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Glutamatergic Projections to the Cochlear Nucleus are Redistributed in Tinnitus

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Abstract—Tinnitus alters auditory–somatosensory plasticity in the cochlear nucleus (CN). Correspondingly, bimodal auditory–somatosensory stimulation treatment attenuates tinnitus, both in animals and humans (Marks et al., 2018). Therefore, we hypothesized that tinnitus is associated with altered somatosensory innervation of the CN. Here, we studied the expression of vesicular glutamate transporters 1 and 2 (VGLUT1 and VGLUT2) in the CN, which reveals glutamatergic projections from the cochlea as well as somatosensory systems to this brainstem auditory center. Guinea pigs were unilaterally exposed to narrowband noise and behaviorally tested for tinnitus using gap-prepulse inhibition of the acoustic startle. Following physiological and behavioral measures, brain sections were immunohistochemically stained for VGLUT1 or VGLUT2. Puncta density was determined for each region of the ipsilateral and contralateral CN. Tinnitus was associated with an ipsilateral upregulation of VGLUT2 puncta density in the granule cell domain (GCD) and anteroventral CN (AVCN). Furthermore, there was a tinnitus-associated interaural asymmetry for VGLUT1 expression in the AVCN and deep layer of the dorsal CN (DCN3), due to contralateral downregulation of VGLUT1 expression. These tinnitus-related glutamatergic imbalances were reversed upon bimodal stimulation treatment. Tinnitus-associated ipsilateral upregulation of VGLUT2-positive projections likely derives from somatosensory projections to the GCD and AVCN. This upregulation may underlie the neurophysiological hallmarks of tinnitus in the CN. Reversing the increased ipsilateral glutamatergic innervation in the CN is likely a key mechanism in treating tinnitus.

Key words: auditory, cross-modal compensation, noise exposure, synaptopathy, VGLUT1, VGLUT2.

INTRODUCTION

Tinnitus, or ringing in the ears, is defined as an auditory sensation in the absence of a corresponding external sound source and affects approximately 10–15% of the world's population (Bhatt et al., 2016; Shore et al., 2016). Tinnitus appears to be correlated with aberrant neural activity along the central auditory pathway, including in the cochlear nucleus (CN) (Eggermont and Roberts, 2015; Shore et al., 2016). In animal models of tinnitus, fusiform cells, the principal output neurons of the dorsal CN (DCN), show increased spontaneous firing rates, enhanced synchrony, and enhanced bursting (Brozoski et al., 2002; Kaltenbach et al., 2004; Dehmel et al., 2012; Wu et al., 2016a).

In addition to processing auditory information from the auditory nerve, the CN also receives input from other sensory modalities including brainstem nuclei of the somatosensory system, the spinal trigeminal nucleus (Sp5) and the cuneate nucleus (Zhou and Shore, 2004; Haenggeli et al., 2005; Zeng et al., 2011). Previous studies have shown that auditory–somatosensory plasticity in the DCN is altered following tinnitus (Dehmel et al., 2012; Koehler and Shore, 2013; Marks et al., 2018). This neural correlate of tinnitus is likely to be associated with "somatic tinnitus", in which tinnitus sufferers can modulate the loudness and pitch of their tinnitus by somatic maneuvers

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Abbreviations: ABR, auditory brainstem response; AVCN, anteroventral cochlear nucleus; CN, cochlear nucleus; DCN, dorsal cochlear nucleus; DCN1, molecular layer of the dorsal cochlear nucleus; DCN3, deep layer of the dorsal cochlear nucleus; ET, exposed tinnitus; ENT, exposed no tinnitus; ET_T, exposed tinnitus treated; GCD, granule cell domain; GI, gap index; GPIAS, gap-pre pulse inhibition of the acoustic startle reflex; icp, inferior cerebellar peduncle; N, sham-exposed control animals; PBS, phosphate-buffered saline; PFA, paraformaldehyde; PVCN, posteroventral cochlear nucleus; Sp5, spinal trigeminal nucleus; sp5, spinal trigeminal tract; TTS, temporary threshold shift; tz, trapezoid body; VCN, ventral cochlear nucleus; VGLUT1, vesicular glutamate transporter 1; VGLUT2, vesicular glutamate transporter 2.

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such as jaw clenching (Levine, 1999; Sanchez et al., 2002; Ostermann et al., 2016).

Projections from the somatosensory system to the CN terminate primarily in the granule cell domain (GCD), which encompasses regions that contain granule and small cells that surround the ventral CN (VCN) and form a layer between the molecular and deep layers of the DCN. Somatosensory projections terminate to a lesser extent in the magnocellular regions of the anteroventral CN (AVCN) and posteroventral CN (PVCN) and in the deep layer of the DCN (DCN3) (Zhou and Shore, 2004; Haenggeli et al., 2005; Zeng et al., 2011). The somatosensory-to-CN projection is glutamatergic (Haenggeli et al., 2005; Zhou et al., 2007; Zeng et al., 2012) and can be distinguished from auditory nerve glutamatergic projections by the subtype of the vesicular glutamate transporter (VGLUT), which mediates pre-synaptic uptake of glutamate into synaptic vesicles (Takamori et al., 2000, 2001; Fremeau et al., 2002). Type I auditory nerve fibers, the myelinated component of the primary sensory nerve, co-label exclusively with the subtype VGLUT1, whereas somatosensory projections primarily co-label with the subtype VGLUT2 and to a minor extent with VGLUT1 in normal-hearing animals (Zhou et al., 2007; Zeng et al., 2012). Thus, studying the distributions of glutamatergic markers, VGLUT1 and VGLUT2, across regions of the CN provides insight into the relative innervation of the CN by the auditory nerve and other, non-cochlear systems, including the somatosensory system.

Previous studies have shown that severe cochlear damage results in a redistribution of VGLUT subtypes in the CN: auditory nerve-associated VGLUT1 expression decreases and non-auditory nerve-associated VGLUT2 expression increases (Zeng et al., 2009; Barker et al., 2012; Heeringa et al., 2016). In particular, the increases in VGLUT2 expression following unilateral cochlear damage corresponds to an upregulation of somatosensory projections to the CN (Zeng et al., 2012). We hypothesized that similar cross-modal compensation in the CN contributes to altered auditory–somatosensory plasticity in tinnitus. Here, we studied VGLUT1 and VGLUT2 expression across CN regions in unilaterally noise-exposed animals with and without behavioral evidence of tinnitus. Tinnitus animals showed an ipsilateral upregulation of VGLUT2 puncta, possibly derived from somatosensory projections to the CN, which was reversed following bimodal auditory–somatosensory stimulation treatment that can reverse tinnitus in animals and humans (Marks et al., 2018).

EXPERIMENTAL PROCEDURES

Experimental set-up

Twenty-four adult pigmented guinea pigs of either sex (Elm Hill Laboratories, 2–3 weeks of age) were used in this study. Animals were socially housed and had *ad libitum* access to food and water. Animals were unilaterally noise- or sham-exposed twice, with a period of four weeks between exposures. To determine the presence of tinnitus, gap pre-pulse inhibition of the acoustic startle reflex (GPIAS) was assessed before and after noise exposures (Turner et al., 2006; Berger et al., 2013; Wu et al., 2016a). Following tinnitus assessment, a subset of tinnitus animals was treated with a custom-designed bimodal stimulation treatment to reverse tinnitus (Marks et al., 2018). For final data analysis, animals were divided into 4 groups: 6 sham-exposed control animals (N), 8 noise-exposed no-tinnitus animals (ENT), and 10 noise-exposed tinnitus animals (ET), of which 4 were treated with the bimodal stimulation paradigm (ET_T). *In vivo* neurophysiological recordings of DCN fusiform cells were performed 12 weeks following the last exposure after which the animals were transcardially perfused and cochleae and brains were collected for further processing. Neurophysiological results are described in previous reports (Wu et al., 2016a; Marks et al., 2018). All procedures were approved by the University's laboratory animal care and use committee, conformed to the NIH Guide for the Care and Use of Laboratory Animals, and followed the Society for Neuroscience's Guidelines for the Use of Animals in Neuroscience Research.

Noise exposure

Animals were anesthetized with ketamine (40 mg/kg, Putney) and xylazine (10 mg/kg, Lloyd) and were unilaterally exposed to the left ear with narrow-band noise (centered at 7 kHz, 0.4 octave bandwidth, 97 dB SPL for 2 h). The exposure was repeated after four weeks. Sham-exposed animals underwent the same procedures, without turning on the intense noise.

Auditory brainstem responses

Cochlear thresholds were determined by auditory brainstem responses (ABR) recorded before (t0), immediately after (t1), and pre-surgery (tf; see Fig. 3A) (Wu et al., 2016a). ABR stimuli (8 kHz, 12 kHz, and 16 kHz tone bursts, 0–90 dB SPL in 10 dB steps, 2 ms $\cos^2$ rise/fall times, 1024 repetitions, 30 Hz presentation rate) were presented using SigGenRP and BioSigRP (Tucker-Davis Technologies Inc. [TDT]). Subdermal electrodes were placed on the vertex and behind each pinna for reference, recording, and grounding, respectively. ABR waveforms were visually inspected for threshold, defined by the lowest stimulus level in which wave 4 (the largest wave) was clearly detectable. Wave 1 amplitude, representing auditory nerve firing (P1-N1), was determined for each stimulus level and frequency using a custom MatLab program.

Tinnitus assessment

The presence of tinnitus was determined using GPIAS as previously described (Turner et al., 2006; Koehler and Shore, 2013; Wu et al., 2016a; Marks et al., 2018). Briefly, the animal's startle reflex in response to a 20-ms, 95-dB SPL broadband noise pulse was measured by video tracking the pinna Preyer reflex (Berger et al., 2013; Wu et al., 2016a). A 50-ms silent gap in a 65-dB SPL constant background carrier (band limited at 8–10, 12–14, or 16–18 kHz) was presented 100 ms before the startle pulse.

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