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Synergistic effects of noise, kanamycin, and hyperoxia on ABR thresholds in CBA/J mice

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**SYNERGISTIC EFFECTS OF NOISE, KANAMYCIN, AND HYPEROXIA
ON ABR THRESHOLDS IN CBA/J MICE**

by

Elizabeth Ann Baum

**A Capstone Project
submitted in partial fulfillment of the
requirements for the degree of:**

Doctor of Audiology

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Approved by:

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Abstract: Young CBA/J mice were exposed to noise, kanamycin, and/or hyperoxia by 30 days post-gestational age in order to determine if a synergistic effect exists on ABR thresholds.

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TABLE OF CONTENTS

Acknowledgements	ii
List of Tables and Figures	2
Abbreviations	3
Epigraph	4
Introduction	5-10
Materials and Methods	10-13
Results	13-15
Discussion	15-17
References	18-19
Appendix	20-21

LIST OF FIGURES AND TABLES

TABLE 1: Number of mice per group and by sex	10
FIGURE 1: CBA/J noise dose-response findings	13
TABLE 2: Average thresholds per group	14
FIGURE 2: Control, kanamycin, kanamycin+noise, and kanamycin+hyperoxia+noise	14
FIGURE 3: Control, noise alone, and hyperoxia + noise	15
FIGURE 4: Noise versus kanamycin + noise	15
APPENDIX A: Average thresholds per group	20
APPENDIX B: ABR Record Form	21

ABBREVIATIONS

ABR	Auditory Brainstem Response
CMV	cytomegalovirus
dB (SPL)	decibels (sound pressure level)
ECMO	extracorporeal membrane oxygenation
Hz	hertz
I.P. or i.p.	intraperitoneal
JCIH	Joint Committee on Infant Hearing
kHz	kilohertz
NICU	neonatal intensive care unit
NIHL	noise-induced hearing loss
NITS	noise-induced threshold shift
OAE	otoacoustic emission
SC	subcutaneous

“Because of improving morbidity and mortality rates for premature human infants, and, since auditory development of humans continues into the third trimester of gestation and beyond, premature humans subjected to the acoustically traumatic environment of high risk intensive care nurseries and potentially ototoxic drugs administered prenatally to the mother or postnatally to the infant himself/herself may be prone to develop long-term pathological sequelae.”

~C.M. Henley and L.P. Rybak, 1995

According to the Centers for Disease Control and Prevention (CDC), the estimated incidence of hearing loss in infants is approximately one to three per one-thousand live births (National Center on Birth Defects and Developmental Disabilities, 2007). Though this number represents hearing loss that is congenital, events that occur in the early part of an infant's life may result in the acquisition of hearing loss. According to the Joint Committee on Infant Hearing (JCIH, 2007), the following are risk factors associated with congenital, acquired, and progressive hearing loss in childhood: family history, neonatal intensive care unit (NICU) stay for more than five days, extracorporeal membrane oxygenation (ECMO), assisted ventilation, exposure to ototoxic medications or loop diuretics, hyperbilirubinemia, in utero infections (CMV, herpes, rubella), craniofacial anomalies, syndromes associated with hearing loss and syndromic stigmata, neurodegenerative disorders, postnatal infections (meningitis), head trauma, and chemotherapy. One potential risk factor for acquired hearing loss that has been under-investigated and is not included in the JCIH report is neonatal noise exposure.

On a daily basis, medically fragile neonates are transported between medical centers via ambulance and aircraft. Many of these infants are premature, are receiving or have received ototoxic antibiotics, and their unprotected ears are commonly exposed to high levels of noise. Although these "once in a lifetime" exposures are usually of relatively short duration and may not pose a risk of hearing loss in neonates by themselves, the potential interactions of these noise exposures in conjunction with other agents (i.e., ototoxic antibiotics, other risk factors) has been underexplored. Several investigators have recorded sound levels in neonatal transit with peaks of up to 125 dB SPL (Skeoch, Jackson, Wilson, & Booth, 2005) and 103 dBA (Shenai, 1977). Although an Australian study reported average sound levels of approximately 82 dBA during helicopter transport (Buckland et al., 2003), a study at Washington University found that infants

were commonly exposed to sounds of 90-100 dBA, and some were as high as 115 dBA during helicopter transport to St. Louis Children's Hospital (Weathers, in press).

Aminoglycoside antibiotics, which are used to treat diseases such as tuberculosis and gram-negative infections (i.e., pneumonia) in humans, are known to result in ototoxicity, vestibulotoxicity, and nephrotoxicity. Studies in the early 1980s indicated that premature infants were more susceptible to aminoglycoside ototoxicity than were adults (as cited in Henley and Rybak, 1995). Aminoglycoside antibiotic treatment in newborn infants ranges in dosage from 5 to 400 mg/kg/day, depending on the drug and the bodyweight and age of the infant. Kanamycin, for example, has a recommended dosage of 15-20 mg/kg/day for infants less than one week of age (Thompson, 1983). These antibiotics are usually administered intravenously or intramuscularly and are divided into two or three equal daily doses. For serious infections, such as bacterial meningitis, aminoglycoside antibiotic treatment may last in excess of three weeks (Finitzo-Hieber, McCracken, Roeser, Allen, Chrane, & Morrow, 1979). One additional factor that many of these infants have in common is the administration of supplemental oxygen. Excessive oxygen administration resulting in oxygen toxicity in humans is known to cause damage to many body systems (Spitzer, 2005). The synergistic affect that oxygen in small amounts, such as for maintenance before and after helicopter transfer, may play in noise-induced hearing loss is unclear. Though some animal studies have shown that oxygen therapy may help to reduce hearing loss associated with acoustic trauma (Ward, 1995 and Fakhry, Rostain, & Cazals, 2007), others have indicated that immediate hyperbaric oxygen therapy may increase hearing loss related to acoustic trauma (Cakir, Ercan, Civelek, Korpinar, Toklu, Gedik, Isik, Sayin, & Turgut, 2006).

Each of the aforementioned conditions, prematurity, aminoglycoside antibiotic treatment, noise exposure, and oxygen therapy, are potential risk factors for hearing loss in and of themselves. We have hypothesized that the combined effects of ototoxic antibiotics, noise, and/or hyperoxia will result in an even greater risk for hearing loss in premature neonates. Ideally, a study to assess such a correlation would record auditory brainstem response (ABR) thresholds and/or otoacoustic emissions (OAEs) in infants both before and after exposure to the aforementioned conditions. Because it is unethical to knowingly expose humans to such potentially damaging agents and events, an animal model is a logical substitution.

The laboratory mouse is a well-established model for human auditory conditions, as it possesses strikingly similar cochlear anatomy and physiology and displays similar patterns of age-related, noise-induced, and ototoxicity-related hearing loss. In addition, the short life span of the mouse allows for easy study of auditory conditions both during early critical periods and throughout the life span (Henry & McGinn, 1992). The CBA/J inbred strain of mice has been widely used as a model of normal hearing and was selected as the model in the present study. Unlike humans, who have an audible frequency range of about 20 to 20,000 Hz, mice hear frequencies from 2 to 100 kHz, with greatest sensitivity between 12 to 24 kHz (as cited in Zheng, Johnson, & Erway, 1999). The auditory system of CBA/J mice remains normal until near the end of its life span, which allows for a more accurate study of ototraumatic manifestations on the normal auditory system.

It is known that young mice are more susceptible to ototoxic and ototraumatic events than are older mice. The first study to assess the susceptibility to noise-induced hearing loss (NIHL) across the entire lifespan of the mouse was completed in 1983 by Kenneth R. Henry. Henry exposed 369 CBA/J mice at various ages to five minutes of an octave band white noise (12-24

kHz) at 124, 114, or 110 dB SPL. Using electrocochleography, Henry assessed auditory nerve function at four days post-exposure. Older mice (≥ 37 days) underwent pre-exposure testing, whereas younger animals (< 37 days) were compared to littermate controls. The major findings were as follows: susceptibility begins at approximately 16 days of age, overall susceptibility was greatest from 20 to 90 days of age, during which time the center frequency demonstrates the greatest threshold shift, and during adulthood (≥ 120 days) a threshold shift is only observed at the highest frequencies (p. 378). Henry concluded that “the CBA/J mouse does not have a sharply defined critical period for NITS, even though the young animal is most severely affected” (p. 381). A more recent noise dose-response study by Ohlemiller, Wright, and Heidbreder (2000) found that CBA/CaJ, C57BL/6J, and BALB/cJ mice aged 1 to 2 months were more susceptible to NIHL than were mice aged 5 to 7 months. The aforementioned study exposed animals to different durations of broadband noise (4-45 kHz) at 110 dB SPL and assessed the amount of ABR threshold shift at two weeks post-exposure. A large proportion of young CBA/CaJ mice had permanent threshold shifts at approximately 3.42 minutes of noise exposure, whereas 63 minutes of noise exposure were required for an equivalent proportion of older CBA/CaJ mice to exhibit permanent threshold shifts.

As with noise exposure, the susceptibility of the mouse to ototoxicity is age-dependent, with the young mouse having the greatest degree of susceptibility. Unlike susceptibility to noise trauma however, the susceptibility to ototoxic drugs does not continue throughout the lifespan of the mouse. A 1983 study by Chen and Saunders sought to establish the critical period for kanamycin ototoxicity in mice. A previous study (Chen & Aberdeen, 1981) had indirectly suggested that the critical period for kanamycin ototoxicity in mice was likely 10 to 14 days of age, as this was the time period during which administration of kanamycin resulted in

susceptibility to audiogenic seizures in BALB/c mice (as cited in Chen & Saunders, 1983). Using the C57BL/6J strain, Chen and Saunders injected mice aged 6-9, 10-13, or 15-18 days with a once daily dose of kanamycin (400 mg/kg, i.p.) or an equivalent volume of saline for four days. At 15 days post-injection regimen, the animals were sacrificed and cochleae were harvested for morphologic analysis. This study supported the previous assumption made by Chen and Aberdeen, as it was discovered that only mice aged 10 to 13 days had significant damage to the outer hair cells, whereas younger and older mice showed little to no structural damage. The investigators also suggested that this period coincides with the rapid period of cochlear development and that age, strain, and duration of treatment may be important factors in the ototoxicity of kanamycin. It is important to note that morphological analysis of ototoxicity in such a case is a more appropriate assessment tool than electrophysiologic responses (such as the ABR) since the auditory system of the mouse is functionally immature until approximately 18 days of age (Henry, 1983).

Finally, it has been established that noise paired with kanamycin results in a greater degree of hearing loss than either factor alone (Dayal, et al., 1971; Quante, 1973; Dayal & Berek, 1975; Marques et al., 1975; Hawkins et al., 1975; Ryan & Bone, 1978; Brown et al., 1980; Brummett, Fox, & Kempton, 1992 (as cited in Humes, 1984)). An example of one such study by Brummett and colleagues (1992) identified a synergistic effect between subclinical doses of white noise (as low as 75 dBA) and subclinical doses of kanamycin (300 mg/kg) on the amount of hair cell loss in adult guinea pigs. All of the aforementioned studies used adult animals, with the overwhelming majority using the guinea pig as a model. In a review paper by Humes (1984), it was suggested that future research “explore the potential interactions resulting from concurrent exposure to multiple agents...in younger, more susceptible animals” (p. 1318).

The current study tested the following hypotheses in an effort to explore such interactions in younger mice: (a) noise exposure following kanamycin treatment will result in an increase in ABR thresholds relative to ABR thresholds in mice exposed to noise alone or kanamycin alone, (b) exposure to 100% oxygen before and after a noise exposure will result in an increase in ABR thresholds relative to those in mice exposed to noise alone, and (c) combined exposures to kanamycin, noise, and hyperoxia will result in an increase in ABR thresholds relative to those in other treatment groups.

MATERIALS AND METHODS

Animals

A total of 27 male and 23 female post-weanling CBA/J mice were utilized. Mice were bred from in-house dam:sire breeding pairs and were housed in the Mechanisms of Cochlear Injury Laboratory at Washington University School of Medicine throughout the experiment. A few of the animals were obtained directly from The Jackson Laboratory. Mice were assigned to one of six groups: control, noise alone, kanamycin alone, kanamycin plus noise, hyperoxia plus noise, and kanamycin plus hyperoxia plus noise. All procedures were approved by the Animal Studies Committee at Washington University School of Medicine. The following table summarizes the number and sex of mice per group:

GROUP	N	MALE	FEMALE
CONTROL	9	4	5
KANAMYCIN	9	5	4
NOISE	9	6	3
KANAMYCIN + NOISE	8	4	4
HYPEROXIA + NOISE	8	5	3
KANAMYCIN + HYPEROXIA + NOISE	7	3	4

Table 1. Numbers of animals per group and by sex

Kanamycin

Mice in groups receiving kanamycin were injected subcutaneously twice daily for ten or eleven days with a 300 mg/kg solution (63.93 mg/mL concentration). Injections began at approximately 20 days post-gestational age and were stopped at approximately 29 or 30 days post-gestational age. Mice were weighed prior to each injection and received 0.006 cc of drug for every gram of body weight. Injections occurred at approximately twelve hour intervals in an attempt to maintain a chronic drug state. The drug regimen was well-tolerated as no mice were lost during the course of treatment.

Noise Exposure

At 30 days post-gestational age, mice in a noise treatment group were exposed to 30 seconds of broadband noise (4 to 45 kHz) at approximately 110 dB SPL. Noise was produced and filtered with General Radio 1310 generators and Krohn-Hite 3550 filters, respectively. Groups of two to three animals were placed in a wired cage suspended between four speakers at 0, 90, 180, and 360 degrees azimuth in a single-walled sound-proof booth with foam treatment (Industrial Acoustics, Bronx, NY). The cage was rotated at approximately 0.013 Hz throughout the duration of the exposure in order to achieve as consistent an exposure as possible. No attempt was made to provide food or water throughout the exposure. For mice receiving exposure to kanamycin and noise, the noise exposure was initiated at 15 minutes following the final dose of kanamycin (injection day 11). A pharmacokinetic analysis of subcutaneous kanamycin injections in adult mice by Sinwat and colleagues (2000) found a peak serum level of approximately 15 minutes and an elimination half-life of one hour (as cited in Wu, Sha,

McLaren, Kawamoto, Raphael, & Schacht, 2001, p. 174), thus the rationale for a 15 minute post-injection noise exposure regimen.

Hyperoxia

Hyperoxic conditions were achieved using an oxygen tank coupled to a modified plastic storage container. Up to six mice at a time were placed in the container and were exposed to 100% oxygen for a total of six hours (three hours pre- and post-noise exposure) at normal atmospheric pressure. No attempt was made to provide food or water during exposures.

ABR Recordings

ABR recording occurred between nine and eleven days post-treatment, or at approximately 39 to 41 days post-gestational age. Tucker-Davis Technologies (TDT) System II hardware and BioSig 33 software were used. Calibration occurred prior to any recordings. Animals were anesthetized with a solution of ketamine and xylazine (80/15 mg/kg, i.p.). Subdermal needle electrodes were placed in the mid-back (ground), medial to the right pinna (active), and at the vertex (reference). Body temperature and heart rate were monitored throughout testing using a rectal probe, and body temperature was maintained at $37.5 \pm 1.0^{\circ}\text{C}$ using an isothermal pad. The right ear of each mouse was stimulated with 5 msec tonebursts (1000 repetitions, 20/second, 1.0 msec rise time) at frequencies of 5, 10, 20, 28.3, and 40 kHz. Filter settings were 100 to 10,000 Hz and speaker distance was 7 cm.

The first wave of the ABR is thought to be generated by cochlear and early auditory nerve activity and is the most robust wave of the mouse ABR (Zheng et al., 1999). Therefore, thresholds were observed as the lowest level that the first negative peak (wave I) could be identified using the following bracketing technique: increase attenuation 10 dB following a

positive response and decrease attenuation 5 dB following a negative response. Following ABR recording, mice were sacrificed and cochleae were harvested for morphologic analysis from a number of the animals in the study. Anesthesia for ABR recording was well-tolerated, as no animals were lost as a result.

RESULTS

Preliminary noise exposures in the current study were four minutes in duration, which was derived from CBA/CaJ data from the previously mentioned noise dose-response investigation by Ohlemiller et al. (2000), as the CBA/J and CBA/CaJ strains of mice are often used interchangeably in research. In the current study, CBA/J mice appear to be markedly more susceptible to NIHL than CBA/CaJ mice. The noise dose-response plot for young CBA/CaJ mice from the Ohlemiller (2000) study can be seen in the top portion of Figure 1. The orange triangles indicate the initial noise dose-response findings for young CBA/J mice in the current study. These results suggest that young CBA/J mice are incredibly more susceptible to noise induced hearing loss than CBA/CaJ mice, as noise exposures of 4, 3, 2, and 0.5 minutes resulted in 100% of the mice having a large amount of hearing loss.

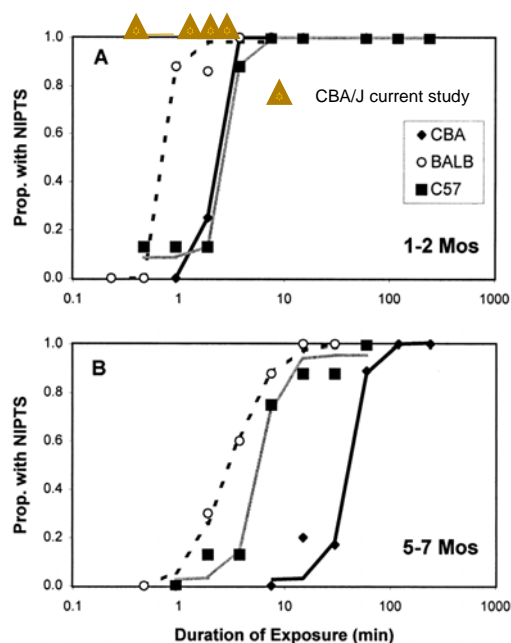


Figure 1. Young CBA/J are markedly more susceptible to NIHL than young CBA/CaJ mice

Reprinted with permission from Ohlemiller et al., 2001

A two-way analysis of variance (ANOVA) was used to determine if a statistical difference in thresholds existed between groups. The following table provides average thresholds per frequency per group with standard deviations in parentheses:

GROUP	5 kHz	10 kHz	20 kHz	28.3 kHz	40 kHz
CONTROL	41.44 (4.64)	23.56 (4.64)	31.67 (7.91)	32.11 (6.51)	38.33 (8.29)
KANAMYCIN	39.22 (3.63)	22.44 (1.67)	25.56 (3.00)	31 (5.00)	36.25 (9.71)
NOISE	68.11 (18.67)	58.56 (20.07)	64.44 (24.30)	73.78 (27.05)	90 (22.91)
KANAMYCIN + NOISE	42.63 (6.23)	22.38 (4.17)	30.63 (4.96)	34.13 (4.58)	53.13 (19.45)
HYPEROXIA + NOISE	53.25 (14.58)	43.63 (16.57)	61.88 (20.34)	81.63 (16.13)	91.25 (21.00)
KANAMYCIN + HYPEROXIA + NOISE	37.71 (6.73)	18.71 (1.89)	27.86 (5.67)	28.14 (4.88)	40.71 (13.67)

Table 2. Average thresholds per group with standard deviations in parentheses

No difference was observed between the control group and the kanamycin alone group, kanamycin plus noise group, or the kanamycin plus hyperoxia plus noise group as can be seen in Figure 2. Little variability was found between the mice in each of these groups. The thresholds of the noise alone and hyperoxia plus noise were significantly different from the control group ($p < 0.001$). On average, the noise alone

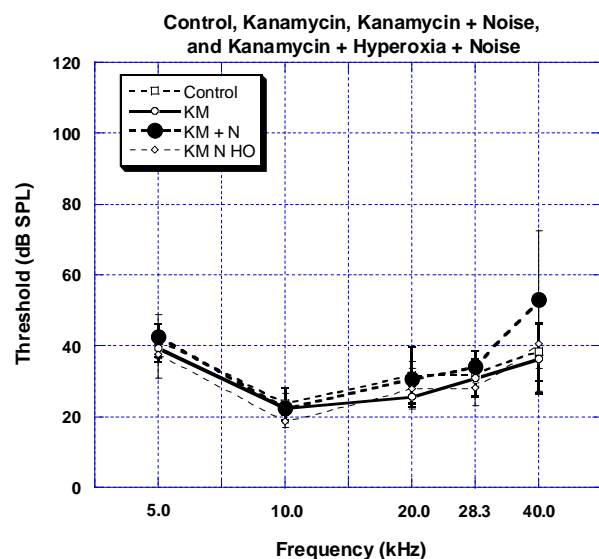


Figure 2. Average thresholds for control, kanamycin, kanamycin + noise, and kanamycin + hyperoxia + noise groups with error bars

group had approximately 35 to 40 dB poorer thresholds than the control group. No significant difference was found between the noise alone group and the hyperoxia plus noise group ($p=0.303$) as can be seen in Figure 3. Both the noise alone and the hyperoxia plus noise groups had a great degree of variability between mice at all frequencies.

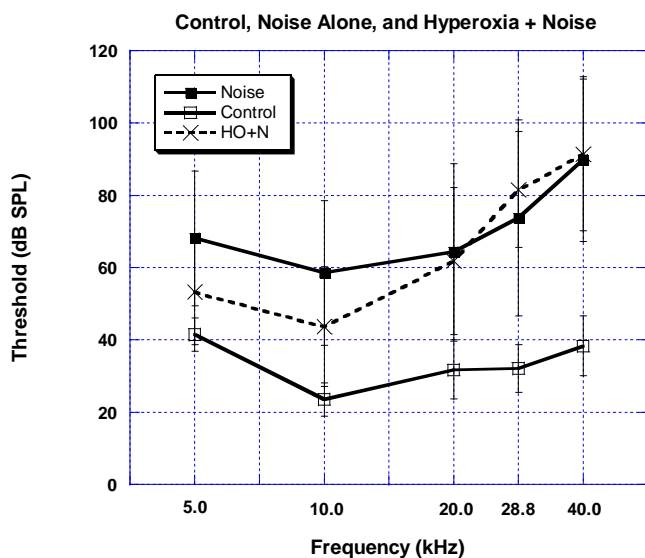


Figure 3. Average thresholds for control, noise alone, and hyperoxia + noise groups. There is a significant difference between the two experimental groups and the control group.

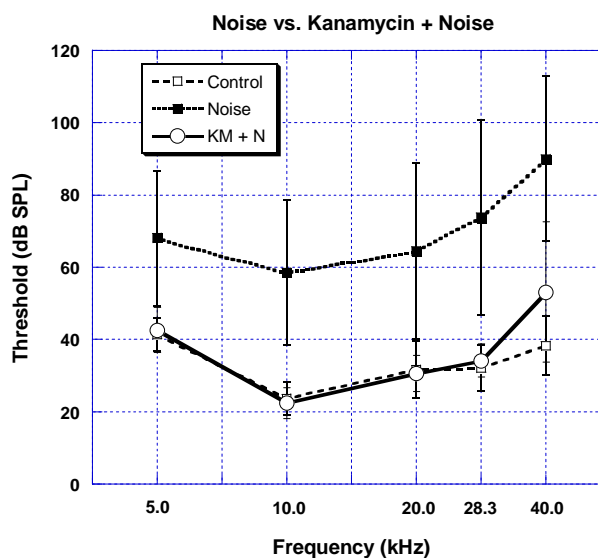


Figure 4. Average thresholds for control, noise, and kanamycin + noise.

DISCUSSION

The purpose of the current study was to determine if a combined exposure to kanamycin, noise, and oxygen would result in a synergistic effect on ABR thresholds in young CBA/J mice. It was hypothesized that (a) noise exposure following kanamycin treatment will result in an increase in ABR thresholds relative to those in mice exposed to noise alone or kanamycin alone, (b) exposure to 100% oxygen before and after a noise exposure will result in an increase in ABR thresholds relative to those in mice exposed to noise alone, and (c) combined exposures to kanamycin, noise, and hyperoxia will result in an increase in ABR thresholds relative to those in other treatment groups. Neither the hypothesis that noise plus kanamycin would result in a

synergistic effect on ABR thresholds nor the hypothesis that kanamycin plus oxygen plus noise would result in a synergistic effect on ABR thresholds was found to be true. In fact, kanamycin not only resulted in a protective affect against noise exposure in these groups, but its administration actually seemed to prevent NIHL completely as can be seen in Figure 4. To the author's knowledge, no other study has shown kanamycin to have such an effect. Also, the addition of oxygen was found to have no significant effect on ABR thresholds in noise-exposed animals ($p=0.303$).

The possibility exists that the protective effect of kanamycin against NIHL is an artifact, and that, in fact, the stress of handling and injecting the mice over 10 or 11 days time is preconditioning the animals against NIHL. Preconditioning refers to an early event that is thought to be mildly injurious initiating protection against a later, more injurious event. A wide body of research exists on preconditioning against NIHL in mice and indicates that factors including heat stress, restraint, and hypoxia result in a cochlear preconditioning against noise exposure (Gagnon, Simmons, Bao, Lei, Ortmann, & Ohlemiller, 2007). This cochlear preconditioning is thought to engage some innate protective cascades, in which factors such as improved blood flow, increased glucocorticoid stress hormones, and heat shock proteins play a role in this protection.

In order to determine if the protective effect of kanamycin is real or is in fact a manifestation of some sort of cochlear preconditioning, several control groups should be added to the current study. One such necessary control group would receive subcutaneous saline injections for eleven days followed by noise exposure to control for the stress of handling. A kanamycin injection control group (11 days of kanamycin followed by ABR testing) would also be required in order to verify that acute hearing loss did not exist from the kanamycin at the time

of noise exposure. Finally, a group receiving a single kanamycin injection followed by a single noise exposure would also be necessary to evaluate the effectiveness of kanamycin of protecting against NIHL in a single dose. To date, two mice have received this treatment (1 kanamycin injection, 300 mg/kg, i.p. followed by 30 sec. noise exposure 15 min. later) and hearing loss was apparent. This finding suggests that the mere presence of kanamycin prior to a noise exposure is not sufficient to be protective against NIHL.

It is unclear how the findings of the current study relate to human neonates. If in fact kanamycin proves to be protective against NIHL, further studies need to be done to address variables such as kanamycin dosing, the levels and duration of noise for which the protective effect remains, and the level of protection in older mice. According to Rybak and Ramkumar (2007), “aminoglycosides persist in the inner ear tissues for 6 months or longer after administration” (p. 932); therefore, it would be of interest to determine the intervals between kanamycin exposure and noise in which the protective effect remains. In addition, the physiological mechanisms of such a protective effect would need to be explored. Finally, future research should address the apparent increased susceptibility to NIHL in young CBA/J mice by performing a noise dose-response study.

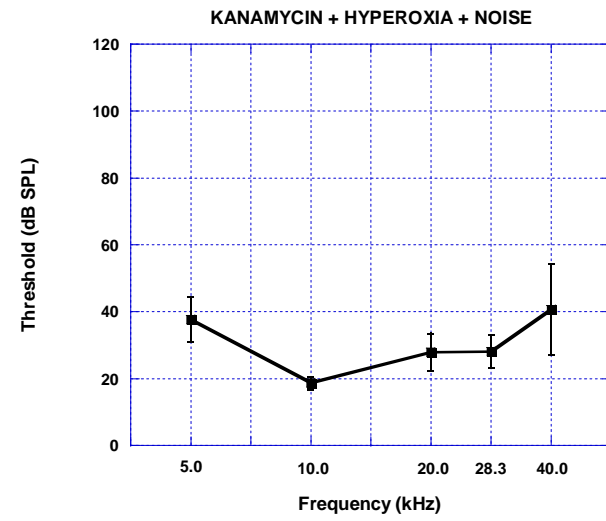
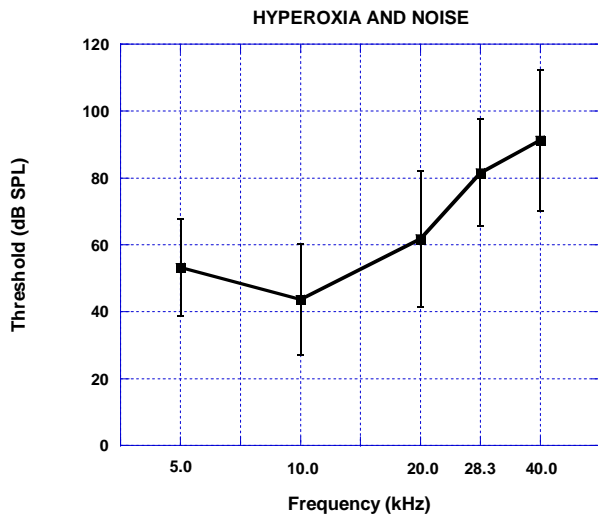
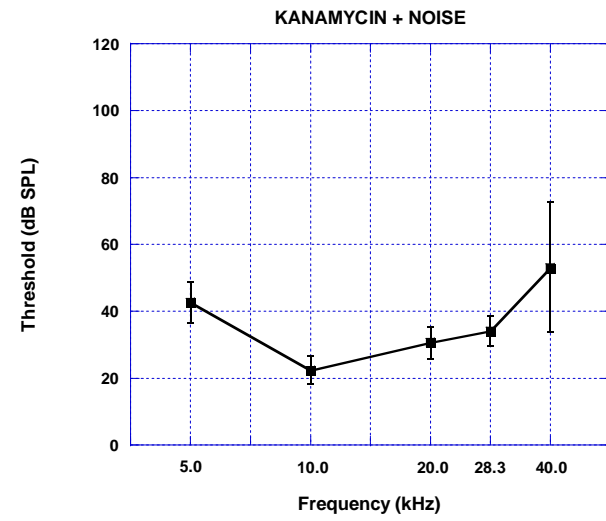
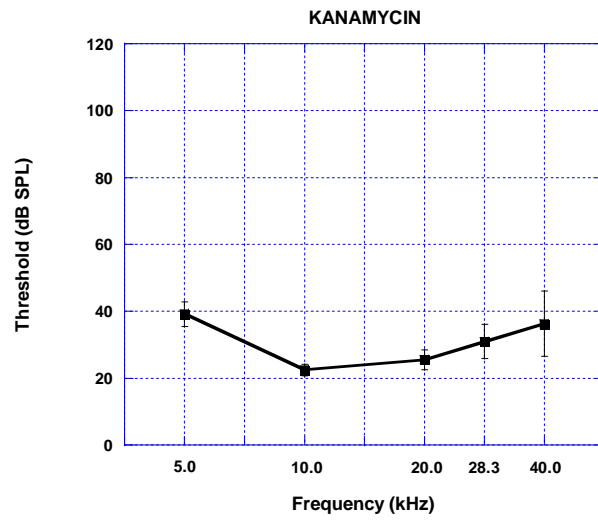
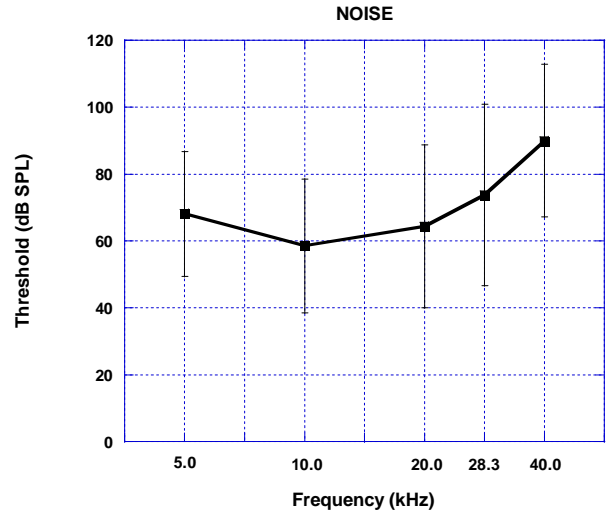
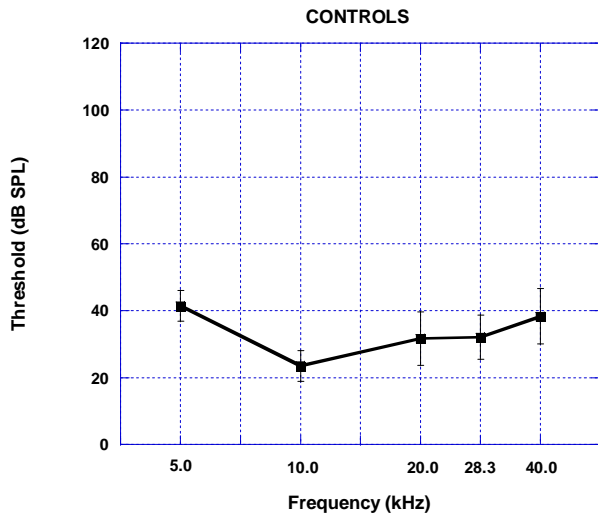
In conclusion, much is unknown about the mysterious auditory system. With so many pharmaceutical options for preventing and treating disease and other medical conditions of many of the body systems, it seems likely that something must exist that can protect the auditory system from the damaging effects of noise. How incredible to think that something as simple as kanamycin, typically considered an ototoxic drug, may in fact hold the key to the prevention of noise-induced hearing loss?

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APPENDIX A. Mean thresholds by group



APPENDIX B. ABR Data Log Form

ABR / CAP Data Log

sheet version date 8/3/04

ID Number _____

Animal type: mouse / rat / gerbil

Strain: _____ **Genotype** (tentative / confirmed): _____

Sac Date _____

Sex: _____ **DOB:** _____ **Age:** _____

Identifying marks: 

Project: _____

Collaborating PI: _____

ABR Conditions: 5 ms tone, 1000 reps, 20/sec, 100-10,000 Hz, x100,000, D/A 30 kHz

Anesthetic: 80/15 mg/kg (Ket/Xyl) / other: _____ **Rise Time:** 1.0 ms / other: _____

Ear: Right / Left **Speaker Distance:** 7 cm / 10 cm

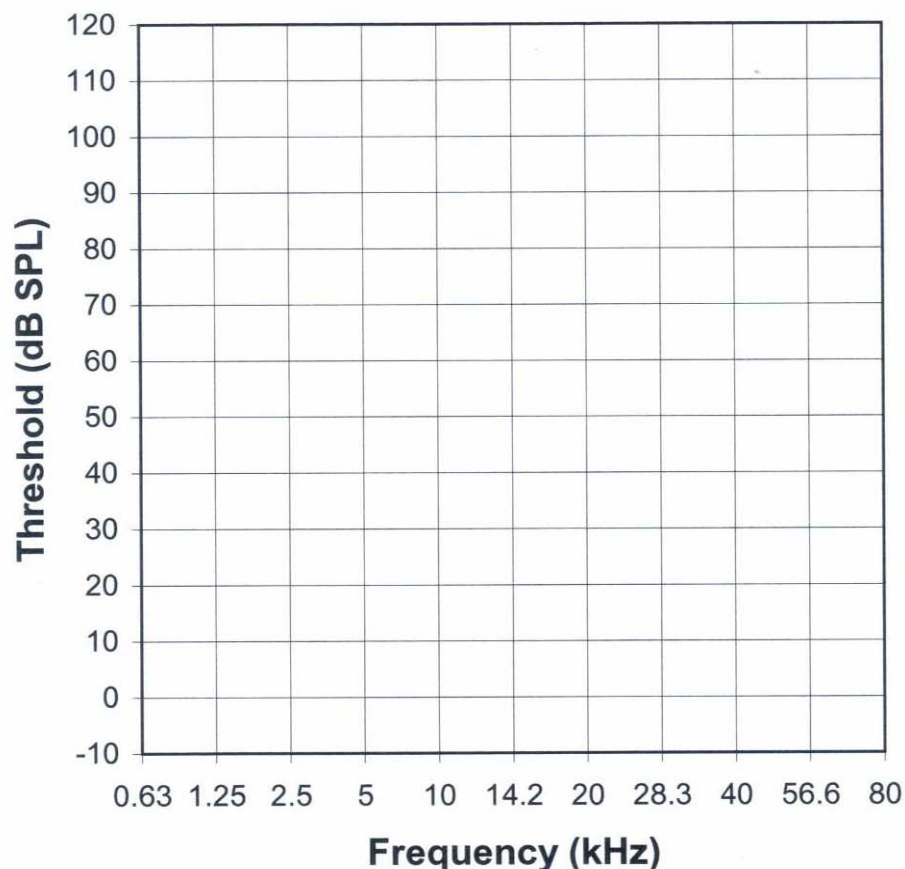
Ground: Back/Other: _____ **Reference:** Vertex/Other: _____

CAP Conditions: 5 ms tone, 100 reps, 3/sec, 30-3,000 Hz, x100,000, D/A 30 kHz

Anesthetic: Pentobarb (60 mg/kg) / other: _____ **Rise Time:** 1.0 ms / other: _____

Round window of: Right / Left **Speaker Distance:** 7 cm / 10 cm

Ground: Hindleg / Other: _____ **Reference:** Neck musculature/Other: _____



Date / Time: _____
 Tester (Initials): _____
 Condition: _____

Date / Time: _____
 Tester (Initials): _____
 Condition: _____

Date / Time: _____
 Tester (Initials): _____
 Condition: _____

Date / Time: _____
 Tester (Initials): _____
 Condition: _____

Notes: _____