

# Physiological and Histological Evaluations of the Cochlea between 3xTg-AD Mouse Model of Alzheimer's Diseases and R6/2 Mouse Model of Huntington's Diseases

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## Abstract

Patients with Alzheimer's diseases (AD) and Huntington's diseases (HD) are known to have abnormal auditory processing, but the physiological and histological evaluations of the cochlea between AD and HD have not been thoroughly assessed. Thus we assessed the auditory brainstem response (ABR) and distortion product otoacoustic emissions (DPOAE), and then examined spiral ganglion neurons (SGNs) and hair cells in the cochlea using 3xTg-AD mouse model of AD and R6/2-HD mouse model of HD. We found that the threshold of ABR, but not DPOAE, was significantly increased in AD mice from 9 months of age and thereafter. The significant loss of SGNs, but not hair cells, was observed in the cochlea of 9- and 12-month AD mice. On the other hand, we found that both ABR and DPOAE thresholds were significantly increased in HD mice from 2 months of age and thereafter. The large loss of hair cells and the small loss of SGNs were observed in the cochlea of 3-month HD mice. Furthermore, the prestin expression in outer hair cells (OHCs) was significantly decreased in HD mice from 2 months of age and thereafter, and the loss of prestin expression was earlier before OHCs death in HD mice. Different from HD mice, the prestin expression in OHCs in AD mice was not changed even at 12 months of age. Our data suggest that cochlear pathology contributing to hearing loss is quite different between transgenic mice of AD and HD. More detailed pathological mechanisms for hearing loss between AD and HD need further study.

**Key Words:** Alzheimer's disease, auditory brainstem response, cochlear pathology, distortion product otoacoustic emissions, hearing loss, Huntington's disease, prestin, transgenic mice

## Introduction

Alzheimer's disease (AD) is the most common dementia of mental decline in the elderly those characterized by cognitive, motor, and behavioral dysfunction (1, 10). While Huntington's disease (HD) is an inherited, neurodegenerative disorder that usually appears in mid-adult life and leads to uncoordinated body movements and cognitive decline (16). Coincidentally, patients with AD and HD are found to have abnormal hearing problems. Clinically typical dementia AD is associated with deficient perceptual and semantic pro-

cessing of environmental sounds and melodies (7, 11). High correlation between hearing loss and dementia was observed in elderly populations (21). HD patients are often troubled with abnormal auditory memory and hearing impairment (8, 13). However, physiological and histological divergence of hearing impairment between AD and HD patients has not been thoroughly assessed.

In the mammalian cochlea, the inner and outer hair cells (IHCs and OHCs) are mechanoreceptors that convert sound waves into electro-potentials leading to firing of action potential of auditory nerve fibers.

The IHCs are involved in the transduction of motion of basilar membrane into a neural code in the auditory nerve during sound stimulation; OHCs undergo rapid somatic length changes when the voltage across their basolateral membranes is altered (9). This somatic electromotility enhances the vibration of the basilar membrane resulting in increased cochlear sensitivity and frequency selectivity (4, 5). Previous studies have shown that prestin, which is abundant in the basolateral membrane, is responsible for somatic electromotility of the OHCs (22). This protein is essential for normal hearing sensitivity and frequency selectivity of mammals and elimination of this membrane protein leads to OHCs death (12, 22).

In the present study, the cochlear pathology between AD and HD was compared utilizing 3xTg-AD and R6/2-HD mouse models. We assessed the hearing function between AD and HD mice using auditory brainstem response (ABR) and distortion product otoacoustic emissions (DPOAE) techniques. The cochlear histology between AD and HD mice was examined by hematoxylin and eosin stain (H&E stain). Particularly, hearing-related prestin protein expression in the OHCs was also compared, between AD and HD mice, by using immunohistochemistry stain. Our observations suggested that cochlear pathology of hearing loss is different between transgenic mice of AD and HD.

## Materials and Methods

### *Animals*

In this study, 32 male 3xTg-AD and 39 male R6/2-HD mice were used to compare cochlear pathology of hearing loss. The 3xTg-AD mouse model has been generated to exhibit plaque and tangle pathology, as well as synaptic dysfunction (18). The 3xTg-AD mouse model is unique from previous models as it expresses 3 dementia-related transgenes, namely APPSWE, PS1M146V, and tauP301L, and demonstrates a clear age-dependent onset of AD neuropathology. Pathology of 3xTg-AD mice is very similar to that seen in human AD patients (17). While the R6/2-HD mice are one of the first transgenic mouse models developed to study HD. The R6/2-HD mice express exon 1 of the human HD gene with around 150 CAG repeats (15). They develop progressive behavioral deficits, characterized by hindlimb clasping on tail suspension, impaired performance on motor tests, as well as weight loss, striatal atrophy and reduced hippocampal neurogenesis (6).

In this study, the survival time, body weight, ABR thresholds, DPOAE thresholds and cochlear histology were evaluated and compared between homozygous 3xTg-AD mice ( $n = 17$ ) and their Non-AD mice

(C57B/6J mice,  $n = 15$ ). The same items were also measured and compared, between homozygous R6/2-HD mice ( $n = 22$ ) and their Non-HD mice (littermate wild-types,  $n = 20$ ). All mice were group housed during the duration of testing, and were provided food and water *ad libitum*. They were maintained in a 12:12 light cycle and all auditory testing was conducted during the light phase of the animal's sleep-wake cycle. All animal experimental procedures were approved by the Committee on Animal Research of National Taiwan Normal University and carried out in accordance with the guidelines of the Committee.

### *Functional Hearing Assessment*

Both thresholds of ABR and DPOAE were assessed and compared, between AD and their Non-AD mice, and HD and their Non-HD mice. Animals were treated with atropine (0.5 mg/kg im) and then anesthetized with urethane (1.2 g/kg ip; Sigma, St. Louis, MO, USA). The rats were considered to be fully anesthetized when the withdrawal reflex was absent during application of pressure to the paws or tail with a hemostat. Anesthesia was supplemented (0.06 g/kg) if any reflex activity was observed. Rectal temperature was maintained at  $37 \pm 1^\circ\text{C}$  by a heating lamp.

The ABR were recorded using digital signal processing hardware and software of Tucker Davis Technologies (TDT, Alachua, FL, USA). Acoustic stimuli were generated digitally using SigGenRP software (TDT) and RX6 Piranha Multifunction Processor hardware (TDT). Prior to the ABR recording, stimuli were calibrated using SigCal software (TDT) and an ER-10B+ low noise microphone system (Etymotic Research Inc., Elk, Grove, IL, USA). The stimuli were delivered monaurally (right ear) into the external auditory meatus of the mice using an EDC1 electrostatic speaker driver (TDT) through an EC-1 electrostatic speaker (TDT). The evoked potentials were filtered (0.3-3.0 kHz), averaged (500 waveforms) and stored in computer hardware for offline analysis later. To determine the thresholds of ABR, the evoked responses were recorded in a 10 dB-step descending from a maximum stimulus intensity of 100 dB sound pressure level (SPL). The background activity was measured before the stimulus onset. The ABR threshold was defined as the stimulus intensity that evoked waveforms with a peak-to-peak voltage greater than 2 standard deviations (SD) of the background activity (2). The ABR thresholds were obtained for sound frequency of 16 kHz in this study.

As suggested by previous study (14), the DPOAEs were also recorded using TDT digital signal processing hardware and software. All stimulation of DPOAE were created using TDT SigGen software (TDT), while ear canal recordings were conducted

using TDT BioSig software (TDT). The DPOAEs were generated by simultaneously two tones differing in frequency (the lower-frequency labeled  $f_1$  and the higher-frequency  $f_2$ ) into the sealed ear canal mice. The primary tones ( $f_1$  and  $f_2$ ) are presented simultaneously into the sealed ear canal. The primary tones are always presented at a fixed ratio ( $f_2/f_1 = 1.2$ ). The distortion product at the  $2f_1 - f_2$  frequency is a reliable indicator of outer hair cell function (14). The place of distortion generation in the cochlea appears to be close to the frequency place of  $f_2$ . Consequently, we plotted the distortion threshold curve as a function of  $f_2$ . DPOAE threshold curves were measured for  $f_2$  frequencies from at 16 kHz. By keeping  $f_2$  constant and varying  $f_1$  the optimum stimulus separation (best ratio  $f_2/f_1$ ) was determined, which produced maximum DPOAE levels at low stimulus levels. With the primary tones set at best ratio, growth functions of the  $2f_2/f_1$  distortion were measured by step-wise increasing the stimulus levels. The level of  $f_2$  sufficient to elicit a DPOAE was defined as the threshold criterion.

#### *Tissue Preparation and Immunostaining*

Anesthetized mice with urethane (1.2 g/kg ip; Sigma, St. Louis, MO, USA) were cardiac perfused first with phosphate-buffered saline (PBS) containing 4% formaldehyde then fixed with 4% formaldehyde (EM grade). The specimens were then washed with PBS and decalcified with 4% EDTA in PBS (pH 7.4) for 3-5 days at 4°C. Decalcified cochlea specimens were then embedded in paraffin, cut into sections (5  $\mu$ m), and placed on coated slides. For immunohistochemistry, sections were stained for room temperature 1 h with prestin polyclonal goat antibody (1:2000 diluted, Santa Cruz, Dallas, TX, USA) using the heat-induced epitope retrieval method. Detection was performed by incubation with biotinylated secondary antibodies (Novolink™ polymer detection system I), 30 min at room temperature, followed by 30 min incubation with avidin–biotin–HRP complex (Novolink™ polymer detection system I). Visualization was performed with DAB Chromogen (Novolink™ polymer detection system I) and counterstained with hematoxylin (Novolink™ polymer detection system I) following suppliers protocol.

#### *Quantification and Statistics Analysis*

As suggested by previous study (13), the relative SGCs density, OHCs and IHCs number in the basal, mid-basal, middle and apical cochlear regions were normalized to a percentage between transgenic mice and their WT mice at different lifespan stage. The OHCs, IHCs were identified by the presence of

a nucleus. The OHCs survival percentage was calculated as the number of intact OHCs present among the 3 OHCs that should be observed in each turn of one cochlea in tissue sections of WT mice with normal hearing. The IHCs survival percentage was calculated as the number of intact IHCs present of the one IHC that should be observed in each turn of one cochlea in tissue sections of mice with normal hearing. Using mid-modiolus sections, the SGCs survival was calculated as the number of SGCs per  $\text{mm}^2$ . The corresponding area of Rosenthal canal was measured on digital photomicrographs of each canal profile. The computer then calculated the area within the outline. For evaluation of prestin expression in OHCs between AD and Non-AD mice, and HD and Non-HD mice, the size and intensity of prestin staining images of OHCs were processed with imaging software (SigmaScan software; Systat Software Inc.) into 8-bit grey tone scale. Evaluation and assembly of images were done with Adobe Photoshop. The relative prestin levels in OHCs were normalized to a percentage between transgenic mice and their WT mice at different lifespan stage.

Statistical evaluations were done by multiple comparisons test. One-way or two-way ANOVA was first performed, and then Student-Neuman-Keuls was completed as the posteriori test (20). All average values are presented as means  $\pm$  standard error of the mean (SEM). Significance was set at  $P < 0.05$ .

## **Results**

#### *Comparison of Survival Time and Body Weight between AD and HD Mice*

In this study, AD and HD mice exhibit a lifespan much shorter than their Non-AD and Non-HD mice (Fig. 1, A and C). AD mice exhibit normal body weight compared to their Non-AD mice at 6 and 9 months of age (Fig. 1B). AD mice lose their body weight from 12 months of age and thereafter. HD mice exhibit normal body weight compared to their Non-HD mice only at 1 month of age (Fig. 1D). HD mice lose their body weight from 2 months of age and thereafter.

#### *Comparison of Auditory Function between AD and HD Mice*

We physiologically assessed and compared the auditory function between AD and HD mice by using the ABR and DPOAE analysis. We have determined thresholds of ABR and DPOAE in sound frequency at 8, 16, 32 kHz. Generally, thresholds of ABR and DPOAE were most obvious when they were determined in sound frequency at 16 kHz. For easy comparison, we selected thresholds of ABR and DPOAE

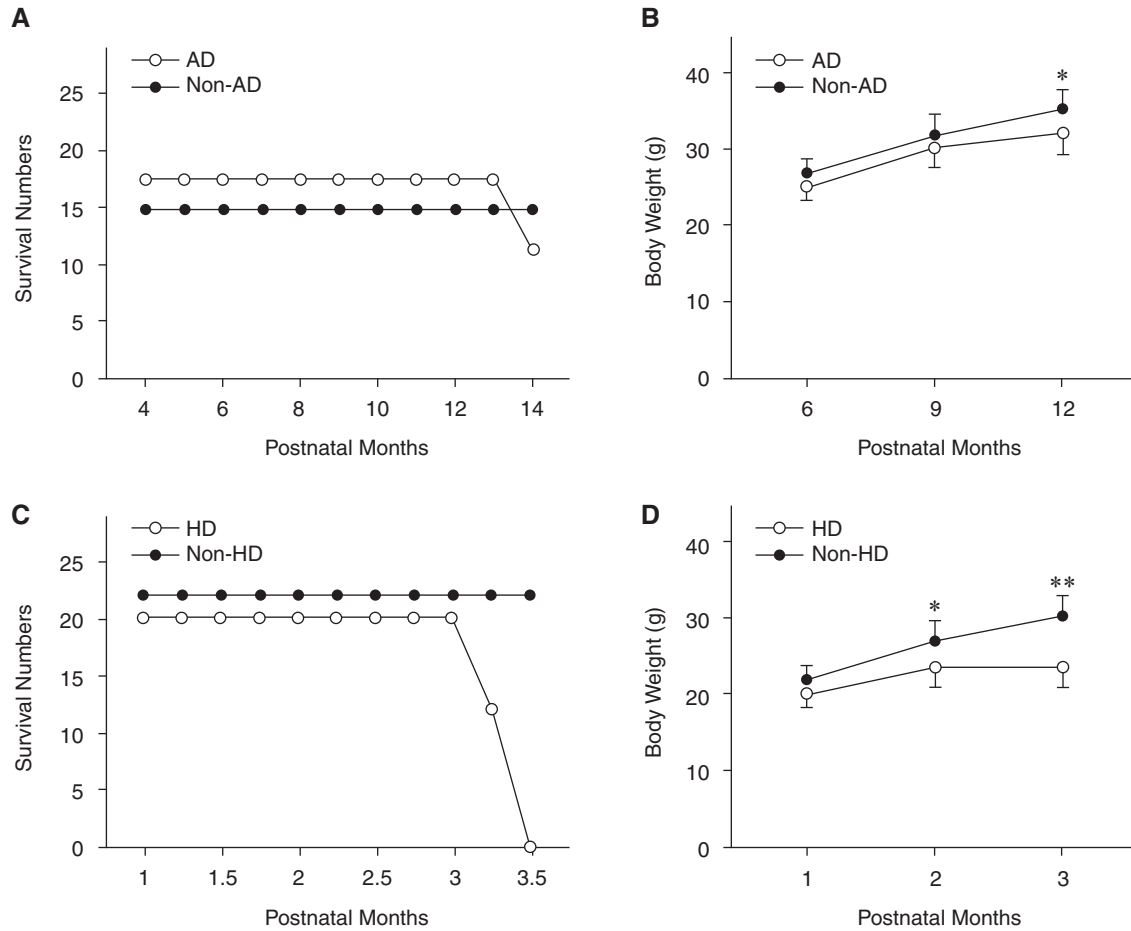


Fig. 1. The survival time and body weight of 3xTg-AD and R6/2 HD mice at different lifespan stage. A, C: The number of survival was drastically reduced from 13 months of age in AD mice and from 3 months of age in HD mice. B, D: The difference of body weight was significant from 12 months of age in AD mice and from 2 months of age in HD mice. Animal numbers are as follow: AD mice  $n = 17$ , Non-AD mice  $n = 15$ , HD mice  $n = 22$  and Non-HD mice  $n = 20$ . Values are mean  $\pm$  SEM, \* $P < 0.05$ ; \*\* $P < 0.01$ , two-way repeated-measures ANOVA followed by a student-Newman-Keuls multiple comparisons post-test were used.

that were determined in sound frequency at 16 kHz between AD and HD mice. AD mice exhibit increased ABR thresholds at 9 and 12 months of age as compared with their Non-AD mice (Fig. 2A,  $P < 0.01-0.05$ ) respectively, but not changed their DPOAE thresholds (Fig. 2B,  $P = 0.35$ ). Unlike the AD mice, HD mice exhibit increased ABR and DPOAE thresholds at 2 and 3 months of age as compared with their Non-HD mice (Fig. 2, C and D,  $P < 0.01$ ).

#### Comparison of Spiral Ganglion Neurons (SGNs) and Hair Cells in the Cochlea between AD and HD Mice

We compared the relative density of SGNs and hair cells in the cochlea of transgenic mice and their WT. AD mice at 9 and 12 months of age exhibit greatly reduction of the relative densities of SGNs in the cochlea as compared with their Non-AD mice (Fig. 3B,  $P < 0.01$ ), but not damaged their hair cells

(Figs. 3A, 4B). Unlike the AD mice, HD mice at 3 months of age exhibit slight reduction of the relative densities of SGNs in the cochlea as compared with their Non-HD mice (Fig. 3D,  $P < 0.05$ ) and great damage of hair cells in the cochlea (Figs. 3C, 4C, D).

#### Comparison of Prestin Expression in Outer Hair Cells between AD and HD Mice

As described in previous study, prestin expression in OHCs is essential for normal hearing sensitivity and frequency selectivity in mammals (22). We compared the prestin expression in OHCs of transgenic mice and their WT. By using immunostaining analysis, we compared and normalized the relative prestin expression in the OHCs to a percentage between transgenic mice and their WT mice at different lifespan stage. AD mice at 6, 9 and 12 months of age exhibit similar the relative prestin expression

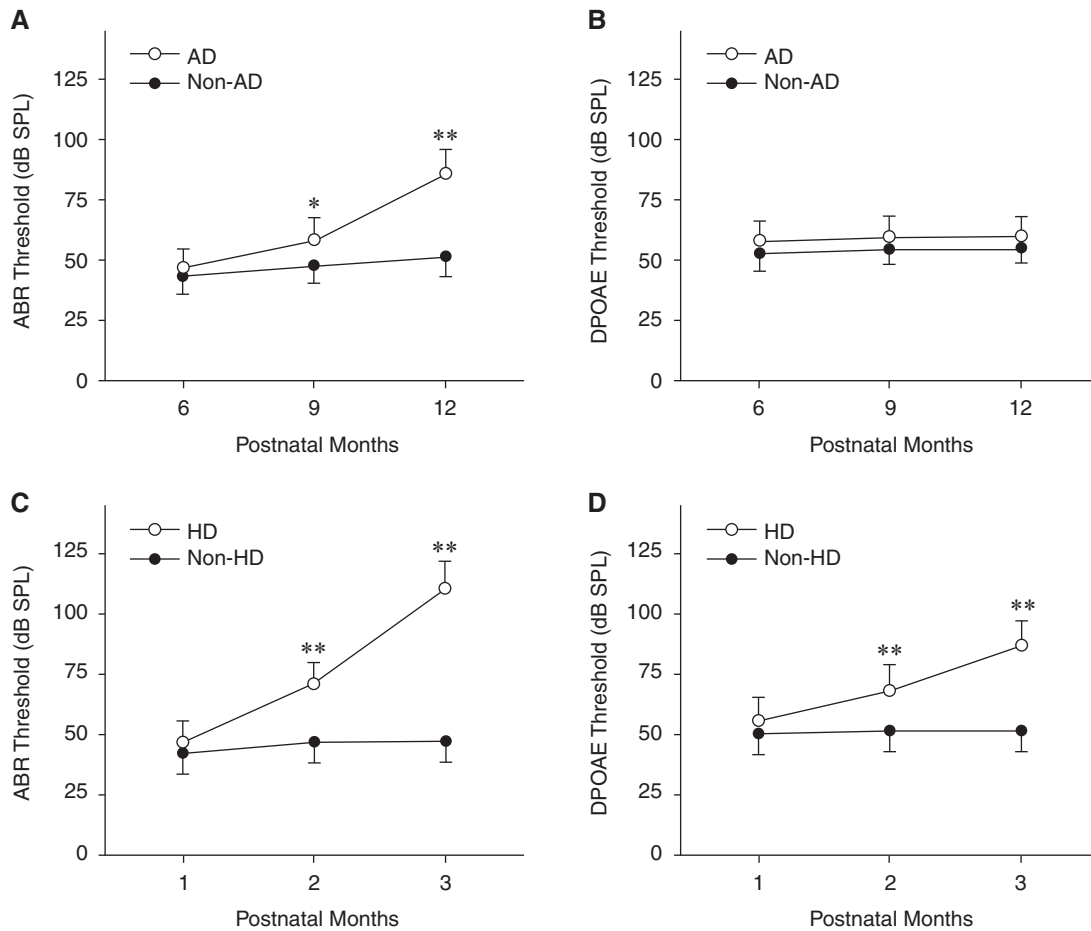


Fig. 2. The thresholds of ABR and DPOAE of 3xTg-AD and R6/2 HD mice at different lifespan stage. A, C: The difference of ABR thresholds was significant from 9 months of age in AD mice and from 2 months of age in HD mice. B, D: The difference of DPOAE thresholds was significant from 2 months of age in HD mice. However, there is no difference in the thresholds of DPOAE at different lifespan stage of AD mice. AD mice  $n = 17$ , Non-AD mice  $n = 15$ , HD mice  $n = 22$  and Non-HD mice  $n = 20$ . Values are mean  $\pm$  SEM,  $*P < 0.05$ ;  $**P < 0.01$ , two-way repeated-measures ANOVA followed by a student-Newman-Keuls multiple comparisons post-test were used.

in the OHCs as compared with their Non-AD mice (Fig. 4A). Unlike AD mice, HD mice exhibit reduced the relative prestin expression in OHCs with age as compared with their Non-HD mice (Fig. 4, C and D). It is worth noting that the reduction of prestin expression in OHCs was earlier before death of OHCs for HD mice (Fig. 4D).

### Discussion

This study elaborates the possible causes of hearing dysfunction between 3xTg-AD and R6/2-HD mice. AD and HD mice exhibit hearing loss because the thresholds of ABR were increased significantly with age (Fig. 2, A and C). However, the thresholds of DPOAE were increased significantly with age only for HD mice (Fig. 2D), but not for AD mice (Fig. 2B). The ABR recording is a neurologic test of auditory brainstem function to auditory stimuli that generates

a response from the cochlea (23). DPOAEs are generated in the cochlea in response to two tones of a given frequency and sound pressure level presented in the ear canal. They are an objective indicator of normally functioning OHCs in the cochlea (14). Abnormal ABRs in AD and HD mice may suggest there are some problems in central or peripheral auditory system. Abnormal DPOAEs in HD mice suggest there are some problems in OHCs in the cochlea.

In AD patients, neurofibrillary tangles and neuritic plaques have been found in the primary auditory cortex and the auditory association cortex (3, 19). It seems reasonable to explain why AD mice have abnormal ABRs. Large reduction of SGNs in the cochlea of AD mice may also provide a reasonable explanation for abnormal ABRs. Our preliminary results suggest that tau hyperphosphorylation and p-tau aggregation, and mitochondria- and endoplasmic-reticulum stress-mediated apoptosis may play a role in degeneration

Fig. 3---color

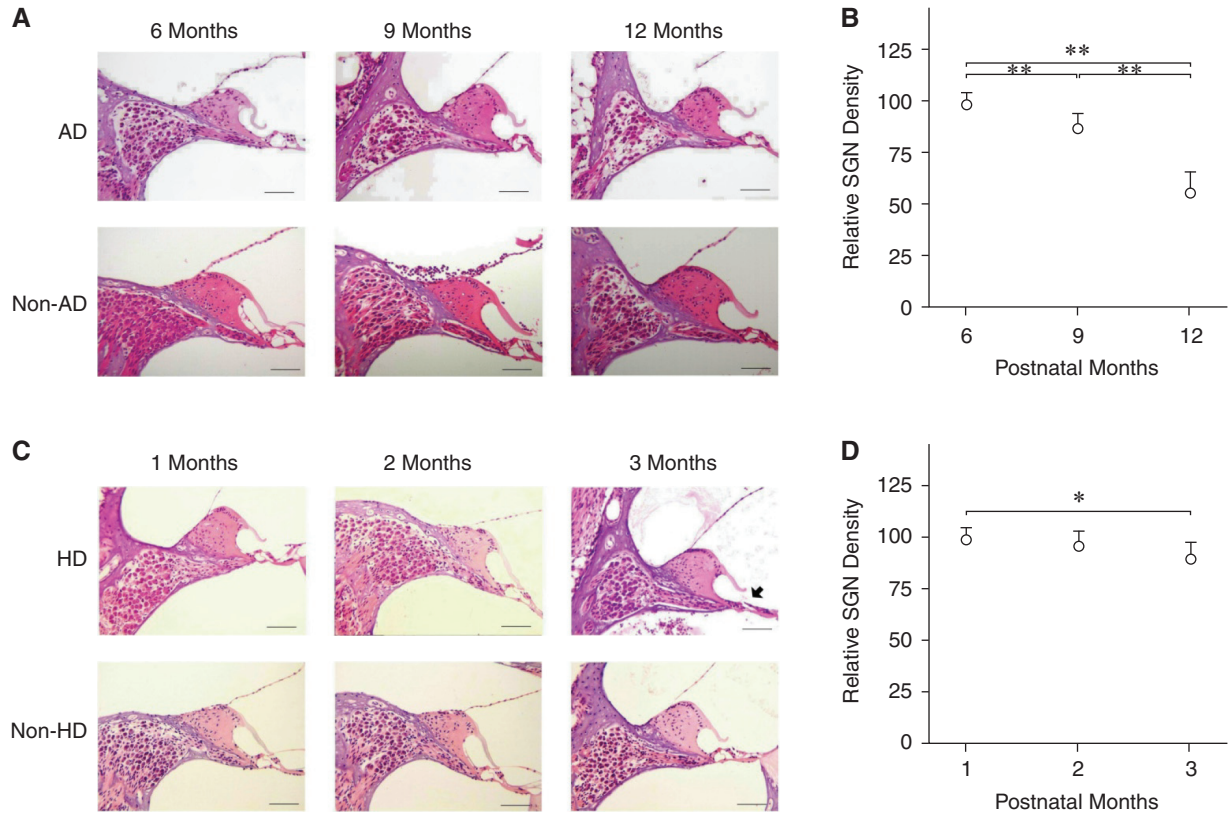


Fig. 3. The H&E staining and spiral ganglion neurons (SGNs) in the basal cochlear region of 3xTg-AD and R6/2 HD mice at different lifespan stage. A: The H&E staining of basal cochlear regions show obvious SGNs damage in AD mice at 9 and 12 months. B: The relative SGNs densities of AD mice were significantly reduced from 9 months of age and thereafter. C: The H&E staining of basal cochlear regions show obvious hair cells damage (marked by the arrow) and slightly SGNs damage in HD mice at 3 months. D: The relative SGNs densities of HD mice were only significantly reduced at 3 months. Animal numbers are as follow: AD mice at 6 months  $n = 7$ , at 9 months  $n = 8$ , and at 12 months  $n = 7$ ; Non-AD mice at 6 months  $n = 8$ , at 9 months  $n = 8$  and at 12 months  $n = 8$ ; HD mice at 1 month  $n = 9$ , at 2 months  $n = 8$  and at 3 months  $n = 8$ ; Non-HD mice at 1 month  $n = 8$ , at 2 months  $n = 8$  and at 3 months  $n = 8$ . Values are mean  $\pm$  SEM, \* $P < 0.05$ ; \*\* $P < 0.01$ , two-way ANOVA followed by a student-Newman-Keuls multiple comparisons post-test were used.

of SGNs in the cochlea of AD mice. Possible pathological mechanisms for auditory SGNs degeneration in AD mice are worth further studied.

Intact OHCs in the cochlea of AD mice provide a reasonable explanation for their normal DPOAEs. Unlike AD mice, older HD mice exhibit abnormal ABRs and DPOAEs. It has been reported that HD patients have abnormalities in central auditory processing (19). The results provide a reasonable explanation for abnormal ABRs. As described earlier, DPOAEs are an objective indicator of normally functioning OHCs in the cochlea. Large reduction of OHCs in the cochlea of HD mice may provide a reasonable explanation for abnormal DPOAEs. We believe that possible pathological mechanisms for auditory hair cells degeneration in HD mice are worth further studied.

As illustrated in Introduction, prestin in OHCs is essential for normal hearing sensitivity and elimi-

nation of this protein leads to OHCs death (12, 22). The reduction of prestin levels in the OHCs were observed in HD mice at 2 and 3 months of age (Fig. 4D). The result may partly explain why HD mice exhibit abnormal DPOAEs at 2 and 3 months of age. For HD mice at 2 months of age, the averaged ABR threshold was elevated at around 30 dB, but the averaged DPOAE threshold only elevated at around 20 dB. Even though we did not find apparent changes in the relative SGN densities of HD mice at 2 months of age, the reduction of prestin levels in the OHCs of HD mice might partly explain why ABR and DPOAE thresholds were elevated. In R6/2 HD mice, as suggested in previous studies, aggregates/inclusions in the striatum and the cortex is first appeared around 3-4 weeks of age. Despite few SGNs actually dying in the cochlea of HD mice, there is ample evidence that their brains do not function in a normal manner. For example, there are clear changes in mutant huntingtin expres-

Fig. 4--color

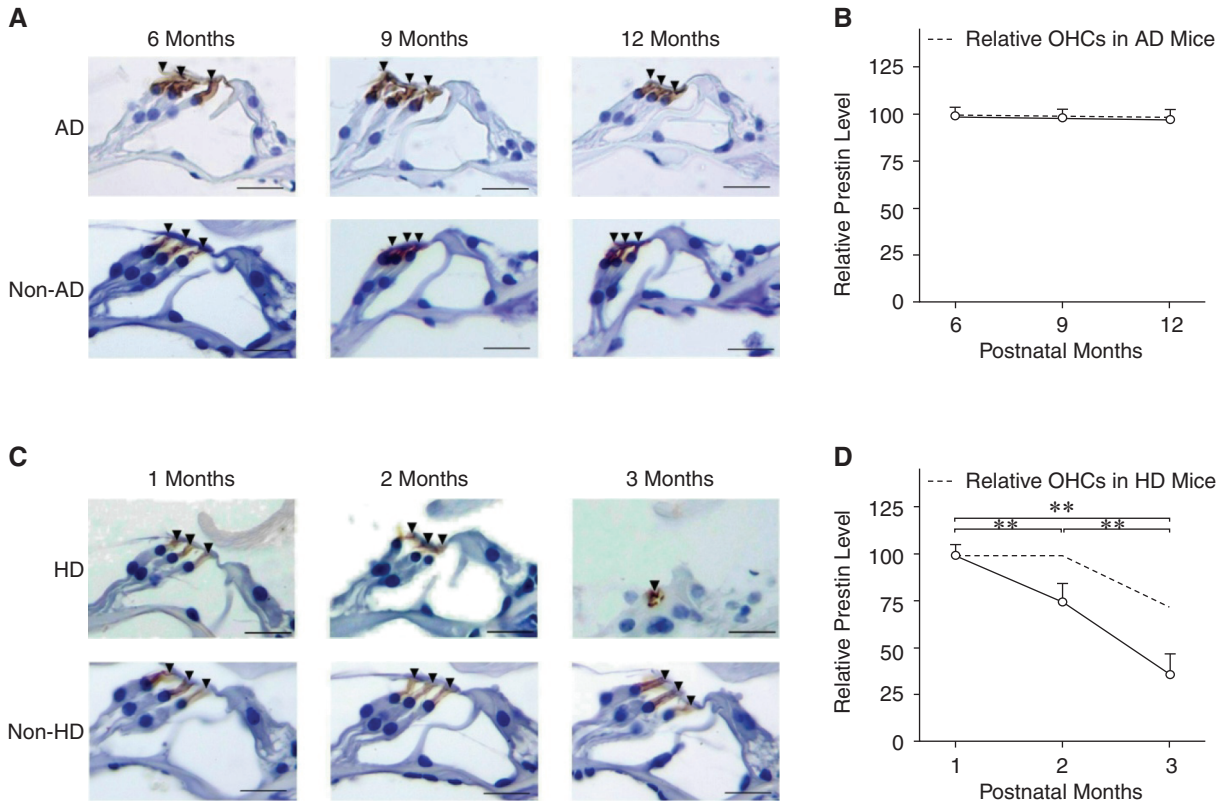


Fig. 4. Expression of hearing-related prestin in outer hair cells (OHCs) of 3xTg-AD and R6/2 HD mice at different lifespan stage. **A**: Immunostaining of prestin expression (brown color marked by arrows) in OHCs of AD and their Non-AD mice were obvious at different lifespan stage. **B**: The relative prestin levels in OHCs of AD and Non-AD mice were similar among different lifespan stage. **C**: Immunostaining of prestin expression (brown color marked by arrows) were significantly reduced from 2 months of age and thereafter. **D**: The relative prestin levels in OHCs of HD mice were significantly reduced from 2 months of age and thereafter in compared with their Non-HD. It is worth noting that the reduction of relative prestin level was earlier before OHCs death. Animal numbers are as follow: AD mice at 6 months  $n = 6$ , at 9 months  $n = 7$ , and at 12 months  $n = 6$ ; Non-AD mice at 6 months  $n = 6$ , at 9 months  $n = 6$  and at 12 months  $n = 7$ ; HD mice at 1 month  $n = 7$ , at 2 months  $n = 8$  and at 3 months  $n = 7$ ; Non-HD mice at 1 month  $n = 8$ , at 2 months  $n = 8$  and at 3 months  $n = 6$ . Values are mean  $\pm$  SEM,  $*P < 0.05$ ;  $**P < 0.01$ , two-way ANOVA followed by a student-Newman-Keuls multiple comparisons post-test were used.

sion in the striatum and cortex of HD mice that have been documented as early as at 6 weeks and become more pronounced with age (6, 15). The result may partly explain why HD mice exhibit greater ABR thresholds at 2 months of age.

In addition, we have clarified that the reduction of prestin expression in OHCs of HD mice is frequency-specific modification. For HD mice at 3 months of age, we found their hearing loss to higher frequency sound is greater than those to lower frequency sound. We also found that the loss of the number to OHCs with sensing higher frequency sound at the lower basal turns of cochlea is greater than those with sensing lower frequency sound at the apical turns of cochlea. Moreover, our immunohistochemistry analysis demonstrates the reduction of prestin stain was greater in the OHCs with sensing higher frequency sound than those with sensing lower frequency sound. We measured the relative prestin expression in OHCs at the apical

turns of cochlea is greater than those OHCs at the lower basal turns of cochlea as compared to their Non-HD mice ( $65 \pm 12$  vs.  $47 \pm 0.11$ ,  $P < 0.01$ ). The frequency-specific modification of prestin expression in OHCs of the cochlea of HD mice might provide a good model for auditory physiologists and otolaryngologists to research presbycusis.

In conclusion, our findings suggest that both older AD and HD mice exhibit hearing dysfunction because of abnormal increases of ABR thresholds. Abnormalities in central auditory processing and SGNs damage may be involved in hearing dysfunction of AD and HD mice. Unlike AD mice, older HD mice show great damage of OHCs in the cochlea. The earlier reduction of prestin expression occurs before OHCs death that may explain some reasons for increasing DPOAE thresholds in HD mice. Possible pathological mechanisms for auditory neurodegeneration between AD and HD are worth further studied.

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## Conflicts of Interest

There are no conflicts of interest.

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