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Short communication

Vesicular glutamate transporter 3 is strongly upregulated in cochlear inner hair cells and spiral ganglion cells of developing circling mice

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HIGHLIGHTS

- IHC VGLUT3 IR was higher in circling mice at P14.
- SGC VGLUT3 IR was higher in circling mice at P14.
- VGLUT3 expression in protein level was most prominent in circling mice at P14.

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ABSTRACT

Vesicular glutamate transporter 3 (VGLUT3) plays a major role in hearing, and mice lacking the VGLUT3 are congenitally deaf due to absence of glutamate release at the inner hair cell afferent synapses. However, whether VGLUT3 is expressed normally in the cochleae of developing circling mice (homozygous (*cir*/*cir*) mice), the animal model for human deafness type DFNB6, has not been established. In this study, we investigated the developmental expression of VGLUT3 in cochlear inner hair cells (IHCs) and spiral ganglion cells (SGCs) of homozygous (*cir*/*cir*) mice from postnatal day (P)1 to P14 using immunofluorescence (IF) staining and Western blot. VGLUT3 immunoreactivity (IR) and protein expression increased progressively with age in homozygous (*cir*/*cir*) and control mice (heterozygous (+/*cir*) mice and ICR mice). The rank order of VGLUT3 IR in IHCs and SGCs in P14 mice was homozygous (*cir*/*cir*) mice = heterozygous (+/*cir*) mice > ICR mice. The rank order of total protein expression was homozygous (*cir*/*cir*) mice > heterozygous (+/*cir*) mice = ICR mice at P14. IF staining and Western blot analysis indicated that developmental VGLUT3 expression in cochleae was most prominent in homozygous (*cir*/*cir*) mice. The possible contribution of VGLUT3 upregulation in the cochlear degeneration is discussed.

1. Introduction

Glutamatergic transmission requires transport of the excitatory amino acid into secretory vesicles by a family of vesicular glutamate transporters (VGLUTs) [1,7,21]. Three isoforms of VGLUTs, named VGLUT1–3, have been identified in the brain [5,6,20]. Among them, VGLUT3 is the most important isoform for hearing; the lack of VGUT3 leads to seizure and deafness in mice [19] and the absence of acoustic startle reflex and vestibular-ocular reflex in zebra fish [16]. It is also reported that DFNA25 type deafness is attributed to a mutation in the gene encoding VGLUT3 in human [18].

Auditory VGLUT3 expression is regulated developmentally or environmentally. In cochlea, inner hair cells (IHCs) express VGLUT3 by embryonic day 19 in mice [19], and its expression is gradually upregulated by P60 in both IHCs and SGCs in rats [17]. In brainstem auditory circuits, VGLUT3 is expressed transiently in different superior olivary complex nuclei between P0 and P12 [2], and its expression is upregulated by cochlear damage in the lateral superior olive [11] in rats. These reports raise a possibility that developmental VGLUT3 expression might be different in the auditory system of genetically abnormal animals, such as mouse models of human deafness.

The circling mouse is a mouse model of human deafness (DFNB6 type). The main cause of deafness is the relatively early degeneration of the organ of Corti and the loss of SGCs [4,12,13]. The stereociliary defects in the cochlear hair cells can be observed as

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early as P10 and the earliest complete hair cell degeneration is reported at P21 [4]. It has not been reported what the expression pattern of VGLUT3 is like in hair cells or SGCs during this early degenerating period in circling mice. The aim of this study was to investigate the temporal expression pattern of VGLUT3 in the cochleae of developing circling mice aged under P14.

2. Material and methods

2.1. Cochlear preparations

Female heterozygous (*+/cir*) mice were mated with male homozygous (*cir*/*cir*) mice, and their offspring (P1–P2 and P13–P14 mice) were used for this study. As homozygous (*cir*/*cir*) mice were discovered within a group of ICR out-bred mice, ICR mice of the same age were also used as control. At least three animals were included in each experiment. The genotype of homozygous (*cir*/*cir*) mice was assessed with polymerase chain reaction analysis according to our previous report [9]. The animals were maintained at the Animal Facility of Dankook University. The Dankook University Institutional Animal Care and Use Committee approved this study.

After mice were deeply anesthetized with isoflurane, their cochleae were removed in ice-cold phosphate buffered saline (0.19 M PBS), fixed in 4% paraformaldehyde (PFA) for 2 h, and decalcified in 5% EDTA for 3 h. After serial treatment with cold sucrose solutions, cochleae were embedded in frozen section compounds, and kept frozen at −80 °C until use.

2.2. Staining and analysis

Immunofluorescence (IF) staining was conducted using anti-VGLUT3 antibody (1:5000, AB5421; Millipore, Milford, MA, USA). Mid-modiolar cochlear sections of 10 μm were prepared (Leica CM 1900; Nussloch, Germany) for IF staining. Sections were incubated overnight at 4 °C with anti-VGLUT3 antibody in PBS-based blocking buffer containing 0.3% Triton X-100, and 1% normal goat serum. The next day, the sections were washed with PBS and incubated at room temperature with a secondary antibody (Alexa Fluor 555 goat anti-guinea pig IgG, A21435; Invitrogen, Carlsbad, CA, USA) diluted 1:250 in PBS containing 0.3% Triton X-100. Nuclei were stained with DAPI (46-diamidino-2-phenylindole).

The stained sections were observed under a confocal laser scanning microscope (LSM700; Carl Zeiss Meditec, Jena, Germany). The same parameter setting used to scan the cells of higher fluorescence was used in scanning the cells of lower fluorescence. The staining intensities were measured with the LSM700 image program. The border of the hair cell to be analyzed was outlined by a graphic tool provided by LSM700 image program to produce mean intensity. To measure the VGLUT3 intensities of SGCs, images were taken using a higher magnification and the averaged intensities per field were used as mean intensities. The final intensity was obtained by subtracting the background intensity. The background intensity was the averaged value of the intensities of three different spots outside the region of interest. The modified images with a graphics program (Adobe Photoshop 7.0) are presented in this manuscript. Statistical analysis was performed with Origin 7.0 (Origin Lab). Data are expressed as mean ± standard error. The independent *t*-test was used for comparisons, and a $p < 0.05$ was considered significant.

2.3. Western blots

Mice cochleae were mixed with T-PER® tissue protein extraction reagent (Thermo Scientific, Rockford, IL, USA). The homogenized samples were boiled in Laemmli sample buffer. The samples were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to a polyvinylidene difluoride membrane (Millipore). Protein transfer was confirmed by staining the membrane with Ponceau S red and the membrane was incubated in Odyssey® blocking buffer for 1 h at room temperature. The membrane was stained with anti-VGLUT3 antibody (1:1000, AB5421; Millipore, Milford, MA, USA) overnight at 4 °C. After

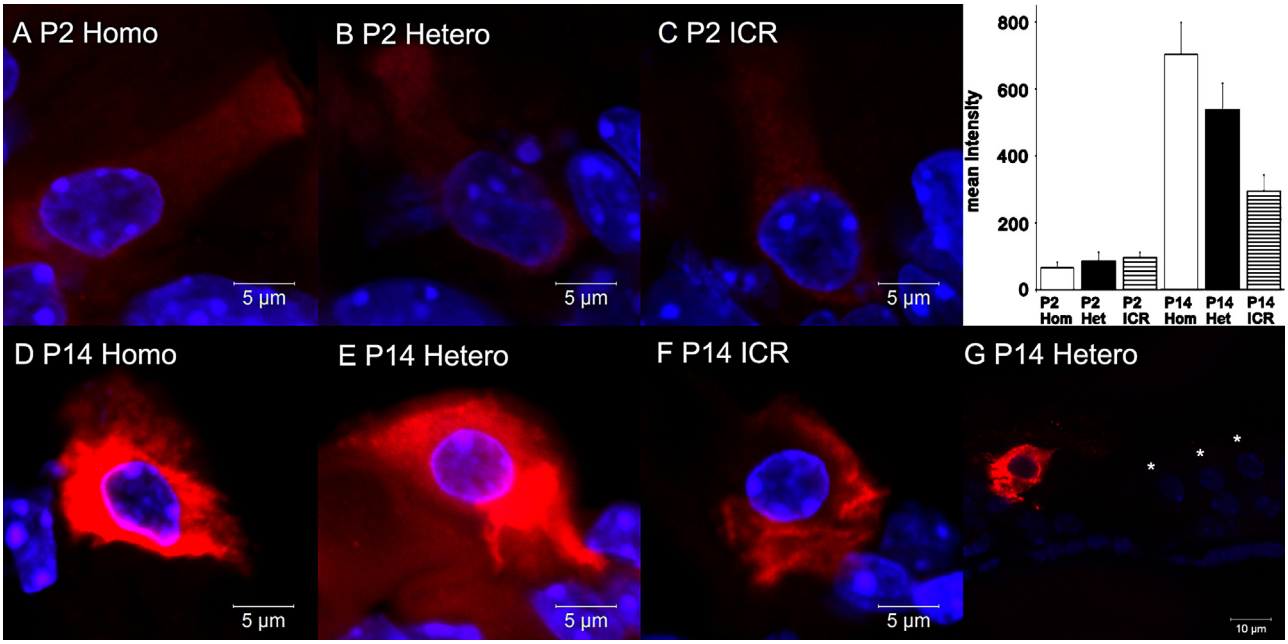

Fig. 1. VGLUT3 IR confined to IHCs. At P2, VGLUT3 IR (red signal) was weak in IHCs of homozygous (*cir*/*cir*) mice (A: homo), heterozygous (+/*cir*) mice (B: hetero), and ICR mice (C), but became prominent at P14 in all mice tested (D: homozygous (*cir*/*cir*) mice, E: heterozygous (+/*cir*) mice, F: ICR mice). The intensity of VGLUT3 IR was weakest in IHCs of ICR mice (F). G is a low magnification view showing VGLUT3 IR confined to IHC in P14 heterozygous (+/*cir*) mice. *Indicates outer hair cells. Nuclei were stained with DAPI (blue signal). Graph above G shows the temporal expression pattern of VGLUT3 IR. *Y* axis indicates mean intensities of VGLUT3 IR and *X* axis indicates ages of mice tested (Hom: homozygous (*cir*/*cir*) mice, het: heterozygous (+/*cir*) mice). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)