Hearing Research 314 (2014) 20–32

Contents lists available at ScienceDirect

Hearing Research

journal homepage: www.elsevier.com/locate/heares

Research paper

Localization of kainate receptors in inner and outer hair cell synapses

Taro Fujikawa [a, b], Ronald S. Petralia [c, *], Tracy S. Fitzgerald [d], Ya-Xian Wang [c], Bryan Millis [a], José Andrés Morgado-Díaz [e], Ken Kitamura [b], Bechara Kachar [a]

[a] Laboratory of Cell Structure and Dynamics, National Institute on Deafness and Other Communication Disorders, National Institutes of Health, Bethesda, MD 20892, USA
[b] Department of Otolaryngology, Tokyo Medical and Dental University, Bunkyo-ku, 113-8519 Tokyo, Japan
[c] Advanced Imaging Core, National Institute on Deafness and Other Communication Disorders, National Institutes of Health, Bethesda, MD 20892, USA
[d] Mouse Auditory Testing Core Facility, National Institute on Deafness and Other Communication Disorders, National Institutes of Health, Bethesda, MD 20892, USA
[e] Cellular Biology Division, National Cancer Institute, Rio de Janeiro CEP 20230-050, Brazil

ARTICLE INFO

Article history:
Received 12 December 2013
Received in revised form
25 March 2014
Accepted 7 May 2014
Available online 21 May 2014

ABSTRACT

Glutamate plays a role in hair cell afferent transmission, but the receptors that mediate neurotransmission between outer hair cells (OHCs) and type II ganglion neurons are not well defined. A previous study using *in situ* hybridization showed that several kainate-type glutamate receptor (KAR) subunits are expressed in cochlear ganglion neurons. To determine whether KARs are expressed in hair cell synapses, we performed X-gal staining on mice expressing *lacZ* driven by the *GluK5* promoter, and immunolabeling of glutamate receptors in whole-mount mammalian cochleae. X-gal staining revealed GluK5 expression in both type I and type II ganglion neurons and OHCs in adults. OHCs showed X-gal reactivity throughout maturation from postnatal day 4 (P4) to 1.5 months. Immunoreactivity for GluK5 in IHC afferent synapses appeared to be postsynaptic, similar to GluA2 (GluR2; AMPA-type glutamate receptor (AMPAR) subunit), while GluK2 may be on both sides of the synapses. In OHC afferent synapses, immunoreactivity for GluK2 and GluK5 was found, although GluK2 was only in those synapses bearing ribbons. GluA2 was not detected in adult OHC afferent synapses. Interestingly, GluK1, GluK2 and GluK5 were also detected in OHC efferent synapses, forming several active zones in each synaptic area. At P8, GluA2 and all KAR subunits except GluK4 were detected in OHC afferent synapses in the apical turn, and GluA2, GluK1, GluK3 decreased dramatically in the basal turn. These results indicate that AMPARs and KARs (GluK2/GluK5) are localized to IHC afferent synapses, while only KARs (GluK2/GluK5) are localized to OHC afferent synapses in adults. Glutamate spillover near OHCs may act on KARs in OHC efferent terminals to modulate transmission of acoustic information and OHC electromotility.

Published by Elsevier B.V.

1. Introduction

Hearing requires both the transduction of sounds into electrical impulses by cochlear hair cells and the relaying and processing of that information along a cascade of neurons (nerve cells) in the brain via chemical synapses between them. Defects in any of these processes can lead to hearing loss. The first synapses in this sequence link the sound-transducing hair cells with the processes of the primary neurons, called spiral ganglion cells, which relay information to the brainstem. Synaptic transmission between the hair cell and the ganglion cell processes depends on release of a chemical neurotransmitter, mainly glutamate, and its binding to neurotransmitter receptor molecules.

About 95% of auditory afferent neurons are type I ganglion neurons (Spoendlin, 1969). Each type I neuron sends a peripheral projection to an inner hair cell (IHC), forming a ribbon synapse (Liberman, 1980, 1982; Moser et al., 2006; Safieddine et al., 2012), where the presynaptic neurotransmitter glutamate and postsynaptic AMPA (α-amino-3-hydroxy-5-methyl-isoxazolepropionic acid)-type glutamate receptors (AMPARs) mediate excitatory transmission (Matsubara et al., 1996; Ruel et al., 1999; Glowatzki and Fuchs, 2002). Multivesicular release at this synapse achieves

Abbreviations: ACh, acetylcholine; GABA, Gamma amino butyric acid; AMPAR, AMPA-type glutamate receptor; GluR, glutamate receptor; KAR, kainate-type glutamate receptor; IHC, inner hair cell; MOC, medial olivocochlear; OHC, outer hair cell

* Corresponding author. 35A Convent Drive, 35A/1E614, NIDCD/NIH, Bethesda, MD 20892-3729, USA. Tel.: +1 301 496 3804.
E-mail address: petralia@nidcd.nih.gov (R.S. Petralia).

http://dx.doi.org/10.1016/j.heares.2014.05.001
0378-5955/Published by Elsevier B.V.

frequent excitatory postsynaptic currents (EPSCs) in type I afferent neurons and causes continuous and rapid transmission of acoustic information. Type II neurons constitute the remaining 5 percent of afferent neurons. Each type II neuron forms a thin, unmyelinated dendrite contacting many outer hair cells (OHCs). Each OHC has 3 to 15 afferent terminals (Liberman et al., 1990) and some of them do not bear a ribbon synapse (Hashimoto and Kimura, 1988; Liberman et al., 1990; Huang et al., 2012). OHC/type II afferent transmission is also glutamatergic, but EPSCs in type II neurons are much smaller in frequency and amplitude, and significantly slower in kinetics compared to averaged EPSCs recorded in type I neurons (Weisz et al., 2009), so that summated stimulation is required to produce an action potential in type II afferent neurons (Weisz et al., 2012). The specific receptor involved remains unknown, because there is no immunoreactivity for AMPARs in type II afferent nerve terminals (Matsubara et al., 1996; Liberman et al., 2011).

OHCs also are innervated by myelinated fibers from the medial olivocochlear (MOC) efferent projections, forming several synapses at the base (Liberman and Brown, 1986). The MOC system is cholinergic and suppresses the electromotile response of OHCs (Wersinger and Fuchs, 2011; Elgoyhen and Katz, 2012), providing a feedback system to optimize cochlear amplification (LePage, 1989). Acetylcholine (ACh) is the only well-defined neurotransmitter in OHC efferent synapses, but additional molecules are proposed to be involved in the transmission as neuromodulators, including gamma amino butyric acid (GABA). It is suggested that GABAergic signaling contributes to the long-term maintenance of hair cells or normal OHC electromotility (Maison et al., 2006, 2009). Furthermore, a recent study shows that released GABA acts on presynaptic $GABA_B$ receptors expressed in OHC efferent terminals to downregulate the release of ACh (Wedemeyer et al., 2013).

Ionotropic glutamate receptors (GluRs) include three major families; NMDA-type, AMPA-type and kainate-type receptors (KARs). GluRs mediate neurotransmission at excitatory synapses, but neuronal KAR-mediated EPSCs have distinct physiological feature of smaller amplitude and slower deactivation kinetics, compared to AMPA-mediated EPSCs and are shaped by auxiliary KAR subunits, such as tolloid-like 1 (NETO1) and NETO2 proteins (Lerma and Marques, 2013). In addition, presynaptic KARs can act as neuromodulators of synapse transmission to control transmitter release in a bidirectional manner; frequency-dependent facilitation and depression of transmitter release via presynaptic KARs have been demonstrated at mossy fiber synapses in the hippocampus (Lerma, 2003) and at parallel fiber synapses in the cerebellar cortex (Delaney and Jahr, 2002). Moreover, postsynaptic KARs are thought to regulate neuronal excitability both through slowly deactivated ionotropic KARs and through G protein-coupled metabotropic KARs (Lerma and Marques, 2013).

A previous study using *in situ* hybridization showed that several KAR subunits (GluK1, GluK2, GluK4, GluK5) are expressed in cochlear ganglion neurons (Niedzielski and Wenthold, 1995). Another study reported that KARs are expressed in IHC afferent synapses and suggested that KARs contribute to hair cell acoustic transmission, based on physiological data using a GluK1-specific antagonist (Peppi et al., 2012). These findings suggest that KARs are involved in normal cochlear function for synaptic transmission or modulation. In order to determine whether KARs are expressed in synapses of IHCs and OHCs, we performed immunolabeling of all the subtypes of KARs in the adult mammalian cochlea. We also performed auditory testing on mice that lacked GluK5. We found that KARs (GluK2/GluK5) are the main postsynaptic GluRs in OHC afferent synapses and that the expression pattern of KARs shows developmental changes. Interestingly, KARs are also expressed in OHC efferent terminals. Moreover, we detected both pre- and postsynaptic KARs in IHC afferent synapses.

2. Materials and methods

2.1. Animals

GluK5 knockout mice (GluK5 KO; strain B6.129P2-*Grik5*^{tm1Dgen}/J), was obtained from the Jackson Laboratories (generated by Deltagen). This strain has been backcrossed to C57BL/6 mice and has a targeted deletion inside the GluK5 (*Grik5*) gene (from base 1936 to base 2006) that is replaced with a reporter gene, *lacZ*. We confirmed that X-gal staining in brain sections of the mice (data not shown) is consistent with previous studies of GluK5 cellular distribution in the brain (Darstein et al., 2003). All the animals used in this study were euthanized in accordance with National Institutes of Health (NIH) guidelines.

2.2. X-gal staining of cochleae

Cochleae from GluK5 KO mice were fixed in 4% paraformaldehyde with 0.02% Nonidet P-40 in phosphate buffered saline (PBS) for ~1 h at 4 °C. Cochleae were decalcified in 5% EDTA for three days at 4 °C, washed in rinse buffer (0.01% sodium deoxycholate, 0.02% Nonidet P-40, 2 mM magnesium chloride in PBS) for 1 h and stained in 1 mg/ml X-gal/5 mM potassium ferrocyanide/5 mM potassium ferricyanide in rinse buffer overnight at 37 °C (Bianchi et al., 2002; Corradi et al., 2003). Cochleae were dissected for whole-mount preparation or processed further for sections; cochleae were cryoprotected in 20% sucrose overnight at 4 °C before embedding in Tissue-Tek O.C.T. compound (Sakura) using a vacuum to remove bubbles inside, and cut at 16 μm thickness on a Leica Cryostat (OT-15 °C, CT-15 °C). After post-fixation with 4% paraformaldehyde for 10 min, sections were incubated in BLOXALL (Vector Laboratories), blocked in 5% bovine serum albumin (BSA) and incubated in mouse anti-neurofilament-H (clone RT97; Sigma–Aldrich, 1:1000) overnight at 4 °C. Sections were then incubated in HRP-conjugated secondary antibody (Santa Cruz Biotechnology, 1:500) and DAB stain was developed with ImmPACT DAB (Vector Laboratories).

2.3. Whole-mount immunofluorescence

Temporal bones of Sprague–Dawley rats (4–6 weeks old or postnatal day (P) 8) were quickly removed and fixed in 2% paraformaldehyde for 10 min at room temperature, if not otherwise specified (Grati et al., 2012). Cochlear tissues were then dissected, permeabilized with 0.5% Triton X-100 for 30 min and blocked with 5% BSA. Primary antibody was incubated as described below. Synaptic markers were also used; ribeye (Synaptic Systems, 1:200), CtBP2 (clone 16/CtBP2; BD Biosciences, 1:200), PSD-93 (Alomone labs, 1:200), PSD-93 (clone N18/30 and clone N18/28; NeuroMab, 1:200), VAMP2 (clone 69.1; Synaptic Systems, 1:200), Na^+/K^+-ATPase α3 (Santa Cruz Biotechnology, 1:50), synapsin (Millipore, 1:500). The tissue then was incubated in Alexa Fluor 488- and 568-conjugated secondary antibodies and Alexa Fluor 674 phalloidin (Molecular Probes) and then fixed again by immersion in 4% paraformaldehyde for ~15 min before mounting using Prolong Anti-fade reagent (Invitrogen). Confocal images were acquired using a Nikon TE-2000 inverted microscope outfitted with a CSU-21 Yokogawa spinning disk (Perkin–Elmer), Plan Apo 100×/1.40 NA objective, and Hamamatsu Orca-ER CCD. Image acquisition, including stacks, was managed through Volocity imaging software (Perkin–Elmer). All image analyses, including fluorescence intensity quantification, peak distance measurement, maximum intensity projection, and 3D reconstruction, were performed with