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Research paper

Impaired auditory processing and altered structure of the endbulb of Held synapse in mice lacking the GluA3 subunit of AMPA receptors

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ABSTRACT

AMPA glutamate receptor complexes with fast kinetics conferred by subunits like GluA3 and GluA4 are essential for temporal precision of synaptic transmission. The specific role of GluA3 in auditory processing and experience related changes in the auditory brainstem remain unknown. We investigated the role of the GluA3 in auditory processing by using wild type (WT) and GluA3 knockout (GluA3-KO) mice. We recorded auditory brainstem responses (ABR) to assess auditory function and used electron microscopy to evaluate the ultrastructure of the auditory nerve synapse on bushy cells (AN-BC synapse). Since labeling for GluA3 subunit increases on auditory nerve synapses within the cochlear nucleus in response to transient sound reduction, we investigated the role of GluA3 in experience-dependent changes in auditory processing. We induced transient sound reduction by plugging one ear and evaluated ABR threshold and peak amplitude recovery for up to 60 days after ear plug removal in WT and GluA3-KO mice. We found that the deletion of GluA3 leads to impaired auditory signaling that is reflected in decreased ABR peak amplitudes, an increased latency of peak 2, early onset hearing loss and reduced numbers and sizes of postsynaptic densities (PSDs) of AN-BC synapses. Additionally, the lack of GluA3 hampers ABR threshold recovery after transient ear plugging. We conclude that GluA3 is required for normal auditory signaling, normal ultrastructure of AN-BC synapses in the cochlear nucleus and normal experience-dependent changes in auditory processing after transient sound reduction.

1. Introduction

Acoustic information is processed within the auditory system. Unlike other sensory systems, parts of the auditory pathway are equipped with neurons possessing unique morphological and biophysical specializations that facilitate the transmission of information with great temporal precision (Trussell, 1999). In the anteroventral cochlear nucleus (AVCN), bushy cells (BC) receive their main excitatory input from auditory nerve axons, which contact the BC cell body through exceptionally large terminals (endbulbs of Held) (Brawer and Morest, 1975). The size of the endbulb facilitates rapid transmission of the presynaptic signal to the BC bodies. In addition, endbulb synapses contain AMPA glutamate receptors (AMPARs) with fast kinetics and high calcium permeability; such properties are crucial for the temporal precision of the auditory signal to the bushy cell (Gardner et al., 1999). These AMPAR complexes contain very little GluA2 and are mainly composed of GluA3 and GluA4 subunits (Gardner et al., 1999; Petralia et al., 2000; Wang et al., 1998). Previously we reported that transient sound reduction leads to an increase of GluA3 immunogold labeling at the postsynaptic density of AN-BC synapses (Clarkson et al., 2016; Whiting et al., 2009). Based on these observations, we hypothesized that GluA3 is involved in normal auditory processing and in experience-related changes in the auditory brainstem. To further assess the role of GluA3 in normal auditory processing, we used young adult GluA3-KO mice. We recorded auditory brainstem responses (ABRs) to assess auditory sensitivity and used electron microscopy to evaluate the ultrastructure of the AN-BC synapse.

The auditory system is able to adapt to changing conditions during development, aging and auditory experience (Brugge, 1992;

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Kandler et al., 2009; Froemke and Schreiner, 2015). Auditory experience can drive changes in sensory processing mechanisms in developing and adult auditory systems (Fallon et al., 2008; Syka, 2002). The ability of the brain to process auditory information despite altered input (e.g. after sound deprivation) is usually attributed to plastic changes in the auditory pathway (Fallon et al., 2008; Syka, 2002; Caras and Sanes, 2015; Chambers et al., 2016). Furthermore, unilateral ear plugging of adult listeners is sufficient to induce experience-dependent changes in acoustic reflex threshold in the auditory brain stem (Munro and Blount, 2009). The mechanisms contributing to these changes may include the modification of AMPAR number and/or subunit composition. Indeed, AMPAR modulation is a major mechanism for the control of efficient synaptic transmission and underlies many forms of synaptic plasticity (Song and Huganir, 2002). In the present study we assessed how the lack of GluA3 affects the ability of the auditory brainstem to adapt to changes in sensory experience. We inserted one ear plug into WT and GluA3-KO mice, and evaluated the recovery of ABR thresholds and peak amplitudes for 60 days after ear plug removal.

2. Methods

2.1. Animals

All experiments were performed using C57BL/6 male mice that were one to three months of age. These mice hear normally as young adults at the age of 1–2 months and exhibit progressive high-frequency hearing loss beginning between 2 and 3 months of age (Willott, 2006). At least 10 mice of each genotype were used per group. All experimental procedures were carried out in accordance with the National Institute of Health guidelines and approved by the University of Pittsburgh and Niigata University Institutional Animal Care and Use Committees.

2.2. Generation and identification of GluA3 knockout mice

Mice deficient in GluA3 were produced by homologous recombination using the C57BL/6 ES cells. We isolated GluA3 (*Gria3*) genes by genomic PCR from the C57BL/6 mouse genome. A GluA3 targeting vector contained exon 11 of the Gria3 gene with the 4.2 kb upstream and 7.0 kb downstream homologous genomic DNA fragments and the diphtheria toxin gene for negative selection. A DNA fragment, which carried a loxP sequence and pgk-1 promoter-driven neomycin phosphotransferase gene (Neo cassette) flanked by two Flp recognition target (frt) sites, was inserted into site 107 bp upstream of exon 11. A polyadenylation (poly-A) signal sequence of pgk-1 was inserted downstream of Neo cassette. The other loxP site was introduced into site 113 bp downstream of exon 11 in order to eliminate the putative transmembrane domain after Cre-mediated recombination. Homologous recombinant ES clones (*Gria3*$^{flox/+}$) were identified by Southern blot analysis. *Eco*RV-digested DNA hybridized with 5′ probe, 15.4 kb for wild-type and 14.3 kb for targeted genome; *Nde*I-digested DNA hybridized with neo probe, 16.5 kb for targeted allele; *Nde*I-digested DNA hybridized with 3′ probe, 14.6 kb for wild-type and 16.5 kb for targeted allele (Fig. 1).

Culture of ES cells and generation of chimeric mice were performed as described previously (Mishina and Sakimura, 2007). Briefly, to establish the homologous recombinants, we introduced the linearized targeting vector into the C57BL/6 derived ES lines and then selected recombinant clones under the medium containing 175 μg/mL G418. Targeted clones were microinjected into 8 cell-stage embryos of the CD-1 mouse strain. The resulting chimeric embryos were developed to the blastocyst stage by incubation for more than 24 h and then transferred into the pseudopregnant CD-1 mouse uterus. Germline chimeras were crossed with C57BL/6 female mice and the heterozygous offspring were crossed with TLCN-Cre mice (Nakamura et al., 2001; Fuse et al., 2004) to establish the GluA3-KO mouse line.

The mouse colony was maintained in an inbred C57BL/6 genetic background strain and housed in an on-site colony at the Hillman Cancer Center Animal Facility, a branch of the Division of Laboratory Animal Resources facility at the University of Pittsburgh. As GluA3 comes from an X-linked gene, both wild type and heterozygous males were used as control mice (hereafter referred as WT). Genotypes were confirmed by PCR using standard conditions. Genomic DNA from tail snips was isolated and used directly for PCR (Fig. 1C). Primers were selected to generate products for both WT and GluA3-KO alleles. Using the forward primer gria3KO-NeoPCR-F1 (5′-TAGAACCCACTGAATGACCC-3′) and reverse primer gria3KO-loxPCR-R1 (5′-TTTAGCCCCTTGGCAAATGC-3′), two PCR products were generated. A 350-bp for the WT allele and an 850-bp for the GluA3-KO allele PCR products were generated.

The absence of GluA3 protein was confirmed by standard Western blotting (Fig. 1D). Two mice of each genotype were used for the biochemical analysis. After CO_2 anesthesia and decapitation, entire brains were dissected rapidly and placed in cold 0.05 M Tris-HCl buffer pH 7.4 with a protease inhibitor cocktail (Pierce, Thermo Fisher Scientific, Rockford, IL, USA). Tissues were homogenized in the same buffer with 10% sucrose. Protein concentration was determined using a micro BCA protein assay (Pierce). The samples were separated in an SDS gel, as described previously (Wang et al., 2011). The affinity-purified rabbit antibody against GluA3 (1:100; AGC-010; Alomone, Jerusalem, Israel) and the mouse monoclonal antibody β-actin (1:10,000; A1978, Sigma-Aldrich;) were used. The blot membranes were developed by the chemiluminescence-enhanced method.

2.3. Auditory brainstem response (ABR)

All recordings were conducted in anesthetized mice (Isoflurane: 3% induction, 1.5% maintenance) in a soundproof chamber and using a Tucker-Davis Technologies (Alachua, FL) recording system. ABR stimuli were presented through a calibrated TDT CF1 closed-field speaker connected to a 2-mm diameter plastic probe. ABRs were recorded by placing needle electrodes subcutaneously at the vertex of the head, at the right cheek and at the left cheek. ABR stimuli were broadband noise clicks (0.1 ms) or tone pips of 4, 8, 12, 16, 24 and 32 kHz (5 ms). Stimuli were presented with alternating polarity at a rate of 21 presentations per second, with an inter-stimulus interval of 47.6 ms. Responses of 512 sweeps were averaged, band-pass filtered (0.3–3 kHz) and amplified 20× through a RA4LI preamplifier (maximum sampling rate ~25 kHz) and transferred through an optical fiber port to a RZ6 processor (maximum sampling rate ~200 kHz). The stimulus intensity was varied from 90 dB to 10 dB, in decreasing steps of 10 dB.

Hearing threshold levels were determined offline by identifying the lowest intensity level at which clear reproducible wave peak forms P1, P2 and P3 were visible in the averaged traces (P4 and P5 were not always visible, particularly in the GluA3-KO mice). Latencies and amplitudes of P1, P2 and P3 were compared between WT and GluA3-KO mice. We used the same nomenclature as Sergeyenko et al. (2013), designated to differentiate peaks or waves from larger mammals (Waves I–V). For measurements of amplitudes and latencies, the peaks and troughs from individual click-evoked ABR traces (temporal resolution 40 μs) were selected manually in BioSigRZ software and exported to an Excel file, which contained the values for every peak and every trough. The peak amplitude was calculated as the distance (in microvolts) from the