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The FBN rat model of aging: investigation of ABR waveforms and ribbon synapse changes

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ABSTRACT

Age-related hearing loss is experienced by one-third of individuals aged 65 years and older and can be socially debilitating. Historically, there has been poor correlation between age-related threshold changes, loss of speech understanding, and loss of cochlear hair cells. We examined changes in ribbon synapse number at four different ages in Fisher Brown Norway rats, an extensively studied rat model of aging. In contrast to previous work in mice/Wistar rats, we found minimal ribbon synapse loss before 20 months, with significant differences in 24- and 28-month-old rats at 4 kHz. Significant outer HC loss was observed at 24 and 28 months in low- to mid-frequency regions. Age-related reductions in auditory brainstem response wave I amplitude and increases in threshold were strongly correlated with ribbon synapse loss. Wave V/I ratios increased across age for click, 2, 4, and 24 kHz. Together, we find that ribbon synapses in the Fisher Brown Norway rat cochlea show resistance to aging until ~60% of their life span, suggesting species/strain differences may underpin decreased peripheral input into the aging central processor.

1. Introduction

Presbycusis, or age-related hearing loss, is arguably the third most common malady of industrialized populations. It is a complex, multifaceted condition involving primary changes to the auditory periphery and maladaptive, compensatory changes in the central auditory pathway. Epidemiologic studies have shown that the prevalence of hearing loss in the United States (US) doubles every decade of life, affecting 30% of the US population aged 65–74 years and 50% of the population over 75 years of age (Lin et al., 2011a). US census data suggest that the population of those over 65 will grow from 14% in 2014 to near 21% by 2040 (https://aoa.acl.gov/), dramatically increasing the number of patients suffering from age-related hearing loss. Loss of speech understanding resulting from presbycusis is socially debilitating and can lead to isolation and depression (Dalton et al., 2003).

The peripheral auditory system, housed in the cochlea of the inner ear, contains outer hair cells (OHCs) and inner hair cells (IHCs), which convert sound waves into electrical signals. IHCs are connected to type I spiral ganglion neurons (SGNs) by ribbon synapses (also known as IHC-SGN synapses), which are a unique glutamatergic synapse found in hair cells (HCs), retinal photoreceptors, and pinealocytes (for review Matthews and Fuchs, 2010). In the mammalian cochlea, each type I SGN synapses with 1 IHC (Kiang et al., 1982; Liberman, 1982; Spoendlin, 1969), but each IHC is connected to 10–30 SGNs by a ribbon synapse (Bohne et al., 1982; Liberman, 1980; Stamataki et al., 2006). Within each synapse, the ribbon complex is made of proteins such as ribeye, basson, and C-terminal binding protein 2 (Ctbp2) and allows a large number of vesicles to dock at the presynaptic terminal to facilitate rapid release of glutamate in response to Ca^{2+} influx (reviewed in Moser et al., 2006). On the postsynaptic side, bipolar SGNs, whose dendrites are unbranched, express α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors (Glowatzki and Fuchs, 2002; Matsubara et al., 1996; Safieddine and Eybalin, 1992). Loss of IHC-SGN synapses, caused by aging, toxic drugs, or acoustic trauma, reduces spontaneous and driven excitatory input to the central auditory nervous system (CANS) resulting in complex, compensatory plastic changes that are frequently characterized by a downregulation of glycinergic and GABAergic

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inhibition (Auerbach et al., 2014; Caspary et al., 2008; Gold and Bajo, 2014; Roberts et al., 2010). In a normal, young adult cochlea, the majority (>95%) of Ctbp2-labeled presynaptic regions overlap with the postsynaptic density on axon terminals of SGNs that express α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors containing the GluR2 subunit (Furman et al., 2013; Matsubara et al., 1996; Sergeyenko et al., 2013).

The auditory brainstem response (ABR) is an evoked far-field potential elicited by acoustic stimuli, whose sequential waves reflect acoustic transmission from the acoustic nerve to precollicular or collicular auditory structures (Buchwald and Huang, 1975; Starr and Hamilton, 1976). Tonotopic organization is a shared feature among most CANS structures, reflecting the cochlea's frequency-specific place map, with responses to higher frequencies at the base and lower frequencies at the apex (Greenwood, 1990; Muller, 1991). ABR responses to pure-tone and click stimuli have historically been used in human and animals to assess acoustic thresholds, with responses to click stimuli thought to most closely approximate behavioral thresholds in human subjects (Frisina et al., 2016; Gorga et al., 1985; Jerger and Mauldin, 1978; Williamson et al., 2015; van der Drift et al., 1987).

Historically, auditory research has found poor correlations between age-related loss of speech understanding, age-related changes in pure-tone auditory thresholds, and age-related loss of cochlear HCs (Starr et al., 1996). Hearing threshold sensitivity, as measured by pure tones, is also not directly affected by degeneration of SGNs when scattered across the cochlea (Schuknecht and Woellner, 1953, 1955). However, recent studies suggest that loss of ribbon synapses connecting IHCs and SGNs may be a more salient marker for functional auditory losses (Kujawa and Liberman, 2009). Thus, age-related loss of IHC-SGN synapses, which decreases excitatory auditory input to the brain, may be a critical factor signaling CANS compensatory changes, where in an attempt to "re-up" the gain, inhibitory neurotransmission is selectively downregulated (Auerbach et al., 2014; Caspary et al., 2008; Gold and Bajo, 2014; Roberts et al., 2010). Studies in CBA/CaJ and UM-HET4 mice, as well as Wistar rats, have shown that age-related loss of IHC-SGN synapses could be detected many weeks before there were changes in auditory thresholds measured by ABR (Altschuler et al., 2015; Mohrle et al., 2016; Sergeyenko et al., 2013).

The present study correlated age-related IHC-SGN synapse changes with evoked auditory potential changes from the CANS of Fisher Brown Norway (FBN) rats. In 1994, the National Institute on Aging (NIA) recommended the FBN strain as a superior model for aging research. FBN rats are a F1 hybrid of Fischer 344 (F344) and Brown Norway rats and have a longer median life span relative to mice and other rat strains. Specifically, FBN rats have a 50% longer median life span than F344 rats (34 vs. 25 months, respectively) with fewer pathologic lesions late in life (Lipman, 1997; Lipman et al., 1996). The FBN rat model has been extensively used to demonstrate age-related changes in inhibitory neurotransmission and temporal processing of acoustic information in central auditory structures (Caspary et al., 1999, 2005, 2006, 2008; de Villers-Sidani et al., 2010; Gold and Bajo, 2014; Hughes et al., 2010; Ling et al., 2005; Milbrandt and Caspary, 1995; Richardson et al., 2011, 2013; Schatteman et al., 2008; Turner and Caspary, 2005; Turner et al., 2005; Wang et al., 2009, 2011). Previous studies have shown that the FBN strain has severe presbycusis at low frequency regions with ~75% OHC loss observed in the apical turn and less than ~25% OHC loss in the high-frequency basal turn at 32 months (Caspary et al., 2008; Keithley et al., 1992). This differs from humans and other rodent strains, which have presbycusis in high frequency regions (Frisina and Frisina, 1997; Frisina and Walton, 2006; Willott, 1991).

The present study recorded ABR potentials from rats at 4 different ages (4–6 months, 20, 24, and 28 months) followed by analysis of HCs and IHC-SGN synapses in the same ears using methods similar to Sergeyenko et al. (2013).

2. Methods

2.1. Animals

Male FBN rats aged 4–6 months (n = 9), 20 (n=9), 24 (n=8), and 28 months (n=7) were obtained from the NIA aged rodent colony (Bethesda, MD, USA) and housed by Charles River Laboratories (Wilmington, MA, USA) before arriving at Southern Illinois University School of Medicine (SIUSOM). Ambient sound levels were measured by Charles River personnel with no information regarding frequency spectra provided. Sound pressure levels (SPLs) varied in rooms housing FBN rats at Charles River depending on the closeness of the racks to the washer and vacuum systems. With both systems off, ambient SPLs varied between 56 and 60 dB. A cage washer was on 5–6 h/d, while a vacuum system ran a total of 1–2 h/d in "spurts" of 20 minutes. SPLs with the washer alone were between 72 and 75 dB. With both systems on, SPLs at the nearest rack could reach as high as 81 dB. It is assumed that most of the energy was at low frequencies, not likely to damage rodent hearing. This was the case for SIUSOM animal facilities, which showed ambient, unweighted SPLs between 2.0 and 49 kHz at 39 dB. No energy above 30 dB was observed in the 1.0–2.0 kHz bin. A distant cage washer had little effect on background levels down to 1.0 kHz, but levels below 1.0 kHz could reach 79 dB with cage washer and air handler rumble. The SIUSOM animal rooms housing FBN rats were assessed using a Bruel & Kjaer (B&K, Norcross, GA, USA) pulse sound measurement system (Pulse 13.1 software) with a 3560C module and a B&K 4138 microphone. All animal studies were performed in accordance with approved animal protocols from the Institutional Animal Care and Use Committee at SIUSOM.

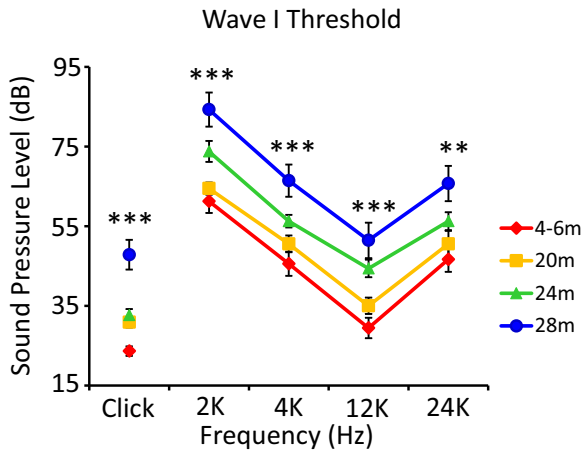

Fig. 1. ABR thresholds of FBN rats increased with age. All groups showed lowest threshold at 12 kHz. Significant differences were detected between groups for click (2-way ANOVA followed by Tukey's post hoc test, $p < 0.001$) and all frequencies tested ($p < 0.001$ for 2, 4, and 12 kHz; $p < 0.01$ for 24 kHz). N = 9 (4–6 months), 9 (20 months), 8 (24 months), and 7(28 months). Data are presented as mean ± standard error of the mean. $^{**}p < 0.01$; $^{***}p < 0.001$. Detailed between group comparisons (Table 1). Abbreviations: ABR, auditory brainstem response; ANOVA, analysis of variance; FBN, Fisher Brown Norway.

Table 1
Wave I threshold comparison among different age groups

Statistical method	*p*-value	Click	2K	4K	12K	24K
Paired comparison by Tukey's post hoc test	4–6 mo vs. 20 mo	0.055[c]	0.830	0.542	0.472	0.798
	4–6 mo vs. 24 mo	**0.015**[a]	**0.021**[a]	**0.044**[a]	**0.003**[b]	0.18
	4–6 mo vs. 28 mo	**0.001**[b]	**0.001**[b]	**0.001**[b]	**0.001**[b]	**0.002**[b]
	20 mo vs. 24 mo	0.899	0.105	0.463	0.101	0.595
	20 mo vs. 28 mo	**0.001**[b]	**0.001**[b]	**0.002**[b]	**0.002**[b]	**0.017**[a]
	24 mo vs. 28 mo	**0.001**[b]	0.078	0.084	0.346	0.234
ANOVA	Total	**F(3,29) = 24.12 4.96e-08**[d]	**F(3,28) = 12.46 2.342e-05**[d]	**F(3,29) = 9.95 0.0001**[d]	**F(3,29) = 11.71 3.375e-05**[d]	**F(3,29) = 5.96 0.0027**[b]

Two-way analysis of variance (ANOVA) followed by a Tukey's post hoc test. Bold values indicate the comparison reached statistical difference.
[a] $p < 0.05$.
[b] $p < 0.01$.
[c] *p*-value very close to statistical significant.
[d] $p < 0.001$.

2.2. Auditory brainstem response (ABR) measurements

Before ABR recordings, rats were anesthetized with an intramuscular injection of a 3:1 mixture of ketamine and xylazine at a dose of 105-mg/kg ketamine and 7-mg/kg xylazine (dose for 20- and 24-month rats were reduced by 10% and 28 months rats by 20%). Anesthesia depth was checked by heart rate and toe pinch. After reaching a stable anesthesia state (~220 pulses/min for 4–6 months rat, ~200 pulses/min for 20 and 24 months, and ~180 pulse/min for 28 months rat), the rat's head was shaved. One recording electrode was inserted into the skin at the vertex, with a second reference electrode inserted just under the mastoid of the left ear. A ground wire was attached to the hind leg. An electrostatic speaker (EC1, Tucker Davis Technologies [TDT] System III, Alachua, FL, USA) was fitted to a tube placed and fixed in the left ear canal. ABRs were recorded in a double-wall soundproof booth (Industrial Acoustic, Bronx, NY, USA).

Acoustic signals were generated using a 16-bit D/A converter (RX6, TDT System III), controlled by customized Auditory Neurophysiology Experiment Control Software (ANECS, Blue Hills Scientific, Boston, MA, USA). Clicks and pure tones for generating ABRs were delivered in 5 dB steps between 0 and 80 dB. Stimuli were clicks and 2, 4, 12, and 24 kHz pure tone bursts presented 512 times at a rate of 20/s, 3 ms duration with a 1 ms rise/fall

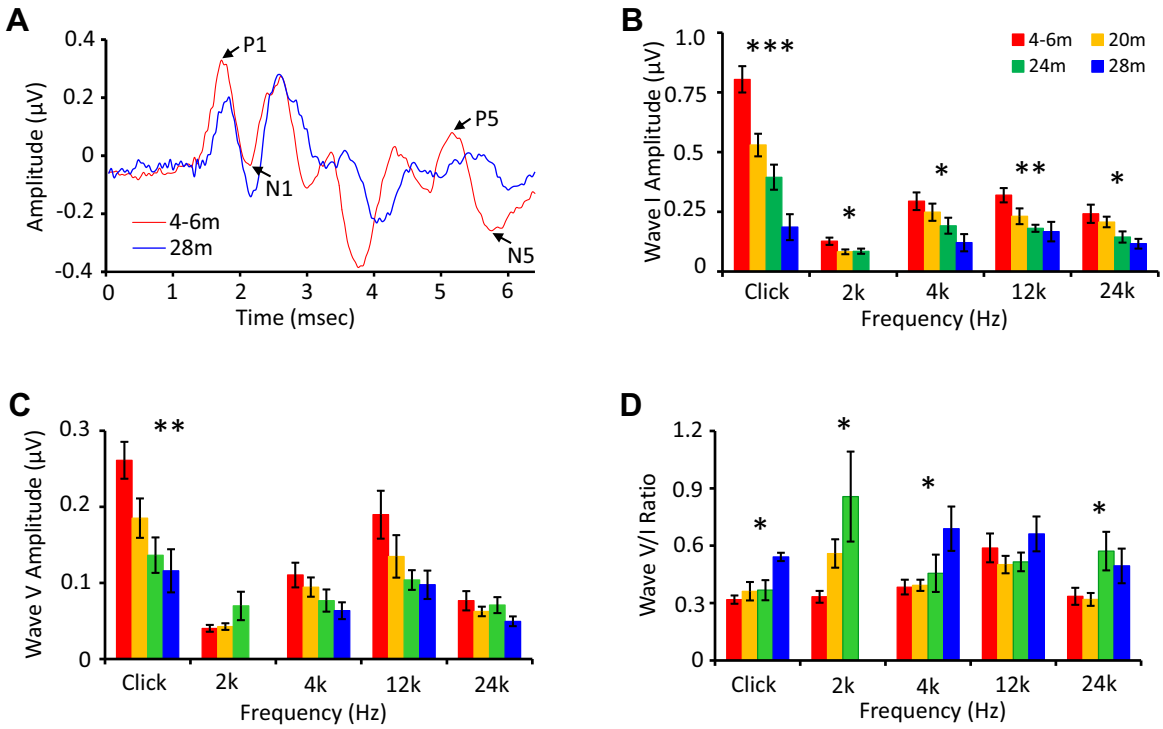

Fig. 2. ABR waveform amplitudes of FBN rats at 4 different ages. (A) Sample ABR waveforms elicited by 12 kHz at 80 dB from a young (4–6 months) and an aged (28 months) rat. Decreased amplitude of all waves was detected when comparing waveforms between young and aged rats. Arrows indicated the peak (P) and trough (N) measurements of wave I (P1, N1) and V (P5, N5) for the young animal waveform. (B and C) Wave I and V amplitudes from click, 2, 4, 12, and 24 kHz for the 4 age groups (no data from 28 months at 2 kHz presented due to the high threshold of the aged rats), respectively. There were significant age-related differences in wave I amplitudes for click ($p < 0.001$), 2 ($p < 0.05$), 4 ($p < 0.05$), 12 ($p < 0.01$), and 24 kHz ($p < 0.05$). Wave V amplitude remained relatively less changed except for the click-evoked response ($p < 0.01$). There was a $p = 0.05$ decrease at 12 kHz among 4 groups. (D) Wave V/I amplitude ratio for click, 2, 4, 12, and 24 kHz from the 4 age groups. Wave V/I ratio for 12 kHz showed no age-related change. There were significant age-related increases in the wave V/I ratios for click, 2, 4, and 24 kHz ($p < 0.05$). Data are presented as mean ± standard error of the mean. Two-way ANOVA followed by a Tukey's post hoc test, $^{*}p < 0.05$; $^{**}p < 0.01$; and $^{***}p < 0.001$. N = 9 (4–6 months), 9 (20 months), 8 (24 months), and 7(28 months). Detailed group comparisons (Tables 2–4). Abbreviations: ABR, auditory brainstem response; ANOVA, analysis of variance; FBN, Fisher Brown Norway.

Table 2
Wave I amplitude comparison among different age groups

Statistical method	*p*-value	Click	2K	4K	12K	24K
Paired comparison by Tukey's post hoc test	4–6 mo vs. 20 mo	**0.0030**[b]	**0.04525**[a]	0.7451	0.2468	0.6660
	4–6 mo vs. 24 mo	**0.0010**[b]	**0.08381**	0.0864	**0.0111**[a]	0.09522
	4–6 mo vs. 28 mo	**0.0010**[b]	**N/A**	**0.0115**[a]	**0.0065**[b]	**0.0252**[a]
	20 mo vs. 24 mo	0.2698	0.8999	0.4356	0.4455	0.5410
	20 mo vs. 28 mo	**0.0018**[b]	**N/A**	0.0915	0.2997	0.2178
	24 mo vs. 28 mo	0.0940	**N/A**	0.7607	0.8999	0.8999
ANOVA	Total	**F(3,27) = 21.02 3.1325e-07**[c]	**F(2,18) = 4.04 0.0356**[a]	**F(3,29) = 4.53 0.0101**[a]	**F(3,29) = 5.59 0.0038**[b]	**F(3,29) =3.72 0.0222**[a]

Two-way analysis of variance (ANOVA) followed by a Tukey's post hoc test. Bold values indicate the comparison reached statistical difference.
[a] $p < 0.05$.
[b] $p < 0.01$.
[c] $p < 0.001$.

time. Electroencephalographic far-field potentials were amplified 200,000 times and filtered between 300 Hz and 10 kHz with data collected and analyzed offline. ABR thresholds, latencies, and amplitudes were obtained based on the average waveforms. The peak of wave I was used to measure the threshold and latency. The amplitude change between the peak and trough of wave I at 80 dB was considered the wave I amplitude. The same criteria were applied to wave V. The ratio of wave V to wave I was calculated at the same intensity for each animal at tested pure-tone frequencies and click. Aged rats with thresholds higher than 80 dB at certain frequencies were excluded from amplitude analysis. Statistical analysis was performed using GraphPad Prism 6 (San Diego, CA, USA).

2.3. Immunostaining

Temporal bones were postfixed in 4% paraformaldehyde (Polysciences, Inc, Warrington, PA, USA) for 2–3 hours at room temperature, followed by decalcification in 120-mM ethylenediaminetetraacetic acid (Fisher Scientific, Waltham, MA, USA) for 3–4 weeks as previously described (Montgomery and Cox, 2016). Samples were stored in 10-mM phosphate-buffered saline (Sigma-Aldrich, St. Louis, MO, USA) at 4 °C until use. Cochlea were dissected into apical, middle, and basal turns using a whole-mount method, and routine immunostaining procedures were performed on free-floating cochlear turns as previously described (Montgomery and Cox, 2016) with the 1 exception that the primary antibody incubation was performed at 37 °C in a hybridization oven. The following primary antibodies were used: mouse IgG1 anti-Ctbp2 (1:500, cat#BDB612044, BD Biosciences, San Jose, CA, USA), mouse IgG2a anti-GluR2 (1:200, cat#MAB397, Millipore, Billerica, MA, USA), and rabbit anti-myosin VIIa (1:200, cat#25-6790, Proteus BioSciences, Inc, Ramona, CA, USA). The following secondary antibodies were purchased from Invitrogen (Waltham, MA, USA) and used at a 1:1000 dilution: Alexa-488 goat anti-mouse IgG2a (cat#A21131), Alexa-568 goat anti-rabbit (cat#A11036), and Alexa-647 goat anti-mouse IgG1 (cat #A21240). Nuclei were stained with Hoechst 33342 (1:2000, cat#H1399, Molecular Probes, Eugene, OR, USA). Images were taken using a Leica SP5 confocal microscope, and image analysis was performed using Leica LAS AF LITE software.

2.4. Ribbon synapse and HC quantification

IHC-SGN ribbon synapses and HCs from the same ear were quantified in immunostained cochlear whole mounts in a subgroup of ABR-tested animals (4–6 months [n = 5], 20 months [n = 4], 24 months [n = 5], and 28 months [n = 5]). Samples chosen for immunostaining were randomly chosen by a researcher blinded to the ABR results. Confocal microscopy was used to identify the 2, 4, 12, and 24 kHz regions using low-magnification 10× images and a rat cochleogram (Viberg and Canlon, 2004). In each frequency region, 100× z-stack images were taken for quantification of synapses, and 40× z-stacks were taken for quantification of HCs. IHC-SGN synapses (containing both presynaptic and postsynaptic components) and orphan synapses (containing only the presynaptic protein) were quantified in 7 IHCs in each frequency region. HC quantification was counted in a 200-μm segment for each frequency region. For each set of counts, the N value represents an individual animal. Statistical analysis was performed using GraphPad Prism 6 (San Diego, CA, USA). Percent of life span for FBN rats was calculated using survival data provided by the NIA aged rodent colony handbook which was based on the study by Turturro et al. (1999).

3. Results

In auditory studies, ABR wave I is thought to represent the far-field response from the acoustic nerve and to be an accurate

Table 3
Wave V amplitude comparison among different age groups

Statistical method	*p*-value	Click	2K	4K	12K	24K
Paired comparison by Tukey's post hoc test	4–6 mo vs. 20 mo	0.1539	0.8999	0.8283	0.4493	0.5543
	4–6 mo vs. 24 mo	**0.0090**[a]	0.1386	0.2456	0.0857	0.8999
	4–6 mo vs. 28 mo	**0.0040**[a]	N/A	0.1448	0.0714	0.3327
	20 mo vs. 24 mo	0.5335	0.1647	0.6645	0.7209	0.6818
	20 mo vs. 28 mo	0.2335	N/A	0.4847	0.6440	0.8999
	24 mo vs. 28 mo	0.8676	N/A	0.8999	0.8999	0.4556
ANOVA	Total	**F(3,27) = 6.27 0.0023**[a]	**F(2,18) = 2.47** 0.1124	**F(3,29) = 2.11** 0.1210	**F(3,29) = 2.92** 0.0508[b]	**F(3,29) = 1.38** 0.2693

Two-way analysis of variance (ANOVA) followed by a Tukey's post hoc test. Bold values indicate the comparison reached statistical difference.
[a] $p < 0.01$.
[b] *p*-value very close to statistical significant.

Table 4
Wave V/I ratio comparison among different age groups

Statistical method	*p*-value	Click	2K	4K	12K	24K
Paired comparison by Tukey's post hoc test	4–6 mo vs. 20 mo	0.8999	0.41	0.8999	0.8999	0.8999
	4–6 mo vs. 24 mo	0.8548	**0.0291**[a]	0.8770	0.8999	0.0555[c]
	4–6 mo vs. 28 mo	**0.0090**[b]	N/A	0.0503[c]	0.8999	0.2984
	20 mo vs. 24 mo	0.8999	0.2516	0.8999	0.8999	**0.0377**[a]
	20 mo vs. 28 mo	**0.0357**[a]	N/A	0.0614	0.7452	0.2273
	24 mo vs. 28 mo	0.0500[c]	N/A	0.22473	0.8999	0.8554
ANOVA	Total	**F(3,27) = 4.32**	**F(2,18) = 3.98**	**F(3,29) = 3.06**	**F(3,29) = 0.32**	**F(3,29) = 3.86**
		0.0131[a]	**0.0371**[a]	**0.0437**[a]	0.8126	**0.0194**[a]

Two-way analysis of variance (ANOVA) followed by a Tukey's post hoc test. Bold values indicate the comparison reached statistical difference.
[a] $p < 0.05$.
[b] $p < 0.01$.
[c] *p*-value very close to statistical significant.

reflection of the magnitude of synchronized peripheral input to brainstem auditory structures. Later ABR waves IV/V (from pre-inferior colliculus [IC] and IC) likely reflect time-locked peripheral excitatory input and a combination of synchronized ascending excitatory activity in homeostatic balance with inhibitory events. With aging, the later ABR waves reflect an age-related compensatory downregulation of brainstem inhibitory function in response to the age-related loss of peripheral input (Auerbach et al., 2014; Caspary et al., 2008; Gold and Bajo, 2014; Roberts et al., 2010).

3.1. ABR measurements

The present study examined wave I ABR thresholds and latencies across 4 age groups of FBN rats. All groups showed shallow "U" shaped ABR audiometric thresholds (Fig. 1), similar to those seen in other rodent ABR threshold studies (Borg, 1982; Heffner et al., 1994; Kelly and Masterton, 1977). For 4- to 6-month-old FBN rats, click stimuli had the lowest threshold (23.6 ± 1.2 dB) compared to other frequencies tested. Lowest threshold among pure tone frequencies was at 12 kHz (29.4 ± 2.6 dB), with higher

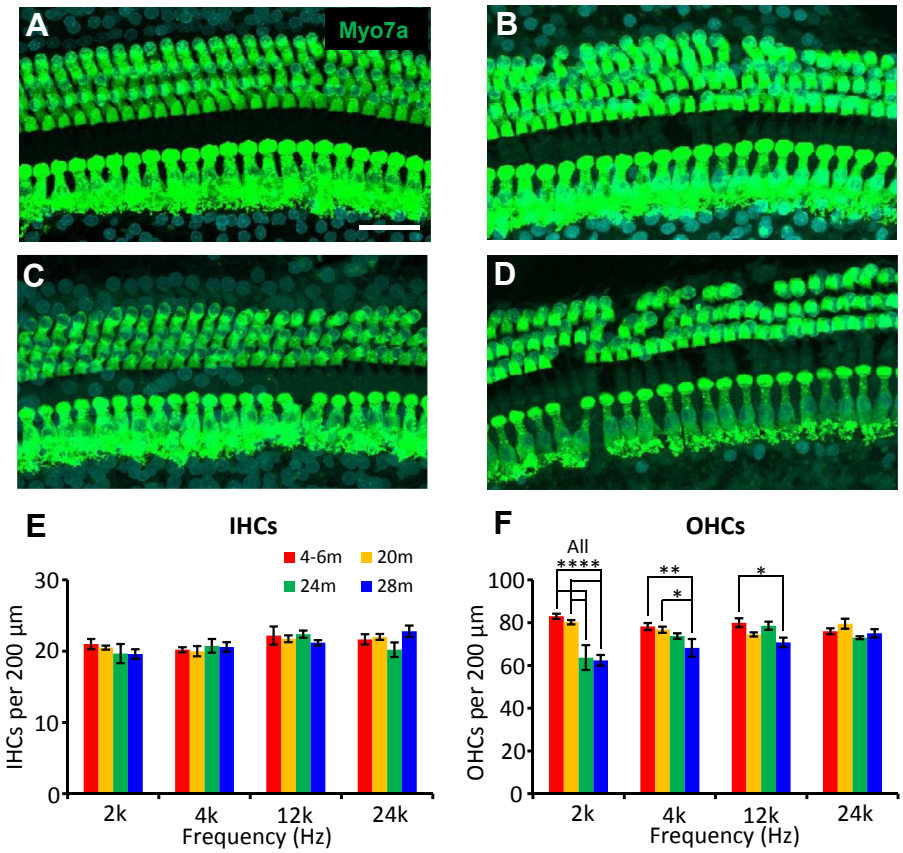

Fig. 3. Cytocochleogram of FBN rat cochleae at 4 different ages. Representative maximum projection confocal images show a well-organized organ of Corti with 3 rows of OHCs and 1 row of IHCs at (A) 4–6 months, (B) 20 months, (C) 24 months, and (D) 28 months of age. HCs are labeled by myosin VIIa (myo7a, green). (E) No significant change in IHC number was observed at any age or frequency distribution along the cochlea [2-way ANOVA $F(9, 45) = 1.454$]. (F) Significant age-related changes in OHC number was detected at 24 months in the 2 kHz region and at 28 months in the 2, 4, and 12 kHz regions. Data are presented as mean ± standard error of the mean. N = 5 for 4–6 months; 4 for 20 months; 5 for 24 months, and 5 for 28 months. Two-way ANOVA [$F(9, 45) = 6.698$] followed by a Tukey's post hoc test, $^{*}p < 0.05$; $^{**}p < 0.01$, and $^{****}p < 0.0001$. Abbreviations: ANOVA, analysis of variance; FBN, Fisher Brown Norway; HCs, hair cells; IHCs, inner hair cells; OHCs, outer hair cells. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

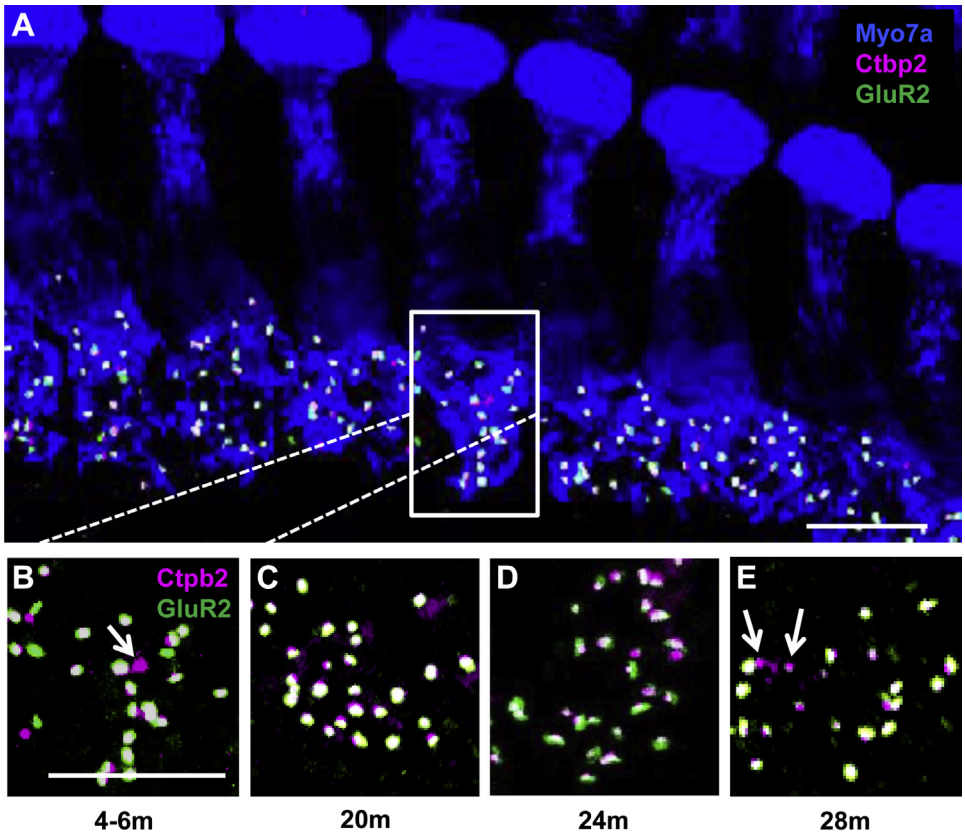

Fig. 4. IHC-SGN synapse changes at 4 kHz in the FBN rat cochlea at 4 different ages. Representative maximum projection confocal images showing age-related changes in synapses located on IHCs (myosin VIIa, Myo7a [blue]). Presynaptic regions are labeled by Ctbp2 (magenta), and postsynaptic glutamate receptors are labeled by GluR2 (green). (A) Merged image showing 7 IHCs from the 4 kHz region in a 4- to 6-month-old rat. Box indicates synapses in 1 IHC, with the higher magnification image shown in (B). Representative high-magnification images of IHC-SGN synapse from the 4 kHz region of 20 months (C), 24 months (D), and 28 months (E) FBN rat cochleae. Arrows indicate orphan synapses. Scale bar in A = 10 μm and B = 5 μm. Abbreviations: Ctbp2, C-terminal binding protein 2; FBN, Fisher Brown Norway; IHC-SGN, inner hair cell–spiral ganglion neuron. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

thresholds at 4 and 24 kHz (45.6 ± 3.1 dB and 46.7 ± 3.1 dB, respectively), and highest threshold at 2 kHz (61.3 ± 3.0 dB) (Fig. 1). There was an expected, significant, and progressive age-related increase in thresholds for clicks and all frequencies (Fig. 1). Table 1 summarizes the individual differences among the different age groups. Statistical differences were found between the 4- to 6- and 24-month groups for clicks ($p < 0.05$), 2 kHz ($p < 0.05$), 4 kHz ($p < 0.05$), and 12 kHz ($p < 0.01$), with no differences at 24 kHz. Significant differences were also seen between the 4- to 6- and 28-month-old groups for all frequencies tested (Table 1, $p < 0.01$). Data also indicate a trend of age differences at 20 months ($p = 0.055$) in response to click, but frequency-specific changes were not detected until 24 months in FBN rats (Table 1).

Age-related changes were seen in the shape of the ABR waveforms. Fig. 2A shows exemplar 12 kHz-evoked ABR waveforms from a 4–6 and a 28-month-old FBN rat. Note the significant overall decrease in waveform amplitude between young and aged ABRs (Fig. 2A). Wave I ABR amplitudes between different ages of FBN rats are summarized in Fig. 2B. Significant, progressive, and age-related decreases were found across ages for click-evoked wave I ABR amplitudes (2-way analysis of variance [ANOVA] followed by a Tukey's post hoc test, $p < 0.001$; Fig. 2B, Table 2). Pure tone–evoked ABRs showed smaller amplitude changes, but statistically significant differences were still detected in all frequencies tested at 28 months (Fig. 2B, Table 2, 2-way ANOVA followed by a Tukey's post hoc test, $p < 0.05$ for 4 and 24 kHz, $p < 0.01$ for 12 kHz). Similar to wave I click-evoked amplitude changes, click-evoked wave V ABR waveforms showed significant decreases with age (Fig. 2C, Table 3, 2-way ANOVA followed by a Tukey's post hoc test, $p < 0.01$). No age-related changes in pure tone–evoked wave V amplitude were noted across the frequencies investigated (Fig. 2C, Table 3). Relatively unchanged wave V amplitudes and significantly decreased wave I amplitudes resulted in a significant increase in the wave V/I ratios. Significant age-related increases (group main effect) in the V/I ratio were found for click-evoked ABRs and for 2, 4, and 24 kHz (Fig. 2D, Table 4, 2-way ANOVA, $p < 0.05$).

Analyses performed on wave I latency revealed no significant differences among the 4 groups ($p > 0.05$) or between specific group pairs, with the exception of across frequency differences between 20- and 28-month-old animals (2-way ANOVA, $p < 0.05$, data not shown).

3.2. Cochlear pathology

After completion of ABR studies, temporal bones were collected for preparation of HC and IHC-SGN ribbon synapse quantification using immunostaining and confocal microscopy. HCs were identified using the well-characterized HC marker myosin VIIa (Hasson et al., 1995), and a rat cochleogram was used to convert the 2, 4, 12, and 24 kHz regions to distance along the cochlear duct (Viberg and Canlon, 2004).

Previous studies from 32-month-old FBN rat cochlea using a method developed by Dr. Barbara Bohne (Eldredge et al., 1973; Ou et al., 2000) described profound OHC loss and minimal IHC loss in

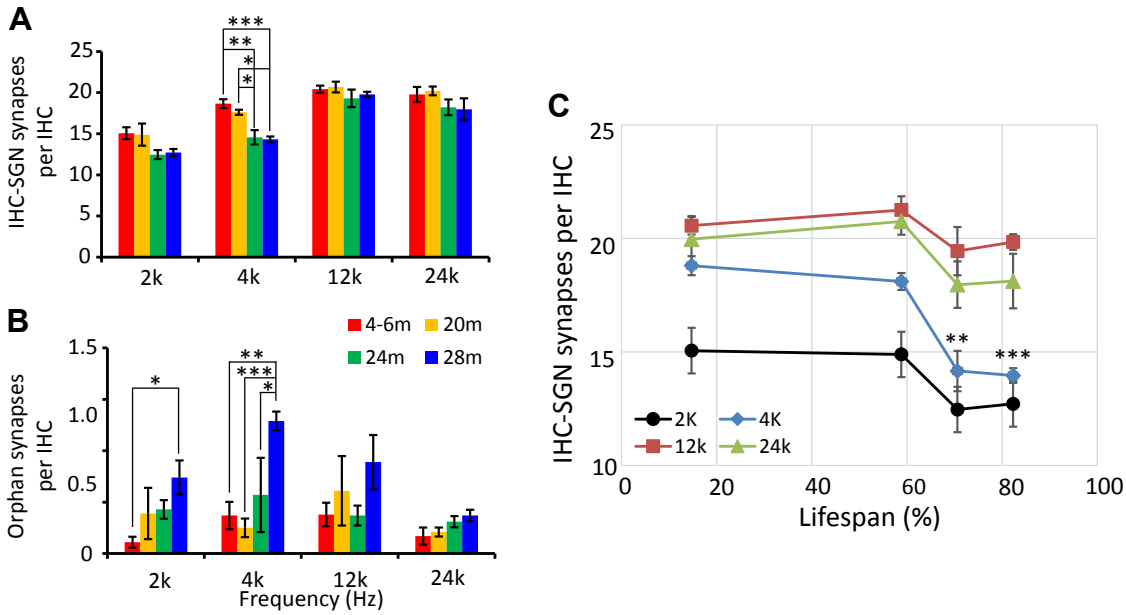

Fig. 5. Quantification of IHC-SGN synapse number in FBN rat cochleae at 4 different ages. Number of IHC-SGN synapses (A) and orphan synapses (B) per IHC at 2, 4, 12, and 24 kHz in FBN rats at 4 different ages. There was a significant reduction in IHC-SGN synapses at 24 months ($p < 0.001$) and 28 months at 4 kHz ($p < 0.001$). In the 2 kHz region, there were significantly more orphan synapses in 28-month samples compared to 4- to 6-month samples ($p < 0.05$). In the 4 kHz region, there were significantly more orphan synapses in 28-month samples compared to samples of 4- to 6-months ($p < 0.01$), 20 months ($p < 0.001$), and 24 months ($p < 0.05$). (C) The number of IHC-SGN synapses was plotted against the percentage of life span. Significant synapse loss was observed starting at 75% of the FBN life span at 4 kHz. Data are presented as mean ± standard error of the mean. N = 5 for 4–6 months; 4 for 20 months; 5 for 24 months, and 5 for 28 months. Two-way ANOVA [$F(9, 45) = 0.8485$ for A and $F(9, 45) = 1.190$ for B] followed by a Tukey's post hoc test, $^{*}p < 0.05$; $^{**}p < 0.01$; and $^{***}p < 0.001$. Abbreviations: ANOVA, analysis of variance; FBN, Fisher Brown Norway; IHC-SGN, inner hair cell–spiral ganglion neuron.

the apical turn (Caspary et al., 2005; Turner and Caspary, 2005). The present study done in 4- to 28-month-old FBN rats found no age-related loss of IHCs and modest OHC loss across all ages examined (Fig. 3). All ages showed a well-organized organ of Corti with 3 rows of OHCs and 1 row of IHCs (Fig. 3A–D). No significant change in IHC number was observed at any age or frequency distribution along the cochlea (Fig. 3E). Significant age-related changes in OHC number were detected at 24 months in the 2 kHz region (23.3 ± 3.3% loss) and at 28 months in the 2 kHz region (29.4 ± 1.5% loss), 4 kHz region (12.8% ± 2.1% loss), and 12 kHz region (11.5% ± 1.1% loss) (Fig. 3F).

Age-related changes to IHC-SGN ribbon synapses were examined in 4- to 28-month-old FBN rats using myosin VIIa to label the cytoplasm of IHCs (Fig. 4A). Quantification of intact IHC-SGN synapses per IHC required the presence of both the presynaptic component (labeled by Ctbp2) and the postsynaptic component (labeled by GluR2). Quantification of orphan synapses included those with the presynaptic ribbon without an opposing postsynaptic receptor. These measurements provide a quantitative assessment of the peripheral input to the central auditory pathway.

IHC-SGN synapse loss was only detected in the 4 kHz region, which correlated with significant age-related increases in ABR thresholds at 24 and 28 months of age. At 4 kHz, a significant 24.7% ± 1.9% age-related loss of synapses was observed for 24-month-old rats with a 25.7% ± 0.7% loss seen for 28-month-old animals

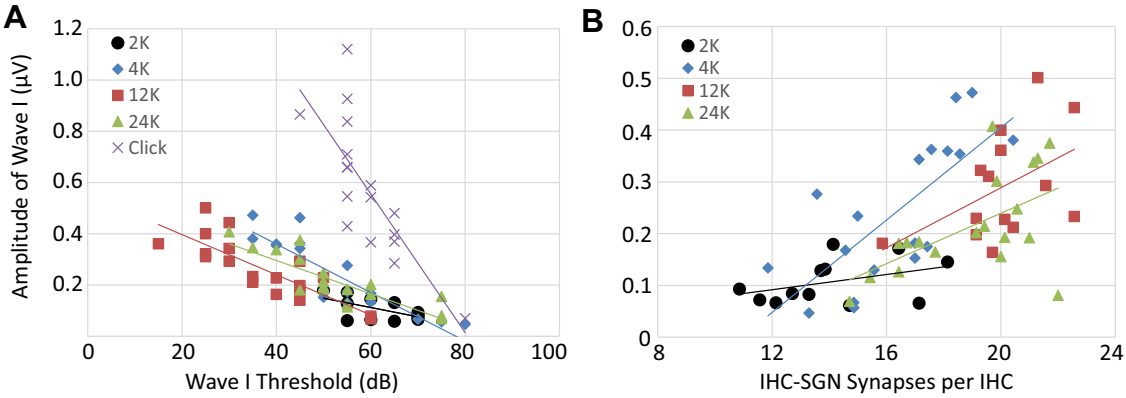

Fig. 6. ABR wave I amplitude significantly correlates with wave I threshold and the number of IHC-SGN synapses regardless of age. (A) Negative correlations were found between wave I amplitude and ABR threshold with the highest correlation at 4 kHz ($p < 0.05$ for 2 kHz; $p < 0.001$ for 4, 12, and 24 kHz and click). (B) Positive correlations were detected between wave I amplitude and the number of IHC-SGN synapses for 4 kHz ($R^2 = 0.54$, $p < 0.001$), 24 kHz ($R^2 = 0.32$, $p < 0.05$), but not 2 kHz ($R^2 = 0.15$, $p = 0.21$). Data were analyzed using a Pearson correlation coefficient test. N = 12 for 2 kHz, 18 for 4 kHz, 19 for 12 kHz, and 19 for 24 kHz. (Table 5). Abbreviations: ABR, auditory brainstem response; IHC-SGN, inner hair cell–spiral ganglion neuron.

Table 5
Correlation between wave I amplitude, ABR threshold, and IHC-SGN synapses

Wave I amplitude	2K	4K	12K	24K	Click
Threshold	$R^2 = 0.37$, [a] **$p = 0.02708$**	$R^2 = 0.78$, [b] **$p = 1.24E-06$**	$R^2 = 0.68$, [b] **$p = 1.35E-05$**	$R^2 = 0.70$, [b] **$p = 9.14E-06$**	$R^2 = 0.61$, [b] **$p = 2.2E-04$**
IHC-SGN Synapse	$R^2 = 0.15$, $p = 0.21$	$R^2 = 0.54$, [b] **$p = 5.1E-04$**	$R^2 = 0.20$, [c] $p = 5.8E-02$	$R^2 = 0.32$, [a] **$p = 1.2E-02$**	N/A

Pearson correlation coefficient test. Bold values indicate the correlation reached statistical difference.
[a] $p < 0.01$.
[b] $p < 0.001$.
[c] p-value very close to statistical significant.

(Figs. 4A–E and 5A). There was a 3.5-fold increase in orphan synapses in the 4 kHz region in 28-month-old rats compared to 4- to 6-month-old animals (Fig. 5B). At 2 kHz, the only significant difference detected was in orphan synapses which increased 6.5-fold in 28-month-old rats compared to 4- to 6-month-old animals (Fig. 5B). When the number of ribbon synapses was graphed as percent of life span, ribbon synapse loss at 4 kHz was observed at 75% of the FBN life span (Fig. 5C), which is approximately 60 years of age in humans (Sengupta, 2013).

3.3. Correlation of ABR with ribbon synapses

Since quantification of IHC-SGN ribbon synapses and HCs were performed on the same ears in a subgroup of rats that had the ABR testing (n = 4–5 for each age group), regression analyses were conducted in these matched data to determine any possible correlations among ABR wave I amplitude, ABR threshold, IHC-SGN synapse number, and HC number.

As shown in Fig. 6A, significant correlations between wave I amplitudes and wave I ABR thresholds were seen across frequencies. Without dividing animals into different age groups, highly positive correlations were detected for clicks and each pure-tone frequency, with p values all less than 0.05 (Table 5, $p < 0.05$ for 2 kHz, $p < 0.001$ for click, 4, 12, and 24 kHz, Pearson correlation coefficient test). Significant positive correlations were also found between wave I amplitudes and IHC-SGN synapse number at 4 kHz ($p < 0.001$) and 24 kHz ($p < 0.05$, Pearson correlation coefficient test) for all ages (Fig. 6B, Table 5).

In addition to these correlations, we also found notable negative correlations between wave V/I ratios with OHC number at 4 kHz (Fig. 7A, $p < 0.001$, Pearson correlation coefficient test) and wave V/I ratio with IHC-SGN synapse number at 24 kHz (Fig. 7B, $p < 0.001$, Pearson correlation coefficient test). In the present study, no data indicated any correlation among IHC number with any ABR wave parameters (data not shown).

4. Discussion

The present study found significant age-related IHC-SGN ribbon synapse loss at 4 kHz, which correlated with age-related increases in wave I ABR thresholds at 24 and 28 months of age. Age-related increases in ABR thresholds were also observed at 2 and 12 kHz in 24-month rats and for all tested frequencies in 28-month rats with no significant IHC-SGN synapse changes. Age-related loss of OHCs was detected at 24 months for 2 kHz and at 28 months for 3 frequencies, whereas IHCs remained intact for all frequencies regardless of age. As expected, ABR wave I amplitudes, representing the far-field response from the acoustic nerve complex, showed relatively larger age-related reductions than did the later wave V, which reflects responses from pre-IC and/or IC generators. This differential is reflected by age-related increases in the ABR wave V/I ratio. Similar to previous reports, regression analysis showed a strong correlation between IHC-SGN synapse numbers and ABR wave I amplitudes measured in the same animals (Altschuler et al., 2015; Kujawa and Liberman, 2009; Mohrle et al., 2016; Sergeyenko et al., 2013). There was little correlation between ABR wave I amplitudes and OHC counts. Together these data demonstrate that IHC-SGN synapses in the FBN rat may be more resistant to aging than in CBA/CaJ and UM-HET4 mice and female Wistar rats (Altschuler et al., 2015; Mohrle et al., 2016; Sergeyenko et al., 2013), suggesting that there are likely species and strain differences underpinning the age of onset and magnitude of the decreased peripheral input caused by age-related IHC-SGN synapse loss.

The present study was carried out in the NIA supplied FBN F1 hybrid strain (F344 × Brown Norway [F344BN]), which has a long life span with 50% mortality at 36 months of age (Lipman, 1997; Lipman et al., 1996). This strain has been extensively used in studies of central auditory aging and has been compared with other rat models of aging (Caspary et al., 1999, 2005, 2006, 2008; de Villers-Sidani et al., 2010; Gold and Bajo, 2014; Hughes et al., 2010; Ling et al., 2005; Milbrandt and Caspary, 1995; Richardson

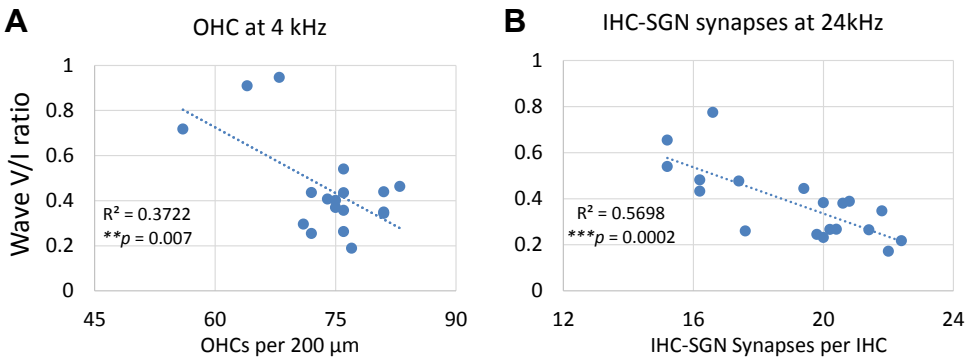

Fig. 7. ABR wave V/I ratio significantly correlates with OHC and IHC-SGN synapses. Correlation between waves V/I ratio and OHC numbers or IHC-SGN synapses was calculated at 2, 4, 12, and 24 kHz regardless of age. Significant negative correlations were found between wave V/I ratio and OHC numbers at 4 kHz (A) ($p < 0.01$, Pearson correlation coefficient test, N = 17); and between wave V/I ratio and IHC-SGN synapse number at 24 kHz (B) ($p < 0.001$, Pearson correlation coefficient test, N = 19). Abbreviations: ABR, auditory brainstem response; IHC-SGN, inner hair cell–spiral ganglion neuron; OHC, outer hair cell.

et al., 2011, 2013; Schatteman et al., 2008; Turner and Caspary, 2005; Turner et al., 2005; Wang et al., 2009, 2011). The present study was driven by the need to better understand the relative timing and magnitude of the impact of aging on the auditory peripheral in the FBN rat, a strain where the impact of age-related "hidden hearing loss" has been extensively described as a compensatory loss of inhibitory function at multiple levels of the CANS (Burianova et al., 2009; Caspary et al., 2008; Richardson et al., 2013).

Similar to previous rodent ABR threshold and aging studies, young FBN rats showed click and pure tone thresholds between 20 and 40 dB SPL with groups showing best thresholds near the rodent acoustic fovea between 12 kHz and 24 kHz (Altschuler et al., 2015; Bielefeld et al., 2008; Caspary et al., 2005; Hunter and Willott, 1987; Mikaelian, 1979; Mohrle et al., 2016; Popelar et al., 2006; Sergeyenko et al., 2013; Tang et al., 2014; Wang et al., 2009). Consistent with many of the rodent aging studies noted above, we found an age-related 22 dB SPL progressive parallel elevation between young (4–6 months) and old (28 months) rats. ABR wave I and V amplitudes progressively decreased with age with greatest changes seen for click, 2, 4, and 24 kHz (consider the V/I ratio in Fig. 2D). As first suggested by Hunter and Willott and confirmed by others across species, the V/I ratio increased progressively with age and showed the largest increases for click at 80 dB SPL (Willott, 1991). However, the unchanged V/I ratio suggests that 12 kHz is the frequency most likely resistant to the effects of aging.

Nearly, 12% OHC loss was detected in cochleae from 28-month-old FBN rats in the 4 and 12 kHz regions. At 2 kHz, a greater OHC loss (~23%) was detected at 24 months, which increased to ~29% at 28 months, consistent with previous studies which reported that FBN rats have severe presbycusis at low frequency regions (Caspary et al., 2008; Keithley et al., 1992) In contrast, IHCs remained intact at all ages and for all frequency regions examined. IHC findings for FBN rats were similar to findings for Sprague-Dawley, F344, and Long-Evans rats, suggesting that aged rats maintained IHC numbers throughout their life span (Keithley and Feldman, 1979; Popelar et al., 2006; Turner and Caspary, 2005). However, the present study observed a far lower level of OHC loss than previously reported (Caspary et al., 2005; Turner and Caspary, 2005). This difference may have occurred because the FBN cytocochleograms in the 2 earlier studies were from 32-month-old animals as opposed to 28-month-old animals in the present study. In addition, the present study used immunostaining with a known HC marker and confocal microscopy, while the previous report used an older method where HCs were identified by location and presence of a cuticular plate without the use of markers (Boettcher et al., 1992; Spongr et al., 1992). It is also possible that genetic strain drift occurred between the time periods when the 2 FBN studies were conducted since this more commonly occurs in hybrid strains, like FBN, than in inbred strains (http://www.informatics.jax.org/nomen/strains.shtml).

IHC-SGN ribbon synapse numbers remained constant at 12 and 24 kHz across all ages examined. Yet at 4 kHz, there was a ~25% loss of synapses observed in 24- and 28-month-old rats, as well as an increase in orphan synapses in 28 months rats. There was also an increase in orphan synapses seen at 2 kHz in 28-month rats. Orphan synapses, where the presynaptic ribbon is present but the postsynaptic glutamate receptor is not detected, are likely transient signs of damage. Previous studies have shown that immediately following noise exposure that induces a temporary ABR threshold shift, orphan synapses increase and then return to baseline by 1 week post noise (Liberman et al., 2015a,b; Wan et al., 2014). This suggests that postsynaptic receptors degenerate first and it takes several days for the presynaptic ribbon complex to degrade. Thus, it is surprising that we were able to detect any increases in orphan synapses in the present study since no damaging insult, noise or drug, was present in these studies.

The presence of orphan synapses and the loss of paired IHC-SGN synapses suggests degeneration of SGN fibers; however, several studies have shown that it takes months to years before degeneration of SGN cell bodies is detected (Kujawa and Liberman, 2009; Lin et al., 2011b; Sergeyenko et al., 2013). We did not measure SGN cell bodies in the present study since all analyses were conducted on the same ear and different methods are needed for ribbon synapse and SGN quantifications. Although there are no reports of SGN quantification in FBN rats, previous studies have shown different patterns of age-related SGN loss in the 2 parental strains of the FBN line, Brown Norway and F344 rats. Aged (36 months) Brown Norway rats maintained SGNs in most frequency regions with significant (~25%) SGN loss observed in the low frequency apical region (Keithley et al., 1992). F344 rats (aged 24–27 months) showed significant loss of SGNs across frequency regions except for the apex (Keithley et al., 1992).

Quantification of IHC-SGN synapses in the present study covered ~70% of the FBN cochlea. Although previous studies have shown that FBN rats have a low frequency, apical presbycusis with little to no HC loss in basal regions (Caspary et al., 2005, 2008; Keithley et al., 1992; Turner and Caspary, 2005), it is unknown what, if any, changes may occur in IHC-SGN synapses at frequencies higher than 24 kHz. We were not able to address this question in the present study due to technical challenges in the whole mount dissection of the basal cochlear turn.

The present ABR findings show age-related wave I amplitude and wave V/I ratio changes that correlated well with the age-related IHC-SGN synapse loss observed. This relationship between IHC-SGN synapses and ABR wave I amplitudes in the FBN rat differed from previous studies in the timing and magnitude of IHC-SGN synapse loss. Previous studies have defined "hidden hearing loss" as the loss of IHC-SGN synapses and acoustic nerve fibers with no detectable change in ABR thresholds (Liberman and Kujawa, 2014). In FBN rats, IHC-SGN synapse loss was detected in 1 frequency region at the same age as the increase in ABR threshold for that frequency. In addition, there were no significant changes in IHC-SGN synapses at the 3 other tested frequencies that did show an increase in ABR threshold. However in CBA/CaJ mice, Sergeyenko et al. (2013) showed synapse loss at 32 weeks of age (~8 months) at 4 kHz, with an age-related progression in severity and spread to other frequency regions, while ABR thresholds showed no change until 96 weeks of age (~22 months). Similarly in UM-HET4 mice, IHC-SGN synapse loss was detected across cochlear frequency regions at 22–24 months, whereas ABR thresholds only increased at 4 kHz at this age. The ABR thresholds later increased in all frequency regions at 27–29 months. Younger ages, between 7 and 22 months were not examined in UM-HET4 mice (Altschuler et al., 2015). In female Wistar rats, only click ABRs were examined showing remarkably low, 10 dB thresholds for this rat strain. Increased thresholds were seen at 19–21 months, with IHC-SGN synapse loss described in 6.5- to 10-month-old rats (Mohrle et al., 2016). These conflicting results are most likely attributable to mouse versus rat species and rat strain differences. However, age-related differences in IHC-SGN synapses and ABR thresholds may also reflect the differences in the animal facility environment, including ambient noise, vibration, and ultrasonic noise produced by ventilated cage racks and motion detectors, respectively (Jeremy Turner personal communication). To better compare the percentage of synapse or cell loss between species, conversion to percent of life span was performed. The oldest age analyzed in the current FBN rat study was 80% of life span, which is comparable to 80-week-old CBA/CaJ mice in the Sergeyenko et al. (2013) study. At this age, there was a ~25% loss of IHC-SGN synapses in the 4 and 12 kHz regions

and a ~20% loss at 30 kHz in CBA/CaJ mice, which is comparable to the ~25% loss of synapses seen at 4 kHz in FBN rats. As detailed in the following paragraph, the present findings in FBN rat are suggestive of complex and multiple causes of presbycusis including metabolic/strial changes, which could explain differences between the studies described previously.

The causes of presbycusis were classically described by Schukenecht as resulting from 4 sources: sensory (loss of HCs), neural (loss of SGNs), metabolic (atrophy of the stria resulting in loss of endocochlear potential), or mechanical (stiffening of the basilar membrance or middle ear changes) (Schuknecht, 1969). Later, mixed causes were also documented (Schuknecht and Gacek, 1993). The observed increase in ABR thresholds in 24- and 28-month-old rats is only partially explained by the peripheral damage detected for OHCs and IHC-SGN synapses. The evidence of hearing loss at 12 and 24 kHz in 24- and 28-month-old rats in the absence of IHCs, OHCs, or IHC-SGN synapse loss is suggestive of other age-related pathologies. Previous studies in quiet-raised gerbils have shown an age-related degeneration of the stria vascularis, along with a decreased endocochlear potential (Gratton et al., 1996; Schulte and Schmiedt, 1992; Schulte et al., 1995). Both the FBN and F344 rat models of aging show age-related parallel shifts in their ABR threshold measures, suggestive of strial pathology (Bielefeld et al., 2010) even in the face of data suggesting age-related loss of OHCs and IHC-SGN synapses in the apical turn. The F344 rat, one of the parental strains of FBN, shows strial degeration at 24 months (Buckiova et al., 2006, 2007), while the study by Bielefeld et al. (2008) showed relatively minor endocochlear potential changes at this age. This suggests that metabolic changes within the stria vascularis of 24- to 28-month-old FBN rats combined with the observed low frequency OHC and IHC-SGN synapse loss may underpin the observed hearing loss.

Taken together, our data suggest that IHC-SGN synapses in the FBN rat are more resistant to aging than CBA/CaJ and UM-HET4 mice, and there are likely species and strain differences underlying the cause of decreased peripheral input in age-related hearing loss. As all these studies suggest, but rarely state, the relative maintenance of the amplitude of later ABR waves likely reflects significant age-related downregulation of inhibitory processes in the auditory brainstem and at higher levels resulting in larger than expected super-threshold responses at multiple levels of the CANS with age (Amenedo and Diaz, 1998; Burianova et al., 2009; Caspary et al., 2008; Gold and Bajo, 2014; Syka, 2002; Wang et al., 2009; Willott et al., 1997). This loss of inhibition, in part, underpins the loss of temporal resolving power and loss of speech understanding seen in the elderly (Alain et al., 2004; Bertoli et al., 2002; Caspary et al., 2008).

Disclosure statement

The authors have no actual or potential conflicts of interest.

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