Hearing Research 335 (2016) 94–104

Contents lists available at ScienceDirect

Hearing Research

journal homepage: www.elsevier.com/locate/heares

Research paper

Perinatal thiamine deficiency causes cochlear innervation abnormalities in mice

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A R T I C L E I N F O

Article history:
Received 28 September 2015
Received in revised form
3 February 2016
Accepted 12 February 2016
Available online 2 March 2016

Keywords:
Thiamine
Development
Auditory neuropathy
Olivocochlear

A B S T R A C T

Neonatal thiamine deficiency can cause auditory neuropathy in humans. To probe the underlying cochlear pathology, mice were maintained on a thiamine-free or low-thiamine diet during fetal development or early postnatal life. At postnatal ages from 18 days to 22 wks, cochlear function was tested and cochlear histopathology analyzed by plastic sections and cochlear epithelial whole-mounts immunostained for neuronal and synaptic markers. Although none of the thiamine-deprivation protocols resulted in any loss of hair cells or any obvious abnormalities in the non-sensory structures of the cochlear duct, all the experimental groups showed significant anomalies in the afferent or efferent innervation. Afferent synaptic counts in the inner and outer hair cell areas were reduced, as was the efferent innervation density in both the outer and inner hair cell areas. As expected for primary neural degeneration, the thresholds for distortion product otoacoustic emissions were not affected, and as expected for subtotal hair cell de-afferentation, the suprathreshold amplitudes of auditory brainstem responses were more affected than the response thresholds. We conclude that the auditory neuropathy from thiamine deprivation could be produced by loss of inner hair cell synapses.

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1. Introduction

Auditory neuropathy is a type of sensorineural hearing loss resulting from disorders affecting inner hair cells (IHCs), cochlear sensory neurons or the synapses between them (for review, see Starr and Rance, 2015). It can be congenital (Bahmad et al., 2007; Santarelli et al., 2009; Pangrsic et al., 2010) or acquired (Beutner et al., 2007), and it is diagnosed based on the presence of otoacoustic emissions despite an absent or highly disorganized auditory brainstem response (Hood, 2015). A number of risk factors have been identified. For example a high incidence of auditory neuropathy is found in infants born prematurely (Xoinis et al., 2007). Recently, a study of infant temporal bones revealed that 30% of preterm babies who died in the NICU had selective inner hair cell (IHC) loss, while only 3% of full-term infants presented such a phenotype (Amatuzzi et al., 2011). This high prevalence for such a rare histopathology (Schuknecht, 1993) suggested that selective IHC loss might be a common cause of auditory neuropathy.

Our group recently reported a link between IHC loss and thiamine, a water-soluble vitamin (B1) required for intermediary metabolism. Patients with the syndromic disorder known as Thiamine-Responsive Megaloblastic Anemia (TRMA) have hearing loss in addition to the pathognomonic anemic symptoms (Fleming et al., 1999, 2001). Genetic analysis revealed a mutation in one of the two transporters involved in trafficking thiamine across cell membranes: *SLC19A2*. Targeted deletion of *SLC19A2* in mice causes auditory neuropathy and selective IHC loss when animals are challenged with a diet low in thiamine (Liberman et al., 2006).

Further evidence for a link between thiamine and auditory neuropathy was provided by a study of children who were bottle-fed from birth with an infant formula completely lacking in thiamine due to a manufacturing error. As infants, these children were hospitalized with a variety of neurological symptoms consistent with thiamine deficiency, collectively known as Wernicke's encephalopathy (Fattal-Valevski et al., 2005). Continued follow-up showed an extraordinarily high percentage of auditory neuropathy (Attias et al., 2012).

Combining all these observations, we wondered if the link

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http://dx.doi.org/10.1016/j.heares.2016.02.006
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between prematurity and auditory neuropathy might reflect an immaturity of thiamine transporters in preterm infants coupled with varying standards for thiamine supplementation during NICU stays (Friel et al., 2001). To gain insight into the effects of thiamine deprivation in the perinatal period, we deprived mice of dietary thiamine during pregnancy and/or nursing. We let the offspring mature to varying ages, measured cochlear function by ABRs and DPOAEs and evaluated the degeneration of hair cells and their afferent and efferent innervation. We found no evidence for selective IHC loss; however, many thiamine-deprived animals showed dramatic abnormalities in the cochlear afferent and efferent innervation, and associated cochlear functional abnormalities consistent with the diagnosis of auditory neuropathy.

2. Materials and methods

2.1. Animals, procedure and statistical analysis

CBA/CaJ mice were used in all experiments. To control thiamine intake, a thiamine-free chow was obtained from TestDiet® (TestDiet.com) in powdered form. The formulation was identical, in all other respects, to that established for use in mice by the American Institute of Nutrition. The desired thiamine concentration was adjusted by addition of powdered thiamine diluted in water. The mixture was provided as a moist gruel.

Mice were assigned to one of three groups: 1) *Control* animals, which were fed with regular chow (22 mg/kg thiamine); 2) *Low-Thiamine* animals, in which thiamine-free chow was supplemented with 0.2 mg/kg of thiamine and 3) *No Thiamine* animals, which received a thiamine-free chow. For the last two groups, thiamine deprivation was either implemented pre-natally (*Pre*) or post-natally (*Post*) for durations ranging from 2 to 6 weeks (Fig. 1).

Cochlear function was assessed bilaterally in each mouse reaching at least 6 wks of age via auditory brainstem responses (ABRs) and distortion product otoacoustic emissions (DPOAEs). After the final cochlear function test, cochleas were fixed by intracardiac perfusion and removed for histological processing and light-microscopic analysis of cochlear degeneration. All procedures were approved by the IACUC of the Massachusetts Eye and Ear Infirmary. Two-way repeated-measure ANOVAs, adjusted with the Holm-Bonferroni correction, were used to assess the statistical significance of group differences.

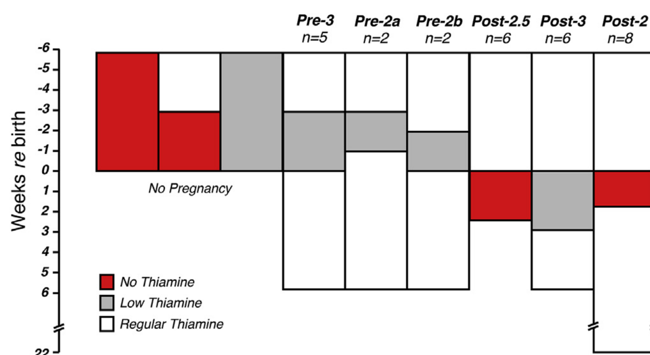

Fig. 1. Schematic of the thiamine diet protocols. Mice were assigned to one of three groups: 1) Control animals, fed with regular chow (22 mg/kg thiamine); 2) Low-thiamine animals, fed with 0.2 mg/kg thiamine and 3) No-thiamine animals, fed with thiamine-free chow. Thiamine restriction was either applied prenatally (***Pre***) or postnatally (***Post***) for durations ranging from 2 to 6 wks. Females on a thiamine-free diet for ≥3 wks failed to become pregnant. On a low-thiamine diet, pregnancies went to full term if diet durations were ≤3 wks. Pups fed by mothers on a thiamine-free diet survived only if thiamine restriction was ≤18 days. Group sizes (n's) are indicated as number of ears evaluated.

2.2. Cochlear function tests

For measuring ABRs and DPOAEs, animals were anesthetized with xylazine (20 mg/kg, i.p.) and ketamine (100 mg/kg, i.p.) and placed in an acoustically and electrically shielded room maintained at 32 °C. Acoustic stimuli were delivered through a custom acoustic system consisting of two miniature dynamic earphones used as sound sources (CUI CDMG15008-03A) and an electret condenser microphone (Knowles FG-23329-PO7) coupled to a probe tube to measure sound pressure near the eardrum.

Digital stimulus generation and response processing were handled by digital I-O boards from National Instruments driven by custom LabVIEW software. For ABRs, stimuli were 5-msec tone pips (0.5 msec $\cos^2$ rise-fall) delivered in alternating polarity at 35/sec. Electrical responses were sampled via Grass needle electrodes at the vertex and pinna with a ground reference near the tail and amplified 10,000× with a 0.3–3 kHz passband. Responses to as many as 1024 stimuli were averaged at each sound pressure level, as level was varied in 5 dB steps from below threshold up to 80 dB SPL. For DPOAEs, stimuli were two primary tones f_1 and f_2 ($f_2/f_1 = 1.2$), with f_1 level always 10 dB above f_2 level. Primaries were swept in 5 dB steps from 20 to 80 dB SPL (for f_2). The DPOAE at $2f_1$-f_2 was extracted from the ear canal sound pressure after both waveform and spectral averaging. Noise floor was defined as the average of 6 spectral points below, and 6 above, the $2f_1$-f_2 point. Threshold was computed, by interpolation, as the primary level (f_2) required to produce a DPOAE of 0 dB SPL.

2.3. Cochlear processing, immunostaining and histological analysis

Mice were perfused intracardially with 4% paraformaldehyde in phosphate buffer. Cochleas were processed in one of two ways. For evaluation of all structures of the cochlear duct, ears were post-fixed in osmium tetroxide (1%) and then decalcified, plastic-embedded and serially sectioned at 40 microns (Wang et al., 2002). For hair cell counts and innervation analysis, cochleas were decalcified, dissected into half-turns and permeabilized by freeze/thawing. The half-turns were blocked in 5% normal horse serum (NHS) with 0.3% Triton X-100 (TX) in PBS for 1 h, followed by incubation for ~19 h at 37 °C in primary antibodies diluted in 1% NHS with 0.3% TritonX. Primary antibodies included 1) mouse (IgG1) anti-CtBP2 (BD Biosciences #612044) at 1:200 to visualize presynaptic ribbons, 2) mouse (IgG2a) anti-GluA2 (Millipore #MAB397) at 1:2000 to visualize postsynaptic glutamate receptor patches, and 3) rabbit anti-VAT (Vesicular Acetylcholine Transporter; Abcam #ab68986) at 1:200 to allow quantification cochlear efferent terminals. The vast majority of efferent fibers are cholinergic and therefore VAT-positive. We did not immunostain the small dopaminergic population (Darrow et al., 2006) because it is very sparse, and therefore difficult to assess. Primary incubations were followed by two sequential 60-min incubations at 37 °C in species-appropriate secondary antibodies with 0.3% TritonX.

Histological analysis of cochlear epithelial wholemounts was based on high-power confocal z-stacks of the sensory epithelium obtained at half-octave intervals along the cochlea from 5.6 to 64 kHz. To identify regions of interest, cochlear lengths were obtained for each case by tracing low-power images of the dissected pieces using a custom ImageJ plug-in that translates cochlear position into frequency using the mouse frequency map (Taberner and Liberman, 2005). Confocal z-stacks were obtained with a glycerol-immersion objective (63×, numerical aperture = 1.3) at 3.17× digital zoom on a Leica TCS SP5. Image spacing in the z plane was 0.25 μm, and the z-span was adjusted for each stack to include all synaptic elements in all of the 9–12 hair cells from each row

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