

ACTIVATION OF CASPASE-2 FOLLOWING NOISE EXPOSURE AND THE EFFECT
OF TREATMENT WITH DIETARY AGENTS ON NOISE-INDUCED OXIDATIVE
STRESS AND ACTIVATION OF CASPASES-2 AND -8

By

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To those in hearing research who may benefit from my modest contribution

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Abstract of Thesis Presented to the Graduate School
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Noise-induced hearing loss (NIHL) is a growing problem due to increased noise exposure in military, occupational, and recreational settings. Noise induces the formation of free radicals in the cochlea due to increased metabolic demand and reduction of cochlear blood flow. This ultimately damages and/or destroys sensory cells causing permanent hearing loss. One consequence of oxidative stress is the initiation of apoptotic signaling pathways via activation of caspases. Caspases -3, -8, and -9 are known to be up-regulated in the inner ear following noise exposure, and caspase-2 activation has been described as an initiator and/or executioner of apoptosis in other systems. Treatment with dietary antioxidants (β -carotene, and vitamins C and E) delivered in combination with the mineral magnesium (Mg) was previously shown to be effective for the reduction of NIHL. Due to the fact that β -carotene is metabolized to vitamin A, this treatment will subsequently be referred to as ACEMg.

The purpose of this study was two-fold: 1) to investigate the activation of caspase-2 following noise exposure, and 2) to determine the effect of ACEMg treatment on noise-induced production of reactive nitrogen species (RNS) and activation of

caspases-2 and -8. Caspase-2 immunolabeling was initially observed in the supporting cells of the guinea pig organ of Corti 2 hours following noise exposure, and moved transiently to the outer hair cells at 4 hours post-noise. Nitrotyrosine (3-NT), a biomarker for production of RNS, and activation of caspase-8 were assessed in order to determine the effect of ACEMg treatment on noise-induced oxidative stress and initiation of the extrinsic (death receptor mediated) apoptotic pathway respectively. The ACEMg treatment effect which was observed, while not statistically reliable under the current study design, supports further studies at later post-noise time points, when oxidative stress is at a maximum. Taken together, these immunohistochemical data support the possibility that caspase-2 plays a role in NIHL, and that the protective effect observed with ACEMg treatment involves attenuation of production of RNS and activation of caspases-2 and -8.

CHAPTER 1

INTRODUCTION

An Overview of Noise-Induced Hearing Loss

Noise-induced hearing loss (NIHL) represents a growing medical problem with far-reaching economic and social impacts. Due to the expansion of technology, we are now exposed to higher levels of noise than ever before, both in occupational as well as recreational settings. Education regarding the potentially harmful effects of noise, occupational hearing preservation programs, and the technology of hearing protection devices (HPDs) have not kept up with the increase in noise exposure. This disparity is made evident by numerous studies citing widespread detrimental effects of noise in various settings[1-24].

It is estimated that 5-10% of the hearing loss burden in the U.S. is caused by noise exposure in the workplace [1], and that number rises to approximately 16% on a global scale [2]. According to the National Institute for Occupational Safety and Health (NIOSH), approximately 30 million people are exposed to hazardous levels of noise on the job [25]. NIHL was also found to increase the risk of work-related accidents [3, 4]. In a Michigan study, 29.9% of those with hearing loss reported work-related noise as its cause [5]. Those with highest risk of NIHL include construction workers, miners, musicians, disk jockeys, law enforcement officers, and military personnel. Across all trades, 59.7% of construction workers were found to have at least moderate NIHL [6]. Hearing loss among symphony musicians was double the rate that would be expected for corresponding age [7, 8]. Among disc jockeys studied, 70% reported temporary threshold shift, and 74% reported frequent tinnitus (ringing in the ears) after spending time in the dance club [9]. A ten-year longitudinal study of police officers reveals the

detrimental effects of impulse noise from gunfire despite the use of dual protection (earplugs and earmuffs) [10].

Diagnosis of NIHL is based upon a history of noise exposure combined with the presence of a “noise notch” on the patient’s audiogram. While varying definitions exist, a “noise notch” is generally an increase in hearing threshold in the 4000 Hz range (Figure 1-1), which expands to include progressively higher and lower frequencies as the exposure to noise progresses. Coles et al. defines a noise notch as a high-frequency notch where the hearing threshold at 3, 4, and/ or 6 kHz is at least 10 dB greater than at 1 or 2 kHz and at least 10 dB greater than at 6 or 8 kHz [26]. Additionally, Niskar et al. gives criteria which require threshold values at 0.5 and 1 kHz to be \leq 15 dB, the greatest threshold value at 3, 4, or 6 kHz to be at least 15 dB higher than the worst (highest) threshold value at either 0.5 or 1 kHz, and a threshold at 8 kHz at least 10 dB better than the worst threshold at 3, 4, or 6 kHz [20]. Age-related hearing loss (ARHL), or presbycusis also causes a threshold increase at the higher frequencies. However, the audiogram of a person with purely ARHL is downward-sloping with progressively higher thresholds at higher frequencies, and lacks the characteristic notch indicating the contribution of noise. Different notch metrics can be used for diagnosis, and one study found that these metrics can agree with expert clinical judgments [27].

Perhaps the greatest proportion of individuals with NIHL is found in the military. Studies have shown that members of all branches of military personnel are at greater risk for NIHL than the general population [11-14]. In 1970, 20% of all Army veterans were entering claims for hearing loss, and the Veterans Administration (VA) paid over \$52 million in compensation. This figure does not include compensation for hearing

loss with a concurrent disability, cost of hearing aids, batteries, or repairs. Medical evacuations for complaints related to hearing loss were conducted at an average rate of one soldier per day during the first year of the war in Iraq. In 2004, the VA spent \$108 million in disability payments to former Navy personnel, which represents a \$65 million increase from 1999. In 2006, the combined total of disability payments for hearing loss and tinnitus were over \$1 billion. This is a 319% increase since the beginning of the war in Afghanistan in 2001 [13, 14].

According to the Occupational Safety and Health Administration (OSHA) standard for noise exposure, a workplace hearing conservation program is required by law if average noise levels are at or above 90 dBA as an 8-hour time-weighted average using a 5 dB exchange rate [28]. This means that for every 5 dB increase in exposure above 90 dB, the permissible exposure time is cut in half. In 1972, NIOSH recommended a more conservative occupational noise exposure limit of 85 dBA as an 8-hour time-weighted average using a 3 dB exchange rate [25]. Humans have the greatest sensitivity to sound in the range of 1000 – 5000 Hz, and sound measurements can be “A weighted” which attenuates noise outside of this range. The sound level is then reported in terms of dBA [15].

Although attempts have been made to implement noise exposure guidelines and hearing conservation programs, there are many barriers which prevent these efforts from being fully effective. One survey of 29 foundry companies found that all were out of compliance with hearing conservation regulations. Furthermore, the noise exposures that workers received during their average shift routinely exceeded 85 dBA as a time-weighted average. Members of the management, as well as employees from each

company were interviewed, and a positive correlation was found between management and employee knowledge of hearing conservation. In other words, as management education with regards to hearing conservation increased, employee knowledge increased as well [16]. These data imply that lack of knowledge has the potential to increase the risk of occupational NIHL. There are also compliance issues such as the failure of workers to wear hearing protection devices (HPDs) properly. One example of this could include removing HPDs in order to communicate. This repeated removal makes it more likely that the HPD could become dirty or be inserted poorly, both of which would compromise the seal within or around the ear thus decreasing the level of sound attenuation. Another factor affecting compliance is that people do not notice an immediate hearing loss, and it is therefore difficult to convince them that they are indeed at risk [1]. Finally, many fear that wearing HPDs may affect their ability to perform, as is the case with symphony musicians [17], to communicate, or to hear warning signals.

Although occupational hearing loss has received much attention, this is not the only source of harmful noise. Recreational sources of noise can include hunting, skeet shooting, personal music players, fireworks, nightclubs, and concerts. One survey found that most adolescents routinely listen to music at maximum volume, and feel that they are not vulnerable to the damaging effects of noise [19]. It was estimated that 12.5% (approximately 5.2 million) of individuals age 6 – 19 years old and 40% of students age 16 – 25 years old exhibit noise-induced threshold shifts [20]. Furthermore, the average sound levels for a concert are between 120 – 140 dBA, while bars and taverns can reach 95 dBA on a busy night [21-23]. In a web-based survey, 61% of concert attendees reported experiencing tinnitus or temporary hearing loss, and 59%

said that they would be more likely to use hearing protection if it were recommended by a doctor or nurse [24]. It is also noteworthy that while occupational and recreational noise exposure are often considered separately in research studies and for the definition of occupational noise exposure limits, one must bear in mind that the same person who receives their full legal noise dose at work may then proceed to come home and mow the lawn while listening to a personal music player.

Limiting exposure and appropriate use of HPDs are considered the most effective methods of hearing loss prevention. However, even when worn according to the manufacturer's standards, there are many instances in which HPDs cannot reduce the level of exposure below recommended limits. The noise reduction rating (NRR), which represents the level of sound attenuation measured in a laboratory setting, must be stated by the manufacturer for all types of HPDs. To date, the most sophisticated hearing protection technology provides an NRR of approximately 30dB. Additionally, many studies have shown that real-life attenuation is nowhere near the stated NRR. In fact, NIOSH recommends the following subtractions from the manufacturer's labeled NRR: for earmuffs subtract 25%, for formable ear plugs subtract 50%, and for all other ear plugs subtract 70%. Using dual protection (ear plugs and earmuffs) only adds 5-10dB of protection.[25].

It is evident that NIHL is a growing problem which must be addressed. Efforts should include increased education, occupational hearing conservation programs, improving HPDs, and further research into pharmacological preventative or even hearing rescue methods. It has been said that, "prevention of NIHL would probably do

more to reduce the societal burden of hearing loss than medical and surgical treatment of all other ear diseases combined” [1].

The Cochlea

Anatomy and Physiology

The cochlea is the organ which is responsible for the transduction of mechanical energy in the form of a sound stimulus into electrical energy in the form of nerve impulses which are sent to the brain via the auditory nerve (cranial nerve VIII). The structure and function of the cochlea has been extensively studied in many species. A brief overview of cochlear function is provided here, however for detailed review of current understanding the reader is referred to the following references [29-31].

In mammals, the cochlea is spiral shaped and resides within a bony encasing (the otic capsule) in the temporal bone. The lumen of the cochlea is divided into three fluid filled chambers called the scala vestibuli, scala media, and scala tympani. The basilar membrane separates the scala media from the scala tympani, while Reissner's membrane forms the partition between the scala media and the scala vestibuli. A layer of sensory epithelium known as the Organ of Corti houses the main sensory cells called hair cells, and lies within the scala media atop the basilar membrane. The hair cells, so named because of tiny hair-like projections called stereocilia at the apical end of each cell, are divided into two types: inner hair cells (IHCs) and outer hair cells (OHCs). There are three times as many OHCs as IHCs, the two types differ in location as well as function. The tectorial membrane is composed of acellular connective tissue, and forms a covering over the Organ of Corti. The fluid within the scala vestibuli and scala tympani (perilymph) has a different ion concentration than the fluid within the scala media (endolymph). The resulting difference in current potential, called the

endocochlear potential is important for sensory cell signal transduction. This endocochlear potential is generated and maintained by a rich vascular bed in the lateral wall of the scala media called the stria vascularis. (Pertinent structures of the cochlea are depicted in figures 1-2 and 1-3)

The range of frequencies that can be heard by young humans with normal hearing is approximately 40 Hz – 20 kHz, and a sound stimulus entering the ear can contain many frequencies which must be decoded by the cochlea. In 1862, Helmholtz suggested that the basilar membrane is composed of fibers arranged radially which each resonate at a different frequency analogous to the strings of a harp [32]. It was later discovered that there are physical gradations in many structures of the cochlea. For instance, the tectorial membrane and basilar membrane both become gradually wider and thicker from the basal to the apical end of the cochlea. These gradations do indeed confer a tonotopic organization allowing different areas of the basilar membrane to resonate at specific frequencies. This led Bekesy to formulate his traveling wave model, which earned him the Nobel Prize in 1961 [33]. Additionally, the hair cell stereocilia increase in length from base to apex, and stereociliary stiffness is inversely correlated with length [34]. This “tuning” of the tectorial membrane, basilar membrane, and Organ of Corti allows for transduction of high frequency sound waves at the base, and low frequency sound waves at the apex.

The hair cells are classified as either inner hair cells (IHCs) or outer hair cells (OHCs). In the human cochlea, there are approximately 3,500 IHCs arranged in a single row, and 11,000 OHCs arranged in three rows. Afferent signal transduction to the auditory nerve is the main function of IHCs. These cells receive afferent innervation

from peripheral processes of the auditory nerve called spiral ganglion cells, and glutamate is the neurotransmitter of the IHC synapse. A spiral ganglion cell contacts only one IHC, while each IHC is connected to multiple spiral ganglion cells. Movement of the endolymph causes deflection of stereocilia at the apical end of the IHC, which in turn causes ion channels to open and close according to the frequency of the sound stimulus. Entry of K^+ and Ca^{2+} generates a transduction current which activates voltage sensitive Ca^{2+} channels and Ca^{2+} activated K^+ channels leading to the release of neurotransmitter into the afferent synapse at the basal end of the cell [30]. The IHCs also receive efferent innervation from the lateral olivocochlear complex. Originating in the auditory brainstem, these neurons synapse on the peripheral processes of the spiral ganglion cells as opposed to the IHC body itself (Figure 1-4) [30]. The efferent synapse contains both excitatory (acetylcholine, dynorphin, and CGRP) and inhibitory (dopamine, enkephalin, and GABA) neurotransmitters which modulate the afferent sensitivity to glutamate. This may be a protective mechanism to prevent overstimulation [35].

The role of the OHC is more complex. After studying the fluid dynamics of the cochlea, it was recognized that a model relying on passive resonance such as that proposed by Helmholtz and Bekesy was not sufficient. The viscosity of the endolymph would dampen the resonance of the basilar membrane leading to a lower sensitivity than that which is actually observed. Thus, it was proposed that the cochlea must perform some active amplification of the incoming sound stimulus [36, 37]. Accumulating evidence suggested that the OHCs provide mechanical amplification by vibrating at the same frequency as the sound stimulus in order to prevent damping of

the traveling wave. Thus OHCs can be thought of as the mechanical effectors of the cochlear amplifier [31]. Afferent innervation of OHCs accounts for only 5% of auditory nerve dendrites, while they receive rich efferent innervation from the olivocochlear bundle. Unlike the IHCs, the OHC efferents synapse on the cell body itself which provides support for an efferent feedback mechanism (Figure 1-5). Additionally, the concept of an afferent signal leading to efferent stimulus and further amplification lends itself to the analogy of “feedback” in a modern sound system. This idea eventually led to the discovery of otoacoustic emissions, or sounds generated by vibration of the inner ear which can be recorded in the external auditory canal [38]. Later, OHC motility was clearly shown, as hyperpolarization causes the cells to lengthen, and depolarization leads to cell shortening [39]. This movement of OHCs was captured by video microscopy in 1986 [40]. The next puzzle to be solved was the mechanism of OHC motility. In 2000, a new kind of motor protein was discovered in the OHC membrane. This protein was named prestin, and is a member of the SLC26 family of anion-bicarbonate transporters. The name prestin is derived from “presto” which means fast in Italian, and was given due to its ability to operate on a microsecond timescale [40]. Overall, OHC motility serves to amplify the resonance caused by the sound stimulus, which enhances the selectivity and specificity of cochlear tuning.

Cellular Mechanisms of Noise-Induced Cochlear Pathology

Exposure to high intensity noise has the potential to damage the cochlea, and to impede its function in various ways. This damage can occur in response to impulse noise such as gunfire, or continuous noise exposure, as is potentially experienced with personal music players. Overexposure can cause both mechanical damage and metabolic damage of the hair cells and surrounding structures. The OHCs are more

susceptible to damage than IHCs, and spiral ganglion neuron degeneration occurs following IHC loss [41]. Furthermore, OHCs at the basal end of the cochlea are more susceptible than those at the apex [42]. Hair cell death by both necrosis and apoptosis simultaneously was shown one hour post-noise in chinchillas exposed to a continuous noise insult of 110 dB SPL centered at 4 kHz for a duration of one hour [43]. Necrosis is a passive form of cell death which is characterized by nuclear swelling, rupture of the plasma membrane, and spilling of cell contents. This causes damage to surrounding tissue, and initiates an inflammatory response. On the other hand, apoptosis is a programmed pathway to cell death characterized by nuclear condensation and fragmentation, which is essential in normal growth and development for the elimination of unwanted or damaged cells [44]. Unlike necrosis, apoptosis does not cause damage to the surrounding tissues. Apoptosis can also be triggered inappropriately causing the death of necessary cells [45]. One study found that 30-50% of hair cells can be lost before any measurable hearing loss can be detected [46].

Mechanical damage and glutamate excitotoxicity

Following acoustic overexposure there is an immediate loss of hearing sensitivity. Depending upon the intensity and type of exposure, this loss of sensitivity can recover fully or partially with time. This immediate hearing loss which recovers with time is commonly known as temporary threshold shift (TTS), and any persistent loss is termed permanent threshold shift (PTS). Less intense noise exposure is usually associated with TTS alone, and mechanical damage to hair cell stereocilia [47], as well as glutamate excitotoxicity are mechanisms which contribute to TTS. Glutamate excitotoxicity refers to the release of large amounts of glutamate into the IHC afferent synapse. This over stimulates the nerve causing swelling and damage which can

usually recover with time [48]. However, recent data indicate that even when hearing sensitivity and hair cell function fully recover with time, neural degeneration without concurrent loss of hair cells (primary neural degeneration) may still occur[49, 50]. As the intensity of exposure increases above approximately 125 dB, cochlear injury is primarily caused by mechanical rather than biochemical mechanisms. Additionally, research suggests that impulse noise is more harmful than continuous noise of the same intensity [51]. Any damage which causes significant hair cell death induces a PTS because mammalian hair cells cannot be regenerated.

Oxidative stress

The production of reactive oxygen and reactive nitrogen species (ROS/RNS) in the cochlea following noise exposure has been well documented and reviewed [52-56]. ROS and RNS include free radicals such as superoxide, peroxynitrite, and hydroxyl radicals. Excess production of free radicals can have detrimental effects because an unpaired electron makes these molecules extremely reactive, and capable of damaging many cellular components. For a review of free radical mechanisms see [57]. Free radical damage has also been implicated in a number of human neurodegenerative diseases [58].

Following noise exposure in chinchillas, there was a marked increase in ROS seen in the OHCs [59], and administration of paraquat (which produces superoxide) to the round window membrane resulted in PTS and hair cell loss [60]. The cochlea contains many endogenous antioxidants such as superoxide dismutase (SOD), catalase, and glutathione (GSH) [61-64]. Glutathione directly scavenges free-radicals. The function of SOD is to convert the harmful superoxide anion to molecular oxygen and hydrogen peroxide, while catalase converts hydrogen peroxide to molecular oxygen and water.

Exposure to intense sound, especially at high frequencies, exerts a greater metabolic demand on the outer hair cells, which by nature have a high energy requirement under normal circumstances. Superoxide, which is a byproduct of mitochondrial respiration, is produced in greater quantities under high metabolic demand and can react with nitric oxide to generate the highly destructive peroxynitrite radical. Additionally, the generation of hydrogen peroxide by SOD can participate in the Haber Weiss and Fenton reactions to produce hydroxyl radicals [55]. Cochlear damage occurs when large scale production of free radicals overwhelms endogenous antioxidant defenses.

Glutathione peroxidase prevents oxidative damage by reducing lipid peroxides and catalyzes endogenous GSH production. One study showed that noise-exposed glutathione peroxidase (Gpx1) knockout mice had a higher PTS, and greater IHC and OHC loss than wild-type controls [65]. A four-fold increase in hydroxyl radicals was also seen within 1-2 hours after noise exposure [66]. Finally, ROS and RNS production was shown to peak 7-10 days following noise exposure, but hair cell loss progressed for approximately 2 weeks. This suggests that there is a window of time following exposure during which treatment with exogenous antioxidant or endogenous antioxidant bolstering agents may be effective [67]. The link between ROS production and cell death is not fully understood. However, there is widespread evidence that ROS play a major role in the following cell death mechanisms [68].

Ischemia/ reperfusion injury: Due to their motility, the OHCs have a high energy requirement and thus a high rate of aerobic respiration. As energy demand increases with noise exposure, mitochondrial efficiency decreases, and superoxide is released as an unwanted byproduct of oxidative phosphorylation. The decrease in mitochondrial

efficiency can be compounded when noise-induced damage of the lateral wall vasculature causes a decrease in cochlear blood flow [69]. This state of ischemia, when demand for oxygen is at its highest, further increases the production of ROS. Furthermore, as the vasculature is repaired, and the site is reperfused, the sudden increase in oxygen once again fuels the production of ROS [52]. This mechanism can run in a vicious cycle, as ROS production can cause further damage to the stria vasularis and decrease cochlear blood flow [70].

Lipid peroxidation: Lipid peroxidation is another self-perpetuating process in which free radicals catalyze the breakdown of lipid molecules within cellular membranes. A byproduct of lipid catalysis is 8-isoprostaglandin-F2 α (8-iso-PGF2 α) which causes vasoconstriction, and again leads to decreased cochlear blood flow. In guinea pigs, there was a 30 fold increase in 8-iso-PGF2 α following noise exposure, and the extent of hair cell loss corresponded to the level of 8-iso-PGF2 α production [70].

Extrinsic vs. intrinsic pathways to apoptosis: The extrinsic and intrinsic pathways are two primary signaling cascades leading to apoptosis. The extrinsic pathway is mediated by cell surface death receptors, while the intrinsic pathway is mediated by the release of pro-apoptotic factors from the mitochondria (Figure 1-6) [71]. There is evidence for the utilization of both pathways in noise-induced cell death in the inner ear. Additionally, apoptotic hair cell death can take place through either caspase-dependent, or caspase-independent pathways. Caspases are aspartate specific cysteine proteases which can propagate a cell death signaling cascade. Caspases -8 and -9 are classified as initiators of the apoptotic signaling pathway, and caspases -3, -

6, and -7 are apoptotic effectors [72]. Caspases -8, -9, and -3 were shown to be activated in chinchilla OHCs following noise exposure [59].

The extrinsic pathway is initiated when the death receptor (Fas) and its associated adaptor protein, Fas-associated death domain (FADD) dimerize with Fas ligand, which is a member of the TNF family, to form the death inducing signaling complex (DISC). This in turn activates the initiator caspase-8 which can either directly activate the effector caspase-3, or cleave BID which facilitates the release of cytochrome C mediated by the insertion of Bax or Bak into the mitochondrial membrane. Both of these circumstances result in cell death [72]. There is evidence that many inflammatory cytokines, including those of the TNF family which can act as a death receptor ligand, are upregulated following noise exposure [73]. Active caspase-8 was also shown to be upregulated in hair cells following noise exposure [59].

The intrinsic pathway can be initiated by a variety of mechanisms following noise-induced ROS production and cell damage. Damage to membrane transport proteins leads to the influx of Ca^{2+} [74, 75] which in turn causes phospholipase A₂ (PLA₂) activation and calpain dependent cleavage of calcineurin. Subsequently, PLA₂ hydrolyzes phospholipids to pro-inflammatory mediators which lead to caspase activation and cell death [76]. Cleavage of calcineurin allows for phosphorylation of nuclear factor of activated T-lymphocytes (NFAT), which is a transcription factor controlling many genes involved in regulation of cell death [77]. Calcineurin can also dephosphorylate the pro-apoptotic regulator Bcl-2-associated death promoter (BAD), which translocates to the mitochondria, downregulates anti-apoptotic members of the

Bcl-2 family and activates Bcl-2-associated X protein (Bax). This causes mitochondrial membrane permeabilization, release of cytochrome C, and ultimately cell death [78].

It is clear that many of these pathways converge at the point of mitochondrial membrane permeabilization. It is well known that the Bcl-2 family of proteins regulate mitochondrial membrane permeability, and ultimately cell death. There are pro-apoptotic (Bax, Bak, Bcl-X_S, Bid, Bad, and Bim), and anti-apoptotic (Bcl-2 and Bcl-X_L) Bcl-2 proteins. When the ratio shifts in favor of pro-apoptotic proteins, Bax translocates from the cytoplasm to the mitochondria, causes membrane permeabilization, and release of cytochrome C into the cytoplasm. Cytochrome C can then associate with apoptotic protease-activating factor-1 (APAF-1), dATP, and procaspase-9 to form the apoptosome. This causes activation of caspase-9, which activates effector caspases leading to cell death [79]. Evidence of the importance of the Bcl-X_L/Bak ratio in cell survival was shown in the inner ear using two noise exposure groups. One group was exposed to noise of a lower intensity designed to induce only TTS, while the other group was exposed to intense noise causing PTS. Bcl-X_L was upregulated in hair cells of the TTS group, while Bak was expressed in the PTS group [80].

Other factors thought to be involved in noise-induced cell death are p53 and c-jun NH₂-terminal kinases (JNKs). The tumor suppressor protein p53 is activated by DNA damage, and also causes loss of mitochondrial membrane integrity [81]. JNKs are thought to function upstream of caspase activation or cytochrome C release. MAP kinases are activated by cellular insult causing phosphorylation of the transcription factor c-jun, which controls many genes regulating cell death [82-84].

The intrinsic pathway to cell death also consists of caspase-independent mechanisms. For review of these mechanisms see [85]. Apoptosis inducing factor (AIF) and endonuclease G (endo G) are two mitochondrial proteins which, like cytochrome C, can be released upon loss of mitochondrial membrane integrity. Endo G is a sequence non-specific DNase which normally functions in mitochondrial DNA replication and repair [86]. AIF was identified in 1999, and is thought to have an essential role in development [87]. Following their release, AIF and endo G translocate to the nucleus and cause DNA fragmentation leading to cell death [88-92].

Ironically, these “caspase-independent” pathways may actually activate a unique pathway involving caspase-2. Caspsase-2 is different than its other family members because it has characteristics of both an initiator, and an effector caspase. It is thought that activated caspase-2 acts upstream causing mitochondrial membrane permeabilization and cytochrome C release. To date, caspase-2 activity in response to noise has not been characterized in the inner ear. However, caspase-2 is known to play a role in cell death subsequent to DNA damage [93-95]. Due to the DNA damage caused by translocation of AIF and endo G, it is reasonable to think that caspase-2 may also be involved in hair cell death secondary to noise exposure. Furthermore, one study found that ROS formation led to activation of caspase-2 in a human leukemic T cell line [96].

Experimental Treatment for Noise-Induced Hearing Loss

Although its prevalence continues to increase despite hearing conservation programs, there are currently no FDA approved pharmacological agents for the prevention or treatment of NIHL. However, there has been a great deal of preliminary translational research driven by the recent increase in understanding of the cellular and

molecular pathways underlying NIHL. The complexity of these pathways implies many possible therapeutic targets and points of intervention. Numerous drugs have entered, or have been proposed for, clinical trials based on demonstration of protective efficacy or even rescue of hearing loss across various animal species (For thorough review see [97]). Most of these drugs fall into one of three categories: direct inhibitors of cellular stress pathways, neurotrophic factors/ neurotransmission blockers, or direct inhibitors of oxidative stress [98]. A significant barrier to clinical applicability is that many of these treatments have only been shown to be effective when applied locally in the inner ear. This is obviously not practical for humans anticipating, or having recently been exposed to harmful noise levels. Thus, the ideal treatment would be one that can be given systemically (preferably orally), and still achieve a therapeutic level in the inner ear.

Inhibitors of Cellular Stress Pathways

The noise-induced cellular stress response can lead to inflammation, disruption of calcium homeostasis, and activation of kinase signaling cascades leading ultimately to cell death. Glucocorticoids have long been used as nonspecific inhibitors of the stress-induced inflammatory response. Dexamethasone, a synthetic glucocorticoid, was infused via implanted osmotic mini-pumps directly into the scala tympani of guinea pigs, and a significant reduction of noise-induced PTS and hair cell loss was observed. Dexamethasone binds to glucocorticoid receptors in the inner ear and regulates transcription of inflammatory mediators, thus reducing ischemia and ROS production [99]. However, systemic administration of glucocorticoids is known to be associated with many untoward side effects, making it an impractical treatment for NIHL.

As mentioned previously, noise-induced calcium influx causes neuronal damage and phospholipase A₂ activation leading to lipid peroxidation and ROS production.

Calcium dysregulation also leads to calpain dependent cleavage of calcineurin and activation of the transcription factor NFAT leading to apoptosis. Oral administration of the calcium channel blockers trimethadione and ethosuximide [100], or diltiazem [101] was carried out in mice and guinea pigs respectively. Trimethadione and ethosuximide exhibited a protective effect when given after noise exposure, and trimethadione reduced PTS when given prior to noise exposure. Diltiazem protected OHCs from impulse noise only when applied both before and after exposure. Inhibition of calpain or calcineurin using leupeptin [82, 102] or cyclosporin A and FK506 [103, 104] respectively also achieved a protective effect.

Finally, inhibition of two kinase signaling pathways was explored. These signaling cascades regulate many genes involved in cell death, and include the Src protein tyrosine kinase (PTK), and c-Jun N-terminal kinase (JNK) pathways. One of the detrimental effects of Src-PTK signaling is the activation of NADPH oxidase which causes increased superoxide production. Reduction of PTS and hair cell loss was achieved following application of Src-PTK inhibitor KX1-004 to the round window membrane of chinchillas [105]. Similarly, the JNK inhibitors D-JNK-1 (aka: AM-111) [106, 107] and CEP-1347 [84] exhibited a protective effect following local administration in various species.

Neurotrophic Factors/ Neurotransmission Blockers

Neurotrophic factors are secreted proteins which promote the survival, growth, and differentiation of neurons. Several neurotrophic factors such as neurotrophin-3 (NT-3) and brain-derived neurotrophic factor (BDNF) are expressed in hair cells [108]. Qiang et al., 2004 cultured mouse spiral ganglion neurons and exposed them to high concentrations of glutamate in order to simulate glutamate excitotoxicity. Less neuronal

cell death was seen when basic fibroblast growth factor (bFGF) was added to the media. Next, they showed that systemic injection of bFGF in guinea pigs decreased PTS and hair cell loss following noise exposure. The interaction of bFGF with growth factor receptors on spiral ganglion cells and hair cells is proposed to increase expression of oxidase, decrease NO production, modulate intracellular calcium, and interfere with expression of pro-apoptotic proteins [109]. Similarly, regrowth of spiral ganglion neurons following deafferentiation with aminoglycoside antibiotics was observed with BDNF and bFGF treatment in guinea pigs.

Another approach aimed at neuroprotection involves the attenuation of glutamate excitotoxicity using glutamate receptor antagonists, or glutamate neurotransmission blockers. Carbamathione and caroverine are glutamate receptor antagonists which reduced PTS following noise with systemic injection in chinchillas and local administration in guinea pigs respectively [110, 111]. Carbamathione downregulates N-methyl-D-aspartate (NMDA) receptor activity, while caroverine acts on both NMDA and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors. Another agent which exhibits NMDA receptor antagonistic properties is the mineral magnesium [35]. Riluzole (2-amino-6-trifluoromethoxy benzothiazole) is a glutamate neurotransmission blocker which exhibited a protective effect following local and systemic administration in guinea pigs [112].

Inhibitors of Oxidative Stress

Acoustic overstimulation decreases mitochondrial efficiency, stimulates excess glutamate release into the afferent IHC synapse, and induces a state of cochlear ischemia, all of which increase production of ROS. Many approaches are being explored which enhance endogenous cochlear antioxidant defenses, inhibit production

of ROS, or directly scavenge free radicals. Antioxidant treatments are potentially appealing due to the fact that many of these compounds have been shown to be safe when given orally.

Early attempts to augment endogenous antioxidant defenses used R-phenylisopropyladenosine (R-PIA) and glutathione monoethyl ester (GSS) [113] applied locally to the round window membrane of chinchillas. R-PIA, which increases GSH and superoxide dismutase levels, decreased PTS and OHC loss. The cell permeable enzyme glutathione synthetase (GSS), which is involved in GSH production also provided a significant reduction in PTS. Other compounds which increase endogenous GSH, including n-acetyl-cysteine (NAC) and D-methionine (D-met), reduce NIHL as well. In addition to its ability to directly scavenge free radicals, NAC is also one of the amino acids composing GSH, and is thus able to replenish this endogenous antioxidant when depleted following intense noise exposure (For review see [114]). NAC has been shown effective for the reduction [110, 115, 116] and rescue [54, 117, 118] of NIHL in various species. It was also effective when given orally, but its efficacy was greatly decreased [119]. D-Met also acts primarily as an indirect antioxidant by increasing intracellular GSH levels. This is possible because methionine is used in the synthesis of cysteine, a component of GSH. D-Met also attenuates the increase in SOD levels which is regularly observed in the cochlea following noise exposure [120]. SOD is responsible for the conversion of superoxide to hydrogen peroxide, which is subsequently removed by catalase. The increase in SOD following noise is not accompanied by an increase in catalase, therefore the excess hydrogen peroxide creates favorable conditions for ROS production. Administration of D-Met was found to

prevent decreases in Na/K ATPase and Calcium ATPase activity, decrease intracellular NO concentration, and prevent lipid peroxidation following noise [79].

Agents recognized for their ability to inhibit the production of ROS include allopurinol, acetyl-L carnitine (ALCAR), and 2-phenyl-1,2-benzisoselenazol-3(2H)-one (ebselen). Allopurinol, also used for its ability to inhibit uric acid synthesis in chronic conditions such as gout, attenuated PTS in systemically injected rats [121]. The mitochondrial membrane component ALCAR serves as a precursor for acetyl-CoA and L-carnitine which carry lipids into mitochondria for β -oxidation and enhance ATP production. Therefore, this compound improves mitochondrial efficiency, and decreases ROS production. ALCAR was effective for reduction of hair cell loss, and for prevention [110, 115] and rescue [118] of PTS. Ebselen is a glutathione peroxidase mimetic which decreases hydroperoxide formation, scavenges peroxynitrite, inhibits NO synthase, and prevents lipid peroxidation and cytochrome C release. Ebselen is effective for the reduction of TTS, PTS, and hair cell loss [122-124]. It also reduced noise-induced swelling of the stria vascularis in rats [125].

Finally, multiple antioxidant compounds which directly scavenge free radicals were found to attenuate PTS and hair cell loss to varying degrees. Among these are mannitol, salicylate, resveratrol, coenzyme Q₁₀, and 4-hydroxy phenyl N-tert-butylnitrone (4-OHPBN). Mannitol, a scavenger of hydroxyl radicals, attenuated PTS when injected systemically [126]. In addition to scavenging hydroxyl radicals, salicylate forms the iron chelator dihydrobenzoate, which prevents ROS formation by inhibiting the iron catalyzed Fenton reaction [117]. Resveratrol, which is naturally present in grapes and red wine, acts as an anti-inflammatory, vasodilator, and neuroprotectant in addition

to its antioxidant properties [121, 127]. Coenzyme Q₁₀ participates in oxidative phosphorylation as an integral member of the electron transport chain, and also has potent antioxidant activity. Efficacy of oral administration was increased when given as coenzyme Q₁₀ Terclatrate (Q-Ter), a form which is highly water soluble [128][129]. Finally, 4-OHPBN, scavenges hydroxyl radicals and superoxide anions, showed a dose dependent reduction in PTS and OHC loss in chinchillas [130]. This compound has entered phase III clinical trials as a treatment for stroke [129].

To date, no treatment has been identified which is completely effective for the prevention or rescue of NIHL. Significant advancements have been made in identifying agents which are effective when given systemically, and are thus more clinically applicable. Due to their different mechanisms of action, and the fact that no single agent has provided complete protection, it may be beneficial to explore various combinations of these agents.

Beta-Carotene, Vitamins C and E, and Magnesium

In 2007, Le Prell et al. administered either a combination of the antioxidants β-carotene and vitamins C and E, magnesium alone (Mg), or β-carotene and vitamins C, and E plus magnesium in guinea pigs exposed to 5 hours of 120dB SPL octave band noise centered at 4 kHz. Given the fact that β-carotene is metabolized to vitamin A, this treatment will be subsequently referred to as ACEMg. Treatments were given once daily beginning 1 hour prior to noise exposure, and continuing until day 5 post-noise. Auditory brainstem response (ABR) thresholds were measured at day 10 following noise exposure. Threshold shifts for the ACEMg group were significantly decreased compared to saline control animals at 4, 8, and 16 kHz. There was no significant difference in ABR threshold between control animals and those treated with either ACE

or Mg (Figure 1-6). Hair cell loss was also significantly reduced in the ACEMg group compared to control and ACE or Mg alone (Figure 1-7) [131]. These data suggest a synergistic protective effect between agents.

Each compound in this combination treatment has a distinct site and mechanism of action for the reduction of cellular damage secondary to oxidative stress. Beta-carotene is a fat-soluble nutrient which effectively scavenges singlet oxygen. As previously mentioned, β-carotene is metabolized to vitamin A. Once adequate reserves of vitamin A have been established, excess β-carotene in the blood is free to directly scavenge free radicals. Singlet oxygen reacts with membrane lipids to form lipid hydroperoxides, thus β-carotene prevents lipid peroxidation [132]. Vitamin C (ascorbic acid) is a water soluble essential nutrient which can directly reduce free-radicals in the aqueous phase [133]. As well as scavenging superoxide, hydroxyl radicals, and singlet oxygen, ascorbic acid can regenerate α-tocopherol (vitamin E) from the α-tocopheroxy radical which results from the reduction of ROS by vitamin E [134]. Dietary or systemically injected vitamin C reduced PTS, prevented hair cell loss, increased endogenous antioxidants in guinea pigs and rabbits [135, 136]. Ascorbic acid also reduced NO production by 56% in the lateral wall and 37% in the organ of Corti of guinea pigs [137]. Vitamin E (α-tocopherol) is another lipophilic nutrient which reduces peroxy radicals, peroxy nitrite radicals, and inhibits the propagation of lipid peroxidation [132]. Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), a water soluble and cell permeable derivative of α-tocopherol is often used for treatment. Pretreatment with vitamin E significantly reduced PTS and hair cell loss, and post-treatment had a protective effect when initiated up to 3 days post-noise [138]. Dietary vitamin E showed

a dose-dependent reduction in PTS in fathead minnows (*Pimephales promelas*) [139].

The protective role of magnesium in NIHL is most likely due to its role as a vasodilator.

Magnesium inhibits calcium influx into vascular smooth muscle cells by blocking calcium channels, and also activates adenylyl cyclase which forms adenosine 3',5'-monophosphate within vascular smooth muscle cells leading to dilation [140].

Magnesium may also reduce glutamate excitotoxicity due to its NMDA-antagonistic effect on afferent dendrites [35, 141]. The noise-induced reduction of cochlear blood flow, as well as the associated decrease in perilymph oxygenation, was attenuated in guinea pigs maintained on a high magnesium diet [142]. Also in guinea pigs, PTS and susceptibility of hair cell stereocilia to noise damage showed a negative correlation with perilymph magnesium concentration, and there was a marked reduction in TTS as well[143, 144]. Human trials with magnesium for NIHL have also been shown to reduce PTS as well as TTS with no side effects [145, 146].

Preliminary data from Le Prell et al. showed that type II fibrocytes, stria cell density, and threshold sensitivity were preserved in noise-exposed CBA/J mice maintained on a high ACEMg diet[147]. Thus, this micronutrient combination has been shown to be effective for reduction of NIHL as a dietary supplement, and each of its components has a very high safety profile based on AREDS seven year ACE trials in humans.

Study Design

The aim of this study was: 1) to evaluate activation of caspase-2 following noise exposure, and 2) to further characterize the effect of ACEMg treatment on free radical production, and caspases -2, and -8 activation subsequent to noise exposure. The difference in expression pattern of these target molecules known to be upregulated

following intense noise were assessed in antioxidant treated and control animals using immunohistochemistry. There are two splice variants of caspase-2 which play opposite roles in initiation of apoptosis. Caspase-2_L induces cell death, while caspase-2_S suppresses cell death [148]. Caspase-2_L was assessed in this study; there are no selective antibodies for caspase-2_S. Caspase-2 expression has been described in the inner ear of newborn rats, where it plays a role in apoptotic cell death during development [149]. However, whether it is activated secondary to noise exposure remains to be determined. Production of 3-nitrotyrosine (3-NT) was assessed as an indicator of oxidative stress in antioxidant treated and control animals. 3-NT is known to accumulate in conditions involving oxidative stress such as Huntington's disease and ischemic brain injury. It is also used as a reliable marker of RNS activity [67, 138, 150]. Caspase-8 expression was evaluated to determine the effect of antioxidant treatment on the extrinsic (death receptor mediated) apoptotic pathway. We hypothesized that free radical production (as assessed by production of 3-NT) would be reduced in antioxidant treated ears, caspase-2 would be activated in noise exposed animals confirming its role in noise-induced cell death, and noise-induced caspase-8 activation would not be effected by ACEMg treatment because antioxidant treatment presumably should not interfere with the ligation of death receptors.

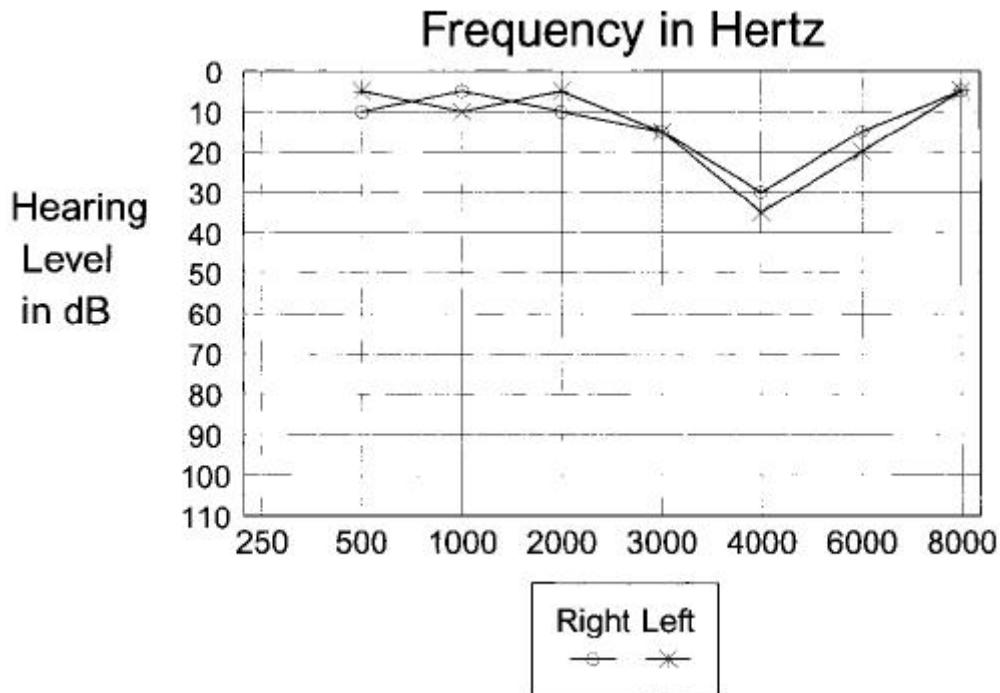


Figure 1-1. Typical audiogram exhibiting early NIHL. The bilateral decrease in hearing sensitivity at 4000 Hz forms the characteristic “noise notch” [15].

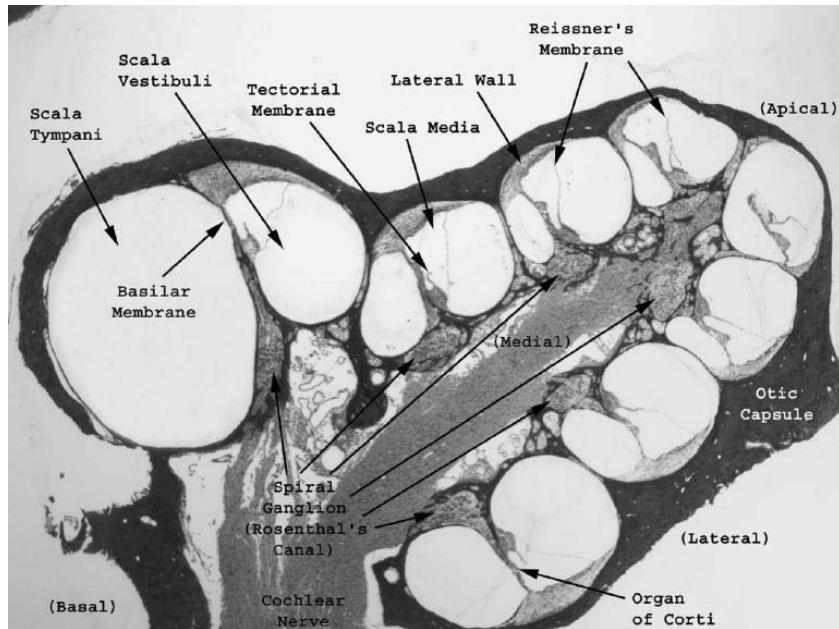


Figure 1-2. Light micrograph of a cross-section of the guinea pig cochlea. Major structures are labeled, and anatomical directions are noted in parenthesis [30].

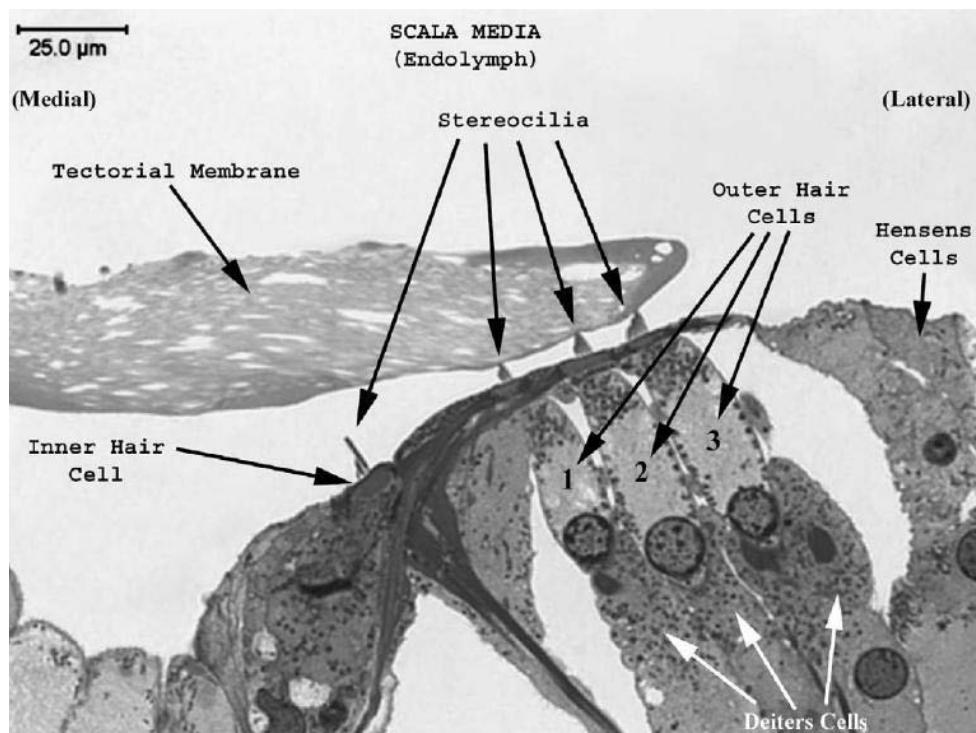


Figure 1-3. Cross-section of the organ of Corti with tectorial membrane covering the hair cells [30].

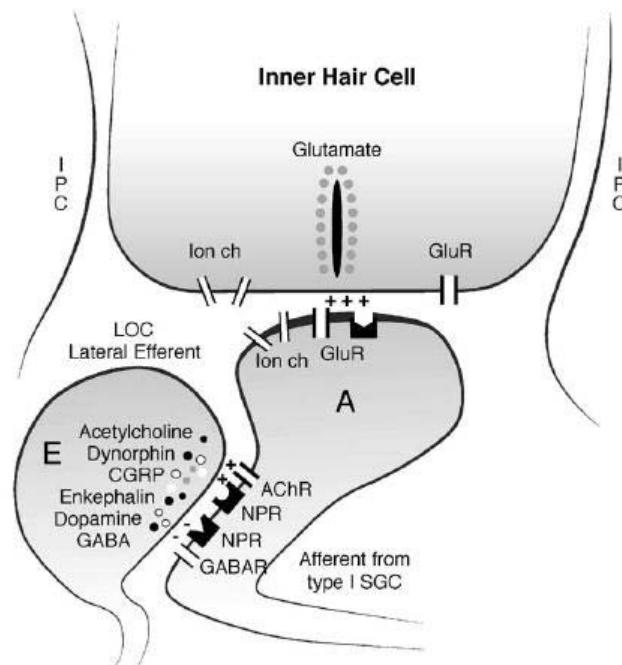


Figure 1-4. Diagram showing efferent (E) and afferent (A) innervation of the IHC. Excitatory (+) and inhibitory (-) neurotransmitters are shown along with their receptors. NPR denotes neuropeptide receptors with various functions, and IPC represents the inner phalangeal cells which surround the IHC [30].

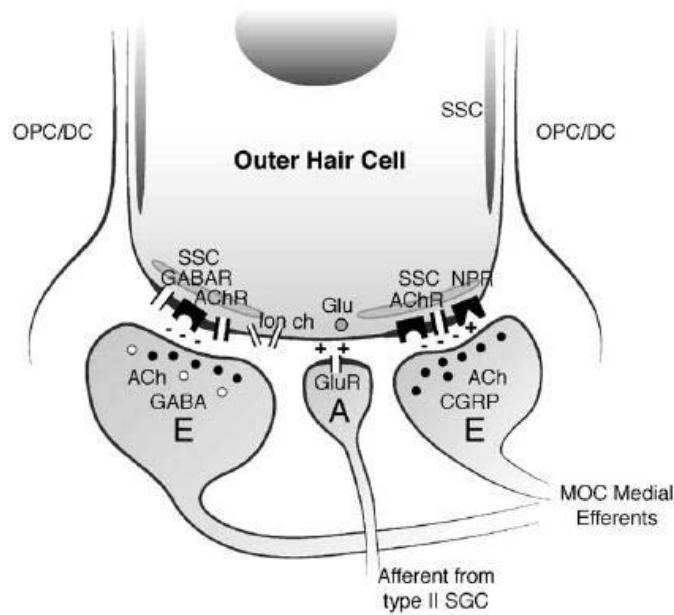


Figure 1-5. Diagram showing afferent (A) and efferent (E) innervation of the OHC. Excitatory (+) and inhibitory (-) neurotransmitters and receptors are shown. SSC denotes sub-synaptic cisternae. Outer phalangeal cells/ Deiters cells are represented laterally (OPC/DC) [30].

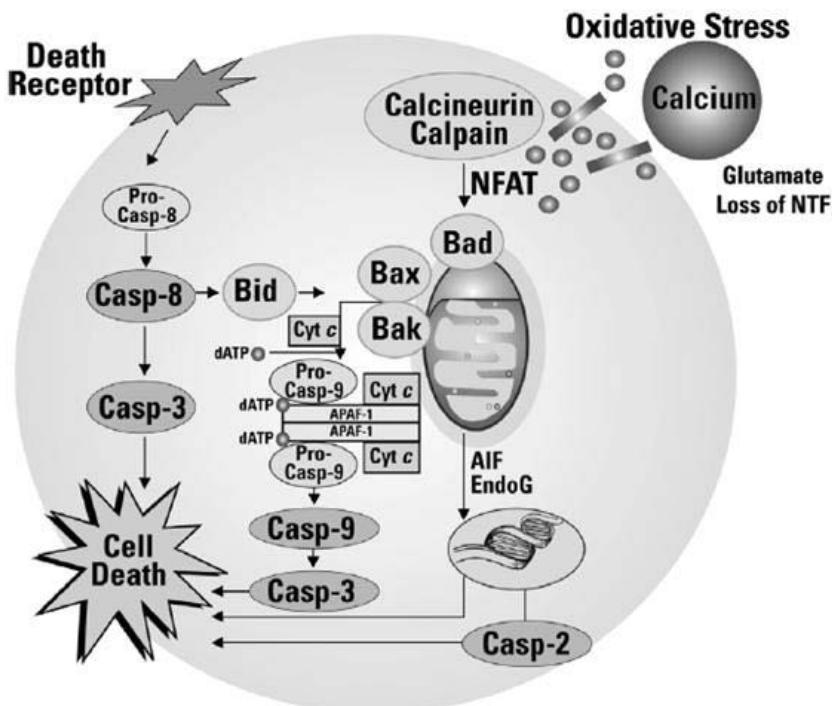


Figure 1-6. Apoptotic pathways. Initiation of apoptosis can occur through either the extrinsic (death-receptor) pathway or the intrinsic (mitochondria mediated) pathway[56].

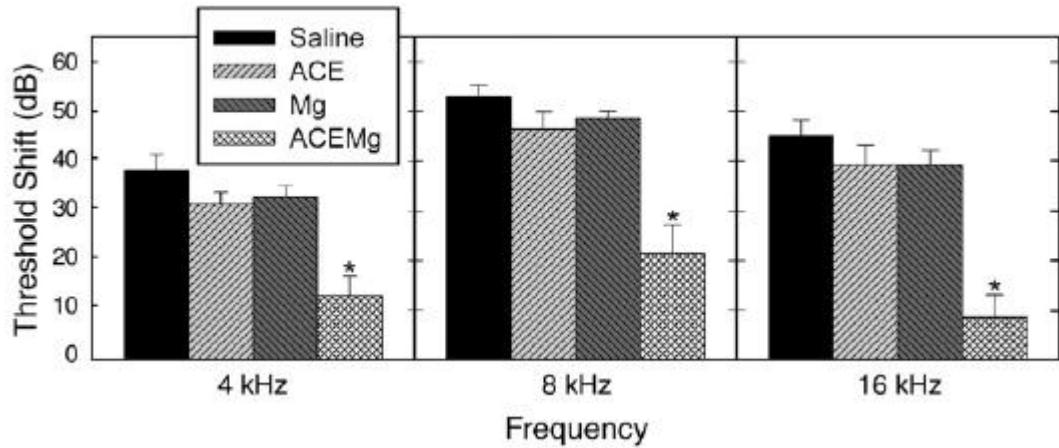


Figure 1-7. Effect of ACEMg treatment on NIHL. Auditory brainstem response (ABR) threshold shift before and 10 days after noise exposure were significantly reduced with ACEMg treatment, but not with either ACE, or Mg alone [131].

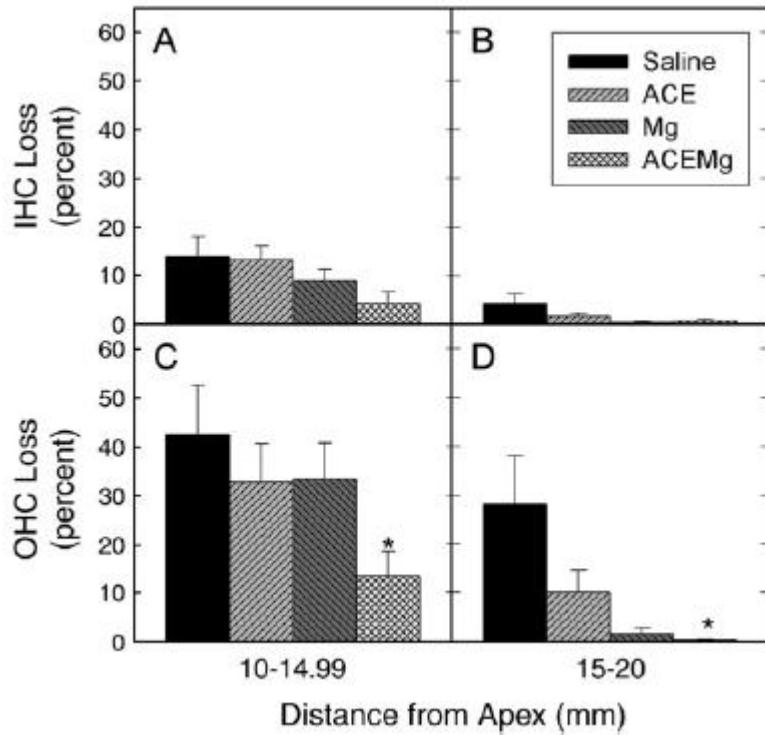


Figure 1-8. Effect of ACEMg treatment on IHC and OHC loss. Outer hair cell loss was significantly reduced with ACEMg treatment, but not with either ACE, or Mg alone [131].

CHAPTER 2 MATERIALS AND METHODS

Subjects

Albino male guinea pigs (250-350 grams) from an approved laboratory animal supplier (Charles River, Wilmington, MA) were used. Guinea pigs were housed in the Animal Care Facility at the University of Florida, and were identified using ear clips. Following arrival, all animals were given at least 48 hours to acclimate to the environment and recover from transportation-related stress. All experimental protocols regarding the use and care of animals were reviewed and approved by the University of Florida Institutional Animal Care and Use Committee.

Noise Exposure

At the onset of the study, speakers were calibrated by placing microphones at the level of the animals' heads (while the cages were unoccupied). Animals were exposed four at a time, each in separate cages. The cages were arranged in the sound booth so that the exposure each animal received was 114 ± 4 dB SPL octave band noise centered at 4 kHz, depending on location within cage. Noise exposure lasted exactly four hours. For exposure of treated and control animals, two antioxidant treated and two saline control animals were included in each noise exposure group. The cage positioning of treated and control animals was alternated with each noise exposure. Animals were unrestrained and were not anesthetized during the noise exposure period.

Electrophysiological Tests

All subjects were screened for normal hearing sensitivity at 2, 4, 8, 16, and 24 kHz in the right and the left ear using the sound-evoked auditory brainstem response (ABR); left ear ABR tests were repeated on one treated and one control animal per exposure

group post-noise, prior to euthanasia to verify hearing loss obtained using this exposure. During ABR tests, animals were anesthetized with ketamine (40 mg/kg, s.c.) and xylazine (10 mg/kg, s.c.) and neural activity in response to brief, tone pips was measured using sterile, 27-gauge electrodes inserted subcutaneously posterior to each pinna and at the vertex of the skull. Tone levels were decreased from 90 dB SPL to 0 dB SPL in 10-dB increments. Each pip was 10 milliseconds in duration and tones were repeated at a rate of 17/second until 1026 responses were acquired. Threshold was independently determined using a 25- μ V Wave III response criterion. Animals were placed on a water-circulating heating pad to maintain body temperature and lubrication was applied to the eyes to prevent dryness during ABR procedures. The depth of the anesthesia was measured using the pedal withdrawal reflex and additional anesthetics administered as needed. All animals received an overdose of sodium pentobarbital following ABR tests, and were euthanized for immunohistochemical assays.

Antioxidant Treatment

All antioxidant treated animals received a total of two treatments. The first treatment was administered 24 hours prior to noise exposure, and the second treatment was given 1 hour prior to noise. Control animals received saline injections equivalent to the dose of micronutrient cocktail given in treated animals. The micronutrient cocktail of β -carotene, vitamins C and E, plus magnesium was given as follows: vitamin A (2.1 mg/kg β -carotene, po), vitamin C (71.4 mg/kg L-threoascorbic acid, sc), vitamin E (26 mg/kg (\pm)-6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid, "Trolox," sc), magnesium (343 mg/ kg MgSO₄, sc). Trolox is a cell-permeable, water-soluble derivative of vitamin E. All test substances were purchased from Sigma-Aldrich (St. Louis, MO, USA) (β -carotene, C9750, CAS 7235-40-7; L-threoascorbic acid, A5960,

CAS 50-81-7; Trolox, Fluka Chemika, 56510, CAS 53188-07-1; magnesium sulfate, M7506, CAS 7487-88-9).

Immunohistochemistry

Following anesthesia with ketamine (40 mg/kg, s.c.) and xylazine (10 mg/kg, s.c.), animals were euthanized at various time points via sodium pentobarbital overdose and were decapitated. Cochlear tissues were immediately harvested and perfused with 4% methanol-free formaldehyde. Tissues remained in fixative for 3 hours before being rinsed and stored in phosphate buffered saline (PBS) until immunolabeling began. In general, tissues were blocked with normal serum, permeabilized with Triton-X-100, incubated with primary antibody, and labeled with secondary antibody. After immunolabeling was complete, tissues were rinsed (in PBS), dissected for surface preparations, and mounted on glass slides using VectaShield mounting medium. To assure an accurate representation of labeling, two images were taken from each turn of the cochlea. Images were collected using a Leica DM5500B epifluorescence microscope, and processed with ImagePro 6.3 software. Images were acquired as a Z-stack, deconvolved using a nearest neighbor algorithm, and tinted after they were acquired in monochrome.

Nitrotyrosine Immunolabeling

Antioxidant treated (N=5) and saline control (N=5) tissues were harvested 2 hours after noise exposure. All tissues were fixed as mentioned above, rinsed with PBS, permeabilized with 0.5% Triton-X-100 for 1 hour, and rinsed again. Subsequently, tissues were blocked with Power Block for 5 minutes. After blocking, tissues were rinsed with PBS and incubated with anti-3-nitrotyrosine mouse monoclonal antibody (clone 39B6; Alexis Biochemicals, 1:500 for 24 hours at 4°C). The primary antibody

was omitted in negative control tissues. Following PBS rinse, tissues were incubated in the secondary antibody (1:100 AlexaFluor 488 goat anti-mouse IgG) for 1 hour at room temperature.

Caspase-8 Immunolabeling

Antioxidant treated (N=5) and saline control (N=5) ears were processed in the same manner as described for nitrotyrosine immunolabeling. Tissues were incubated in mouse anti-caspase-8 monoclonal antibody (Santa Cruz Biotechnology; sc-5263, 1:500 for 48 hours at 4°C). The primary antibody was omitted in negative control tissues. Following PBS rinse, tissues were incubated in the secondary antibody (1:100 AlexaFluor 488 goat anti-mouse IgG) for 1 hour at room temperature.

Caspase-2 Immunolabeling

The purpose of caspase-2 immunolabeling was two-fold: 1) to determine if caspase-2 immunolabeling would be observed post-noise since this has not been described previously, and 2) to gather preliminary data concerning the effect of ACEMg treatment on caspase-2 expression post-noise (ACEMg, N=2; saline control N=2). All tissues were labeled for caspase-2_L (long variant). Animals used for the initial characterization of caspase-2 expression were euthanized at 2 (N=4), 4 (N=4), and 24 (N=3) hour post-noise time points. Animals euthanized with no noise-exposure served as controls (N=4). All antioxidant treated and saline control animals were euthanized at 2 hours post-noise.

Fixed and rinsed tissues were permeabilized with 0.5% to 1% Triton-X-100 for 30 to 60 minutes. Subsequently or simultaneously, tissues were blocked with 10% Normal Goat Serum (with or without 1% BSA) for 30 to 60 minutes, or with Power Block for 5 minutes. After blocking, tissues were rinsed in PBS, and then incubated for 24 hours in

mouse anti-caspase-2 antibody (BD Transduction Laboratories #611022, ICH-1L) at 4°C. Preliminary tests with 1-10 µg/ml initial concentrations revealed the 2.5µg/ml concentration to produce the most specific labeling. After incubating in the primary anti-caspase-2 antibody, tissues were rinsed in PBS. All tissues were then incubated in the secondary antibody (1:100 Alexaflour 488 goat anti-mouse IgG) for 1 hour at room temperature (20°C).

Statistical Analysis

In order to determine antioxidant treated versus saline control group differences in 3-NT and caspase-8 expression, seven observers who were blind to study conditions were asked to rank image sets in order from least immunolabeling within the hair cells to most. These sets were generated by pooling images from treated and control animals according to the section of the cochlea from which they were taken. Thus, there were three image sets from each target molecule (3-NT and caspase-8) corresponding to the first, second, and third turns of the cochlea. Each image set consisted of twenty images, as there were ten animals (ACEMg treated n=5; saline control n=5), and two images were taken from each turn of the cochlea. Each observer was given uniform training and instruction on how to judge the images. Additionally, a significantly positive ($p<0.01$) Spearman's rank correlation coefficient was observed between each of the observers' rankings. The rank numbers assigned to each image were averaged across all observers to give average image scores. Subsequently, the mean of the two average image scores from each animal was taken to give average section scores. Finally, the means of the average section scores were taken to give average ear scores for each of the ten animals. These data were then employed to detect group differences using the Wilcoxon-Mann-Whitney two-sample rank-sum test,

which tests the null hypothesis that the probability distributions associated with the two populations (ACEMg treated and saline control) are equivalent. The data were analyzed for treatment effect within each section, as well as within the ear as a whole.

CHAPTER 3 NITROTYROSINE

Production of 3-nitrotyrosine (3-NT) was assessed as an indicator of oxidative stress in antioxidant treated (N=5) and saline control (N=5) animals. The increased metabolic demand on hair cells due to noise-exposure causes an increase in the production of superoxide. Superoxide then reacts with nitric oxide to generate the peroxynitrite anion, which modifies cellular proteins to form 3-nitrotyrosine. We hypothesized that antioxidant treatment would decrease the production of RNS, and thus decreased labeling would be observed within the hair cells of treated ears upon comparison with those of saline control ears.

Results

Upon initial observation, the results appeared encouraging due to the apparent difference in immunolabeling in the hair cells between the ACEMg and saline control groups (Figure 3-1). However, this difference was not consistently observed (Figure 3-2), and did not prove to be statistically significant with the current methods of analysis and sample size. Negative control tissues in which the primary anti-3-NT antibody was omitted did not show significant non-specific labeling (Figure 3-3). The results of the Mann-Whitney U test which was conducted for the detection of differences in the probability distributions between the treated and control groups were as follows: median section scores in the first turn for the ACEMg and saline control groups were 11.43 and 12.71 respectively (Mann-Whitney U = 10.0, p = 0.345 one-tailed); median section scores in the second turn for ACEMg and saline control groups were 11.29 and 8.79 respectively (Mann-Whitney U = 12.0, p = 0.50 one-tailed); median section scores in the third turn for the ACEMg and saline control groups were 7.00 and 14.29 respectively

(Mann-Whitney U = 11.0, p = 0.421); finally, the median average ear scores for the ACEMg and saline control groups were 9.90 and 10.83 respectively (Mann-Whitney U = 10.0, p = 0.345). These results are summarized in Table 3-1.

Discussion

Nitrotyrosine is known to accumulate in conditions involving oxidative stress such as Huntington's disease and ischemic brain injury [151]. It has also been used as a reliable biomarker of RNS activity [67, 138]. The reaction of superoxide with nitric oxide (NO) generates the highly reactive peroxynitrite anion, which modifies cellular proteins to generate nitrotyrosine [150]. Treatment with this antioxidant cocktail was expected to reduce the production of RNS, and thus 3-NT. This hypothesis is based on the ability of ascorbic acid to scavenge superoxide and to reduce noise-induced NO production [137], and also the overall efficacy of ACEMg treatment for the reduction of noise-induced PTS as was demonstrated previously [131].

However, as was previously mentioned, we did not observe a statistically reliable difference in 3-NT production between the treated and control groups using the current study design. The lack of statistical significance seen in our results may be caused by temporal and spatial variation in 3-NT production following noise. Evidence for this theory is based on a varying pattern of 3-NT production at different post-noise time points. A previous study showed that immunostaining for 3-NT following noise exposure was initially low, and localized to the supporting (Hensen and Claudius) cells. Additionally, significant immunostaining did not appear in the hair cells until later time points (day 7-10), when 3-NT production reached a maximum (Figure 3-4) [67]. All animals in this study were sacrificed two hours following noise exposure, which corresponds to the time at which Yamashita et al. described 3-NT production only in the

supporting cells. Our images were analyzed with respect to immunolabeling within the outer hair cells alone, and this may be the reason for our failure to detect a significant treatment effect. Given this information, it may be that an appreciable treatment effect would be more readily detectable in the 7-10 day post-noise range during the time of peak 3-NT production. These studies are in progress.

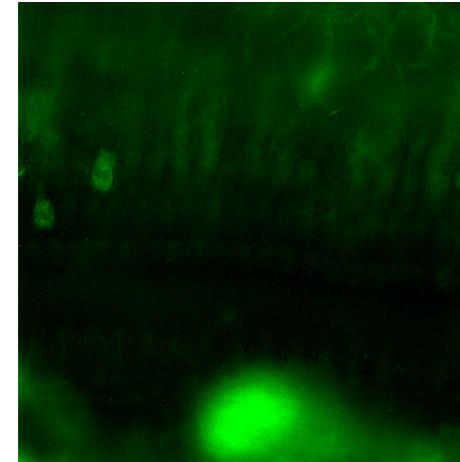
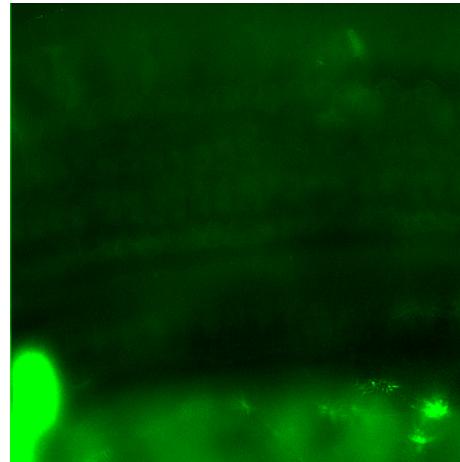
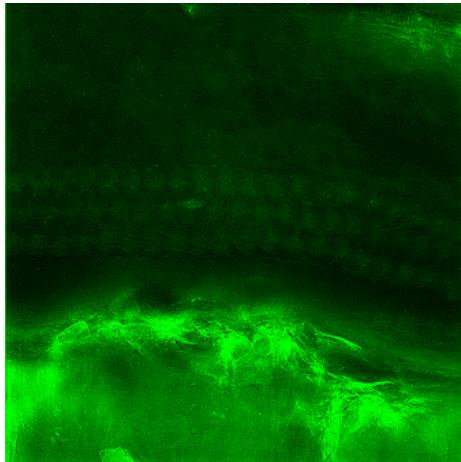
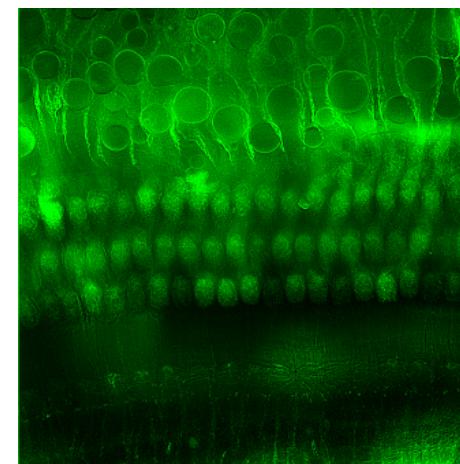
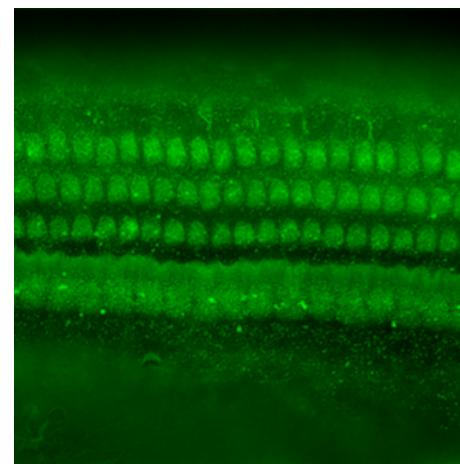
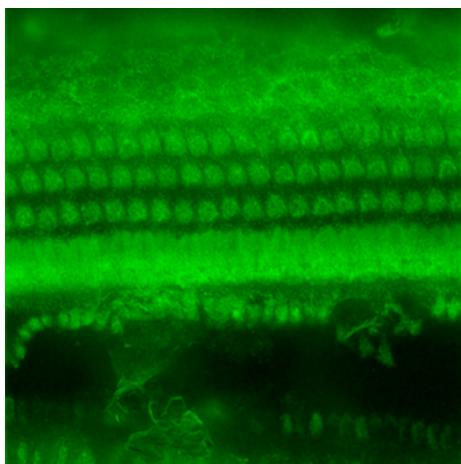
A**B**

Figure 3-1. Epifluorescence micrographs of ACEMg treated (A) and saline control (B) sections of the organ of Corti labeled with anti-3-nitrotyrosine antibody showing the greatest observed treatment effect. Sections in the left, middle, and right columns were taken from the 1st, 2nd, and 3rd, turns of the cochlea respectively. These images were selected from ears with the lowest treated and the highest saline average rank score.

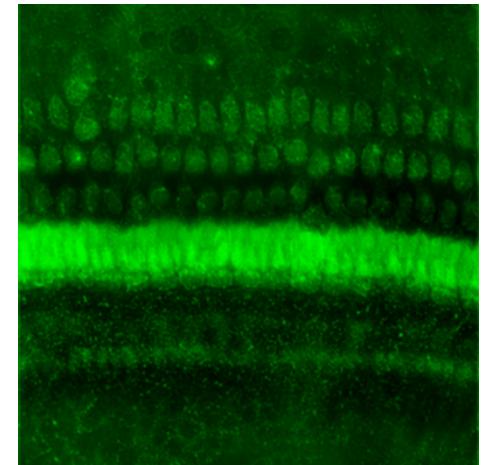
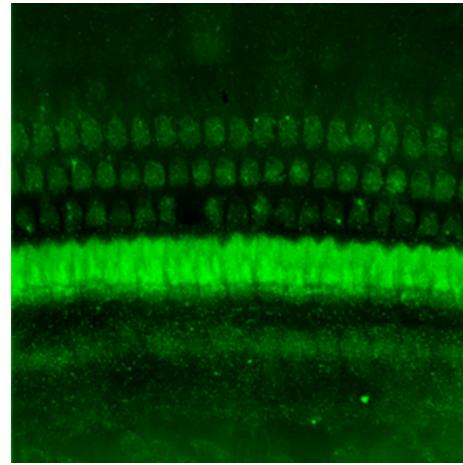
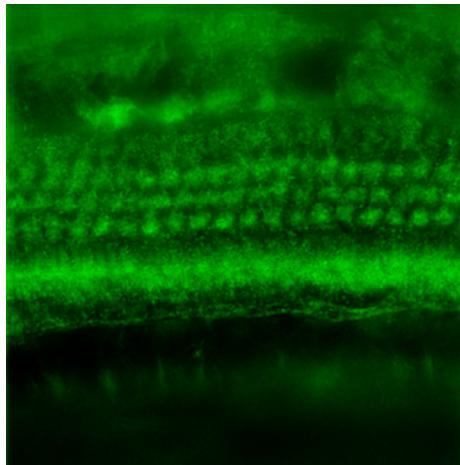
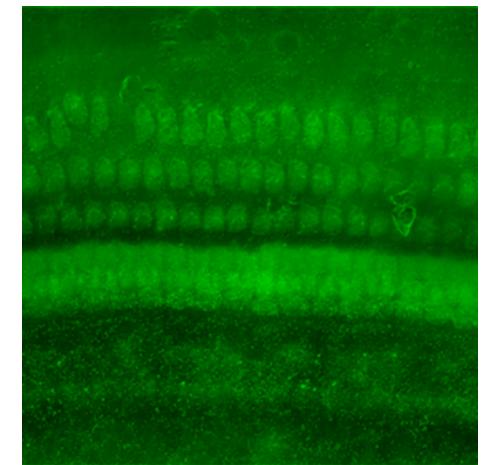
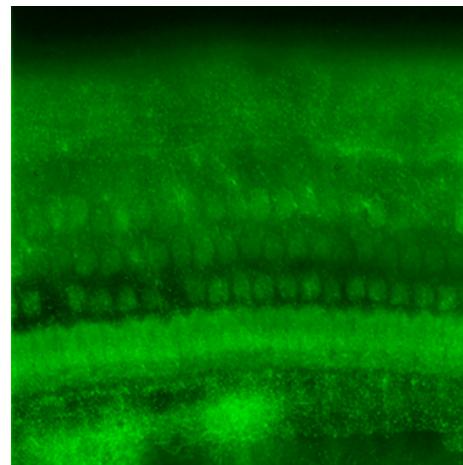
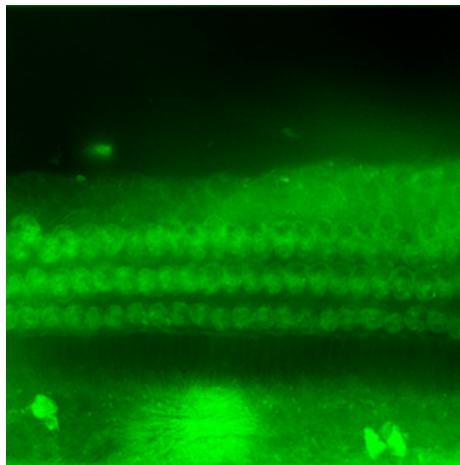
A**B**

Figure 3-2. Epifluorescence micrographs of ACEMg treated (A) and saline control (B) sections of the organ of Corti labeled with anti-3-nitrotyrosine antibody showing median treated and control images. Sections in the left, middle, and right columns were taken from the 1st, 2nd, and 3rd, turns of the cochlea respectively. These images were selected from ears with the median treated and saline rank score.

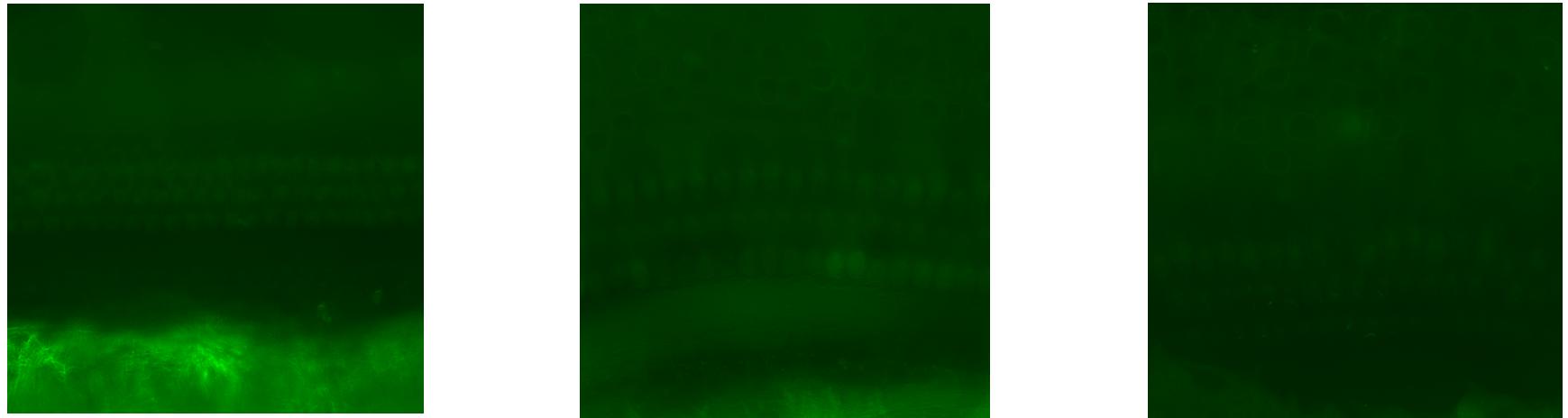


Figure 3-3. Negative control ear in which the primary 3-NT antibody incubation step was omitted.

Table 3-1. Difference in distribution of ACEMg treated and saline control 3-NT image rank scores

	1 st Turn	2 nd Turn	3 rd Turn	Ear Average
Median ACEMg Score	11.43	11.29	7.00	9.90
Median Saline Score	12.71	8.79	14.29	10.83
Mann-Whitney U	10.0	12.0	11.0	10.0
P-value (one-tailed)	0.345	0.500	0.421	0.345

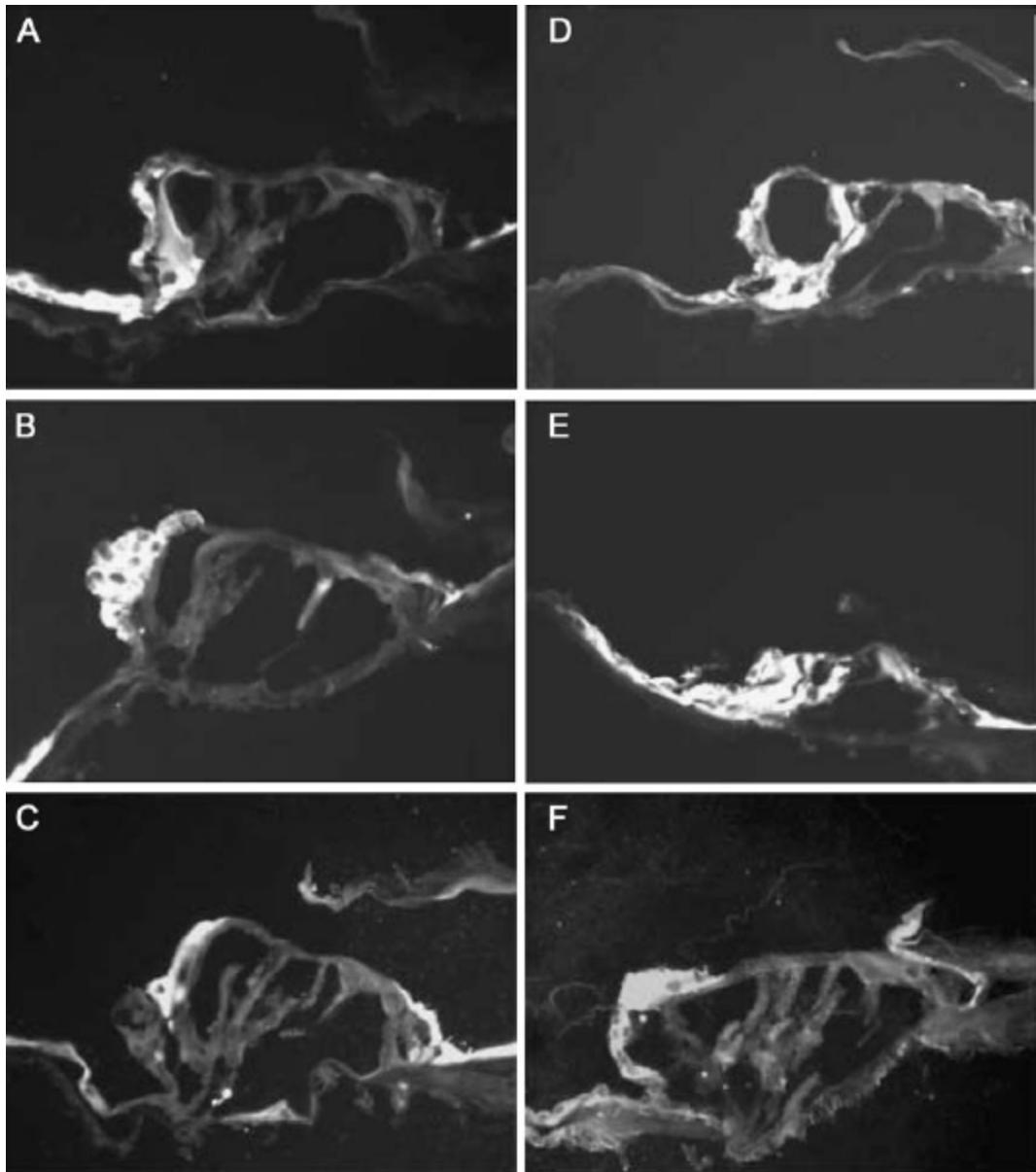


Figure 3-4. Immunostaining for NT shifts from supporting cells (Hensen, Claudius) to OHCs, including Deiters, with a maximum at 7–10 days. Sections are from an area approximately one half turn apical of the main lesion (A: control, B: immediate, C: Day 3. D: Day 7 E: Day 10, F: Day 14). All guinea pigs were exposed to octave band noise centered at 4 kHz and 120 dB SPL for 5 hours [67].

CHAPTER 4 CASPASE-8

Activation of caspase-8 following noise exposure was assessed in ACEMg treated (N=5) and saline control (N=5) ears to determine the effect of antioxidant treatment on the extrinsic (death receptor mediated) apoptotic pathway which is characterized by activation of caspase-8 following ligation of cell surface death receptors. We hypothesized that ACEMg treatment would not have a considerable effect on post-noise expression of caspase-8 due to the fact that antioxidant treatment should not prevent the ligation of death receptors.

Results

Surprisingly, although still not statistically reliable, we observed a greater treatment effect on caspase-8 expression than on 3-NT production (Figures 4-1 and 4-2). Control tissues in which the primary anti-caspase-8 antibody was omitted did not show significant non-specific labeling (Figure 4-3, A). Similarly, no caspase-8 expression was detected in control ears which were not exposed to noise (Figure 4-3, B). The results of the Mann-Whitney U test which was conducted for the detection of differences in the probability distributions between the treated and control groups were as follows: median section scores in the first turn for the ACEMg and saline control groups were 6.75 and 14.69 respectively (Mann-Whitney U = 7.0, p = 0.155 one-tailed); median section scores in the second turn for ACEMg and saline control groups were 7.94 and 14.31 respectively (Mann-Whitney U = 10.0, p = 0.345 one-tailed); median section scores in the third turn for the ACEMg and saline control groups were 12.56 and 6.69 respectively (Mann-Whitney U = 11.0, p = 0.579); finally, the median average ear scores for the ACEMg and saline control groups were 9.60 and 11.98 respectively

(Mann-Whitney U = 5.0, p = 0.076). These results are summarized in Table 4-1. The treatment effect was greatest in the first turn, and decreased through the second and third turns. When the cochleae were analyzed as a whole, the difference in distributions between the treated and control groups approached statistical significance.

Discussion

The apoptotic initiator, caspase-8, can either directly activate the effector caspase-3, or cleave BID which facilitates the release of cytochrome C mediated by the insertion of Bax or Bak into the mitochondrial membrane. Both of these circumstances result in cell death [72]. There is evidence that many inflammatory cytokines, including those of the TNF family which can act as a death receptor ligand, are upregulated following noise exposure [73]. Caspase-8 was also shown to be activated in hair cells following noise exposure [59]. Our observations confirm this finding, and suggest the possibility that ACEMg treatment may attenuate caspase-8 activation. This protective effect could be mediated in part by the vasodilatory properties of magnesium. Magnesium prevents noise-induced reduction of cochlear blood flow by dilation of the vasculature within the stria vascularis [142]. This in turn prevents ischemic injury and production of inflammatory mediators such as those of the TNF family which, as previously mentioned, can act as death receptor ligands [73].

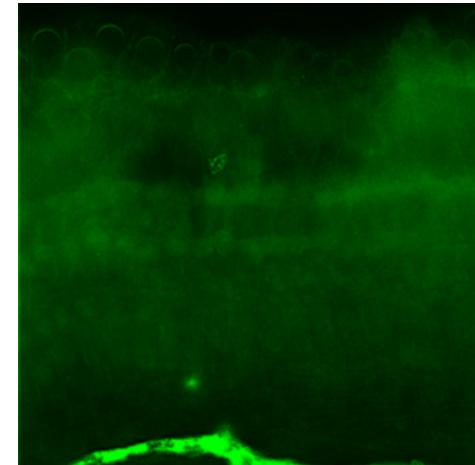
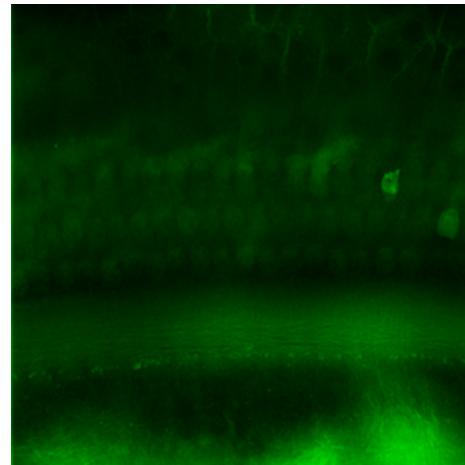
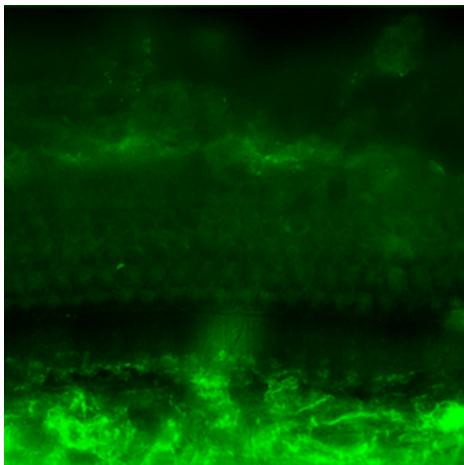
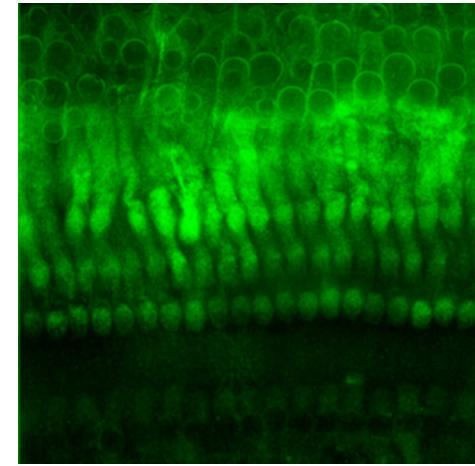
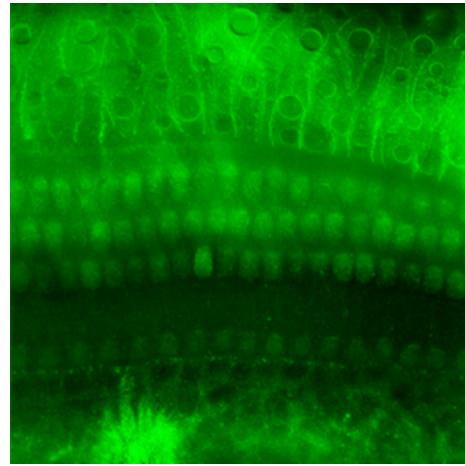
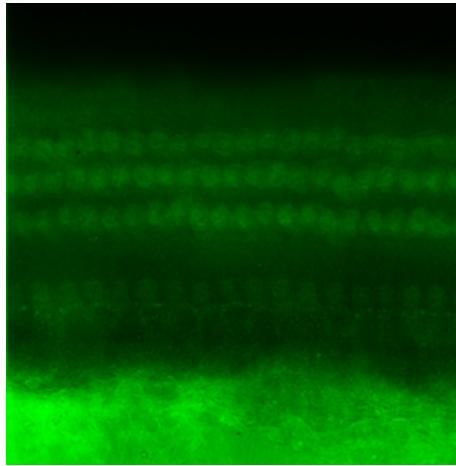
A**B**

Figure 4-1. Epifluorescence micrographs of ACEMg treated (A) and saline control (B) sections of the organ of Corti labeled with anti-caspase-8 antibody showing the greatest observed treatment effect. Sections in the left, middle, and right columns were taken from the 1st, 2nd, and 3rd, turns of the cochlea respectively. These images were selected from ears with the lowest treated and the highest saline average rank score.

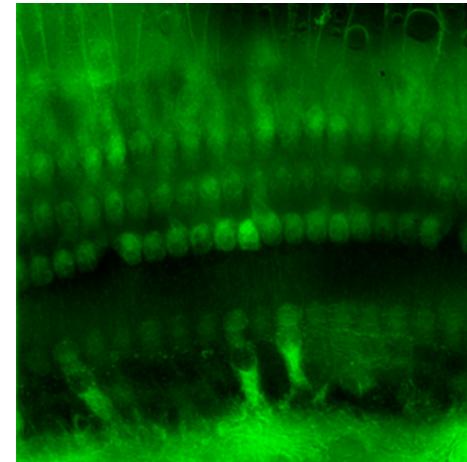
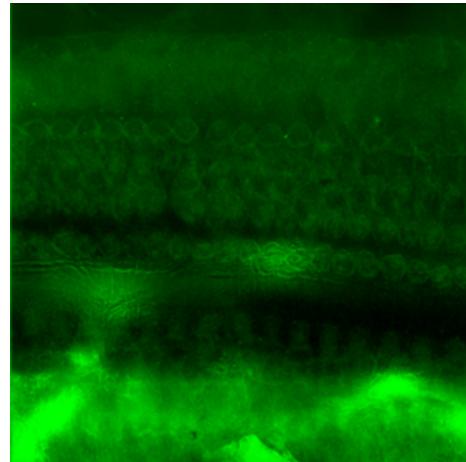
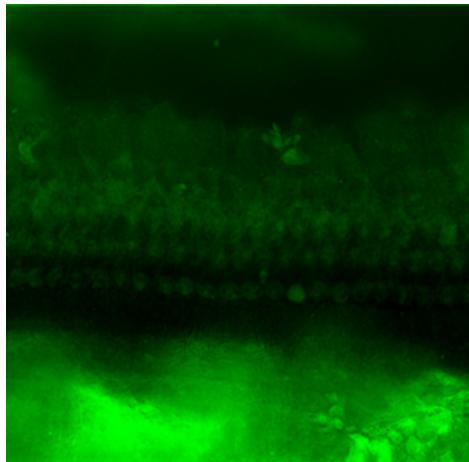
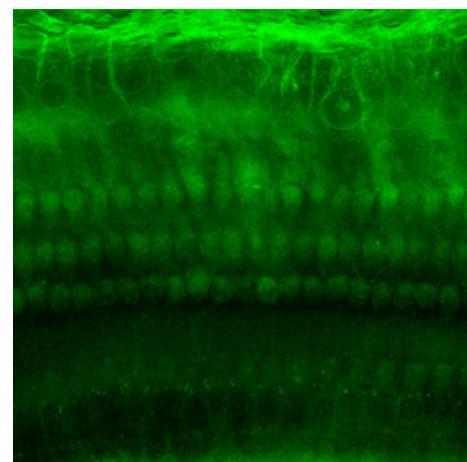
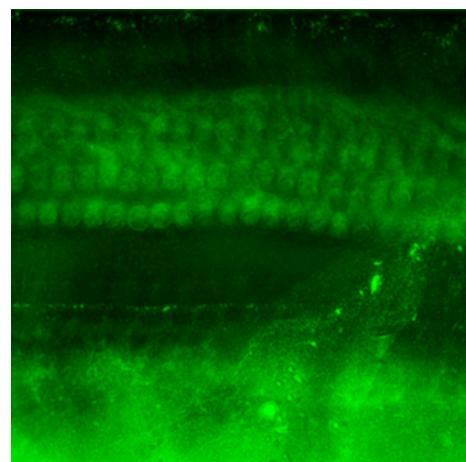
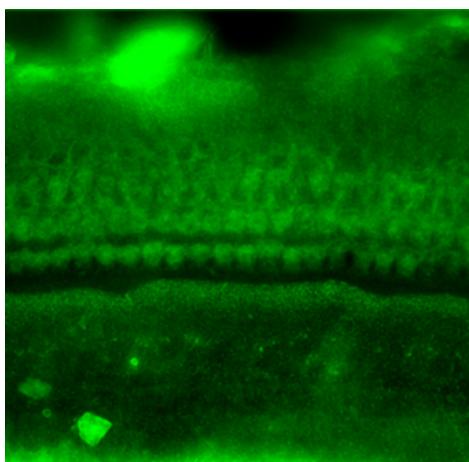
A**B**

Figure 4-2. Epifluorescence micrographs of ACEMg treated (A) and saline control (B) sections of the organ of Corti labeled with anti-caspase-8 antibody showing median treated and control images. Sections in the left, middle, and right columns were taken from the 1st, 2nd, and 3rd, turns of the cochlea respectively. These images were selected from ears with the median treated and saline rank score.

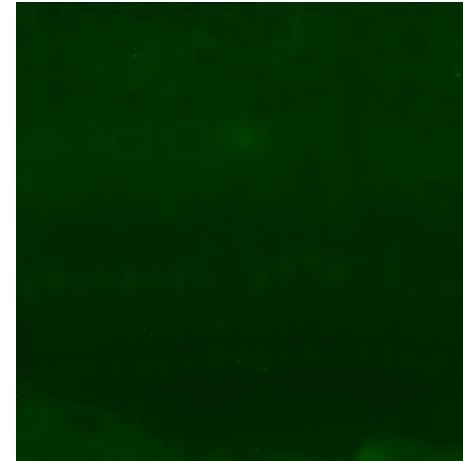
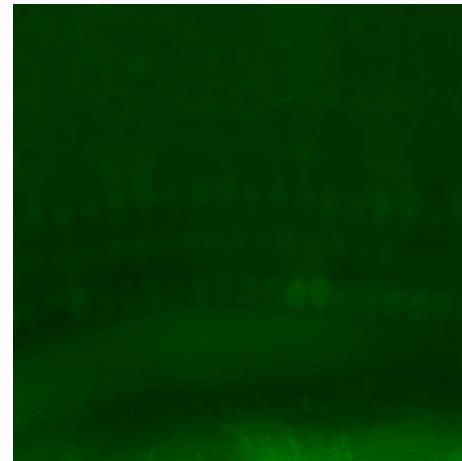
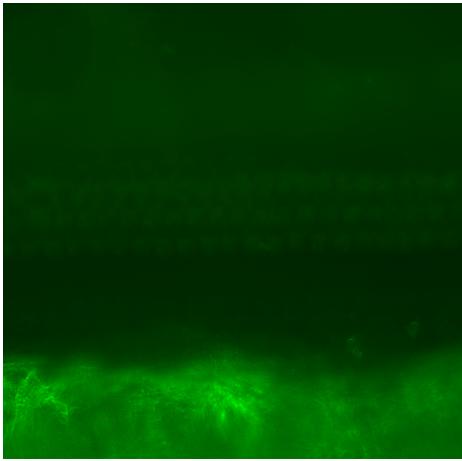
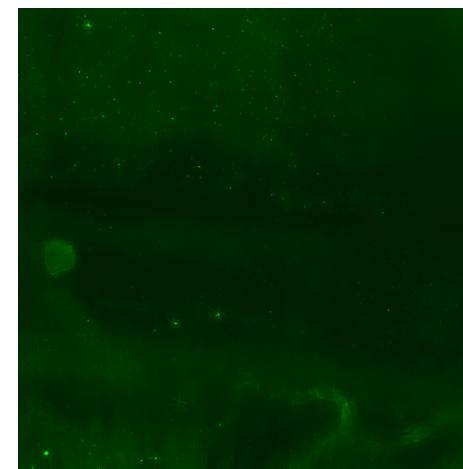
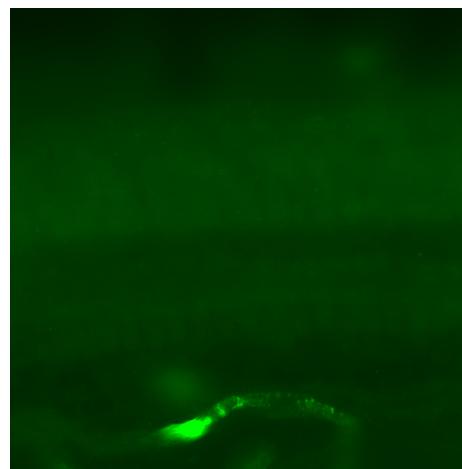
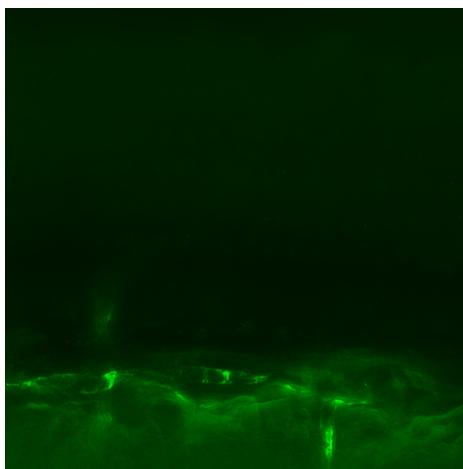
A**B**

Figure 4-3. Negative control in which the primary anti-caspase-8 antibody incubation was omitted (A) and no noise control (B).

Table 4-1. Difference in distribution of ACEMg treated and saline control caspase-8 image rank scores

	1 st Turn	2 nd Turn	3 rd Turn	Ear Average
Median ACEMg Score	6.75	7.94	12.56	9.60
Median Saline Score	14.69	14.31	6.69	11.98
Mann-Whitney U	7.0	10.0	11.0	5.0
P-value (one-tailed)	0.155	0.345	0.579	0.076

CHAPTER 5 CASPASE-2

Given the fact that caspases -3, -8, and -9 have an established role in noise-induced damage to the inner ear [59], and that caspase-2 activation has been described in response to oxidative stress [96, 152], we sought to determine the extent of caspase-2 activation in the cochlea following noise exposure. Caspsase-2 differs from its other family members because it has characteristics of both an initiator, and an effector caspase. To date, caspase-2 activity in response to noise has not been characterized in the inner ear. Evidence which shows caspase-2 activation following ROS formation [96] led us to hypothesize that similar activation would be observed in response to noise insult.

Results

These data provide the first evidence that caspase-2 is activated in response to acoustic overexposure, and imply a possible role for caspase-2 in NIHL. There was no significant non-specific labeling of negative control tissues in which the primary anti-caspase-2 antibody was omitted (Figure 5-1, A and B). Unexpectedly, at the 2 hour post-noise time point, caspase-2 labeling appeared to be localized within the supporting cells (phalangeal process of outer pillar cells and Deiters cells). This was the case in 6 of 7 ears (Figure 5-1, C and D). By the four hour post-noise time point, caspase-2 expression was observed in supporting cells (2 of 7 ears, Figure 5-1, E), but more often in the OHCs (5 of 7 ears, Figure 5-1, F). Finally, 24 hours following noise exposure caspase-2 immunolabeling was again observed most commonly in supporting cells (4 of 5 ears, Figure 5-1, G and H). Labeling in all control tissues not exposed to noise was

difficult to detect, was diffuse, and was not localized to a particular cell type (Figure 5-1, I and J).

Due to the fact that the data show noise-induced activation of caspase-2, we chose to conduct a preliminary experiment testing the effect of ACEMg treatment on this activation. Preliminary observations of ears treated with ACEMg (N=2) versus saline control (N=2) did not eliminate the possibility that this treatment reduces activation of caspase-2 following noise. One of the ACEMg treated ears showed diffuse caspase-2 expression which was minimal throughout each turn of the cochlea (Figure 5-2, A). However, the other exhibited the same characteristic pattern of labeling in the supporting cells which was found in untreated ears (Figure 5-3, A). Further investigation is necessary due to the small sample size. Finally, in 2 of 2 ACEMg treated, and 2 of 2 saline control ears, caspase-2 expression decreased with distance from the base of the cochlea.

Discussion

These data show, for the first time, the expression of caspase-2 in the inner ear following noise exposure, and suggest a possible role for caspase-2 in noise-induced cell death. Previously, evidence for caspase-2 in the inner ear was limited to apoptotic cell death during development of neo-natal rats [149]. Caspase-2 activation occurs very early after cellular insult (for review see [94, 95]), and blocking or down-regulating caspase-2 activity inhibits the release of cytochrome c and Smac from mitochondria, prevents translocation of Bax from the cytosol to mitochondria, and prevents translocation of apoptosis inducing factor (AIF) from mitochondria to the nucleus [93, 94, 153]. Smac increases caspase activity by inhibiting the inhibitor of apoptosis protein (IAP).

While much of the focus is usually on the susceptibility of hair cells to noise damage, there is considerable evidence that supporting cells are susceptible as well. Supporting cells, such as Hensen's cells and the outer space of Nuel was shown to collapse at 24 hours post-noise [112]. Additionally, free-radicals were detected in Hensen's and Claudius cells in the guinea pig after noise, and labeling spread to hair cells as time progressed [67]. Our observation that caspase-2 expression is initially localized to the supporting cells and progresses to OHCs, coupled with the knowledge that caspase-2 is activated by oxidative stress [96, 152] suggests the possibility that caspase-2 contributes to noise-induced cell death in the inner ear, and that treatment with ACEMg may inhibit this cell death pathway. Additionally, the decreasing gradient of caspase-2 expression from base to apex which we observed is in line with the common knowledge that the structures at the base are more susceptible to noise-induced damage than those at the apex.

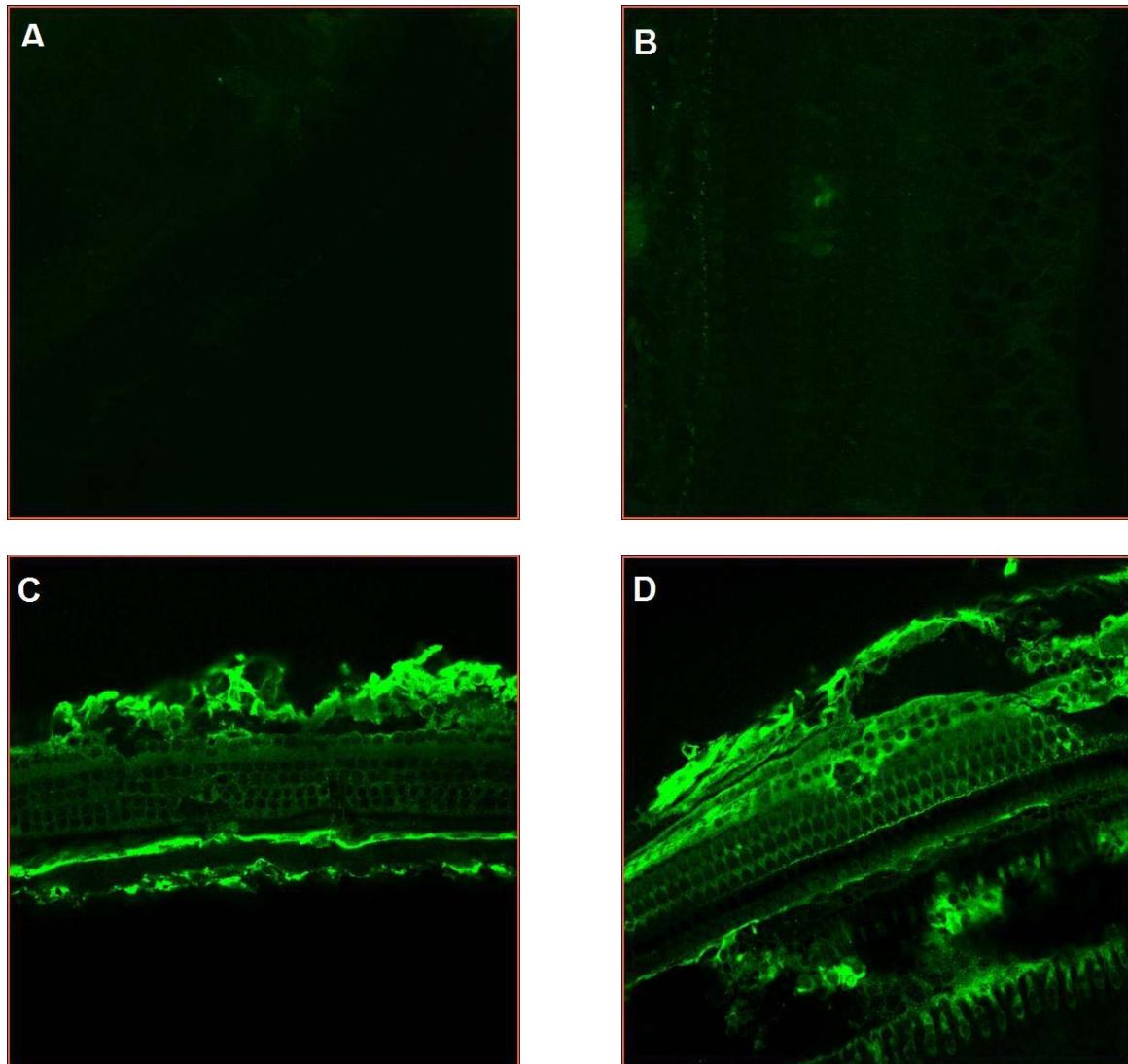


Figure 5-1. Epifluorescence micrographs of the organ of Corti from the 1st turn of the cochlea labeled with anti-caspase-2_L antibody showing temporal difference in post-noise expression. Negative control in which the primary antibody was omitted (A and B). Tissues harvested: 2 hours post-noise (C-D) showed distinct labeling in the supporting cells in 6 of 7 ears, 4 hours post-noise exhibited expression in supporting cells in 2 of 7 ears (E) and in the OHCs in 5 of 7 ears (F), and 24 hours post noise (G-H) found caspase-2 expression in the supporting cells in 4 of 5 ears and only one ear with expression in the hair cells. There were 4 no-noise control ears. The samples shown here (I-J) had the most labeling of any no-noise control tissues.

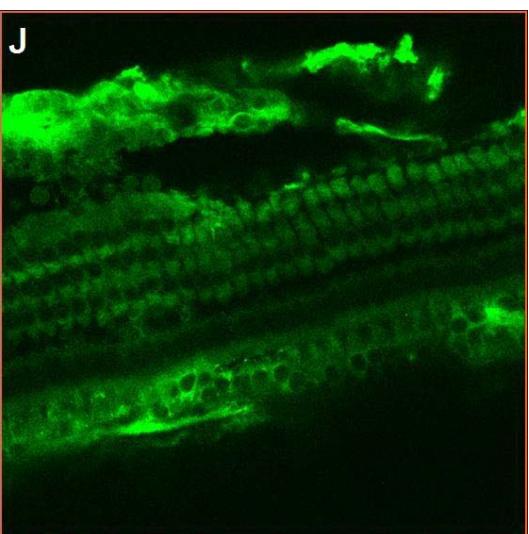
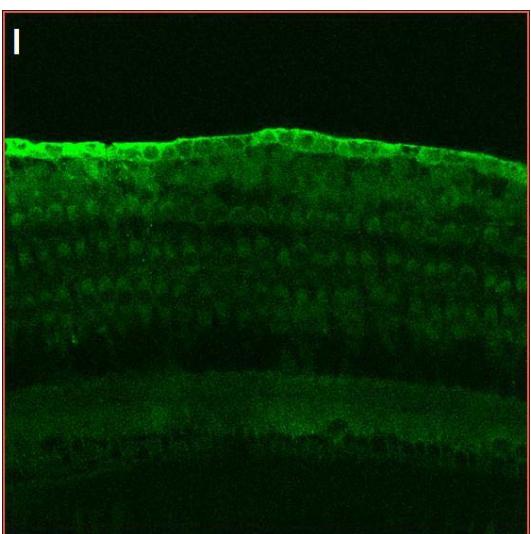
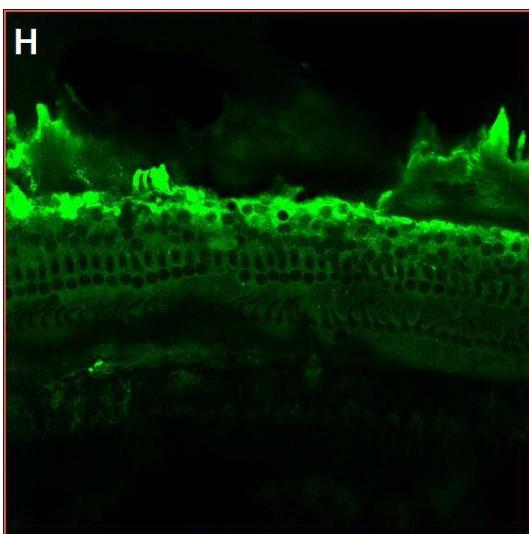
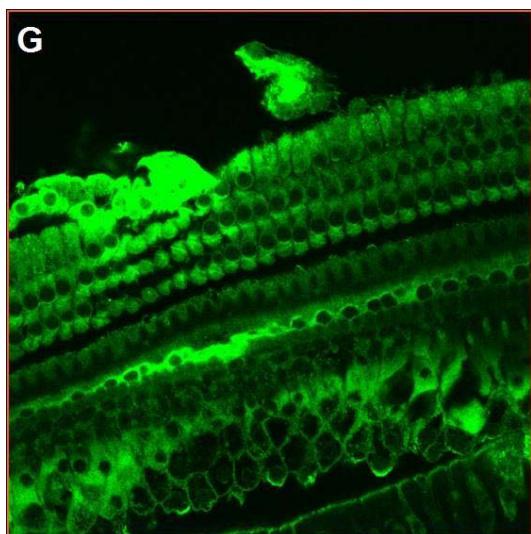
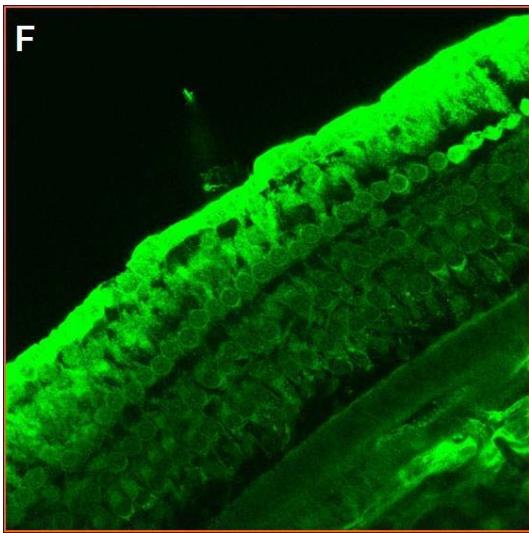
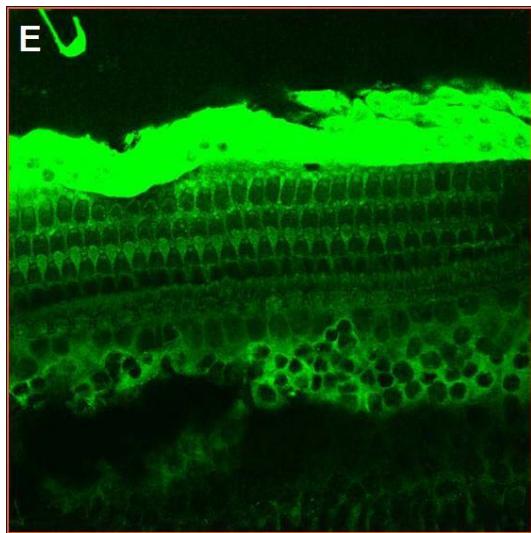


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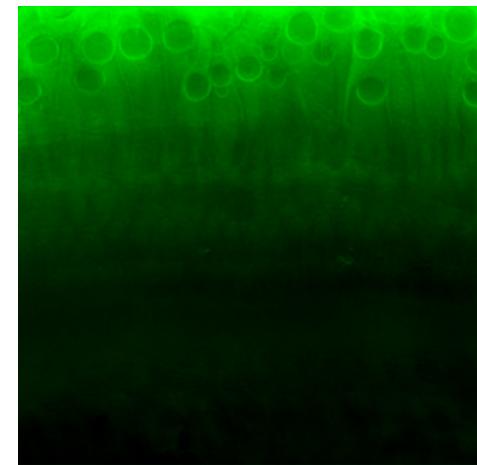
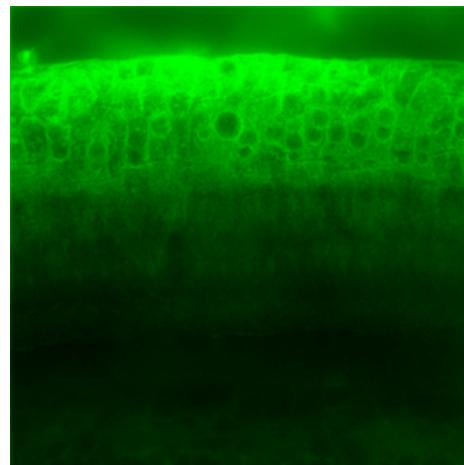
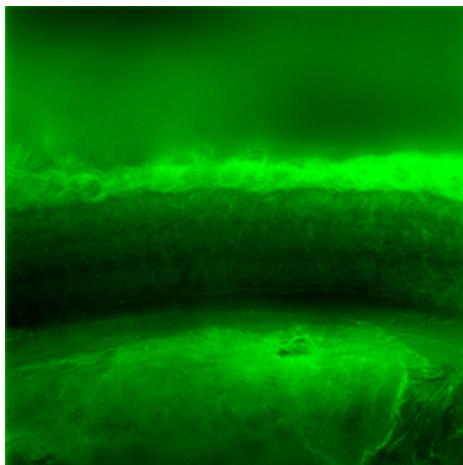
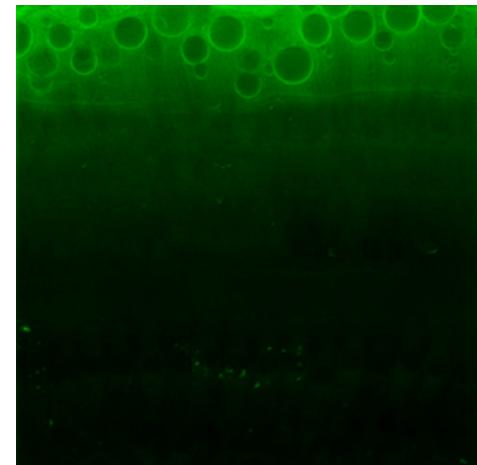
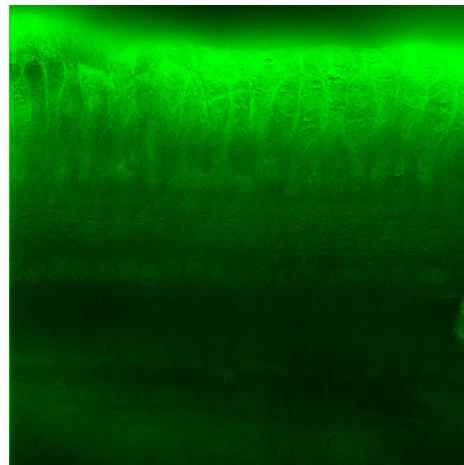
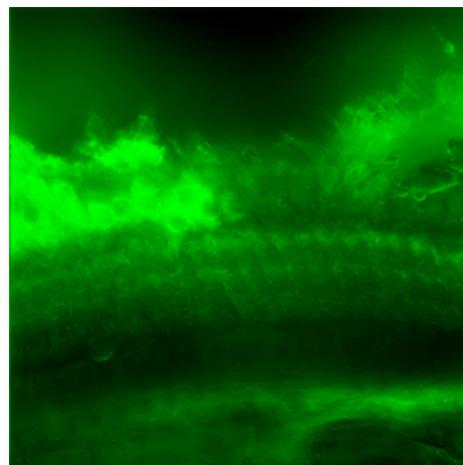
A**B**

Figure 5-2. Epifluorescence micrographs of ACEMg treated (A) and saline control (B) sections of the organ of Corti labeled with anti-caspase-2_L antibody showing diffuse labeling in treated and control tissues. Sections in the left, middle, and right columns were taken from the 1st, 2nd, and 3rd, turns of the cochlea respectively. Both treated and control tissues show diffuse immunolabeling, which decreases with distance from the base.

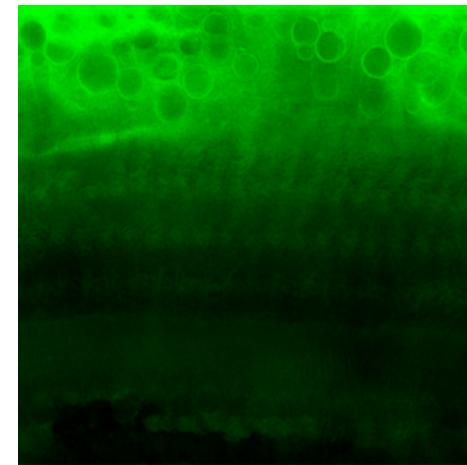
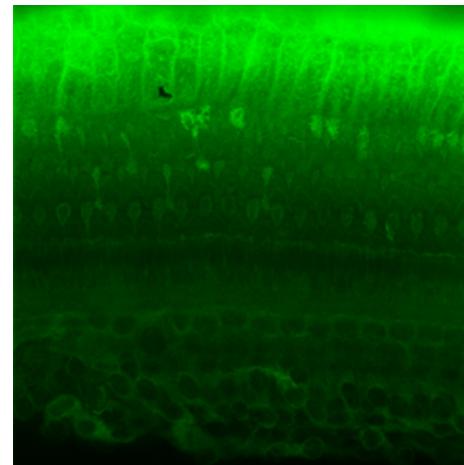
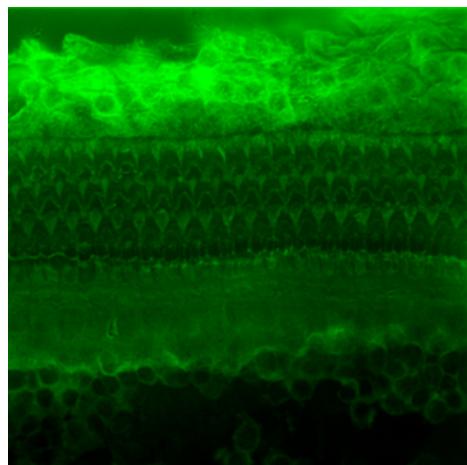
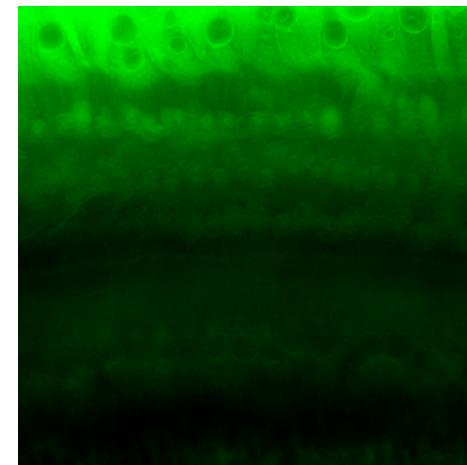
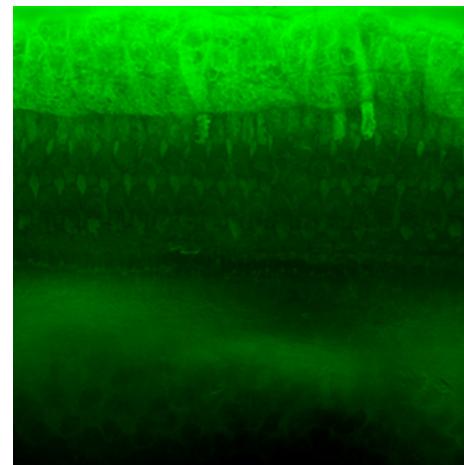
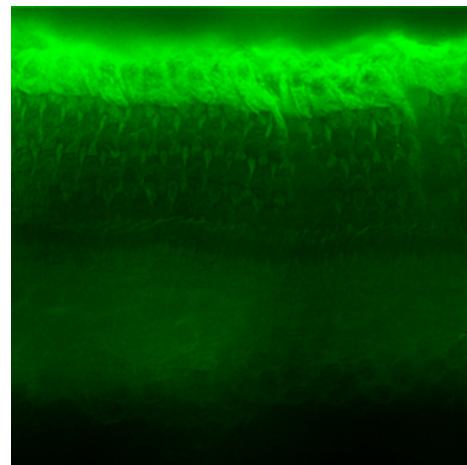
A**B**

Figure 5-3. Epifluorescence micrographs of ACEMg treated (A) and saline control (B) sections of the organ of Corti labeled with anti-caspase-2 antibody showing immunolabeling concentrated in the supporting cells in 1st and 2nd turns, with labeling becoming more diffuse in the 3rd turn.. Sections in the left, middle, and right columns were taken from the 1st, 2nd, and 3rd, turns of the cochlea respectively.

CHAPTER 6

CONCLUSIONS AND FUTURE DIRECTIONS

Despite efforts to educate the public about the harmful effects of noise, NIHL continues to be a growing problem which causes significant decrease in the individual's quality of life, and a collective economic burden. A great deal of progress has been made in understanding the cellular and molecular mechanisms underlying this disease. Greater understanding has sparked the development of experimental therapeutics which intervene at various points of cell death pathways, with the ultimate goal of prevention of hair cell loss. Despite varying degrees of success in animal models, as well as clinical trials, there is still no FDA approved pharmacological agent for the prevention or treatment of NIHL.

This study has taken another step in elucidating the pathways which lead to NIHL by providing the first evidence that caspase-2 is up-regulated following intense noise exposure. Caspase-2 expression was observed in the supporting cells of the organ of Corti 2 hours post-noise, with the amount of expression decreasing from base to apex. At 4 hours post-noise, expression appears to move transiently to the OHCs, with expression once again in the supporting cells at later time points.

Noise damage is usually mediated, at least in part, by oxidative stress resulting from high metabolic demand and noise-induced reduction of cochlear blood flow. We also investigated the effect of the micronutrient treatment combination of β -carotene, vitamins C and E, plus magnesium on the activation of caspases -2 and -8, as well as the production of 3-nitrotyrosine, a marker of RNS activity. These data support the possibility that ACEMg treatment may attenuate the noise-induced activation of caspases -2 and -8 as well as RNS production. Given the fact that ROS and RNS

production are known to peak at 7-10 days following noise exposure, further studies are warranted which explore this treatment effect at later post-noise time points.

Future research will seek to characterize the effect of ACEMg treatment on other mediators of apoptotic signaling pathways such as caspases -3 and -9 as well as caspase-independent mediators such as endonuclease G and apoptosis inducing factor (AIF). Given the fact that free-radical production has been implicated in hearing loss caused by aminoglycoside antibiotics, chemotherapeutics, and age-related hearing loss, ACEMg treatment should be studied for application in these areas as well.

APPENDIX: COMMENTS ON METHODS AND STATISTICAL ANALYSIS

In this study, individual images of noise exposed, sectioned organ of Corti from treated and control groups were pooled and ranked in order from least immunolabeling within the hair cells to most by a series of observers who were blind to study conditions. A nonparametric statistical test was then used to analyze the difference in ranks between the treated and control groups. The following discussion will correlate previously obtained hearing data from noise exposed animals (114 dB SPL centered at 4 kHz for 4 hours) with what we would expect to see in terms of ROS production/caspase activation, and what we actually observed. Limitations associated with the ranking design and statistical analysis will also be commented upon.

The effect of the noise exposure used in this study on hearing sensitivity in guinea pigs has been well characterized in our lab by measurement of auditory brainstem response (ABR) thresholds. Figure A-1 shows average noise-induced threshold shifts at various post-noise time points. The fact that there is a large decrease in hearing sensitivity across all frequencies at the early post-noise time points justifies our analysis of the entire ear taken as a whole. All of the treated and control animals in this study were euthanized at the two hour post-noise time point. Due to the threshold elevation at this time across all frequencies, we would expect there to be increased free-radical production and possibly caspase activation throughout the cochlea. Our data support this in that there is increased labeling for all targets in each section when compared to control tissues which have not been exposed to noise. For future studies which will examine labeling at the later post noise time points, we would expect to find the most labeling in the high frequency (basal) region of the cochlea.

Turning now to the statistics, the nonparametric statistical test was chosen because we are analyzing ordinal data, or qualitative data that can be ranked in order of magnitude. Parametric statistical tests rely on certain assumptions such as that the data are sampled from a normally distributed population. Nonparametric tests on the other hand do not depend on the distribution of the sampled population, and are thus referred to as distribution-free tests. Additionally, nonparametric methods are concerned with the location of the probability distribution of the population rather than on specific parameters of the population, such as the mean.

Specifically, the Wilcoxon-Mann-Whitney two-sample rank sum test was used to analyze the difference in image ranks between the treated and control groups. This is a nonparametric method which tests the null hypothesis that the probability distributions associated with the treated and control populations are equivalent. The conditions required for a valid rank sum test are as follows: 1) the two samples are random and independent, and 2) the two probability distributions from which the samples were drawn are continuous so that there are no ties. If the treated and control populations were identical, we would expect the ranks to be randomly mixed between the two samples. On the other hand, if the treated population tends to have less labeling for a particular target (as hypothesized) we would expect the smaller ranks to be mostly in the treated sample and the larger ranks to be mostly in the control sample. This experiment defined the one-tailed alternative hypothesis to be that the distribution of treated ranks would be less than (shifted to the left of) the distribution of control ranks. The test statistic is calculated based on the totals of ranks (rank sums) for each of the two samples. The greater the difference between rank sums, the greater the evidence

indicating a difference between the probability distributions of the two populations.

Once the test statistic has been calculated for a particular trial, the observed significance level, or p-value, can be calculated based upon the sampling distribution of the test statistic under the null hypothesis. The p-value is the probability (assuming that the null hypothesis is true) of observing a value of the test statistic that is at least as contradictory to the null hypothesis, and supportive of the alternative hypothesis, as the actual one computed from the sample data. More specifically, in the case of the Wilcoxon-Mann-Whitney test, the p-value answers this question: if the treated and control populations really have the same median image rank score, what is the chance that random sampling would result in a sum of ranks as far apart or more so as observed in this experiment? We have made the assumption that the saline control images would be ranked higher and therefore, 1-tailed p-values were reported. If the p-value is small (<0.05), one could conclude that the treated and control populations have different medians. If the p-value is large, there is not sufficient evidence to reject the null hypothesis and conclude that the medians differ. This does not necessarily mean that the medians are the same; it just means that under the current experimental conditions there is not enough evidence to say that they differ.

One limitation was the small sample size used in this experiment. With small sample sizes, rank tests often have little statistical power. The power of a statistical test is defined as the probability of correctly rejecting the null hypothesis when in fact the alternative hypothesis is true. The probability of type II error (β) is defined as the probability of incorrectly accepting the null hypothesis when the alternative is true. Therefore the statistical power of a test can be calculated as $1 - \beta$. For the rank sum

test, power calculations can be performed in the absence of a priori knowledge of population variance using an odds parameter (γ) to show the relationship between the two distributions. For example, when $\gamma = 1$, the distributions of ranks are identical for the two groups, and when $\gamma = 4$, the odds are 4:1 that the control animals had higher ranks than treated [154]. In our case, the ear average data for 3-NT had a γ of 1.5 and the ear average data for caspase-8 had a γ of 4. Figure A-2 plots the odds parameter (γ) against power for our two sample case with five observations per group. From this, we can clearly see that we were not adequately powered to detect even our greatest difference in primary outcome measure seen in the caspase-8 ear average data where $\gamma = 4$. Studies are ongoing which will both increase the sample size at the early post noise time points, and assess the treatment effect at later post noise times.

Another possible limitation is that working with ordinal data sets in which the images were ranked from least to most labeling may mask large differences between individual images. For instance, the difference between images ranked 1 and 2 may be much greater or much smaller than that between images ranked 3 and 4. This may be alleviated by having observers score each image on a pre-determined numerical scale. Also, an alternative to image rank analysis could be explored for future studies in which the image collection protocols are rigorously standardized to allow for exact measurements of either area or intensity of labeling within the hair cells to be determined using quantitative image analysis software.

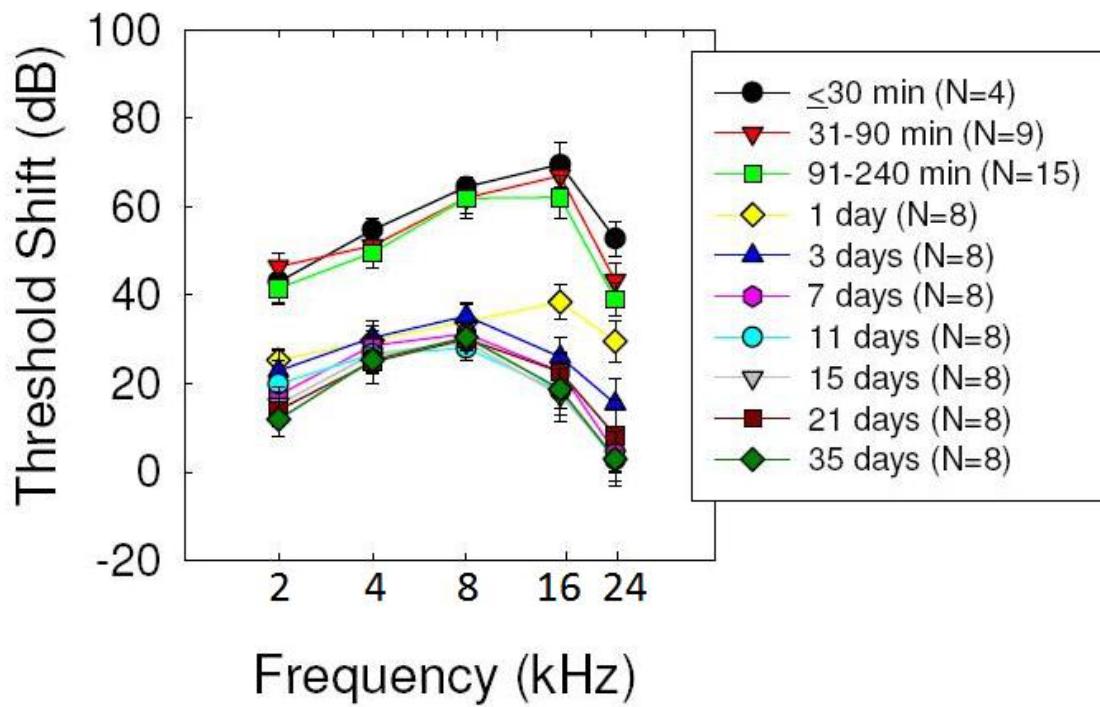


Figure A-1. Auditory brainstem response (ABR) threshold shift at various post-noise time points.

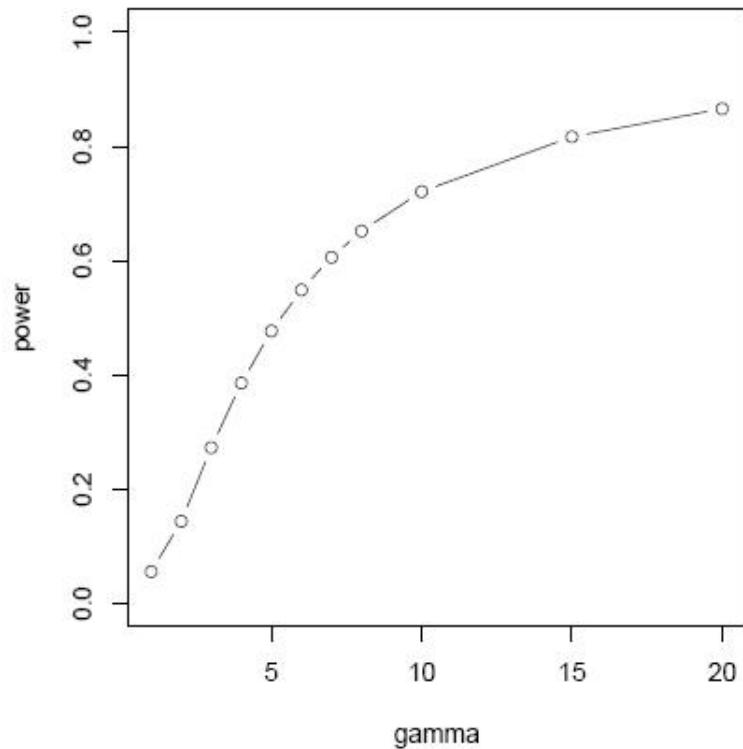


Figure A-2. Plot of statistical power vs. the odds parameter γ

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BIOGRAPHICAL SKETCH

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