Published in final edited form as:

Hear Res. 2017 February ; 344: 284–294. doi:10.1016/j.heares.2016.12.006.

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

Sofía García-Hernández¹, Manabu Abe², Kenji Sakimura², and María E. Rubio^{1,3,4}

¹Department of Otolaryngology, University of Pittsburgh, School of Medicine, Pittsburgh, PA, USA

²Niigata University Brain Research Institute, Japan

³Department of Neurobiology, University of Pittsburgh, School of Medicine, Pittsburgh, PA, USA

⁴Center for the Neural Basis of Cognition, University of Pittsburgh, School of Medicine, Pittsburgh, PA, USA

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.

Corresponding author: María E. Rubio M.D./Ph.D, Department of Otolaryngology and Neurobiology, University of Pittsburgh Medical School, BST3 Building, 3501 Fifth Avenue #10015, Pittsburgh, PA 15261, Phone: 412-624-6939, Fax: 412-383-5298.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Authors contributions:

MA and KS produced the GluA3 knockout mice; SGH and MER conceived the experiments; SGH performed data acquisition and analysis; MER analyzed data for Figure 6F; SGH wrote the manuscript with feedback from MER; SGH, MA, KS and MER approved the final version of the manuscript; MER provided funding.

Conflict of interest statement

The authors declare no competing financial interests.

Keywords

Auditory brainstem; cochlear nucleus; endbulb of Held synapses; ultrastructure; postsynaptic density; auditory experience

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 (Trussel, 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 (AMPA) 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; 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 Hugarir, 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 2–3 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*^{fllox/+}) were identified by Southern blot analysis. *EcoRV*-digested DNA hybridized with 5' probe, 15.4 kb for wild-type and 14.3 kb for targeted genome; *NdeI*-digested DNA hybridized with neo probe, 16.5 kb for targeted allele; *NdeI*-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'-TTTAGCCCCCTTGGCAAATGC-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 CO₂ 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 interstimulus interval of 47.6 ms. Responses of 512 sweeps were averaged, band-pass filtered (0.3 – 3 kHz) and amplified 20x 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 microseconds) 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 maximum positive peak to the following maximum negative trough. The latency was calculated as the interval (in milliseconds) from the acoustic stimulus (0 microvolts) to the corresponding maximum positive peak. The values were plotted in GraphPad Prism software.

2.4 Ear plugging

After anesthetizing the animals with isoflurane and placing them on a controlled temperature pad, a foam ear plug (E-A-R™Classic™) was compressed and placed into the right external ear canal at postnatal day 30 (P30). We have established this model as an efficient way to attenuate by ~40 dB the passage of sound through the ear canal (Whiting et al., 2009; Wang et al., 2011). Since the ear canal of mice is around 6mm in length (Saunders and Crumling, 2001), the length of the plug never exceeded 3mm, so that contact with the tympanic membrane was avoided. Two stitches were carefully placed at the base of the pinna to prevent the mice from removing the plug. The plug was left in place for 10 days. ABRs elicited with clicks and tones were recorded immediately before inserting the ear plug and once again immediately before the ear plug was removed. After removing the ear plug, the ear canal was cleared of any debris and inflammation status of the ear canal was monitored daily. By two days to one week after removing the ear plug, the ear canal looked normal. ABRs were recorded 10, 20 and 60 days after ear plug removal. At equivalent times, ABRs were recorded from age-matched controls that were not ear plugged (see Fig. 3A).

2.5 Electron microscopy and image analyses

Brain tissues of three mice of each genotype were processed for electron microscopy, as described previously (Gómez-Nieto and Rubio, 2009). The mice were anesthetized with ketamine and xylazine (100 mg/kg each) and transcardially perfused with 0.1M phosphate buffer pH 7.2, containing 3% paraformaldehyde and 1.25% glutaraldehyde. Brains were post-fixed overnight in the same fixative and sectioned at a thickness of 100 µm with a vibratome (Leica VT1000S; Vienna, Austria). Sections were further post-fixed with 1% osmium tetroxide in 0.1% cacodylate buffer pH 7.4. After washing with the same buffer to remove excess osmium, the sections were dehydrated with graded alcohols and propylene oxide, then infiltrated with EMBed-812 resin, flat-embedded and polymerized for 48 hours at 80°C. Sections containing the AVCN were trimmed and mounted on resin blocks. Ultrathin sections (silver-gold interference, ~70 nm) were cut with an ultramicrotome (Leica EM UC7) and collected on copper grids (G300-Cu, Electron Microscopy Sciences, Hatfield, PA, USA) and counterstained with 1% uranyl acetate and 0.4% lead citrate.

Bushy cells (BCs) from caudal and rostral AVCN were analyzed in this study, as BCs of the mouse do not show the rostro-caudal differences reported in other rodents and mammals (Lauer et al., 2013; Trettel and Morest, 2001). We considered only cell bodies that were large and oval to round-shaped, and had an eccentric nucleus. The ultrastructure of the cell body of the BC in the mouse is similar to the BC in other rodents (Lauer et al., 2013; Gómez-Nieto and Rubio, 2009). The cell body of each BC received at least one large end bulb profile which made multiple asymmetric synaptic contacts on the postsynaptic cell membrane. The end bulb profiles contained large, clear and round synaptic vesicles, as described previously for other animal species (Gómez-Nieto and Rubio, 2009; Lauer et al., 2013). Additionally, the cell bodies received numerous bouton-like terminals that made asymmetric synaptic contacts on the postsynaptic cell membrane and that contained large, clear and round synaptic vesicles, similar to those observed in endbulb profiles. We considered all these terminals for the ultrastructural analysis, as they represent excitatory inputs from the AN (Cant and Morest, 1979; Tolbert and Morest, 1982; Wang et al., 1998).

Ultrathin sections were examined using a JEM-1400 Transmission Electron Microscope (JEOL Ltd., Akishima Tokyo, Japan). Images were acquired with a side mounted ORIUS™ SC200 CCD camera (Gatan Inc., Warrendale, PA, USA) at magnifications of 4000x for cell bodies, 12–30000x for AN endings, and 60000x for PSDs.

ImageJ software (1.43u, NIH, <https://imagej.nih.gov/ij/>) was used for morphometric analyses. The contour of each auditory nerve ending was manually traced and its area calculated (referred as AN ending profile area). The PSD length was manually traced from single images, in which the pre-synaptic membrane was parallel to the post-synaptic plasma membrane. The PSDs were classified as flat or dome-shaped, and the respective percent of total PSDs was calculated for individual mice of each genotype. Images were assembled with Adobe Photoshop, modifying brightness and contrast, if necessary.

2.6 Statistical analyses

All statistical analyses were performed using GraphPad Prism software. Values are expressed as mean $\pm$ SEM and compared between groups using a repeated measures two-way ANOVA. Significant differences were analyzed further with a Bonferroni post hoc test. For morphometric analyses we used an unpaired Student's *t* test. In all tests, means were considered significantly different if *P* 0.05.

3. Results

3.1 Auditory processing in the absence of GluA3

To determine whether the GluA3 AMPAR subunit is necessary for normal hearing, we used mice lacking GluA3. We tested wild type, heterozygote and GluA3-KO mice at 60 days of age, when they are sexually mature and their auditory system is anatomically (Limb and Ryugo, 2000) and functionally mature (Huangfu and Saunders, 1983). We recorded ABRs and determined auditory thresholds (Fig. 2A and 2B). We found that ABR thresholds for clicks and tone-pip frequencies are similar between WT and GluA3-KO mice (Fig. 2B). The lower thresholds were obtained with click-evoked stimuli (~ 20 dB) followed by 8 kHz and 12 kHz- evoked stimuli (~ 45 dB; data shown only for 8 kHz). This is consistent with the low sensitivity for middle and high frequencies reported for the C57 strain (Frisina et al., 2011).

Since normal thresholds do not necessarily indicate normal auditory processing, and the peaks 4 and 5 are not discernible in most of the ABR traces from GluA3-KO mice, we measured the amplitudes and latencies of peaks 1, 2 and 3 (P1, P2 and P3, respectively), as they were reliably identified at the presented stimulus levels (see Fig. 2A). The amplitudes of ABR peaks have been interpreted as the sound-evoked summed neural responses from specific regions of the auditory pathway. In mice, it has been proposed that P1 corresponds to the response of the auditory nerve (AN). P2 to P5 represent the neural responses in the AVCN, the superior olivary complex (SOC), the lateral lemniscus (LL) and the inferior colliculus (IC), respectively (Parham et al., 2001). We computed absolute peak latencies as a function of stimulus intensity, and found that the latency of P2 is significantly larger at all stimulus intensities in GluA3-KO as compared to WT mice (Fig. 2C), suggesting a

decreased response of the AN fibers, slowed transmission from the AN to the CN or slowed processing of the incoming auditory signals in the CN. We also recorded peak amplitudes as a function of stimulus level using click-evoked ABR. We found that P1 amplitude is lower in GluA3-KO, as compared to WT (Fig. 2D), suggesting either degeneration or de-synchronization of the AN fibers (Schaette and McAlpine, 2011; Sergeyenko et al., 2013). Amplitudes of P2 and P3 (Fig. 2E, 2F) are significantly reduced for 80 dB SPL in the GluA3-KO, reflecting deficient neural signaling in both AVCN and SOC nuclei at higher sound levels.

3.2 Auditory threshold after transient sound reduction

Since the auditory system is plastic and can reorganize its structure and function after auditory deprivation during development and in adulthood (Munro and Blount, 2009; Kandler et al., 2009; Syka, 2002), we transiently reduced the acoustic signal entering one ear of WT and GluA3-KO mice at the age of 30 days (Fig. 3A). As shown in Figure 3B, inserting a foam plug in one ear resulted in a significant increase in ABR thresholds for all tested frequencies in both WT and GluA3-KO mice, as compared with their age-matched controls. ABR thresholds were not significantly different between genotypes, confirming that the ear plug reduced the sound intensity in both WT and GluA3-KO equally.

To follow the recovery from 10 days of sound reduction in WT and GluA3-KO mice, we recorded ABRs from ear plugged mice at 10, 20 and 60 days after the ear plug was removed; ABRs from age-matched control WT and GluA3-KO mice were also recorded. By 10 days after ear plug removal, ABR thresholds for WT mice in response to clicks and 8 kHz tone pips remain significantly elevated by ~15 dB; thresholds for all the other frequencies returned to levels comparable to age-matched controls (Fig. 3C). In contrast, thresholds for GluA3-KO mice in response to clicks and tones at all the frequencies are significantly elevated when compared to age-matched control KO mice (Fig. 3C).

By 20 days after ear plug removal, ABR thresholds in WT mice are similar to the age-matched controls (Fig. 3D). Conversely, thresholds in GluA3-KO mice in response to clicks and 8k Hz tone pips are significantly elevated by ~ 20 dB with respect to the age-matched control KO mice (Fig. 3D). This suggests that the absence of GluA3 hinders plastic changes that contribute to the recovery of normal auditory thresholds after transient sound reduction.

By 60 days after ear plug removal, ABR thresholds in WT mice are similar to those in the age-matched controls (Fig. 3E). In contrast, ABR thresholds in the GluA3-KO mice did not further improve (Fig. 3E). In fact, threshold values at 60 days after ear plug removal are similar to those at 20 days after ear plug removal (Fig. 3D). This implies that the capacity of the lower brainstem to fully recover auditory function after transient sound reduction is compromised in the absence of GluA3, as summarized in Figure 3F. In addition to the lack of threshold recovery in GluA3-KO mice that were ear plugged, we observed that thresholds in GluA3-KO controls are also elevated with respect to WT controls (Fig. 3E). This suggests an early onset of hearing loss in the absence of GluA3.

3.3 ABR peak amplitudes after transient sound reduction

Since ABR peak amplitude measurements are more sensitive in detecting subtle changes in central processing than ABR thresholds (Kujawa and Liberman, 2009), we measured peak amplitudes in ear plugged and age-matched control WT and GluA3-KO mice. Because with the ear plug inserted, thresholds reach up to 80 dB, we plotted amplitudes and latencies only for intensities of 80 dB. With the ear plug inserted for 10 days, amplitudes of P1, P2 and P3 are significantly reduced compared to those of age-matched controls (Fig. 4A). Latencies of P1, P2 and P3 are significantly increased in both genotypes (Fig. 4B) when mice have the ear plug inserted. Thus, ear plug-induced threshold elevation is accompanied by decreased amplitude and increased latency of ABR peaks, reflecting sound attenuation and subsequent reduced signal transmission along the brainstem auditory pathway.

By 10 days after ear plug removal, amplitudes of P1 in WT mice are similar to those in age-matched controls (Fig. 4C), while P1 amplitudes in GluA3-KO mice are reduced at 40 dB. Amplitudes of P2 are similar to those in the age-matched controls in both genotypes (Fig. 4D), except for the increased amplitude in GluA3-KO mice at 80 dB. Amplitudes of P3 are similar to those in the age-matched controls in both genotypes, except for increased amplitudes in WT mice at 80 dB (Fig. 4E). These results show that the GluA3-KO mice have a different pattern of ABR peak amplitude recovery after transient sound reduction.

By 20 days after ear plug removal, P1-P3 amplitudes in WT mice are similar to those of the age-matched controls (Fig. 4F–H). This indicates that the increased amplitude of P3 in WT is reversed in a period of ten days, which is consistent with the plasticity of the brainstem (Munro and Blount, 2009). By contrast, in GluA3-KO mice, amplitudes of P1 and P2 are increased at intensities of 60 and 80 dB, when compared to age-matched controls (Fig. 4F–G). The lengthier period of increased amplitudes in the GluA3-KO suggests slower adaptation to normal sound environments after transient sound reduction.

By 60 days after ear plug removal, P1-P3 amplitudes in WT mice are similar to those in the age-matched controls (Fig. 4I–K). However, P1 amplitudes in GluA3-KO mice remain elevated at 60 dB. The complete recovery of normal amplitudes in WT mice by 20 days after ear plug removal together with the more persistent elevations of P1 and P2 amplitudes in GluA3-KO mice indicates that the lack of GluA3 hampers the regulatory capacity of auditory pathways and impedes its full recovery after transient sound reduction.

The reduction of ABR P1 amplitude elicited at suprathreshold stimulus levels can be used as a measure to quantify neural degeneration in mice (Shaheen et al., 2015). The ABR P1 amplitudes plotted in Figure 4I, 60 days after ear plug removal, indicate that there were no reductions in amplitude; the responses of the ear plugged mice either matched those of the age-matched controls or were increased. Based on this ABR data, we deduce that the presence of the ear plug did not result in any measurable cochlear or cochlear nerve degeneration.

3.4 Onset of hearing loss in mice lacking GluA3

The C57BL/6 mice used in this study exhibit a progressive, high frequency hearing loss that begins between 60 – 90 days of age (Willott, 2006; Zheng et al., 1999). As previously

mentioned, we observed that thresholds in control GluA3-KO were elevated when mice reached 100 days of age (Fig. 3E). Therefore we re-plotted the ABR thresholds from control mice that were 30 and 100 days of age (Fig. 5). We found that in WT mice only thresholds in response to 32 kHz tones are significantly increased at the age of 100 days, compared to mice of 30 days of age. However, in GluA3-KO mice thresholds in response to 8 kHz, 24 kHz and 32 kHz are significantly elevated at the age of 100 days as compared to 30 day old mice. This suggests that the lack of GluA3 promotes early onset of hearing loss in the lower and mid-frequency range.

3.5 Ultrastructure of the AN-BC synapse

The consistently altered responses of ABR P2 in the GluA3-KO mice, together with the fact that ABR P2 reflects neural responses in the CN (Melcher and Kiang, 1996), and GluA3 is the most abundant subunit in the AN-BC synapse (M.E. Rubio, unpublished data), led us to evaluate whether the lack of GluA3 affects the ultrastructure of the AN-BC synapse of the AVCN. We analyzed BCs from caudal and rostral AVCN (see methods). We performed morphometric analyses of the endbulb synapse in WT (Fig. 6A) and GluA3-KO (Fig. 6B) adult mice. We calculated the area of individual AN ending profiles and found no significant differences between genotypes (Fig. 6C). We also quantified the number of PSDs per profile and found that the number of PSDs was significantly reduced by ~30% in the GluA3-KO, compared to WT mice (Fig. 6D). We also measured the length of the PSDs and found that those from GluA3-KO mice were significantly smaller by ~30% (Fig. 6B inset and E) as compared to WT mice (Fig. 6A inset and E). Additionally, most of the PSDs from GluA3-KO mice lose the prototypical dome-shaped of the AN-BC synapse (Reed et al., 2000; Fig. 6F). These observations indicate that the deletion of GluA3 affects the post-synaptic structure of the AN-BC synapse.

4. Discussion

Little is known about the role of specific AMPAR subunits in the auditory system. Here we studied the role of GluA3 in normal and experience-related changes in auditory signaling. By using ABRs, we evaluated functional changes in GluA3-KO mice. ABRs peak 2 was consistently altered in the GluA3-KO mice, what led us to analyze ultrastructurally the synapse of auditory nerve on bushy cells (endbulb synapses).

We found that GluA3-KO mice at the age of 60 days have normal ABR thresholds but reduced peak 1 to 3 (P1-P3) amplitudes and increased latency of P2. The decreased amplitude of P1 suggests reduced and/or less-synchronized activity in the AN, as P1 amplitude represents the sound evoked summed neural responses of the AN fibers and their synchronized activity (Kujawa and Liberman, 2009; Schaette and McAlpine, 2011). This might reflect cochlear synaptopathy as indicated by the reduction of P1 amplitude (Sergeyenko et al., 2013). The decreased amplitude of P2 and P3 could be a direct consequence of the reduced activity in the AN. Likewise the increased latency of P2 could arise from the reduced amplitude of P1, since changes in the amplitude of P1 influence the latency of the subsequent peaks (Mehraei et al., 2016). Furthermore, the increased latency of P2 in the GluA3-KO could represent slowed signaling in the CN. Since the CN is the first

central station that encodes the timing of auditory signaling (Trussell, 1999), it is likely that timing is deficient in the absence of GluA3. A recent study by Yang et al. (2011) showed that the lack of GluA4 affects ABR amplitudes and latencies of P3 and P4. Together with our findings, these observations suggest that GluA3 and GluA4 are each necessary for normal auditory signaling within defined synapses of the auditory pathway. Since in the MNTB, GluA4 is essential for fast synaptic transmission (Yang et al., 2011), it is likely that GluA3 plays a similar role in the CN. This is supported by our findings on increased latency of P2 (CN). In contrast, we did not detect an increased latency of P3 (SOC) in the GluA3-KO, presumably because GluA4 may be sufficient to compensate for the slowed signaling in the CN. It has been proposed that the subunit composition of postsynaptic AMPARs determines the functional differences in auditory neurons (Geiger et al., 1995). However this hypothesis seems to be more complex because the CN and the SOC have in common the highest levels of the fast kinetics GluA3- and GluA4 AMPAR subunits, as compared to the levels of GluA1 and GluA2 (Koike-Tani et al., 2005; Petralia et al., 2000; Schmid et al., 2001; Wang et al., 1998). Regardless the similarity of AMPAR subunit composition, it is clear that the CN and the SOC respond differentially to changes in the acoustic environment. Clarkson et al. (2016) showed that GluA3, but not GluA4, mediates auditory-experience plasticity at the end bulb synapse in the AVCN. Yang et al. (2011) showed that the deletion of GluA4, but not of GluA3, alters greatly the synaptic transmission at the MNTB. Pilati et al. (2016) showed that GluA4 in the lateral superior olive decreases in response to auditory damage. Taken together, these studies suggest that GluA3 in the CN, and GluA4 in the SOC, are selectively modified by auditory experience. Such selectivity may stem from intrinsic circuitry connections, the relative expression of specific AMPAR subunits, the diversity of AMPAR- interacting proteins as well as the intracellular signaling pathways in specific auditory neurons. Further research is required to investigate the glutamatergic synaptic mechanisms underlying functional differences in these auditory circuits.

Our findings of deficient amplitude and increased latency of P2 in the GluA3-KO also correspond well with the ultrastructural changes we observed in the PSDs of the AN-BC synapse. The PSD complex is essential for the anchoring of neurotransmitter receptors and associated signaling proteins (Sheng and Hoogenraad, 2007), and any modification of its structure alters synaptic transmission (Freche et al., 2011). Our findings demonstrate that in the absence of GluA3, the number, size and shape of the PSDs in AN-BC synapses are significantly reduced.

The reduced number and smaller PSDs in GluA3-KO mice may be correlated with aging, since fewer synapses and reduced area of PSDs in the brain of aged rats has been reported (Nicholson et al., 2004; Wong et al., 2000). The lack of PSDs with a dome-shape in GluA3-KO mice may also correspond with age-related hearing loss, since curved synapses are thought to be more active (Markus and Petit, 1989) and the loss of curvature of PSDs is correlated with deafness in cats and mice (Reed et al., 2000; Lee et al., 2003). These ultrastructural alterations of the AN-BC synapses correspond well with our findings of decreased ABR peak amplitudes and early onset of hearing loss in GluA3-KO mice. Both, decreased peak amplitude and hearing loss are correlated with auditory aging (Hunter and Willott, 1987; Sergeyenko et al., 2013). Given that the structural modifications in PSD size and number observed in the GluA3-KO mice (present study) are very similar to those found

in aging, and there is an age-dependent increase of GluA3 in the hippocampus of rats (Blair et al., 2013) and WT mice (Cantanelli et al., 2014), we propose that the presence of GluA3 is necessary to prevent premature age-related hearing loss. In humans, mutations of the *GRIA3* gene (encoding for GluA3) have been reported to occur naturally (Wu et al., 2007). However, the effect of *GRIA3* mutations in human hearing loss has not been studied.

GluA3 may also be critical for maintaining the ultrastructure of AN-BC synapses during postnatal development, since the AN-BC synapse undergoes ultrastructural modifications during the first post-natal month of age in mice and cats (Limb and Ryugo, 2000; Ryugo et al., 2006). We speculate that GluA3 promotes the assembly and/or trafficking of specific AMPAR subunits to the PSD. The absence of GluA2 subunit in mice results in the assembly of AMPARs with abnormal combinations of the remaining receptor subunits (Petrálie et al., 2004). Such AMPAR combinations are not efficiently trafficked to the synapse (Sans et al., 2003). Hence it is possible that in the AVCN of our GluA3-KO mice, the remaining AMPARs are not efficiently trafficked to the PSD. The functional implications in the AVCN are reflected in the slower sound evoked activity of the peak 2.

It has been shown that auditory perception is modified by variations within the ambient sound environment (Formby et al., 2003) and that the auditory system adapts to sound variations through plastic changes in the brainstem (Munro and Blount, 2009). We reasoned that the selective regulation of AMPAR subunits might be a mechanism underlying experience-dependent plasticity of the auditory brainstem. Support for this idea comes from evidence that transient sound reduction resulted in an up-regulation of GluA3 in the AN-BC synapse of the CN (Clarkson et al., 2016; Whiting et al., 2009). Then GluA3 may be the AMPAR subunit that is required for adaptive plasticity in the brainstem after partial sound reduction. Therefore, in this study we plugged one ear for ten days in young adult WT and GluA3-KO mice to promote reversible changes in auditory brainstem responses. Relatively few studies have followed the recovery of ABR threshold after non-invasive transient hearing deprivation and most of them address the outcome of sound deprivation during developmental sensitive periods (e.g. Mowery et al., 2015). In this study we followed the ABR threshold and peak amplitude recovery for up to 60 days after cessation of sound reduction to assess adaptive plasticity in auditory brainstem responses. We found that elevation of ABR threshold due to transient ear plugging is accompanied by decreased amplitude and increased latency of P1-P3. In WT mice, auditory thresholds recovered to normal by 20 days after cessation of sound reduction. In contrast, in GluA3-KO mice, thresholds recovered more slowly and incompletely, compared to those in WT mice. In addition, the increase of P1 and P2 amplitudes during the recovery period lasted for a longer time in the GluA3-KO as compared to WT. This indicates a more prolonged and hyper-responsive activity in auditory signaling in the absence of GluA3. Therefore, it is evident that the GluA3-containing AMPARs are required for the adaptive changes underlying the normal recovery of auditory signaling after transient sound reduction.

In summary, the present study demonstrates that GluA3-containing AMPARs are necessary for normal auditory processing, for maintaining the structural integrity of postsynaptic densities in the AN-BC synapse and for adaptive plastic changes after transient sound reduction.

Acknowledgments

This work was supported by the National Institutes of Deafness and Others Communication Disorders (R01DC013048, M.E.R.), the Pennsylvania Lions Hearing Research Foundation (M.E.R.) and by internal funding from the Department of Otolaryngology of the University of Pittsburgh (M.E.R.). We thank Megan Lambert and the Hillman Cancer Center Animal Facility for maintaining the GluA3-KO mice colony; Lester Torres and Minyoung Park for technical support; Drs. Ana M. Estrada-Sánchez, Karl Kandler and Steven J. Potashner for critical comments on this manuscript; Dr. Steven J. Potashner for editing the manuscript.

References

- Blair MG, Nguyen NN, Albani SH, L'Etoile MM, Andrawis MM, Owen LM, Oliveira RF, Johnson MW, Purvis DL, Sanders EM, Stoneham ET, Xu H, Dumas TC. Developmental changes in structural and functional properties of hippocampal AMPARs parallels the emergence of deliberative spatial navigation in juvenile rats. *J Neurosci*. 2013; 33(30):12218–12228. [PubMed: 23884930]
- Brawer JR, Morest DK. Relations between auditory nerve endings and cell types in the cat's anteroventral cochlear nucleus seen with the Golgi method and Nomarski optics. *J Comp Neurol*. 1975; 160(4):491–506. [PubMed: 1091667]
- Brugge, JF. An Overview of Central Auditory Processing. In: Popper, AN.; Fay, RR., editors. *The Mammalian Auditory Pathway: Neurophysiology*. Springer-Verlag; 1992. p. 1-33.
- Cant NB, Morest DK. The bushy cells in the anteroventral cochlear nucleus of the cat. A study with the electron microscope. *Neurosci*. 1979; 4:1925–1945.
- Cantaneli P, Sperduti S, Ciavardelli D, Stuppia L, Gatta V, Sensi SL. Age-Dependent Modifications of AMPA Receptor Subunit Expression Levels and Related Cognitive Effects in 3xTg-AD Mice. *Front Aging Neurosci*. 2014; 6(200):1–11. [PubMed: 24478697]
- Caras ML, Sanes DH. Sustained Perceptual Deficits from Transient Sensory Deprivation. *J Neurosci*. 2015; 35(30):10831–10842. [PubMed: 26224865]
- Chambers AR, Resnik J, Yuan Y, Whitton JP, Edge AS, Liberman MC, Polley DB. Central gain restores auditory processing following near-complete cochlear denervation. *Neuron*. 2016; 89(4):867–879. [PubMed: 26833137]
- Clarkson C, Antunes FM, Rubio ME. Conductive Hearing Loss Has Long-Lasting Structural and Molecular Effects on Presynaptic and Postsynaptic Structures of Auditory Nerve Synapses in the Cochlear Nucleus. *J Neurosci*. 2016; 36(39):10214–10227. [PubMed: 27683915]
- Fallon JB, Irvine DR, Shepherd RK. Cochlear implants and brain plasticity. *Hear Res*. 2008; 238(1–2):110–117. [PubMed: 17910997]
- Formby C, Sherlock LP, Gold SL. Adaptive plasticity of loudness induced by chronic attenuation and enhancement of the acoustic background. *J Acoust Soc Am*. 2003; 114(1):55–58. [PubMed: 12880017]
- Freche D, Pannasch U, Rouach N, Holcman D. Synapse geometry and receptor dynamics modulate synaptic strength. *PLoS One*. 2011; 6(10):e25122. [PubMed: 21984900]
- Frisina RD, Singh A, Bak M, Bozorg S, Seth R, Zhu X. F1 (CBA×C57) mice show superior hearing in old age relative to their parental strains: hybrid vigor or a new animal model for “golden ears”? *Neurobiol Aging*. 2011; 32(9):1716–1724. [PubMed: 19879021]
- Froemke RC, Schreiner CE. Synaptic plasticity as a cortical coding scheme. *Curr Opin Neurobiol*. 2015; 35:185–199. [PubMed: 26497430]
- Fuse T, Kanai Y, Kanai-Azuma M, Suzuki M, Nakamura K, Mori H, Hayashi Y, Mishina M. Conditional activation of RhoA suppresses the epithelial to mesenchymal transition at the primitive streak during mouse gastrulation. *Biochem Biophys Res Commun*. 2004; 318(3):665–672. [PubMed: 15144889]
- Gardner SM, Trussell LO, Oertel D. Time course and permeation of synaptic AMPA receptors in cochlear nuclear neurons correlate with input. *J Neurosci*. 1999; 19(20):8721–8729. [PubMed: 10516291]

- Geiger JR, Melcher T, Koh DS, Sakmann B, Seeburg PH, Jonas P, Monyer H. Relative abundance of subunit mRNAs determines gating and Ca²⁺ permeability of AMPA receptors in principal neurons and interneurons in rat CNS. *Neuron*. 1995; 15(1):193–204. [PubMed: 7619522]
- Gómez-Nieto R, Rubio ME. A bushy cell network in the rat ventral cochlear nucleus. *J Comp Neurol*. 2009; 516(4):241–263. [PubMed: 19634178]
- Huangfu M, Saunders JC. Auditory development in the mouse: structural maturation of the middle ear. *J Morphol*. 1983; 176(3):249–259. [PubMed: 6887258]
- Hunter KP, Willott JF. Aging and the auditory brainstem response in mice with severe or minimal presbycusis. *Hear Res*. 1987; 30(2–3):207–218. [PubMed: 3680066]
- Kandler K, Clause A, Noh J. Tonotopic reorganization of developing auditory brainstem circuits. *Nat Neurosci*. 2009; 12(6):711–717. [PubMed: 19471270]
- Koike-Tani M, Saitoh N, Takahashi T. Mechanisms underlying developmental speeding in AMPA-EPSC decay time at the calyx of Held. *J. Neurosci*. 2005; 25(1):199–207. [PubMed: 15634782]
- Kujawa SG, Liberman MC. Adding insult to injury: cochlear nerve degeneration after “temporary” noise-induced hearing loss. *J Neurosci*. 2009; 29(45):14077–14085. [PubMed: 19906956]
- Lauer AM, Connelly CJ, Graham H, Ryugo DK. Morphological characterization of bushy cells and their inputs in the laboratory mouse (*Mus musculus*) anteroventral cochlear nucleus. *PLoS One*. 2013; 8(8):e73308. 26. [PubMed: 23991186]
- Lee DJ, Cahill HB, Ryugo DK. Effects of congenital deafness in the cochlear nuclei of Shaker-2 mice: an ultrastructural analysis of synapse morphology in the endbulbs of Held. *J Neurocytol*. 2003; 32(3):229–243. [PubMed: 14724386]
- Limb CJ, Ryugo DK. Development of primary axosomatic endings in the anteroventral cochlear nucleus of mice. *J Assoc Res Otolaryngol*. 2000; 1(2):103–119. [PubMed: 11545139]
- Mehraei G, Hickox AE, Bharadwaj HM, Goldberg H, Verhulst S, Liberman MC, Shinn-Cunningham BG. Auditory Brainstem Response Latency in Noise as a Marker of Cochlear Synaptopathy. *J Neurosci*. 2016; 36(13):3755–3764. [PubMed: 27030760]
- Markus EJ, Petit TL. Synaptic structural plasticity: role of synaptic shape. *Synapse*. 1989; 3(1):1–11. [PubMed: 2645686]
- Melcher JR, Kiang NY. Generators of the brainstem auditory evoked potential in cat. III: Identified cell populations. *Hear Res*. 1996; 93(1–2):52–71. [PubMed: 8735068]
- Mishina M, Sakimura K. Conditional gene targeting on the pure C57BL/6 genetic background. *Neurosci. Res*. 2007; 58:105–112. [PubMed: 17298852]
- Mowery TM, Kotak VC, Sanes DH. Transient Hearing Loss Within a Critical Period Causes Persistent Changes to Cellular Properties in Adult Auditory Cortex. *Cereb Cortex*. 2015; 25(8):2083–2094. [PubMed: 24554724]
- Munro KJ, Blount J. Adaptive plasticity in brainstem of adult listeners following earplug-induced deprivation. *J Acoust Soc Am*. 2009; 126(2):568–571. [PubMed: 19640020]
- Nakamura K, Manabe T, Watanabe M, Mamiya T, Ichikawa R, Kiyama Y, Sanbo M, Yagi T, Inoue Y, Nabeshima T, Mori H, Mishina M. Enhancement of hippocampal LTP, reference memory and sensorimotor gating in mutant mice lacking a telencephalon-specific cell adhesion molecule. *Eur. J. Neurosci*. 2001; 13(179–189)
- Nicholson DA, Yoshida R, Berry RW, Gallagher M, Geinisman Y. Reduction in size of perforated postsynaptic densities in hippocampal axospinous synapses and age-related spatial learning impairments. *J Neurosci*. 2004; 24(35):7648–7653. [PubMed: 15342731]
- Parham, K.; Sun, XM.; Kim, DO. Noninvasive Assessment of Auditory Function in Mice: Auditory Brainstem Response and Distortion Product Otoacoustic Emissions. In: Willott, JF., editor. *Handbook of Mouse Auditory Research: From Behavior to Molecular Biology*. Boca Raton; 2001. p. 37–58.
- Petralia RS, Rubio ME, Wang YX, Wenthold RJ. Differential distribution of glutamate receptors in the cochlear nuclei. *Hear Res*. 2000; 147(1–2):59–69. [PubMed: 10962173]
- Petralia RS, Sans N, Wang YX, Vissel B, Chang K, Noben-Trauth K, Heinemann SF, Wenthold RJ. Loss of GLUR2 alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor subunit differentially affects remaining synaptic glutamate receptors in cerebellum and cochlear nuclei. *Eur J Neurosci*. 2004; 19(8):2017–2029. [PubMed: 15090029]

- Pilati N, Linley DM, Selvaskandan H, Uchitel O, Hennig MH, Kopp-Scheinpflug C, Forsythe ID. Acoustic trauma slows AMPA receptor-mediated EPSCs in the auditory brainstem, reducing GluA4 subunit expression as a mechanism to rescue binaural function. *J Physiol.* 2016; 594(13): 3683–3703. [PubMed: 27104476]
- Reed EE, Pongstaporn T, Ryugo DK. The effects of congenital deafness on auditory nerve synapses and globular bushy cells in cats. *Hear Res.* 2000; 147:160–174. [PubMed: 10962182]
- Ryugo DK, Montey KL, Wright AL, Bennett ML, Pongstaporn T. Postnatal development of a large auditory nerve terminal: the endbulb of Held in cats. *Hear Res.* 2006; 216–217:100–115.
- Sans N, Vissel B, Petralia RS, Wang YX, Chang K, Royle GA, Wang CY, O’Gorman S, Heinemann SF, Wenthold RJ. Aberrant formation of glutamate receptor complexes in hippocampal neurons of mice lacking the GluR2 AMPA receptor subunit. *J Neurosci.* 2003; 23(28):9367–9373. [PubMed: 14561864]
- Saunders, JC.; Crumling, MA. The Outer and Middle Ear. In: Willott, JF., editor. *Handbook of Mouse Auditory Research: From Behavior to Molecular Biology*. Boca Raton; 2001. p. 99–115.
- Schaette R, McAlpine D. Tinnitus with a normal audiogram: physiological evidence for hidden hearing loss and computational model. *J Neurosci.* 2011; 31(38):13452–13457. [PubMed: 21940438]
- Schmid S, Guthmann A, Ruppersberg JP, Herbert H. Expression of AMPA receptor subunit flip/flop splice variants in the rat auditory brainstem and inferior colliculus. *J Comp Neurol.* 2001; 430(2): 160–171. [PubMed: 11135253]
- Sergeyenko Y, Lall K, Liberman MC, Kujawa SG. Age-related cochlear synaptopathy: an early-onset contributor to auditory functional decline. *J Neurosci.* 2013; 33(34):13686–13694. [PubMed: 23966690]
- Shaheen LA, Valero MD, Liberman MC. Towards a Diagnosis of Cochlear Neuropathy with Envelope Following Responses. *J Assoc Res Otolaryngol.* 2015; 16(6):727–745. 2015. [PubMed: 26323349]
- Sheng M, Hoogenraad CC. The postsynaptic architecture of excitatory synapses: a more quantitative view. *Annu Rev Biochem.* 2007; 76:823–847. [PubMed: 17243894]
- Song I, Huganir RL. Regulation of AMPA receptors during synaptic plasticity. *Trends Neurosci.* 2002; 25(11):578–588. [PubMed: 12392933]
- Syka J. Plastic changes in the central auditory system after hearing loss, restoration of function, and during learning. *Physiol Rev.* 2002; 82(3):601–636. [PubMed: 12087130]
- Tolbert LP, Morest DK. The neuronal architecture of the anteroventral cochlear nucleus of the cat in the region of the cochlear nerve root: electron microscopy. *Neuroscience.* 1982; 7(12):3053–3067. [PubMed: 7162626]
- Trettel, J.; Morest, DK. Cytoarchitectonic Atlas of the Cochlear Nucleus of the Mouse. In: Willott, JF., editor. *Handbook of Mouse Auditory Research: From Behavior to Molecular Biology*. Boca Raton; 2001. p. 279–296.
- Trussell LO. Synaptic mechanisms for coding timing in auditory neurons. *Annu Rev Physiol.* 1999; 61:477–496. [PubMed: 10099698]
- Wang H, Yin G, Rogers K, Miralles C, De Blas AL, Rubio ME. Monaural conductive hearing loss alters the expression of the GluA3 AMPA and glycine receptor $\alpha 1$ subunits in bushy and fusiform cells of the cochlear nucleus. *Neuroscience.* 2011; 199:438–451. [PubMed: 22044924]
- Wang YX, Wenthold RJ, Ottersen OP, Petralia RS. Endbulb synapses in the anteroventral cochlear nucleus express a specific subset of AMPA-type glutamate receptor subunits. *J Neurosci.* 1998; 18(3):1148–1160. [PubMed: 9437035]
- Whiting B, Moiseff A, Rubio ME. Cochlear nucleus neurons redistribute synaptic AMPA and glycine receptors in response to monaural conductive hearing loss. *Neuroscience.* 2009; 163(4):1264–1276. [PubMed: 19646510]
- Willott JF. Measurement of the auditory brainstem response (ABR) to study auditory sensitivity in mice. *Curr Protoc Neurosci.* 2006; 8 Unit8.21B.
- Wong TP, Marchese G, Casu MA, Ribeiro-da-Silva A, Cuello AC, De Koninck Y. Loss of presynaptic and postsynaptic structures is accompanied by compensatory increase in action potential-dependent synaptic input to layer V neocortical pyramidal neurons in aged rats. *J Neurosci.* 2000; 20(22):8596–8606. [PubMed: 11069968]

- Wu Y, Arai AC, Rumbaugh G, Srivastava AK, Turner G, Hayashi T, Suzuki E, Jiang Y, Zhang L, Rodriguez J, Boyle J, Tarpey P, Raymond FL, Nevelsteen J, Froyen G, Stratton M, Futreal A, Gecz J, Stevenson R, Schwartz CE, Valle D, Huganir RL, Wang T. Mutations in ionotropic AMPA receptor 3 alter channel properties and are associated with moderate cognitive impairment in humans. *Proc Natl Acad Sci.* 2007; 104(46):18163–18168. [PubMed: 17989220]
- Yang YM, Aitoubah J, Lauer AM, Nuriya M, Takamiya K, Jia Z, May BJ, Huganir RL, Wang LY. GluA4 is indispensable for driving fast neurotransmission across a high-fidelity central synapse. *J Physiol.* 2011; 589(17):4209–4227. [PubMed: 21690196]
- Zheng QY, Johnson KR, Erway LC. Assessment of hearing in 80 inbred strains of mice by ABR threshold analyses. *Hear Res.* 1999; 130(1–2):94–107. [PubMed: 10320101]

Highlights

- The lack of GluA3 leads to decreased ABR peak amplitudes, increased latency of peak 2 and early onset hearing loss.
- The number and size of PSDs in the synapse of the auditory nerve on bushy cells is reduced in GluA3-KO mice.
- The absence of GluA3 hinders ABR threshold recovery after transient sound reduction.

Figure 1. Production of GluA3 (*Gria3*-flox) knockout mice

A, Schematic representation of *Gria3* genomic DNA (*Gria3*⁺) and targeted genomes (*Gria3*^{flox}). The closed boxes indicate the coding exons. The filled ovals in the targeted genomes delineate the 5' and 3' termini of the targeting vectors. The blue and red bars indicate probe regions (5', neo and 3') for Southern blot analysis. Two *flp* sequences (semicircles) are attached to remove the neomycin resistant gene (Neo). Triangles indicate *loxP* sequences. pA, polyadenylation signal sequence. **B**, Southern blot analysis of genomic DNA prepared from wild-type ES cells (C57BL/6) and targeted clone (No. 183-J13). Positions of DNA size markers (kb) are indicated to the left. **C**, PCR of genomic DNA from WT, heterozygote (Het) and homozygote (KO) mice. **D**, Western blot from WT and GluA3-KO brain tissue. The corresponding 100 kDa band of GluA3 is absent in the GluA3-KO mice. Actin (42 kDa) is observed in both WT and GluA3-KO.

Figure 3. Elevation of ABR threshold due to transient sound reduction is reversible in WT but not in GluA3-KO mice

A, Experimental design (see methods for details) for data shown in 2B-2F and in Figure 3. **B**, Mean ABR thresholds ($\pm$ SEM) for both WT and GluA3-KO mice immediately before ear plug (EP) removal are significantly elevated with respect to age-matched controls (WT: $F_{(1,35)}=426.80$, $p<0.0001$; GluA3-KO: $F_{(1,40)}=528.85$, $p<0.0001$). **C**, 10 days after EP removal, thresholds remain elevated in the GluA3-KO ($F_{(1,40)}=91.38$, $p<0.0001$) and, in the WT ($F_{(1,35)}=25.49$, $p<0.0001$), when ABRs are elicited with clicks and 8 kHz tones. **D**, 20

days after EP removal, thresholds in the WT recovered to normal levels ($F_{(1,35)}=3.72$, $p=0.062$), while those in the GluA3-KO remain elevated for ABRs elicited by clicks and 8 kHz tones ($F_{(1,40)}=22.45$, $p<0.0001$). **E**, 60 days after EP removal most thresholds have recovered to normal levels, except for those in the GluA3-KO elicited with clicks (WT: $F_{(1,35)}=0.32$, $p=0.576$; GluA3-KO: $F_{(1,40)}=17.76$, $p<0.0001$). **F**, Summary of recovery of thresholds in response to clicks; broken lines correspond to threshold values from age-matched controls. Asterisks in **B–E** indicate significant differences between ear-plugged and age-matched control mice of the same genotype at the given stimulus (Bonferroni post hoc test: * $p<0.05$, ** $p<0.01$, *** $p<0.001$).

Figure 4. Sound-evoked activity in the brainstem is altered after transient sound reduction in the absence of GluA3

P1 and P2 in the GluA3-KO are hypersensitive to ABR stimuli after transient sound reduction. **A**, mean amplitudes ($\pm$ SEM) of P1-P3 elicited with clicks at 80 dB are significantly reduced with the EP inserted in the WT ($F_{(1,24)}=61.17$, $p<0.0001$) and the GluA3-KO ($F_{(1,24)}=61.73$, $p<0.0001$). **B**, latencies are significantly increased in both genotypes (WT: $F_{(1,24)}=170.82$, $p<0.0001$; KO: $F_{(1,24)}=319.77$, $p<0.0001$) as compared to respective genotype age-matched controls. **C–E**, 10 days after EP removal, P1 and P3

amplitudes in the GluA3-KO are similar to those of controls, except in P1 at the 40 dB stimulus level (P1: $F_{(1,12)}=11.41$, $p=0.005$; P3: $F_{(1,12)}=1.7$, $F_{(1,12)}=0.216$). P2 amplitudes in the ear plugged GluA3-KO are similar to controls ($F_{(1,12)}=3.21$, $p=0.098$), except at the intensity of 80 dB ($F_{(2,12)}=4.39$, $p=0.037$), hence there is interaction between variables ($F_{(1,12)}=5.96$, $p=0.016$). In the WT, P3 is significantly increased ($F_{(1,15)}=13.66$, $p=0.002$), as compared to controls. **F–H**, 20 days after EP removal, amplitudes of P1 and P2 in the GluA3-KO are significantly increased (P1: $F_{(1,27)}=9.15$, $p=0.0054$; P2: $F_{(1,21)}=21.74$, $p=0.0001$; P3: $F_{(1,18)}=1.84$, $p=0.191$), while those in the WT are similar to age- matched controls (P1: $F_{(1,27)}=2.00$, $p=0.169$; P2: $F_{(1,27)}=0.69$, $p=0.412$; P3: $F_{(1,27)}=0.00$, $p=0.982$). **I–K**, 60 days after EP removal P1 in the GluA3-KO remains elevated at the 60 dB stimulus level ($F_{(1,15)}=13.13$, $p=0.0025$); P2 and P3 are similar to controls. In the WT, P1-P2 are similar to controls. In all panels, asterisks denote significant differences between ear-plugged and age-matched control mice of the same genotype at the given stimulus (Bonferroni post hoc test: * $p<0.05$, ** $p<0.01$).

Figure 5. ABR thresholds in GluA3-KO mice are prematurely elevated

A, Mean ABR thresholds ($\pm$ SEM) in WT and GluA3-KO mice are similar at the age of 30 days. **B**, ABR thresholds in the GluA3-KO mice are elevated with respect to WT at the age of 100 days ($F_{(1,25)}=52.23$, $p<0.0001$) for 8, 16 and 24 kHz frequencies ($F_{(4,25)}=82.37$, $p<0.0001$). Asterisks denote differences between ages at the given stimulus (Bonferroni post hoc test: (** $p<0.01$, *** $p<0.001$).

Figure 6. Lack of GluA3 affects the number and structure of AN-BC synapses

A and B: Fine structure of AN terminals on the cell body of one bushy cell (BC) of the AVCN of a WT (A) and a GluA3-KO (B) mouse. Insets show details of one PSD (boxed area) from the WT (A) and the GluA3-KO (B). Asterisks indicate PSDs; d, desmosomes. Scale bars: A and B, 0.5 μm ; insets, 1 μm . **C,** Mean ($\pm$ SEM) of AN ending profile area is similar between the WT (n=121) and the GluA3-KO (n=135). **D,** Mean ($\pm$ SEM) number of PSDs per profile is significantly reduced in the GluA3-KO (n=102) as compared to the WT (n=137). **E,** Mean ($\pm$ SEM) PSD length is significantly reduced in the GluA3-KO (n=102) as

compared to the WT (n=137). **F**, The percentage of dome-shaped PSDs is significantly reduced in the GluA3-KO (n=102) as compared to the WT (n=137). Data for each genotype were compared using Student's t test. Asterisks denote differences between genotypes (* $p < 0.05$, *** $p < 0.001$; ns, not significant).