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Research paper

Deletion of *Shank1* has minimal effects on the molecular composition and function of glutamatergic afferent postsynapses in the mouse inner ear

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ABSTRACT

Shank proteins (1–3) are considered the master organizers of glutamatergic postsynaptic densities in the central nervous system, and the genetic deletion of either Shank1, 2, or 3 results in altered composition, form, and strength of glutamatergic postsynapses. To investigate the contribution of Shank proteins to glutamatergic afferent synapses of the inner ear and especially cochlea, we used immunofluorescence and quantitative real time PCR to determine the expression of Shank1, 2, and 3 in the cochlea. Because we found evidence for expression of Shank1 but not 2 and 3, we investigated the morphology, composition, and function of afferent postsynaptic densities from defined tonotopic regions in the cochlea of Shank1$^{-/-}$ mice. Using immunofluorescence, we identified subtle changes in the morphology and composition (but not number and localization) of cochlear afferent postsynaptic densities at the lower frequency region (8 kHz) in Shank1$^{-/-}$ mice compared to Shank1$^{+/+}$ littermates. However, we detected no differences in auditory brainstem responses at matching or higher frequencies. We also identified Shank1 in the vestibular afferent postsynaptic densities, but detected no differences in vestibular sensory evoked potentials in Shank1$^{-/-}$ mice compared to Shank1$^{+/+}$ littermates. This work suggests that Shank proteins play a different role in the development and maintenance of glutamatergic afferent synapses in the inner ear compared to the central nervous system.

1. Introduction

Excitatory glutamatergic transmission in the cochlea occurs between the sensory inner hair cells (IHCs) and their afferent fibers and relies on specialized multi-molecular, pre- and postsynaptic structures. Presynaptic ribbons are electron-dense structures that tether glutamate-filled synaptic vesicles and enable multi-vesicular release (see recent review by Safieddine et al., 2012). Glutamate activates postsynaptic AMPA receptors (Glowatzki and Fuchs, 2002) that are part of postsynaptic densities (PSDs) on the afferent dendrites (Meyer et al., 2009). Although recent work has contributed greatly to our understanding of the molecular components that shape release from the hair cell ribbons, we know considerably less about how the molecular organization of the PSD shapes afferent responses.

PSDs of the cochlear afferent dendrites are morphologically (Nouvian et al., 2006) and molecularly (Davies et al., 2001) similar to glutamatergic PSDs found in the central nervous system (CNS; reviewed in Sheng and Hoogenraad, 2007): they are electron dense, oppose presynaptic structures, and contain similar proteins, including a variety of glutamate receptor subtypes and canonical postsynaptic density (PSD) proteins. These PSD proteins include signaling and scaffolding proteins that have long been appreciated to shape AMPA receptor expression (including localization, recruitment, and recycling) and, thereby, the diversity of responses

Abbreviations: IHC, inner hair cell; AMPAR, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor; PSD, postsynaptic density; CNS, central nervous system; ABR, auditory brainstem response; VsEPs, vestibular evoked potentials; CTBP2, C-terminal-binding protein 2; DIC, differential interference contrast; GKAP, guanylate kinase-associated protein

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seen in glutamatergic synapses of the CNS (reviewed in Sager et al., 2009). Like central glutamatergic synapses, across mammals, auditory afferents, even those contacted by the same hair cell, can display enormous diversity in their responses, including differences in thresholds and spontaneous firing rates (Liberman, 1982; el Barbary, 1991; Tsuji and Liberman, 1997; Taberner and Liberman, 2005). Recent work identified differences in AMPA receptor expression that may contribute to differences in auditory nerve thresholds and spontaneous firing rates (Liberman et al., 2011). Moreover, auditory sensitivity *in vivo* has been shown to be regulated by reversible changes in surface AMPAR expression in the cochlea (Chen et al., 2007). These previous findings suggest that, as in the CNS, differences in PSD composition shape glutamatergic responses in the cochlea.

Of the variety of proteins comprising the PSD, Shank proteins (1–3) are found in nearly all glutamatergic synapses in the CNS and are considered the "master" organizers of the PSD (reviewed in Sheng and Kim, 2000). Shank proteins constitute a significant part of the overall protein content of the PSD and, via various protein–protein interaction and multimerization domains, link AMPA and other glutamate receptor subtypes to the cytoskeleton. In the CNS, shank proteins are also involved in the dynamic structural and molecular reorganization of dendritic spines (Sala et al., 2001). Knockout mice for Shank1 (Hung et al., 2008), 2 (Schmeisser et al., 2012) and 3 (Peca et al., 2011; Schmeisser et al., 2012) are viable and their molecular and behavioral phenotypes have been examined. Compared to wild type mice, Shank1 knockout mice display altered molecular composition of postsynaptic density proteins, reduced number and size of dendritic spines and thinner PSDs, and decreased AMPA receptor-mediated synaptic strength (Hung et al., 2008). Since comparable synaptopathies are observed in Shank2$^{-/-}$ and Shank3$^{-/-}$ mice, there is likely only partial redundancy in the function of Shank family members.

These observations from the CNS coupled with the recent identification by immunofluorescence of Shank1 in the afferent PSDs of the developing cochlea (Huang et al., 2012), led us to hypothesize that Shank proteins are also essential components of cochlear afferent PSDs and that the absence of Shank proteins would disrupt the structural and molecular organization of the PSD and result in auditory deficits. To investigate this hypothesis, we examined the expression of Shank1, 2, and 3 in the cochlear inner ear by both immunofluorescence and quantitative real time PCR (qPCR). Because we identified only Shank1 in the cochlear inner ear, we then examined for changes in afferent synaptic organization and function in Shank1$^{-/-}$ mice, which presumably lack all known Shank isoforms. To our surprise we observed only subtle changes in the morphology and composition of IHC afferent PSDs and no changes in auditory brainstem responses (ABRs) in Shank1$^{-/-}$ mice compared to Shank1$^{+/+}$ littermates. Similarly, there was no observed deficit in the vestibular function of Shank1$^{-/-}$ mice compared to Shank1$^{+/+}$ littermates.

2. Materials and methods

2.1. Animals

All experimental procedures were carried out in accordance with the Institutional Animal Care and Use Committees (IACUCs) at both the University of North Carolina Wilmington and the University of Nebraska Lincoln. C57BL/6 were used for initial experiments (Fig. 1) and were obtained from the The Jackson Laboratory. For all other experiments (Figs. 2-6), 129S4/SvJae Shank1^{tmShng}-heterozygous (Shank1$^{+/-}$) mice were obtained from The Jackson Laboratory. Homozygous wild type (Shank1$^{+/+}$) and knockout (Shank1$^{-/-}$) mice were obtained from crosses of heterozygous (Shank1$^{+/-}$) mouse matings. Genotyping was performed using a protocol described previously (Truett et al., 2000; Silverman et al., 2011). All data were collected from mice aged 4 weeks and from littermates originating from at least three different litters for each experimental condition.

2.2. Immunofluorescence of auditory and vestibular sensory epithelia

Mice were anaesthetized via halothane inhalation before being sacrificed. Auditory and vestibular sensory epithelia were isolated

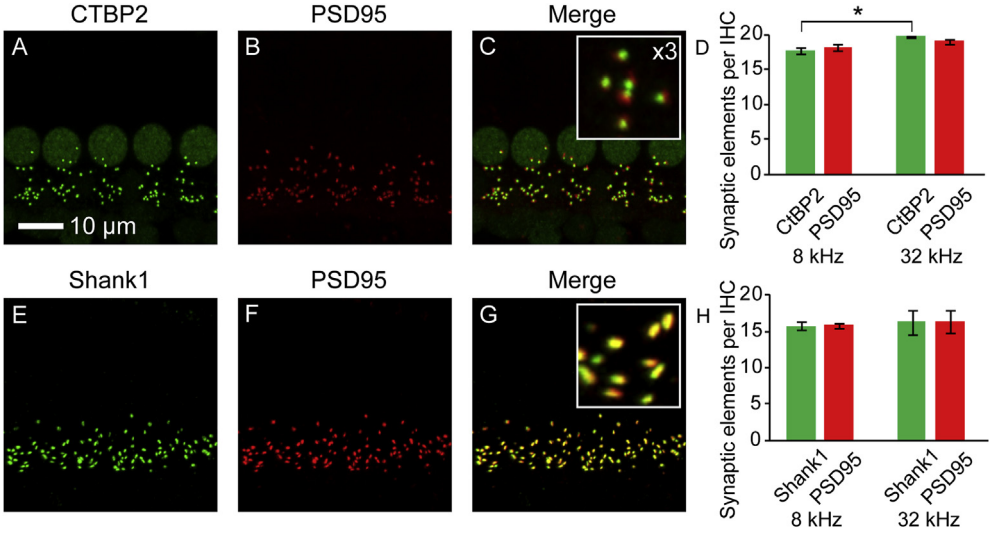

Fig. 1. Shank1 is a component of inner hair cell (IHC) afferent postsynaptic densities (PSD). Organs of Corti from 4 week old mice were immunostained with either a mouse monoclonal IgG1 antibody against CTBP2 (green, A) or rabbit polyclonal antibody against Shank1 (green, E) and a mouse monoclonal IgG2A antibody against PSD95 (red, B,F). Observations of individual samples revealed that almost every presynaptic CTBP2-positive ribbon was juxtaposed to a PSD95-positive PSD and vice versa (C) and that almost every PSD95-positive PSD also expressed Shank1 immunoreactivity and vice versa (G). Images are presented as Z-projections of a stack of confocal micrographs from the 32 kHz region. Mean values (±SEM) of CTBP2-positive presynaptic ribbons (D) or Shank1-positive PSDs (H) and PSD95-positive PSDs across samples are compared at two tonotopic regions (8 and 32 kHz). Statistically significant differences are indicated with an asterisk. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)