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A pattern of otoferlin expression interrupted by gentamicin exposure in ribbon synapse of Inner Hair Cell in C57BL/6J Mice

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Abstract

Despite ototoxic effect of aminoglycosides on auditory sensory system has been well documented, the mechanism of ototoxic damage on synaptic connection (ribbon synapse) between inner hair cells (IHCs) and spiral ganglion neurons (SGNs) is still largely unknown. Otoferlin is essential for a late step in exocytosis of neurotransmitter at ribbon synapses. In this study, C57BL/6J mice were intraperitoneally injected with gentamicin (100 mg/kg) once a day, the apical coils of organ of Corti were obtained at the 4th day, the 7th day and the 10th day respectively (n = 10/per group), the normal mice without a Gentamicin exposure were set for control (n = 10/per group). Otoferlin was detected by both immunofluorescence and western blotting. The hearing thresholds were measured by auditory brainstem responses (ABR). The expression of Otoferlin was up-regulated during the period of first 7 days with a Gentamicin exposure. The down-regulation of Otoferlin expression was found after the 7th day with a gentamicin exposure. The hearing thresholds showed a linear increase. Gentamicin exposure may enhance transiently the expression of Otoferlin in ribbon synapse in C57BL/6J mice. This property might correlate with the ribbon synaptic plasticity in C57BL/6J mice. It may provide us a new insight for a further detailed study of the ribbon synaptic plasticity under the condition of aminoglycosides application.

Key words: Cochlea; inner hair cell; ribbon synapse; gentamicin exposure; otoferlin; ABR; immunofluorescence; western blotting.

Introduction

It's known that every part of whole hearing pathway in which a voice signal is encoded and transferred to the central neuron system need to work precisely. Ribbon synapses, which act as the first synaptic afferent neuron connection, play a critical role in the process of voice encoding. For a research in sensory synaptic plasticity, the analysis to the function of ribbon synapses is always being an important part in an entire project. The auditory inner hair cell (IHC) ribbon synapse displays molecular specificities. It lacks major neurotransmitters found at conventional synapses (Jahn *et al.*, 2003) including synaptophysins (Eybalin *et al.*, 2002), synapsins (Mandell *et al.*, 1990), and synaptotagmins I and II (Safieddine and Wenthold, 1999).

We have identified gene, OTOF, underlying an autosomal recessive, nonsyndromic prelingual deafness, DFNB9. It has been verified that otoferlin, encoded by OTOF, was involved in vesicle membrane fusion. Otoferlin mRNA was detected in inner hair cells (IHCs) of Cochlea and type I, II hair cells of vestibule in the adult mouse (Yasunaga *et al.*, 1999). Otoferlin is a transmembrane protein of synaptic vesicles in IHCs and interacts with the SNARE complex proteins syntaxin-1 and SNAP25. Those proteins are critical for fusion between synaptic vesicle membrane and presynaptic membrane during the progress of neurotransmitter release. Otoferlin was also demonstrated to be combined in a calcium-dependent manner with the presynaptic SNARE proteins of IHCs. Hence, it indicates that otoferlin is essential for a late step in neurotransmitter exocytosis in ribbon synapses (Roux *et al.*, 2006).

Aminoglycosides, a bactericidal antibiotic, works by creating fissures in the outer membrane of the bacterial cell. Despite the ototoxic effects of aminoglycosides on auditory sensory system were well documented, the ototoxic damage on synaptic connection between IHCs and SGNs was still largely unknown.

Here, we report our recent study about how gentamicin exposure interrupts the expression of otoferlin and its significant role to reveal the mechanism of ribbon synapse plasticity in hearing impairment.

It may provide us a new insight for a further detailed study of the ribbon synaptic plasticity in the condition of aminoglycosides application.

Methods and Materials

1. Animals preparation

Male C57BL/6J mice with documented dates of birth were obtained from laboratory animal centre of China medical university. All of mice were studied at 8 weeks of age. No outer or middle ear pathology was encountered in any of the animal studied. All procedures were conducted in accordance with animal protocol approved by the animal care and use committee at china medical university. Mice were divided into 4 groups, the first group was set for control group, and other three groups were intraperitoneally injected with gentamicin once a day (100 mg/kg, Invitrogen, USA) for 4 days, 7 days, 10 days respectively.

2. Assessment of hearing function

Auditory brainstem response (ABR) performed with 1% pentobarbital sodium (95 mg/kg, Sigma, USA) sedation was applied to determine auditory threshold. Threshold was based on the visibility and reproducibility of wave III according to Bourre *et al.* (1999). During the animals were under sedation, ABR testing was performed in response to 8 kHz tone bursts. A computer-based signal-averaging system from SmartEP (Intelligent Hearing Systems, USA) was applied to collect ABR data. The ABR was recorded by three needle electrodes placed subdermally over the vertex (positive), the testing mastoid (negative), and the contralateral mastoid (ground) of the animal. Sound was presented through an insert earphone (Intelligent Hearing Systems, USA), which was placed directly in the ear canal. The ABR threshold began at 90 dB and decreased in 5-dB steps, and each response was repeated.

3. Cochlear tissue processing

C57BL/6J mice were killed by decapitation, according to national ethical guideline. The number and suffering of animals were kept as low as possible. The cochleas were removed from temporal bones and their apexes were fenestrated with a fine pin to facilitate the flow of fixative. The stapes and their footplate were removed from the round window through which the solution of ice-cold 4% paraformaldehyde in 1 × PBS (PH 7.4) was slowly perfused followed by an overnight post-fixation. Under the dissecting microscope, contralateral cochleas which were not perfused with fixation fluid were microdissected further in cold 0.01 mM PBS, PH 7.4. The cochlea shell was separated from basal turn; the stria vascularis and the spiral ligament were carefully removed; and the apical coil of organ of Corti was separated from the osseous spiral lamina. The apical coils were stored at -80°C.

4. Immunostaining procedure

The fixed cochlear were carried on frozen sections. The slides produced by frozen section were washed three times in 0.01 mM PBS and preincubated for 30 min in 0.5% Triton X-100 followed 2 h in 5% goat serum, 2% bovine serum albumin(BSA), PBS blocking solution at room temperature. Primary antibodies to otoferlin (1:50, Santa Cruz, USA) were applied overnight at 4°C. After washed with 0.01 mM PBS (3 × 10 min), the organs of Corti were incubated with bovine anti-goat IgG FITC (1:100, Santa Cruz, USA). The preparation were washed 3 × 10 min in 0.01 mM PBS and 1 × 10 min in double distilled water (DDW), placed onto the glass microscope slides with a drop of glycerol PBS and covered with glass coverslips.

5. Western blotting

For specific detection of otoferlin protein in apical coils of basement membrane, 100 mg tissue per group were prepared in 1 ml of ice cold lysis buffer (100 mmol/LTris/HCl, pH7.5; 100 mmol/Lsodium chloride; 0.5% deoxycholate sodium; 1 mmol/L eathylenediaminetetraaceticacid, EDTA; 1% Nonidet P40; 0.1% sodium dodecyl sulfate , SDS and protease inhibitor), a equal amount of protein (50 μg each) were loaded onto 7.5% SDS-polyacrylamide gels and electrophoretically transferred to PVDF membranes (Roth, Germany). Otoferlin protein was detected with anti-otoferlin polyclonal antibody using chemiluminescence ECL substrate (Amersham, UK). Stripped blots were re-probed with anti-β-actin polyclonal antibody (Santa Cruz, USA).

6. Statistical analysis

All data are presented as mean ± SD. Statistical analysis for all the experiments was done by one way ANOVA Student-Newman-Keuls test. P values of < 0.05 were considered to indicate statistically significant differences.

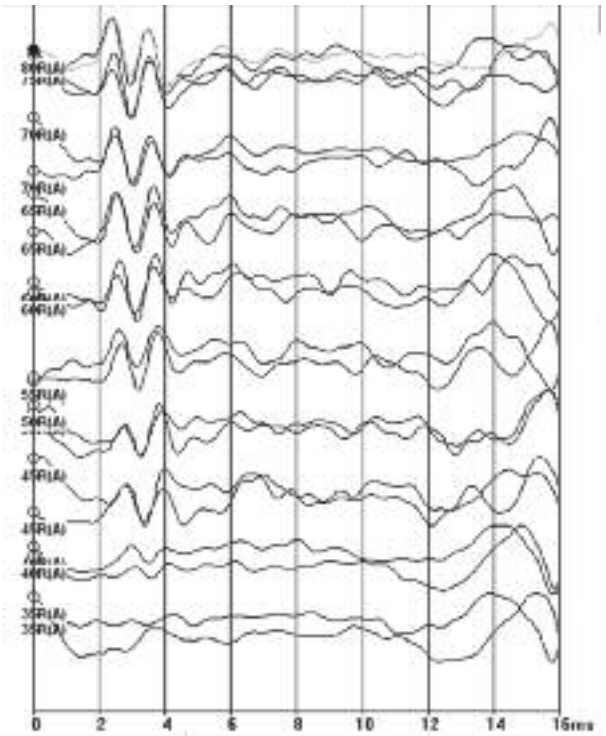

FIG. 1. — Examples of ABR recordings at 8 kHz of C57BL/6J mice with 10 day's gentamicin exposure. The hearing threshold at 8 kHz was 40 dB.

Results

1. GENTAMICIN-INDUCED ABR THRESHOLD SHIFT IN C57BL/6J MICE

ABR was recorded at 8 kHz. Gentamicin exposure (100 mg/kg injected intraperitoneally for 4, 7, 10 days respectively) caused significant ABR threshold shift at 8 kHz. The hearing thresholds were shown a linear increased during the process of gentamicin exposure (Fig. 1, Table I). Threshold was based on the visibility and reproducibility of wave III.

Mean ABR thresholds (and mean ± SEM) in the control, the 4th day, the 7th day and the 10th day groups. ABR threshold increased with continual gentamicin exposure. ABR hearing threshold was tested from each group and statistically significant differences were identified among 4 groups ($P < 0.01$).

2. EXPRESSION OF OTOFERLIN PROTEIN

The analysis of C57BL/6J mice organ of Corti represented a prominent staining. Expression of otoferlin was shown in highest level at the 7th day after gentamicin exposure, and it decreased rapidly at the 10th day. Absorbency ratio of otoferlin at different time with gentamicin exposure. A significant difference of absorbency ratio was observed among the groups (Student's t test, $P < 0.05$) (Fig. 2).

3. DISTRIBUTION OF OTOFERLIN

Images were acquired using a fluorescence microscope (Olympus, Japan) with 488 nm for excitation. 40 × objective was used and local region was digitally double zoomed in. The target region was scanned and the images were taken. Otoferlin was found in the IHCs but not in the outer hair cells (OHCs) (Fig. 3).

Discussion

Hearing relies on faithful synaptic transmission at the ribbon synapse of cochlea IHCs. The afferent synapses of IHCs in cochlea are specialized for encoding sound information with temporal precision over long period (Fuchs *et al.*, 2003). Presynaptic active zone in mature IHCs usually contain a single synaptic ribbon which is electron-dense structure tethered synaptic vesicles (Smith and Sjostrand, 1961). Ribbon synapses of IHCs encode the complexity of voice signals by fast and tonic release through fusion of nuerotransmitter-containing vesicles (Griesinger *et al.*, 2005). Each SGN only recieves one input from specific IHC synapse (Liberman, 1982). Despite many study of the function of presynaptic ribbons have been reported, the molecular machinery of ribbon synapses is still largely undiscovered (Fuchs *et al.*, 2003; Sterling and Matthews, 2005).

Table I

ABR threshold at each time point

	Control	4th day	7th day	10th day
8 kHz ABR threshold (dB SPL)	23.5 ± 2.96	30.0 ± 2.73	37.5 ± 2.45	42.0 ± 2.34

Mean ABR thresholds (and mean ± SEM) in the control, the 4th day, the 7th day and the 10th day groups. ABR threshold increased with continual gentamicin exposure.

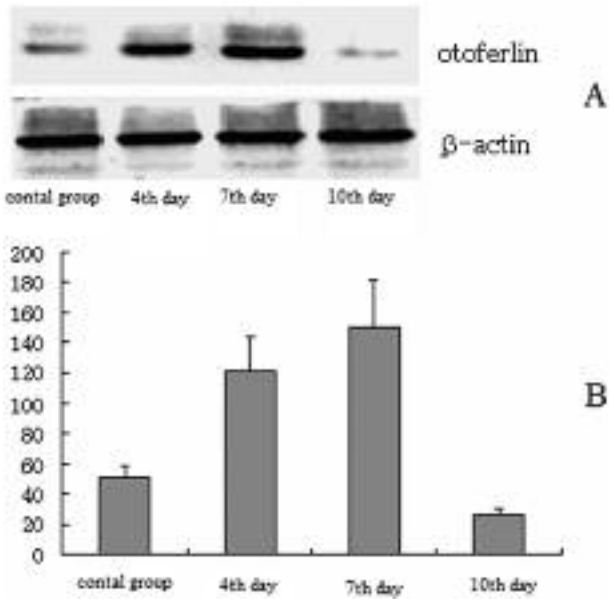

FIG. 2. — Expression of otoferlin using western blotting. (A) Expression of otoferlin at the control, the 4th, the 7th and the 10th day groups respectively with gentamicin exposure. Otoferlin was predominantly expressed at the 7th day; (B) Absorbency ratio of otoferlin at different time with gentamicin exposure.

Otoferlin was reported a predicted C2-domain transmembrane protein, which is defective in DFNB9. Otoferlin localizes on synaptic vesicles associating with ribbon synapse (Nalefski and Falke, 1996). It binds Ca^{2+} and displays interactions with SNARE proteins syntaxin1 and SNAP25 (Chapman *et al.*, 1995). Exocytosis in otoferlin deficient mice is almost completely abolished. Therefore, Otoferlin is essential for a late step of synaptic vesicle exocytosis and may funtion as the major Ca^{2+} sensor to trigger membrane fusion at the IHC ribbon synapse (Eybalin *et al.*, 2002; Safieddine and Wenthold, 1999).

A ribbon synapse, formed between IHC and SGN, was documented to work as the first synaptic afferent neuronal connection. The physiological study revealed that ribbon synapses are characterized by a high rate of tonic neurotransmitter release (Glowatzki and Fuchs, 2002) which is usually thought to act as a rapid supply of synaptic vesicles. The ribbon synapse involves in a fast grade transmission at special sensory synapses (Juusola *et al.*, 1996). Because the sensory systems, including vision and hearing, need the highest rate of information transfer as well as the finest sensory discrimination; it indicated the importance of ribbon synapses for the normal function of the vertebrate brain.

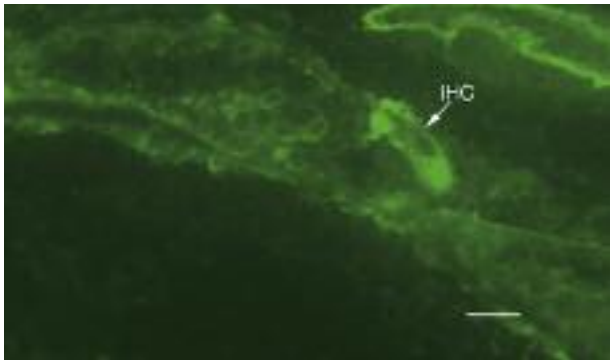

FIG. 3. — Immunofluorescence analysis of Otoferlin (shown in green) in IHCs. 40× oil immersion objective was used and local region was digitally double zoomed in. The target region was scanned and the images were taken. Otoferlin was detected in IHCs, but not in OHCs. Scale bar: 30 μm.

In our study, we have found no depletion of inner hair cell for all mice during the application of Gentamicin exposure; however, we can see a discrepancy in expression of otoferlin in IHCs. The expression of otoferlin reached the highest level at the 7th day with Gentamicin exposure, then the expression of otoferlin decreased greatly after the 7th day with gentamicin exposure (at the 10th day). A reasonable explanation could due to the synaptic self-protected mechanism that probably minimize the toxic damage to the ribbon synapses during the period of gentamicin exposure, however, many detailed investigations need to accomplish in order to demonstrate this hypothesis.

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