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Genetic basis for susceptibility to noise-induced hearing loss in mice

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Abstract

The C57BL/6J (B6) and DBA/2J (D2) inbred strains of mice exhibit an age-related hearing loss (AHL) due to a recessive gene (*Ahl*) that maps to Chromosome 10. The *Ahl* gene is also implicated in the susceptibility to noise-induced hearing loss (NIHL). The B6 mice (*Ahl*/*Ahl*) are more susceptible to NIHL than the CBA/CaJ (CB) mice ($+^{Ahl}$). The B6×D2.F_1 hybrid mice (*Ahl*/*Ahl*) are more susceptible to NIHL than the CB×B6.F_1 mice (+/*Ahl*) [Erway et al., 1996. Hear. Res. 93, 181–187]. These genetic effects implicate the *Ahl* gene as contributing to NIHL susceptibility. The present study demonstrates segregation for the putative *Ahl* gene and mapping of such a gene to Chromosome 10, consistent with other independent mapping of *Ahl* for AHL in 10 strains of mice [Johnson et al., 2000. Genomics 70, 171–180]. The present study was based on a conventional cross between two inbred strains, CB×B6.F_1 backcrossed to B6 with segregation for the putative +/*Ahl*:*Ahl*/*Ahl*. These backcross progeny were exposed to 110 dB SPL noise for 8 h. All of the progeny were tested for auditory evoked brainstem responses and analyzed for any significant permanent threshold shift of NIHL. Cluster analyses were used to distinguish the two putative genotypes, the least affected with NIHL (+/*Ahl*) and most affected with PTS (*Ahl*/*Ahl*). Approximately 1/2 of the backcross progeny exhibited PTS, particularly at 16 kHz. These mice were genotyped for two D10Mit markers. Quantitative trait loci analyses (log of the odds = 15) indicated association of the genetic factor within a few centiMorgan of the best evidence for *Ahl* [Johnson et al., 2000. Genomics 70, 171–180]. All of the available evidence supports a role for the *Ahl* gene in both AHL and NIHL among these strains of mice. © 2001 Elsevier Science B.V. All rights reserved.

Key words: Mouse; Noise-induced; Age-related; Hearing loss; Genetic

1. Introduction

Susceptibility for noise-induced hearing loss (NIHL) can be defined in two ways: one organism is said to be more susceptible than another when equal acoustic energy exposures result in a greater permanent threshold shift (PTS) in the susceptible individual; or alternately, to produce equal PTS in a susceptible individual requires less acoustic energy than the non-susceptible individual. A genetic basis for age-related hearing loss

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Abbreviations: +, wild type gene; ABR, auditory evoked brainstem response; AHL, age-related hearing loss; *Ahl*, the gene encoding age-related hearing loss; B6, inbred mouse strain C57BL/6J; CB, inbred mouse strain CBA/CaJ; *dfw*, deaf waddler gene; LOD, log of the odds; NIHL, noise-induced hearing loss; *mdfw*, modifier of the deaf waddler gene; PTS, permanent threshold shift; QTL, quantitative trait loci; TS, threshold shift; TTS, temporary threshold shift

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(AHL) and for susceptibility to NIHL in mice appear to be correlated (Erway et al., 1996).

Differing susceptibility to the damaging effects of noise has been documented in human populations. For example, Taylor et al. (1965) demonstrated this phenomenon among jute weavers. Female jute weavers who had been exposed to the same constant noise (99 or 102 dB) for long periods of time (1–54 years), when adjusted for age, showed as much as 70 dB difference in auditory threshold between the least affected and the most affected workers with NIHL.

Erway et al. (1993) studied AHL among five inbred strains of mice and the resulting 10 F_1 hybrid strains of mice. They utilized the auditory evoked brainstem response (ABR) to determine hearing thresholds up to 3 years of age. Some strains developed AHL much earlier than other strains, suggesting some genetic basis for accelerating presbycusis. Johnson et al. (1997) made genetic crosses between the normally hearing CAST/Ei strain and the C57BL/6J (abbreviated B6) strain which exhibits AHL. The backcross mice segregated with about 50% developing AHL by 12–18 months and about 50% with nearly normal hearing. A recessive gene designated *Ahl* for AHL, has been mapped to mouse Chromosome 10. The same *Ahl* gene with an early-onset AHL has been mapped in 10 other inbred strains, on Chromosome 10 around 24 centiMorgan (cM) (Johnson et al., 2000).

Erway et al. (1996) compared the susceptibility of mice to NIHL in two inbred strains, CBA/CaJ (abbreviated CB) wild type (+/+) and C57BL/6J (*Ahl*/*Ahl*), and in two F_1 hybrid strains: CB × B6 (+/*Ahl*) and B6 × D2(*Ahl*/*Ahl*). The +/+ and +/*Ahl* mice exhibited no PTS. The inbred B6(*Ahl*/*Ahl*) and the B6 × D2.F_1(*Ahl*/*Ahl*) mice exhibited identical patterns of 20–50 dB PTS. Moreover, both inbred and F_1 hybrid strains (*Ahl*/*Ahl*) exhibited a very significant and similar onset of AHL.

In order to demonstrate segregation and association of the posited *Ahl* gene, F_1 hybrids (CB × B6.F_1+/*Ahl*) were backcrossed to the inbred B6(*Ahl*/*Ahl*) mice with an expected ratio of 1/2 less susceptible (+/*Ahl*) to 1/2 noise-susceptible (*Ahl*/*Ahl*) progeny. The present study was conducted to demonstrate association between the *Ahl* gene mapped to Chromosome 10 and the susceptibility of the *Ahl*/*Ahl* mice to NIHL. All of the backcross progeny were exposed to noise. The mice were characterized by their response to noise exposure as revealed by threshold shifts (TS) from before exposure compared to 1, 3, 7 and 14 days after exposure.

All mice exposed to noise were genotyped for heterozygosity or homozygosity via two linked microsatellite markers located on either side of *Ahl* on mouse Chromosome 10. Any significant correlations between the NIHL response and the *Ahl* genotypes would support the role of the *Ahl* gene in susceptibility to NIHL.

2. Materials and methods

2.1. Subjects

Both inbred mouse strains were from stock originally obtained from The Jackson Laboratory (Bar Harbor, ME, USA). Inbred mice of the C57BL/6J (B6, *Ahl*/*Ahl*) strain were crossed with CBA/CaJ (CB, +/+^{Ahl}). The resulting F_1 progeny (CB × B6, +/*Ahl*) were then backcrossed to the B6 strain, producing an N2 generation consisting of approximately half heterozygous (+/*Ahl*) and half homozygous (*Ahl*/*Ahl*) progeny.

Sixty-one backcross progeny were used in this investigation. The backcross study group included mice of both sexes, approximately 4 months old. Mice at this chronological age have fully mature auditory systems, but do not yet exhibit signs of AHL (Erway et al., 1993). The breeding, maintenance, transport, and experimental protocols were approved by the Institutional Animal Care and Use Committees for The University of Cincinnati and for The National Institute for Occupational Safety and Health, Cincinnati, OH, USA.

2.2. Noise exposure

Mice were exposed awake. Cages were constructed from stainless steel mesh and contained six compartments. Each mouse was confined to a compartment with opportunity to move about within the compartment. Such separation of mice minimized huddling and shadowing with maximally uniform exposure to noise. The cages were placed into each of two noise exposure chambers. Noise exposures were limited to approximately 20 subjects per session. When it became necessary to expose for 8 h, fresh apple slices were provided as a source of food and water of which some was consumed.

The exposure facility has been previously described (Davis and Franks, 1989). The acoustic system consisted of a General Radio Type 1310 random noise generator, whose output signal was controlled by a Wilsonics BSIT Tone Switch and PATT attenuators. The output of the attenuators was amplified by a Soundcraftman 4 × 300 Power Amplifier, a single channel of which drove four Realistic #40-1310B super-tweeters built into a removable chamber cover. A graph of the one third octave analysis of the broad-band noise has been presented elsewhere (Erway et al., 1996). It is virtually flat between 8 and 20 kHz.

Calibration and monitoring of the sound within each chamber was performed by a Sennhauser MKE 2–3

electret microphone whose input was displayed and analyzed on a Brüel and Kjær 2133 Real Time Frequency analyzer. All measurements were made without frequency weighting, using the linear scale. Calibrations were confirmed pre- and post-exposure by a Brüel and Kjær 4230 Sound Level calibrator.

Sixty-one mice received a broad-band noise exposure of 110 dB SPL for 8 h. To further distinguish the heterozygotes (+/*Ahl*) and (+/+), 1–2 months after the first noise exposure 14 mice with somewhat ambiguous NIHL were re-exposed to 110 dB for 4 h.

2.3. ABR measures

Prior to and following noise exposure, the ABR was used to determine auditory threshold among the backcross progeny. Mice were anesthetized with an i.p. injection of tribromoethanol, 3.5 mg/10 g body weight. Anesthesia was supplemented throughout testing as needed. The animal was placed on a heating pad during testing to maintain body temperature and on a warming tray during recovery from anesthesia. Mice exhibiting abnormal ABRs or threshold elevations of greater than or equal to 20 dB in excess of the strain average prior to exposure were excluded from the study.

The ABR was evoked and averaged by an Intelligent Hearing System ABR unit (North Miami, FL, USA) connected to a laptop computer. An auditory stimulus was delivered by headphones (AKG-K340) and presented binaurally via plastic funnels. Grass stainless steel needle electrodes were inserted subcutaneously at the vertex (active), ventrolateral to the left ear (inverting) and the dorsum (ground). A Grass 511 preamplifier boosted the biological signal before presentation to the ABR unit for analysis and display. The signal was averaged for 512 sweeps or until a reproducible waveform could be seen, whichever came first.

Auditory threshold was determined by the lowest stimulus intensity ± 5 dB where at least two peaks of the ABR waveform could be visually detected. Individual hearing loss was quantified based upon the TS between pre- and post-exposure ABR thresholds. TS were measured for tone pips at 8, 16 and 32 kHz (3 ms duration, 1 ms rise and decay time) and click stimuli (0.1 ms duration). Mice were evaluated 1 day before and 1, 3, 7, and 14 days after the initial noise exposure. A subgroup of 14 mice from the main study was re-exposed and measured at the same post-exposure time intervals.

ABR thresholds were determined for every mouse 1–3 days before transport and exposure to noise.

2.4. Phenotype evaluation

Subjects exhibiting a positive TS of at least 20 dB lasting at least 14 days post-exposure, were classified as susceptible to NIHL (the putative *Ahl*/*Ahl* genotype). Subjects exhibiting temporary threshold shift (TTS) of similar magnitude, but lasting less than two weeks post-exposure, were considered normal hearing (the putative +/*Ahl* genotype). In addition, classifications of auditory phenotype were made using cluster analysis (SAS® Proc Cluster, Ward's Minimum-Variance method) of TS to 16 kHz tone pips at 1, 2, 7 and 14 days following noise exposure. Mice were clustered into their most likely phenotypic groups (NIHL vs. normal hearing) based upon the similarity of their TS over time.

Analysis of variance was also used to analyze the data. ABR TS were compared with respect to genotype, days post-exposure, noise stimulus condition, experiment, gender and coat color (agouti (*A*/*a*) vs. black (*a*/*a*)). The criterion level for statistical significance was set at $P < 0.05$.

2.5. Genotype evaluation

Tail-tip samples were collected from all mice, preserved in 95% ethanol in separate vials, and shipped to author G.C. DNA was prepared from the tail-tip tissue using the proteinase K method (Blin and Stafford, 1976). Primer pairs for microsatellite markers D10Mit3 and D10Mit31 of Chromosome 10 were from Research Genetics (Huntsville, AL, USA). (D10Mit3 located at $\sim$21 cM and D10Mit31 at $\sim$36 cM on Chromosome 10). The polymerase chain reaction (PCR) was performed in a reaction volume of 30 μl, with 50 ng of each mouse DNA, 15 pmol of each of two primers, one of which was end-labeled with γ-ATP, 0.25 mM dNTPs and 1 unit of *Taq* DNA polymerase in PCR buffer. After denaturation at 94°C for 3 min, 25–28 cycles of PCR were carried out. Each cycle consisted of denaturing at 94°C for 1 min, annealing at 54–58°C for 1 min and extension at 72°C for 30 s. PCR products were electrophoresed through 8% denaturing polyacrylamide gels, which were subsequently exposed to X-ray film (Kodak X-O-Mat). Fig. 1 includes a representative gel for analysis of the D10Mit3 locus in 15 backcross mice (numbers 45–59).

Genetic distance between the gene affecting the susceptibility to NIHL phenotype and the D10Mit3 and D10Mit31 markers was estimated by determining the recombination frequencies between them. One percent recombination frequency is defined as 1 cM of genetic distance.

Following genetic mapping of the mice, the quantitative trait loci (QTL) analysis (MAPMAKER/QTL, version 1.1b) was used to establish a relation between the 20 measured phenotypic traits for each mouse (ABR thresholds for clicks, 8, 16, and 32 kHz tone pips over test days 1, 3, 7 and 14 days post-exposure). These

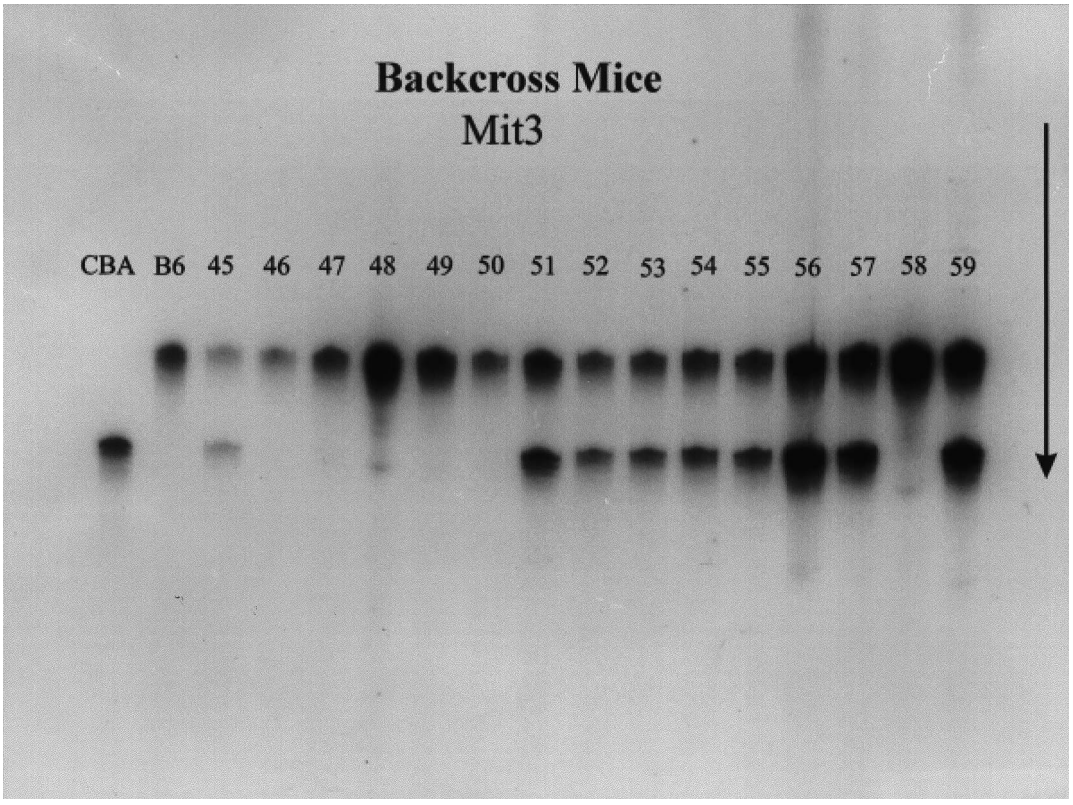

Fig. 1. Example of results of microsatellite analysis of D10Mit3 in 15 backcross mice. Note in the two leftmost lanes are the controls for CBA/CaJ and B6 (C57BL/6J). Each lane represents a single mouse. Those lanes with a single band are homozygous for the B6 type (presumably *Ahl*/*Ahl*) (lanes 46–50, and 58). Those lanes with two bands are heterozygous for the B6 and CBA type (presumably *Ahl*/+) (lanes 45, 51–57, and 59).

measured phenotypes were analyzed by computations of log of the odds (LOD) scores over the map distances (cM intervals) between the D10Mit3 (~21 cM) and D10Mit31 (~36 cM) genetic markers which flank the *Ahl* gene on Chromosome 10. LOD scores of 3.0 or more are considered evidence for genetic linkage of the measured phenotypes with gene(s) within this region of Chromosome 10.

3. Results

3.1. Preliminary results for intensity of noise exposure in the CB×B6.N2 generation of mice

Erway et al. (1996) showed that the C57BL/6J and B6×D2.F1 (*Ahl*/*Ahl*) mice exhibited NIHL after a 2 h exposure to 110 dB SPL, whereas the CB and CB×B6.F_1 mice did not exhibit any NIHL. In preparation for the study of NIHL among the CB×B6.N2 generation of mice, preliminary regimes of noise exposure were undertaken, and are summarized here. This included a cohort of the CB×B6.N2 mice exposed to 110 dB SPL for 2 h. However, none of the mice exhibited any PTS. The same cohort of mice was subjected to another exposure of 110 dB for 2 h, but they still failed to exhibit any significant NIHL. A new cohort of mice was exposed to 110 dB for 4 h; they also failed to exhibit any significant PTS. Finally, a cohort of these mice was exposed to 110 dB for 8 h. Among this co-

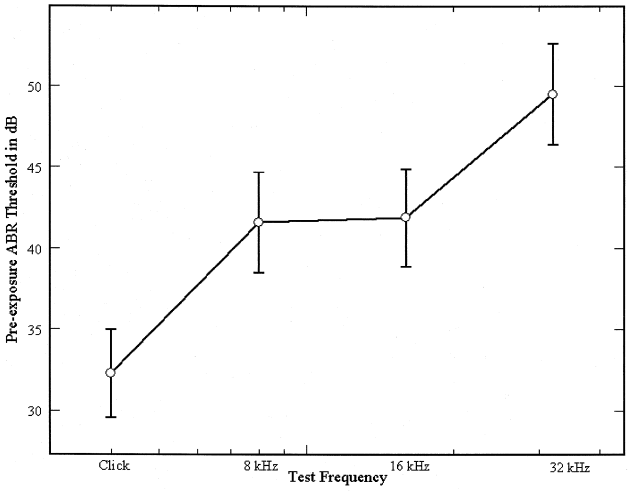

Fig. 2. Pre-exposure ABR threshold for all 61 mice. Error bars indicate standard deviation. Mice were tested with a click and tone pips at 8, 16 and 32 kHz.

hort, and then among all successive cohorts, about half of the CB×B6.N2 progeny exhibited significant PTS, whereas approximately equal numbers did not exhibit NIHL.

3.2. Results for NIHL

Sixty-one mice of the CB×B6.F_1 backcross (N2) progeny exhibited the average pre-exposure ABR thresholds for clicks and tone pips of 8, 16 and 32 kHz shown in Fig. 2. All 61 of these mice were exposed for 8 h to 110 dB broad-band noise. When these mice were finally retested at 14 days after noise exposure, they exhibited a bimodal distribution for threshold differences (−5 to +40 dB) for the 16 kHz stimulus (see Fig. 3). About half (32) of the mice had recovered ABR thresholds within normal range (≤15 dB) assuming a ±5 dB error for testing. The other half (29) exhibited 20–40 dB PTS. This bimodal distribution is consistent with the presumed $\frac{1}{2}$ +/*Ahl*: $\frac{1}{2}$ *Ahl*/*Ahl* genotypes. The CB×B6.N2 mice differed considerably with regard to the effect of noise exposure on the sensitivity to the four stimuli: clicks, and 8, 16 and 32 kHz. TSs were determined for each mouse as the difference between the pre-exposure threshold and any elevated threshold determined at 1, 3, 7 and 14 days after the exposure to noise. A PTS was defined as TS ≥ 20 dB at 14 days post-exposure. (1) For clicks, only three mice exhibited PTS. (2) For 8 kHz, 35 mice exhibited TS at 3 and/or 7 days,

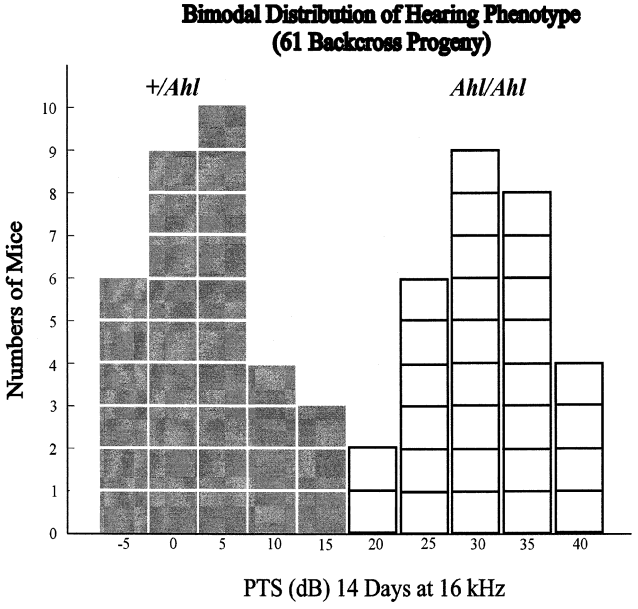

Fig. 3. PTS at 16 kHz, 14 days post-exposure. The *y*-axis shows the number of mice with that level of PTS. Note the bimodal character of the distribution suggesting segregation of the Ahl gene into heterozygous and homozygous groups. Based on reaction to noise, the shaded bars represent heterozygotes and the open bars represent homozygotes.

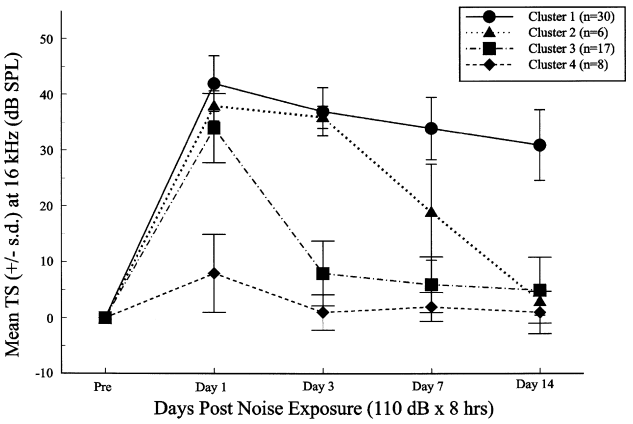

Fig. 4. Results of cluster analysis of TS. The *x*-axis displays the time post-exposure. The *y*-axis shows the TS to a 16 kHz tone pip at each time post-noise exposure. The cluster analysis was able to demonstrate four clusters based on the shapes of the recovery curves. These curves could be further reduced to two curves. The error bars indicate standard deviation.

but only four mice exhibited PTS. (3) Similarly for 32 kHz, many mice exhibited TS, but only four mice exhibited PTS.

By contrast, the TS for the 16 kHz sensitivity were much greater than for the other stimuli (see Fig. 4).

By day 1 after noise exposure, 51 (87%) of the mice displayed TTSs in the range of 25–50 dB. Over the course the next 2 weeks, 31 (50%) of the mice recovered to normal hearing thresholds.

The cluster analysis for all of the TS at 1, 3, 7 and 14 days for 16 kHz identified four clusters of mice which differed in their response to and recovery from noise exposure (Fig. 4). By 1 day after noise exposure, clusters 1, 2 and 3 displayed elevated mean TS of 34–42 dB, whereas cluster 4 ($n=8$) displayed an average TS of 8 dB. The major differences among the clusters emerged in their patterns of hearing recovery over the 2 weeks of testing. By day 3 after noise exposure, mice in clusters 1 ($n=30$) and 2 ($n=6$) continued to display average TS of approximately 40 dB, while those in cluster 3 ($n=17$) had recovered to less than 10 dB of TS. The mice in cluster 2 were recovering by day 7 and complete by day 14. The mice in cluster 3 ($n=17$) recovered rapidly (days 1–3) from 34 dB TS to less than 10 dB.

Despite the noted differences among the four clusters, the next to the last step of this analysis identified similarities that permitted their arrangement of all 61 of the N2 progeny into the two major NIHL susceptibility phenotypes. The first major group was comprised of the 30 mice in cluster 1. Mice within this major group demonstrated the more susceptible phenotype, with ∼30 dB PTS (cluster 1).

Cluster 2 mice ($n=6$) had TS comparable to cluster 1 at days 1 and 3, but they completely recovered through days 7 and 14. Cluster 3 ($n=17$) with ∼35 dB TS at

day 1, and cluster 4 ($n = 8$) with < 10 dB TS at day 1 exhibited no PTS. The sum of these three clusters equals 31, the posited 1/2 for the +/*Ahl* genotype.

These cluster analyses provided a more objective classification that complemented the classification using the bimodal distribution based only on the final PTS at day 14. There is no significant difference between the phenotypic ratios derived using the objective cluster analysis (30 susceptible to PTS: 31 normal) and the more subjective bimodal distribution based on the final ABR thresholds at day 14 (32 susceptible: 29 normal).

3.3. Genotype analysis

The DNA of the 61 backcross progeny was typed for the D10Mit3 and D10Mit31 marker on Chromosome 10. Polymorphisms exist between the two markers with respect to their parental source (CB vs. B6 mouse strains). Homozygosity for the B6 isoform (only one band) or heterozygosity for both isoforms (two bands) was used to determine the genotype of each of the backcross progeny (Fig. 1).

A total of 25 mice displayed homozygosity for the parental B6 form of the D10Mit3 and D10Mit31 markers, with presumed homozygosity (*Ahl*/*Ahl*). Similarly, 24 mice which were heterozygous for both the CB and B6 markers displayed the parental (CB × B6) pattern of inheritance, indicative of heterozygosity (+/*Ahl*) for the *Ahl* alleles. The remaining 12 N2 mice exhibited recombination between the D10Mit3 and D10Mit31 markers. Given the estimated 15 cM between these two D10Mit markers one would expect ($0.15 \times 61 \cong 9$ mice). Single crossovers between the two D10Mit markers could affect seven different D10Mit3 _*Ahl*_D10Mit33 genotypes.

The D10Mit genotypes and hearing phenotypes of the backcross progeny were compared. Seven classes of mice were identified which exhibited either concordance or discordance between their D10Mit genotypes and their observed hearing phenotypes (Fig. 5). A total of 41 (87%) mice were concordant for phenotype and genotype.

Among the parentals (homozygous for both D10Mit markers), 22 mice with the putative *Ahl*/*Ahl* genotype exhibited PTS. Among the non-parentals (heterozygous for both D10Mit markers) 19 mice with the +/*Ahl* genotype, heard normally. This 87% concordance is strong evidence for *Ahl* being located between these two D10Mit markers.

There were 12 single crossover events between the two D10Mit markers. That result is equivalent to 20% (20 cM), but it is not significantly different from the predicted nine ($0.15 \times 61 \cong 9$) recombinants between these two markers. The other 8 (13%) mice that were

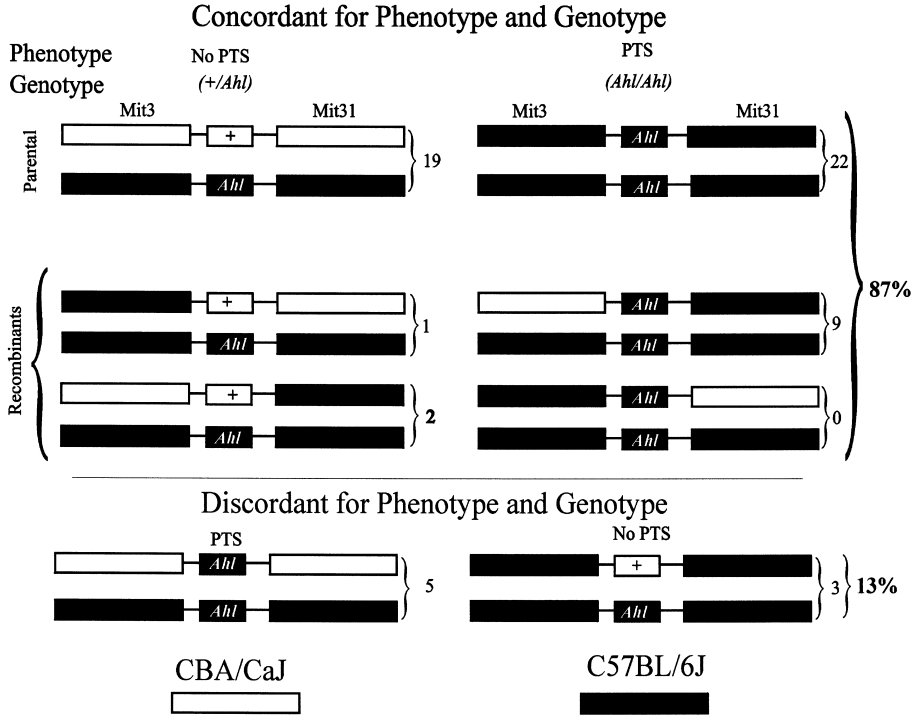

Fig. 5. Schematic diagram for genotype and phenotype. The left column displays mice which showed no PTS, the right displays mice with a PTS. Each row shows the paired chromosomes-one from each parent. The top rows show the parental types, which account for the majority of mice. The 2nd and 3rd rows show presumed recombinants with the corresponding phenotype. The 4th row demonstrates genotypes with discordant phenotypes and which, perhaps, indicate a double crossover.

discordant for the D10Mit genotypes and AHL phenotypes. These would have required an explanation based on double crossover events, which is most unlikely ($P < 0.01$). It is more likely that the ability to identify the noise susceptible phenotype is limited, especially at the cut-off shown in the bimodal distribution (Fig. 2).

QTL analyses were conducted on ABR TS to 20 measured traits among backcross progeny typed for the D10Mit3 and D10Mit31 markers. Of these QTL analyses, the 16 kHz data were significant. A QTL analysis was conducted for all TS determined at day 14, for the 16 kHz frequency for all 61 backcross progeny. (The QTL analysis computes a LOD score for intervals between the chosen D10Mit markers, in this case between D10Mit3 ($\sim$21 cM) and D10Mit33 ($\sim$36 cM).) LOD scores of 15 were computed for 4 of the 12 computed intervals between these two D10Mit markers. This is the strongest evidence for linkage of the putative *Ahl* gene between these two D10Mit markers.

The data were subsequently re-analyzed to rule out the influence of extraneous variables including sex, experimental run, and coat color. Separate ANOVAs were performed on TS at 16 kHz for each of these variables. The models included terms for strain, test, and all possible interactions. The main effect of test and all interactions with test were within-subject terms. The other main effects and interactions were between-subjects terms. The main effect of sex [$F(1, 50) = 0.63$, $P = 0.43$] and interactions involving sex were not statistically significant. Due to the logistics of testing, the study was separated into three identical experiments of 20 mice each on different dates. These different run dates were analyzed to rule out any effect of experimental run. The main effect of experimental run was not statistically significant [$F(2, 43) = 0.69$, $P = 0.51$], nor were any interactions involving experimental run. Finally, the gene for agouti coat color (*A*/*A*) also segregates, so it was necessary to rule out the possibility of association between the *Ahl* allele and another genetic locus. The main effect of coat color [$F(1, 48) = 2.05$, $P = 0.16$] and interactions involving coat color were not statistically significant.

4. Discussion

One may question the possibility that a gene identified for its contribution to AHL could also affect NIHL. Clearly the ear is most sensitive to sound stimulation, and it is especially vulnerable to the most intense and acute acoustic stimuli. We have deduced from the following genetic studies that one gene among highly inbred strains of mice contributes to AHL and to NIHL:

The final ABR threshold at day 14 post-exposure is informative for distinguishing the less and more susceptible phenotypes. However, the cluster analysis has objectively clustered the 61 progeny into the single, largest (30) N2 progeny with a uniform pattern of PTS and minimal recovery to PTS in the putative *Ahl*/*Ahl* segregants of the N2 progeny. By contrast, the heterogeneity among clusters 2, 3, and 4 of the putative +/*Ahl* N2 progeny, may be related to the genetic heterogeneity as the discussed basis for the required 8 h exposure to induce the demonstrated incidence of PTS.

The *Ahl* gene affecting AHL in 10 inbred strains has been mapped to Chromosome 10 at about 24 cM (Johnson et al., 2000). The CBA/2J(CB,+^{Ahl}) mice hear normally during their lifetime and are less susceptible to NIHL. The C57BL/6J (B6, *Ahl*) mice exhibit AHL by mid-life and are more susceptible to noise. The CB$\times$B6.F$_1$(+/*Ahl*) mice did not exhibit either AHL or susceptibility to NIHL. The DBA/2J (D2, *Ahl*) mice are deaf by 4 months of age. The B6$\times$D2.F$_1$ (*Ahl*/*Ahl*) mice exhibit AHL and are susceptible to NIHL (Erway et al., 1996). In the present study the CB$\times$B6.F$_1$ (+/*Ahl*) mice backcrossed to B6(*Ahl*/*Ahl*) yield progeny: 1/2+/*Ahl* less susceptible to NIHL and 1/2 *Ahl*/*Ahl* exhibiting susceptibility to NIHL. Furthermore, the putative *Ahl* gene(s) for AHL and for NIHL map(s) very closely on mouse Chromosome 10 (between D10Mit3 ($\sim$21 cM) and D10Mit31 ($\sim$36 cM), about a 15 cM region).

One of the primary objectives of the current study was to determine if susceptibility to NIHL is strongly associated with the putative *Ahl* gene. Susceptibility to NIHL was previously reported among inbred mouse strains or hybrids whose genomes are heterozygous or do not contain the *Ahl* gene (Erway et al., 1996). While this information strongly supports such association, the possible role of a genetic factor (presumably the *Ahl* gene) in noise susceptibility, it does not prove it. Such proof must come from demonstrating equal segregation and assortment of the *Ahl* gene among backcross progeny. If the gene conferring increased susceptibility to NIHL segregates equally during meiosis, one half of the progeny should be susceptible and one half should be less susceptible.

The challenge for demonstrating phenotypic segregation involved selecting a noise exposure sufficient in intensity and duration to produce a TS in the susceptible mice while not producing a significant loss in the less susceptible mice. Due to the large inter-animal variability (Cody and Robertson, 1983), this has presented a problem in the past. The genetically well-defined inbred mouse models allow for better selection of these parameters. Even so, the CB$\times$B6:N2 backcross generation required a much longer (8 h vs. 2 h) noise expo-

sure than in the inbred and F_1 hybrid mice in the previous experiments (Erway et al., 1996, Davis et al., 1999).

Highly inbred strains of mice (30+ generations of brother×sister matings) are as genetically homogeneous as are monozygotic twins. Inbred mice are all genetically homogeneous with homozygosity at tens-of-thousands of genetic loci. By comparison, all crosses between any two inbred strains yield F_1 hybrid mice that are genetically homogeneous. However, the F_1 hybrids are homozygous for alleles that the two inbred strains shared, and the F_1 hybrids are heterozygous for all loci for which the inbred parents had different alleles. Such complete genetic homogeneity, plus heterozygosity for many loci contribute most to the uniform phenotypes and 'F_1 hybrid vigor'. Such F_1 homogeneity plus 'heterozygous advantage' persists only with deliberate and repeated crosses between the same two inbred strains. If the F_1 hybrids are allowed to interbreed, the F_2 progeny will exhibit maximal genetic heterogeneity. Similarly, backcrossing any F_1 hybrid strain to either parent strain will yield maximal genetic heterogeneity, plus equal segregation for any recessive alleles among the original parents.

As noted above, the B6 and the B6×D2.F_1 (*Ahl*/*Ahl*) mice exhibited NIHL in response to exposure to 110 dB SPL for 2 h. In the present study the CB×B6.F_1 backcross mice (*Ahl*/*Ahl*) required a noise exposure of 110 dB SPL for 8 h before any of the N2 progeny exhibited NIHL (PTS to 14 days). This four-fold increase in noise exposure (+6 dB SPL) that was required to obtain a comparable NIHL may be related more to the genetic heterogeneity of the N2 progeny, than directly to the *Ahl*/*Ahl* genotype. Such genetic heterogeneity may also have contributed to most of the variability observed among and within clusters 2, 3, and 4. Nevertheless, it is remarkable that any single gene, whatever its actual molecular role, makes a demonstrable major contribution to NIHL susceptibility. The variation for onset and severity of AHL among the *Ahl*/*Ahl* N2 generations of 10 different inbred strains, is consistent with the variability found for NIHL among the CB×B6.N2 generation of mice. Such genetic heterogeneity among non-inbred and line-bred stocks may be why other researchers see such high variability in other species (e.g. Cody and Robertson, 1983).

Why is the test frequency of 16 kHz most affected in the mouse? The maximum auditory sensitivity for the mouse is located approximately at 16 kHz (Fay, 1988). Also, we have shown that if the exposure stimulus does not contain enough acoustic energy at 16 kHz that no measurable PTS will result (Davis, 2001). Thus the maximum PTS at 16 kHz is probably similar to the 4 kHz 'notch' seen in humans due to occupational noise exposures.

Yoshida et al. (2000) reported that one (129S6/SvEv-Tac) of the sixteen 129 inbred strains 'is exceptionally resistant to NIHL' compared to in the standard well-hearing CBA/CaJ inbred strain. Some of the 129 strains exhibit AHL (Zheng et al., 1999). The '129' strains are notably polymorphic for many genes (Simpson et al., 1997; Threadgill et al., 1997).

Erway et al. (1996) demonstrated that the CBA/CaJ and CB×B6.F1 mice are resistant to NIHL after 2 h exposures to 110 dB SPL, whereas the C57BL/6J and B6xD2.F1 mice were equally susceptible to the same level of noise exposure. Furthermore, Davis et al. (1999) showed that NIHL in the CBA/CaJ mice was quantitatively shifted ~3 dB SPL higher than in the C57BL/6J mice.

Given the known genetic heterogeneity among the 129 strains, we will not postulate a particular genetic basis for the greater resistance to NIHL in the 129S6 strain than in the normally well-hearing (no AHL) CBA/CaJ mice. The Yoshida et al. (2000) parameters of noise exposure (16–32 kHz octave band) and evaluation (DPOAEs) were nevertheless different than our broad-band noise exposures and ABR thresholds for the CBA/CaJ and C57BL/B6 mice. Resolution of any major genetic effects on NIHL will require appropriate backcrosses as demonstrated herein for the CB×B6.F1 backcrossed to B6. Such differences are more likely to involve two-gene interactions as demonstrated between modifier of the deaf waddler gene (*mdfw*) and PMCA2 (Kozel et al., 2001; Noben-Trauth et al., 1997).

The present study demonstrates that the susceptibility to NIHL is associated with the *Ahl* gene. These data indicate that the noise susceptibility phenotype and the *Ahl* genotype are so highly associated (as evidenced by the high LOD score for 16 kHz) that they are virtually indistinguishable.

The *Ahl* gene maps to the same portion of Chromosome 10 as the waltzer (*v*) mutation (Johnson et al., 1997) and may map to the same allele as the modifier gene (*mdwf*) for the deaf waddler gene (*dwf*) mutation on Chromosome 6 (DiPalma et al., 2001). Deaf waddler is a mutation of the gene encoding Plasma Membrane Calcium ATPase 2 (PMCA2) (Street et al., 1998). The physiological basis of how *Ahl* interacts with *dwf*, or how it confers NIHL susceptibility, is not presently known and awaits further molecular biological study.

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