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Glutamate co-transmission from developing medial nucleus of the trapezoid body – Lateral superior olive synapses is cochlear dependent in kanamycin-treated rats

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ABSTRACT

Cochlear dependency of glutamate co-transmission at the medial nucleus of the trapezoid body (MNTB) – the lateral superior olive (LSO) synapses was investigated using developing rats treated with high dose kanamycin. Rats were treated with kanamycin from postnatal day (P) 3 to P8. A scanning electron microscopic study on P9 demonstrated partial cochlear hair cell damage. A whole cell voltage clamp experiment demonstrated the increased glutamatergic portion of postsynaptic currents (PSCs) elicited by MNTB stimulation in P9–P11 kanamycin-treated rats. The enhanced VGLUT3 immunoreactivities (IRs) in kanamycin-treated rats and asymmetric VGLUT3 IRs in the LSO of unilaterally cochlear ablated rats supported the electrophysiologic data. Taken together, it is concluded that glutamate co-transmission is cochlear-dependent and enhanced glutamate co-transmission in kanamycin-treated rats is induced by partial cochlear damage.

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1. Introduction

The inhibitory GABA/glycinergic MNTB–LSO synapses, which are involved in sound localization in the mammalian auditory brainstem, co-release glutamate transiently during development in rats [1]. We have demonstrated elevated glutamatergic co-transmission in developing circling mice [2], an animal model for human hereditary deafness (DFNB6) [3,4]. As spontaneous cochlear degeneration occurs relatively early in circling mice, we reasoned that enhanced glutamatergic transmission might be related to cochlear degeneration. However, we could not argue convincingly that glutamatergic co-transmission was cochlear-dependent because circling mice are genetically abnormal and the hair cells are relatively intact until P10 [3]. To prove the cochlear dependency of glutamatergic co-transmission, we had to reproduce the enhanced glutamatergic co-transmission in genetically normal animals, whose cochleae were partially destroyed.

As aminoglycosides have been reported to be not as strongly effective during the first postnatal week [5–8], we expected that large doses of aminoglycoside antibiotics in rats might produce partial damage of cochlear hair cells, which might reproduce enhanced glutamatergic co-transmission. In this study, we observed enhanced glutamatergic co-transmission at MNTB–LSO synapses in kanamycin-treated rat pups, which was quite similar to that observed in circling mice. The detailed results and meaning of glutamatergic transmission are discussed.

2. Materials and methods

2.1. Animals and slice preparations

Pregnant female Sprague-Dawley rats were purchased from a domestic company (Samtako BioKorea, Osan, Korea) and their pups were used. The pups were treated with kanamycin (700 mg/kg, subcutaneous injection, twice a day) from P3 to P8 and they were evaluated after P9. The animals were maintained in the Animal Facility of Dankook University. The Dankook University

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Institutional Animal Care and Use Committee (DUIAC) approved this study.

After the pups received deep anesthesia with isoflurane, the brains were removed in ice-cold artificial cerebrospinal fluid (aCSF) with 1 mM kynurenic acid, and 300-μm thick coronal slices were cut with a vibratome (LEICA VT1000s; LEICA Microsystems, Heidelberger, Germany). The slices were allowed to recover for at least 30 min in an interface chamber before recording. The slices were transferred to a submersion-type chamber mounted on an upright microscope and perfused continuously with Mg^{2+}-free aCSF consisting of the following (in mM): NaCl (124), KCl (5), KH_2PO_4 (1.25), glucose (10), $NaHCO_3$ (26), and $CaCl_2$ (2).

2.2. Electrical stimulation and electrophysiology

Low-resistance electrodes (<2 MΩ filled with aCSF), positioned at the lateral end of the MNTB, were used to stimulate LSO inputs (Master 8 and Isoflex; A.M.P.I., Israel). Whole cell patch clamp recordings were obtained from the visualized principal type of LSO neurons. Only one cell was tested for every slice. Because we selected the single best slice per rat, the number of cells presented in the electrophysiologic study equaled the number of animals tested. In minimal stimulation experiments, the electrical stimulation intensity was adjusted to ensure a failure rate of >50%. At this intensity, 100–200 responses were evoked every 10 s. Identification of successful responses and failures was performed off-line by eye. All failures were excluded when calculating average peak amplitudes. The recording electrodes (2–3 MΩ) were filled with solution consisting of the following (in mM): K-gluconate (22), EGTA (10), KCl (91), HEPES (20), Na_2GTP (0.3), Na_2ATP (1), KOH (35), and QX 314 (5). All chemicals, with the exception of QX 314, CNQX, and bicuculline (Tocris) were purchased from Sigma. The data were filtered at 5 kHz (EPC-8; HEKA, Germany), digitized at 10 kHz, and stored on a computer using a homemade program (R-clamp 1.23).

2.3. Cochlear ablation

Hypothermia was used to induce and maintain anesthesia in pups (P2–P3). A small incision was made to the ventral side of the pinna, exposing the external auditory canal and tympanic membrane. After removing the tympanic membrane, the middle ear mesodermal tissue and ossicles were removed. Using a pipette, cochlear contents were aspirated after breaking the thin otic capsular bone covering the cochlea. After the incision was sutured, the rats were kept in a cage floating over a warm bath. After recovery, the rats were returned to their home cage. The completely damaged cochlea was reconfirmed while harvesting the brains.

2.4. Immunohistochemistry

For immunohistochemistry, the animals were sequentially perfused transcardially with cold 0.02 M phosphate buffered saline (PBS; pH 7.4) and ice-cold 4% paraformaldehyde. The brains were cryoprotected in a series of cold sucrose solutions. The samples were then embedded in OCT, stored at −20 °C, and cut with a microtome at 10–20 μm thick (Leica CM 1900; Nussloch, Germany). The mounted samples on coated slides were washed by PBS and permeabilized with 0.3% Triton X-100. Sections were incubated overnight at 4 °C with primary antibodies in PBS-based blocking buffer containing 1% bovine serum albumin, 0.3% Triton X-100, and 1% normal horse serum. Primary antibodies included rabbit monoclonal anti-NMDAR1 (diluted to 1:500, AB9864; Millipore, CA, USA), rabbit polyclonal anti-NMDAR2A/2B (1:1000, AB1548; Millipore), rabbit polyclonal anti-glycine receptor $\alpha_1 + \alpha_2$ (1:250, ab23809; Abcam, Cambridge, UK), and guinea pig polyclonal anti-VGLUT3 antibodies (1:100,000, AB5421; Millipore, CA, USA). The next day, samples were washed with PBS and incubated at room temperature with secondary antibodies (Alex Fluor 488 goat anti-rabbit IgG, A-11008; and Alexa Fluor 555 goat anti-guinea pig IgG, A21435; Invitrogen, CA, USA) diluted 1:200 in PBS-based buffer containing 0.3% Triton X-100. Nuclei were stained with DAPI (4′,6-diamidino-2-phenylindole). The staining patterns were observed using a confocal laser scanning microscope (LSM700; Carl Zeiss Meditec, Jena, Germany). The LSM700 image program was used to determine the staining intensities of VGLUG3, glycine receptors, NMDAR1, and NMDAR2A/2B.

2.5. SEM

At P9 (after kanamycin treatment for 6 days), intracardiac perfusions were performed with 4% paraformaldehyde while the animals were under deep anesthesia with zoletil (Virbac, Carros, France). After perfusion, animals were decapitated and the cochleae were removed immediately. The bony capsule was removed and the lateral wall tissues, as well as the membranous structures, were separated. The dissected specimens were rinsed with 0.1 M PBS and fixed in 1% osmium tetroxide. After that, the specimens were gently rinsed with 0.1 M PBS and dehydrated in graded series of ethanol. After immersing in absolute ethanol for 1 h, the specimens were dried in a Critical point dryer (HCP-2; Hitachi, Tokyo, Japan). The dried specimens were attached to aluminum stubs with aluminum paint and coated with platinum–palladium using E-1030 ion sputter (Hitachi, Tokyo, Japan). The surfaces of the organs of Corti were examined with S-4300 SEM (Hitachi, Tokyo, Japan).

2.6. Statistical analysis

In the analysis of immunohistochemical data, we took pictures of three different areas with a high-magnification view (680×) and the averaged intensities per field for each signal (red or green) were used as mean intensities. The analysis of the electrophysiologic data was performed with Clampfit 9.2 (Molecular Devices) and Origin 7.0 (OriginLab Corporation). The electrophysiologic data and immunohistochemical data were analyzed with Origin 7.0. Data are expressed as the mean ± standard error of the mean throughout the text. Comparisons were made using the Student's *t*-test, and a p-value < 0.05 was considered statistically significant.

3. Results

3.1. Enhanced glutamatergic transmission in kanamycin-treated rat pups

As glutamate co-transmission from MNTB–LSO synapses has been reported to decrease after P8 in rats [1], rats were treated with high-dose kanamycin from P3 to P8 and the cochlear damage was evaluated with a SEM at P9. The SEM study revealed partially missed hair cells from the base to the apex (Fig. 1). We then evaluated the glutamatergic co-transmission at MNTB–LSO synapses using the whole cell voltage clamp technique on P9–P11. In the control group, the 10-min average of steady state PSCs after glutamate receptor antagonist treatment (50 μM DL-APV + 50 μM CNQX) was 54.3 ± 8.2% (control group, $n = 9$, Fig. 2A), and 26.6 ± 5.8% of the control (kanamycin-treated group, $n = 16$; Fig. 2B). Two values were significantly different. We even observed almost complete block of PSCs with the glutamate receptor blocker mixture in 2 of 16 cells in kanamycin-treated rats. One example is demonstrated in Fig. 2B. Next, we used a minimal stimulation technique to further analyze the glutamate co-transmission from single fiber. Minimal stimulation elicited PSCs of variable amplitudes

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