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Expression of the neuron-specific potassium chloride cotransporter KCC2 in adult rat cochlear

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ABSTRACT

Auditory transduction in the cochlear is subject to modulate higher auditory centers in the brain via the efferent systems, which provide protection against damage caused by excessive excitation during auditory over stimulation. GABA is a proven inhibitory neurotransmitter in the efferent systems in mammalian cochlear. KCC2 is a neuron-specific potassium chloride cotransporter whose role in mature central neurons is to maintain the low intracellular Cl^- concentrations required for the hyperpolarizing responses to the inhibitory amino acids GABA and glycine. However, there is a lack of information concerning KCC2 expression in the mammalian cochlear. In this study, reverse-transcription polymerase chain reaction (RT-PCR) and immunohistochemistry were used to detect the expression and localization of KCC2 in the mammalian cochlear. The results showed that these neuron-specific KCC2 transporters were present in most spiral ganglion neurons (SGNs) corresponding to the distribution of $GABA_A$Rs. In addition, less intense reactions were observed on the organ of Corti, stria vascularis, and fibrocytes of the spiral ligament. These data suggest that KCC2 may play an important role in the modulation of a GABA neurotransmitter's function in a mammalian cochlear. Moreover, the presence of KCC2 on the organ of Corti and its surrounding tissues may contribute to maintaining normal K^+ cycling. It is also presumed to be related to Cl^- transportation in hair cells.

Gamma-aminobutyric acid (GABA) is thought to be one of the major "inhibitory" neurotransmitters in the central nervous system (CNS). In the mammalian auditory system, GABA is reported to be distributed in the olive-cochlear bundles (OCB) that constitute the cochlear efferent system in many species. It can mediate fast inhibition in the adult cochlear nucleus via $GABA_A$ receptors ($GABA_A$Rs) [4,22], which involves activation of intrinsic Cl^- conductance. While PCR amplifications have revealed a complicated expression pattern of $GABA_A$Rs in the cochlear [5], electrophysiological studies also confirmed that functional $GABA_A$Rs and $GABA_B$ receptors ($GABA_B$Rs) are present on spiral ganglion neurons (SGNs), the primary afferent neurons in the cochlear [11,12]. It has been reported that GABA receptors (GABARs) influence cochlear afferent neurotransmission induced by glutamate (Glu) [1]. The abundance of functional GABARs on SGNs and the organ of Corti [23] indicate that this Cl^--mediated neurotransmission most likely plays an important role in modulating cochlear function.

It is known that hyperpolarizing fast inhibitory neurotransmission by GABA requires an efficient chloride extrusion mechanism in mature postsynaptic neurons [14]. The major effector of this task in adult animals is the neuron-specific potassium chloride cotransporter 2 (KCC2) [17]. KCC2 reduces neuronal Cl^- concentrations and shifts the GABA reversal potential toward more negative potentials, thus promoting hyperpolarizing GABA responses [13].

Although it is reported to be a neuron-specific isoform, there is a lack of information concerning KCC2 expression in the mammalian cochlear. Whether or not the KCC2 mRNA is expressed in the cochlear and how this functional protein is distributed is thus far unknown. The present work investigates the presence of KCC2 in the cochlear of the adult healthy male Wistar rats using reverse-transcription polymerase chain reaction (RT-PCR) and immunohistochemistry. Furthermore, the functional role of KCC2 in adult mammalian cochlear is also discussed.

All animal procedures were performed in accordance with the National Institutes of Health guidelines and were approved by the Committee on animal Research, University of Wuhan, China. Adult male Wistar rats (200–250 g), which had a normal pinna reflex and were free from middle ear infection, were acquired from the Experimental Animal Center of Hubei Province (SCXK: 2003–2007), China.

To detect KCC2 mRNA expression in the cochlear, we used RT-PCR. Animals were anesthetized with sodium pentobarbital (80 mg/kg). The cochleae were rapidly removed, immersed in liquid nitrogen, and stored at −80 °C. The brains were also extracted as

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positive controls. Total RNA from cochleae and brains was extracted (TRIzol; Life Technology, U.S.A.) according to standard protocols and reverse transcribed (ThermoScript RT-PCR system; Invitrogen). cDNA was synthesized from 1 μg/μl total RNA with 100 pmol/μl oligo(dT) 20 in a total of 20 μl with RNase Free H_2O. Total RNA was denatured at 65 °C for 10 min and then cooled on ice. Ten microliters of the mix was added to a 10 μl reaction volume containing final concentrations of 1× first-strand buffer, 10 mM dithiothreitol (DTT), 2 mM dNTPs, and 40 U ribonuclease inhibitor (RNaseOUT; Invitrogen). Fifteen units of reverse transcriptase (ThermoScript; Invitrogen) were added to the positive control mix. cDNA synthesis was performed at 42 °C for 60 min and the enzyme was deactivated by a 5-min incubation at 99 °C. The cDNA mix (1.5 μl) was used for PCR amplification. The amplification reaction mixture contained 1× PCR buffer, 0.2 mM dNTPs, 1.5 mM $MgCl_2$, 2 U *Taq* DNA polymerase (Platinum; Invitrogen), and 0.2 μM sense and antisense primers specific for rat KCC2 (sense, 5′-AGGGAAGCAAAGAG-CACGAA-3′; antisense, 3′-TTACCACAACACGGACGACC-5′). After an initial denaturation for 2 min at 94 °C, the KCC2 products were amplified by the following thermocycling conditions: 94 °C (30 s), 54 °C (30 s), and 72 °C (30 s) for 35 cycles. After a final elongation step at 72 °C for 7 min, the PCR products were analyzed by electrophoresis on 1% agarose gels and visualized by ethidium bromide staining. As a negative control, 35 cycles of PCR with cochlear were performed without prior RT.

In order to detect the distribution of this neuron-specific KCC2 protein in the rat cochlear, the immunohistochemical staining method was used in our study. Double immunofluorescence staining was explored to detect the coexpression of KCC2 and $GABA_A$Rs. Animals were deeply anesthetized with sodium pentobarbital (80 mg/kg). After surgical exposure of the heart, the vascular tree was immediately flushed with 0.9% warm saline, followed by fixation with 4% paraformaldehyde in phosphate buffered saline (PBS). The bullae were exposed rapidly, the stapes removed, the round window perforated, and 0.5 ml of fixative infused gently through the oval window. The apical portion of the bony cochlear was gently opened to allow the fixative to perfuse through the tissues. After 24 h at 4 °C, the tissues were rinsed with saline, and decalcified by immersion in 10% EDTA (pH 7.0) for 72 h at room temperature with gentle stirring. The EDTA solution was changed daily. The tissues were then soaked in 20% sucrose solution for 8 h and finally in 10% sucrose overnight. The cochleae were placed in Tissue-Tek ornithine carbamyl transferase (OCT) compound (Tissue-Tekfi, Labonord, France) for cryostat sectioning. Serial midmodiolar sections were cut with a 10 μm thickness and mounted on glass slides. For specific detection of the KCC2, a polyclonal antibodies (Santa Cruz Biotechnology, Santa Cruz, U.S.A.), which is directed against a peptide mapping within an internal region of KCC2, was raised in goat and used in this study. Sections were rinsed in PBS-T (0.01 M PBS, 0.05% Triton, 0.9% saline) for 15 min and non-specific binding was blocked with 2% normal donkey serum. Thereafter, sections were incubated in a solution containing a combination of primary anti-KCC2 (dilution 1:200) and rabbit anti-$GABA_A$Rs antibody (dilution 1:100, Wuhan USCN Science Co., Ltd., Wuhan, China) overnight at 4 °C. After 3 PBS washes (5 min each), sections were processed with fluorescein isothiocyanate (FITC)-conjugated donkey anti-goat (dilution 1:300, Santa Cruz Biotechnology, U.S.A.) and Rhodamine (TRITC)-conjugated donkey anti-rabbit antibodies (dilution 1:200, Santa Cruz Biotechnology, U.S.A.). Sections were washed in PBS, placed on slides and mounted with nulli-fluorimetric glycerine (Lingfei technological company, China).

Immuno-positive staining was examined with a fluorescent microscope (Olympus BX51). The coexpression was analyzed with a Leica TCS SP2 AOBS Confocal microscope System (Leica Microsystems, Heidelberg, Germany). Control experiments were carried out with omission of primary antibody and absorption with antigen.

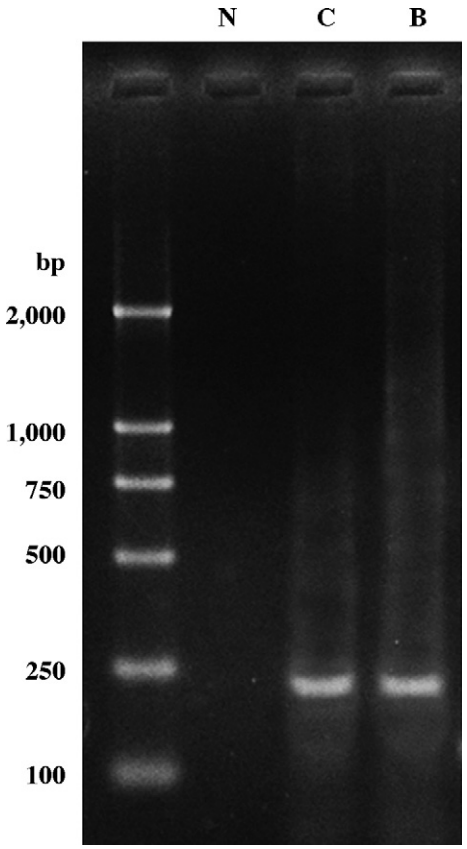

Fig. 1. KCC2 mRNA expression in adult rat cochlear. Analyzed by electrophoresis on 1% agarose gels, PCR products with length corresponding to the KCC2 cDNA fragment (248 bp) were detected. N: negative control, C: cochlear ($n = 2$), B: brain (used as a positive control).

We examined the presence of KCC2 mRNA in rat cochlear. As is shown in Fig. 1, PCR products with lengths corresponding to the KCC2 cDNA fragment (248 bp) were obtained from the rat cochlear and brain. To prove that the PCR products were derived from mRNA, we performed a PCR using cochlear without prior RT that resulted in no bands.

With immunohistochemical staining, in cryostat sections of adult rat inner ear, anti-KCC_2 primary antibody produced an intense immunostaining reaction in the SGNs (Fig. 2A–C). Strong staining was also observed in the supralimbal region and tectorial membrane (Fig. 2A and C). In addition, less intense reactions were observed on the outer hair cells (OHCs), inner hair cells (IHCs), Deiters' cell (DC) (Fig. 2C), stria vascularis, and fibrocytes of the spiral ligament (Fig. 2A and C). At a higher magnification, the entire plasma membrane of the SGNs appeared to be stained (Fig. 2B). Double-labeling experiments with anti-KCC2 and $GABA_A$R antibodies on cryostat sections of adult rat cochlear revealed that most SGNs expressed both KCC2 and $GABA_A$Rs (Fig. 3). No immunostaining was detected in the rat cochlear when primary antibody was omitted and absorption with antigen (data not shown). These results suggest that this type of neuron-specific potassium chloride cotransporter may play an important role in the functioning of the GABA neurotransmitter in mammalian cochlear.

SGNs are the primary afferent neurons in mammalian cochlear. It is believed that SGNs are the first relay neurons in the pathway that convey auditory signals from hair cells in the inner ear to the CNS via synaptic transmission. On the other hand, auditory