

Methionine sulfoxide reductase A provides age-dependent protection from early-onset hearing loss in the mouse cochlea: anatomical study.

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Abstract

Background: Methionine sulfoxide reductase A (*MsrA*) protects the biological activity of proteins from oxidative stress damage and fights onset of age-related hearing loss (Alqudah, ARO 2015 and 2016). In the current study, we attempted to determine the defect in the protein expression of *MsrA* in adults *MsrA* knockout mice and the role of *MsrA* in preventing loss of the cellular structures from aging.

Method: Mid-modiolar sections from cochlea were incubated overnight at room temperature with the primary antibody for *MsrA* (Abcam, cat# ab16803, diluted 1:100). Next day, the slices were incubated with a biotinylated goat anti-rabbit IgG secondary antibody (Vector, cat # BA-1100 diluted 1:400) for 3 h at room temperature, then with a streptavidin Alexa 488 (Thermo Fisher, cat# A20000 diluted 1:1000) for 1 h. The stained sections were examined under a fluorescence microscope.

For histology analysis, 6-month-old wild-type and *MsrA* knockout mice were perfused intracardially with a fixative solution containing 2.5% glutaraldehyde and 1.5% paraformaldehyde. Both inner ears were dissected, postfixed in osmium, embedded in Araldite, and sectioned at 20 μm on a microtome. Sections were then mounted on microscope slides and spiral ganglion neurons counted.

Results: Although both mutants and controls showed the same staining intensity of the cochlear tissues, there was a clear difference in the immunolabeling of the stained brain sections. This observation suggests a relatively low abundance of *MsrA* protein within the cochlea.

Semiquantitative analysis of plastic sections of cochlea from 6-month old *MsrA* knockout mice showed loss of cochlear spiral ganglion neurons by 15% in the 16-26 kHz frequencies region,

and 60-70% loss of fibrocyte type IV cells when compared to age-matched control mice.

Conclusion: In this study, we raise the possibility that *MsrA* represents one component of the protective mechanism that may be responsible for cochlear protection from aging.

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Introduction

Cumulative and irreversible loss of sensory cells (hair cells) and auditory neurons in the cochlea, which leads to reductions in threshold sensitivity and speech discrimination, have a tremendous negative impact on age-induced hearing loss (AIHL) (Schmiedt, Mills, & Boettcher, 1996). It is well recognized that the combination of noise exposure and aging can cause the loss of additional sensory cells and exacerbate threshold shifts compared with the effects of aging alone (Kujawa & Liberman, 2009; Sergeyenko, Kujawa, & Liberman, 2013). The observations that noise-damaged ears age differently than undamaged ones (Kujawa & Liberman, 2009), and that exposure to noise is more detrimental at younger than at older ages (Kujawa & Liberman, 2009), suggest that there is a protective and age-related mechanism against damage from exposure to noise in the cochlea (Kujawa & Liberman, 2009; Sergeyenko et al., 2013).

One of the main protagonists in the pathophysiology of aging and noise exposure is oxidative stress. In the cochlea, oxidative stress can trigger accumulation of oxidized proteins, leading to damage of the sensory cells (hair cells) and auditory neurons, and exacerbate hearing loss (Fujimoto & Yamasoba, 2014; Henderson, Bielefeld, Harris, & Hu, 2006).

Methionine (Met) is an amino acid that is highly susceptible to oxidation, particularly under conditions of oxidative stress and aging (Levine & Luo, 2008; Stadtman et al., 2005; Stadtman, Moskovitz, & Levine, 2003; Stadtman, 2002). Oxidation of Met to methionine sulfoxide (MetO) is reversible, and the reverse reaction is catalyzed by a group of genes called methionine sulfoxide reductase system (Msr). This system is subdivided into two different subgroups: methionine S-sulfoxide reductase A (*MsrA*) and methionine R-sulfoxide reductase B (*MsrB1-3*). *MsrA* reduces both free and protein-based methionine-S-sulfoxide while *MsrB* is special for protein-based methionine-R-sulfoxide. The function of the Msr system is to provide

necessary protection against oxidative stress by removing the oxygen atoms to repair the damaged molecules, which will restore the natural activity of proteins in the cell (Moskovitz, 2007; Moskovitz et al., 1998; Moskovitz et al., 2001; Salmon et al., 2009).

Methionine oxidation in proteins of the cochlea is not well understood. To advance this field, we will study a biological tool, the *MsrA* knockout mouse (Oien et al., 2010), which shows phenotypes linked with abnormal levels of oxidative stress (Brennan & Kantorow, 2009; Liu et al., 2008; Moskovitz et al., 2002; Oien et al., 2010; Oien et al., 2008; Sreekumar, Hinton, & Kannan, 2011). More specifically, *MsrA* mutant mice have shown a phenotypic manifestation characterized by progressive neural degeneration (Brennan & Kantorow, 2009; Cabreiro, Picot, Friguet, & Petropoulos, 2006; Moskovitz, 2005) and hearing loss (Alqudah, 2016). The abolished expression of *MsrA* gene in mutant *MsrA* mice will help to determine the hearing functions controlled by this gene. It is also a useful tool for looking to the ability of the *MsrB* family of genes to regulate methionine oxidation and their involvement at the onset of hearing loss.

The role of the Msr system, particularly *MsrA*, in reducing the damage effect of oxidative stress in multiple parts of body, has been studied and published by other laboratories (Oien et al., 2010; Oien et al., 2008; Sreekumar et al., 2011; Moskovitz et al., 2002; Brennan & Kantorow, 2009; Cabreiro, Picot, Friguet, & Petropoulos, 2006; Moskovitz, 2005; Liu et al., 2008). As *MsrA* assists in preventing formation and accumulation of oxidative stress toxicity in the cells (Moskovitz et al., 1998; Novoselov et al., 2010; Oien et al., 2008; Pal, Oien, Ersen, & Moskovitz, 2007), production of proteins containing damaged (oxidized) methionine residue (MetO) becomes restricted, slowing down the aging process and preventing age-dependent neurodegenerative diseases (Moskovitz et al., 1998). Although several investigations reported

the presence of oxidative stress markers in the inner ear which are linked to cellular toxicity (Fujimoto & Yamabasa, 2014; Henderson, Bielefeld, Harris, & Hu, 2006; Staecker, Qing Yin, & Thomas, 2001), the repairing process of damaged proteins, which is a unique feature of the *MsrA* activity, has not been investigated until now due to the lack of a proper animal model. As a consequence, studies of the role of the *Msr* genes in the hearing system have been limited. Using *MsrA* knockout mice to study the effect of all *Msr* (*MsrA* and *MsrB*) genes in protecting the cochlea from acoustic overexposure and aging damage is highly innovative and significant.

Recently, Ahmad et al. (2011) reported that a mutation of the *MsrB3* gene causes autosomal recessive prelingual human deafness (DFNB74). The mutation also increases accumulation of MetO proteins in the cells, causing harm to hearing function from birth. Absence of *MsrB3* in mice triggers cell loss in the form of apoptotic cell death inside the cochlea, degeneration of hair cells stereocilia, and profound hearing impairment by age of 20 days. These findings suggest an association between mutation of *MsrB3* and genetic hearing loss. It is important to note, however, that the information regarding the involvement of *MsrB3* in age-related and noise-induced hearing loss is still missing and needs further investigation.

Regarding the expression of *MsrA* gene in the auditory system, a recent study illustrated the presence of *MsrA* in the supporting cells of the organ of Corti and in the spiral ganglion neurons (Kwon et al., 2014). *MsrA* was reported to prevent age-related central nervous system (CNS) modifications associated with neurodegenerative diseases (Oien et al., 2008) by sustaining proper function of dopaminergic fibers. In the auditory system, these fibers are reported to innervate the cochlea arising from the olivocochlear (OC) efferent system in the brainstem. Studies have described that dopaminergic fibers project to sensory cells in the inner ear to protect hearing function from the damaging effect of acoustic trauma (Gaborjan et

al.,1999; Maison et al., 2012). As these dopaminergic neurons are protected by *MsrA*, it is a good candidate to study how Msr genes reduce age-induced hearing loss (AIHL) and the incidence of noise-induced hearing loss (NIHL).

MsrA is highly expressed in the liver and kidney of animals and humans (Moskovitz, 2007; Moskovitz et al., 2002; Novoselov et al., 2010). In the brain, the *MsrA* gene is expressed in the glial cells and down-regulated during aging (Liu et al., 2008; Pal et al., 2007), which triggers the onset of age-related neurodegenerative diseases such as Parkinson's disease (Liu, et al. 2008). The latter disorder is pathologically associated with damage and death of dopaminergic neurons in the brain (Naoi & Maruyama, 1999; Surmeier, 2010). Ortiz and his colleagues (2011) observed that *MsrA* knockout mice exhibit neurodegenerative brain pathologies and malfunction of brain dopaminergic neurons due to the loss of dopamine receptor 2 (D2DR) activity. This observation suggests that *MsrA* knockout mice may have impaired dopaminergic signaling pathways also in the cochlea (Maison et al., 2012) and that *MsrA* might play a pivotal role in protecting the auditory dopaminergic neurons from neurodegeneration triggered by aging and noise.

Studies published in the last two decades have shown that the cochlea also contains dopaminergic afferent fibers from dopaminergic neurons placed in the cochlear nuclei (Darrow, Simons, Dodds, & Liberman, 2006; Gaborjan et al.,1999; Ruel et al., 2001), and that these fibers control the cochlear responses evoked by sounds (Maison, 2012). Felix and Ehrenberger (1992) provided evidence that the firing rate of afferent fibers in contact with the inner hair cells is suppressed following application of dopamine and that dopamine action is mediated by dopamine receptor activation. Inoue et al. (2006) demonstrated the expression of several dopamine receptors beneath the inner hair cell region, and Darrow et al. (2006) suggested that

dopaminergic neurons from the olivocochlear nuclei extend beneath the inner hair cells to form dopaminergic endings. However, Maison et al. (2012) recently studied the function of dopaminergic fibers in the cochlea using acoustic trauma and showed that mice lacking dopamine receptors exhibit exacerbated damage from acoustic trauma, suggesting that dopaminergic innervation is most likely essential to reduce the glutamate-induced excitotoxicity, neural death causing by increasing the glutamate release after acoustic trauma (Darrow, Simons, Dodds, & Liberman, 2006; Gaborjan et al., 1999; Inoue et al., 2006; Liu et al., 2008; Maison et al., 2012). Despite the fact that many lines of evidence demonstrate that inner ear dopaminergic fibers mediate auditory function, there is no broad indication that suggests these fibers are impacted by Parkinson's disease, an age-related degenerative disorder which induces loss of dopaminergic neurons of the brain.

The importance of understanding the role of *MsrA* in the hearing system (using the *MsrA* knockout mouse as a model) rests on the possibility that such research might unveil new approaches of treating acoustic trauma through Msr-based therapy. Additionally, more investigations to determine the specific function of *MsrA* protection from acoustic trauma and aging will provide more clues about a poorly understood phenomenon (i.e., the exacerbation of hearing loss in those patients that have both age-related and noise-induced hearing loss). Furthermore, using *MsrA* knockout mice, an animal model widely used to study age-related pathologies of the central nervous system (CNS), may provide evidence for the likely development of hearing loss in patients suffering from age-related neurodegenerative pathologies, such as in Parkinson's disease.

For the reasons described above, the preliminary project was designed to define the specific involvement of *MsrA* in protecting against age-related hearing loss, high levels of noise,

and the combined effect of these adverse conditions in mice species. We were successful in confirming that *MsrA* defends against oxidative stress generated by aging and noise exposure. The related data (see Figure1) showed that, with evaluation of the hearing of old and young, wild-type and knockout mice using Audiometry brainstem responses (ABR) and Distortion Product Otoacoustic Emissions (DPOAE), mutant animals developed progressive sensorineural hearing loss (SNHL) earlier than the controls (starting at 5 weeks versus 16 weeks in wild-type mice). This finding suggests that *MsrA* is involved in protection against age-related hearing loss (ARHL). Figure2 illustrates the tendency of the normal and mutant subjects to suffer harmful effects from the direct exposure to noise. As seen from this figure, genetic deficiency of *MsrA* causes permanent threshold shift (PTS) up to 30 dB at the frequency region of the noise stimuli, compared to 17 dB in controls. While applying a noise stimulus that induces temporary threshold shift (TTS) did not noticeably change the hearing ability in wild-type mice, the *MsrA* knockout mice exhibited delay in the recovery of TTS after exposure to loud sound (Alqudah et al., 2015). Therefore, we conclude that the protection against noise-induced TTS and PTS are compromised in *MsrA* mutant mice.

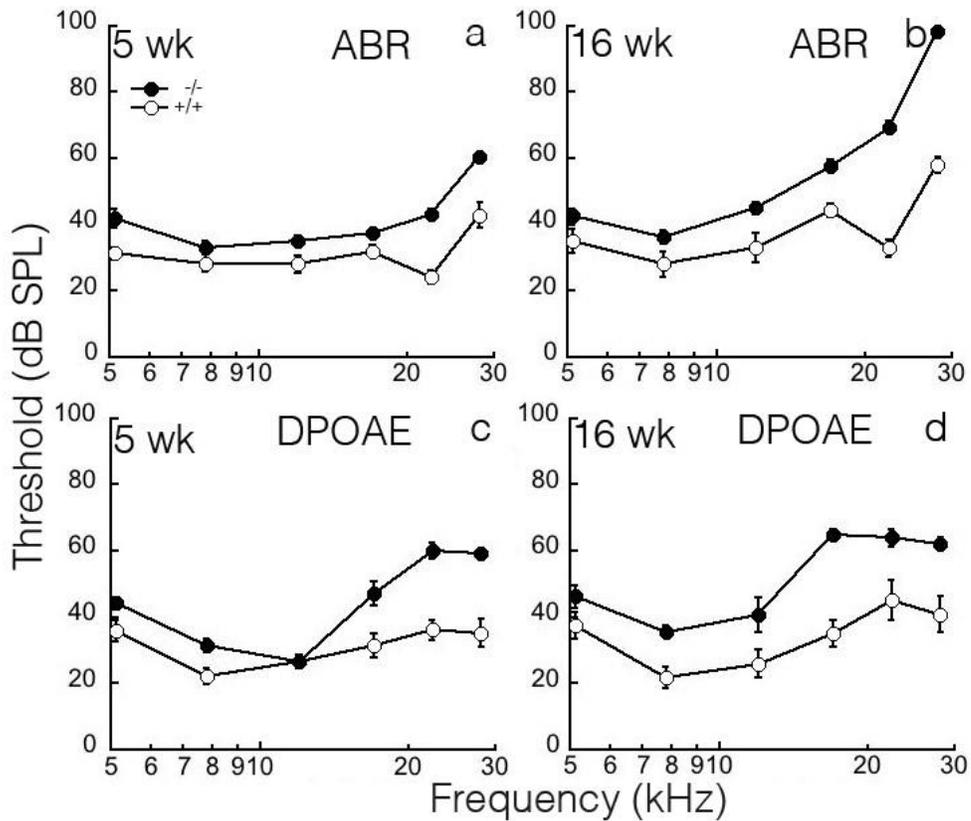


Figure 1: Hearing loss is more severe as *MsrA* knockout mice get older. Baseline ABR and DPOAE thresholds in young (5 weeks) and aged (16 weeks) *MsrA* mutant and wild-type mice. Thresholds for groups of animals (*MsrA*-knock out (-/-) and wild-type (+/+)) at 5 (a, c) and 16 weeks (b, d) of age are shown for ABRs (top panel) and DPOAEs (lower panel). Both ABR and DPOAE thresholds worsened in 16-week-old mice compared to 5-week-old mice. The data are expressed as the mean +/- s.e. Each point represents data from 8-11 mice for each group. The key in panel "a" applies to all panels.

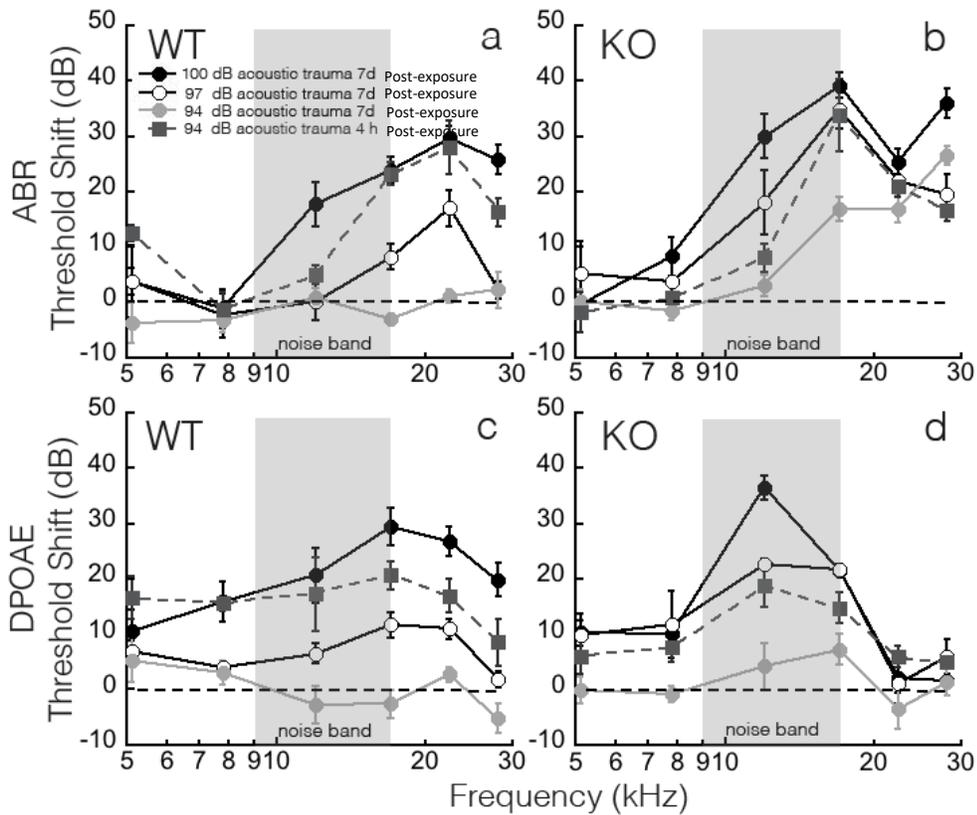


Figure 2: *MsrA* mediates protection to stimuli that generate PTS and TTS damage. Five-week-old wild-type mice exposed to a 100 dB SPL noise exposure (2 h at 9-17 kHz) show 18-30 dB permanent threshold shifts (PTS) compared to non-exposed mice ($p < 0.0001$ by ANOVA). PTS was reduced to 8-17 dB following an application of 97 dB noise level ($p < 0.05$ by ANOVA) and exposure to 94 dB noise showed no threshold elevation, suggesting temporary thresholds shift (TTS) when compared to the same condition of 4 hrs after the noise (a and c) ($p > 0.05$ by ANOVA). In contrast, 5-week-old *MsrA* knockout mice exposed to the same noise conditions show larger threshold shifts (25-40 dB for 100 dB SPL noise and of 19-35 dB for 97 dB SPL). TTS-generating stimuli (94 dB SPL) (right panel: b and d) still generated 17-26 dB threshold shifts seven days following the noise (gray circle; $p < 0.0001$ by ANOVA). The data are expressed as the mean \pm S.E. Each point represents data from 5-11 mice for each group. The key presented for panel “a” applies also to all other panels.

Accordingly, in this dissertation project, an anatomical follow-up study is presented and discussed to validate the previous electrophysiological findings that indicate *MsrA* protects against neurodegeneration in the cochlea and controls the sensitivity to acoustic trauma. The following aims are proposed to investigate and define the biological changes that are caused by the loss of *MsrA* by using two methods: histological tissue analysis and immune-labeling.

Aim 1: Defining the cellular changes caused by *MsrA* deficiency by counting the cell number for the following structures: spiral ganglion neurons (SG), inner hair cells (IHC), outer hair cells (OHC), and fibrocytes. Since the previous data showed loss of hearing and changes in gene expression of *MsrB1* and *MsrB3* in *MsrA* mutant mice, we assume that the mutation in *MsrA* gene is targeting the main cochlear structures and causes losses in the cell populations composed these structures.

Aim 2: Detecting the defect in protein distribution inside *MsrA* mutant cochleas by staining midmodular sections of wild-type and knockout mice with *MsrA* antibody that recognizes the presence of *MsrA* antigens in the studied tissues. Our assumption is that *MsrA* protein is expressed in the organ of Corti and the spiral ganglion neurons, which are potential sites of the damage generated by *MsrA* mutation, in older aged populations. Consequently, we expect to detect a defect in the *MsrA* protein distribution inside the mutant cochleas by visually recognizing less *MsrA* localization and intensity staining than in the controls.

Materials and Methods

General experimental approach

Animals

To analyze and report the cochlear histopathology and anatomy in *MsrA* knockout mice, we have designed a breeding colony from a group of mice given by Dr. Moskovitz from the University of Kansas. Knockout *MsrA* mice on a C57BL6/129sv background were backcrossed for more than 10 generations into a C57BL6 background. All procedures described for this study were approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Kansas Medical Center (KUMC). *MsrA* knockout mice (initially provided by Dr. Moskovitz) and wild-type mice (initially obtained from The Jackson Laboratories, Bar Harbor, ME) were used to expand our own mouse colony and to generate the presented data.

During non-experiment times, the animals were maintained in the animal care facility of KUMC with all conditions (temperature, moisture and light-dark cycles) set for ideal environment and under the supervision of a skilled veterinarian.

Histology

After the final physiological testing, we conducted the experiment on 6-month-old wild-type and *MsrA* knockout mice. The selected age is the time point when we ensured that the phenotype of hearing loss is expressed very strongly in the *MsrA* knockout mice. The mice were deeply anesthetized and perfused intracardially with a fixative solution containing 2.5% glutaraldehyde and 1.5% paraformaldehyde in phosphate buffered saline (PBS) for 10 min. Both inner ears were dissected out, and the round and oval windows were opened to allow intralabyrinthine perfusion of the same fixative. After overnight post-fixation in the same fixative at 4°C, the inner ears were osmicated (1% OsO₄ in deionized H₂O) for 1 h and then decalcified in 0.1 M EDTA (in PBSA) for two days. Inner ears were then dehydrated in ethanol and propylene

oxide, embedded in araldite, and sectioned at 20 μm on a microtome with a steel knife. Sections were then mounted on microscope slides, cover slipped, and observed under a bright-field microscope (Nikon, Eclipse 80i).

Cell counting

The spiral ganglion neurons, hair cells, and fibrocytes were counted in serial sections, beginning from section number 29 to 46. Our preliminary data of frequency mapping shows that these sections cover the high frequency end of the cochlea. The main reason for selecting that area of the cochlear partition is because we found the worst hearing thresholds caused by *MsrA* mutation there. For each subject, all 20 sections were examined under an electron microscope (Nikon, Eclipse 80i) using a magnification lens (20X). By focusing the vision throughout the entire section thickness, we were able to specify the location of the cell nuclei. Localizing the nuclei is essential to make precise determinations of fractional loss for each cell type. Furthermore, accuracy was assured by marking the xy position of each nucleus while repeatedly rolling the focus to image the entire depth of the section.

Immunohistochemistry

Several studies have shown that oxidative stress is one of the possible causes of cell death, as it targets the microelements of the cellular structures, such as proteins (Brennan & Kantorow, 2009; Moskovitz, 2005; Pal et al., 2007; Moskovitz, 2007). Thus we first evaluated the distribution of the *MsrA* antigens in the intact cochlear tissue, and then, compared the protein expressions of normal and *MsrA* mutant mice. This study comprised three major steps as described below.

a) Processing the tissue

Animals were sacrificed and the inner ears were isolated and fixed with 4% paraformaldehyde in PBS for 1 day at 4°C. The specimens were decalcified using 0.1 M EDTA (in PBS) for two days at 4°C. The specimens were then dehydrated using a graded series of ethanol solution, cleared in xylene, and embedded in paraffin. Next, the cochleas were placed into a plastic mold and left for 1 h in a 60°C oven, oriented with their brain side facing the bottom of the mold, and then left to cool slowly overnight.

b) Sectioning the paraffin blocks

We prepared the paraffin blocks by trimming the excess paraffin around the tissue. The blocks were fixed firmly on a plastic holder by melting the edges of paraffin blocks using heated metal chuck. We cut the tissue to 12 µm thick sections and gently dropped them in heated water bath containing 2 liters of H₂O at 39-41°C.

c) Immunostaining

Mid-modiolar cochlear sections were cut (12 µm thickness) and placed on a warm plate overnight at 39-40°C. were dewaxed in xylene, rehydrated through alcohol into PBS, and incubated for 10-20 min with 3% hydrogen peroxide (H₂O₂) in -20 °C to block endogenous peroxidase activity. Next, the sections were incubated for 1 h in PBS with 5% goat serum and 0.3% Triton X100 at room temperature and then incubated overnight at room temperature with the same solution to which the primary antibody for *MsrA* (Abcam, cat# ab16803, diluted 1:100) was added. The following day, the slices were washed in PBS and then incubated with a biotinylated goat anti-rabbit IgG secondary antibody (Vector, cat # BA-1100 diluted 1:400) for 3 h at room temperature, then with a streptavidin Alexa 488 (Thermo Fisher, cat# A20000

diluted 1:1000) for 1 h. The slide was then observed under a fluorescence microscope (Nikon, Eclipse 80i) by two trained testers to determine the staining intensity and localization of *MsrA* antibody staining.

Statistical Analysis

According to resource equation, the sample size was assigned to be eight mice (five knockouts and three wild-types). Our goal is to ensure obtaining an adequate power necessary to detect statistical significance in our research, as well, using the minimum number of animals to achieve efficiently the assigned objectives. The spiral ganglion neurons and fibrocytes numerical assessments for the two groups were calculated by determining the percentage of the present cells in the modular sections, where the basal and upper turns meet. We decided to select the arithmetic mean for the controls as the average cell counting number for normal hearing C57BL6 mice, leading us to have almost 100 percent of spiral ganglion cellular density for *MsrA* wild-type mice. Then the statistical differences between the means of controls and subjects was measured using an unpaired Student's t-test.

Results

Histology

We examined plastic-embedded cochlear cross-sections for evidence of histological changes in the mutant mice using a light microscope. It has been recently suggested that the spiral ligament fibrocytes, which interconnect with the basal cells of the stria vascularis via gap junctions, may be critical in maintaining cochlear homeostasis (Delprat et al., 2005). As shown in Figure 3 (adopted from Lieberman's and Hirose's study (2003), these types of cells are divided into four classes dependent upon their distinctive orientations and morphologies. Type I fibrocytes are situated near the stria vascularis, where the clusters travel to the spiral prominence. The bodies of these cells have widespread interdigitating bulging methods to make contact with adjacent cells through a group of membranes. The type II fibrocytes aid the function of type I fibrocytes and stria vascularis cells in recycling the potassium ions since these cells are a continuum for type I fibrocytes and the basal cells composed stria vascularis. Although both types I and II have the same physiological function, they are different in the cellular shape where is type II fibrocytes are less extended and have more fine processes than type I (Spicer & Schulte 1991; Schulte & Steel, 1994). Type III fibrocytes are surrounded by bony otic and type I and II fibrocytes. Their shapes are variably flattened with honeycomb like processes. Lastly, the spindle-shaped type IV fibrocytes are located within the triangular area under the basilar crest.

When compared to the control group, our results showed that fibrocytes in six-month-old knockout mice showed no difference in the population for type I, II, and III. The only exception was in fibrocyte type IV cells, which demonstrated a 60-70% loss in the knockout group across

the low frequencies (5-8 kHz) and high frequencies (16-26 kHz) regions, when compared to age-matched control mice ($p < 0.001$) (Figure 4 A, B, C).

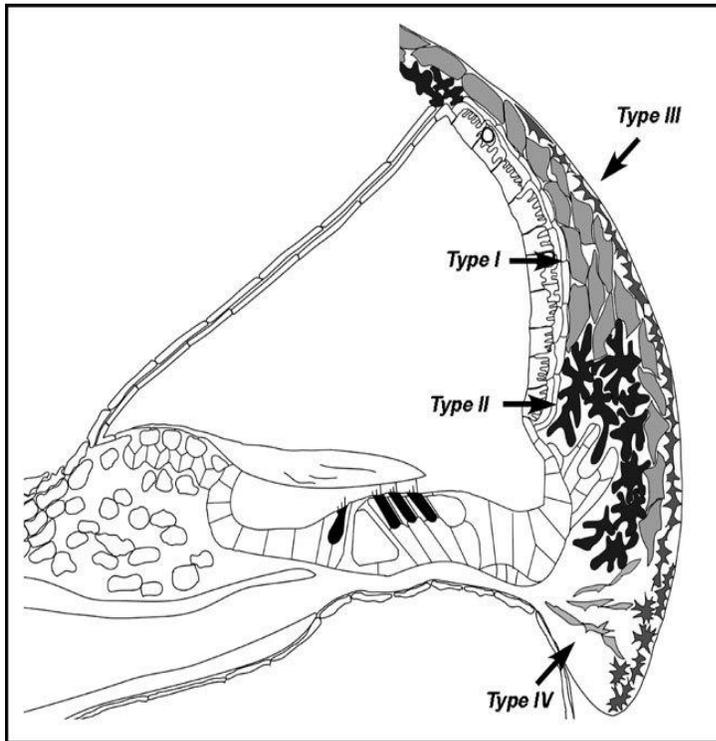


Figure 3: Schematic drawing of the cochlear duct shows the spatial organization and cellular shape of the four fibrocyte types in the lateral wall. Adapted from “Lateral wall histopathology and endocochlear potential in the noise damaged cochlea,” Hirose, K. and Liberman, M. C, 2003, JARO - Journal of the Association for Research in Otolaryngology, 4(3), 339-352.

While there is no loss of inner and outer hair cells (Figure 4D, E), six-month old *MsrA* knockout mice demonstrated a 20% loss of spiral ganglion neurons in the frequency region of 16-26 kHz when compared to age-matched control mice ($p < 0.001$) (Figure 4G, H, I). Loss of spiral ganglion neurons was not significantly different between wild type and *MsrA* knockout mice when counted in the frequency region of 5-8 kHz.

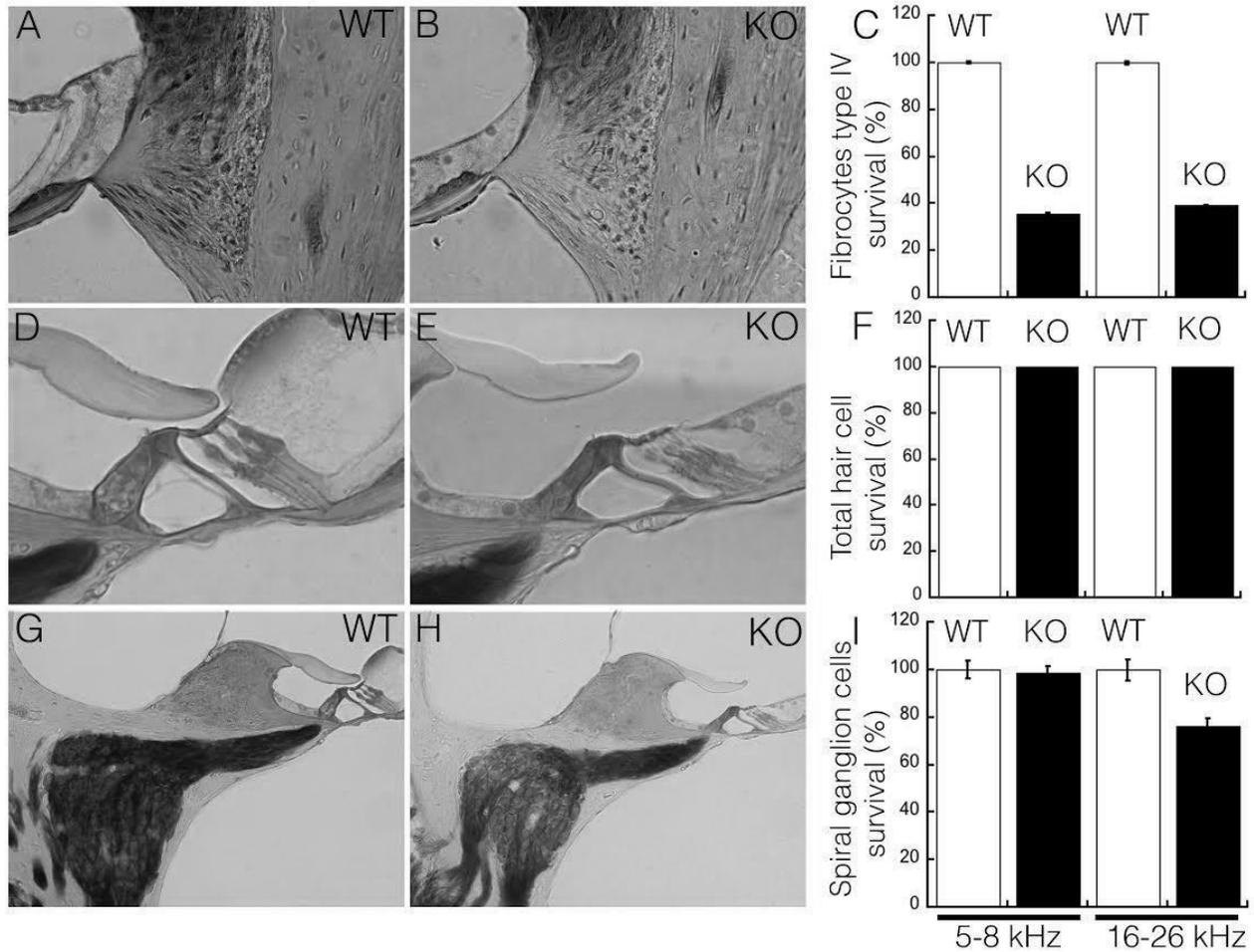


Figure 4: *MsrA* knockout mice show severe loss of type IV fibrocytes and Spiral ganglion neurons but do not show loss of hair cells. Loss of fibrocytes (A, B, and C) and spiral ganglion neurons (G, H and I) was seen as decreased density of cell population, although total hair cells (inner and outer hair cells) were not different from age matched wild type mice (D, E, and F). The data are expressed as the mean +/- S.E. Each point represents data from 3-5 mice for each group. Magnification 10X

Immunohistochemistry (IHC)

Both the wild-type and knockout groups showed the same degree of staining, suggesting the *MsrA* antibody was not able to detect the difference in *MsrA* protein in the cochlea that is expected to exist between these two genotypic groups (Figure 5 C and D). To troubleshoot this, we tested the following potential factors that could contribute to negative or non-specific staining: (1) Antigen retrieval. We tried lowering the retrieval temperature from 97 °C to 70 °C, decreasing the duration of the retrieval process from 40 minutes down to 20, and changing from heating the tissues to adding enzymes (citrate buffer) to aid the lysis of unwanted proteins. (2) Tissue sections. Cryostat sections were used initially, then we changed to paraffin blocks to optimize conditions for antibodies to detect the *MsrA* protein. Despite several trials with these new conditions, the same results were observed. It is also possible that, due to the polyclonal nature of the *MsrA* antibody that was prepared using the full-length *MsrA* protein, it may recognize region(s) that are shared among *Msr* isoforms inside the cochlea. As the result, similar staining patterns are noticed in wild-type and *MsrA* knockout samples.

To examine the possible presence in the *MsrA* knockout mice of other antigenic target proteins which are homologous to *MsrA*, we aligned the peptide sequence of *MsrA* with each of the *MsrB* isoforms individually. The results showed there is no resemblance between *MsrA* and *MsrB2* or *MsrB3*. However, we found 14 common amino acid residues between *MsrB1* and *MsrA* proteins (see Appendix A). If present at high levels, *MsrB1* proteins could generate signals as detected by the *MsrA* antibody.

Lastly, we confirmed the efficiency of the *MsrA* antibody in IHC by detecting *MsrA* in the brain, a tissue in which *MsrA* is highly expressed. Our results have shown that the *MsrA* antibody can detect *MsrA* protein in the brain of wild type mice, whereas no signals detected in

the *MsrA* knockout counterparts (Figure 5 A and B). This suggests that the low abundance of *MsrA* protein in the normal cochlea is most likely the reason for negative IHC staining, and this method is not sensitive one for studying the presence of *MsrA* proteins in the organ of Corti and spiral ganglion neurons.

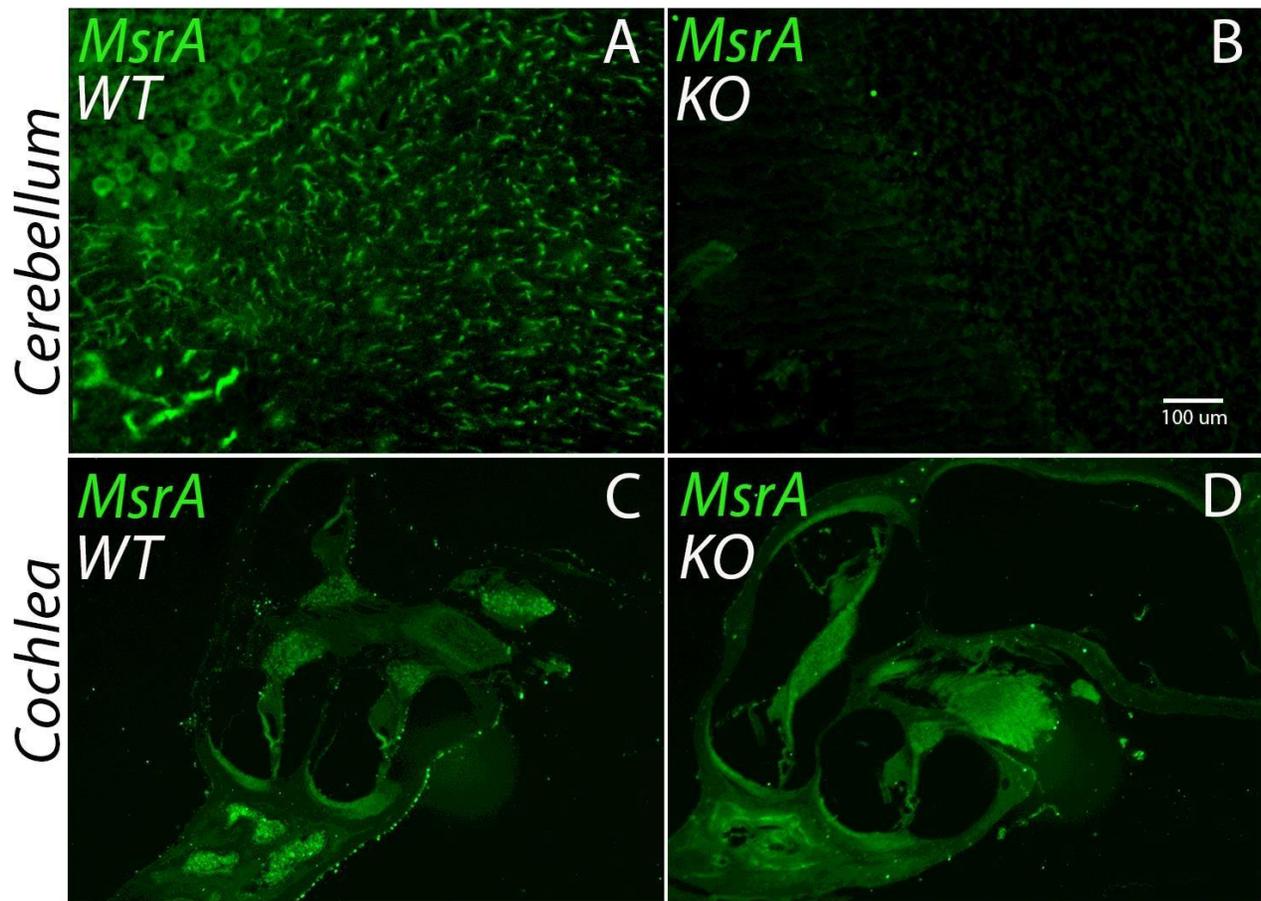


Figure 5: Intense *MsrA* immunoreactivity observed in the brain of wild-type mice (A), but not in the *MsrA* knockout mice (B), indicates the efficacy and specificity of the *MsrA* antibody. In the brain, strong label is observed in the cerebellum. However, nonspecific labeling was observed in the cochlear tissue (C and D), suggesting undetectable amount of *MsrA* protein within cochlear structures. Sections in A-D were immune-reacted together and images were taken at identical exposure levels. Magnification: 10X (top panel; A and B) and 4X (lower panel; C and D).

Discussion

The main goal of this dissertation project was to further support our previous finding that the role of *MsrA* is to protect the cochlea from the damaging effects of noise and aging (Alqudah et al., ARO 2015 and 2016). The hypotheses were as follows: 1) Reporting changes in the cell population of the *MsrA* mutant cochleas. The cellular densities for the studied structures are supposed to be less in *MsrA* knockout mice compared with wild-type controls. 2) Observing more detectable signals generated from *MsrA* antibody/antigens complexes in the normal *MsrA* cochleas than those present in the mutants. This discussion will offer explanations with details and observations to clarify the final conclusions. Potential future research will also be discussed, including an illustration of the possible human clinical application.

Many studies have indicated *MsrA* as a major player in inhibiting neurological disorders like Alzheimer's disease and Parkinson's disease (Brot, Weissbach, Werth, & Weissbach, 1981; Gabbita, Aksenov, Lovell, & Markesbery, 1999). *MsrA* knockout mice showed impaired motor ability and abnormal behavior caused most likely by cerebellar dysfunction (Moskovitz et al., 2001). Oxidative stress is a common process that occurs both in noise exposure and in aging. In the cochlea, oxidative stress can trigger accumulation of oxidized proteins, leading to damage of the sensory cells (hair cells) and auditory neurons, and exacerbating hearing loss.

Methionine (Met) is an amino acid that is highly susceptible to oxidation, particularly under conditions of oxidative stress and aging (Levine & Luo, 2008; Stadtman et al., 2005; Stadtman, Moskovitz, & Levine, 2003; Stadtman, 2002;). Oxidation of Met to methionine sulfoxide (MetO) is reversible, and the reverse reaction is catalyzed by the methionine sulfoxide reductase system (Msr), comprising methionine *S*-sulfoxide reductase A (*MsrA*) and methionine *R*-sulfoxide reductase B (*MsrBI-3*). Consequently, the *Msr* system provides efficient protection

against oxidative stress by repairing oxidative damaged proteins, reinstating normal function to the repaired proteins, and reducing the accumulation of oxidized proteins in cells (Moskovitz, 2007; Moskovitz et al., 1998; Moskovitz et al., 2001; Salmon et al., 2009).

The combination of higher protein oxidation, induced by a constant oxidative stress, and compromised *Msr* system may induce more damage, leading to changes in protein structure and dysfunction of sulfoxidized proteins and cells. These resulting changes may eventually lead to permanent cell damage.

A recent study reported *MsrA* expressing in the cells of organ of Corti, spiral ganglion neurons, and lateral wall (Maison et al., 2012). *MsrA* provides protection against age-related central nervous system (CNS) changes that are associated with neurodegenerative diseases (Moskovitz et al., 2002; Oien et al., 2008). *MsrA* also maintains proper function of neurons of the brain dopaminergic system (Liu et al, 2008; Oien et al, 2010; Oien et al, 2008; Ortiz, Oien, Moskovitz, & Johnson, 2011), a class of neurons that projects neurofibers within the cochlea and protect it from acoustic trauma (Gaborjan et al., 1999; Maison et al., 2012). Thus, *MsrA* was a strong candidate for studying the role of *Msr*s in protecting against AIHL and noise-induced hearing loss (NIHL).

Prior to this study, we reported that *MsrA* mRNA levels of the cochlea increase during aging, while age-dependent expression of *MsrBI* in *MsrA* knockout mice is compromised in comparison with wild-type controls. Furthermore, we showed that young *MsrA* knockout mice have normal neural hearing capabilities, which sharply worsen at older ages. Finally, we demonstrated that *MsrA* knockout mice exposed to noise show an increased vulnerability to acoustic trauma at both young and older ages; suggesting that *MsrA* mediates protection from noise also at older age (Alqudah et al., ARO 2015 and 2016).

Yet, information regarding histopathology of the cochlea in mice that lack *MsrA* is unknown. This study attempted to investigate the histological changes in normal and mutant *MsrA* mice following aging as a means of explaining the phenotype of hearing impairment. The results revealed that the six-month-old *MsrA* knockout mice had significant loss of spiral ganglion neurons mainly manifested at high frequencies and widespread loss of type IV fibrocyte cells in the lateral wall of the cochlea.

We speculate that the loss of spiral ganglion neurons is the possible cause for the deterioration in the ABR responses in the mice that lack *MsrA*. This assumption stems from observing that the latency for ABR wave I in mice lacking *MsrA*, which originates from spiral ganglion neurons, is longer than in the wild-type mice (Melcher, Guinan, Knudson, & Kiang, 1996). This means slower neural conduction velocity for processing the auditory signal delivered to the brain. Moreover, the sensory component of hearing loss present in *MsrA* mutant mice and characterized by abnormal DPOAE amplitudes and thresholds could be the result of loss of type IV fibrocytes. Previous studies reported that these fibrocytes are necessary to maintain the homeostasis of the endolymphatic fluid in scala media by regulating the flow of potassium ions in the endolymphatic fluid (Delprat et al., 2005).

To examine the distribution of *MsrA* in the cochlea, we determined the cellular distribution of *MsrA* using immunohistochemistry in adult both wild-type and mutant *MsrA* mice. In this study, the protein expression was measured as the level of labeling intensity. While Intense *MsrA* immunoreactivity was observed in the brain of *MsrA* wild-type mice but not in the brain of knockout mice (Figure 5A and B), all cochlear samples from knockout and wild-type mice showed moderate to strong staining regardless of the genotype (Figure 5 C and D).

Therefore, we considered this result as non-specific staining. Various trials were conducted to improve the staining quality, yet, the same results were reported by two professional testers. The most likely interpretation to explain this non-specific staining is that the *MsrA* protein is not detectable in the mouse cochlea. Although our data revealed *MsrA* antibody labeling the supporting cells, spiral ligament, spiral limbus, Reissner's membrane and the spiral ganglion neurons (as shown in Kwan's 2014 study) we were unable to differentiate the staining intensity between the wild-type and knockout mice and figure out any change in protein abundance inside the cochlea caused by mutation in *MsrA* gene.

Details regarding tissue distribution differences between wild-type and knockout mice are still unclear since the IHC experiment was not powerful enough to detect the presence of *MsrA* in the cochlear tissue. Therefore, Western blot assay and in situ hybridization are planned for future experiments to identify the genotypic difference in protein expression and localization.

MsrA mutant mice showed higher sensitivity to noise exposure than controls mice (Alqudah et al., ARO 2015); therefore, histological analysis of the cochlea of knockout mice exposed to noise would be helpful to investigate the molecular mechanism that are regulated by *MsrA* in protecting mice from noise damage. This will be accomplished by identifying the level of cellular damage caused by noise in mice which lack *MsrA*.

Acquiring basic knowledge of the role of *MsrA* in hearing loss (using the *MsrA* knockout mouse as a tool) will greatly assist in understanding the age-dependent interactions between noise- and age-induced hearing-loss. Additionally, possible investigations into the specific role of *MsrA* protection from acoustic trauma may prompt a new line of studies to treat acoustic trauma through Msr-based therapy. Furthermore, these investigations may provide evidence for the possible roles of the Msr system in patients exhibiting age-related neurodegenerative pathologies of the central nervous system (CNS).

One of the limitations of this study is that the *MsrA* mutation is originally present in the C57BL/6 (B6) mouse strain, which is a poor model for the study of age-related hearing loss. The B6 mouse develops early age-related hearing loss due to a mutation in the gene for cadherin-23, and B6 mice are considered to have extreme variability in thresholds at high frequencies. Although B6 mice have normal hearing at younger ages (1-2 months), they have poor auditory responses at 12 months of age. We realize that the sound responses of this strain are so hard to be tracked with aging so that we restricted our study within four to six months of age.

Conclusion

Our results indicated that *MsrA* is an essential factor in the encoding process of the protein that maintains the basic cellular structures in the cochlea, including spiral ganglion neurons and the type IV fibrocytes. These structures have been reported as main contributors in processing the auditory signals in mammals. Moreover, *MsrA* mutant animals showed a 20% loss of spiral ganglion cells and a loss of 60% of fibrocystic class IV, which has been shown as a cause of sensorineural hearing loss in the high-frequency region (supported by electrophysiological tests). More investigation should be conducted to measure the *MsrA* protein level in wild-type and mutant mice in an effort to associate the observed phenotype, caused by the *MsrA* mutation, with microbiological modifications affecting the protein abundance inside the studied tissues. The ultimate goal for this study is to acquire basic knowledge of the role of *MsrA* in preventing hearing loss by using the *MsrA* knockout mouse model. This investigation will be useful to develop gene-based therapy that will replace, to a certain degree, the conventional approach to restore hearing function such as hearing aids and cochlear implants.

Review of the Literature

Methionine Sulfoxide Reductase (Msr) System

MsrA as a protective mechanism against neurodegeneration in the brain and the cochlea

Prior studies have linked *MsrA* to protection against the onset of age-related neurodegenerative pathologies in the brain (Moskovitz et al., 2002; Oien et al., 2008) and the retina (Brennan & Kantorow, 2009; Sreekumar et al., 2011). This dissertation study tested the notion that *MsrA* might play a key role in the cochlea as well.

Prior studies have shown that *MsrA* knockout mice have impaired dopaminergic signaling pathways (Ortiz et al, 2011; Salmon et al., 2009), suggesting a pivotal role for *MsrA* in protecting dopaminergic neurons from age-related neurodegeneration (Oien, et al, 2010). *MsrA* knockout mice exhibit neurodegenerative brain pathologies (Oien, 2008) and malfunction of brain dopaminergic neurons, probably due to the observed loss of dopamine receptor 2 (D2DR) activity (Liu et al., 2008; Oien et al., 2010; Oien et al., 2008; Ortiz et al, 2011). Studies published in the last two decades show that the cochlea contains dopaminergic innervation and regulates cochlear responses to sound. Felix and Ehrenberger (1992) provided evidence that the firing rate of afferent fibers contacting the inner hair cells is suppressed following application of dopamine and that dopamine action is mediated by dopamine receptors activation. Inoue et al. (2006) demonstrated the expression of several dopamine receptors in the cochlear neurons beneath the inner hair cell region, and Darrow, Simons, Dodds, and Liberman (2006) suggested that dopaminergic neurons from the olivocochlear nuclei extend towards beneath the inner hair cells to form dopaminergic endings. However, only recently, Maison et al. (2012) confirmed that mice lacking dopamine receptors exhibit exacerbated damage from acoustic trauma, suggesting that dopaminergic innervation might act to minimize glutamate-induced excitotoxicity resulting from acoustic trauma (Maison et al., 2012; Ruel et al., 2001)

Mechanisms of Msr-mediated protection in the inner ear

The possible role of *MsrB1* in hearing loss has not yet been investigated due to the lack of relevant mouse models and insufficient knowledge about the possible mutations of this gene in humans. However, Kwon et al. (2014) reported the expression of *MsrB1* in the inner, outer hair cells of the organ of Corti, and in the spiral ganglion, suggesting a possible role of *MsrB1* in protection from cochlear oxidative stress. Another report indicated that *MsrB1* is expressed in the liver, which declines with age (Moskovitz et al., 2002). In our previous study, we observe that *MsrB1* expression in young *MsrA* knockout mice is also compromised (see Appendix B), suggesting that *MsrB1* might be likely regulated by *MsrA* in an age-dependent fashion and/or cochlea protection might be mediated by *MsrA* or *MsrB1* or both (Alqudah et al., ARO 2015-2015).

Possible role of Msr-mediated protection from acoustic trauma

The published literature regarding acoustic injury is in agreement with the general observation that a narrow band (8-16 kHz) of sound exposure for 2 h at a sound pressure level equal to or less than 100 dB triggers the mechanical stereocilia damage but not sensory cell loss (Karadaghy et al., 1997). Furthermore, the protective process from the oxidative stress generated by this type of noise is still ambiguous and need further investigation.

MsrB3 has been linked to deafness in humans and mice, suggesting a crucial role of *MsrB3* in the development or functioning of the inner ear. Ahmad and his colleagues (2011) reported that *MsrB3* is present at the base of hair cell stereocilia and controls the maturation, thus suggesting a role of *MsrB3* in the maintenance of stereocilia structure. Consistent with the location of *MsrB3* and with the generation of stereocilia damage following acoustic trauma,

MsrB3 could elicit protection against generation of acoustic injury by repairing R-MetO containing proteins in stereocilia.

Immunohistochemistry of Msr

Previous studies indicated that the expression profiles of the Msr genes do not follow systematic pattern and vary from one part of the body to another (Kim & Gladshev, 2004; Marchetti et al., 2005; Taungjaruwina, Bhawan, Keady, & Thiele, 2009). These variations in the expression of Msr genes were observed even in the cellular level, where *MsrA* and *MsrBs* are distributed unequally in different subcellular structures. According to Kim and Gladshev (2004), *MsrA* is expressed in nucleus, cytosol and mitochondria while *MsrB1* in cytosol and nucleus, *MsrB2* in mitochondria, and *MsrB3* in endoplasmic reticulum or mitochondria. Among various tissues and organs, *MsrA* is mostly expressed in the kidney, cerebellum, and liver; *MsrB1* is highly expressed in liver, pancreas, and leukocytes; *MsrB2* is predominantly in the heart, liver, and skeletal muscle; and *MsrB3* largely in the bladder, aorta, heart, and skeletal muscle.

In some areas of human body, Msr family members were detected in different proportions within the same system, for example, in the skin. Taungjaruwina, Bhawan, Keady, and Thiele (2009) reported in his study that *MsrA* strongly expresses in all keratinocytes, except the stratum corneum. Moreover, *MsrA* is detectable in melanocytes, sebaceous gland, eccrine gland, hair follicles, and blood vessels. Although the *MsrB* is divided to three subsets, it is common to see the expressions of all *MsrBs* at the same spot, but in a compressed level for each one of them. The results of the study revealed that *MsrB1* and *MsrB3* expression was detected in blood vessels, while *MsrB2* was observed within melanocytes. In the ocular system, *MsrB1* and *MsrB3* are weakly expressed in both the epithelium and fiber cells. Whereas, *MsrA* normally is

more expressed in the epithelium than fiber cells, and *MsrB2* expression was higher in the fiber cells than in epithelium (Marchetti et al., 2005).

The presence and distribution of Msr family members in the hearing system has been studied by one group of researchers so far (Kwon et al., 2014). Their findings indicated that these genes have been defined in both cochlear and vestibular organs of inner ear. Interestingly, as in skin and ocular systems, each Msr is limited to specific cochlear structures and different Msr genes are expressed at various levels. Moreover, they showed that *MsrA* was highly expressed in the supporting cells, spiral ligament, spiral lamina, and Reissner's membrane, with the lowest expression level in the spiral ganglion cells. They also suggested that *MsrB1* is expressed largely in hair cells and the spiral ganglion region, whereas *MsrB2* is likely expressed in the spiral ganglion, tectorial membrane and stria vascularis, and none in the organ of Corti. The researchers verified that *MsrB3* protein is present in rod-like patterns at the base of each stereocilium but poorly expressed in hair cell bodies.

To further advance the understanding about the location and function of Msr genes in the auditory system, Kwon et al. also analyzed the vestibular tissues. In addition, they documented that *MsrA*, *MsrB1* and *MsrB2* are also detectable in varied ratios. Their research indicated that *MsrA* and *MsrB1* are strongly expressed in almost all the parts of vestibule: the sensory and neural cells of the crista ampullaris, utricle, and saccule, while *MsrB2* is not expressed in the hair cell of the vestibule and scattered in the vestibular ganglion neurons. Similar localization of *MsrB3* protein at the base of stereocilia was also observed in vestibular hair cells.

Histopathology of age related hearing loss

In the mouse model, the age-related hearing loss causes histological changes accompanied with alternations in the physiological function of hearing system. Early studies

have been focused on the integrity of two types of cell populations, hair cells and spiral ganglion neurons, within the scala media structures to explain the pathophysiological modifications associated with ARHL. In the C57BL/6J mouse, ARHL starts initially in the high frequency region at about 3 to 6 months of age and then continues to include all frequencies by 15 months (Henry, 1983; Willot, Parham, & Hunter, 1991). White and her colleagues (2000) documented the pattern and the sequence of degeneration in the hair cells and spiral ganglion neurons as the C57BL/6J mice are aging. They showed that, by age 8 months, the C57BL/6J mouse has only 4% of the outer hair cells remaining. At age of 18 months, massive loss of inner hair cells was reported in these mice and only 29% of the cells were present. By 26 months of age, more than 80% of the OHCs were absent across the whole cochlea while IHC losses were approximately 100% close to the base of the cochlea and about 20% in the apical turn. Regarding the peripheral neural component of the hearing system, there was a substantial decline with age in spiral ganglion cell density, which was strongly correlated with surviving inner hair cells previously defined. In general, age-related progressive degeneration of spiral ganglion cells was worsening from distal location in the osseous spiral lamina (near the organ of Corti) than at a proximal location (near the spiral ganglion).

Liberman and Hequembourg (2001) investigated the role of structures such as spiral lamina, stria vascularis, and spiral ligament in the histopathology of aging in mice. They confirmed conclusions from previous studies that found hair cell loss follows a basal to apex gradient. They also observed that the spiral ganglion neurons underwent degeneration as a result of the aging process. This conclusion was based on an observation that spiral ganglion damage is steadily increasing from apex to the midcochlear region in aged ears, which is opposing the direction of gradual inner hair cells death. In addition, this study also showed that ARHL is

targeted through fibrocyte type IV that are located in a triangular region of spiral ligament just below the point of insertion of the basilar membrane (Henson & Henson, 1988). Heumen, Claxton, and Pickles (2000) suggested that this class of fibrocytes is essential to maintaining the hemostasis of scala media, based on the fact that these fibrocytes have a large expression of tyrosin kinase receptors that are highly selective receptors to the ions entering the cochlea. The type IV fibrocyte loss started earlier than that for the loss of hair cells, specifically by the age of 3 months, and extended further beyond the OHC loss region. Thus, the presence of early and severe degeneration in the fibrocytes predicts the decline in the hair cell population during aging process. This study provides the evidence that defects in the spiral ligament, especially at the fibrocytes type IV, might initiate the neural degeneration and other forms of sensory hearing loss.

Age-dependent sensitivity to noise

It is known that ears exhibiting noise damage age differently than those without damage (Kujawa & Liberman, 2006), and that noise exposure is more detrimental at younger ages than later (Kujawa & Liberman, 2006). These observations suggest that there is a protective mechanism against damage from exposure to noise in the cochlea that is also tightly regulated with age. Ohlemiller, Wright and Heidbreder (2000), and then Kujawa and Liberman (2006) provided direct evidence that cochlear sensitivity to noise is an age-dependent phenomenon. Notably, these researchers have shown that young mice are more sensitive to noise exposure/damage than older mice and that this sensitivity in younger mice is followed by a sharp reduction in sensitivity in adulthood (between 8 and 16 weeks of age). Kujawa and Liberman (2006) suggested that metabolic changes happening within the cochlea might explain this phenomenon. This notion is supported by the observation that attenuation of the middle ear

sound transmission has little influence on sensitivity to noise between 8 and 16 weeks (Rosowski, Brinsko, Tempel, & Kujawa, 2003).

Degeneration of the cochlear synaptic terminals

Literature is in agreement with the observation that the most distinct pathological site for both noise-induced hearing loss and age-related hearing loss are the spiral ganglion neuron terminals that contact the inner hair cells (IHC) (Lin, Furman, Kujawa, & Liberman, 2011; Liberman & Kujawa, 2009; Liberman & Kujawa, 2014; Perez & Bao, 2011; Sergeyenko et al., 2013). In aging animals, loss of afferent synapses of the IHCs is progressive throughout life and across the whole cochlea (Perez & Bao, 2011). However, after noise, loss of synapses during aging is severe and especially in the frequency region where noise exposure generated high thresholds (Lin et al., 2011; Liberman & Kujawa, 2009; Liberman & Kujawa, 2014; Sergeyenko et al., 2013). The mechanism of damage of afferent synapses is based on the observation that following noise exposure IHC synapses swell and rupture within hours after exposure (Pujol & Puel 1999; Wang et al., 2002). Synaptic swelling is the most likely result of excitotoxicity from a large release of glutamate from the IHC during the noise exposure (Kujawa & Liberman 2009).

Numerous studies have suggested that degeneration of synapses target a specific group of fibers in the cochlea that have a low spontaneous discharge firing rate (Buran et al., 2010; Furman et al., 2013; Schmiedt et al., 1996; Sergeyenko et al., 2013). For instance, cochlear neurons exhibit a range of spontaneous discharge firing rates: from low spontaneous discharge rate neurons (activated when in presence of high level of sound) to high spontaneous discharge rate neurons (activated when in presence of a low level of sound) (Liberman & Dodds 1984). There is no evidence as to why neurons with low spontaneous discharge firing rates are more vulnerable to noise exposure than those with high spontaneous

discharge rates. However, it is possible that low spontaneous discharge firing rates fibers are more sensitive to overload of Ca^{2+} (which its cellular concentration increase during profuse release of neurotransmitter glutamate), triggering cellular excitotoxicity and cell death.

In order to answer this and other questions relating to noise effects, researchers need to explore the areas that will assist to acquire more knowledge about noise and aging combined damage.

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Appendix A

BLAST®

Basic Local Alignment Search Tool

[NCBI/ BLAST/ blastp suite-2sequences/ Formatting Results - C9NX101V11R](#)

▶ [Formatting options](#)

▶ [Download](#)

[Blast report description](#)

Blast 2 sequences

unnamed protein product (233 letters)

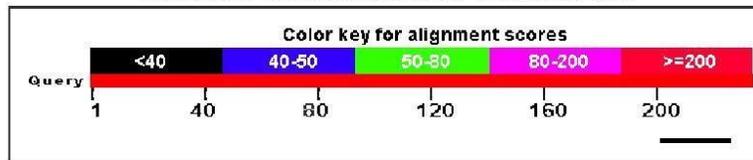
RID [C9NX101V11R](#) (Expires on 02-19 03:27 am)

Query ID lcl|Query_137889
Description unnamed protein product
Molecule type amino acid
Query Length 233

Subject ID lcl|Query_137891
Description None
▶ [See details](#)
Molecule type amino acid
Subject Length 116
Program BLASTP 2.3.1+

[Graphic Summary](#)

Distribution of 1 Blast Hits on the Query Sequence



Graphical display of BLASTP results of protein sequence alignment of *MsrB1* with *MsrA*. The black line represents a mathematical score of weak similarity between *MsrA* and *MsrB1* polypeptides. Sequence similarities are indicated by scores associated with various colors (left to right: weak to strong).

⊕ Dot Matrix View



⊖ Descriptions

Sequences producing significant alignments:

Description	Max score	Total score	Query cover	E value	Ident	Accession
unnamed protein product	13.9	13.9	10%	1.5	24%	Query_137891

⊖ Alignments

unnamed protein product

Sequence ID: lcl|Query_137891 Length: 116 Number of Matches: 1
Range 1: 46 to 78

Score	Expect	Method	Identities	Positives	Gaps	Frame
13.9 bits(24)	1.5()	Compositional matrix adjust.	8/33(24%)	13/33(39%)	8/33(24%)	

Features:

```
Query 201 YAEDYHQYLSKNPDG-----YCGLGGTGV 225
      + E H ++K P+ CG G G+
Sbjct 46 FTETIHPDSVTKCPEKNRPEALKVSCGKCGNGL 78
```

The BLASTP result of the strongest alignment between *MsrB1* and *MsrA*, showing 8 identical and 5 chemically similar (+) residues spanning a stretch of 25 residues, which corresponds to 24% identity. The definition of nomenclatures can be found at the following website: <https://blast.ncbi.nlm.nih.gov/Blast.cgi>.

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[Blast report description](#)

Blast 2 sequences

unnamed protein product (233 letters)

RID [C9P9TJRY11R](#) (Expires on 02-19 03:34 am)

Query ID lc|Query_261273
Description unnamed protein product
Molecule type amino acid
Query Length 233

Subject ID lc|Query_261275
Description None
▶ [See details](#)
Molecule type amino acid
Subject Length 175
Program BLASTP 2.3.1+

⊖ [Descriptions](#)

⊖ [Alignments](#)

Negative BLASTP search result of *MsrB2*- *MsrA* alignment.

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Basic Local Alignment Search Tool

[NCBI/ BLAST/ blastp suite-2sequences/ Formatting Results - C9P4ZRXC11R](#)

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[Blast report description](#)

Blast 2 sequences

unnamed protein product (233 letters)

RID [C9P4ZRXC11R](#) (Expires on 02-19 03:31 am)

Query ID lc|Query_214209
Description unnamed protein product
Molecule type amino acid
Query Length 233

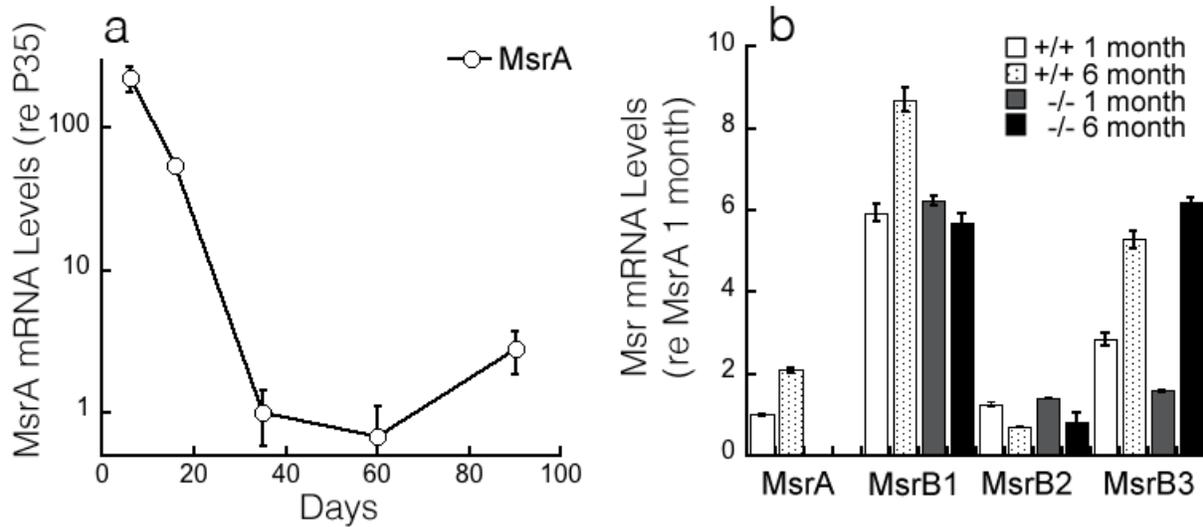
Subject ID lc|Query_214211
Description None
▶ [See details](#)
Molecule type amino acid
Subject Length 186
Program BLASTP 2.3.1+

⊖ [Descriptions](#)

⊖ [Alignments](#)

Negative BLASTP search result of *MsrB2*- *MsrA* alignment.

Appendix B



MsrA mRNA post-birth and in adults. Quantitative RT-PCR of cochleae (organ of Corti and spiral ganglia) indicated *MsrA* mRNA levels are high right after birth but decrease rapidly thereafter (around 12 days), reaching the lowest level at 60 days (a) ($p < 0.0001$ compared to 6- days post birth using the unpaired Student's t-test). Aging increases cochlear Msrs mRNA levels in the adult by 6 months of age (b and c) ($p < 0.0001$ compared to 1month using the unpaired Student's t-test); except for *MsrB2*, which decreases by half (b)($p < 0.0001$). Msrs mRNA changes in *MsrA* knockout mice. Quantitative RT-PCR of cochleae indicated *MsrB1* mRNA levels did not increase at 6 months of age ($p > 0.05$ compared to 1-month post birth using the unpaired Student's t-test), whereas *MsrB3* increases ($p < 0.0001$), suggesting that the lack of *MsrA*, and possibly aging, might regulate *MsrB1* in the cochlea but not *MsrB3*. The mean \pm S.E. are plotted. Each point in a-c represents data from 4 and 5 mice, respectively