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Genetic and temporal aspects of protection by kanamycin against cochlear noise injury

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GENETIC AND TEMPORAL ASPECTS OF PROTECTION BY KANAMYCIN AGAINST COCHLEAR NOISE INJURY

by

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Abstract: Experiments explored the minimal kanamycin dosing regimen that renders protection against noise induced hearing loss in young CBA/J mice. We also tested the age-dependence of protection in CBA/J as well as the dependence of protection on a particular genetic background in experiments using young C57BL/6J and CBA/CaJ mice.

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ABBREVIATIONS

ABR	auditory brainstem response
ANOVA	analysis of variance
B6	C57BL/6J
dB SPL	decibel sound pressure level
kHz	kilo hertz
IHC	inner hair cell
i.p.	intraperitoneal
JAX	The Jackson Laboratory
kg	kilogram
KM	kanamycin
mg	milligram
min	minute
ml	milliliter
mos	months
ms	millisecond
NIPTS	noise induced permanent threshold shift
OHC	outer hair cell
s	seconds
SD	standard deviation

INTRODUCTION

Aminoglycoside antibiotics are highly effective for treating and preventing life-threatening gram-negative bacterial infections. They also show tissue specific cytotoxicity that includes the cochlea. Both functional and morphological cochlear damage can occur from sufficiently high doses. Cochleotoxicity manifests as a hearing loss that initially increases thresholds at higher frequencies, and affects mostly outer hair cells (OHCs). Basal OHCs may be selectively exposed to higher concentrations of the drug, and these cells may have a greater inherent susceptibility than the apical OHCs (Rizzi & Hirose, 2007). Most cells take up aminoglycosides, however, cells of the kidney and cochlea preferentially retain them following systemic administration (Li & Steyger, 2009). Proposed mechanisms of cochlear injury by aminoglycosides include penetration of the drug into the endolymphatic fluid of scala media, uptake by hair cells through stereociliary transduction channels, and generation of toxic reactive oxygen species leading to hair cell death.

Exposure to loud noise also induces permanent functional and morphological cochlear damage. The cellular pattern of noise injury overlaps extensively with that of aminoglycosides. While damage may be seen in many cells and structures, key injury involves disruption of the OHCs as well as swelling and rupture of afferent auditory nerve fiber terminals that innervate the inner hair cells (IHCs) (Clark, 2008). Both types of injury are thought to operate via oxidative stress (Le Prell et al., 2007).

Decades of research support the contention that noise and aminoglycoside antibiotics act together in a synergistic manner, resulting in a greater hearing loss effect than when either agent is used alone. Presently there is little evidence bearing on synergism between ototoxins and noise in humans. On a daily basis, humans are exposed to loud noise while simultaneously being

treated with ototoxic aminoglycosides. Such synergy may particularly impact two groups: adults exposed to occupational noise and children. Children appear to be more susceptible than adults to both noise and ototoxic compounds (Bernard, 1981; Henley & Rybak, 1995; Li & Steyger, 2009). Infants, moreover, may represent a special risk category. Newborns in neonatal intensive care units often receive aminoglycosides while exposed to high noise levels produced by mechanical ventilation or air transport (Bernard, 1981; Li & Steyger, 2009; Shenai, 1977; Skeoch, Wilson & Booth, 2005). A study conducted at Washington University found medically fragile neonates treated with gentamicin are exposed to 90-100 dBA during helicopter transport to Saint Louis Children's Hospital (Weathers, 2008). These infants may be particularly at risk of early NIHL. The mechanisms of enhanced injury to the inner ear caused by combined ototoxic and noise exposure, and the presumed enhancement of injury in the young, continues to be intensive areas of research.

Mouse models in ototoxin and noise research

It is difficult—and often ethically prohibitive—to carry out well-controlled experiments that address the cellular and molecular bases of cochlear injury in humans. Instead, experiments on animals, and mice in particular, have been widely applied in relating molecular and histologic aspects of pathology to clinical findings. Malakoff (2000) has referred to the mouse as "biomedicine's model mammal," owing to their fast reproductive rates, relatively low maintenance costs, and scientists' growing ability to engineer genetic variations. Most human genes appear to have mouse homologues. This makes it possible to gain insights into human diseases via gene-altered mouse models.

Mice are commonly employed for investigations of inner ear development, function, and injury (Ohlemiller, 2006). Henry, Chole, McGinn, & Frush (1981) found the manifestations of kanamycin (KM) ototoxicity in mice to be similar to those in humans, although after the first month of life, greatly increased doses are needed in mice. Threshold elevation appears most severe at higher frequencies, corresponding to greater hair cell loss at the basal turn of the cochlea. Recent work by Schacht and colleagues (Wu et al., 2001) further developed the mouse ototoxicity model by comparing necessary dosing parameters and injury patterns across three popular inbred strains (C57BL/6J, BALB/cJ, and CBA/J). CBA/J and CBA/CaJ inbred mice are popular "good hearing" strains. C57BL/6J (B6) mice are also of particular interest as they carry the *Ahl* allele of cadherin 23 (*Cdh23*^{Ahl}), predisposing them to accelerated age-related sensorineural hearing loss and noise injury (Ohlemiller, Wright, & Heidbreder, 2000). Clear differences distinguish mouse strains with regard to vulnerability to ototoxicity and noise. Differences in ototoxicity may reflect differences in metabolism, cellular uptake mechanisms, or differential expression of antioxidant enzymes (Wu et al., 2001).

Critical period for ototoxicity and noise

Saunders and Bock (1978) were among the first to report a "critical period" whereby the immature cochlea has a higher degree of susceptibility to injurious events that are innocuous to the adult cochlea. Animal research including mice has shown that ototoxic drugs (Henry et al., 1981; Bernard, 1981) and acoustic trauma (Henry, 1983; Henry, 1984; Ohlemiller et al., 2000) pose a greater threat to the immature rather than the adult cochlea. Henry et al. (1981) reported that auditory nerve evoked potential thresholds at high frequencies in preweanling mice were severely affected by KM, while adult mice were only minimally affected at the highest frequency

tested (64 kHz). Histologic examination of cochleas revealed greater OHC loss in the basal turn of the cochlea, as well IHC loss in the preweanling mice compared to adults. In a similar study (Sha, Zajic, Epstein, & Schacht, 2001) 10 day old mice received KM (400 mg/kg/day) for 10 days. Thresholds determined at one month were 45-50 dB higher in the KM treated group than in the saline-injected controls. A preliminary screening conducted by this research team found mature mice tolerated much higher levels (700 mg/kg/day) of KM without ototoxic effects. In an effort to achieve more sustained drug serum levels, twice daily injections were employed in adult mice of three different strains, including CBA/J, B6, and BALB mice (Wu et al., 2001). All three strains displayed functional and structural pathology with a base to apex pattern, similar to ototoxic injury in humans. It is clear from the mentioned studies that ototoxicity is not only dependent on age of treatment, but also on dosage levels.

The critical period for noise susceptibility appears longer and less sharply defined than that for ototoxicity (Henry, 1983). In one prominent early study, Henry (1983) exposed CBA/J mice to 5 minutes of 12-24 kHz octave band of noise at 124 dB SPL at 20, 90, and 360 days of age. All mice showed a noise induced permanent thresholds shift (NIPTS), yet the injury varied by age, with the youngest mice most severely affected. A later study conducted by Ohlemiller et al. (2000) showed that the noise dose response relationship is steeper on a log-time axis in young adult mice than older adults. Histological analysis further showed that OHC loss was more extensive in the lower base of the younger animals, even when similar NIPTSs were found. Thus the critical period for both ototoxicity and noise seem to reflect particular vulnerability of OHCs.

Observations regarding the critical period in animals are only potentially clinically useful, of course, if they possess a human parallel. Humans do show differing degrees of susceptibility

throughout their lifespan to noise induced damage seen particularly in the higher frequencies (Sataloff et al., 1967; Fausti et al., 1981; Dieroff, 1982 (as cited in Henry, 1983)). Additionally, newborns and pediatrics are deemed more susceptible to ototoxins (Henley & Rybak, 1995), as well as those with kidney and liver problems. Specifically, aminoglycoside ototoxicity is dependent upon the frequency, duration and amount of dosage per bodyweight.

Interactions between aminoglycoside ototoxicity and noise

It has been established that combined effects of aminoglycoside ototoxicity and acoustic trauma are intensified versus either agent alone. Most supporting research utilized adult animals for these discoveries. For example, Brummett, Fox, & Kempton, (1992) administered a subclinical dose of KM to adult guinea pigs, followed by 10 hours of noise at various levels. This was repeated 7 times for each animal. Results showed the percent hair cell loss in animals receiving KM plus noise to be greater than the sum effect of KM or noise alone. When the animals were treated with KM in conjunction with low-level noise (45 dB SPL), permanent cochlear damage was not observed. Synergy in the form of exacerbated basal turn OHC loss was also found in young guinea pigs treated with both low-dose gentamicin and noise at 76 dB SPL (Dodson, Bannister, & Douek, 1982). One principle that emerges from studies such as these is that the extent of ototoxin and noise synergy seems dependent on the clearance rate of aminoglycosides from the cochlea, and thus the amount of ototoxin present at the time of exposure.

Not all evidence points to exacerbation of injury by combined ototoxins and noise. Recently, Fernandez, Ohlemiller, Gagnon, & Clark (2010) discovered that repeated subclinical doses of KM can have a protective effect against noise induced injury. In that study, 20-day-old

CBA/J mice were injected with KM or saline (300 mg/kg) every 12 hours for 10 consecutive days. On the eleventh day the mice were exposed to 30 seconds of 110 dB SPL broadband noise. Ten days following the exposure, auditory brainstem response (ABR) measures indicated the noise exposed saline treated mice had significantly elevated threshold shifts of about 30-40 dB SPL compared to the saline treated, no noise exposed controls. Surprisingly, the mice treated with KM and noise had statistically normal hearing thresholds (See Figure 1). Hair cell counts indicated that preservation of outer hair cells was the most prominent anatomic correlate of protection. Fernandez et al. (2010) further reported that protective effects of the KM extend to at least 48 hours following the last dose. Additionally, mice receiving a single dose of KM only 15 minutes prior to the noise exposure did not exhibit protection. It was concluded the mere presence of KM is not adequate for protection.

The Fernandez et al. study applied an intensive, yet apparently sub-toxic, KM dosing regimen. It remained unclear how small an amount, or how infrequent the dose of KM could be protective. In a follow-up study, Rybak Rice (2009) investigated the shortest KM dosing interval necessary to produce complete protection for the same mouse age, strain, and type of exposure. CBA/J mice were injected with KM at varying intervals of once daily, once every other day, and once every third day for a span of 10 days. Results indicated all three treatment groups produced protective results, though the sample size in the every third day treatment group was small. Further, Rybak Rice (2009) included a treatment group that received KM daily (1 dose/day for 10 days), with no noise exposure to uncover any toxic effects of KM. ABR threshold testing concluded the KM did not cause hearing loss, as no threshold shifts were seen.

Collectively, these studies support the contention that KM engages a form of preconditioning, whereby a non-damaging or minimally damaging stressor offers protection against a later more injurious stressor. Preconditioning against NIPTS in mice has been shown utilizing hypoxia (Gagnon et al., 2007), heat stress (Yoshida, Kristiansen, & Liberman, 1999), and even simple restraint (Wang & Liberman, 2002). Each of these may set in motion overlapping protective cascades potentially involving increased levels of heat shock proteins and glucocorticoid stress hormones, and may also involve improved blood flow.

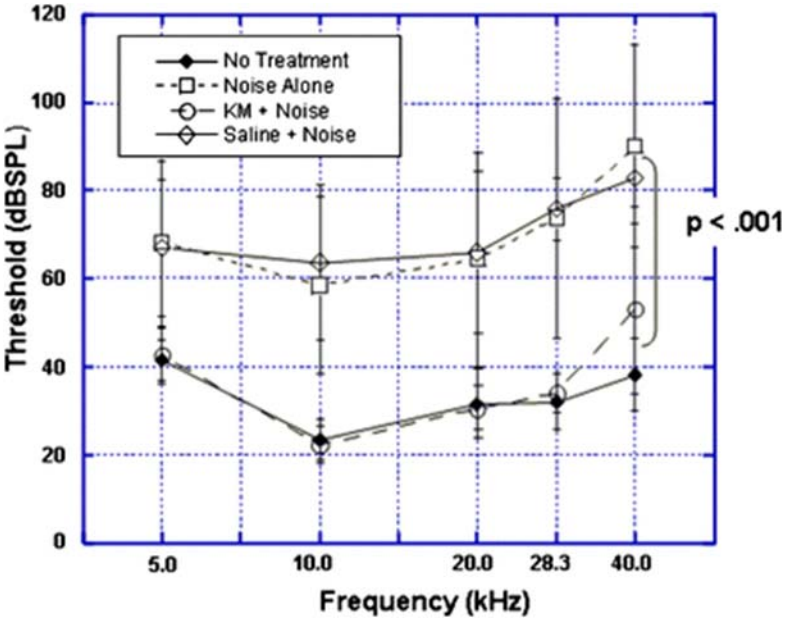

Figure 1: Mean (±SD) ABR thresholds for mice receiving either No Treatment, Noise Alone, KM + Noise, or Saline + Noise. Mice receiving Saline + Noise showed significantly elevated ABR thresholds versus unexposed controls (two-way ANOVA). Thresholds in saline-treated mice were not significantly different from the Noise Alone group. Mice receiving KM + Noise showed ABR thresholds not significantly different from the unexposed controls.

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Purpose of the present study

The generality and limits of KM related protection against noise remains underexplored. Research going forward has been aimed at the molecular mechanisms of the enigmatic protection by KM, in order to better define the conditions under which protection is found. Rybak Rice

(2009) did not determine the limits of protection afforded by KM. Further, it is imperative to study the genetic variability among mouse strains to determine KM sensitivity and protection. Whether the reported protection depends on a particular genetic background can be tested by examining other inbred mouse strains. Next, if adult CBA/J mice are found to be protected, it would suggest KM related protection is not restricted to the early vulnerability window or critical period for both noise and ototoxin induced hearing loss known to exist in animals. Finally, it is necessary to enhance the findings of the Rybak Rice (2009) study in whether we have to inflict injury within the cochlea to get protection, as a preconditioning stressor would suggest. With this in mind, the current research had four aims:

The first was to continue defining the minimal KM dosing regimen that affords protection against a NIPTS in young CBA/J mice. We sought to verify the results of the Rybak Rice (2009) study by increasing the sample size, and to test the effects of a single dose, 24 and 48 hours pre-noise exposure, as well as two doses applied over 3 days at 72 and 24 hours prior to exposure.

The second aim was to test for any strong genetic background dependence of KM related protection in different strains of young mice using B6 and CBA/CaJ inbred mice. These experiments applied a somewhat aggressive (1 dose/day for 10 days) paradigm in order to help detect any protective effects. We reasoned that if other inbred strains are not protected by KM, it would suggest that particular alleles carried by CBA/J mice at unknown loci may be critical. Such results would also offer the possibility of subsequent genetic analyses aimed at determining the protective pathways involved.

The third aim was to determine whether KM related protection against NIPTS in CBA/J mice is limited to young mice by treating mice at 2 months of age with KM followed by an

optimal noise exposure time that will consistently produce a NIPTS. Any strict age-dependence of protection would be taken to indicate that the key pathways engaged by KM are somehow linked to the very injury mechanisms unique to the critical period.

Lastly, the fourth aim was to uncover any toxic effects of KM levels that also confer protection. If sub-clinical injury is critical for protection, in keeping with the mechanisms of preconditioning, then it is important to establish whether we can protect with KM without measurable injury. To identify cochlear injury in the extreme lower basal turn of the cochlea, the ABR test frequencies were extended to include 56.6 kHz. Although not employed for all experiments, all treatment groups were tested at this frequency within a given experiment. These experiments included a treatment group receiving KM alone to study the effects of the sub-chronic low dose on the cochlea.

MATERIALS AND METHODS

Animals

Experiments used inbred CBA/J, B6 and CBA/CaJ mice, all either purchased from The Jackson Laboratory (JAX) or derived from breeders purchased from JAX. A total of 67 CBA/J, 23 C57BL/6J, and 5 CBA/CaJ mice were used. Thresholds in all saline treated control CBA/J mice resembled those in similar-aged untreated archival control mice. In some cases, archival data were added to saline-treated control data for statistical purposes. All mice were housed in the Central Institute for the Deaf Animal Colony. During treatment and recordings the mice were housed in the Mechanisms of Cochlear Injury Laboratory at Washington University School of Medicine. Mice had free access to food and water. All procedures were approved by the Animal Studies Committee at Washington University School of Medicine.

Kanamycin and saline dosing

Mice received KM sulfate subcutaneously suspended in a 0.9% commercial saline solution containing 63.93 mg/ml of KM to yield 300 mg/kg per dose. The KM solution was prepared on a weekly basis. For controls, saline was administered subcutaneously at an equivalent volume/weight dose. Drug administration was randomized within litters and sex, so that each litter contained control and experimental mice. Body weight was monitored daily and the administered drug dosages were adjusted accordingly. Drug treatment was well tolerated as no mice were lost during the course of the study. For experiments involving younger mice (< 1 month), injections began at approximately 20 days post-gestational age. For older mice, dosing began at 60 days post-gestational age. Experiments in B6, CBA/CaJ and adult CBA/J mice all adhered to a regimen of 1 dose/day for 10 days. Injections were administered within the same hour (±1 hour) for each treatment day for all cohorts.

Noise exposure

Noise exposures were carried out in a foam-lined, single-walled soundproof room. Two to three mice from different treatment groups were placed in a 21x21x11 cm wired cage mounted on a turntable pedestal that rotated 1 revolution/80 seconds to provide a homogeneous sound field. Four speakers surrounded the cage at 0°, 90°, 180° and 270° azimuth. The amplitude of the loudspeaker is flat within 10 dB from 5-40 kHz with a peak at 10 kHz.

On the eleventh day of treatment, or at 30 days of age, young CBA/J, B6, and CBA/CaJ mice received broadband, 4-45 kHz, noise exposure at 110 dB SPL. Young CBA/J mice were exposed to 30 seconds of noise, determined from previous research that concluded 30 seconds

will reliably produce a NIPTS (Rybak Rice, Gagnon, & Ohlemiller, 2009). B6 and CBA/CaJ mice received 4 minutes of noise exposure (Ohlemiller et al., 2000) 15 minutes after the final dose of KM or saline.

For experiments involving older mice, adult CBA/Js received 15 minutes of noise exposure, 15 minutes following the final dose of KM or saline on the eleventh day of treatment, or at 70 days of age. Pre-experimental data were collected on 6 adult CBA/J mice separated by receiving either 15 or 30 minutes of noise. Fifteen minutes was found to adequately and reliably produce a NIPTS and was therefore employed in the current study.

Auditory Brainstem Response recordings

Auditory thresholds were determined from evoked ABRs, a noninvasive measure of cochlear function that corresponds well with behavioral thresholds. Mice were anesthetized with an intramuscular injection of a ketamine and xylazine solution (80/15 mg/kg) and positioned dorsally in a custom headholder 7 cm from the speaker. Body temperature was maintained at 37.5±1°C with the use of a controlled heating pad and a rectal probe. Subdermal needle electrodes were inserted behind the right ear (active), at the vertex (reference), and contralaterally in the back (ground). The left ear was clamped with a clip to ensure recorded thresholds were only from the right ear.

Threshold was defined as the lowest stimulus level at which a positive Wave I in the evoked response tracing was evident using a 5 dB minimum step size. Wave I was used because it is the most robust wave of the mouse ABR, and thought to be generated exclusively by cochlear auditory nerve activity (Zheng, Johnson, & Erway, 1999). All thresholds were verified twice at each frequency tested. Due to the fragility of pre-weanling mice at the time of the initial

experiment, recordings were obtained only once, post noise exposure. To track only permanent threshold shifts, rather than temporary, the ABR recording was carried out 10 days post noise exposure, or 40 or 80 days post-gestation for the young and adult mice, respectively. Stimuli were presented 1000 times to the right ear in 5 ms tonebursts. Stimulus presentation and data acquisition used Tucker Davis Technologies System II hardware and Biosig 32 software.

Previous research and initial experiments in the current study determined ABR thresholds over the frequency range 5-40 kHz (Fernandez et al., 2009; Rybak Rice, 2009). Latter experiments in this study added an additional test frequency of 56.6 kHz, one half octave above 40 kHz. This frequency extended the testing into the cochlear hook region (Muller, von Hunerbein, Hoidis, & Smolders, 2005), which may be more sensitive to both KM and noise. Two-way analysis of variance (ANOVA) followed by Bonferroni multiple comparisons tests were applied to test for significant ABR threshold differences by experimental group and frequency.

Sacrifice and tissue processing for histology

Following ABR threshold recordings, mice were overdosed using sodium pentobarbital at 4 times the surgical dose (240 mg/kg, i.p.). When no toe-pinch response was present, mice were transcardially perfused with 2.0% paraformaldehyde and 2.0% glutaraledehyde solution in 0.1 M phosphate buffer. Cochleas were quickly isolated and immersed in fixative for the removal of the stapes. At a later date, they were decalcified using an EDTA sodium solution, stained with Osmium, dehydrated using acetone, and finally embedded in Epon-Araldite for histologic analyses. All middle ears were inspected for signs of otitis media. When found present, related data were excluded from further analyses.

RESULTS

Testing the minimal optimal KM dosing paradigm

Previous research (Rybak Rice, 2009) using a small sample (n=3) suggested that young CBA/J mice receiving KM every third day are completely protected from NIPTS that results from the 30 s noise exposure. The current study applied an additional 6 mice to the every third day paradigm. As shown in Figure 2, mice injected every third day were protected at low frequencies, but only somewhat protected at high frequencies. Thresholds were statistically different from the noise exposed controls ($p < .001$), but were also significantly elevated compared to no-noise controls. Since more frequent dosing (e.g., every other day) had appeared completely protective in previous experiments, this indicated that the effective 'dose integration time' for KM-related protection cascades is less than 72 hours.

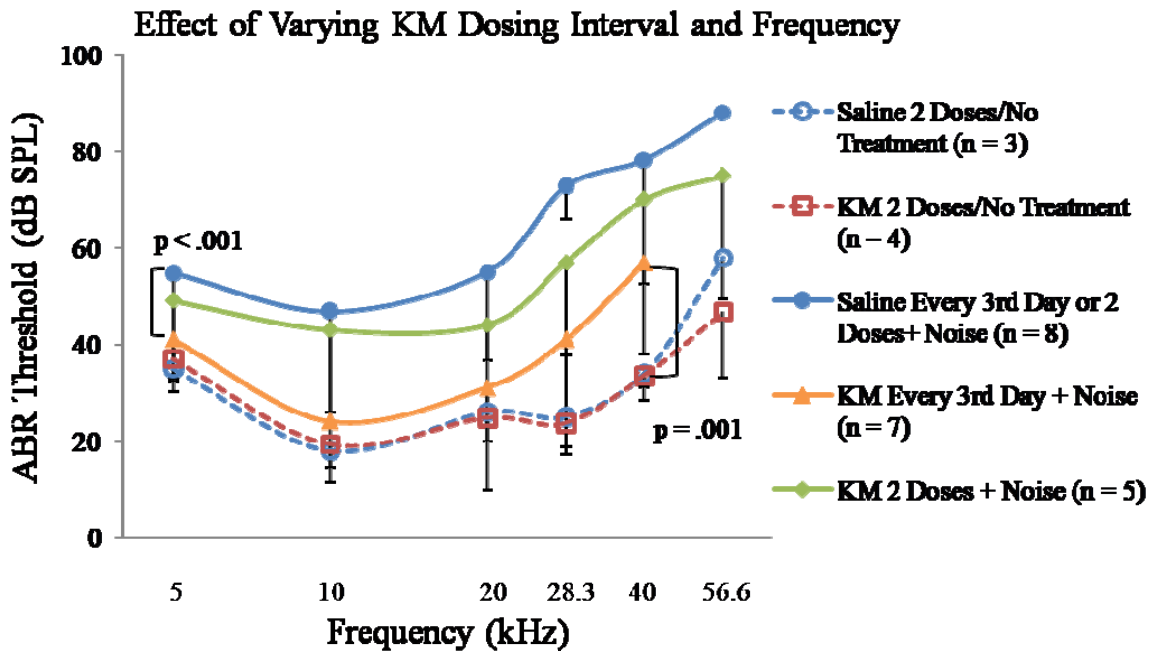

Figure 2: ABR thresholds for young KM and saline treated CBA/J mice following 30 s noise exposure, measured 10 days after noise. Reducing the KM dosing frequency to every 3rd day is only somewhat effective, while 2 doses are ineffective for protection from noise.

We also tested the effect of reducing the number of doses, while holding the interval fixed at every other day (72 and 24 hours prior to exposure). As shown in Figure 2, mice receiving only 2 doses of KM, even when applied at the apparent minimum optimal dose interval, were little protected from noise. This indicated that KM-related protection arises through innate responses that must build up over an exposure period of more than 3 days. Additional experiments whereby KM was given only once either 24 or 48 hours prior to noise exposure also supported this conclusion. As shown in Figure 3, these mice showed thresholds that were statistically indistinguishable from mice receiving only saline prior to noise.

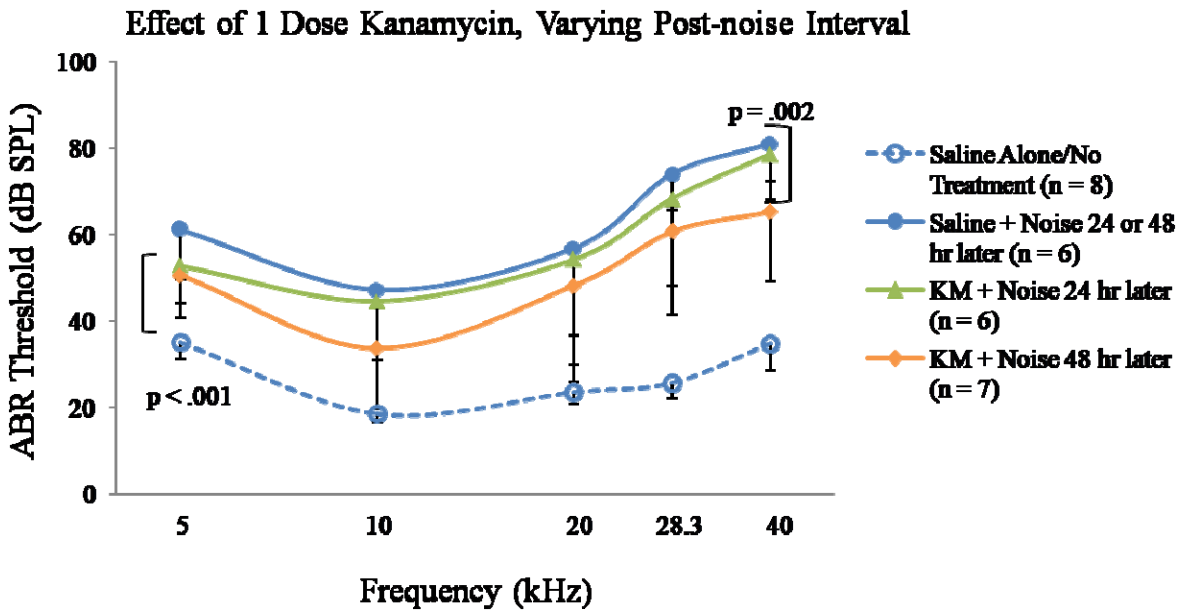

Figure 3: ABR thresholds in mice given a single dose of KM or saline before noise exposure, measured 10 days after noise. A single dose of KM is ineffective for protection from noise.

Testing the 'Genetic Tolerance' of KM protection

Assuming that protection by KM acts through widely common mechanisms, it should be possible to demonstrate protection on genetic backgrounds other than CBA/J. This was tested by

applying a KM treatment regimen that is completely protective in CBA/J to B6 and CBA/CaJ mice. B6 mice receiving KM once daily for 10 days did not exhibit significant KM related protection (Figure 4). Thresholds in these mice did not differ significantly from the saline treated noise exposed control mice.

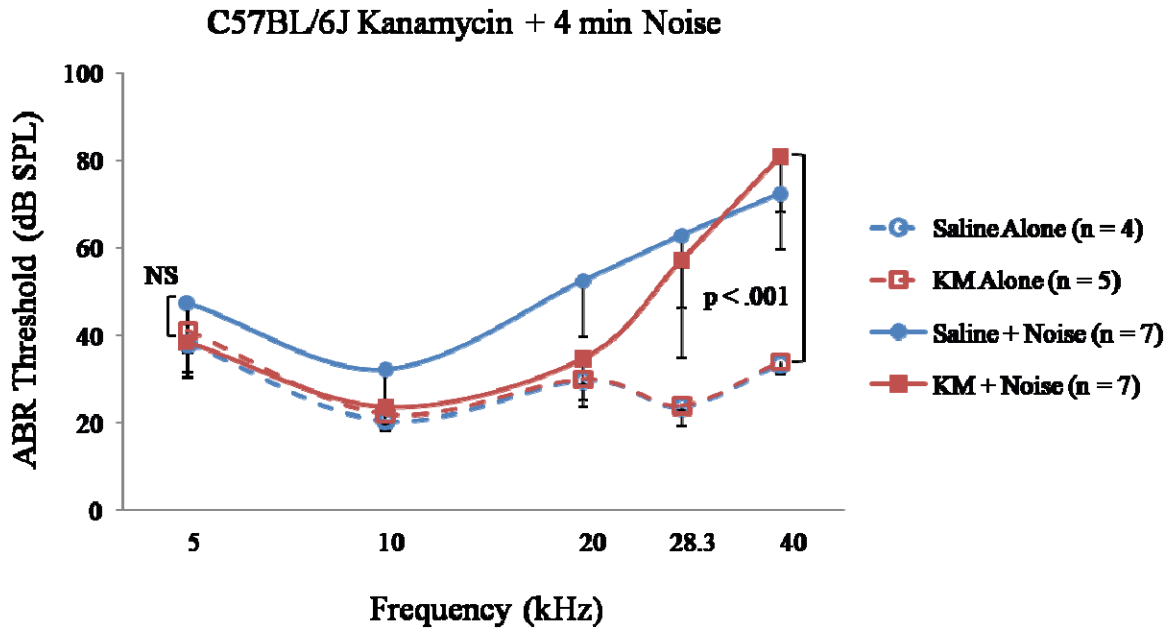

Figure 4: ABR thresholds for young KM and saline treated C57BL/6J mice after 4 min noise exposure, measured 10 days after noise. Young C57BL/6J mice did not exhibit significant KM related protection.

One interpretation is that KM protection depends explicitly on alleles at unknown loci that CBA/J mice carry and B6 mice do not. However, it could simply mean that the optimal KM treatment paradigm for CBA/J and B6 is different, although this too would presumably reflect allelic differences.

Due to the small number of CBA/CaJ mice utilized, and the small amount of NIPTS in these mice, a statistical analysis could not be performed for this experiment. Protection by KM

was not evident (Figure 5). The saline noise exposed controls were not exposed to an adequate duration of noise to produce a significant NIPTS.

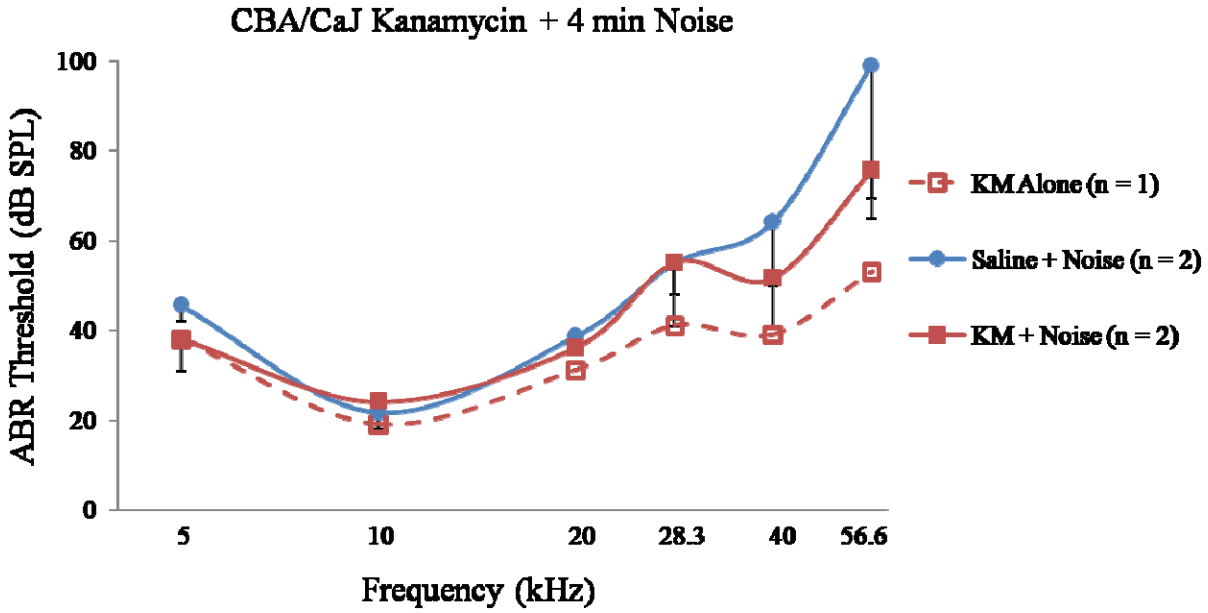

Figure 5: ABR thresholds for young KM and saline treated CBA/CaJ mice after 4 min noise exposure, measured 10 days after noise. Young CBA/CaJ mice did not exhibit significant KM related protection.

Testing the age requirements of KM protection

Previous studies (Fernandez et al., 2010; Rybak Rice, 2009) showed that young CBA/J mice are exquisitely sensitive to noise, requiring only a 30 s exposure in the first month of life to sustain both moderate hearing loss and hair cell loss. It could be the case that some cellular process, that is active only during the first month of life, is also essential for the protective effects of KM against noise in these mice. This was tested by repeating our experiments in older CBA/J mice, which are less sensitive to noise injury and much less sensitive to KM. As shown in Figure 5, significant protection was found for 2 month old CBA/J mice treated with KM (1 dose/day for 10 days) and noise, compared with the thresholds of the mice receiving saline and

noise (p<.001) and the mice treated with saline and no noise exposure (p<.001). As previously stated, note that these mice were given 15 minutes of noise exposure, the time determined to reliably produce a NIPTS.

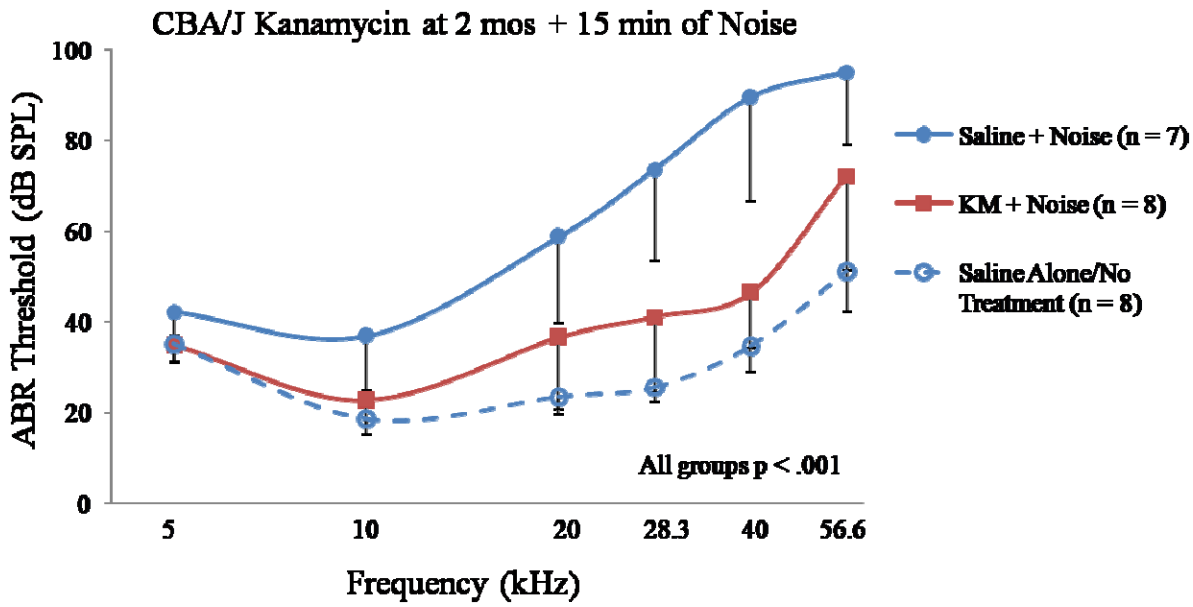

Figure 6: ABR thresholds for adult (2 mos) KM and saline treated CBA/J mice after 15 min of noise exposure, measured 10 days after noise. KM related protection is not restricted to the early vulnerability window for ototoxicity.

Testing the toxic effects of KM

The basal turn of the cochlea was tested at 56.6 kHz in the experiments involving mice receiving the two doses of KM or saline, with and without noise, for the CBA/CaJ experiment, and for the 2 month old CBA/J experiment. The experiment involving noise exposed CBA/CaJ mice did not have a control group, and therefore KM related protection versus injury remains ambiguous (Figure 5). The two dose paradigm and the 2 month old CBA/J data suggest that while 56.6 kHz is elevated when KM and saline are given alone, it is increasingly elevated when

noise is introduced. Therefore, the KM may not be inflicting injury to produce an ototoxin induced hearing loss apparent on the ABR thresholds.

Appendix A provides average thresholds per frequency per group with standard deviations in parentheses for all experimental mice.

DISCUSSION

Earlier studies in other models (e.g., Brummett et al., 1992) found that aminoglycosides exacerbate NIPTSs in adult animals, and the general finding of protection by KM in young mice might seem to contradict many earlier findings. While the present experiments do not attempt to explain this seeming contradiction, it remains possible that protection in mice reflects their young age, the particular low doses of KM used, or some property exclusive to mice. We doubt the ultimate explanation is the latter, and would anticipate that protection will be found for a range of species and conditions. Because of the broadly applicable principles that are likely to be revealed, it is worthwhile to identify key molecular 'players' in protection of hearing in mice. Ideally, these will be factors that can be engaged pharmacologically, since no practical therapy that results is likely to actually involve KM (or any other physical triggers of preconditioning). Toward the ultimate goal of identifying molecular cascades and therapeutic targets, the present study aimed at narrowing the conditions under which KM is protective.

Previously, young CBA/J mice were administered a repeated low dose of KM; 2 dose/day for 10 days (Fernandez et al., 2010). The mice were then subjected to loud broadband noise sufficient to cause both moderate permanent threshold shift and hair cell loss. The KM completely protected the mice from a NIPTS, and significantly preserved OHCs. A later study determined KM administered in 1 dose/day and 1 dose every other day for 10 days was also

ample for protection (Rybak Rice, 2009). Conclusions support the hypothesis that KM engages a form of preconditioning. The current study sought to define the minimal KM doses that can be given to young CBA/J mice to still afford protection against a NIPTS. In addition, the generality of the previous results were explored to determine if the protection was restricted to the CBA/J strain and to young mice. It is important to define the minimal conditions for this protection as a foundation for future studies of molecular mechanisms. Presumably, increasing the level of KM engages both cellular protective and injury pathways. If we were to study mechanisms at KM levels far higher than actually required for protection, the activation of pathways not needed for protection could pose confounds for the interpretation of results. Similarly, if KM-related protection is in some way tightly tied to either the CBA/J strain, or to very young ages, such restrictions tell us something about the essential protective mechanisms. Moreover, such differences could be used to help identify essential mechanisms, through experiments aimed at what is critically different at later ages, or in other strains. The latter could apply a gene mapping approach as a 'back door' route to mechanisms.

Clues to mechanisms from the minimal effective paradigm

The minimal paradigm tests conducted in this study demonstrated that reducing the KM dosing frequency to once every third day was only somewhat effective, as protection was only seen in low frequency thresholds. Likewise, two doses applied over 3 days (72 and 24 hours prior to noise) were not sufficient. Further, one dose administered 24 and 48 hours prior to noise exposure was also ineffective for protection from noise. From this array of evidence, we conclude: 1) The mere presence of KM is not protective. Thus, mechanisms such as plugging of hair cell transducer channels are probably not involved. 2) The minimum effective levels for

KM to achieve full protection are those afforded by more than two applications every 72 hours. 3) The optimal KM-initiated protective state requires that KM dwell at sufficient levels for more than 48 hrs. These conclusions place constraints on when we may expect to generate and find the critical protective factors.

Significance of genetic background

We could not identify significant protection in B6 or CBA/CaJ mice using the same protocol that was completely successful in CBA/Js. The sample size for the CBA/CaJ was small and should therefore be enhanced in future studies to determine their ability to utilize KM for protection. The B6 data suggest that differences in genetic background include alleles that either render B6 mice less sensitive to KM related stress, or impair preconditioning mechanisms. It has been established that genetic variability among strains partly determines sensitivity to aminoglycosides (Wu et al., 2001). B6 and CBA/J mice may have differences in metabolism, cellular uptake mechanisms, or differential expression of antioxidant enzymes that alter the ability of KM to establish a preconditioned state. Additional experiments should test other KM treatment paradigms, since such strain differences could simply require different treatment schedules or doses. If other inbred mouse strains are found to not show protection, it may be possible to use inter-strain differences to genetically dissect biochemical pathways involved in KM related preconditioning or the early developmental window for ototoxicity. Muller et al. (2005) suggest this can be executed with transgenic and gene targeting technologies. Alternatively, classic mapping methods could be applied. F1 hybrid mice could be produced by crossbreeding the CBA/J with another strain, such as B6, and the protective potential of KM determined in the F1s. Depending upon whether protection by KM appears dominant or

recessive, the F1s can then be backcrossed to the recessive parental strain. 'Protectability', in the form of threshold shifts after KM with noise in the N2 backcrosss mice, could then potentially be mapped.

Protection versus injury by KM

In preconditioning, a non-damaging or minimally damaging stressor offers protection against a later more injurious event (Gagnon et al., 2007). Because there exists in the cochlea a spatial gradient of vulnerability to noise and ototoxins, it is possible that protection from noise in mid-cochlear regions can only come at the expense of KM-induced injury to the deep cochlear base. Testing the ABR frequency at 56.6 kHz was intended to test such a link. Evidence presented suggests that protection need not coincide with injury. In future experiments, particularly those involving other strains and ages, it will be useful to determine if the growth of protection at ~10-20 kHz with KM dosing is correlated with growth of threshold elevation to KM alone above 40 kHz. It will also be important to perform histologic analyses, in addition to ABR testing.

Conclusions

Results from the current research further support the protective effects of KM—a highly ototoxic compound—against NIPTS. The biochemical pathways involved, as well as similar abilities of other aminoglycosides, merit further exploration. The goal, or course, is not to apply KM itself as a therapeutic, but rather to safely mimic its benefits. Greater understanding of cochlear response to aminoglycosides may lead to the development of novel therapies.

Clinically, we can offer better patient care and perhaps medically introduce the use of ototoxic drugs as a way to protect hearing to the field of otolaryngology and audiology.

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APPENDIX A: Average thresholds per frequency per group with standard deviations in parentheses for all experimental mice

Group	Noise	n	5 kHz	10 kHz	20 kHz	28.3 kHz	40 kHz	56.6 kHz
MINIMAL DOSING								
Saline Every 3rd Day	30 s	6	55.33 (19.15)	53.0 (20.74)	58.5 (18.37)	72.5 (6.89)	72.66 (8.16)	
KM Every 3rd Day	30 s	7	40.57 (10.69)	24.43 (12.49)	31.0 (21.21)	41.43 (23.75)	56.71 (19.02)	
Saline 2 doses	n/a	3	35.33 (2.89)	18.0 (0)	26.0 (0)	25.0 (0)	34.33 (2.89)	58.0 (8.7)
KM 2 doses	n/a	4	37 (4.08)	19.25 (4.79)	24.75 (4.79)	23.75 (4.79)	33.5 (5.0)	46.75 (13.77)
Saline 2 doses	30 s	2	54.5 (10.61)	40.5 (24.75)	51.0 (28.28)	72.5 (3.5)	83.5 (10.61)	88.0 (7.07)
KM 2 doses	30 s	5	49.0 (16.81)	43.0 (20.31)	44.0 (20.80)	57.0 (19.24)	70.0 (17.46)	75.0 (15.65)
Saline 1 dose, 24 hrs	30 s	3	60.3 (15.28)	39.6 (16.07)	54.3 (25.17)	70 (14.14)	78.5 (3.54)	
KM 1 dose, 24 hrs	30 s	6	52.8 (8.61)	44.6 (24.83)	54.3 (24.22)	68.3 (20.17)	78.5 (10.37)	
Saline 1 dose, 48 hrs	30 s	3	62.0 (10.0)	54.6 (15.28)	59.3 (18.93)	76.6 (2.89)	82.6 (11.55)	
KM 1 dose, 48 hrs	30 s	7	50.6 (9.88)	33.7 (13.97)	48.1 (22.15)	60.7 (19.24)	65.3 (16.18)	

Table 1: Average ABR thresholds by experimental group at each test Hz for the minimal dosing paradigm (Standard deviations in parentheses)

Group	Noise	n	5 kHz	10 kHz	20 kHz	28.3 kHz	40 kHz	56.6 kHz
C57BL/6J								
Saline 1 dose/day	n/a	4	38.25 (4.79)	20.5 (2.89)	29.75 (6.29)	23.75 (4.79)	33.5 (2.89)	
KM 1 dose/day	n/a	5	41.0 (4.18)	22.0 (4.18)	30.0 (5.48)	24.0 (6.52)	34.0 (10.37)	
Saline 1 dose/day	4 m	7	47.43 (17.07)	32.29 (13.97)	52.43 (12.82)	62.86 (16.55)	72.43 (12.82)	
KM 1 dose/day	4 m	7	38.43 (6.90)	23.71 (3.54)	34.57 (9.45)	57.14 (22.15)	81.0 (12.91)	
CBA/J / CaJ								
KM 1 dose/day	n/a	1	38	19	31	41	39	53
SA 1 dose/day	4 m	2	45.5 (3.54)	21.5 (3.54)	38.5 (3.54)	55.0 (14.14)	64.0 (14.4)	99.0 (29.70)
KM 1 dose/day	4 m	2	38.0 (7.07)	24.0 (7.07)	36.0 (0)	55.0 (7.07)	51.5 (10.61)	75.5 (10.61)

Table 2: Average ABR thresholds by experimental group at each test Hz for the C57BL/6J and CBA/CaJ mice (Standard deviations in parentheses)

Group	Noise	n	5 kHz	10 kHz	20 kHz	28.3 kHz	40 kHz	56.6 kHz

CBA/J ADULT								
Saline 1 dose/day	15 m	7	42.23 (5.35)	36.86 (11.85)	58.86 (19.12)	68.33 (15.71)	69.0 (21.79)	75.5 (10.61)
KM 1 dose/day	15 m	8	34.88 (3.72)	22.75 (7.44)	36.63 (17.0)	41.13 (16.65)	46.5 (12.25)	61.33 (5.16)

Table 3: Average ABR thresholds by experimental group at each test Hz for the 2 month old CBA/J mice (Standard deviations in parentheses)

APPENDIX B: Daily Injections/Body Weight Data Form

Todays Date:			Factor =	
ID	Treatment	BW	Inj. Volume	

APPENDIX C: 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:**

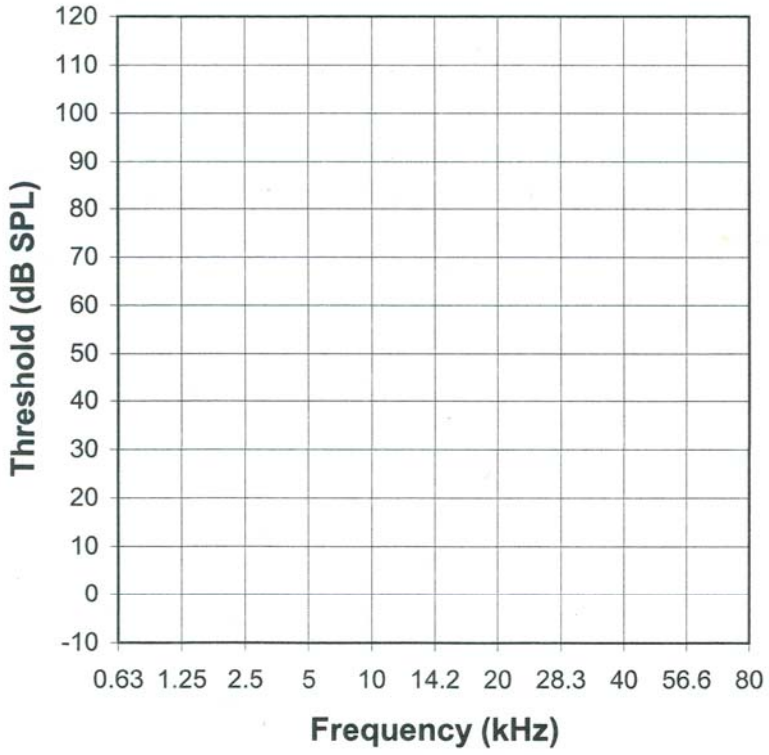
Identifying marks: 

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: