

## Original Article

# Changes of ribbon synapses number of cochlear hair cells in C57BL/6J mice with age<sup>Δ</sup>

Xing-Wang Jiang<sup>1</sup>, Xiao-Rui Li<sup>2</sup>, Yan-Ping Zhang<sup>1</sup>

<sup>1</sup>Department of Otolaryngology, 309th Hospital of Chinese PLA, Beijing 100091, China; <sup>2</sup>Department of Otolaryngology, Yuncheng Central Hospital, Yuncheng 044099, Shanxi, China

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**Abstract:** To investigate the changes of ribbon synapses (RS) number in cochlear hair cells in C57BL/6J mice with age. Basilar membranes within the cochlea of C57BL/6J mice aged 2, 6, 10 and 12 months were harvested (5 mice in each age group). The presynaptic and postsynaptic membranes were subject to double immunohistochemical staining and observed with a laser confocal microscope. The number of RS in each segment of basilar membrane was counted by using 3D reconstruction technique. Compared with 2-month-old mice, reduction of RS number in basilar membrane inside cochlea mainly occurred to the basal turn among C57BL/6J mice aged 6 months. The number of RS in each turn among 10-month-old mice decreased considerably, and such decrease continued in the top turn and middle turn in mice aged 12 months. In contrast, the number of RS in the basal turn increased slightly. Reduction of RS probably takes place in the early stage of C57BL/6J mice presbycusis. Early prevention of presbycusis can be achieved through measures to mitigate the reduction of RS.

**Keywords:** C57BL/6J mice, inner hair cell, ribbon synapse

## Introduction

C57BL/6J mice are senescence accelerated mice with genetic defects and show hearing changes that resemble presbycusis. They are generally used as animal model for the study of presbycusis [1]. Ribbon synapses of cochlear hair cells are the first synapses engaged in centripetal transmission, which derive their name from the ribbon-like distribution pattern. The ribbon synapses play an important role in sound encoding and transmission [2]. Various factors can affect the morphology and number of RS, including aging, continuous noise stimuli, genetic mutations, and the use of ototoxic drugs, all of which can lead to sensorineural hearing loss to varying extent [3-5]. Ultrathin sectioning is generally applied in combination with transmission electron microscope to observe the morphology of ribbon synapses. We combined immunofluorescence staining technique, laser confocal microscopy and 3D reconstruction technique together in observing the dynamic changes of ribbon synapses number of cochlear hair cells in C57BL/6J mice with

age. New experimental findings were provided by the research on the pathogenesis of presbycusis.

## Materials and methods

### Experimental animals

C57BL/6J mice aged 2, 6, 10 and 12 months were provided by Experimental Animal Center of Peking University Health Care Center. Five mice weighted 20-30 g with normal auricle reflex were chosen for each age group regardless of gender. Mice with middle ear and inner ear diseases were excluded.

### Reagents

1% pentobarbital sodium (Laboratory Animal Research Center of 309 Hospital of the PLA); goat anti-mouse CtBp2 antibody (SANTA CRUZ, USA); rabbit anti-mouse glutamate receptor 2/3 (CHEMICON, USA); FITC-labelled donkey anti-goat antibody (Jackson, USA); TRITC-labelled donkey anti-rabbit secondary antibody (Jackson, USA).

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### *Experimental method*

#### *Preparation of basilar membrane of cochlea:*

The mice were anesthetized by intraperitoneal injection of 1% pentobarbital sodium at 40 mg/kg. After the mice were immobilized on the operating table, the chest was opened. The cardiac apex was punctured with a fine needle and fixed with vascular clamps. The blood was replaced by perfusion with 0.01 M PBS until the perfusion fluid turned colorless. Then cardiac perfusion was performed using 4% paraformaldehyde, and the specimens were fixed. The mice were sacrificed by cervical dislocation and the temporal bones were removed to harvest bilateral cochleas. The stapes were removed under the anatomic microscope. The round window and oval window were opened, and the cochlear tip was dripped with fine needle so that a top turn was connected with the basal turn. Next 4% paraformaldehyde was perfused from cochlear top to round window and oval window using a 2 mm plastic straw so as to replace perilymph in scala vestibuli and scala tympani. The tissues and cells in the cochlea were fixed. After perfusion, the cochlea was fixed in 4% paraformaldehyde and placed at room temperature for 1.5 h or in the fridge at 4°C overnight. Then the cochlea was taken out, washed with 0.01 M PBS twice, and then soaked in 10% EDTA (PH=7.4) for 1 d for decalcification until the bony wall was easily pierced with fine needle. The decalcified cochlea was placed in 0.01 M PBS, and the cochlear shell was stripped from the basal turn under the anatomic microscope. Bony wall of cochlea, stria vascularis and spiral ligament were removed. Full-thickness stripping was performed to top turn. Basilar membrane of every turn was separated intact from the cochlear axis with the removal of vestibular membrane and tectorial membrane. The line connecting the cochlear tip and the vestibular window divided the basilar membrane into top, middle and bottom segments. The protocol was approved by the Animal Breeding Center and Use Committee of 309 Hospital of the PLA.

*Double immunohistochemistry staining:* The basilar membranes were incubated in PBST containing 0.25% Triton X-100 for 30 min, and excess PBST was removed with disposable plastic straw. The basilar membrane was washed with PBS buffer for three times, 10 min each time. After that, the basilar membrane

was placed into EP tube containing 3% donkey serum blocking buffer so as to block non-specific sites. Then the blocking buffer was removed and PBS buffer was used to wash the basilar membrane for 3 times, 10 min each time. Next the basilar membranes were incubated with goat anti-mouse CtBp2 antibodies (diluted 1:100 in PBS buffer) and rabbit anti-mouse GluR2/3 antibodies (diluted 1:100 in PBS buffer). The specimens were placed in the fridge at 4°C overnight. The specimens were taken out the next day, and the basilar membranes stained with primary antibodies were washed with PBS buffer for three times, 10 min each time. Later, donkey anti-goat FITC-labelled antibodies (diluted 1:100 in PBS buffer) and donkey anti-rabbit IgG-TRITC antibodies (diluted 1:100 in PBS buffer) were added to incubate the cells at room temperature for 30 min in the dark. Excess secondary antibodies were removed and the specimens were washed with PBS buffer for three times, 10 min each time. This was followed by washing with distilled water once. The specimens were coated to the glass slide with DAPI staining buffer, and sectioning was performed.

*Laser confocal microscopy:* The specimens after immunofluorescence staining were observed by laser confocal microscopy and DAPI was excited at the wavelength of 358 nm. The observation points were selected under the 40x oil immersion objective. The numbers were magnified by 1.5 times locally. First the nuclei of inner hair cells were located. FITC was excited at 494 nm and TRITC at 547 nm. The size of the view window was adjusted. Two-channel observation mode was chosen, and the cells were double stained as orange yellow. After the specific fluorescence was detected, continuous scan was carried out for the basilar membranes at 1.0 µm slice interval until the specific fluorescence disappeared. The 2D images were collected. Image files belonging to one sequence were stored in one folder and numbered successively.

*3D reconstruction:* The 2D images were called in through the top view window under 3DS max2012 in a top-to-bottom sequence, which was the sequence during scanning. First one 2D image was called in, and a sphere was drawn at the position of orange fluorescence as the mark. Then the second image was called in. If the position of orange fluorescence was the

**Table 1.** Total number of RS in each turn of basal membrane of cochlea in C57BL/6J mice belonging to different age groups ( $\bar{x} \pm s$ )

Age (months)	Top turn <sup>ΔΔ</sup>	Middle turn <sup>ΔΔ</sup>	Basal turn <sup>ΔΔ</sup>
2	226.17±12.891 <sup>*</sup>	202.00±14.751	194.17±14.552
6 <sup>Δ</sup>	229.33±15.488	180.00±16.876	108.17±8.472
10 <sup>Δ</sup>	199.67±14.747	148.50±10.075	110.50±11.743
12	159.67±24.704 <sup>**</sup>	132.50±13.795	125.17±22.049

Note: <sup>\*</sup>P<0.05 compared with the number of RS in middle turn and basal turn of mice of the same age group; <sup>Δ</sup>P<0.05 in pairwise comparison between mice aged 6 months and 10 months in terms of total number of RS in top turn, middle turn and basal turn; <sup>\*\*</sup>P<0.05 compared with the number of RS in basal turn of mice of the same age group; <sup>ΔΔ</sup>P<0.05 in pairwise comparison of number of RS in the top turn except P>0.05 among mice aged 2 months and 6 months; P<0.05 in comparison of number of RS in middle turn between mice aged 2 months and those aged 6, 10 and 12 months and between mice aged 6 months and those aged 10 and 12 months; P<0.05 in comparison of the number of RS in basal turn between mice aged 2 months and those aged 6, 10 and 12 months.

same as in the first image, no mark was made, and the same synapse was indicated. If the orange fluorescence occurred at other positions, it was believed that a new synapse was found on this slice. Therefore, a sphere was drawn as the mark [6]. By this method, total number of RS in each segment of basilar membrane and the number of RS in each inner hair cell were calculated. The average number of RS in each inner hair cell was the mean of the sum of average number of RS in each inner hair cell of each segment within each field of view, expressed as mean  $\pm$  standard deviation ( $\bar{x} \pm s$ ).

*Statistical analysis*

Statistical analysis was performed using SPSS 16.0 software. One-way ANOVA was performed for the following values: total number of RS at different positions of basilar membrane among mice in the same age group, total number of RS at the same position of basilar membrane among mice of different age groups, average number of RS in each inner hair cell at different positions of basilar membrane among mice in the same age group, and average number of RS in each inner hair cell at the same position of basilar membrane among mice of different age groups. Tukey's-b test was used for pairwise comparison. For total number of RS at different positions among mice of different age groups and average number of RS in each inner hair

cell at different positions among mice of different age groups, repeated measures analysis of variance was adopted. P<0.05 indicated statistical significance.

**Results**

*Comparison of total number of RS in each segment of basilar membrane of the cochlea among C57BL/6J of different age groups*

Compared with 2-month-old mice, the reduction of RS in the basilar membrane of the cochlea in mice aged 6 months mainly occurred in basal turn; the reduction was less significant in middle turn, and no reduction was found in the top turn. For mice aged 10 months, the number of RS first began to decrease in top turn;

the reduction continued in the middle turn, and the number of RS in basal turn remained stable. In mice aged 12 months, the number of RS continued to decrease in top turn and middle turn, while that in the basal turn increased slightly (**Table 1; Figure 1**).

It can be seen that total number of RS in basilar membrane of the cochlea varied with age of C57BL/6J mice and segments of basilar membrane. The interaction of these two factors had an influence on the total number of RS in basilar membrane.

*Comparison of average number of RS in each inner hair cell in each segment of basilar membrane*

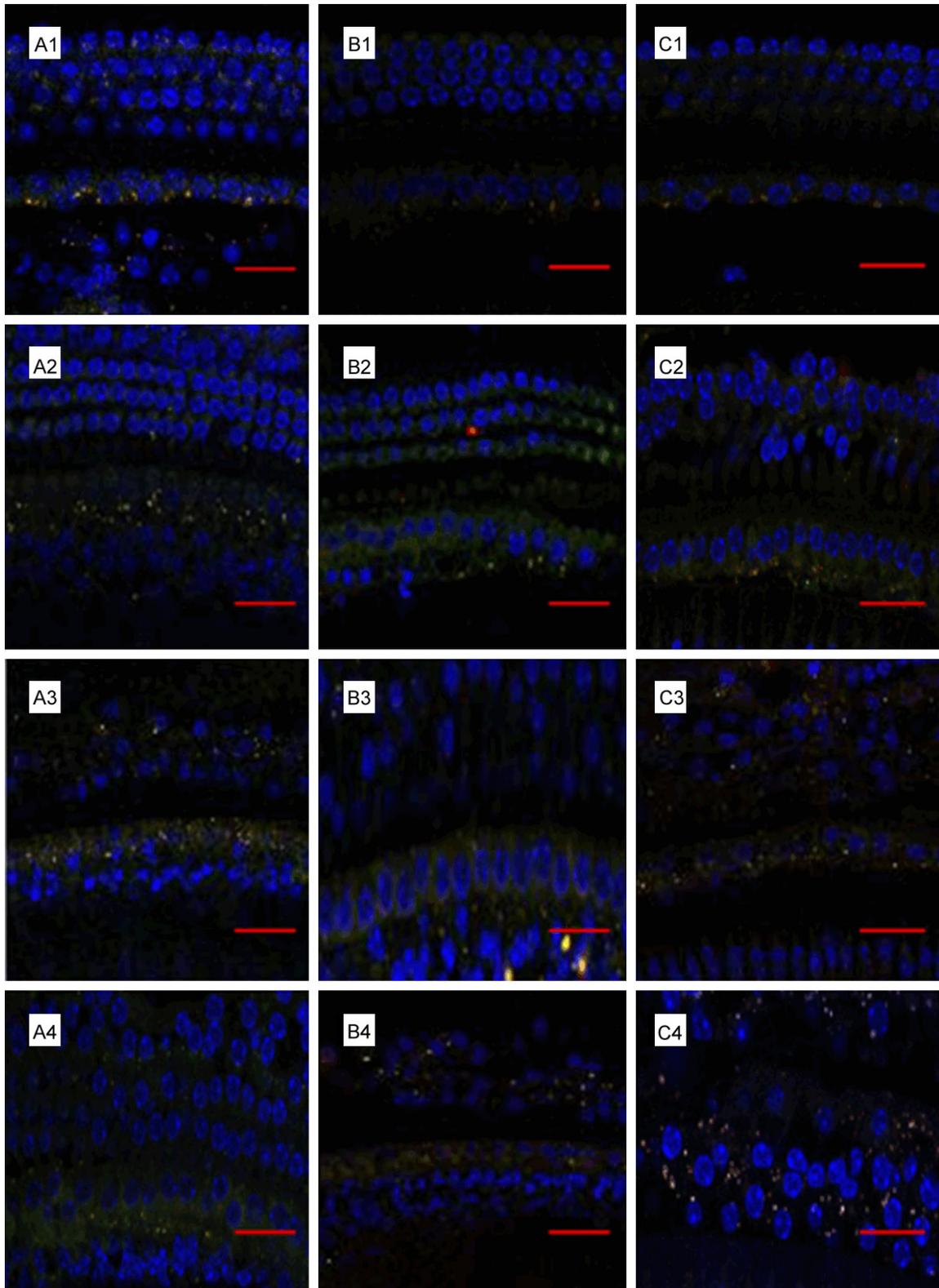
The result of average number of RS in each inner hair cell in each segment of basilar membrane among mice of different age groups was consistent with that of total number of RS in each segment of basilar membrane (**Table 2**).

It was obvious that the number of RS in each inner hair cell varied with age of C57BL/6J mice and segment of the basilar membrane. The interaction between age and segment of the basilar membrane had an influence on the average number of RS in each inner hair cell.

**Discussion**

Cochlear afferent synapses are the first synapses transmitting signals into central nervous

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**Figure 1.** Three-channel synthetic images of RS in different segments of basilar membrane in mice belonging to different age groups after immunofluorescence staining under laser confocal microscope. A-C. Indicate top turn, middle turn and basal turn, respectively; 1-4 indicate mice 2, 6, 10 and 12 months of age. Every orange yellow fluorescence represents one RS. Compared with mice aged 2 months, the reduction of RS in mice aged 6 months

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mainly occurred in basal turn. The number of RS in each turn decreased obviously in mice aged 10 months. The reduction continued in top turn and middle turn in mice aged 12 months, while the number of RS in the basal turn increased slightly. The scale was 15  $\mu$ m.

**Table 2.** Number of RS in each inner hair cell at different positions of basilar membrane in C57BL/6J mice of different age groups ( $\bar{x} \pm s$ )

Age (months)	Top turn <sup>ΔΔ</sup>	Middle turn <sup>ΔΔ</sup>	Basal turn <sup>ΔΔ</sup>
2 months	16.59±0.561 <sup>*</sup>	14.95±0.687	13.92±1.040
6 months <sup>Δ</sup>	16.57±0.565	13.66±1.049	8.29±0.715
10 months <sup>Δ</sup>	14.30±1.230	11.42±0.775	8.76±0.772
12 months	10.12±1.892	10.33±0.978	9.78±0.801

Note: <sup>\*</sup>P<0.05 compared with the number of RS in middle turn and basal turn of mice of the same age group; <sup>Δ</sup>P<0.05 in pairwise comparison between mice aged 6 months and 10 months in terms of total number of RS in each inner hair cell in top turn, middle turn and basal turn; <sup>ΔΔ</sup>P<0.05 in pairwise comparison of number of RS in the top turn except P>0.05 among mice aged 2 months and 6 months; P<0.05 in comparison of number of RS in middle turn between mice aged 2 months and those aged 6, 10 and 12 months and between mice aged 6 months and those aged 10 and 12 months; P<0.05 in comparison of the number of RS in basal turn between mice aged 2 months and those aged 6, 10 and 12 months.

system and play an irreplaceable role in the formation and loss of hearing. Changes of morphology, number and functions of ribbon synapses due to various factors may lead to hearing loss to varying extent.

More than 20 proteins can be detected in mature RS, and RIBEYE is the only specific protein on the presynaptic membrane that has enzymatic activity [7]. RIBEYE consists of A-domain and B-domain. B-domain contains the entire sequence of C-terminal binding protein 2 (CtBP2) except 20 N-terminal amino acids. Anti-CtBP2 antibodies can bind to the B-domain of RIBEYE, thereby labeling presynaptic membrane of ribbon. Glutamate receptor (GluR) between the inner hair cells and the afferent nerve synapses mediates rapid conduction of impulses. GluR2 and GluR3 AMPA (amino-hydroxy-methylxazole propionic acid) receptors are located on the neurotransmitter receptors of postsynaptic nerve endings. Therefore, anti-Glut2/3 antibodies binding to GluR2 and GluR3 can be used to label postsynaptic membrane. In our experiment, C57BL/6J mice 2, 6, 10 and 12 months of age were used for labeling of presynaptic membrane and postsynaptic membrane of ribbon in the basilar membrane of cochlea by double immunofluo-

rescence staining. Synapses were scanned in two-channel mode by laser confocal microscopy slice by slice. An intact RS was co-localized at sites of red and green fluorescence (color of orange yellow on synthetic images). By this method, the number of RS on basilar membrane of cochlea was determined conveniently.

Compared with mice aged 2 months, the reduction of RS in the basilar membrane mainly occurred in the basal turn among mice aged 6 months; the reduction was less significant in the middle turn, but no reduction occurred in the top turn. The number of RS began to decrease in the top turn in 10-month-old mice; the reduction continued in the middle turn and the number of RS remained constant in the basal turn of mice aged 10 months. The number of RS decreased in the top turn and middle turn of mice aged 12 months, while that in the basal turn increased somewhat. It was found by previous studies that C57BL/6J began to show signs of hearing loss at 6-8 months of age. ABR threshold indicated that high-frequency hearing loss was dominated [8, 9]. Consistency of structural and functional changes implied that the reduction of RS was mainly associated with hearing loss of C57BL/6J mice. According to the research by Stamatakis et al. [10], old-aged mice had a much smaller number of RS compared with young mice; the number of RS in the former was about one half that of the latter. Observation along the cochlear axis or at the base of inner hair cells revealed that the afferent nerve endings were distributed in a radial pattern in young animals. However, selective loss of afferent nerve endings in high-frequency region was found in old-aged mice. It was thus speculated that stereocilia of hair cells might have abnormalities of top connections [11, 12]. This damaged the electromechanical energy conversion on the top of cells, leading to an obvious increase of signals during the changes of membrane potential in inner hair cells. As a result, a large amount of glutamic acid is released in the presynaptic membrane, causing neurotoxicity. Moreover, edema, degeneration, necrosis or even excitatory injury of afferent nerves would happen. The long-term

accumulation of such damage may cause changes of the number and structure of RS and finally lead to auditory impairment [13].

Reduction of RS in the basal turn of basilar membrane of the cochlea was the most significantly in 6-month-old C57BL/6J mice. However, no obvious changes of the number of RS were observed in the top turn and middle turn of basilar membrane of the cochlea. This indicated RS first began to decrease in the basal turn. Stamatakis et al. [10] also found that the number of afferent nerve synapses in the inner hair cells of C57BL/6J mice varied with different segments of basilar membrane. RS in the inner hair cells of the basal turn in the elder mice decreased disproportionately, while that in the top turn was preserved. Besides, the residual RS in the basal turn of the elder mice was enlarged. The volume of mitochondria in the nerve endings was obviously larger than that of young animals, and the number of synaptic vesicles decreased. All these structural changes directly reduced the efficiency of exocytosis. According to the changes of morphology and number of RS in the basal turn of basilar membrane, it was inferred that RS of the high-frequency region in the basilar membrane of C57BL/6J mice was more likely to be lost compared with the low-frequency region. This mechanism was consistent with that in patients with presbycusis. Our results indicated that the number of RS in the basilar membrane of the cochlea among C57BL/6J mice aged 10 months and 12 months began to increase slowly. This can be explained by a certain compensatory mechanism involving the increase of the number of RS that is related to the plasticity of the number of RS [14]. More researches are needed among mice of other age groups.

We found that the reduction of RS in inner hair cells of cochlea in C57BL/6J mice probably occurred in the early stage of presbycusis. This provided important experimental basis for early prevention of presbycusis through measures that counteracted the reduction of RS. However, the change of RS number was only studied in C57BL/6J mice aged below 12 months, and more studies on elderly C57BL/6J mice and other strains of mice are needed. During the progression of presbycusis, the morphology, function and number of RS undergo complex changes, and the mechanism underlying these changes requires further study. However, only

RS number could be determined by multiple immunofluorescence staining, whereas the morphology of a single synapse and the changes of sub-cellular structures were not visualized. The use of electron microscopy can make up for this defect.

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### Disclosure of conflict of interest

None.

**Address correspondence to:** Yan-Ping Zhang, Department of Otolaryngology, 309th Hospital of Chinese PLA, #17 Heishanhu Road, Beijing, China. Tel: +8601066775732; Fax: +8601066775732; E-mail: zhangyping68@163.com

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