auditory nerve (CN). The following antibodies are depicted in this figure: msCoxIV (green), rbIBA (red), gtOcm (white/far red), Dapi (blue). The msCoxIV antibody is specific to indicating mitochondria stress. The rbIBA antibody is specific for labeling macrophages. The gtOcm antibody labels Ocm presence in the OHCs. Dapi is a hair cell nuclei marker. The antibodies and markers are not specific to certain frequency regions in the cochlea, rather they label important structures and components within the cochlea for analysis of noise exposure effects. The scale bar is 20 µm.

There was a constitutive presence of tissue-resident macrophages in the SG and SL. The SV is a highly vascular region in the cochlea; thus, it undergoes rapid recruitment of macrophages when under stressful conditions. This is evident by the significantly increased number of macrophages present in the SV of Ocm WT and Ocm KO mice post-noise exposure (Figure 10). A Two-way ANOVA was used to analyze the significant recruitment of macrophages to the SV (p-value <0.0001). The Ocm WT noise-exposed mice had the greatest presence of macrophages in the cochlea than that compared to noise-exposed Ocm KO mice and no-noise control mice (Figure 6). After injury due to noise exposure, macrophages accumulated in predicted areas (Figure 9). This demonstrated that hair cell injury may recruit non-resident macrophages from the bloodstream to the cochlea.

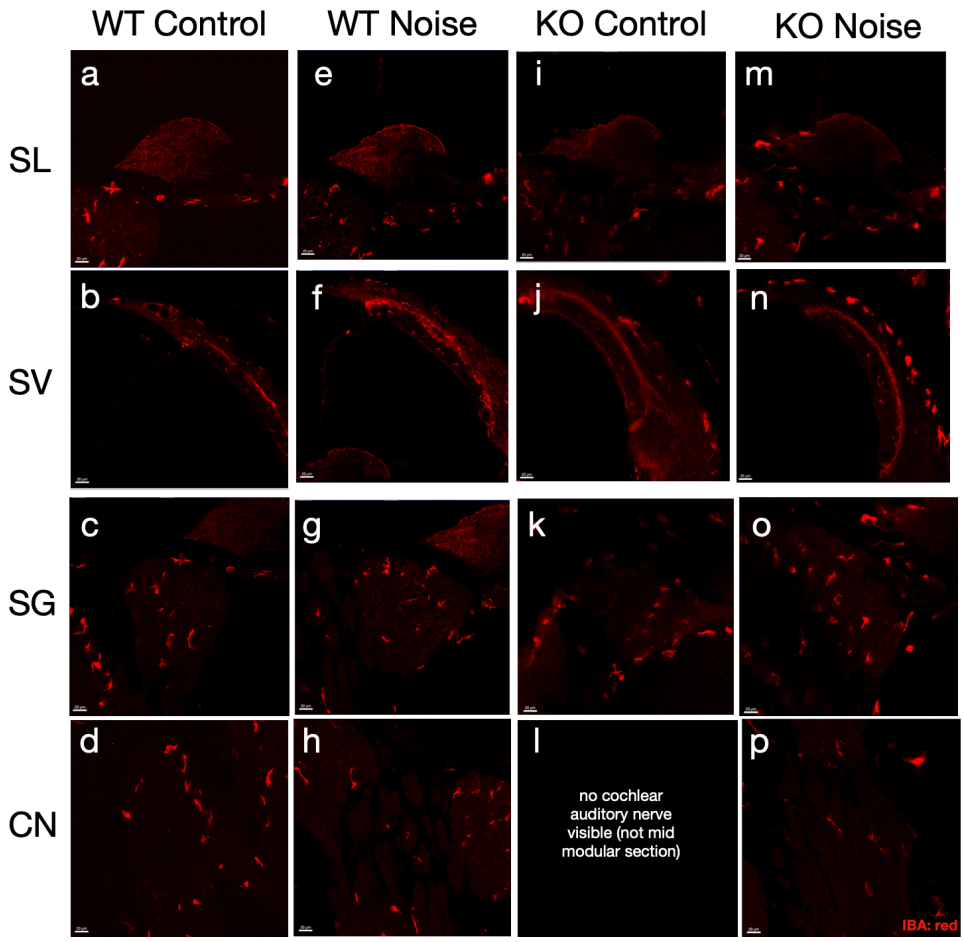

Figure 9. Day 6 confocal images of macrophages at region-II of the cochlea, which refers to the base or basal portion of the cochlea. All images were captured at a magnification of 63x with oil immersion. The images were all captured with the same confocal settings. The four specific locations of the cochlea that were captured are the following: spiral limbus (SL), stria vascularis (SV), spiral ganglion (SG), and the cochlear modular auditory nerve (CN). The first and third columns present control mice, which were not exposed to noise, Ocm WT, and Ocm KO respectively. The second and fourth columns present noise-exposed mice who were exposed to elevated noise, Ocm WT, and Ocm KO respectively. The morphology of the macrophages changes slightly across genotype strain and noise exposure. Macrophage presence increased with exposure to noise levels. To identify the macrophages, rbIBA was the antibody used (red). The scale bar is 20 µm.

A high density of macrophages and mitochondrial stress is noted in the stria vascularis and spiral limbus, post-noise exposure (Figure 8). Under steady conditions, the organ of Corti is devoid of immune cells such as macrophages whereas the surrounding tissues of the cochlea are rich in macrophages (Hu et al., 2018). The organ of Corti lacks

standard immune cell emigration from the bloodstream (Hu et al., 2018) unless it undergoes substantial damage or injury to hair cells from conditions such as noise-induced ER stress (Figure 10). Typically, the macrophages in the cochlea are uniformly distributed along the apex to the base of the cochlea (Hu et al., 2018). The stria vascularis serves as the "battery" of the cochlea. It works both against and with the concentration gradient to simultaneously pump in 1 Ca^{+2} for every 4 K^{+} it pumps out. The stria vascularis does not degenerate as fast as the IHCs and OHCs, because Ca^{+2} regulation speeds up the process of degeneration. The macrophages present in the stria vascularis due not serve any immune functions. They serve an important role in the integrity and permeability of the blood labyrinth barrier. Thus, endothelial cells activate the strial macrophages and contribute to increased vascular permeability post-noise exposure (He et al., 2020). The increase in vascular permeability is a mediator of inflammation as the capacity of the capillaries allows for the flow of small molecules. The increased presence of IBA is clear, but the increase in CoxIV expression is not certain (Figure 8). It is unclear due to the variation in expression across the different regions of the cochlea when comparing the control mice and noise-exposed mice (Figure 8). The organ of Corti presents a decrease in CoxIV expression with noise exposure, while the stria vascularis presents increased CoxIV expression with noise exposure (Figure 8). Thus, the relationship between ER stress and mitochondrial stress pathways cannot be assessed definitively.

Morphology of Macrophages

There are morphology differences between macrophages in no-noise mice cochlea and noise-exposed mice cochlea. The macrophages at rest (Figure 9) are ramified, due to the absence of the noise exposure trigger for inflammation. These are most likely tissue-resident macrophages ready for activation if stress and inflammation are induced to play an active role in the immune system. The activated macrophages (Figure 9) appear more rounded. These could be infiltrated monocytes ready to become macrophages or activated resident and non-resident macrophages recruited by the injured tissue to aid in the inflammatory immune response. Monocytes arrive at the site of inflammation and transform into macrophages for inflammatory or tissue repair purposes (Climer et al., 2019). This transformation is made possible through the release of cytokines in the injured hair cells that signal monocytes to transform into activated macrophages. An uninjured cochlea with no signs of inflammation contains resident proteins that are distributed throughout (Figure 9). In addition to resident macrophages, many macrophages were recruited post-noise exposure (Figure 9) for inner ear surveillance and tissue repair due to inflammation (Rai et al., 2020).

There are two important cells to consider in inflammatory pathways, which are macrophages and neutrophils as each plays a crucial role in inflammation. They are immune cells that are recruited to the site of injury and engage in phagocytosis by engulfing debris and dying cells. At the site of tissue damage, Ocm has been found in both neutrophils and macrophages (Climer et al., 2019). Neutrophils are the first responders of the innate immune system. With the onset of inflammation, the neutrophils enter the tissues and produce high levels of Ocm (Kurimoto et al., 2013). Previous studies

have shown that macrophage activation can persist after the depletion of neutrophils (Kurimoto et al., 2013). However, the experiments for this thesis were focused solely on the presence of macrophages; thus, in this case, macrophages may have been recruited to the noise-exposed tissues of the cochlea following the infiltration of neutrophils. Further research is needed to describe the role of neutrophils in inflammatory responses induced by ER stress.

Oncomodulin's Role in Macrophage Activity

When comparing the Ocm WT and Ocm KO mice post-noise exposure, our results reveal that the Ocm WT mice exposed to noise had a significantly greater number of macrophages than compared to that of Ocm KO mice exposed to noise (Figure 10). The characterization of the inflammatory response in our results was represented by the outnumbered macrophages in the Ocm WT mice compared to the Ocm KO mice (Figure 10). Previous studies have shown that the oncomodulin (Ocm) which is expressed in our Ocm WT mice, is secreted by macrophages that bind to ganglion cells with high affinity via the Ca^{2+}/calmodulin (CaM) kinase-dependent pathway to exert a stronger axon-promoting effects (Yin et al., 2006). It is suggested that Ocm plays a role in axon regeneration by promoting its processes through inhibitory environments such as the optic nerve (Yin et al., 2006), and perhaps the cochlear auditory nerve as well. The inflammatory pathways lead to an upregulation of Ocm, which is a small Ca^{2+} binding protein that serves a key role in inflammation-induced regeneration (Kurimoto et al., 2013). This is evidenced by the increase in Ocm expression in the OHCs of the cochlea from mice exposed to noise (Figure 8). Previous studies have shown that Ocm is a macrophage-derived growth factor (Yin et al., 2006); thus, it is essential for macrophage

activation for positive processes such as repair and regeneration (Yin et al., 2006). Thus, the difference between the increase in macrophages of noise-exposed Ocm WT and Ocm KO may be explained by the ability of Ocm to play a role in inflammatory and immune responses. However, there was no notable presence of macrophages specifically in the organ of Corti (Figure 10).

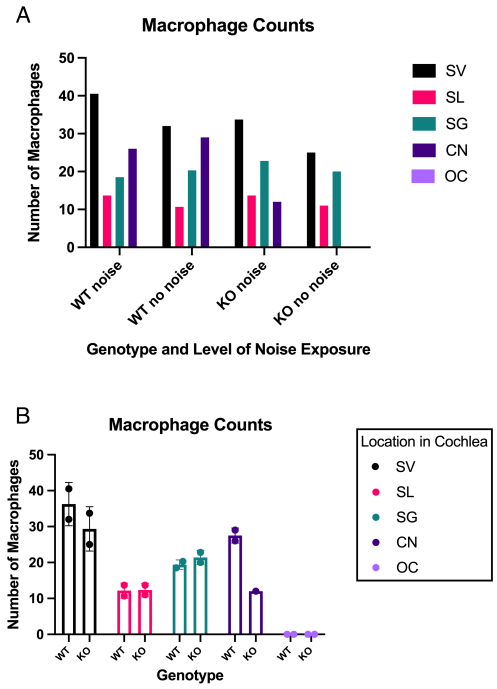

Figure 10. Macrophage Counts. Macrophages were accounted for in four major areas: stria vascularis (SV), spiral limbus (SL), spiral ganglion (SG), and the cochlear modular auditory nerve (CN). The macrophages were most abundant in the SV (One-way ANOVA; n = 9 cochlea, *p-value <0.0001). In (A), the number of macrophages present is classified by location with each color representing a different location of the cochlea. Also, (A) allows for a comparison within each genotype to analyze where the macrophages are most present in the control compared to the noise-exposed mice. The purpose of (A) is to compare the number of macrophages between the genotypes and to interpret if noise exposure has impacted macrophage presence. In (B), we see an average number of macrophages present in each location of the cochlea, regardless of noise exposure. The error bars indicate the numerical difference of macrophages between the control mice and noise-exposed mice. The dot at the top of each error bar represents the number of macrophages for the noise-exposed mice. The dot at the bottom of the error bar represents the number of macrophages for the control mice. The purpose of (B) is to identify where macrophages are most present in the cochlea. Overall, noise-exposed Ocm WT mice had the greatest number of macrophages present in the cochlea.

Upregulation of Unfolded Protein Response due to Noise Exposure

The unfolded protein response (UPR) is a key process in ER stress. ER stress engages UPR when proteins await chaperones who assist in proper folding (Figure 11). The purpose of UPR is to assist in properly folding misfolded or unfolded proteins in the endoplasmic reticulum. The accumulation of unfolded or misfolded proteins can be due to stressful conditions in the cells. Thus, UPR is described as an adaptive reaction that reduced the unfolded or misfolded protein load (Hetz, 2012). The process of UPR maintains cell viability (Hetz, 2012).

Manf plays a crucial role in ER stress. As an endoplasmic reticulum chaperone resident protein, Manf serves a critical role in the homeostasis of the endoplasmic reticulum. This protein is primarily localized in the luminal endoplasmic reticulum (Figure 11). The presence of Manf can be upregulated in ER stress due to its promoter being recognized by ATF6 and XBP1 (Figure 11). Manf primarily interacts with BiP and Reticulon 1-C (Figure 11). The expression of Manf is heavily increased by the presence of other ER stress markers such as transcription factor 6 (ATF6) and transcription factor X-box binding protein 1 (XBP1) (Yu et al., 2021). The pathway of Manf serves a special role in the programming of transcription factors that are involved in UPR (Figure 11).

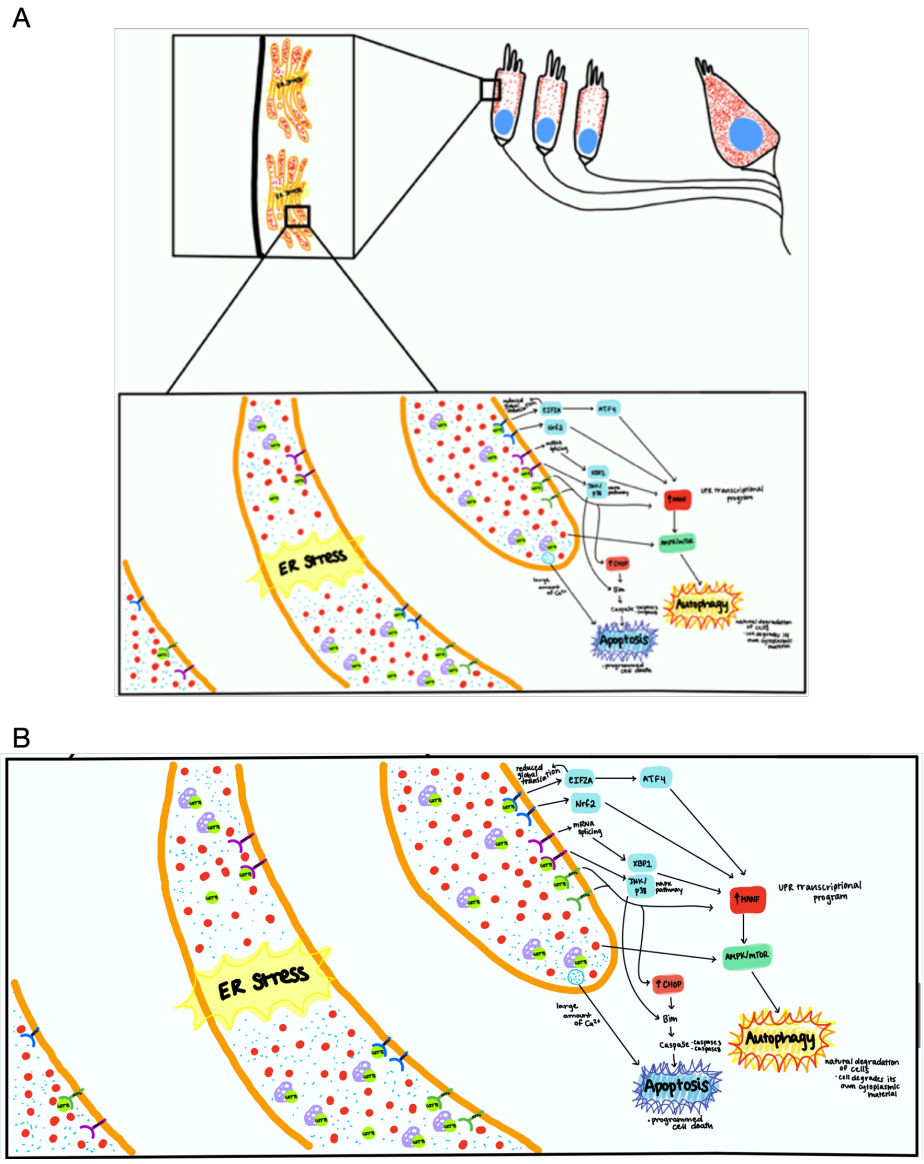

Figure 11. Schematic of Manf pathway in tissue experiencing ER stress. (A) begins with a simple illustration of the organ of Corti with the IHC and OHCs, then it leads to a more detailed focus on the rough endoplasmic reticulum (RER). During ER stress, there is an increased presence of Manf (red dots). This schematic displays the unfolded protein response (UPR) during ER stress. During UPR, there are unfolded proteins (lavender bundles) awaiting chaperones (lime green spheres: GRP78) to assist in proper protein folding. After assisting in protein folding, the chaperones (lime green spheres: GRP78) then bind to intracellular receptors (blue, olive green, magenta: PERK, IRE1α, ATF6 respectively) bound to the RER. The UPR upregulates transcriptional factors (light blue squares: eTF2A, Nrf2, ATF4, XBP1, JNK/p38). Alongside this pathway, increased amounts of cytoplasmic Ca^{+2} can contribute to apoptosis, which results in hair cell death. Eventually, the presence of ER stress may lead to apoptosis or autophagy due to the upregulation of CHOP and Manf. (B) is a magnified view of the specific components that play a role in the pathway of Manf in OHCs undergoing ER stress.

Presence of Manf Varies among Mice Depending on Noise Exposure

Manf is more strongly expressed in the IHCs than compared to OHCs (Figures 12, fsdfadf). Manf is most concentrated along the borders of the OHCs, which is where the ER of the cell is present (Figure 12). The role of Manf is to promote ER homeostasis as an ER-resident protein that is part of UPR. Thus, it implies that the increased Manf expression in the noise-exposed cochlea is due to its function in promoting survival and normal hearing function (Figures 13 and 14). Manf can promote hair cell survival and normal hearing function via protecting against inflammatory responses by ER stress-related proteins and the NF-kappaB pathway (Figure 11). A notable increase in the presence of Manf was seen in the cochlear OHCs of mice exposed to noise compared to the control mice (Figure 13). After completing a Two-way ANOVA, a significant increase in Manf expression of Ocm WT mice post-noise exposure was seen with a p-value <0.0001. This is supported by recent studies that have shown progressive OHC death starting soon after the onset of hearing function in Manf-inactivated mice (Herranen et al., 2020). Manf expression is more prominent in the IHCs and supporting cells than compared in the OHCs of the cochlea.

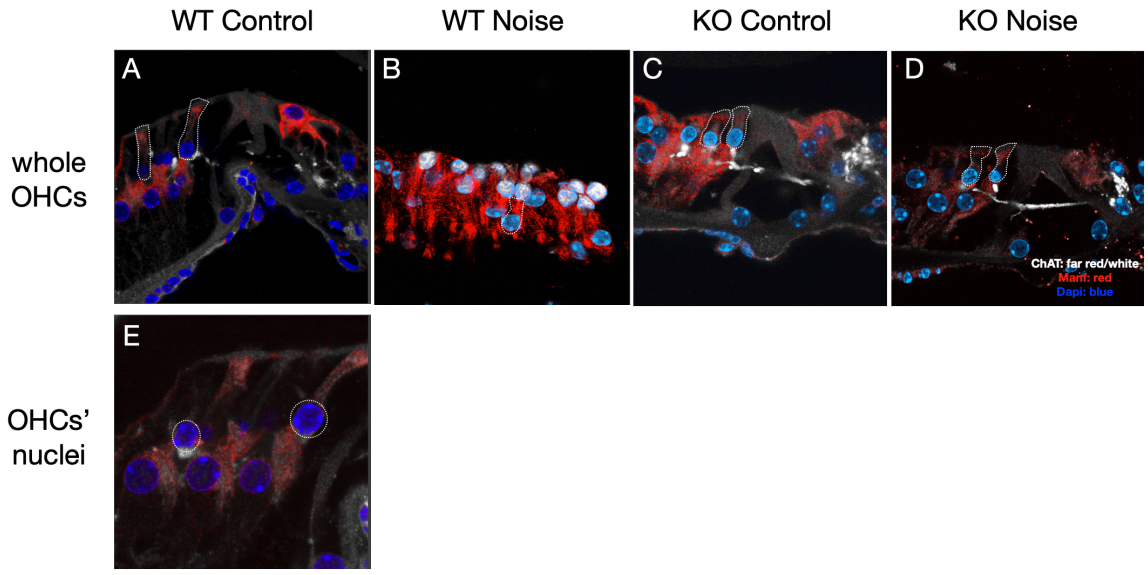

Figure 12. Confocal images of Manf expression in cochlea via immunostaining. All images were taken at a magnification of 63x with oil immersion. The images were all captured with the same confocal settings. All the images show a strong expression of Manf in the Deiters cells, which are the supporting cells in the organ of Corti located below the OHCs (A-E). The expression of Manf in the outer hair cells (OHC) is outlined in the organ of Corti images (A-D) with a scale bar of 7 µm; the stipulating in white presents individual OHCs (A-D). (A) was captured at region-III of the cochlea, which is the apical portion of the cochlea. (B) was captured near region-III of the cochlea at the cochlea's apex. (C) was captured from region-II of the cochlea, which is the basal portion of the cochlea (image flipped to have OHCs on the left side as seen in other images for comparison). (E) is an airyscan of the OHCs at the region I of the cochlea, which is located near the base of the cochlea. The airyscan had a scale bar of 2 µm, to emphasize the Manf expression specifically near the nuclei of the OHCs which are stipulated in white (E). The following antibodies were used: gtChAT (white), rbManf (red), and Dapi (blue). The gtChAT antibody labels the neurotransmitter acetylcholine to highlight the efferent and efferent connections within the organ of Corti. The rbManf antibody is a marker of ER stress as it labels the endoplasmic reticulum resident protein itself Manf. Dapi is a hair cell nuclei marker. The antibodies and markers are not specific to certain frequency regions in the cochlea, rather they label important structures and components within the cochlea for analysis of noise exposure effects.

The location of Manf expression in the OHCs is significant. Specifically, in the cochlea, Manf was expressed along the borders of OHCs which is where the ER is located and very close to the OHCs' nuclei (Figure 12). Also, Manf is strongly expressed in supporting cells, specifically Deiters cells (Figures 12, 15, and 16). A lack of Manf

secretion from supporting cells such as Deiters cells can promote damage in the OHCs (Herranen et al., 2020). There is a presence of Manf expression at the reticular plate located between the OHCs and their hair cell projections (Figure 12). The location of the ER along the borders of the OHCs is evident in the immunofluorescent experiments (Figure 12). The expression of Manf in the nuclei of OHCs post-noise exposure is increased compared to that of control mice (Figures 13 and 14). A Two-way ANOVA reaffirmed the significant increase in Manf expression of Ocm WT mice post-noise exposure with a p-value of <0.0001, even when assessing only the nuclei of OHCs. Thus, this confirms the existence of ER stress in the OHCs.

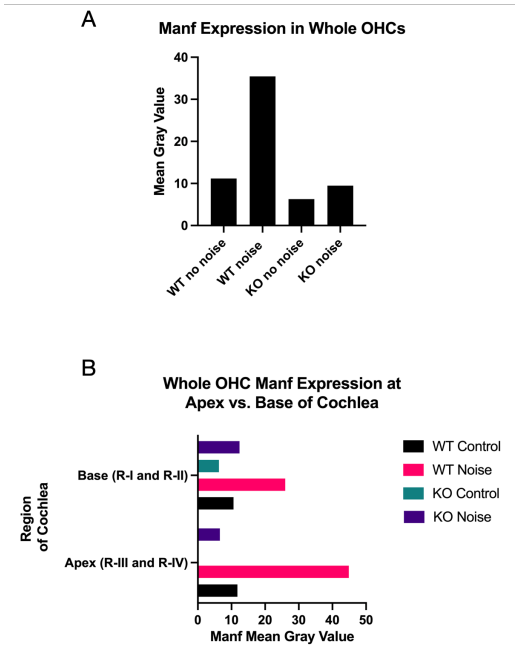

Figure 13. Manf expression in whole OHCs of the noise-exposed cochlea and non-noise exposed cochlea. As seen in (A), the expression of Manf was greatest in Ocm WT mice post-noise exposure (One-way ANOVA; n = 9 cochlea, *p-value <0.0001). (B) presents a comparison of Manf expression between the OHCs in the apex (apical regions with are regions III and IV) and the base (basal regions which are regions I and II). The comparison between control Ocm WT and noise-exposed Ocm WT is seen with more Manf expressed in the Ocm WT mice post-noise exposure (black and purple, respectively). Likewise, the effect of noise on Ocm KO mice revealed that Ocm KO mice exposed to noise had an increased expression of Manf (pink and magenta, respectively).

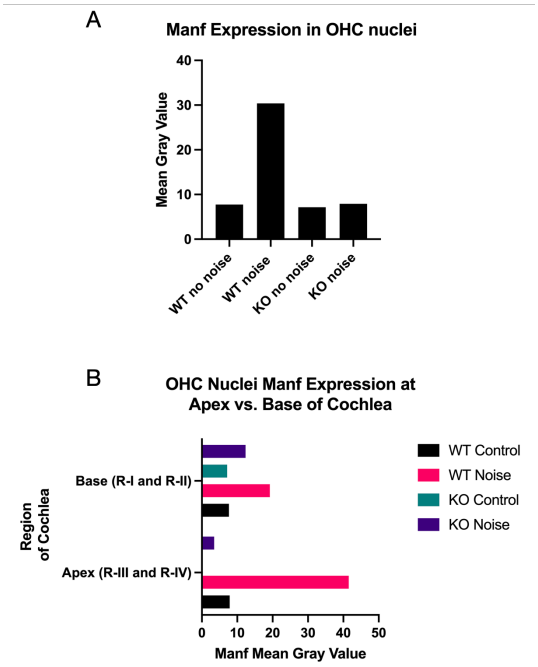

Figure 14. Manf expression in nuclei of OHCs of the noise-exposed cochlea and non-noise exposed cochlea. Manf is more strongly expressed near the nuclei of hair cells in addition to its abundance in supporting cells and IHCs. As seen in (A), the expression of Manf was greatest in OHC nuclei of Ocm WT mice post-noise exposure (One-way ANOVA; n = 9 cochleae, *p-value <0.0001). (B) presents a comparison of Manf expression between the OHCs' nuclei in the apex (apical regions with are regions III and IV) and the base (basal regions which are regions I and II). The comparison between control Ocm WT and noise-exposed Ocm WT is seen with more Manf expressed in the nuclei of OHCs of Ocm WT mice post-noise exposure (black and purple, respectively). Likewise, the effect of noise on Ocm KO mice revealed that Ocm KO mice exposed to noise had an increased expression of Manf in their OHCs' nuclei (pink and magenta, respectively).

Oncomodulin's Role in Manf Expression

When comparing the Ocm WT and Ocm KO mice post-noise exposure, our results reveal that the Ocm WT mice exposed to noise had a significantly higher expression of Manf compared to that of Ocm KO mice exposed to noise (Figures 13 and 14). Previous studies have shown that the oncomodulin (Ocm) serves a crucial role in inflammatory pathways, specifically in the recruitment of immune cells such as neutrophils and macrophages (Kurimoto et al., 2013). Manf plays a major role in UPR,

which can be described as a repair pathway for properly folding misfolded proteins. Ocm serves a role in axon regeneration (Yin et al., 2006) and inflammation-induced regeneration (Kurimoto et al., 2013); thus, the increased Manf expression post-noise exposure in Ocm WT mice who contain Ocm compared to Ocm KO mice who do not have Ocm may be explained by the suggestion that Ocm has a key role in inflammatory pathways.

Additionally, Ocm expression is increased in the basal regions or the base of the cochlea than compared to apical regions or the apex of the cochlea (Simmons et al, 2010). Our results imply that Manf is expressed more strongly in the apical portion of the cochlea, specifically in region 3, than in the basal part of the cochlea consisting of regions 1 and 2 (Figures 13 and 4). The decreased expression of Ocm in the basal regions of the cochlea may have called for the increased presence of Manf, due to the high cytoplasmic Ca^{2+} levels in the OHCs from ER stress. Also, the expression of Manf is abundant in IHCs (Figure 12). Previous studies have shown that Manf is predominantly expressed in supporting cells and IHCs (Herranen et al., 2020) and that Ocm has varied expression in IHCs from the apex (apical regions) to the base (basal regions) of the cochlea (Simmons et al, 2010). The presence of Ocm is reduced in the apical regions of the cochlea compared to the basal regions or base of the cochlea (Simmons et al, 2010). Thus, it can be inferred that the presence of Ocm can influence Manf expression. Since ER stress entails elevated cytoplasmic Ca^{2+} levels, the expression of Manf is increased in the apex to account for the decreased presence of Ocm in the apical regions of the cochlear. The increased expression of Manf further aids in returning the endoplasmic reticulum of injured hair cells to homeostasis. This may help explain why Manf

expression is increased in Ocm WT mice who contain Ocm than in Ocm KO mice who do not have Ocm present.

Manf and CHOP Expression depict UPR

Alongside the expression of Manf, an increase in CHOP expression was observed in the cochlea of mice post-noise exposure (Figure 15). As CHOP serves an important role in UPR, the presence of CHOP indicates protein translation. Additionally, CHOP is a pro-apoptotic component that indicates the onset of apoptosis induced by ER stress. The presence of Manf leads to an upregulation of CHOP (Herranen et al., 2020). The immunofluorescence experiments revealed an upregulation of CHOP and Manf, inferring the increased function of UPR in the cochlea of noise-exposed mice (Figure 15). The co-expression of Manf and CHOP best supports the presence of ongoing UPR in damaged hair cells. This links the process of UPR to hair cell injury and loss in the cochlea.

By comparing the expression of Manf and CHOP between the OHCs of control mice and noise-exposed mice, it is evident that the mice exposed to noise had upregulation of CHOP and Manf expression (Figure 15). This is seen through the more intensified expression of Manf and CHOP in the organ of Corti for Ocm WT mice and Ocm KO mice exposed to noise (Figure 15).

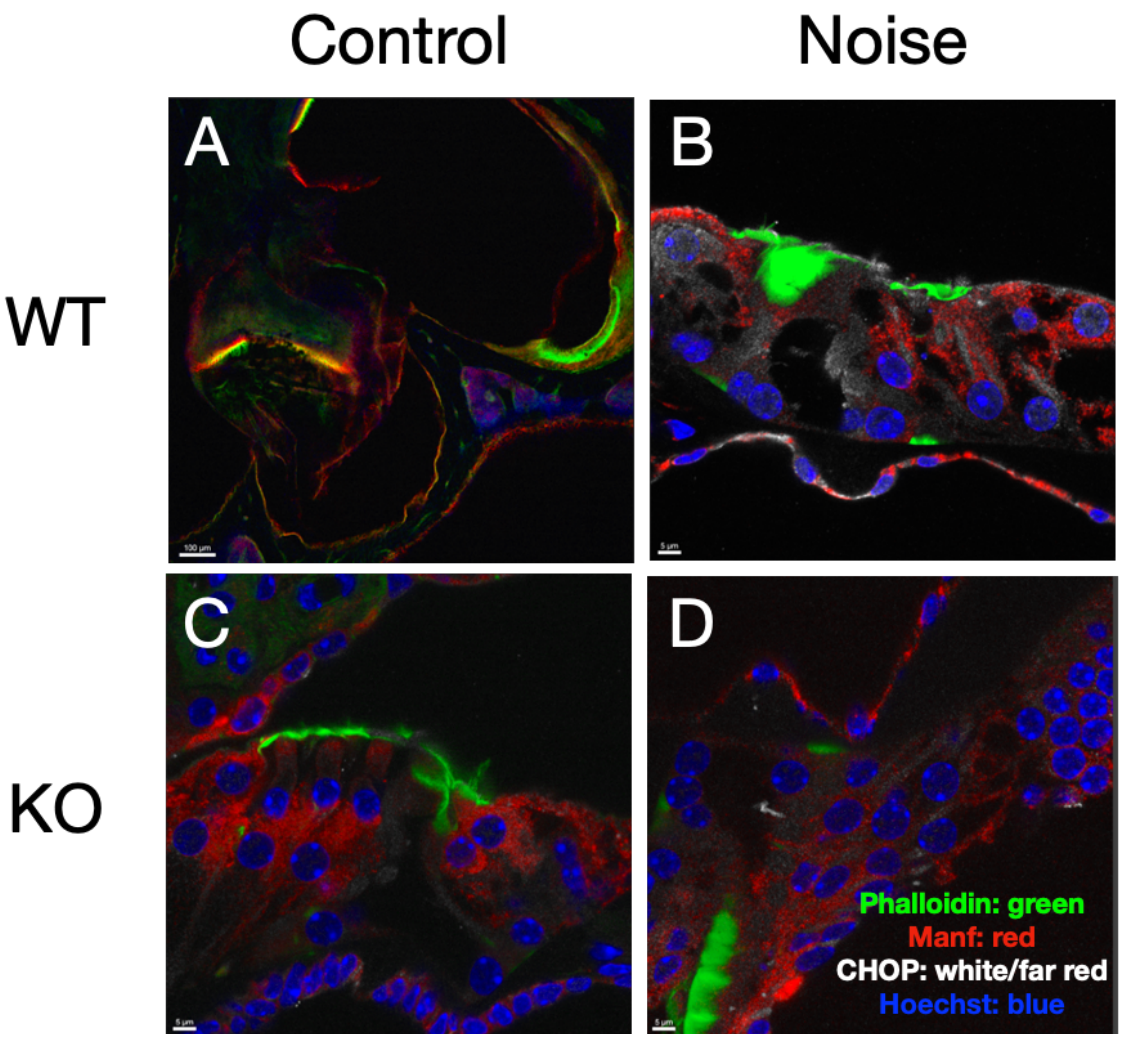

Figure 15. Day 6 Confocal images of Manf and CHOP expression in Ocm WT and Ocm KO mice cochlea via immunostaining (all images were taken at a magnification of 63x with oil immersion, except A). The images were all captured with the same confocal settings. (A) is a 10x image of the Ocm WT control. A visible 63x image of the organ of Corti for the OCm WT control was unable to be collected from the cochlear sections since there was an issue with slide mounting. The expression of Manf and CHOP in Ocm WT mice post-noise exposure is seen in (B). Manf and CHOP expression in Ocm KO control mice not exposed to noise is seen in (C). The Manf and CHOP expression of Ocm KO mice post-noise exposure is seen in (D). The following antibodies were used for this figure: phalloidin (green), rbManf (red), msCHOP (white/far red), and Hoechst (blue). The rbManf antibody labels the endoplasmic resident protein itself Manf. The msCHOP antibody labels the pro-apoptotic protein itself CHOP. Phalloidin serves as a hair cell marker. Hoechst serves as a hair cell nuclei marker. The scale bar is 5 µm.

CHAPTER FIVE

Discussion

Activation of Pro-Apoptotic Components

ER stress occurs when the ER is overwhelmed by protein folding. The saturated presence of unfolded proteins triggers the onset of UPR. The UPR process requires an accumulation of chaperones that assist in protein folding. The increased pressure on the cell to undergo such a process due to traumatic injury from noise may lead to hair cell death. This can be described as apoptosis. Apoptosis in the cochlea leads to the loss of hair cells that are crucial for hearing. This is evidenced by the changes in expression of certain proteins in the organ of Corti between noise-exposed and control no-noise mice.

Elevated noise exposure can result in prolonged ER stress, which may activate pro-apoptotic components affecting OHC survival. The pro-apoptotic components activated are proteins such as CHOP and proteolytic enzymes such as caspases, which trigger programmed hair cell death in the cochlea. Immunofluorescent experiments have shown that noise exposure leads to an upregulation of CHOP which has been accompanied by increased expression of Manf. Previous studies have shown that Manf can suppress the ER calcium depletion-independent CHOP apoptotic pathway. In addition to apoptosis, the expression of Manf can activate UPR transcription signaling. This entails the upregulation of ER-signal transduction of two key UPR pathways that involve IRE1 and PERK (Park et al., 2019). The presence of Manf reveals the relationship between UPR and ER stress, which can induce apoptosis.

The prolonged duration of ER stress accompanied by a lack of adaptive responses can lead to apoptosis as hair cells are further injured and die. Noise exposure places cochlear tissue under ER stress, which activates UPR. The onset of UPR may lead to apoptosis, which results in hair cell injury and loss. ER stress may induce the cell death mechanism of apoptosis, which can be a major contributor to many chronic diseases. It portrays the potential three-way connection between ER stress, UPR, and apoptosis.

Inflammatory Pathways Upregulated by Noise

The effects of noise are related to prolonged ER stress. The ER machinery is a major regulator of intracellular Ca^{+2} homeostasis. Thus, ER stress is associated with elevated levels of cytoplasmic Ca^{+2}. Cochlear damage involves an inflammatory component (Frye et al., 2019). Pro-inflammatory responses cause more oxidative stress due to the unregulated excess levels of Ca^{+2} in the cell. The presence of oncomodulin (Ocm) helps mitigate such inflammation due to its regulation of extra Ca^{+2} in the cell. Ocm is a major protein found in sensory cells, specifically the OHCs.

Through immunofluorescence, the increased presence of Manf which plays a role in UPR indicates the existence of ER stress induced by elevated noise levels. Thus, it can be implied from our experiments that Ocm KO mice showed signs of elevated stress and inflammation post-noise exposure; however, it was not to the same extent as Ocm WT mice. This may be since Ocm KO mice already have poor hearing due to the lack of Ocm, but further experiments are needed to ensure this is valid.

The Interplay between Mitochondrial Stress and ER Stress

The co-labeling of mitochondrial stress markers and ER stress markers implies a relationship between the two in terms of adapting cellular functions. This is best represented by the immunofluorescent labeling of CoxIV, IBA, and Manf. Noise exposure can increase stress levels in both of the following organelles: the mitochondria and endoplasmic reticulum. The simultaneous reactions of ER stress and mitochondrial stress alter the normal functionality of tissue, which can lead to an upregulation of reactive oxygen species which can lead to ototoxic injury of the cochlear tissue. The noise exposure compromised the ER and mitochondria of the cochlear cells by inducing hair cell injury. This is represented by the increased expression of Manf and IBA in the noise-exposed cochlea compared to the non-noise exposed, control cochlea. The difference in CoxIV expression was not unanimous across the different locations of the cochlea. However, when studied specifically at the organ of Corti alongside Manf expression (Figure 8), the slightly prevalent decrease in CoxIV expression may indicate a reduction in mitochondrial stress due to ongoing pathways activated by ER stress. Previous studies have shown that autophagy can attenuate NIHL by reducing oxidative stress, which primarily targets the mitochondria (Yuan et al., 2015). Noise exposure results in ER stress, which leads to an upregulation of Manf expression. Since the increase in Manf expression can lead to the onset of autophagy, this process may be reducing mitochondrial stress within cells of the cochlea as evidenced by decreased expression of CoxIV. The possible communication between Manf and CoxIV can help highlight the interplay between ER stress and mitochondrial stress in NIHL.

Also, like inflammatory pathways and ER stress, the influx of calcium plays a major role in mitochondrial stress. It implies a connection between the three conditions of cochlear hair cells post-noise exposure injury. Further experiments are necessary to confirm the potential connection and role of calcium in ER stress and mitochondrial stress.

CHAPTER SIX

Conclusion

Studies have shown that hair cell injury or death may be a result of elevated noise exposure, ototoxic injury, or a mere consequence of normal aging. We hypothesized that elevated noise exposure would induce ER stress to activate apoptosis and inflammatory pathways. The onset of inflammation is apparent by the increased presence of macrophages due to the pro-inflammatory conditions induced by the elevated noise levels. The injury due to noise exposure leads to an increased inflammatory response. This is demonstrated by an enhanced number of macrophages present post-noise exposure. Similarly, the onset of apoptosis is evidenced by the presence of pro-apoptotic components such as CHOP in mice cochlea post-noise exposure. Through immunofluorescent experiments, we recognized the inflammatory pathways through the infiltration of macrophages into the cochlear tissue post-noise exposure. Additionally, the immunofluorescent experiments revealed an upregulation of key components such as Manf and CHOP revealing the activation of autophagy and apoptosis, respectively, due to the onset of UPR.

The pathways activated by ER stress occur in cells that have been injured due to stressful conditions. In this case, the exposure to elevated noise levels has induced stress specifically in the endoplasmic reticulum. The hair cell injury may have been addressed by the activation of inflammatory pathways as a form of repair, despite the damage that contributes to NIHL. This is evidenced by the increase in macrophages as seen through IBA antibody labeling. The further damage to hair cells may have been addressed by pro-

apoptotic components like CHOP. CHOP can induce apoptosis of damaged hair cells leading to hair cell loss in the cochlea, which can be evidence of NIHL. Additionally, the hair cells under ER stress may require more Manf for proper protein folding to maintain the endoplasmic reticulum. This is represented by the increase in Manf expression in the cochlea of mice exposed to noise. The two distinct pathways help imply the relationship between NIHL and ER stress.

Implications

Exposure to elevated noise levels can lead to cochlear damage in the form of OHC injury and/or death. Immediately following the engagement with persistent and prolonged noise at elevated levels, the cellular damage in the cochlea caused by such noise may be masked. This means that the immediate effects of the injury or loss of hair cells may not be sufficiently pronounced for an individual to notice and recognize symptoms of noise-induced hearing loss. Through the natural process of aging, the synaptic connections present within the inner ear may be lost as the brain begins to experience a structural loss of synapses. The process of synapse elimination is termed synaptophagy because it entails the phagocytosis of viable synapses. Over time, the symptoms of hearing loss are more pronounced allowing them to be more recognizable to humans. This hearing loss was initially due to noise exposure, which induced ER stress leading to the injury and loss of cochlear hair cells. The loss of synaptic connections may contribute to more pronounced effects of hearing loss. The death of cochlear hair cells can be followed by a loss of spiral ganglion neurons (Cunningham et al., 2017), which may lead to the loss of synaptic connections in the inner ear. It can be implied that there is a relationship between noise-induced hearing loss and synaptophagy.

Application

Noise Exposure Therapies

Previous studies have shown that ER stress and UPR activation mediate NIHL in mice. Our results have shown that ER stress from noise can injure and damage OHCs, leading to pathways that may result in hair cell death. As an endoplasmic reticulum resident protein for endoplasmic reticulum homeostasis, Manf may serve as a candidate for therapeutic treatment.

ER stress may be antagonized by Manf to reduce or delay the loss of OHCs, which is triggered by exposure to elevated noise levels. Manf has the potential to prevent ER-stress-mediated cell death. By attenuating apoptosis, Manf may have the ability to activate repair pathways in cells under stressful conditions. Due to the protective function of Manf, this protein may have potential therapeutic value in treating ER stress-related conditions. Several studies indicate that Manf is protective for cells in various pathological conditions such as inflammatory, metabolic, and neurological diseases (Danilova et al., 2019). As ER stress plays a major role in several neurological and metabolic conditions, Manf may serve a protective and therapeutic role in aiding cells to restore homeostasis. ER stress is linked to conditions such as Usher syndrome, Parkinson's disease, Alzheimer's disease, diabetes, and stroke (Herranen et al., 2020).

Next Steps

Hearing loss is more pronounced at higher frequencies (Cunningham et al., 2017). Humans tend to lose hearing in higher frequency regions of the cochlea, which comprises

the basal regions or base of the cochlea. NIHL has become a substantial burden impacting approximately 104 million people (Cunningham et al., 2017).

Our study revealed that the apex or apical regions of the cochlea, such as regions III and IV, are most affected by noise exposure. This is evidenced by the increased presence of Manf in the apical regions of the cochlea post-noise exposure, regardless of genotype strain. Our results imply that the cochlea is properly functioning by increasing the presence of Manf to activate ER stress pathways to address hair cell injury due to noise exposure. Since high-frequency hearing loss is more common and involves the basal regions of the cochlea, the implication of our results could be that the cochlea has diminished protective capabilities in the base compared to the apex. This is evidenced by the difference in Manf presence between the basal and apical regions. Future research is necessary to develop treatments and interventions that address high-frequency regions by possibly mirroring the pathway of Manf as seen in the low-frequency regions. This is especially important for individuals who are more prone to NIHL, as their surroundings and lifestyle may consist of more persistent and prolonged exposure to elevated noise levels. However, this conclusion requires a deeper understanding of the relationship between NIHL and pathways involved in ER stress. From a broader perspective, research focusing on the hair cells in the lower frequency regions of the cochlea would be most relevant to the trend we recognized regarding NIHL.

Manf has implied its capabilities as serving a protective role. Further research focused on studying the Manf pathway within ER stress in sensory hair cells may lead to new avenues into novel therapeutic interventions and treatments for humans experiencing NIHL.

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