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Research paper

Inner hair cells of mice express the glutamine transporter SAT1

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ABSTRACT

Glutamate has been implicated in signal transmission between inner hair cells and afferent fibers of the organ of Corti. The inner hair cells are enriched in glutamate and the postsynaptic membranes express AMPA glutamate receptors. However, it is not known whether inner hair cells contain a mechanism for glutamate replenishment. Such a mechanism must be in place to sustain glutamate neurotransmission. Here we provide RT-PCR and immunofluorescence data indicating that system A transporter 1 (SLC38A1), which is associated with neuronal glutamine transport and synthesis of the neurotransmitters GABA and glutamate in CNS, is expressed in inner hair cells. It was previously shown that inner hair cells contain glutaminase that converts glutamine to glutamate. Thus, our finding that inner hair cells express a glutamine transporter and the key glutamine metabolizing enzyme glutaminase, provides a mechanism for glutamate replenishment and bolsters the idea that glutamate serves as a transmitter in the peripheral synapse of the auditory system.

1. Introduction

Several lines of evidence point to glutamate as the likely transmitter of inner hair cells (IHCs) of the organ of Corti (OC) (Eybalin and Altschuler, 1990; Hakuba et al., 2000; Ottersen et al., 1998; Takumi et al., 1997). These cells are enriched with glutamate and glutaminase (GLNase) – a glutamate synthesizing enzyme – and are presynaptic to dendrites that express high densities of AMPA glutamate receptors (Matsubara et al., 1996; Takumi et al., 1999; Usami et al., 1992). However, before conclusions can be drawn on transmitter identity it needs to be shown how the transmitter pool can be sustained. In the central nervous system, transmitter glutamate is replenished by recycling of the glutamate carbon skeleton. Essential steps in this "glutamate–glutamine cycle" are uptake of released glutamate in glial cells, conversion of glutamate to glutamine by glial glutamine synthetase, and shuttling of glutamine from glia to neurons for conversion to glutamate by neuronal GLNase. Several of these steps appear to be in place in the OC, with supporting cells substituting for CNS glia. What is missing is evidence for glutamine uptake in IHCs. Specifically, there is a need to show that IHCs are equipped with a glutamine transporter. Recently, several glutamine transporters have been described (Chaudhry et al., 2008) but none of these have been investigated for their possible presence in the OC.

Here we show that the glutamine transporter SAT1 (Solbu et al., 2010) is strongly and specifically expressed in IHCs and that this transporter is concentrated at the apical aspect of these cells. In contrast, the vesicular glutamate transporter VGLUT3 is found in the basal part of the hair cells, at the site of glutamate release. Thus, IHCs show a clear functional polarization in regard to glutamine and glutamate transport.

The mechanisms that underlie release and handling of transmitters in the OC are fundamental to our understanding of auditory processing and also bear on a number of pathological conditions including acoustic trauma (Hakuba et al., 2000; Robertson, 1983; Saunders et al., 1985). Our data support the idea that IHCs and adjacent supporting cells form a glutamate processing unit similar to that formed by glutamate releasing terminals and adjacent astrocytes in the CNS.

Abbreviations: AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; BSA, bovine serum albumin; CNS, central nervous system; GP, Guinea Pig; GS, glutamine synthetase; HSA, human serum albumin; IHCs, inner hair cells; NGS, normal goat serum; OC, organ of Corti; GLNase, glutaminase; PB, phosphate buffer; PBS, phosphate buffered saline; PCR, polymerase chain reaction; RT, reverse transcription; SAT1, system A transporter 1; TBNT, Tris buffer (5 mM) containing 0.9% NaCl and 0.1% Triton X-100; VGLUT, vesicular glutamate transporter.

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2. Materials and methods

2.1. Tissue preparation

C57BL/6 mice, 8 weeks old, were used in the present study. The mice were deeply anesthetized with sodium pentobarbital (50 mg/kg i.p.).

For the confocal microscopic analysis, 1 ml of fixative [4% formaldehyde in 0.1 M phosphate buffer (PB), pH 7.4] was injected through the tympanic membrane. Subsequently, the mice were perfused transcardially at 8 ml/min with 2% dextran in 0.1 M PB (pH 7.4, 4 °C, 15 s) followed by the fixative used for tympanic injection (room temperature, 5 min). After perfusion the temporal bones were removed and postfixed in the same fixative (4–8 h, 4 °C). The cochlear tissues were dissected from the temporal bone under an operation microscope, and rinsed in 0.1 M PB (pH 7.4, 4 °C, overnight). The specimens were immersed in 10% sucrose, 20% sucrose and 30% sucrose in 0.1 M PB (pH 7.4, 8 h each) and then sectioned at a cryostat at 14 μm.

For postembedding immunogold analysis, 1 ml of fixative consisting of 4% formaldehyde and 0.1% glutaraldehyde in 0.1% PB (pH 7.4, 4 °C) was injected into the labyrinth. The mice were then perfused transcardially at 8 ml/min with 2% dextran in 0.1 M PB (pH 7.4, 4 °C, 15 s) followed by the fixative used for labyrinth injection (room temperature, 5 min). After perfusion the temporal bones were removed and postfixed in the same fixative (4–8 h, 4 °C). The cochlear tissues were dissected from the temporal bone under an operation microscope, rinsed in 0.1 M PB with 4% glucose (pH 7.4, 4 °C, overnight) and cryoprotected in 10% glycerol, 20% glycerol and 30% glycerol in 0.1 M PB (pH 7.4, 8 h each). Freeze substitution and low-temperature embedding in a methacrylate resin were carried out as described previously (Matsubara et al., 1996).

For reverse transcription-polymerase chain reaction (RT-PCR), the mice were perfused transcardially at 8 ml/min with 2% dextran in 0.1 M PB (pH 7.4, 4 °C, 5 min) and decapitated. Temporal bones were quickly removed and the cochlear tissues were dissected out from the temporal bone and then rapidly frozen in liquid nitrogen for storage at −80 °C.

2.2. Antibodies

Antibodies to rabbit anti-SAT1 were prepared and characterized as described. The specificity has already been demonstrated (Buntup et al., 2008; Solbu, et al., 2010). Other primary antibodies used were: Guinea Pig (GP) anti-VGLUT1 (1:200; Millipore, Bedford, MA, USA), GP anti-VGLUT2 (1:200; Millipore, Bedford, MA, USA) and GP anti-VGLUT3 (1:2000; Millipore, Bedford, MA, USA). Secondary antibodies were Cy3-conjugated donkey anti-rabbit IgG and Cy5-conjugated donkey anti-GP (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA).

2.3. Immunofluorescence staining of cryostat sections

Immunocytochemical staining of SAT1 and VGLUTs was performed using an indirect immunofluorescence method. The sections were rinsed twice for 10 min in 0.01 M phosphate buffer saline (PBS) (pH 7.4) and incubated for 1 h in blocking buffer [10% normal goat serum (NGS), 1% bovine serum albumin (BSA) and 0.5% Triton X100 in 0.01 M PBS (pH 7.4)]. The sections were rinsed three times for 10 min each in 0.01 M PBS (pH 7.4) and incubated 2 h at room temperature with the primary antibodies (anti-SAT1 2 μg/ml and VGLUTs) in primary antibody solution [3% NGS, 1% BSA, 0.5% Triton X-100, 0.05% NaN_3 in 0.01 M PBS (pH 7.4)]. After three 10-min rinses in PBS (pH 7.4), the sections were incubated for 1 h with secondary antibody diluted 1:1000 in 0.01 M PBS (pH 7.4) containing 3% NGS, 1% BSA, and 0.5% Triton X-100. The sections were rinsed three times for 10 min each in 0.01 M PBS (pH 7.4) and mounted in ProlongGold antifade reagent premixed with the nuclear stain, DAPI (4′, 6-diamidino-2-phenylindole) (Invitrogen, Carlsbad, CA, USA). The sections were viewed and photographed with a Zeiss LSM 5 Pascal confocal microscope (Carl Zeiss, Jena, Germany). For control, the primary antibodies were absorbed with the synthetic peptides used for immunization. As an additional control, parallel sections were incubated without primary antibodies (to test for possible spurious staining due to the detection system). These controls showed faint and homogeneous background fluorescence, attesting to the selectivity of the immunocytochemical procedure (data not shown).

2.4. Postembedding immunogold analysis

The Postembedding immunogold analysis procedure has been described (Matsubara et al., 1996). In brief, ultrathin sections were incubated in the following solutions at room temperature: (1) 0.1% sodium borohydride and 50 mM glycine in Tris buffer (5 mM) containing 0.9% NaCl and 0.1% Triton X-100 (TBNT; 10 min); (2) 2% human serum albumin (HSA) in TBNT (10 min); (3) rabbit polyclonal antibodies to SAT1 (2 μg/ml) in TBNT containing 2% HSA (8 h); (4) 2% HSA in TBNT (10 min); (5) goat anti-rabbit IgG coupled to 10 nm gold particles (GAR10, Abcam, United Kingdom) diluted 1:20 in TBNT containing 2% HSA and polyethyleneglycol (5 mg/ml, 2 h). The sections were counterstained by 0.7% uranyl acetate (90 s) and 0.3% lead citrate (90 s) and examined with a Philips Tecnai 12 transmission electron microscope at 60 kV.

2.5. Reverse transcription-polymerase chain reaction (RT-PCR) analysis

Total RNA was extracted from cochlear tissues with the Catrimox-14 RNA Isolation Kit Ver. 2.11 (Iowa Biotechnology, Urbandale, IA, USA). The yield of total RNA was determined by Agilent 2100 Bioanalyzer RNA 6000 Nano Assay (Agilent Technologies, Palo Alto, CA, USA). RT-PCR assay was performed with the aid of an RNA PCR Kit (Takara, Tokyo, Japan). Reactions without the reverse transcriptase enzyme (−RT) were performed as control, and reactions using the dissection medium as template were used to control for contamination from lysed cells. The +RT and −RT reaction products were used for RT-PCR. Primer sequences are as follows: SAT1 forward (agaagtagaaaacggccagataaat) and reverse (atacttacatactcgtcgcatttcc), 111 bp; VGLUT1 forward (cagtattcaggatggagttctgtct) and reverse (tgttaaacttcgtaacagggttcat), 190 bp; VGLUT2 forward (taggattcagtggatttgctatctc) and reverse (agtgcataaaatatgactccaccat), 229 bp; and VGLUT3 forward (actctgaacatgtttatccccttctg) and reverse (cccaaacataccgtagatgtaaaag), 230 bp. PCR steps were denatured at 94 °C for 2 min, followed by 30 cycles of 94 °C for 30 s, 60 °C for 30 s, and 72 °C for 1 min, and then processed with a final extension at 72 °C for 5 min. After amplification, expected sizes of PCR products were confirmed on 2% agarose gel, and the bands were visualized by ethidium bromide upon exposure to an ultraviolet transilluminator.

All studies were carried out in accordance with Shinshu University School of Medicine and National Institutes of Health guidelines for the ethical treatment of experimental animals. Care was taken to minimize number of mice used as well as their suffering.

3. Results

By use of RT-PCR we could show that the cochlear tissues contains mRNA encoding SAT1. In addition we confirmed that the cochlea expresses the genes that code for the VGLUT1–3 (Fig. 1; cf. Seal et al., 2008).