2002

The effects of noise exposure on nitric oxide synthase knockout mice

Emily Timmermann

THE EFFECTS OF NOISE EXPOSURE ON NITRIC OXIDE SYNTHASE KNOCKOUT MICE

by

Emily Timmermann

An independent study submitted in partial fulfillment of
the requirements for the degree of

Master of Science in Speech and Hearing

Emphasis in Audiology

Washington University
Department of Speech and Hearing

May 24, 2002

Approved by: Brian Faddis, Ph.D. and Kevin Ohlemiller, Ph.D., Independent Study Advisors

INTRODUCTION

Hearing loss is a prevalent and most times irreversible disability affecting a growing segment of our society. Noise-induced hearing loss (NIHL), resulting from chronic moderate noise exposure or acute noise toxicity, is the second leading type of sensorineural hearing loss today (Rabinowitz, 2000). Americans are bombarded with noise all the time, and this noise is often unavoidable. Exposure to noise causes the hair cells in the inner ear to degenerate, leading to a permanent hearing loss (Bahadori and Bohne, 1993). Because of the amount exposure to occupational and recreational noise, increasing life span, and increasing age of the United States work force, NIHL will to continue to effect more and more people unless some type of prevention or treatment techniques are developed.

In order to develop prevention and treatment techniques, we must first understand the cellular and molecular mechanisms that mediate NIHL. One mechanism that has been studied by many researchers is the role of free oxygen radicals. Free radicals can be useful if present in low levels, but they become harmful if present in large concentrations (Ohlemiller, 2001). Increased production of free radicals has been demonstrated following noise exposure (Yamane et al., 1995; Ohlemiller et al., 1999), cochlear ischemia (Seidman et al., 1991; Seidman and Quirk, 1991), and application of ototoxic agents (Kopke et al., 1997; Hirose et al., 1997). The production of antioxidants serves as a safeguard, protecting the cochlea from free radical damage. Research has revealed that increasing antioxidant defenses serves to protect the cochlea from further damage (Yamasoba et al., 1998; Seidman et al., 1993; Seidman and Quirk, 1991; Seidman et al., 1991).

Another mechanism that a lot of research has focused on is nitric oxide (NO). NO is a molecule involved in many different biochemical cascades throughout all the cells of the body,

including those of the cochlea. NO plays a role in normal cochlear functioning. It is formed by the enzyme nitric oxide synthase (NOS) and plays a role in many physiologic and pathophysiologic processes (Bentz et al., 2000). When produced in large amounts, NO can be toxic. Some of the most studied roles of NO include modulation of neurotransmission, blood flow regulation, and induction of cytotoxity. Each of these roles can impact the physiology and/or pathophysiology of the mammalian cochlea (Fessenden et al., 1994).

Nitric oxide is synthesized by three isoforms of nitric oxide synthase. Each of these isoforms is characterized by its original characteristics and differs in location, regulation, and physiological function. The three NOS isoforms are: (1) neuronal NOS (nNOS or NOS 1); (2) inducible NOS (iNOS or NOS 2); and (3) endothelial NOS (eNOS or NOS 3) (Fessenden and Schacht, 1998). NOS 1, one of two constitutive isoforms, is found in certain neurons and in skeletal muscle (Huang, 2000). NOS 1 is calcium-dependent and primarily acts as a neurotransmitter or neuromodulator (Fessenden and Schacht, 1998). NOS 2 is not constitutively present. It can be induced in macrophages and in vascular smooth muscle by a variety of signals, including trauma and inflammatory cytokines (Huang, 2000). Once NOS 2 is induced, it is continually active and acts as a cytotoxin of the immune response of macrophages. NOS 2 is calcium-independent (Fessenden and Schacht, 1998). The third isoform, NOS 3, is found in endothelial cells (Bentz et al., 2000). Like NOS 1, NOS 3 is calcium-dependent and constitutive. When NOS 3 is activated, NO is formed and diffuses rapidly to the smooth muscle layer of the blood vessels. Once this happens, the cells relax, increasing the diameter of the blood vessel, and thus increasing blood flow (Fessenden and Schacht, 1998).

Much research has been conducted over the past decade localizing NOS isoforms to various structures in the inner ear. Localization studies in the guinea pig cochlea have found

NOS 1 in spiral ganglion cells and cells of the spiral ligament (Franz et al., 1996; Gosepath et al., 1997; Michel et al., 1999). NOS 1 has also been localized to the modiolus (Michel et al., 1999; Gosepath et al., 1997) and the stria vascularis (Gosepath et al., 1997). Inner hair cells (IHCs) and outer hair cells (OHCs) have also shown evidence of NOS 1 (Franz et al., 1996; Gosepath et al., 1997). Research conducted by Popa and colleagues (2001) with human cochleae have localized NOS 1 to spiral ganglion cells, nerve fibers, OHCs, the stria vascularis, and supporting cells of the organ of Corti (Hensen's, Dieters', and Claudius' cells).

NOS 2 is an isoform that is not regularly found in various structures in the cochlea during these localization studies. NOS 2 is the inducible form of nitric oxide, it must be induced or 'turned on' by an appropriate stimulus before it can be found in the inner ear. A localization study conducted by Gosepath and colleagues (1997) failed to find NOS 2 present in any structure of the guinea pig cochlea. These researchers explain this by suggesting that the isoform NOS 2 is not expressed under normal physiologic conditions (Gosepath et al., 1997). According to Forestermann and colleagues (1995), NOS 2 can be expressed in virtually any cell type as long as it is adequately stimulated.

Localization studies have also localized NOS 3 to many structures throughout the cochlea. Studies with guinea pig cochlea have found NOS 3 in both IHCs and OHCs (Franz et al., 1996; Heinrich et al., 1998). NOS 3 has also been localized to the spiral ligament, cells of the organ of Corti, and nerve fibers (Michel et al., 1999). Spiral ganglion cells (Michel et al., 1999; Gosepath et al., 1997) and the stria vascularis (Michel et al., 1999; Heinrich et al., 1998) have also shown evidence of NOS 3. Localization studies of NOS 3 with human cochleae have found this isoform present in OHCs, spiral ganglion cells, and nerve fibers (Popa et al., 2001).

All the major isoforms of nitric oxide synthase are present in the cochlea, suggesting that nitric oxide plays multiple roles in the inner ear. NOS 1 and NOS 3 are both dependent on calcium for functioning, and it is possible that one role these isoforms play in the inner ear is intracellular calcium regulation (Gosepath et al., 1997). Intracellular calcium plays an important role in the initiation of the release of neurotransmitter. If NO restricts the amount of intracellular calcium present, there could be a reduction in the amount of neurotransmitter that is released at the synapses of the auditory nerve and this could affect auditory signal transmission (Gosepath et al., 1997). NOS 1 and NOS 3 isoforms are also expressed in OHCs. Intracellular calcium regulates the motile response of OHCs. It is possible that NO plays a role in the motile response of OHCs by regulating the concentration of free calcium ions in the OHCs. An increase of intracellular calcium leads to a contraction and stiffness of the OHCs. This process may serve to protect the hair cells from noise trauma (Gosepath et al., 1997; Popa et al., 2001). These two examples illustrate how NO could affect the functioning of the inner ear by altering the concentration of intracellular calcium.

Another major role of NO involves the regulation of blood flow throughout the cochlea. NOS 3 plays a major role in this regulation because of its presence in endothelial cells throughout the inner ear. Blood flow in the cochlea increases or decreases by changing the diameter of the blood vessels (Fessenden and Schacht, 1998). Cochlear blood vessels, in general, have a basal vasoconstrictor tone. When this tone is altered, the blood vessel diameter and blood flow change. Nitric oxide is produced in the blood vessels and is basal vasodilatory in nature. Therefore, cochlear blood flow can be rapidly regulated because of the interaction between these constricting and relaxing factors throughout the blood vessels. This indicates that NO is one of the factors contributing to blood vessel tone and, therefore, blood flow (Brechtelsbauer et al.,

1994). A study conducted by Brechtelsbauer and colleagues (1994) looked at the role NO plays in the regulation of blood flow in the guinea pig cochlea. During this experiment, an inhibitor of NOS was applied to the round window of the cochlea. This resulted in a reduction of cochlear blood flow, indicating that NO is produced in the blood vessels of the cochlea and contributes to the regulation of cochlear blood flow.

NO has been suggested to contribute to the functioning of the inner ear in other ways as well. NOS 1 is present in the supporting cells, Deiters' and Hensen's cells. When exposed to high intensity sounds, these supporting cells contract to protect the OHCs. Therefore, NO may play a role in this contraction to protect hearing sensitivity during noise exposure (Popa et al., 2001). NOS 3 may play a role in synaptic transmission because of its presence in nerve cells (Popa et al., 2001).

Not only does NO work toward the proper physiologic functioning of the inner ear, but NO could have pathophysiologic consequences as well. Nitric oxide is a cytotoxin, meaning it can poison, or cause harm to, cells of the inner ear. An overstimulation of NOS-containing neurons will lead to an overproduction of NO and consequently the death of surrounding neurons (Fessenden et al., 1994).

The purpose of this study was to look at the role of NOS 1, NOS 2, and NOS 3 in the mouse cochlea. A previous study with mice genetically deficient for inducible nitric oxide synthase (NOS 2 knockout) found no difference in susceptibility to NIHL. Because the NOS 2 study was negative and because of the suspected functional redundancy or overlap of various functions of NOS isoforms throughout the inner ear, this study focused on mice with two targeted deletions (double knockouts): NOS 1/NOS 2 and NOS 2/NOS 3. Using this combination of double knockout mice will allow us to look more specifically at the roles NOS 1

and NOS 3 play in NIHL without the possibility of the functional redundancy of NOS 2 masking the role of the other NOS isoforms. It is hypothesized that mice genetically deficient for both NOS 1 and NOS 2 will exhibit elevated initial thresholds because of impaired synaptic transmission. It is also hypothesized that mice genetically deficient for both NOS 2 and NOS 3 will show greater temporary and permanent threshold shifts following the noise exposure because there will be a decrease in the blood flow throughout the cochlea. Alternatively, the mouse cochlea may be protected from NIHL because of a decrease in NO produced by NOS 1, NOS 2, or NOS 3.

MATERIALS AND METHODS

Animals

Forty-two mice, both male and female, were used in this study. The animals were 9-11 weeks old at the time of noise exposure. Mice with targeted deletions of the genes coding for NOS 1, NOS 2, and NOS 3 were derived from stock obtained from Jackson Laboratories (Bar Harbor, Maine). NOS1/NOS 2 and NOS 2/NOS 3 double knockout mice and wildtype counterparts were used. The NOS 1 animals were derived on a B6.129 background and the NOS 2 and NOS 3 animals on a C57BL/6 background. The animals were housed in the Bioresource Facilities at Central Institute for the Deaf (CID). They were housed on a 12:12 light:dark cycle with food and water available *ad libitum*. The Animal Care and Use Committees at CID and Washington University approved all procedures.

Noise Exposure

Both the noise exposures and auditory brainstem response recordings (ABRs) were conducted in a foam-lined, double-walled acoustic isolation booth. The noise exposure cage consisted of a 21 x 21 x 11 cm wire cage in a B&K 3921 turntable which rotated at a rate of one revolution/80 seconds. Four separate speakers were positioned around the cage to help ensure uniform sound delivery. Noise was generated by General Radio 1310 generators and bandpassed at 4-45 kHz by Krohn-Hite 3550 filters. The animals were exposed in pairs at 110 dB SPL for 15 minutes.

ABR Recordings

Auditory brainstem responses were recorded immediately prior to noise exposure, 24 hours after exposure, and 14 days after exposure, for each animal. The animals were anesthetized with a mixture of 80 mg/kg ketamine and 15 mg/kg xylazine, given intraperitoneally. The animals were placed on a heating pad and their body temperature was maintained at 37.5 +/- 1.0° C using a rectal probe. Needle electrodes were inserted behind the right pinna (active), at the vertex (inverting), and at the back (ground). The electrodes were led to a Grass P15 differential amplifier (100-10,000 Hz, x100), then to a custom broadband amplifier (0.1-10,000 Hz, x1000), then digitized at 30 kHz using a Cambridge Electronic Design Micro1401 in conjunction with SIGNAL™ and custom signal averaging software operating on a 120 MHz Pentium PC. A Wavetek Model 148 oscillator generated the sine wave stimulus with a 5 ms total duration, including a 1 ms rise time and 1 ms fall time and was amplified by a Crown D150A power amplifier. The speaker was located 7 cm laterally to the animal's right pinna. Toneburst stimuli were presented in the freefield at frequencies of 5, 10, 20, 28.3, and 40 kHz,

and each level 1000 times at 20/second. Five decibels descending minimum step sizes were used at each frequency to visually determine the presence of a response.

Tissue Processing

The animals were euthanized with an overdose of pentobarbital (>200mg/kg) and transcardially perfused with 4% paraformaldehyde and 0.05% glutaraldehyde in phosphate buffer. The bullae were dissected and stored in fixative at 4°C for further evaluation.

Statistical Analysis

Values for thresholds were averaged across animals for each frequency tested. The data were analyzed using a one-way analysis of variance (ANOVA) with repeated measures on one variable (frequency) followed by Tukey's pairwise multiple comparison test (SigmaStat, SPSS Science Inc.). In all analyses, $\alpha = 0.05$.

RESULTS

Visual inspection of each group of mice revealed no differences. NOS 1/NOS 2 double knockout mice and NOS 2/NOS 3 double knockout mice did not differ in physical appearance or weight from each other or their wildtype counterparts. The mice also behaved in a similar manner. This was true for both sexes of mice. The double knockout mice exhibited no behavioral or physical phenotype to distinguish them from their wildtype counterparts.

NOS 1/NOS 2

A one-way ANOVA failed to show a significant effect due to group among the initial or permanent thresholds between wildtype and NOS 1/NOS 2 double knockout mice (Figure 1). Graphical presentation of this data suggests that with increased sample size, the initial thresholds would be statistically greater for the knockout mice compared to their wildtype counterparts.

NOS 2/NOS 3

A one-way ANOVA among the initial threshold values for NOS 2/NOS 3 mice did not show a significant effect due to group, meaning there was no difference between the initial pre-exposure thresholds for the wildtype versus double knockout mice (Figure 2).

One-way ANOVA did show a statistically significant difference due to group ($F=4.831$; $p<0.05$) for the temporary threshold values, indicating that there was a difference in 24-hour post-noise exposure thresholds between wildtype and NOS 2/NOS 3 double knockout mice. The double knockout mice had significantly lower threshold values (i.e. were more protected from noise) than their wildtype counterparts.

One-way ANOVA also revealed a statistically significant difference due to group ($F=19.801$; $p<0.001$) for the permanent threshold values. This indicates that there was a significant difference in permanent threshold values between wildtype and NOS 2/NOS 3 double knockout mice. The double knockout mice exhibited lower permanent threshold values (i.e. were more protected from noise) than their wildtype counterparts. There was also a statistically significant interaction between group and frequency ($p<0.05$) indicating that the difference was localized specifically to responses at 10 and 20 kHz. This indicates that the difference between

wildtype and NOS 2/NOS 3 double knockout mice thresholds was more pronounced at these frequencies.

Another way to assess changes in ABR thresholds is to examine difference scores between two measures. A one-way ANOVA comparing the amount of shift from initial threshold to permanent threshold after the noise exposure revealed a statistically significant effect due to group ($F=8.212$; $p<0.05$). The noise exposure caused a permanent threshold shift that was statistically different between wildtype and NOS 2/NOS 3 double knockout mice. The double knockout mice exhibited a permanent threshold shift that was significantly less than their wildtype counterparts.

Histopathology

Cochlear tissues were fixed and archived. These tissues were not examined as part of the present study.

DISCUSSION

Overall, no significant effects were found among the NOS 1/NOS 2 double knockout animals. It was hypothesized that the NOS 1/NOS 2 double knockout animals would have elevated initial thresholds because of the possible decrease in synaptic transmission. As a whole, the graph suggests that the NOS 1/NOS 2 double knockout animals did have greater initial thresholds than the wildtype animals; however, the difference was not found to be significant. Graphical presentation of the data suggests that with increased sample size, the initial thresholds for the double knockout mice would be significantly elevated.

Prior to the noise exposure, both NOS 2/NOS 3 double knockout mice and their wildtype counterparts exhibited similar thresholds. However, following the noise exposure, the NOS 2/NOS 3 double knockout animals showed less of a temporary threshold shift than the wildtype counterparts. Two weeks following the noise exposure, the NOS 2/NOS 3 double knockout animals had less of a permanent threshold shift and showed more recovery from the noise exposure, especially at 10 and 20 kHz. These results reveal that NO derived by NOS 2 or NOS 3 at least partially mediates the damage that causes both temporary and permanent threshold shifts.

In general, the NOS 2/NOS 3 wildtype and double knockout animals showed less overall variability in threshold data and exhibited more significant recovery from temporary threshold shift compared to the NOS 1/NOS 2 wildtype and double knockout animals. These differences between the two overall groups of animals may be explained by the mixed background of the NOS 1/NOS 2 animals as compared to the NOS 2/NOS 3 animals which came from a C57BL/6 background.

Research completed by Nuttall and colleagues (2001) revealed results that were contradictory to the results obtained in the present study. They compared auditory responses of NOS 1 knockout mice with control mice after significant noise exposure. It was found that NOS 1 knockout mice exhibited less of a permanent threshold shift than the control mice. The control mice also had greater hair cell losses than the knockout mice. These results suggest that NOS 1 knockout mice are resistant to NIHL.

The current results do not support Nuttall's findings; however, some differences between the set up of the two experiments may possibly explain these different findings. First, the total amount of noise exposure the animals in the two studies received was very different. The animals from the current study only received on single exposure of 15 minutes of broadband

noise (4-45 kHz) at 110 dB SPL. On the other hand, the animals in Nuttall's experiment received exposure to broadband noise (4-32 kHz) for 3 hours/day for five consecutive days at 125 dB A. Also, because NOS 2 is the inducible form, and mice genetically deficient for this gene were used in the current study, it may be possible that greater noise exposure would be more likely to reveal an effect with this type of knockout animal. This difference in the total amount of noise exposure between experiments could possibly explain the different results. Second, a difference in age and/or background of the mice between experiments could also result in different findings. Lastly, the fact that Nuttall did not use any double knockout mice may also explain a difference. Nuttall used only NOS 1 knockout mice and the current study examined double knockout mice (NOS 1/NOS 2 and NOS 2/NOS 3). It is possible that with double knockout animals the deletion of one gene counteracts the deletion of the second gene, if the molecules have opposing actions.

Nitric oxide is a neutral free radical. Free radicals, including nitric oxide, play a role in noise-induced hearing loss. Like NO, free radicals can be harmful when present in high concentrations. If free radicals are not eliminated by antioxidants, a significant amount of damage to the sensory cells of the inner ear could result (Yamasoba et al., 1998). Ohlemiller and colleagues (1999) have shown an increase in the level of free radicals in mouse cochlea 1-2 hours following a permanent threshold shift-inducing noise exposure. This level of free radicals remained elevated during the few hours following the noise exposure. Yamane and colleagues (1995) also suggest an increase of production of free radicals in the cochlea following noise exposure in the guinea pig. An increase in the amount of free radicals throughout the cochlea after noise exposure, as shown in these two experiments, can lead to damage of the inner ear.

Antioxidants present throughout the cochlea serve to protect the inner ear from this damage caused by increased levels of free radicals. Research has revealed that temporarily increasing antioxidant defenses could protect the inner ear from noise-induced damage. For example, Seidman and colleagues (1993) treated rats to allopurinol and superoxide dismutase, two antioxidants that serve to scavenge or block the formation of free radicals. Rats that were administered these drugs exhibited thresholds that were better than the thresholds of control animals when exposed to the same amount of threshold-shifting noise. This experiment reveals that increasing antioxidant defenses helps to preserve cochlear sensitivity when exposed to high levels of noise.

Hu et al. (1997) present another example of the protective effects of antioxidants. These researchers treated the round window of chinchillas' right ears with R-phenylisopropyladenosine (R-PIA), an antioxidant enzyme. Saline was applied to the round window of the left ears. The animals were then noise exposed. Immediately following the noise exposure there was no difference in the evoked potential thresholds or distortion product otoacoustic emissions between ears. However, after four days the R-PIA treated ears showed significantly more threshold recovery than the control ears, and 20 days following noise exposure the R-PIA ears exhibited less of a permanent threshold shift and less outer hair cell loss than the control ears. This study indicates that this particular antioxidant facilitates outer hair cell recovery in the inner ear following noise exposure.

Noise-induced hearing loss is a common disability affecting many people. Research with animal models can help us understand the cellular and molecular mechanisms that impact NIHL so that we can develop prevention and treatment techniques. Understanding the relationship between nitric oxide and NIHL will put us one step closer. The current study revealed that mice

genetically deficient of the NOS 2 and NOS 3 genes were protected from a hearing loss following a significant noise exposure, indicating that one or both of these genes may be responsible for producing nitric oxide that damages the inner ear when exposed to harmful levels of noise. These results can help us learn a little more about what damages that cochlea during noise exposure and one day lead us to prevention and treatment for noise-induced hearing loss.

REFERENCES:

Bahadori, R.S. and Bohne, B.A. (1993). Adverse-effects of noise on hearing. *American Family Physician*, 47(5): 1219-1226.

Bentz, B.G., Simmons, R.L., Haines, G.K., and Radosevich, J.A. (2000). The yin and yang of nitric oxide: Reflections on the physiology and pathophysiology of NO. *Head and Neck*: 71-83.

Brechtelsbauer, P.B., Nuttal, A.L., and Miller, J.M. (1994). Basal nitric oxide production in regulation of cochlear blood flow. *Hearing Research*, 77: 38-42.

Fessenden, J.D., Coling, D.E., and Schacht, J. (1994). Detection and characterization of nitric oxide synthase in the mammalian cochlea. *Brain Research*, 668: 9-15.

Fessenden, J.D. and Schacht, J. (1998). The nitric oxide/cyclic GMP pathway: A potential major regulator of cochlear physiology. *Hearing Research*, 118: 168-176.

Forstermann, U., Gath, I., Schwarz, P., Closs, E.I., and Kleinert, H. (1995). Isoforms of nitric oxide synthase: Properties, cellular distribution, and expressional control. *Biochemical Pharmacology*, 50(9): 1321-1332.

Franz, P., Hauser-Kronberger, C., Bock, P., Quint, C., and Baumgartner, W.D. (1996). Localization of nitric oxide synthase I and III in the cochlea. *Acta Oto Laryngologica (Stockh)*, 116: 726-731.

Gosepath, K., Gath, I., Maurer, J., Pollock, J.S., Amedee, R., Forstermann, U., and Mann W. (1997). Characterization of nitric oxide synthase isoforms expressed in different structures of the guinea pig cochlea. *Brain Research*, 747: 26-33.

Heinrich, U.R., Maurer, J., Gosepath, K., and Mann, W. (1998). Immunelectron microscopic localization of nitric oxide synthase III in the guinea pig organ of Corti, *Eur Arch Otorhinolaryngol*, 255: 483-490.

Hirose, K., Hockenbery, D.M., and Rubel, E.W. (1997). Reactive oxygen species in the chick hair cells after gentamicin exposure in vitro. *Hearing Research,* 104: 1-14.

Hu, B.H., Zheng, X.Y., McFadden, S., Kopke, R.D., and Henderson, D. (1997). R-phenylisopropyladenosine attenuates noise-induced hearing loss in the chincilla. *Hearing Research*, 113: 198-206.

Huang, P.L. (2000). Lessons learned from nitric oxide synthase knockout animals. *Seminars in Perinatology*, 24(1): 87-90.

Kopke, R.D., Liu, W., Gabaizadeh, R., Jacono, A., Feghali, J., Spray, D., Garcia, P., Steinman, H., Malgrange, B., Ruben, R.J., Rybak, L., and Van de Water, T.R. (1997). Use of organotypic cultures of Corti's organ to study the protective effects of antioxidant molecules on cisplatin-induced damage of auditory hair cells. *American Journal of Otology,* 18(5): 559-571.

Michel, O., Hess, A., Bloch, W., Stennert, E., Su, J., and Addicks, K. (1999). Localization of the NO/cGMP-pathway in the cochlea of guinea pigs. *Hearing Research*, 133: 1-9.

Nuttall, A., Omelchenko, I., and Xiaorui, S. (2001). Production of NO and noise-induced hearing loss in nNOS knock-out mice. *Association of Research in Otolaryngology Abstracts,* Twenty-fifth Midwinter Meeting, p. 32.

Ohlemiller, K.K., Wright, J.S., and Dugan, L.L. (1999). Early elevation of cochlear reactive oxygen species following noise exposure. *Audiology and Neuro-Otology*, 4: 229-236.

Ohlemiller, K.K. (2001). Oxidative cochlear injury and the limitations of antioxidant therapy. In press.

Popa, R., Anniko, M., Takumida, M., and Arnold, W. (2001). Localization of nitric oxide synthase isoforms in the human cochlea. *Acta Oto Laryngologica*, 121: 454-459.

Rabinowitz, P.M. (2000). Noise-induced hearing loss. *American Family Physician*, 61(9): 2749-2756.

Seidman, M.D., Quirk, W.S., Nuttall, A.L., and Schweitzer, V.G. (1991). The protective effects of allopurinol and superoxide dismutase-polyethylene glycol on ischemic and reperfusion-induced cochlear damage. *Otolaryngology-Head and Neck Surgery,* 105(3): 457-463.

Seidman, M.D. and Quirk, W.S. (1991). The protective effects of tirilated mesylate (U74006F) on ischemic and reperfusion-induced cochlear damage. *Otolaryngology-Head and Neck Surgery,* 105(4): 511-516.

Seidman, M.D., Shivapuja, B.G. and Quirk, W.S. (1993). The protective effects of allopurinol and superoxide dismutase on noise-induced cochlear damage. *Otolaryngology-Head and Neck Surgery,* 109(6): 1052-1056.

Yamane, H., Nakai, Y., Takayama, M., Iguchi, H., Nakagawa, T., and Kojima, A. (1995). Appearance of free radicals in the guinea pig inner ear after noise-induced acoustic trauma. *European Archives of Oto-Rhino-Laryngology,* 252(8): 504-508.

Yamasoba, T., Nuttall, A.L., Harris, C., Raphael, Y., and Miller J.M. (1998). Role of glutathione in protection against noise-induced hearing loss. *Brain Research,* 784: 82-90.

FIGURE LEGENDS

Figure 1. Line graph of ABR thresholds for NOS 1/NOS 2 double knockout mice and their wildtype counterparts. The solid lines are the wildtype thresholds and the dotted lines are the double knockout mice thresholds. The red lines indicate the initial pre-noise exposure data. The blue lines show the temporary threshold values 24 hours after the noise exposure. The green lines show the permanent threshold values two weeks after the noise exposure. No significant differences were found between the wildtype and double knockout mice for any measure.

Figure 2. Line graph of ABR thresholds for NOS 2/NOS 3 double knockout mice and their wildtype counterparts. The solid lines are the wildtype thresholds and the dotted lines are the double knockout mice thresholds. The red lines indicate the initial pre-noise exposure data. The blue lines show the temporary threshold values 24 hours after the noise exposure. The green lines show the permanent threshold values two weeks after the noise exposure. Double knockout mice showed significant protection from NIHL for both temporary ($p<0.05$) and permanent ($p<0.001$) threshold values.

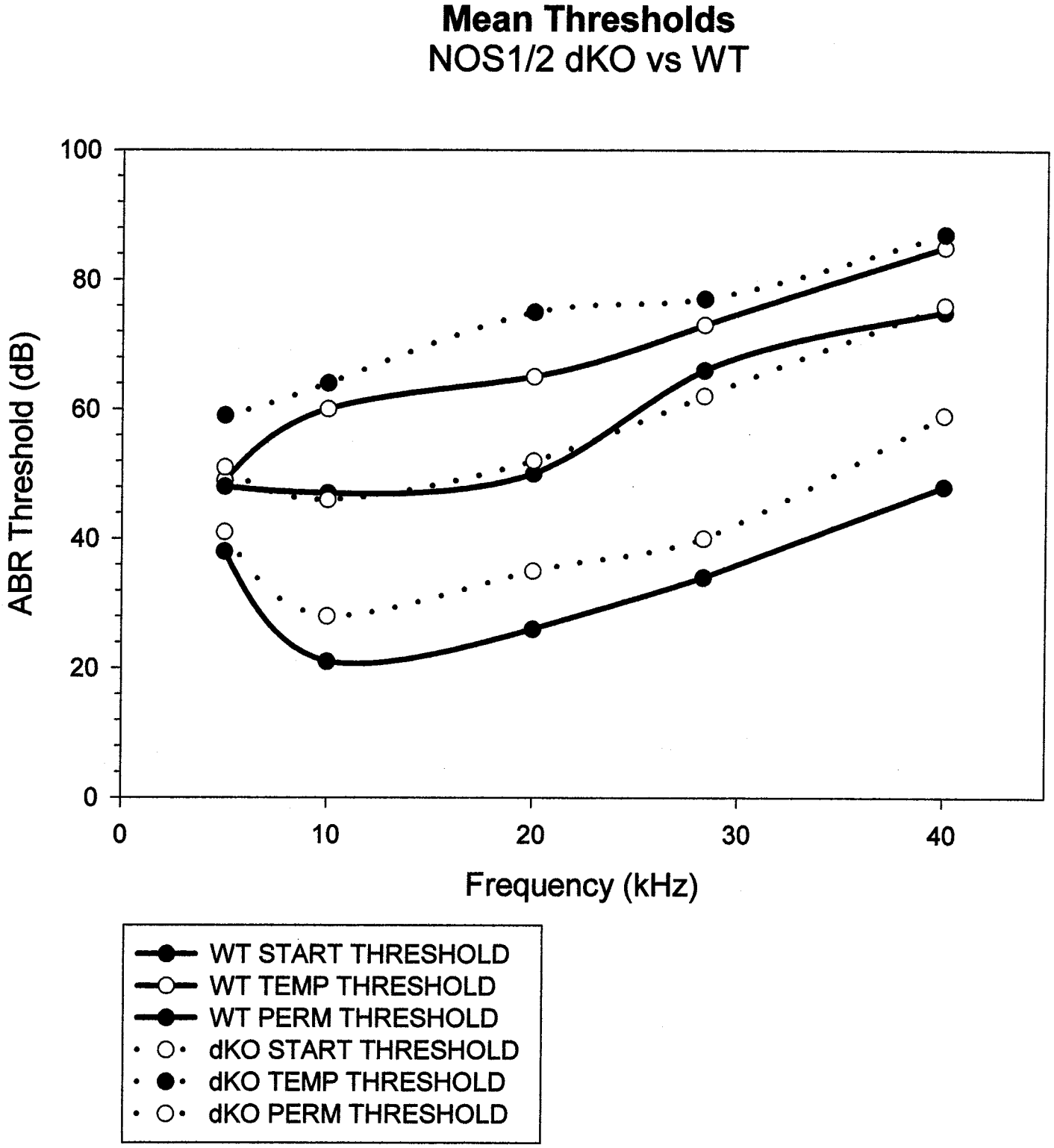
Mean Thresholds
NOS1/2 dKO vs WT
ABR Threshold (dB)
100
80
60
40
20
0
0
10
20
30
40
Frequency (kHz)
WT START THRESHOLD
WT TEMP THRESHOLD
WT PERM THRESHOLD
dKO START THRESHOLD
dKO TEMP THRESHOLD
dKO PERM THRESHOLD

Figure 1

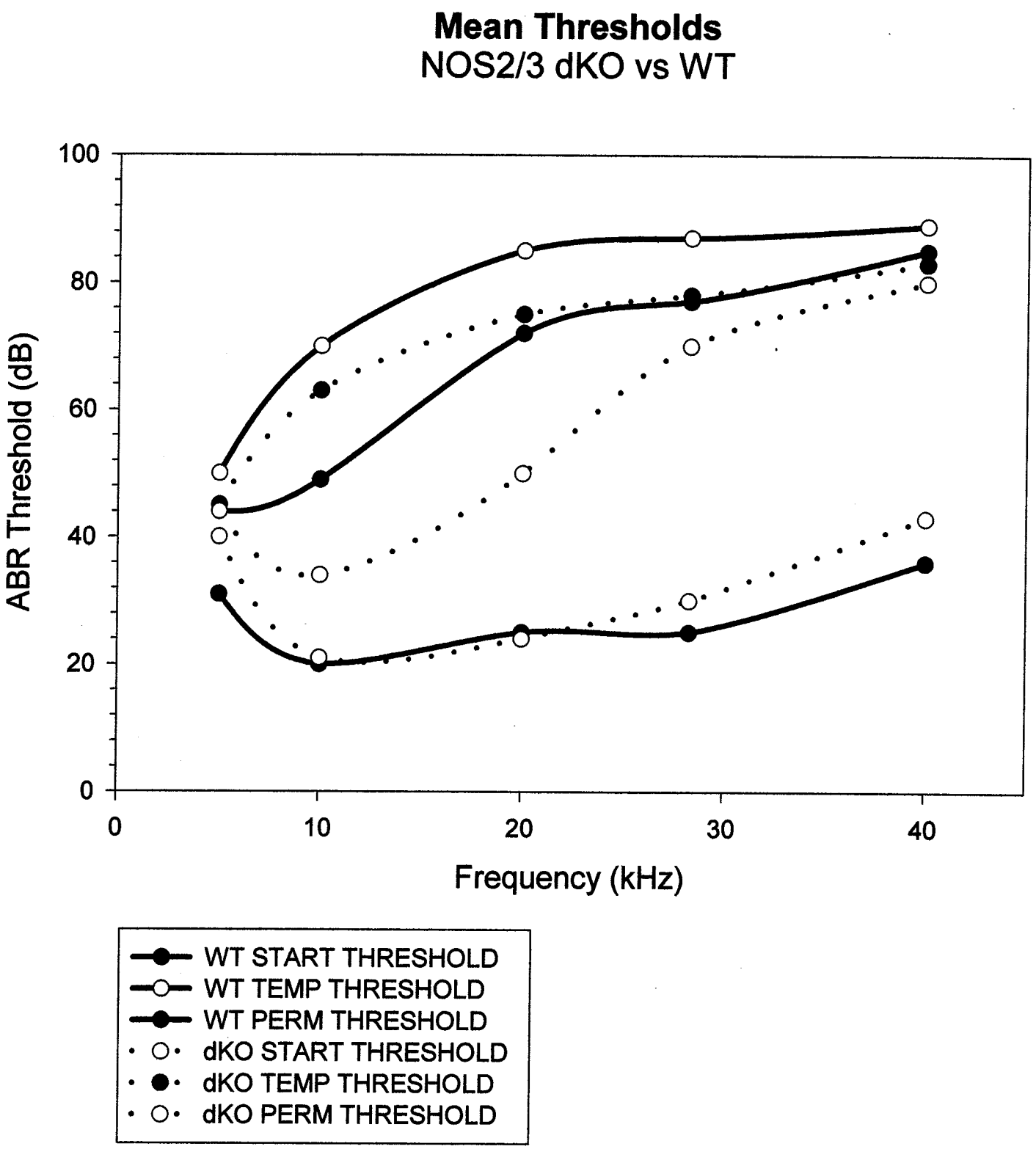
Mean Thresholds
NOS2/3 dKO vs WT
ABR Threshold (dB)
100
80
60
40
20
0
0
10
20
30
40
Frequency (kHz)
WT START THRESHOLD
WT TEMP THRESHOLD
WT PERM THRESHOLD
dKO START THRESHOLD
dKO TEMP THRESHOLD
dKO PERM THRESHOLD

Figure 2