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Research paper

Effects of mitochondrial mutations on hearing and cochlear pathology with age

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ABSTRACT

Age-related hearing loss is a multi-factorial process involving genetic and environmental factors, including exposure to noise and ototoxic agents, as well as pathological processes. Among these is the accumulation of mitochondrial DNA mutations and deletions. The creation of a transgenic mouse with a loss-of-function deletion of the nuclear gene that encodes the polymerase required to repair damaged mitochondrial DNA (*PolgA*) enabled evaluation of age-related cochlear pathology associated with random mitochondrial DNA deletions that accrue over the lifespan of the mouse.

In comparison with their wild-type or heterozygous counterparts, animals with mutated DNA polymerase gamma developed hearing loss most rapidly. Any loss of mitochondrial DNA polymerase function however, resulted in detrimental effects, as evidenced by hearing tests and histological investigation of transgenic heterozygotes. Cochlear pathology in transgenic animals at 10 months of age included loss of neurons and clumping of surviving neurons in the apical turn of the spiral ganglion. Mitochondrial mutations in young animals, on the other hand, were protective against the development of temporary threshold shift in response to relatively low level noise exposure. This supports the idea that temporary threshold shifts are the result of an active process involving mitochondria and respiratory chain activity. Our results indicate that mitochondrial mutation and deletion can certainly contribute to the development of an aging phenotype, specifically age-related hearing loss.

1. Introduction

Presbycusis, or age-related hearing loss, affects an estimated 40% of individuals over the age of 65 years. It is a multi-factorial process involving genetic factors, environmental factors, such as exposure to noise and ototoxic agents, as well as pathological processes. It is experienced as an elevation in hearing thresholds occurring over time (Gates and Mills, 2005) and is accompanied by cochlear pathology including the degeneration of sensory and neural cells as well as the stria vascularis (Schuknecht and Gacek, 1993). The morbidity of this disease is largely due to impairment in speech recognition and discrimination, especially in noisy environments, resulting in social isolation and an inability to compensate for other age-related disabilities.

One of the many hypothetical contributors to systemic and cochlear aging is the accumulation of mitochondrial DNA (mtDNA) damage, both mutations and deletions that lead to mitochondrial inefficiency (Ames, 2004; Fischel-Ghodsian et al., 1997; Seidman et al., 2004; Yamasoba et al., 2007). As mitochondria are responsible for the energy production and therefore survival of each cell, when mtDNA mutations reach a threshold level that renders enough organelles bioenergetically inefficient, the cell dies.

In order to investigate this phenomenon and its role in aging, transgenic mice (D257A) were generated with two altered residues in the nuclear DNA domain of the polymerase gamma gene (POLG) locus, impairing its proofreading ability (Trifunovic et al., 2004; Kujoth et al., 2005). This nuclear-encoded gene produces the catalytic subunit of the mtDNA polymerase, the only polymerase with confirmed activity within the mitochondrial genome (Hübscher et al., 1979). As a consequence of this mutation, mtDNA of transgenic animals accumulates unrepaired damage at a higher rate than in normal mice (Trifunovic et al., 2004; Kujoth et al., 2005; Kujoth and Prolla, 2008). These *PolgA* mice do not produce a measurable

Abbreviations: ABR, auditory brainstem response; ANOVA, analysis of variance; B6, C57BL/6 mouse strain; COX, cytochrome oxidase; DAB, diaminobenzidine-tetrahydrochloride; DPOAE, distortion product otoacoustic emission; D257A, transgenic mice with altered residues in the POLG locus, PolgAD257A/D257A; D257A/+, heterozygous transgenic mice with altered residues in the POLG locus; EDTA, ethylenediaminetetraacetic acid; mtDNA, mitochondrial deoxyribonucleic acid (DNA); POLG, polymerase gamma gene; PTS, permanent threshold shift; ROS, reactive oxygen species; TTS, temporary threshold shift; WT, wild-type transgenic mice with altered residues in the POLG locus.

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increase in reactive oxygen species (ROS) (Trifunovic et al., 2005), but they do display progressive respiratory chain dysfunction (Edgar et al., 2009). By 7–9 months of age transgenic mice demonstrate some of the stigmata of aging: kyphosis, alopecia, weight loss, decreased bone mineral density and content, anemia, and reduction in fertility. They also develop premature presbycusis with elevated auditory brainstem response (ABR) thresholds and a loss of auditory ganglion cells by the age of 9 months (Kujoth et al., 2005; Someya et al., 2008; Niu et al., 2007). Presbycusis in these subjects has been associated with mtDNA transcriptional changes in cochlear tissues correlating with a decline in energy metabolism, cytoskeletal dysfunction, hearing loss and induction of apoptosis (Someya et al., 2008).

The present work supports the proposal that random mtDNA mutations and deletions can lead to hearing loss and cochlear pathology similar to that seen in age-related hearing loss. A cohort of subjects was selected for examination at regular intervals over the course of a year. Animals with double mutations at the nuclear POLG locus and their original background, wild-type B6 animals have been studied (Kujoth et al., 2005; Someya et al., 2008; Niu et al., 2007) with acknowledgement that the background strain carries the *ahl* allele of the Cdh23 gene responsible for early onset hearing loss (Noben-Trauth et al., 2003; Mikaelian et al., 1974). We additionally included genotypic nuclear wild-type animals from our colony that were exposed at conception to the mitochondrial pools of their heterozygotic mothers, as well as nuclear heterozygotes for the *PolgA* gene. Observing representatives of all these genotypic variants allowed for a comparison of the effects of variable amounts of mtDNA damage: damage present in the embryo at conception and damage that would accumulate throughout development and aging.

We hypothesized that widespread, random mtDNA mutation would affect all cell types within the inner ear according to their energy requirements and the random pattern of mutation. We also expected that resultant dysfunction would produce measurable disparities in ABR and distortion product otoacoustic emission (DPOAE) thresholds. We anticipated that the greatest cochlear pathology would occur in cell types with the greatest requirement for metabolic energy such as the hair cells, cells of the stria vascularis, spiral ligament, and auditory neurons. We also examined the activity of one protein encoded in mtDNA, cytochrome oxidase (COX), in cochleas of advancing age. Because we predicted that right and left ears would be affected independently, we measured and compared the two ears in individual animals. In order to test the ability of transgenic mice to respond to and recover from acoustic trauma, we selected a separate group of young animals and exposed them to loud noise, while following their acute and subacute responses as measured by ABR.

2. Materials and methods

2.1. Animals

The experimental cohort utilized for this project was developed from 3 females and 1 male heterozygous D257A mice, *PolgA* mice generously given to us by Drs. Tomas Prolla and Greg Kujoth (University of Wisconsin, Madison, WI, USA). In our animal facility, the mice were bred to establish a colony of homozygous (D257A), heterozygous (D257A/+) and chromosomal wild-type (WT) mice. B6 (C57BL/6) mice were purchased from the Jackson Laboratory (Bar Harbor, ME, USA) for use as wild-type controls.

All animals were housed in standard polyurethane cages with free access to food and water in a temperature and light-regulated room. The use of these animals was performed in accordance with regulations and training established by the Veterinary Medical Unit of the Veterans Affairs San Diego Healthcare System.

2.2. Physiologic assessment of hearing

Bilateral hearing tests were performed in 8 mice from each genotype every month from 2 to 10 months of age. The test consisted of ABRs and DPOAEs collected with SigGen and BioSig computer software and System 3 hardware (Tucker-Davis Technologies, Alachua, FL, USA). Prior to testing, anesthesia was achieved with an initial dose of ketamine hydrochloride (50 mg/kg), xylazine hydrochloride (5 mg/kg) and acepromazine maleate (1 mg/kg). If additional doses were required to maintain anesthesia during testing, they were administered at 30–40% of the initial dose. Anesthetized mice were positioned on a heating pad maintained at 37 °C in a single-walled acoustic booth (Industrial Acoustics Co., Bronx, NY USA). A speculum with 2 EC1 speakers and an ER10B+ microphone probe (Etymotic Research, Elk Grove Village, IL, USA) was placed tightly into the external auditory canal. For the ABR recordings, electrodes (Astro-Med, Inc., Grass Instrument Division, West Warwick, RI, USA) were inserted subdermally at the vertex, mastoid and distal paraspinal regions.

ABRs were elicited with click stimuli (0.1 ms; 10/s, alternating polarity) delivered to the subject via one of the speakers inserted into its ear. Pure-tone stimuli (tone pips at 8, 16 and 32 kHz) were also employed in ABR testing for all subjects during the testing session at the 9th-month of age. The stimulus-locked ABR was amplified and filtered, using a battery-operated amplifier with the output connected to the computer. The click stimuli were presented with progressive attenuation from 90 dB to 0 dB in 5 dB increments, and the recorded stimulus-locked activity was averaged ($n = 512$). Threshold was defined as the stimulus intensity level between the level that induced a clearly visualized brainstem potential and one that did not. If the threshold exceeded 90 dB, 95 dB was assigned as the value of threshold.

Cubic DPOAE were measured at 6, 8, 16, 20, and 28 kHz using equal intensity primary tones with ratios of 0.894 and 1.118 of the center frequencies. The stimulus intensity was increased in 5 dB increments from 0 to 70 dB to establish the 2f1-f2 thresholds. Mean DPOAE thresholds for each frequency were defined as the lowest intensity at which the distortion product was 2–3 dB above the noise floor. If a response was not detected at a measureable intensity, a level of 75 dB was assigned as the threshold.

2.3. COX activity assay

To histologically assess mitochondrial function, we adapted a histochemical assay that demonstrates the relative activity of the mitochondrial enzyme, COX (Wong-Riley, 1979). This enzyme is coded by the mtDNA and is a component of the respiratory chain acting within the mitochondria. Its activity was assessed by incubating tissues with the substrate, cytochrome c and the chromagen, diaminobenzidine (DAB) for visualization in histological sections. Mice of all genotypes that had previously undergone hearing assessment were sacrificed and their cochleas were quickly removed by microdissection. The cochlea with the worst hearing at 10 months of age was selected for the COX assay. In order to ensure that our incubation solution rapidly reached cochlear tissues, the stapes was removed, the oval window punctured and a small section of bone from the apical-lateral aspect of the cochlear capsule was removed. The cochlea was then placed in PBS until each cochlea from a group of 3–4 mice had been harvested. They were subsequently immersed in a solution containing 3,3′-DAB, cytochrome c and catalase (Sigma). Cochleas were incubated for 4 h at 37 °C with continuous gentle agitation. Cochleas were then removed from the DAB solution and fixed by immersion in 1.5% paraformaldehyde/2.5% glutaraldehyde, phosphate buffered fixative for at least 12 h at room temperature. To provide a negative

control, oxidative phosphorylation was blocked by adding 0.1 M potassium cyanide to the solution of the control cochlea run with each assay. Following fixation, the cochleas were decalcified in 10% EDTA for 5–7 days, cryosectioned (9 μm) and mounted on superfrost plus (Fisher Scientific) slides. Half of all sections were subsequently counterstained with cresyl violet in order to clearly delineate relevant structures. Three sections from each enzymatically reacted cochlea (B6 $n = 5$, D257A $n = 6$, D257A/+ $n = 5$, WT $n = 7$ cochleas) were analyzed by one of the authors who did not know which sections were from which genotype. An Olympus light microscope was used to assess the relative density of the DAB staining in the stria vascularis, spiral ligament, Reissner's membrane, hair cells, auditory neurons and limbus. A value of 0–5 (very dark staining) was given to each of these structures in each section. A mean score was calculated for each structure within each genotype. An ANOVA statistical test with a Fisher's Least Significant Difference post-hoc test was used to determine if there was a difference among the genotypes for each labeled structure.

2.4. Tissue preparation for histology

A total of 48 mice ($n = 18$ D257A, $n = 14$ D257A/+, $n = 12$ WT, $n = 4$ B6) including those that were processed for the COX assay, were evaluated for cochlear pathology. These mice ranged from 3 to 16 months of age. All mice were born and raised in our colony. The oldest mice had not been used for prior experimentation. These mice were anesthetized as described above and intracardially perfused with 2% paraformaldehyde and 1% glutaraldehyde in phosphate buffer and kept at 4 °C for 12 h. Cochleas were then dissected from the skull and prepared for frozen or paraffin sectioning (10 μm). Sections were stained with cresyl violet or hematoxylin and eosin and evaluated using an Olympus light microscope.

2.5. Acoustic trauma

In order to evaluate the effect of mtDNA mutations on the cochlear response to loud noise, a separate cohort of 2-month-old mice composed of individuals from each genotypic group (B6, WT, D257A/+, D257A) was selected and exposed to an octave-band noise (8–16 kHz) at an intensity of either 95 dB or 105 dB for 140 min. Bilateral ABRs were measured just prior to the noise exposure, immediately following the exposure and again one month later. Noise exposure was performed by placing subjects in a cage within a custom made Plexiglas sound chamber (Wang et al., 2002). White noise was generated by a Grason & Stadler model 1208 noise generator, filtered through a Krohn-Hite model 3750 filter and amplified with a Crown amplifier. A model 2446H JBL speaker positioned on the top of the sound chamber was used to deliver the sound to 4 mice simultaneously. The sound level at the animals' head was measured using a hand-held Brüel and Kjaer model 2209 sound level meter.

3. Results

3.1. Animals

All subjects were weighed prior to each hearing test session and weights were recorded each month (Fig. 1). Though all mice gained weight through their third month of age, regardless of genotype, only unrelated B6 mice continued to gain weight steadily throughout the 10-month observation period. Homozygous mice (D257A) did not gain weight after their third month. By 7 months of age the D257A mice were significantly lighter than the B6 mice and this difference increased over the next 4 months (ANOVA, $p < 0.001$, Fisher's Least Significant Difference post-hoc test). Although the mean weight of the D257A mice was lower than that of the heterozygotes (D257A/+) and nuclear wild-type transgenic mice (WT) this difference was not statistically different.

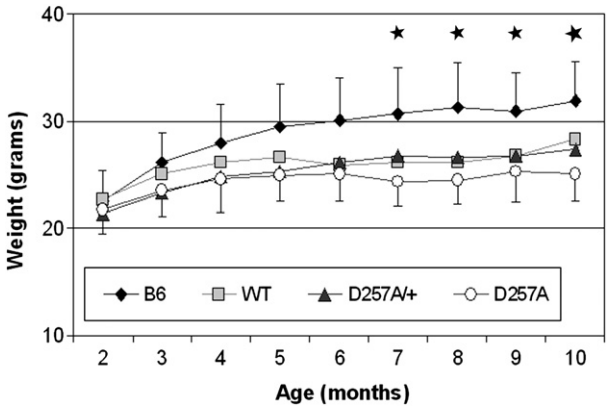

Fig. 1. Transgenic mice with variable amounts of mutated mtDNA polymerase *(PolgA)* do not gain weight after 3 months of age. Transgenic homozygous (D257A), heterozygous (D257A/+) and mice derived from heterozygous mothers (WT) are all affected. The background strain, B6, gains about 8 g after 3 months of age. Stars represent statistically significant difference between D257A and B6 (ANOVA, $p < 0.001$, Fisher's Least Significant Difference post-hoc test).

3.2. Physiologic assessment of hearing

The DPOAE thresholds were measured bilaterally for each subject from the ages of 2–10 months on a monthly basis. At 2 months, the mice were maximally sensitive at 16 kHz with an average threshold of 12 dB. At 28 kHz, mean threshold for all genotypes was 54 dB. There was no difference among genotypes. ABR thresholds persistently increased, especially at high frequencies, with each subsequent monthly testing in all genotypes (Fig. 2). Many of the animals had reached maximum measurable threshold for 20 and 28 kHz by the age of 6 months. By 10 months of age, none of the animals had measureable thresholds at 20 and 28 kHz. The only frequencies that yielded differences among genotypic groups were the relatively low frequencies, 6 and 8 kHz. Though all subjects began with low-frequency thresholds of 20 dB or less, by 7 and at 8 and 9 months D257A mice demonstrated thresholds that were greater than the B6 mice (ANOVA, $p < 0.05$, Fisher's Least Significant Difference post-hoc test). By 10 months however, there was no significant difference among the groups at any frequency.

Bilateral ABR testing performed concurrently with DPOAE tests showed that all 4 genotypes elevations in mean ABR thresholds as they aged (Fig. 3). By 7 months of age, all transgenic genotypes demonstrated some level of hearing loss greater than the B6, control group. The mean threshold of the D257A mice was significantly worse than that of the B6 cohort ($p < 0.05$, ANOVA, Fisher's Least Significant Difference post-hoc test). This pattern was maintained as each group's hearing continued to deteriorate over the subsequent 3-month testing period. At the ages of 8 and 9 months, both D257A/+ and D257A mice had significantly higher thresholds than the corresponding mean B6 threshold ($p < 0.04$, ANOVA, Fisher's Least Significant Difference post-hoc test). At 10 months however, only the D257A/+ group maintained its significant difference ($p < 0.05$) from the B6 group. Overall, B6 subjects experienced an elevation of 15 dB in mean ABR thresholds while mean threshold elevations of 30 and 27 dB were seen in D257A and D257A/+ mice groups, respectively. Transgenic wild-type mice also demonstrated increased thresholds, culminating in a final average elevation of 22 dB.

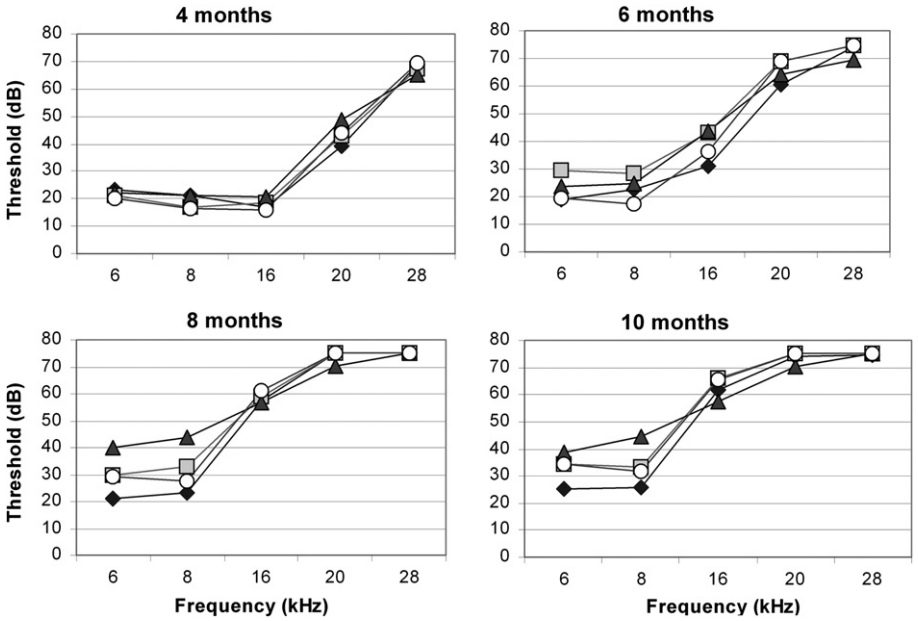

Fig. 2. Mean DPOAE threshold for each genotypic group at 4, 6, 8 and 10 months of age. All groups experienced high frequency DPOAE losses beginning at 6 months of age. Mean DPOAE thresholds at 8 months depict low frequency increases in transgenic animals compared with the background strain, B6 mice (ANOVA $p < 0.05$, Fisher's Least Significant Difference post-hoc test). At 10 months however, these small increases did not reach statistical significance. B6, solid diamonds; WT, solid squares; D257/+, solid triangles; D257A, open circles.

To determine whether the auditory nerve thresholds measured by ABR were greater than the hair cell thresholds from DPOAE measurements, 8 kHz tone-pips were used to test ABR thresholds at 9 months of age. These results are plotted against the mean DPOAE threshold at 8 kHz for each genotypic group (Fig. 4). The results suggest that the loss of neural activity at 8 kHz does not exceed the loss of outer hair cell function at 9 months of age. Losses are greater in all transgenic genotypes than the B6 group.

Right and left cochlear ABR thresholds in the 10-month-old animals were compared to assess expected differences due to random accumulation of mitochondrial mutations. Although some subjects showed equally poor hearing in the two ears, most animals possessed hearing that was worse in one ear or the other. The differences among the genotypes were assessed by taking the absolute value of the difference between the left and right ears for each animal (Fig. 5). As predicted, D257A mice had a larger difference in ABR threshold between the two cochleas than the heterozygous or wild-type mice ($p < 0.05$, ANOVA, Fisher's Least Significant Difference post-hoc test). The *ahl* allele also appears to affect each ear independently, as the B6 mice also had a large inter-ear difference.

3.3. Acoustic trauma

In order to evaluate the consequences of the accumulation of mtDNA defects on acoustic trauma, mice were exposed to loud noise and their recovery measured. A separate cohort of mice representing all four genotypes was selected to undergo noise

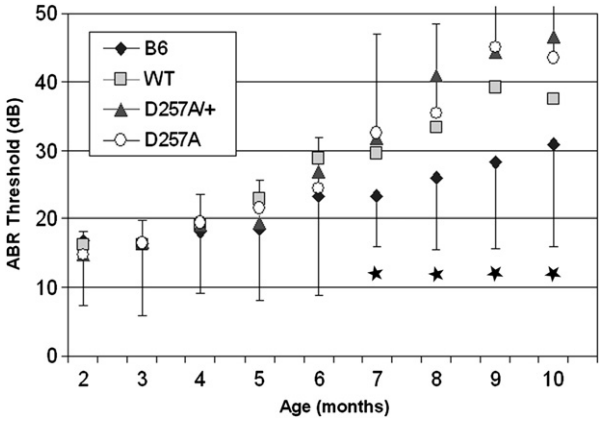

Fig. 3. Mean ABR thresholds for each genotype as a function of age. By 7 months of age, transgenic homozygous (D257A) and heterozygous (D257A/+) mice have higher thresholds than the background, B6 mice, but all transgenic groups experienced some amount of hearing loss and are not significantly different from each other. Negative error bars represent the standard deviation for the B6 mice. Positive error bars represent the standard deviation for the D257A mice. Stars represent ages where the D257A and D257A/+ mice have greater thresholds than the B6 mice (ANOVA, $p < 0.05$, Fisher's Least Significant Difference post-hoc test).

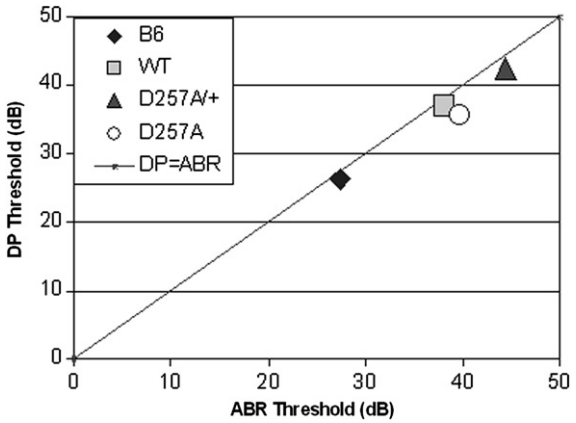

Fig. 4. Relationship between mean DPOAE and ABR thresholds in response to an 8 kHz stimulus at 9 months of age for each genotype. The line represents equal thresholds in the 2 measures. The neural loss is only slightly greater than the sensory loss.

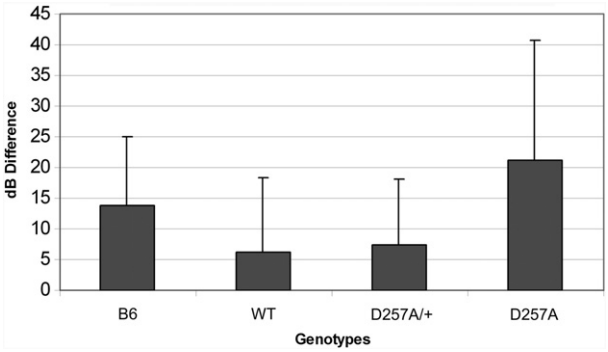

Fig. 5. Mean (standard deviation) ABR threshold differences between the left and right cochlea for individual mice at 10 months of age for each of the 4 genotypes of mice. The D257A mice have a larger absolute difference in ABR threshold between the two cochleas than the heterozygous (D257A/+) or wild-type (WT) mice (ANOVA, $p < 0.05$, Fisher's Least Significant Difference post-hoc test). The B6 mice also had large threshold differences between the 2 ears.

exposure at the age of 2 months. After 95 dB exposure, B6 animals demonstrated a temporary ABR threshold shift (TTS) of 34 dB in both ears (Fig. 6, top panel). D257A mice showed only a mean of 22 dB TTS and this difference was significant at $p < 0.001$ (ANOVA, Fisher's Least Significant Difference post-hoc test). One month later, the threshold shifts had diminished to 8 and 10 dB for the B6 and D257A groups, respectively, a difference that is not significant ($p = 0.061$). Heterozygous and WT transgenic subjects experienced mean TTS and permanent threshold shifts (PTS) between the B6 and D257A values and no differences between these other groups proved significant. Mice that were exposed to 105 dB noise demonstrated TTS between 20 and 80 dB and PTSs between 22 and 50 dB. No significant differences were seen among the 4 genotypes (Fig. 6, lower panel).

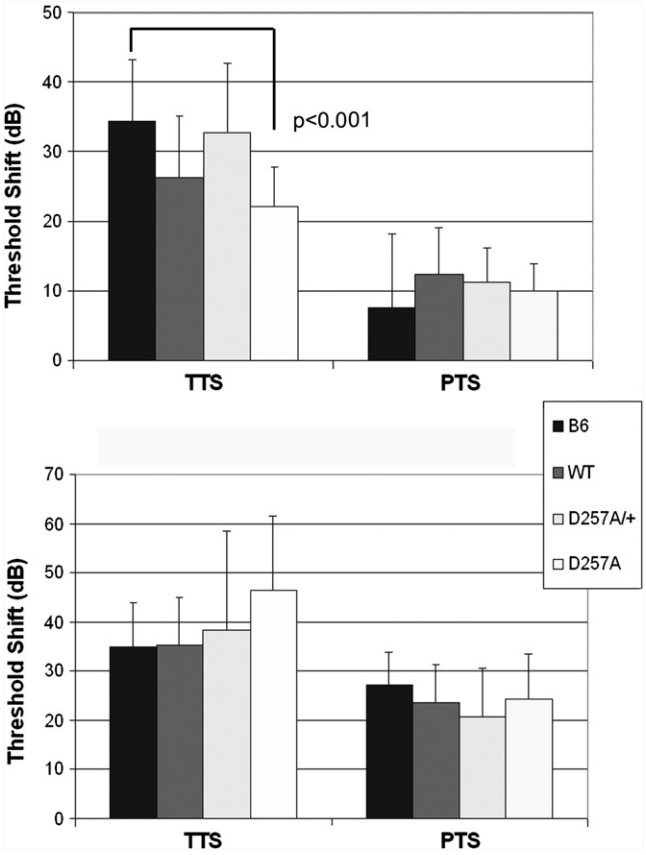

Fig. 6. Mean (standard deviation) ABR threshold shifts in response to 2 h of noise exposure (8–16 kHz) at 95 dB SPL (top panel) or 105 dB SPL (lower panel). All mice were 2 months of age at the time of the noise exposure ($n = 4$ in each group). The amount of TTS is significantly less in D257A mice relative to B6 mice following the 95 dB noise exposure ($p < 0.001$, ANOVA, Fisher's Least Significant Difference post-hoc test), but not at the 105 dB exposure level. The PTS measured a month after the noise exposure, however was no different among the genotypes.

3.4. COX activity

Twenty-three cochleas at 10–11 months of age were used to evaluate the endogenous COX activity of cochlear cells. This activity is recognized as the density of the DAB staining in histological sections. The cells with the densest stain were those of the stria vascularis, followed by the suprastrial and infrastrial cells of the spiral ligament, the neurons and the hair cells. The supralimbal cells that form the junction of Reissner's membrane with the spiral limbus also showed intense staining in all genotypes (Fig. 7). While the marginal, intermediate and basal cells of the stria vascularis all stained with the same density in an individual section, the neuronal staining density within one cross-section of Rosenthal's canal exhibited a large variability in the stain intensity. While the D257A mice and the D257A/+ mice were scored with slightly lower densities for the all the evaluated structures, statistical testing did not support a quantifiable difference in the stain density among the genotypes.

3.5. Pathology

Cochlear sections from each genotype (B6, $n = 4$; WT, $n = 11$; D257A/+, $n = 18$; D257A, $n = 17$) were evaluated in young subjects (3–6 months of age) and older subjects (10–16 months). The cochleas from the young mice appeared normal with the exception that several cochleas from the D257A ($n = 3$ of 6) and D257A/+ groups ($n = 2$ of 8) appeared to have a small loss of neurons at the apical end of the ganglion. This was identified as larger spaces than normal between neurons within Rosenthal's canal. At 10 months of age the most consistent and notable pathology in all genotypes was the loss of the organ of Corti and the auditory neurons in the basal, high frequency, region of the cochlea (Fig. 8). We attribute this to the B6 background strain which has long been known to have this pathology (Mikaelian et al., 1974; Keithley et al., 2004).

The most unusual pathology associated with the transgenic mice was the clumping of neurons at the apical end of Rosenthal's canal (Fig. 7, D257A/+ and D257A; Fig. 8). Rather than maintaining extracellular space between adjacent cells, 3–6 neurons often appeared adherent to one another. Three to 5 "clumps" were seen in 8 of 8 cochleas from D257A mice ranging in age from 10 to 12 months of age. Six of 7 D257A/+ mice aged 10–17 months also had clumped neurons in the apical turn and 0 of 7 WT mice between the ages of 10 and 15 months had clumped neurons. No cochleas from mice less than 10 months had clumps of neurons and none of the B6 mice cochleas had clumped neurons, regardless of age.

The stria vascularis showed no obvious pathology although we did not perform any specific quantitative measures of its area or volume in individual sections. The organ of Corti also appeared without gross pathology in any region other than the basal turn, where it was completely missing in most aged mice of all genotypes. The loss of scattered hair cells is difficult to quantify in frozen sections, but all aged cochleas did show evidence of some loss of hair cells.

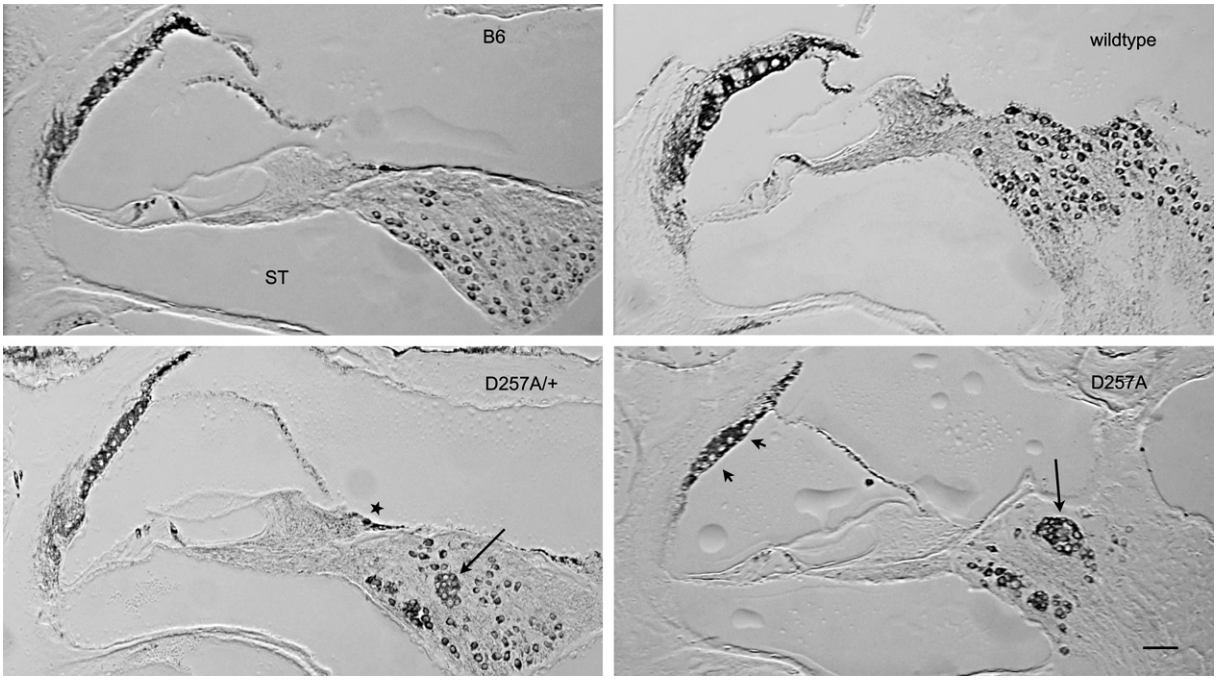

Fig. 7. Photomicrographs (Nomarski optics) of cytochrome oxidase enzymatically labeled cells in 10-month-old mice cochleas from each genotype: B6, wild-type, D257A/+ and D257A. In all 4 genotypes labeled cells include the sensory cells, neurons, cells of the stria vascularis (arrowheads in D257A photo), supra- and infrastrial cells in the spiral ligament and Reissner's membrane. In addition to the cells known to contain high densities of mitochondria, there is dark staining of the supralimbal cells (star in the D257/+ photo) that form the junction of Reissner's membrane with the spiral limbus. In many of the cochleas from the D257A/+ and D257A mice the neurons at the apical end of Rosenthal's canal appeared to be clumped together (arrows). ST, scala tympani, Scale bar for all figures, 50 μm.

4. Discussion

Transgenic mice that lack the nuclear-encoded polymerase gamma necessary to repair mtDNA, have an increased rate of mtDNA point mutation (Trifunovic et al., 2004; Kujoth et al., 2005), large deletions in their mtDNA (Kujoth and Prolla, 2008) as well as perturbations in the mtDNA replication cycle (Bailey et al., 2009). Their production of ROS is normal, although they demonstrate respiratory chain dysfunction (Trifunovic et al., 2005). They also display signs of premature aging including premature hearing loss (Kujoth et al., 2005; Niu et al., 2007; Yamasoba et al., 2007; Someya et al., 2008). This transgenic mouse was originally created as a potential model for human aging. While it possesses many features of aging, this fact alone does not prove that mtDNA mutations are responsible for aging. Certainly, though, such mutations give rise to pathology that is similar to age-related hearing loss or presbycusis and mtDNA mutations exist in aged human cochlear cells (Fischel-Ghodsian et al., 1997; Bai et al., 1997). For this reason these mice are an interesting model for the study of age-related hearing loss and how mtDNA mutations can give rise to it.

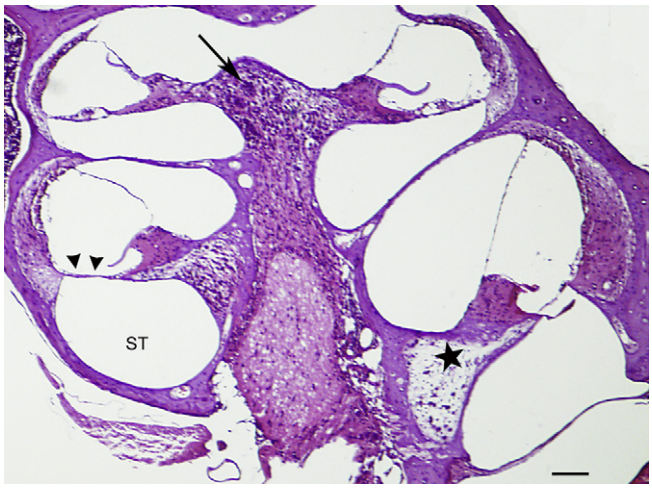

Fig. 8. Photomicragh demonstrating the loss of the organ of Corti (arrowheads) and the auditory neurons in the basal region (star) of the cochlea in a 12-month-old D257A mouse. These pathologies are representative of that normally seen in B6 mice, the background strain, at this age. In addition to this expected, B6 pathology, there are some clumped neurons (arrows) at the apical end of Rosenthal's canal. This is unique to the D257A and D257A/+ mice. Hematoxylin & eosin stained paraffin section, 10 μm. Scale bar, 100 μm.

Though the B6 background strain, that carries the *ahl* allele of the Cdh23 gene, has been well described, the D257A transgenic mouse model is new and complicated. The gene in question, *PolgA,* is transferred on nuclear, chromosomal DNA and is expected to distribute according to Mendelian principles. The manifestation of the transgenic effect, however, is determined in part through maternally-derived mtDNA present in the cytoplasm of the ovum at the time of conception. In mice with only one copy of wild-type *PolgA* (D257A/+), it is unknown how much baseline mtDNA damage may be present. It is known, however, that by the time D257A mice reach the age of 5 months, they demonstrate mtDNA mutation frequencies of 4–10 per mitochondrial genome (Kujoth et al., 2005) and even low levels of deletion may have a physiological effect (Vermulst et al., 2009). It is to be expected, therefore, that animals born to heterozygous mothers will receive mitochondria from the ovum's cytoplasm possessing some existent level of mtDNA mutation. Even if the progeny possess two functional copies of *PolgA*, preexistent mtDNA mutations or deletions inherited from the mother, will not necessarily be restored, and as cells divide, they will pass the mutated mtDNA to each daughter cell. There remains much uncertainty regarding the level of mitochondrial recombination within a cell and how much repair may be

accomplished through these means. While it is unfortunate that B6 mice were used as the background stain for the development of the D257A transgenic mouse, the pathology associated with the *ahl* allele can be separated from the D257A pathology. We did not confirm the presence of *ahl* in any of our mice.

As only heterozygous females were used for breeding purposes in our colony, we chose to follow four cohorts of animals from 2 to 10 months of age, including two different groups with wild-type copies of mtDNA polymerase, one strain derived from our own colony (identified as WT), and the other separate representatives of the background strain (identified as B6). Although body weight measures over time (Fig. 1) support the hypothesis that the D257A mice are phenoltypically distinct from the other transgenic genotypes, the hearing data do not show a statistically significant divergence in loss between transgenic groups raised in our facility. That is, the hearing of the D257A mice does not differ from the D257A/+ or wild-type mice. All groups do demonstrate a larger hearing loss than the B6 mice and there is evidence that low frequency loss is greater in transgenic mice relative to background mice. It appears then, as previously reported, that even a small amount of mutated mtDNA, can affect the progeny to some degree (Vermulst et al., 2009).

Measures of otoacoustic emissions illustrated a progressive hearing loss, most pronounced at the high frequencies of 16, 20 and 28 kHz, in all 4 genotypes. However, it is important to acknowledge that the background strain (C57BL/6 or B6) on which D257A mice were developed, possesses the *ahl* allele which is associated with accelerated age-related hearing loss (Noben-Trauth et al., 2003; Mikaelian et al., 1974; Keithley et al., 2004). Nonetheless, by the age of 7 months, transgenic *PolgA* mice had developed an even larger hearing loss than the B6 mice. In addition, the mtDNA transgenic mice began to demonstrate low frequency hearing loss (4 and 8 kHz) at 7 months of age when the mtDNA are likely to have greater than 4–10 mutations per mitochondrial genome (Kujoth et al., 2005). Measures of ABR thresholds also showed greater hearing loss in transgenic than in B6 mice, though there was no statistical difference between the D257A, heterozygous and wild-type mice. It is possible that a distinction may have become evident as transgenic mice aged past 10 months, since wild-type mice did not appear to be losing their hearing as rapidly as D257A or heterozygous mice. It is also possible that the assembly of larger cohorts would have provided more clear distinctions among the transgenic groups. Our results are similar to what has previously been reported regarding the effect of the *ahl* allele on the time of onset of hearing loss, but the magnitude of the losses were not as great in our sample.

Under histopathological examination, some features were discovered in all genotypic groups, while some were not. The most consistent and notable finding was the loss of the organ of Corti and the auditory neurons in the basal, high frequency region of the cochlea in all genotypes, at 10 months of age and older. A very unique finding to the D257A and D257A/+ mice was the appearance of clumped auditory neurons at the apical end of Rosenthal's canal. To the best of our knowledge, the only previous report of such pathology in the spiral ganglion was in relation to a mutant mouse described as "deafness" (dn/dn) (Webster, 1985). The gene that underlies this pathology has not been identified. Given that there is a great deal of change in the regulation of gene transcription in the *polgA* transgenic mice, it is possible that the gene giving rise to the deafness phenotype is down-regulated in D257A mice. One category of genes that might be responsible is the cell adhesion genes, many of which are down-regulated in these mice (Someya et al., 2008). In contrast to the hearing data that do not allow differentiation between the D257A and wild-type transgenic mice, this clumping was only present in D257A and D257A/+ mice. This observation is consistent with the proposal that a certain load of mtDNA mutations and/or deletions is required to establish a situation where neuronal aggregation may occur.

The enzymatic activity of COX was assessed in our assay with the purpose of identifying specific cell types in histological sections that produce relatively more ATP. Sections of cardiac muscle tissue from transgenic D257A mice have already been shown to stain in a mosaic pattern, rather than a homogeneous pattern, when examined for COX activity indicating that some myocytes had deficient COX activity (Trifunovic et al., 2004). Stained sections of duodenum, heart and brain also implicated individual cells with reduced COX activity in *PolgA* mice (Vermulst et al., 2008). With our application of this assay, cochlear cells with the highest density of mitochondria were the cells that labeled most strongly. These proved to be the cells of the stria vascularis, the supra- and infrastrial fibrocytes of the spiral ligament, the auditory neurons, sensory cells and the cells at the attachment of Reissner's membrane to the limbus. Though we expected to reveal a difference in the enzymatic activity of COX in cochlear cells among our 4 genotypic groups, there was no observable difference. In all subjects, the supralimbal cells that form the junction of Reissner's membrane with the spiral limbus demonstrated intense staining. Though we did not confirm the presence of mitochondria with electron microscopy we may presume that these particular cells contain a significant concentration of mitochondria. As evidence of their importance in cochlear energy production, they are known to express the $\alpha 2$ and $\beta 1$ mRNA subunit isoforms of Na, K-ATPase (Ryan and Watts, 1991). Although in any section the neuronal staining ranged from very dark to very faint, the *PolgA* mice did not possess a greater number of pale neurons than the other genotypes. We interpret this result to reflect the lack of a reliable reduction in the functional COX enzyme present in transgenic mitochondria. Any hearing loss therefore, cannot be attributed to a particular COX deficiency. It is possible that the variation in label pattern among the neurons reflects the variation in spontaneous discharge rate of the neurons. It is also possible that the neurons are very sensitive to decrements in COX activity and degenerate rapidly when this occurs making the loss of neurons a better reflection of the loss of activity than the label density. The 10-month-old *PolgA* mice did demonstrate a loss of neurons at the apical end of the canal that was more pronounced than the other genotypes investigated.

Acoustic trauma was also employed to test the effect of the *PolgA* mutation on hearing loss. There is a growing body of evidence suggesting that cellular damage from traumatic noise exposure is a result of ROS generation in cochlear sensory cells (Yamasoba et al., 2005; Bielefeld et al., 2005; Le Prell et al., 2007; Lin et al., 2009; Vlajkovic et al., 2009). Due to the reputed mitochondrial insufficiency in D257A mice, we hypothesized that cochlear cells would fail to be driven by high intensity noise though they have been shown to generate ROS (Trifunovic et al., 2005). We initially selected a relatively low-level noise exposure in order to be situated at the threshold of the phenomenon rather than to overwhelm it. Immediately following exposure to a 95 dB octave-band noise, threshold increases were, in fact, smaller in the D257A mice relative to the wild-type mice, while exposure to the 105 dB noise band did not show a difference in the magnitude of the threshold shift. The difference in response at 95 dB may be attributable to the metabolic inability (Edgar et al., 2009) of the transgenic cochlea to respond to the stimulus. Because of this inability, fewer ROS are generated and less damage occurs. The higher amplitude stimulus activates a longer length of the organ of Corti and may thereby generate enough ROS to create a threshold shift. While at this point we may only speculate regarding the mechanism underlying this phenomenon, these mice may prove useful for establishing cellular mechanisms of noise-induced hearing loss.

5. Conclusions

Mice with any level of exposure to mutated mtDNA polymerase gamma developed a low-frequency hearing loss at an earlier age than the background strain, B6, but the 3 *PolgA* transgenic mice genotypes did not differ among themselves in the degree of hearing loss. The cochleas of 10-month-old *PolgA* animals had severe sensorineural degeneration in the basal turn as well as neural degeneration in the apical turn. Surviving neurons in the apical turn were often clumped together. This pathology in the apical turn was not seen in wild-type transgenic mice or control, B6 mice. Cellular degeneration in the basal turn of the cochlea was seen in all genotypes and most likely reflects the effect of the *ahl* allele. D257A transgenic mice were protected against the development of TTS when relatively low noise levels were utilized. This finding is consistent with the proposal that oxidative phosphorylation and ROS are involved in acoustic trauma. Though the process of accelerated mtDNA mutation contributes to the development of premature aging, it is unlikely to be the only factor involved in age-related hearing loss in humans or other mammals.

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