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Genetic background effects on age-related hearing loss associated with *Cdh23* variants in mice

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Abstract

Inbred strain variants of the *Cdh23* gene have been shown to influence the onset and progression of age-related hearing loss (AHL) in mice. In linkage backcrosses, the recessive *Cdh23* allele (*ahl*) of the C57BL/6J strain, when homozygous, confers increased susceptibility to AHL, while the dominant allele (*Ahl*+) of the CBA/CaJ strain confers resistance. To determine the isolated effects of these alleles on different strain backgrounds, we produced the reciprocal congenic strains B6.CBACa-*Cdh23*^{Ahl+} and CBACa.B6-*Cdh23*^{ahl} and tested 15-30 mice from each for hearing loss progression. ABR thresholds for 8 kHz, 16 kHz, and 32 kHz pure-tone stimuli were measured at 3, 6, 9, 12, 15 and 18 months of age and compared with age-matched mice of the C57BL/6J and CBA/CaJ parental strains. Mice of the C57BL/6N strain, which is the source of embryonic stem cells for the large International Knockout Mouse Consortium, were also tested for comparisons with C57BL/6J mice. Mice of the C57BL/6J and C57BL/6N strains exhibited identical hearing loss profiles: their 32 kHz ABR thresholds were significantly higher than those of CBA/CaJ and congenic strain mice by 6 months of age, and their 16 kHz thresholds were significantly higher by 12 months. Thresholds of the CBA/CaJ, the B6.CBACa-*Cdh23*^{Ahl+}, and the CBACa.B6-*Cdh23*^{ahl} strain mice differed little from one another and only slightly increased throughout the 18-month test period. Hearing loss, which corresponded well with cochlear hair cell loss, was most profound in the C57BL/6J and C57BL/6N strains. These results indicate that the CBA/CaJ-derived *Cdh23*^{Ahl+} allele dramatically lessens hearing loss and hair cell death in an otherwise C57BL/6J genetic background, but that the C57BL/6J-derived *Cdh23*^{ahl} allele has little effect on hearing loss in an otherwise CBA/CaJ background. We conclude that although *Cdh23*^{ahl} homozygosity is necessary, it is not by itself sufficient to account for the accelerated hearing loss of C57BL/6J mice.

Keywords

age-related hearing loss; *Cdh23*; *ahl*; C57BL/6J; C57BL/6N; CBA/CaJ; inbred mouse strains; congenic mouse strains; cochleograms; ABR thresholds; hair cells

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1. Introduction

Age related hearing loss (AHL), or presbycusis, poses a serious human health concern as it negatively affects the life of many elderly individuals (Dalton et al., 2003). In a 2003 CDC study, presbycusis was found to be second only to arthritis as a handicapping condition within the elderly segment of our society (Bielefeld et al., 2010). Susceptibility to AHL has a significant genetic component although AHL presents with a complex pathophysiology (DeStefano et al., 2003). A range of environmental factors, including exposure to loud noises or ototoxic drugs, can exacerbate and accelerate AHL. The high degree of genetic complexity in human populations coupled with highly variable environmental factors make it difficult to identify the genetic components underlying AHL in the human population, although some limited success has been achieved (Bared et al., 2010; DeStefano et al., 2003; Friedman et al., 2009; Garringer et al., 2006; Rodriguez-Paris et al., 2008; Unal et al., 2005).

Laboratory mice are more amenable to studies of AHL predisposition because of their well-defined genetics, short life span and the ability of researchers to control for environmental factors. Like humans, many inbred mouse strains express variable degrees of AHL, and at least ten quantitative trait loci associated with AHL have been identified in mice (Latoche et al., 2011; Noben-Trauth et al., 2009). C57BL/6J (B6J) and CBA/CaJ (CBA) are the two most widely used inbred strains in hearing research. The B6J strain has been used extensively as a model of early onset AHL (Henry et al., 1980; Hequembourg et al., 2001b; Mikaelian, 1979; Wang et al., 2008; Willott, 1986). B6J mice exhibit a high frequency hearing loss by 3-6 months of age that progresses to a profound impairment by 15 months. In contrast, CBA mice remain resistant to AHL until 15 months of age or older and are often used as good hearing controls (Henry et al., 1980; Li et al., 1991; Zheng et al., 1999). B6J and CBA mice also exhibit dramatically different rates of cochlear hair cell loss (Spongr et al., 1997b).

One of the major genetic factors contributing to hearing loss in B6J mice is the *ahl* locus on Chromosome 10 (Johnson et al., 1997), which has been shown to be a splice variant of the cadherin 23 gene (*Cdh23*) (Noben-Trauth et al., 2003). The CBA strain does not have this predisposing *Cdh23* splice variant, consistent with its much slower rate of hearing loss. Cadherin 23 has been shown to be a component of the tip links of hair cell stereocilia, along with protocadherin 15 (Kazmierczak et al., 2007). Mutations in the genes encoding these two proteins affect tip link formation and stability, which is necessary for normal hair cell mechanotransduction (Alagramam et al., 2011). Mice with an ENU-induced missense mutation in the ectodomain of *Cdh23* show a loss of tip links that correlates with a progressive hearing loss that is similar to, but more severe than the hearing loss associated with the *ahl* splice variant of *Cdh23* (Schwander et al., 2009).

Here we report on the characterization of two reciprocal congenic strains that we developed to evaluate the isolated effects of *Cdh23* allelic variants when on different strain backgrounds. The CBACa.B6-*Cdh23^{ahl}* congenic strain (CBA.B6-*ahl*) is homozygous for the recessive AHL susceptibility allele (*ahl*) of *Cdh23*, which we transferred from the B6J strain onto the CBA background. The reciprocal B6.CBACa-*Cdh23^{Ahl+}* congenic strain (B6.CBA-*Ahl+*) is homozygous for the dominant AHL resistance allele (*Ahl+*) of *Cdh23*, which we transferred from the CBA strain onto the B6J background. To follow the progression of hearing loss in the congenic strain mice and mice of the B6J and CBA parental strains, auditory brainstem response (ABR) thresholds were measured at 3, 6, 9, 12, 15 and 18 months of age. After the final ABR test, inner ears of mice from each strain were examined for cochlear hair cell loss to determine its correspondence with hearing loss..

We also report on hearing loss in mice of the C57BL/6N strain (B6N), an NIH subline of C57BL/6 separated from B6J in 1951. A complete description of the B6N strain at The Jackson Laboratory (C57BL/6NJ, Stock #005304) and its development is given in the JAX mice database (<http://jaxmice.jax.org/strain/005304.html>). Like B6J, the B6N strain carries the *Cdh23^{ahl}* hearing loss susceptibility allele, but it has not previously been assessed for AHL. Because the B6N strain is the source of embryonic stem cells for the International Knockout Mouse Consortium (<http://www.knockoutmouse.org/>), establishment of a hearing profile for this control strain is important for assessing hearing-related phenotypes of the many knockout mice being generated by this program.

2. Materials and methods

2.1 Mice and congenic strain development

All mice examined in this study originated from The Jackson Laboratory (<http://www.jax.org/>), and all procedures involving their use were approved by the Institutional Animal Care and Use Committee. The Jackson Laboratory is accredited by the American Association for the Accreditation of Laboratory Animal Care.

We use the following abbreviated strain designations throughout the paper, which are followed in parentheses by their corresponding full inbred strain names and Jackson Laboratory Stock Numbers: CBA (CBA/CaJ, Stock #000654), B6J (C57BL/6J, Stock #000664), B6N (C57BL/6NJ, Stock #005304), B6.CBA-Ahl+ (B6.CBACa-Cdh23^{CBA/CaJ}, Stock #10615) and CBA.B6-ahl (CBACa.B6-Cdh23^{ahl}, Stock #10614). The region of the *Cdh23* gene that is associated with AHL in inbred mouse strains (Noben-Trauth et al., 2003) was amplified by PCR from the genomic DNA of each of these strains, and *Cdh23^{ahl}* and *Cdh23^{Ahl+}* genotypes were confirmed by DNA sequence analysis (Supplementary Fig.1).

The B6.CBA-Ahl+ and CBA.B6-ahl reciprocal congenic strains were developed by genetic introgression of B6J- and CBA-derived segments of Chromosome 10 (containing the *Cdh23* gene) into the genomes of CBA and B6J, respectively. To accomplish this introgression, mice from the B6J and CBA parental strains were first intercrossed to generate F1 hybrids. The F1 hybrids were then backcrossed to B6J mice to produce N2 generation mice for development of the B6.CBA-Ahl+ strain and to CBA mice to produce N2 mice for development of the CBA.B6-ahl strain. Repeated backcrossing of hybrid progeny (selected on the basis of Chromosome 10 marker genotypes) to the parental (B6J or CBA) strain continued until 10 backcross generations (N10) were completed. Finally, the N10 generation mice of each line were interbred to produce the two homozygous congenic strains. To define the extent of the congenic regions in each strain, single nucleotide polymorphisms (SNPs) along the length of Chromosome 10 were genotyped. The CBA-derived congenic interval of B6.CBA-Ahl+ extends from 53.46 Mb (SNP *rs3696307*) to 105.44 Mb (SNP *rs13480749*), and the B6J-derived congenic interval of CBA.B6-ahl extends from 38.69 Mb (SNP *rs13480581*) to 74.61 Mb (SNP *rs13480654*).

2.2 Hearing assessment: auditory-evoked brainstem response

Hearing in mice was assessed by ABR threshold analysis, essentially as previously described (Zheng et al., 1999). Mice were anesthetized with an intraperitoneal injection of tribromoethanol (0.2 ml of 12.5 mg/ml stock per 10 g of body weight), and then placed onto a 37 °C heating pad in a sound-attenuating chamber. The evoked brainstem responses to 8, 16 and 32kHz tone-bursts stimuli were amplified and averaged. By varying the sound pressure level (SPL) in 5 dB increments, ABR thresholds were defined as the lowest dB SPL level at which an ABR pattern could be clearly recognized with peaks 1-2 being the most prominent at high levels and still recognizable at intensities near threshold. Stimulus

presentation, data acquisition and analysis were performed using computerized equipment from Intelligent Hearing Systems (IHS; Miami, Florida). 100 dB was the maximum SPL presented for all stimuli. With our testing system, average ABR thresholds (in dB SPL) for normal hearing mice are about 40 dB for 8 kHz, 20 dB for 16 kHz, and 45 dB for 32 kHz stimuli.

2.3 Cochlear histology

Cochleae were prepared and evaluated as described in detail in earlier publications (Ding et al., 2001; Johnson et al., 2010; Zheng et al., 2009). Mice were euthanized by CO₂ asphyxiation, and decapitated. The temporal bones were removed, immersed in 4% paraformaldehyde, and shipped to the University of Buffalo for analysis. Cochleae were stained with Ehrlich's hematoxylin solution, the organ of Corti carefully microdissected out into two or three segments and mounted as a flat surface preparation in glycerin on glass slides. One person, blind to the ABR results, dissected the cochleas and prepared the surface preparation. A second person blind to the experimental conditions made the hair cell counts and had no prior knowledge of the ABR results. Surface preparations were examined with a light microscope (Zeiss Standard, 400X magnification). With careful inspection, the hair cells can be readily distinguished from support cells in hematoxylin stained specimens. By moving the focal plane of the microscope up and down, the person making the hair cell counts can determine if the stereocilia bundle, the cuticular plate and the hair cell nucleus, located slightly below the cuticular plate are present. A hair cell was counted as present if both the cuticular plate and hair cell nucleus were present; if either were missing the cell was counted as missing. Alternatively, if the hair cell is missing, the vacated space is replaced by a phalangeal scar. Inner hair cells (IHC) and outer hair cells (OHC) were counted along successive 0.12-0.24 mm intervals of the organ of Corti beginning at the apex. Cochleograms showing percent hair cells missing as a function of percent total distance from the apex were constructed for each animal. Percent hair cell losses were based on laboratory norms for young CBA/CaJ mice (Ding et al., 2001). Locations along the cochlear duct corresponding to acoustic frequencies were determined using a mouse frequency-place map (Muller et al., 2005).

2.4 Statistical analysis

ABR data analysis was performed using the JMP 7.0 interactive statistics and graphics software program (www.JMP.com). Statistical significance of the differences among means was determined by one-way ANOVA with Tukey test corrections for multiple pair-wise comparisons. Hair cell loss data were analyzed using a 2-way ANOVA with cochlear location a repeated measure. A Tukey post-hoc analysis was used to identify significant differences associated with strain and cochlear location (SigmaStat 3.5 for Windows, version 3.5).

3. Results

3.1 Age-related hearing loss

Hearing assessment by ABR threshold analysis was performed for all five strains at 3, 6, 9, 12, 15 and 18 months of age. Table 1 lists the ABR threshold means for each auditory stimulus, the numbers of mice tested, and threshold variance estimates (standard deviations) for each strain and test age. The changes in ABR thresholds for each strain over time are illustrated graphically in Figure 1 for each test stimulus. At 3 months of age all of the strains exhibited normal ABR thresholds for all three of the test frequencies (8, 16, and 32 kHz). B6J and B6N strain mice exhibit a moderate high frequency hearing loss (32 kHz, + 30 dB) by 6 months of age that progresses to almost complete high frequency deafness by 18 months. The 8 kHz and 16 kHz thresholds of B6J and B6N mice remain nearly normal until

9 months of age, but by 12 months of age they are moderately elevated (+ 25-45 dB). Hearing loss in CBA, B6.CBA-*Ahl*⁺ and CBA.B6-*ahl* strain mice occurs later and progresses more slowly than that observed for B6J and B6N mice. CBA, B6.CBA-*Ahl*⁺ and CBA.B6-*ahl* mice exhibit only a mild hearing loss at 12 months of age, which progresses to a moderate impairment (+20-30 dB) by 18 months of age. The degree of ABR threshold variation seen within each strain for all test ages and frequencies (Table 1) reflects the complex nature of AHL, a quantitative trait for which environmental and stochastic factors, as well as genetics, affect the onset time and progression of hearing loss.

Statistical comparisons of ABR threshold means are presented in Table 2 and detailed in Supplementary Fig 2. The threshold means of B6J and B6N strain mice did not significantly differ from one another at any of the ages and frequencies tested, but they significantly diverged from those of the other three strains by 6 months of age for high frequency stimuli (32 kHz), and by 12 months of age for lower frequencies (8 and 16 kHz) as well. Throughout the test period, no statistically significant differences in threshold means were detected between CBA.B6-*ahl* congenic strain mice and CBA mice. At 15 and 18 months of age, mean thresholds of B6.CBA-*Ahl*⁺ mice were greater than those of CBA mice for all test frequencies (Fig. 1), and statistically significantly different for the 8 kHz stimulus (Table 2).

3.2 Cochlear pathology

After completion of hearing assessments for the final 18-month test age, inner ears of mice from the two congenic strains (B6.CBA-*Ahl*⁺ and CBA.B6-*ahl*) and the two parental strains (B6J and CBA) were transported to the University at Buffalo for analysis as noted in Section 2.3. Cochlear surface preparations were made to visualize the hair cells of mice from each of the four strains. Figure 2 shows a series of photomicrographs of hematoxylin-stained surface preparations from the apex (panel A), middle (panel B) and basal (panel C) regions of the cochlea of a B6.CBA-*Ahl*⁺ mouse; the focal plane of the images was adjusted to be near the nuclei of the OHC. In this example, most of the OHC and IHC were present near the apex and middle of the cochlear whereas many OHC and some IHC were missing in the base of the cochlea. Figure 3 shows lower magnification (200X), representative photomicrographs centered roughly 40-60% (~10-20 kHz region) of the distance from the apex of the cochlea of the four strains (Muller et al., 2005). As expected, all the IHC and OHC were present in the CBA parental strain (Fig. 3A) whereas most OHC and some IHC were missing in the B6J parental strain consistent with previously reported results (Spongr et al., 1997a). Like the CBA parental strain, all the IHC and OHC were present in the CBA.B6-*ahl* congenic strain (Fig. 3B). Nearly all the OHC were present in the B6.CBA-*Ahl*⁺ congenic strain; however, surprisingly some IHC were missing in this congenic strain.

The mean ($\pm$ SEM) percentages of outer hair cell (OHC) and inner hair cell (IHC) loss along the length of the cochlea were determined for each strain (Fig. 4A-D). Positions along the cochlea related to test frequencies (lower axis, Fig. 4A-D) were estimated using a mouse frequency-place map (Muller et al., 2005). B6J strain mice showed the highest percentage of OHC and IHC loss; OHC loss was typically greater than IHC (Fig. 4B). Little hair cell loss was observed in the cochleae of CBA (Fig. 3A) and CBA.B6-*ahl* mice (Fig 4C). B6.CBA-*Ahl*⁺ strain mice showed significantly less hair cell loss than B6J mice, but compared with CBA and CBA.B6-*ahl* mice, they showed a higher degree of OHC loss at the most basal and apical regions of the cochlea and greater IHC loss from the middle to the base of the cochlea. Statistical analyses of IHC and OHC losses are presented in Table 3. A two-way, repeated-measures (cochlear location) ANOVA on OHC loss (Table 3A) and IHC loss (Table 3B) showed significant main effects for genetic background, cochlear location and the interaction of genetic background and cochlear location. A Tukey post-hoc analysis

showed significant differences in OHC loss (Table 3A) and IHC loss (Table 3B) among strains as a function of cochlear location.

4. Discussion

4.1 CAST- and CBA-derived *Cdh23* alleles reduce hearing loss in B6 congenic mice

We previously generated and analyzed a B6.CAST-*Ahl*⁺ congenic strain (Johnson et al., 1997; Keithley et al., 2004) analogous to the B6.CBA-*Ahl*⁺ congenic strain described in this paper, but with the AHL protective allele of the CAST/EiJ (CAST) strain, rather than the CBA strain, transferred to the B6J strain background. Mice of both congenic strains exhibit a much slower rate of hearing loss compared with the B6J host strain mice, demonstrating that both CAST and CBA alleles of *Cdh23* can confer AHL resistance in mice with an otherwise B6J genome. The dramatic effect that the *Ahl*⁺ allele has in reducing the severity of hearing loss in congenic B6J mice indicates that the *ahl/ahl* genotype is a major contributor to the early AHL of B6J mice.

Comparisons of our ABR threshold results for B6.CBA-*Ahl*⁺ mice with previously reported results for B6.CAST-*Ahl*⁺ mice suggest a possible difference in hearing loss profiles. At 18 months of age and older, hearing loss in B6.CAST-*Ahl*⁺ mice was intermediate to that of B6J and CAST parental strain mice (Keithley et al., 2004), whereas ABR thresholds of the 18-month-old B6.CBA-*Ahl*⁺ mice examined in the present study are only slightly higher than those of CBA parental strain mice. This apparent difference can be explained by the differences in hearing loss between the parental CAST and CBA strains. CAST mice maintain near normal hearing thresholds throughout 21 months of age (Keithley et al., 2004). In contrast, CBA mice exhibit approximately 20 dB threshold elevations by 18 months of age (Fig. 1), despite the fact that IHC or OHC loss is not evident in the corresponding cochlear regions (Fig. 4A).

4.2 The B6-derived *Cdh23* allele has no effect on hearing in CBA congenic mice

We chose to use the CBA strain in this study because it is widely used in hearing research as a good hearing control strain and because CBA mice are much more docile than CAST mice, a wild-derived strain. The wild nature of CAST mice has prevented us from creating a CAST.B6-*ahl* congenic strain. The CBA.B6-*ahl* congenic strain we developed and describe here has allowed us to test whether homozygosity for the *ahl* variant can worsen the hearing loss in an AHL-resistant strain of mice. Somewhat unexpectedly we found that the hearing loss in CBA.B6-*ahl* strain mice, even at 18 months of age, remains similar to that of CBA mice and is much milder than that observed in B6J mice. This result indicates that the *ahl/ahl* genotype of B6J mice by itself is not sufficient to worsen hearing loss in an otherwise CBA genetic background, and that deleterious B6J alleles at additional loci must be required. Analysis of F1 hybrids between CBA.B6-*ahl* congenic strain and B6J mice will help determine whether these other contributing B6J alleles are recessive or dominant in nature and illuminate what genetic strategy would work best to map the additional loci.

4.3 Correspondence of hearing loss and hair cell loss

Little hair cell loss was observed in the 18-month-old CBA mice and the CBA.B6-*ahl* congenic strain, except in the most apical regions where 20% OHC were missing in both strains and in the most basal region where approximately 10% and 20% OHC were missing in the CBA and CBA.B6-*ahl* strains respectively (Fig. 4). The location and degree of hair cell loss in the CBA parental strain and CBA.B6-*ahl* congenic strain mice were nearly identical to that observed in our earlier study of CBA/J and CBA/CaJ mice (Spongr et al., 1997a). Despite the fact that there was little or no hair cell loss at 8, 16 and 32 kHz regions of the cochlea (Fig. 4A,C), these frequencies all showed mild to moderate ABR threshold

increases of 30 dB (Fig. 1). Hearing loss without accompanying hair cell loss in aging CBA mice may be explained by a loss of marginal cells in the stria vascularis (and consequent decline in endocochlear potential) and a loss of spiral ganglion neurons, as other investigators have previously documented in CBA/CaJ mice older than one year (Ohlemiller et al., 2010).

OHC losses in the B6J parental strain mice declined from roughly 90% near the base of the cochlea to 30-50% near the apex; IHC also declined from 90% near the base to less than 10% near the apex. The location and degree of hair cell loss in these B6J strain mice was nearly identical to our previously reported results for 18-month-old C57BL/6 mice (Spongr et al., 1997a) and consistent with the configuration of the high-frequency hearing loss seen in these mice (Fig. 1). ABR threshold shifts at 8, 16 and 32 kHz were roughly proportional to the degree of OHC and/or IHC loss, suggesting that hair cell loss is a major factor contributing to hearing loss in B6J mice. Others have noted that fibrocytes in the spiral ligament begin to degenerate before hair cells and suggested that this may be a key factor contributing to AHL (Hequembourg et al., 2001a); however, there is no significant decline in endocochlear potential in aging B6J mice (Lang et al., 2002; Ohlemiller, 2009), which argues against this proposed mechanism. Impaired mitochondrial function and energy metabolism also have been implicated in AHL. Young 1.5-month-old C57BL/6N mice expressed much lower levels of a key mitochondrial enzyme, succinate dehydrogenase, in hair cells than CBA mice, months before the onset of hearing loss (Ding et al., 1999). A deletion of the mitochondrial pro-apoptotic gene, *Bak1*, in B6J mice reduced age-related hair cell and spiral ganglion loss and suppressed AHL (Someya et al., 2009). Although *Cdh23^{ahl}* is thought to primarily affect tip link integrity and may not directly impair energy metabolism, tip link dysfunction could increase cellular stress and contribute to the activation of apoptotic pathways.

Perhaps the most novel pathological finding in this report is the unusual pattern of IHC loss in the B6.CBA-*Ahl*⁺ congenic strain mice. These mice exhibited 30-40% IHC loss over the basal 80% of the cochlea, but little OHC loss except in the most basal and apical regions of the cochlea (Fig. 4D). The magnitude of the IHC lesion in the apical half of the cochlea matched that seen in the B6J mice; however, IHC losses in the basal 40% of the cochlea were half as great as in B6J mice (Fig. 4F). OHC losses in the middle 60% of the cochlea of the B6.CBA-*Ahl*⁺ congenic mouse strain were negligible and substantially less than in the B6J parental strain, and these results corresponded well with the ABR threshold differences between these two strains (Fig. 1). The difference in OHC loss between B6J and B6.CBA-*Ahl*⁺ congenic mice was greater than the difference in IHC loss (Fig. 4E, F), suggesting that *Cdh23^{ahl}* primarily affects OHC survival, especially in the mid to basal region of the cochlea, and only partially contributes to IHC loss in the basal region. The *Cdh23^{ahl}* allele appears to have little effect on either low frequency hearing loss (8 kHz, Fig. 1) or hair cell loss in the apical region of the cochlea (Fig. 4E,F). Consistent with this interpretation, Ohlemiller found an identical progression of low frequency hearing loss (< 10 kHz) in B6 and B6.CAST-*Ahl*⁺ congenic mice (Ohlemiller, 2006).

4.4 Value of the B6.CBA-*Ahl*⁺ and CBA.B6-*ahl* congenic strains

While we realize that the congenic segments of the B6.CBA-*Ahl*⁺ strain and CBA.B6-*ahl* strain contain hundreds of genes, we attribute their hearing loss effects primarily to the *Cdh23* gene because of its known variants and their strong associations with the hearing loss phenotypes in inbred strain mice (Noben-Trauth et al., 2003). Although we cannot rule out the possibility of minor contributions from other genes in the congenic intervals, we are aware of no other genetic differences between the B6J and CBA strains in the Chromosome 10 congenic regions that are known or predicted to influence hearing.

B6.CBA-*Ahl*⁺ and CBA.B6-*ahl* congenic strain mice can serve as valuable tools, in comparisons with mice of the B6J and CBA parental strains, to deduce the specific effects of *Cdh23* strain variants in a wide variety of auditory studies. In addition to studies of AHL, these two newly generated congenic strains could be used to investigate the influence of these genetic factors on noise-induced hearing loss and ototoxicity. The B6.CBA-*Ahl*⁺ strain also can be used to control for the effects of the *Cdh23*^{*ahl*} allele in hearing assessments of mutations maintained on the B6J or B6N strain background. The *Ahl*⁺ allele can easily be transferred to B6J or B6N mutant strains by first outcrossing to the B6.CBA-*Ahl*⁺ strain and then intercrossing the resulting hybrids and selecting F2 mice with mutant and *Ahl*⁺ genotypes as future breeders. Although the previously developed B6.CAST-*Ahl*⁺ congenic strain can be used for the same purpose, the new B6.CBA-*Ahl*⁺ strain provides some increased fecundity.

4.5 Hearing loss profile of C57BL/6N mice

Many genetically engineered mouse mutations are maintained on the B6J background, but most mice from the International Knockout Mouse Consortium are being generated on B6N, a different substrain of B6 mice. Although both B6J and B6N substrains have the same *ahl* susceptibility allele, it is possible that their hearing phenotypes may differ due to mutations that have occurred in other genes since their separation. Behavioral differences, for example, have been detected between B6J and B6N substrain mice (Bryant et al., 2008), and a genetic difference with phenotypic consequences has been reported. The B6J strain has a null mutation of the nicotinamide nucleotide transhydrogenase gene (*Nnt*) that is not present in the B6N substrain and that influences diet-induced obesity (Nicholson et al., 2010). Because of the possibility of substrain differences, we examined the hearing phenotypes of B6J and B6N mice. Our results show that B6J and B6N mice have identical hearing loss onset times and rates of progression, which allows for direct comparisons of hearing-related phenotypes of mutant mice that are maintained on either background.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

ABR	auditory brainstem response
AHL	age-related hearing loss
IHC	inner hair cell
OHC	outer hair cell
ANOVA	analysis of variance
st dev	standard deviation
SEM	standard error of the mean

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Highlights

- >We investigated auditory effects of *Cdh23* variants in both C57BL/6J and CBA/CaJ genetic backgrounds.
- >The CBA/CaJ-derived *Cdh23* allele lessens hearing loss and decreases hair cell loss in a C57BL/6J genetic background.
- > The C57BL/6J-derived *Cdh23* allele has no effect on hearing loss or hair cell death in a CBA/CaJ genetic background.
- > Deleterious alleles at other loci in addition to *Cdh23* must contribute to the accelerated hearing loss of C57BL/6J mice.
- > Mice of the C57BL/6J and C57BL/6N substrains have identical hearing loss profiles.

Figure 1. Hearing loss progression

Mice of the B6J, B6N, B6.CBA-Ahl+, CBA.B6-ahl, and CBA strains were tested at 3, 6, 9, 12, 15, and 18 months of age, and ABR thresholds were obtained for pure-tone 8 kHz, 16 kHz and 32 kHz auditory stimuli. For each auditory stimulus, plots of ABR threshold means (dB SPL) for each strain at successive test ages are shown on the left and corresponding plots of hearing loss means (dB) on the right. Numerical values, variance estimates, and numbers of mice tested are given in Table 1, and statistical analyses of mean differences among strains are presented in Table 2 and in Supplementary Figure 2.

Figure 2. Cochlear hair cell loss

Representative photomicrograph of surface preparations from a nineteen-month-old B6.CBA-*Ahl*⁺ mouse taken from (A) apical, (B) middle and (C) basal region of the cochlea (400X magnification). Focal plane of the microscope set near nuclei outer hair cells (OHC) and inner hair cells (IHC). Jagged arrows point to missing OHC and IHC.

Figure 3. Strain-specific patterns of hair cell loss

Representative photomicrographs (400X magnification) taken 40-60% (10-20 kHz region) of the distance from apex of the cochleae of (A) CBA, (B) CBA.B6-*ahl*, (C) B6.CBA-*Ahl*⁺ and (D) B6J mouse strains examined at 18-19 months of age. Most OHCs (yellow bracket) were present in CBA, CBA.B6-*ahl* and B6.CBA-*Ahl*⁺ mice, whereas most OHCs were missing in B6J mice (jagged, gold arrowhead). Nearly all IHCs were present in CBA and CBA.B6-*ahl* mice, whereas many IHCs were missing in B6J mice and B6.CBA-*Ahl*⁺ mice (red arrowhead). Note that there is little if any IHC or OHC loss in CBA or CBA.B6-*ahl* mice, that B6.CBA-*Ahl*⁺ mice show much less OHC loss than B6J mice, but more than CBA or CBA.B6-*ahl* mice, and that IHC loss is similar in B6.CBA-*Ahl*⁺ and B6J mice.

Figure 4. Quantification of cochlear hair cell loss

Mice of the CBA, B6J, CBA.B6-*ahl*, and B6.CBA-*Ahl*⁺ strains were examined at 18-19 months of age. **A-D.** For each strain, mean percent loss ($\pm$ SEM) of IHC and OHC was plotted as a function of percent distance from the apex. **E.** Comparisons of mean ($\pm$ SEM) percent OHC loss as a function of percent distance from the apex among strains. **F.** Comparisons of mean ($\pm$ SEM) percent IHC loss as a function of percent distance from the apex of the cochlea among the strains. Statistical analyses of these data are presented in Table 3. Lower x-axis shows the relationship of test frequency to cochlear place (Muller et al., 2005).

Table 1
ABR threshold means, standard deviations, and numbers of mice tested

8 kHz, 16 kHz and 32 kHz threshold means (in dB SPL) and standard deviations (SD) are given for each strain and age along with the number (N) of mice tested and the gender mix (F, female; M, male). Means presented in bold are 15 dB or greater above those of 3 month old mice.

		B6.CBA-Ah/+	B6J	B6N	CBA.B6-ahl	CBA/CaJ
3 month test age		N = 18 (8F, 10M)	N = 19 (5F, 14M)	N = 30 (14F, 16M)	N = 22 (11F, 11M)	N = 18 (10F, 8M)
	8 kHz	mean	33.61	33.42	32.83	35.23
		SD	7.63	4.43	6.39	4.99
	16 kHz	mean	18.33	16.58	17.00	20.00
		SD	4.85	2.91	8.37	6.17
	32 kHz	mean	42.22	45.00	47.35	46.36
6 month test age		N = 21 (10F, 11M)	N = 16 (10F, 6M)	N = 20 (10F, 10M)	N = 20 (10F, 10M)	N = 23 (11F, 12M)
	8 kHz	mean	35.95	37.50	34.00	38.75
		SD	5.15	6.58	5.76	5.82
	16 kHz	mean	18.10	21.56	21.25	26.00
		SD	5.59	9.08	8.41	6.81
	32 kHz	mean	42.38	78.13	76.75	51.54
9 month test age		N = 21 (5F, 16M)	N = 19 (10F, 9M)	N = 18 (9F, 9M)	N = 19 (10F, 9M)	N = 26 (15F, 11M)
	8 kHz	mean	37.86	45.26	46.11	43.68
		SD	6.81	10.20	8.14	7.23
	16 kHz	mean	22.62	30.53	27.50	26.58
		SD	7.68	14.52	9.74	7.27
	32 kHz	mean	45.24	83.42	81.39	47.63
12 month test age		N = 23 (5F, 18M)	N = 19 (10F, 9M)	N = 17 (8F, 9M)	N = 17 (10F, 7M)	N = 25 (14F, 11M)
	8 kHz	mean	45.22	65.26	59.06	47.35
		SD	10.50	10.86	16.75	8.31
	16 kHz	mean	25.22	53.16	43.24	27.94
		SD	8.59	16.52	21.65	5.88
						11.81

		B6.CBA-Ah1+	B6J	B6N	CBA.B6-ahl	CBA/CaJ
32 kHz	mean	50.22	92.11	87.94	48.53	55.80
	SD	8.19	6.73	8.85	8.06	12.13
15 month test age						
8 kHz	mean	61.39	74.38	70.63	55.36	46.36
	SD	10.55	8.34	7.50	10.28	5.95
16 kHz	mean	35.83	68.13	61.25	36.07	27.27
	SD	9.28	5.44	11.90	15.21	2.61
32 kHz	mean	65.56	97.19	97.81	58.93	57.73
	SD	13.49	4.46	2.56	8.36	7.86
18 month test age						
8 kHz	mean	66.15	84.23	75.00	59.55	52.50
	SD	10.64	5.34	7.56	7.57	9.35
16 kHz	mean	46.54	74.23	74.33	37.27	40.00
	SD	15.33	7.03	12.23	9.32	8.37
32 kHz	mean	70.00	99.23	99.67	66.36	65.83
	SD	15.56	1.88	1.29	8.97	13.93

Table 2

Statistical Analysis of ABR thresholds

One-way ANOVA using the Tukey test was used to determine the statistical significance of ABR threshold mean differences between strains at the 0.05 alpha level (ns, no significant difference). For each auditory stimulus, threshold means were compared between each strain pair combination at each test age.

8 kHz ABR thresholds		3 months	6 months	9 months	12 months	15 months	18 months
B6.CBA-A/h+	B6J	ns	ns	p<0.05	p<0.001	p<0.005	p<0.001
B6.CBA-A/h+	B6N	ns	ns	p<0.05	p<0.005	ns	ns
B6.CBA-A/h+	CBA.B6-ahl	ns	ns	ns	ns	ns	ns
B6.CBA-A/h+	CBA	ns	ns	ns	ns	p<0.05	p<0.005
B6J	B6N	ns	ns	ns	ns	ns	ns
B6J	CBA.B6-ahl	ns	ns	ns	p<0.001	p<0.001	p<0.001
B6J	CBA	ns	ns	ns	p<0.001	p<0.001	p<0.001
B6N	CBA.B6-ahl	p<0.05	ns	ns	p<0.05	p<0.005	p<0.001
B6N	CBA	p<0.05	ns	ns	p<0.001	p<0.001	p<0.001
CBA.B6-ahl	CBA	ns	ns	ns	ns	ns	ns
16 kHz ABR thresholds		3 months	6 months	9 months	12 months	15 months	18 months
B6.CBA-A/h+	B6J	ns	ns	ns	p<0.001	p<0.001	p<0.001
B6.CBA-A/h+	B6N	ns	ns	ns	p<0.001	p<0.001	p<0.001
B6.CBA-A/h+	CBA.B6-ahl	ns	p<0.05	ns	ns	ns	ns
B6.CBA-A/h+	CBA	ns	ns	ns	ns	ns	ns
B6J	B6N	ns	ns	ns	ns	ns	ns
B6J	CBA.B6-ahl	ns	ns	ns	p<0.001	p<0.001	p<0.001
B6J	CBA	ns	ns	ns	p<0.001	p<0.001	p<0.001
B6N	CBA.B6-ahl	ns	ns	ns	p<0.05	p<0.001	p<0.001
B6N	CBA	ns	ns	ns	p<0.05	p<0.001	p<0.001
CBA.B6-ahl	CBA	ns	ns	ns	ns	ns	ns
32 kHz ABR thresholds		3 months	6 months	9 months	12 months	15 months	18 months
B6.CBA-A/h+	B6J	ns	p<0.001	p<0.001	p<0.001	p<0.001	p<0.001
B6.CBA-A/h+	B6N	ns	p<0.001	p<0.001	p<0.001	p<0.001	p<0.001
B6.CBA-A/h+	CBA.B6-ahl	ns	p<0.05	ns	ns	ns	ns

32 kHz ABR thresholds	3 months	6 months	9 months	12 months	15 months	18 months
B6.CBA-Ahl/+	ns	ns	ns	ns	ns	ns
B6J	ns	ns	ns	ns	ns	ns
B6J	CBA.B6-ahl	p<0.001	p<0.001	p<0.001	p<0.001	p<0.001
B6J	CBA	p<0.001	p<0.001	p<0.001	p<0.001	p<0.001
B6N	CBA.B6-ahl	ns	p<0.001	p<0.001	p<0.001	p<0.001
B6N	CBA	ns	p<0.001	p<0.001	p<0.001	p<0.001
CBA.B6-ahl	CBA	ns	ns	ns	ns	ns

Statistical Analysis of Hair Cell Loss

Table 3

A two-way repeated measure ANOVA with Tukey post-hoc analysis was used to determine statistical significance of genetic background and cochlear location on outer and inner hair cell loss and mean differences between strains. DF, degrees of freedom; SS, sum of squares; MS, mean squares; F, F-ratio; P, probability.

A. Outer hair cell loss:						
Source of Variation	DF	SS	MS	F	P	
Genetic Background	3	63624	21208	35	<0.001	
Cochlear Location	4	13392	3348	29	<0.001	
Genetic Background × Cochlear Location	12	15446	1287	11	<0.001	
Strain Comparisons						
% Distance from Apex						
	10	30	50	70	90	
B6.CBA-A ^h /+ vs. CBA	p<0.05	ns	ns	ns	p<0.05	
B6.CBA-A ^h /+ vs. CBA.B6- <i>ahl</i>	p<0.05	ns	ns	ns	p<0.05	
B6.CBA-A ^h /+ vs. B6J	ns	ns	p<0.05	p<0.05	p<0.05	
B6J vs. CBA	p<0.05	p<0.05	p<0.05	p<0.05	p<0.05	
B6J vs. CBA.B6- <i>ahl</i>	p<0.05	p<0.05	p<0.05	p<0.05	p<0.05	
CBA.B6- <i>ahl</i> vs. CBA	ns	ns	ns	ns	ns	
B. Inner Hair Cell Loss:						
Source of Variation	DF	SS	MS	F	P	
Genetic Background	3	37603	12534	50	<0.001	
Cochlear Location	4	18786	4697	37	<0.001	
Genetic Background × Cochlear Location	12	16635	1386	11	<0.001	
Genetic Background × Cochlear Location						
% Distance from Apex						
	10	30	50	70	90	
B6.CBA-A ^h /+ vs. CBA	ns	p<0.05	p<0.05	p<0.05	p<0.05	
B6.CBA-A ^h /+ vs. CBA.B6- <i>ahl</i>	ns	p<0.05	p<0.05	p<0.05	p<0.05	
B6.CBA-A ^h /+ vs. B6J	ns	ns	ns	p<0.05	p<0.05	
B6J vs. CBA	ns	p<0.05	p<0.05	p<0.05	p<0.05	
B6J vs. CBA.B6- <i>ahl</i>	ns	p<0.05	p<0.05	p<0.05	p<0.05	

Genetic Background × Cochlear Location	% Distance from Apex			
	10	30	50	70
CBA.B6- <i>ahl</i> vs. CBA	ns	ns	ns	ns
				90
				ns