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Research Paper

The effect of progressive hearing loss on the morphology of endbulbs of Held and bushy cells

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ABSTRACT

Studies of congenital and early-onset deafness have demonstrated that an absence of peripheral sound-evoked activity in the auditory nerve causes pathological changes in central auditory structures. The aim of this study was to establish whether progressive acquired hearing loss could lead to similar brain changes that would degrade the precision of signal transmission. We used complementary physiologic hearing tests and microscopic techniques to study the combined effect of both magnitude and duration of hearing loss on one of the first auditory synapses in the brain, the endbulb of Held (EB), along with its bushy cell (BC) target in the anteroventral cochlear nucleus. We compared two hearing mouse strains (CBA/Ca and heterozygous *shaker-2*$^{+/-}$) against a model of early-onset progressive hearing loss (DBA/2) and a model of congenital deafness (homozygous *shaker-2*$^{-/-}$), examining each strain at 1, 3, and 6 months of age. Furthermore, we employed a frequency model of the mouse cochlear nucleus to constrain our analyses to regions most likely to exhibit graded changes in hearing function with time. No significant differences in the gross morphology of EB or BC structure were observed in 1-month-old animals, indicating uninterrupted development. However, in animals with hearing loss, both EBs and BCs exhibited a graded reduction in size that paralleled the hearing loss, with the most severe pathology seen in deaf 6-month-old *shaker-2*$^{-/-}$ mice. Ultrastructural pathologies associated with hearing loss were less dramatic: minor changes were observed in terminal size but mitochondrial fraction and postsynaptic densities remained relatively stable. These results indicate that acquired progressive hearing loss can have consequences on auditory brain structure, with prolonged loss leading to greater pathologies. Our findings suggest a role for early intervention with assistive devices in order to mitigate long-term pathology and loss of function.

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1. Introduction

The ability to detect, localize, and perceive sound relies on accurate connectivity, precise timing, and rapid transmission of neural signals throughout the brain. If any part of the auditory pathway is damaged, the result is an impaired perception of the acoustic environment. Sensorineural hearing loss is primarily caused by pathology in the inner ear affecting the sensory receptors or primary auditory neurons. One of the conundrums of hearing loss is the emergence of symptoms such as difficulty hearing sound in noise, loudness distortion, and tinnitus—issues that are not effectively remedied by sound amplification. There is growing evidence that these symptoms are not created in the ear per se but rather by reactive changes within the central nervous system. Indeed, plastic changes are frequently observed in the auditory system as sequelae to deafness (Kral et al., 2001; Ryugo et al., 1997, 1998; Shepherd and Hardie, 2001; Snyder et al., 2000; Tirko and Ryugo, 2012; West and Harrison, 1973). Deafness, however, lies at the far end of the hearing loss spectrum, establishing a worst-case benchmark of changes to auditory pathways resulting from long-term sensory deprivation. It is hypothesized that analogous but less severe brain changes would follow acquired progressive hearing loss.

The auditory nerve (AN) delivers signals from the cochlea to the brain, terminating throughout the cochlear nucleus (CN; Muniak et al., 2016). Thus, the AN is first to experience the consequences of hearing loss due to receptor damage, and any pathologies are likely to reverberate throughout the central auditory system. One

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Abbreviations

ABR	auditory brainstem response
AN	auditory nerve
AVCN	anteroventral cochlear nucleus
BC	bushy cell
CN	cochlear nucleus
DAB	3,3′-diaminobenzadine
DCN	dorsal cochlear nucleus
EB	endbulb of Held
EGFP	enhanced green fluorescent protein
EM	electron microscopy
G	glutaraldehyde
GFP	green fluorescent protein
GlyT2	glycine transporter 2
P	paraformaldehyde
PSD	postsynaptic density
PVCN	posteroventral cochlear nucleus
RMS	root mean square
SD	standard deviation
SEM	standard error of the mean
sh2	*shaker*-2
VCN	ventral cochlear nucleus

particular AN synaptic interface, the endbulb of Held (EB), along with its bushy cell (BC) target in the anteroventral CN (AVCN), has been the subject of extensive studies on deprivation-induced plasticity due to its prominent size and specializations for temporal coding. The EB is a large, complex axosomatic ending created by type I AN fibers (Ryugo and Spirou, 2009) that contains hundreds of active zones in contact with individual BCs (Nicol and Walmsley, 2002; Ryugo et al., 1996). This synaptic junction is designed to allow the auditory spike stream to be transferred from the EB to the BC at rapid rates and with high fidelity (Babalian et al., 2003; Manis and Marx, 1991; Pfeiffer, 1966).

Changes to auditory activity have been shown to affect EB and BC anatomy. When sound-evoked activity in the AN is completely blocked from birth, BCs become smaller (Lesperance et al., 1995; Pasic and Rubel, 1989; Saada et al., 1996; West and Harrison, 1973), and EBs atrophy, losing their complex arborizations (Limb and Ryugo, 2000; Ryugo et al., 1997, 1998; Wright et al., 2014; Youssoufian et al., 2008). In addition, the postsynaptic densities (PSDs) of EB terminals flatten and hypertrophy in congenitally deaf animals (Gulley et al., 1978; Lee et al., 2003; Ryugo et al., 1997). When some residual sound-evoked activity in the AN remains, as noted in a cohort of hard-of-hearing cats, EB structure takes an intermediate form between the large, complex EBs of normal hearing cats and the smaller, atrophied EBs found in congenitally deaf white cats (Ryugo et al., 1998). This observation suggests that EB integrity may be sensitive to graded variations in sound-evoked activity in the AN.

To characterize the effects of acquired progressive hearing loss on EB and BC structure, we utilized the DBA/2 mouse, a strain that exhibits early-onset sensorineural hearing loss. DBA/2 mice begin to display high-frequency hearing loss at 3–4 weeks of age, shortly after hearing onset (P11; Ehret, 1976) and just before sexual maturity (6–7 wks; Green and Witham, 1991); this loss progresses into adulthood to include the remaining mid-to-low frequencies (Henry and Haythorn, 1975; Hultcrantz and Spangberg, 1997; Johnson et al., 2000; Shin et al., 2010; Willott and Erway, 1998; Willott et al., 1984; Zheng et al., 1999). The pattern of hearing loss of the DBA/2 is closely paired to a progressive loss of inner and outer hair cells (Hultcrantz and Spangberg, 1997), and has been attributed to mutations in two separate proteins—cadherin-23 (Noben-Trauth et al., 2003) and fascin-2 (Shin et al., 2010)—associated with stereocilia stability and function. Changes in synaptic transmission have been observed at the EB-BC interface in older DBA/2 mice (Wang and Manis, 2005, 2006), suggesting structural alterations may be present. Use of this mouse allows us to isolate the effects of auditory threshold loss from the potential confound of senescence in studies of "age-related" hearing loss (Frisina and Walton, 2006), as the loss occurs at a relatively young age.

DBA/2 mice were examined at different age-points corresponding to different stages of progressive hearing loss. This approach was adopted in order to build a comprehensive profile of how EB and BC structure changes with varying degrees and durations of hearing loss. For normal hearing controls, we utilized age-matched CBA/Ca mice (Zheng et al., 1999). We also included congenitally deaf homozygous recessive *shaker-2* ($sh2^{-/-}$) mice (Lee et al., 2003; Limb and Ryugo, 2000; Probst et al., 1998), as well as their strain-matched hearing heterozygous $sh2^{+/-}$ counterparts to examine the influence of complete sound deprivation. Deafness in $sh2^{-/-}$ mice is caused by a mutation in myosin-15, another protein implicated in stereocilia and hair cell function (Liang et al., 1999; Probst et al., 1998).

We identified the frequency range that exhibited the greatest change in hearing thresholds with age in our mouse cohorts—the progression of hearing loss in the DBA/2 mouse is highly variable with frequency (Zheng et al., 1999)—and used a frequency model of the mouse CN (Muniak et al., 2013) to constrain our light and electron microscopic (EM) analyses of each mouse to the corresponding sub-region of its CN. We hypothesized that mice with a greater degree and longer duration of hearing loss would exhibit EB and BC morphology that more closely resembled that of congenitally deaf animals.

2. Methods

2.1. Animals

A total of 56 mice of either sex with CBA/Ca, DBA/2, and *shaker-2* backgrounds were used for this study (Tables 1–3). Mice were grouped into 1-, 3-, and 6-month-old cohorts. Subsets of these mice were transgenic animals expressing enhanced green fluorescent protein (EGFP) under the control of the glycine transporter 2 (GlyT2) gene promoter (Zeilhofer et al., 2005), and were used for EM analysis (GlyT2-EGFP mice; Table 3). Parent GlyT2-EGFP mice with a C57Bl/6 background were obtained from Prof. H.U. Zeilhofer (University of Zurich, Switzerland). CBA/Ca transgenic mice (CBGlyT2-EGFP), $sh2^{+/-}$ transgenic mice ($sh2^{+/-}$GlyT2-EGFP), and $sh2^{-/-}$ transgenic mice ($sh2^{-/-}$GlyT2-EGFP) were obtained by backcrossing the transgene into our mouse strains of interest for at least 10 generations. Genotyping of *sh2* and GlyT2-EGFP mice was completed in-house by the Garvan Molecular Genetics Department to verify hetero- and homozygosity as well as the presence of the transgene. To avoid possible ectopic expression of EGFP, only transgenic heterozygotes were used for analysis (Zeilhofer et al., 2005). Animals were housed in individually ventilated cages with ad libitum access to food and water. All procedures were performed in accordance with NHMRC guidelines and approved by the Animal Ethics Committee of the Garvan Institute of Medical Research and St. Vincent's Hospital, UNSW Australia.

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