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Genetic influences on hypoxic conditioning

Amanda Ortmann

Genetic Influences on Hypoxic Conditioning

by

Amanda Ortmann

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Approved by: Kevin K. Ohlemiller, Ph.D., Independent Study Advisor

Abstract

Noise-induced hearing loss (NIHL) involves in part metabolic injury resulting from reactive oxygen species (ROS) and oxidative stress. It is of great importance to prevent the overproduction of ROS in the cochlea. Doing so will protect valuable hair cells from damage, thereby conserving hearing. Recent research has shown that noise injury is attenuated by conditioning with mild whole-body stressors prior to noise exposure. In CBA/J mice NIHL is attenuated by conditioning the mice to a 4 hour episode of mild hypoxia 24 hours prior to noise exposure. However another strain (C57BL/6 *Ahl/Ahl*) exhibited no effect from pre-exposure hypoxia. This study begins the process of determining the number and characteristic of the gene(s) involved in the conditioning response by demonstrating principles of inheritance. CBA/J x C57BL/6 F1 hybrids were examined to see if they were capable of conditioning. There were no significant differences between the hypoxia conditioned group and the controls, which is consistent with involvement of a single recessive gene. Also the B6.CAST$^{+\,Ahl}$ were tested for the hypoxic conditioning response. Post-noise exposure thresholds for control and conditioned groups did not significantly differ, indicating that the *Ahl* gene does not contribute to the conditioning response. Possible mechanisms of the generation of ROS in the cochlea during noise exposure and the activation endogenous defense system by conditioning are reviewed.

Introduction

Noise-induced hearing loss from either chronic moderate noise exposures or acoustic trauma is the second leading type of sensorineural hearing loss (Rabinowitz, 2000). Even with all of the current mandates of hearing conservation programs, it has been estimated that 10 million persons in the United States suffer permanent hearing loss resulting from noise or trauma

(NIDCD 1998). Because of the irreversible nature of NIHL and the nearly incessant amount of occupational and recreational noise, it is important to protect the ears from acoustic injury. Although much action has been taken to promote the use of hearing protection devices, NIHL still remains to affect a large population of workers, recreational shooters, and military personnel. Therefore, other prevention or treatment techniques need to be developed.

Determinants of cell survival after injury include antioxidant, enzymes, proteins that promote cochlear blood flow, and expression of genes that serve to protect the cochlea against adverse conditions such as noise and toxicity (Kopke et al. 2002, Wang and Liberman 2002, Huang et al. 2000, Ohlemiller et al. 2000a, Ohlemiller et al. 1999b). Researchers are working to enhance these stress-defense pathways in the cochlea so that the incidence of NIHL can be minimized. Several studies have demonstrated attenuation of NIHL by administration of antioxidants prior to noise exposure (Kopke et al. 2002, Yamasoba et al. 1998, Quirk et al. 1994, Seidman et al. 1993). These enhance endogenous defense mechanisms in the cochlea and thereby maintain cellular homeostasis under environmental stress (Kopke et al. 1999). Enhancement of innate cellular protective mechanisms has also been demonstrated in the brain and eye by using various sublethal stressors such as hyperthermia and hypoxia to establish ischemic tolerance (Omata et al. 2002, Zhu et al. 2002, Miller et al. 2001, Chandel et al. 2000, Iyer et al. 1998, Feuerstein et al. 1997, Gidday et al. 1994). In the realm of hearing, restraint stress, hyperthermia, and moderate noise prior to toxic noise exposure attenuated noise induced cochlear injury (Wang and Liberman 2002, Yamasoba et al. 1999, Canlon 1997, Dechesne et al. 1992, Myers et al. 1992). As these protective mechanisms underlying conditioning become better understood, it is possible that they may be pharmacologically enhanced in anticipation of noise exposure.

Although studies have shown cerebral and retinal protection by hypoxic conditioning, until recently it had never been examined in the context of hearing. In an abstract for the 2003 Mid-winter ARO meeting, Ohlemiller and colleagues described the effects of hypoxic conditioning in CBA/J mice. Their results demonstrated partial protection from NIHL in mice that were conditioned by exposure to hypoxia (8% oxygen) for 4 hours as opposed to room air only prior to noise exposure. Interestingly, the results also indicated a temporal variation in protective and toxic effects of hypoxic conditioning (figure 1). A 24 hour interval between hypoxic conditioning and noise exposure appeared to render partial protection from NIHL, whereas shorter intervals were more harmful than noise alone. Chen (2002) also demonstrated deleterious effects these shorter intervals by showing that mild hypoxia during noise exposure potentiated NIHL. For time as long as 48 hours, hypoxic conditioning showed no effect on NIHL. The temporal characteristic of the conditioning response may reflect the temporal variation in the interaction of toxins such as reactive oxygen and nitrogen species (ROS and RNS) and expression of protective factors. Another finding from this study is that no protection by hypoxic conditioning was observed in C57/BL6 mice. Thus one or more genes that differentiate these two strains affect the efficacy this conditioning response.

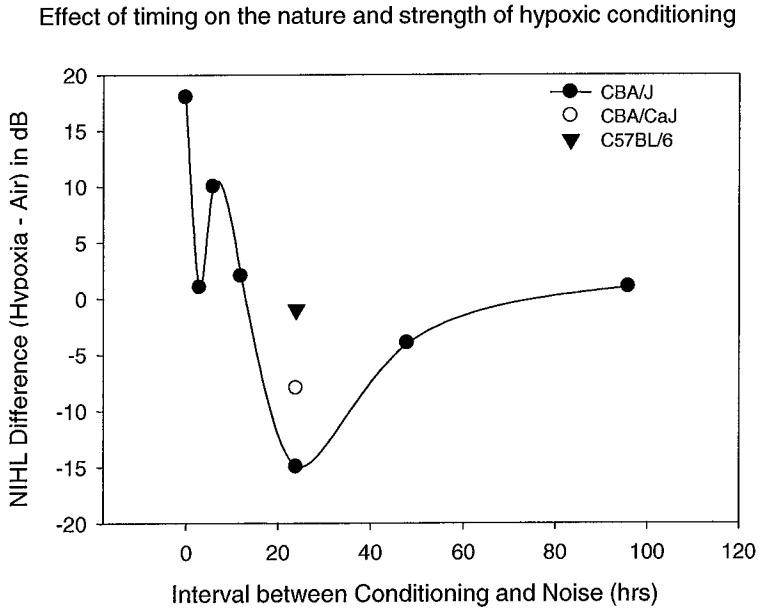

Figure 1: The effect of timing on the nature of hypoxic conditioning in CBA/J mice. Note the C57BL/6 displayed no benefit from conditioning

With the continuing advances in molecular and genetic medicine, it is advantageous to establish the number and characteristics of the gene(s) involved in cochlear hypoxic conditioning and other conditioning paradigms. Many genes contribute to deafness (Steel and Bussoli 1999). With the discoveries of each these unique genes there is an ability to learn more about their patterns of expression and their molecular pathophysiology which can pave the wave for gene therapy (Van De Water et al. 1999). The first goal of this project was to examine the effect of hypoxic conditioning on the B6.CAST$^{+\,Ahl}$ mice. This strain was chosen because they have the same genetic background as C57BL/6 with the exception of one gene: B6.CAST^{+Ahl} do not carry the *Ahl* (age-related hearing loss) mutation which imparts both age related hearing loss and acute sensitivity to noise. If these mice do not exhibit a positive effect from conditioning, then the *Ahl* gene can be ruled out as having an effect on the conditioning response.

The second goal was to examine the inheritance pattern of this trait using CBA/J x C57BL/6 F1 mice. This experiment is a first step in determining the number of genes that play a role in hypoxic conditioning. The outcome is expected to support one of the three possibilities: 1) that the hybrids are capable of conditioning which would be consistent with one single dominant gene responsible for the response, 2) that the hybrids are unable to condition, consistent with involvement of a single recessive gene, or 3) that they are intermediate which is consistent with the involvement of multiple genes in the conditioning response.

Materials and Methods

Animals

This study included a total of 32 mice of both sexes aged 4 months (± 2 weeks) at the time of the noise exposure. The B6.CAST + *Ahl* (n=16), CBA/J, and C57BL/6 mice derived

from stock obtained from the Jackson Laboratories (Bar Harbor, Maine). The CBA/J x C57BL/6 F1 hybrids (n=16) were bred in the Central Institute for the Deaf Bioresource Facilities. All animals were housed on a 12:12 light:dark cycle with food and water available *ad libitum.* Procedures were approved by the Animal Care and Use Committees at CID and Washington University.

Noise Exposure

Noise exposures and auditory brainstem response recordings (ABRs) were performed in a foam-lined, double-walled soundproof test booth (Industrial Acoustics, Bronx, NY). The noise exposure apparatus consisted of a 21 x 21 x 11 cm wire cage mounted on a pedestal inserted into a B&K 3921 turntable which rotated at a rate of one revolution/80s to ensure a uniform sound field. Four Motorola KSN1020A piezo ceramic speakers were positioned on the 42 x 42 cm metal bar frame that was surrounding the cage. The opposing speakers were oriented concentrically, parallel to the cage, and driven by separate channels of a Crown D150A power amplifier. Noise was generated by General Radio 1310 generators and band passed at 4.0-45.0 kHz by Krohn-Hite 3550 filters. B6.CAST$^{+\,Ahl}$ mice were exposed in pairs for 30 min and the F1 hybrids were exposed in pairs for 1.5 h at 110 dB SPL. These noise exposure times and levels have previously shown to evoke permanent threshold shifts (PTS) in both strains (Ou et al. 2000, our own data). Measurements at the center of the cage indicated that the levels ranged from 110 to 113 dB SPL.

Hypoxia and Room Air Treatment

Based on the data of CBA/J mice (figure1), a 24 hour time interval between hypoxia and noise exposure appears to be the point of maximum conditioning by hypoxia. Therefore, all hypoxia and room air treatment were administered 24 hours prior to noise exposure. 16 mice (8

B6.CAST$^{+\,Ahl}$ and 8 F1 hybrids) were treated with a 4 hour hypoxic conditioning period. For the hypoxia treatment, the no more than 4 mice were placed in a 63 x 48 x 22 cm chamber having an inlet hose on one end and a small vent on the opposite end. The hose led to a gas bottle/flow regulator assemble that supplied a mixture of 8% oxygen/92% nitrogen into the chamber at a rate of 2.0 l/min. 16 control mice (8 B6. CAST$^{+\,Ahl}$ and 8 F1 hybrids) were supplied with normal room air using the same duration and rate.

ABR Recording

ABR recordings were recorded were performed before noise exposure and 14 days after exposure. Animals were anesthetized (80 mg/kg ketamine, 15 mg/kg xylazine, IP) and then placed on a thermostatic controlled water heating pad. The core body temperature was maintained at 37.5 ± 1.0° using a rectal probe (YSI 73A). Platinum needle electrodes were inserted subcutaneously behind the right pinna (non-inverting), at the vertex (inverting), and in the back (ground). The left pinna was closed off with a small clamp to ensure testing of the right ear. Electrodes were led to a Grass P15 differential amplifier (100-10,000 Hz x 100), then to a custom amplifier providing another x 1000 gain, then digitized at 30 Hz using a Cambridge Electronic Design Micro 1401 in conjunction with SIGNAL™ and custom signal averaging software operating on a 120 MHz Pentium PC. A Wavetek Model 148 oscillator generated a sine wave stimulus with a 5 ms total duration, including 1 ms rise/fall times. The stimulus was amplified by a Crown D150A power amplifier and output to another KSN1020A piezo ceramic speaker located 7 cm directly lateral to the right pinna. Toneburst stimuli were presented in the calibrated sound field at the frequencies 5, 10, 20, 28.3, and 40 kHz. Stimuli were presented 1000 times at 20/s. The minimum SPL that evoked a response (visual detection of short latency negative wave) was determined at each frequency using a 5 dB minimum step size.

Histology

After the final ABR testing, the cochleas were taken for histological evaluation. Animals were injected with an overdose of sodium pentobarbital (60mg/kg IP) and perfused transcardially with cold 2.0 % paraformaldehyde/2.0 % glutaraldehyde in 0.1 M phosphate buffer. Each cochlea was isolated and immersed in same fixative. The stapes was removed, the lateral vestibular canal was notched, and a small hole was made at the apex of the cochlear capsule. To ensure complete infiltration of the cochlea with fixative, a transfer pipette was used to gently circulate fixative throughout the cochlea. The cochleas were then stored at 4° C for further evaluation. Histological analyses are still in progress.

Statistical Analysis

Pre and post noise exposure ABR threshold values were averaged across animals for each frequency tested (Figure 2 and 3). Averaged permanent threshold shifts (as measured two weeks after noise exposure) between hypoxic conditioned and control groups were analyzed using a two-way analysis of variance (ANOVA). $\alpha = 0.05$ (Figure 4 and 5) For each strain, the average overall permanent threshold difference between hypoxic conditioned and control groups were calculated and plotted (Figure 6)

Results

Initial ABR thresholds for the B6.CAST$^{+\,Ahl}$ and CBA/J x C57BL6 F1 hybrids were within the normal limits based on data obtained by Ohlemiller et al. (2000b) Figures 2 and 3 show average pre and post noise exposure thresholds for both strains. Post noise exposure permanent threshold shifts revealed no significant difference between groups treated with hypoxia 24 hr prior to noise and the group treated with room air 24 hour prior to noise exposure

in either of the two strains (Figure 4 and 5). Figure 6 displays the efficacy of the conditioning response for all the strains at the 24 hour time interval between conditioning and noise exposure.

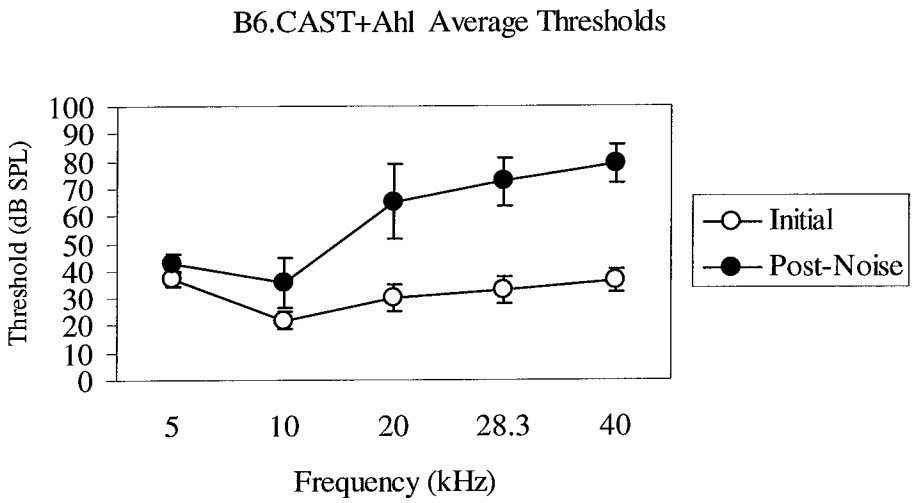

Figure 2: Average ABR Thresholds for B6.CAST $^{+Ahl}$

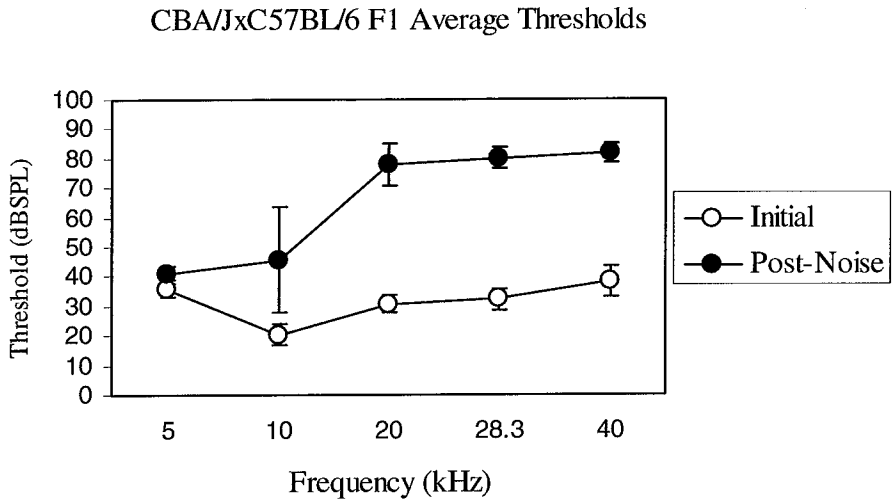

Figure 3: Average ABR Thresholds for CBA/JxC57BL/6 F1

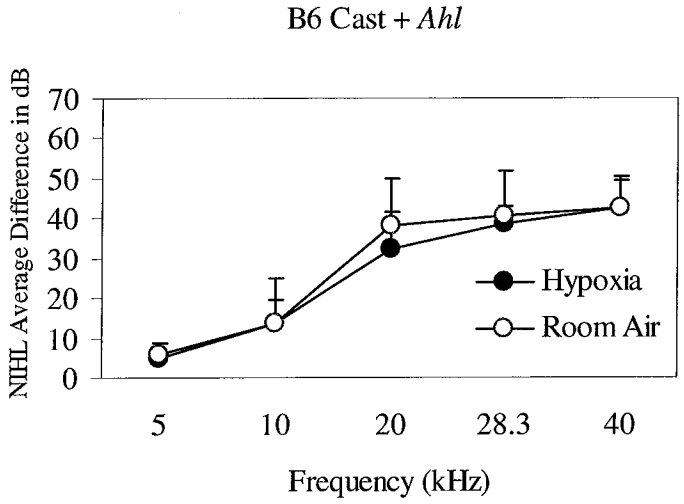

Figure 4: Comparison of average difference in permanent threshold shift between the B6.CAST +Ahl control and hypoxic conditioned groups

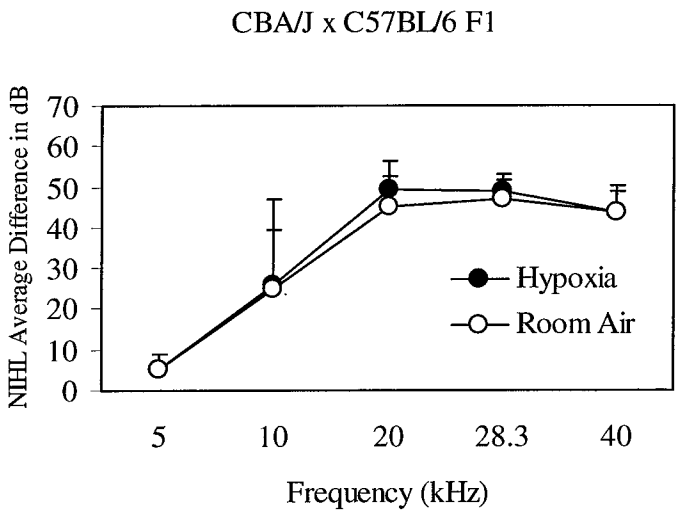

Figure 5: Comparison of average difference in permanent threshold shift between the CBA/JxC57BL/6 F1 control and hypoxic conditioned groups

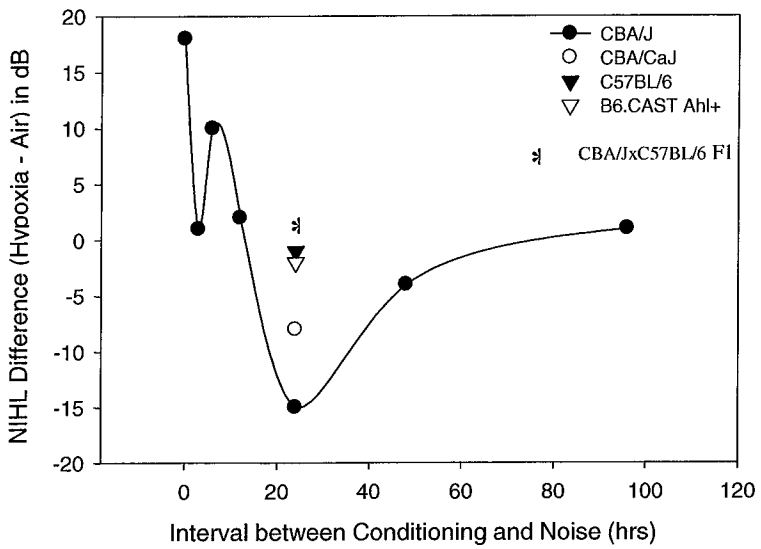

Figure 6: Comparison of the efficacy of the conditioning response at the 24 hour interval between conditioning and noise exposure for different strains of mice. The C57BL/6, B6.CAST +Ahl, and the CBA/JxC57BL6 F1 lack the protective benefit from conditioning that the CBA/J displays at this interval.

Discussion

The B6.CAST^{+Ahl} results indicate that the *Ahl* mutation does not affect conditioning by hypoxia. The results of the F1 hybrids are consistent with involvement of a single recessive gene, although they do not rule out other possibilities. Further studies involving the backcross of the F1s to these two strains need to be performed in order to confirm the involvement of this single recessive allele. If this is true, backcrossing the F1s to CBA/J would be expected to yield a mix of mice in which 50% are capable of conditioning and backcrossing the F1s to C57BL/6 mice should not yield any mice that can be conditioned. Confirming the number and characteristics of the gene(s) involved in the conditioning response to hypoxia may guide the work of molecular biologists in identifying the most important gene(s) and their products.

The result of the study by Ohlemiller et al. (2003) and the present study show that there exists an innate protective mechanism activated during mild stress that is influenced by genetic

expression. Finding the gene(s) and other cellular mechanisms involved in protection may promote development of pharmacological approaches for enhancing these pathways prior to noise exposure.

Involvement of ROS/RNS in noise injury and protection by conditioning

Noise-induced cochlear injury can occur two ways: mechanically with a high level, short duration exposure exceeding 140 dB or metabolically in which exposure levels lie between 90-140 dB (Clark and Bohne 1999). Much research has been performed to discover and define the mechanisms underlying metabolic cochlear injury. This research has indicated that NIHL, ototoxicity, and presbycusis as well as other degenerative diseases such as Alzheimer's, Parkinson's, and Huntington's disease all involve oxidative stress (Kopke et al. 1999, Van De Water 1999, Waters 1999). Cochlear cells are capable of producing both toxins and protective proteins and enzymes that maintain the appropriate homeostasis for cellular survival. Oxidative stress occurs when there is an imbalance between reactive oxygen and nitrogen species (ROS and RNS) and the antioxidants that regulate them. ROS/RNS are free radicals produced during normal metabolism and can serve in regulatory mechanisms. Insufficient regulation of ROS/RNS initiates a chain reaction that causes cellular damage and may lead to apoptosis (Huang et al. 2000, Evans and Halliwell, 1999, Kopke et al. 1999, Waters 1999). Acute and chronic noise exposure activates a cascade of events involving ROS/RNS that eventually leads to destruction of cochlear hair cells, stria vascularis, and neurons. It is important to document the production and regulation of ROS/RNS in the cochlea to various types and degrees of stressors, in order to understand the phenomenon of noise tolerance by conditioning.

Increased production of ROS/RNS and related injury has been demonstrated to take place after numerous stressors such as ototoxins (Kopke et al. 1999), cochlear ischemia-reperfusion

(Ohlemiller et al. 1999a), hypoxia (Chandel et al. 2000, Huang et al. 2000), and noise exposure (Yamane et al. 1995, Ohlemiller et al. 1999c). Acoustic overstimulation of the cochlear hair cells leads to deprivation of oxygenation and vascular supply which causes a generation of ROS (Chen 2002, Lamm and Arnold 2000, Lamm and Arnold 1999, Yamasoba et al. 1999, Hatch et al. 1991). Noise injury also may involve glutamate excitotoxicity, which results from overstimulation of the inner hair cells (IHC). Glutamate is a major neurotransmitter between the IHC and afferent cochlear nerve fiber. In noise, there is an excessive synaptic glutamate concentration, which leads to increased intracellular Ca^{2+} and production of free radicals. Intracellular Ca^{2+} continues to rise as ROS damages proteins that regulate Ca^{2+} homeostasis. This major disruption of cell metabolism due to ROS/RNS production causes further damage by 1) breaking strands of cell DNA and making base modifications, 2) inhibiting antioxidant enzymes, 3) damaging lipid membrane by lipid peroxidation, 4) damaging cellular ion channels, 5) changing regulation of transcription factors and genetic expression, and 5) initiation of apoptosis (Kopke et al. 2002, Evans and Halliwell 1999).

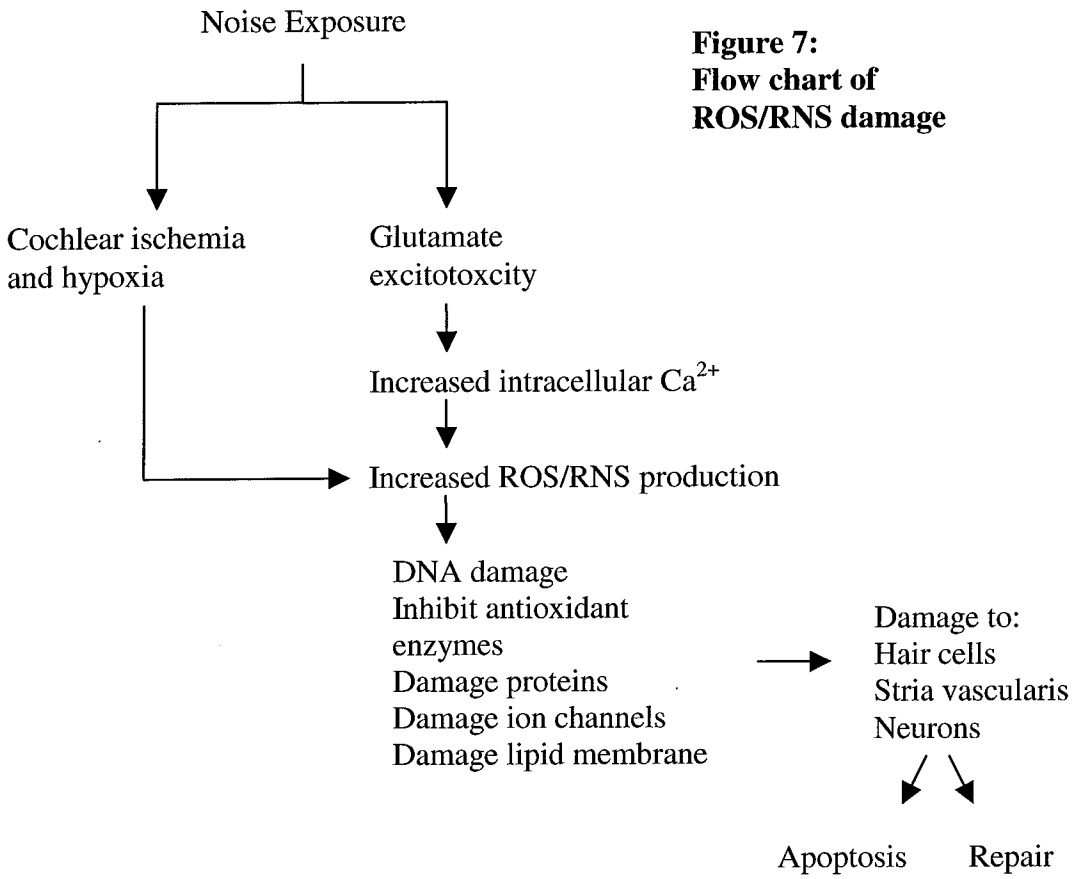

Figure 7:
Flow chart of
ROS/RNS damage

Cells defense against oxidative stress involves upregulating the antioxidant system, which utilizes free radical scavengers to maintain the appropriate balance of cellular proteins (Lu and Liu 2001). Free radical scavengers include superoxide dismutase (SOD), catalase, and glutathione (GSH). However, these antioxidants deplete rapidly and have a short term protective effect. Tolerance of lethal stress by conditioning extends beyond antioxidant production to changes in genetic expression. It is hypothesized that the increased ROS levels inhibit the degradation of the hypoxia-inducible factor 1α (HIF1α) protein (Chandel 2000). HIF1α promotes the expression of genes that encode proteins and other products that provide cellular protection from stress such as the heat shock proteins (HSPs), HIF1α, TNFα, VEGF, Bcl-2, Bcl-xL, glycolytic enzymes, and glucose transporters (Jones and Bergeron 2001, Chandel et al. 2000,

Chien et al. 2000, Gidday et al. 1999, Wang and Liberman 1999, Waters 1999, Yu et al. 1999, Iyer et al. 1998, Feuerstein et al. 1997, Myers et al. 1992). Figure 5 outlines the cellular response to conditioning by mild hypoxia and the role of the protective proteins. During lethal stress activation of these products may occur too slowly. By the time that these proteins are ready to serve and protect the valuable cochlear hair cells, the cells may already be damaged by ROS/RNS. However during conditioning with mild stressors such as hypoxia these genes and proteins may be upregulated and given time to prepare for the upcoming noise exposure. By inducing these protective mechanisms before noise exposure, the intracellular defense is strengthened and ready to regulate the production of ROS/RNS. The early phase of protection which occurs during and several minutes after noise exposure reflect the changes in cellular metabolism through antioxidants. The delayed phase of protection which is invoked by conditioning sustains several hours to a couple of days. This phase may reflect changes in the genetic expression (Omata et al. 2002).

The temporal pattern of the hypoxic conditioning response (figure 6) might reflect the pattern of ROS generation and regulation. Mild hypoxia does evoke the innate mechanisms that protect from noise injury. Because both major and mild stress may both involve oxidative stress, it is important to give the system sufficient time between conditioning and noise exposure for this defense mechanism to reach its fullest potential. When the interval between hypoxic conditioning and noise exposure is too short, it is possible that the ROS/RNS production overlap exacerbating cellular damage (figure 7). However, when enough time is given for conditioning to evoke the changes in genetic expressions ROS/RNS production during noise exposure can be better regulated (figure 8).

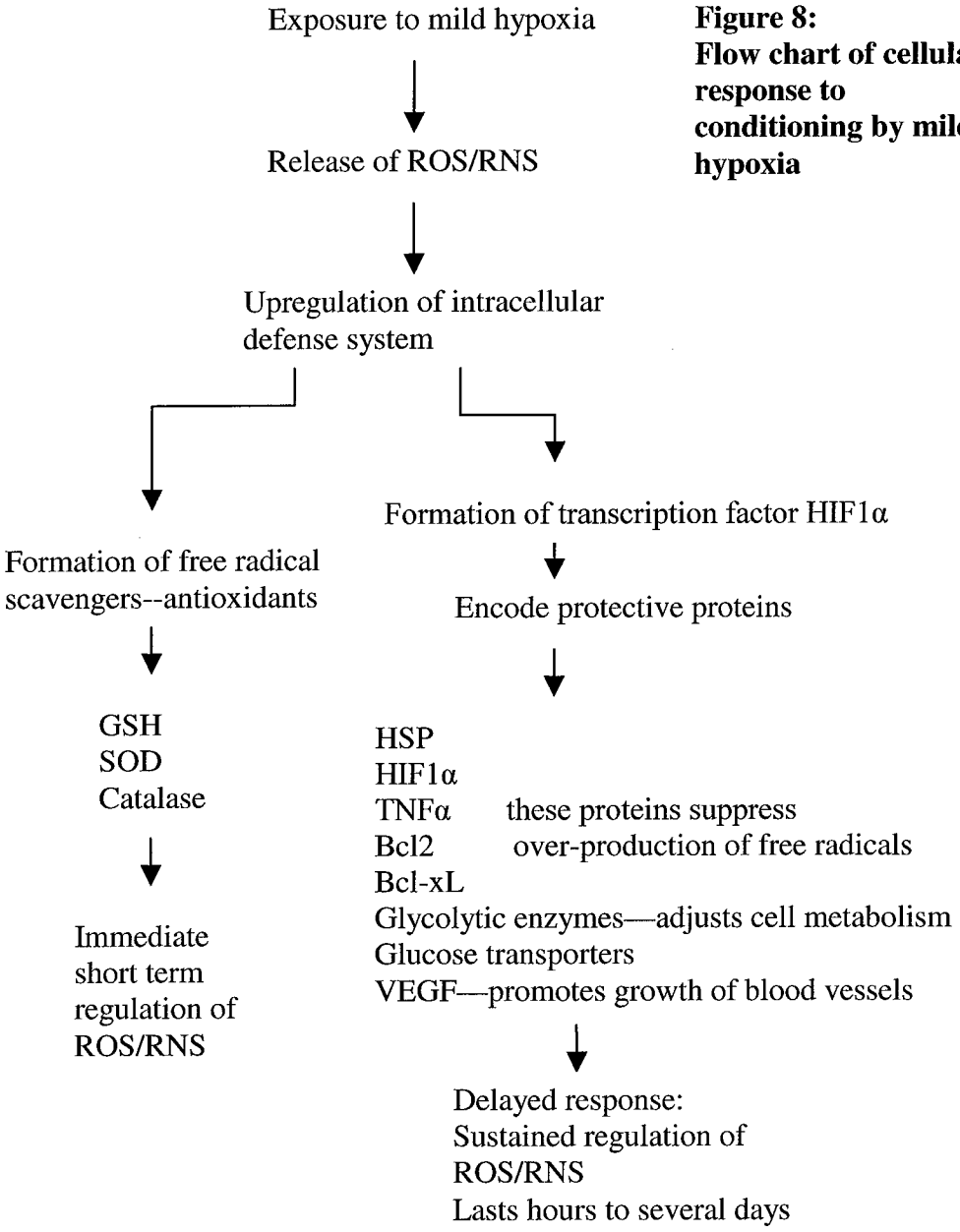

Figure 8:
Flow chart of cellular response to conditioning by mild hypoxia

Conclusion

The protective effects of noise tolerance by hypoxic conditioning is suggested to be mediated by the stress-induced changes in gene expression (refer to figure 6). It would be of great use to determine the number and characteristics of the gene(s) involved in this conditioning response. This study concludes that a single recessive gene may play an important role in this response. Further studies involving the offspring of the F1s backcrossed to their parent strains need to be conducted to confirm this hypothesis.

Further research is also necessary to define the underlying molecular mechanisms of the conditioning phenomenon. Using applications of antioxidants and other ROS/RNS inhibitors during hypoxic conditioning may validate the hypothesis that the conditioning mechanisms are dependant on ROS/RNS involvement. If ROS/RNS production is inhibited, it is supposed that the conditioning response would disappear. Discovering the molecular and genetic mechanism of disorders such as NIHL will allow new developments in gene therapy and therapeutic intervention. Perhaps by pharmacologically modulating a specific gene expression and enhancing protective molecular mechanisms noise induced cochlear injury can be avoided.

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