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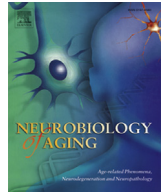http://dx.doi.org/10.1016/j.neurobiolaging.2016.05.001

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Loss of auditory sensitivity from inner hair cell synaptopathy can be centrally compensated in the young but not old brain

Dorit Möhrle [a,1], Kun Ni [a,b,1], Ksenya Varakina [a,1], Dan Bing [a], Sze Chim Lee [a],
Ulrike Zimmermann [a], Marlies Knipper [a], Lukas Rüttiger [a,*]

[a] Department of Otolaryngology, Head & Neck Surgery, Tübingen Hearing Research Centre (THRC), Molecular Physiology of Hearing, University of Tübingen, Tübingen, Germany
[b] Department of Otolaryngology, Head & Neck Surgery, Shanghai Children's Hospital, Shanghai Jiao Tong University, Shanghai, China

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ABSTRACT

A dramatic shift in societal demographics will lead to rapid growth in the number of older people with hearing deficits. Poorer performance in suprathreshold speech understanding and temporal processing with age has been previously linked with progressing inner hair cell (IHC) synaptopathy that precedes age-dependent elevation of auditory thresholds. We compared central sound responsiveness after acoustic trauma in young, middle-aged, and older rats. We demonstrate that IHC synaptopathy progresses from middle age onward and hearing threshold becomes elevated from old age onward. Interestingly, middle-aged animals could centrally compensate for the loss of auditory fiber activity through an increase in late auditory brainstem responses (late auditory brainstem response wave) linked to shortening of central response latencies. In contrast, old animals failed to restore central responsiveness, which correlated with reduced temporal resolution in responding to amplitude changes. These findings may suggest that cochlear IHC synaptopathy with age does not necessarily induce temporal auditory coding deficits, as long as the capacity to generate neuronal gain maintains normal sound-induced central amplitudes.

1. Introduction

Aging people often experience difficulties in perceiving speech in noise, even without elevated audiometric thresholds (Füllgrabe et al., 2014). The development of poor suprathreshold speech processing during aging in humans has previously been linked with the possibility of progressing cochlear synaptopathy, as has been observed in aging animals (Bharadwaj et al., 2014; Bramhall et al., 2015). Recently, it was shown in rodents (Kujawa and Liberman, 2009; Rüttiger et al., 2013; Sergeyenko et al., 2013) and humans (Viana et al., 2015) that afferent auditory neuronal fiber loss may progress with age or after "nontraumatic" loud sound, even when audiometric thresholds are normal, causing hearing deficits independent of the loss of outer hair cells (OHCs).

The hearing disorders that might accompany auditory nerve fiber loss in rodents (Kujawa and Liberman, 2009; Rüttiger et al., 2013; Sergeyenko et al., 2013) presumably reflect impaired processing of acoustic temporal cues in humans, which are critical for sound localization, discrimination of speech, and identification of signals in background noise [see (Rance and Starr, 2015) for a review].

In animal studies, the age-related and noise-induced cochlear damage has been shown to preferentially affect low spontaneous discharge rate (SR), high threshold auditory fibers (Kujawa and Liberman, 2009; Sergeyenko et al., 2013). As this fiber type is assumed to be especially important for hearing well in noise in humans (Bharadwaj et al., 2014), its preferential loss has been suggested to cause age-dependent auditory processing deficits (Bharadwaj et al., 2014; Sergeyenko et al., 2013). On the contrary, in humans, central auditory deficits have been proposed to arise with age (Gates et al., 2010; Parham et al., 2013) including age-dependent plasticity changes (Wingfield and Grossman, 2006) and cognitive decline (Füllgrabe et al., 2014). For any future therapeutic intervention to cure or counteract speech in noise deficits with age, it is crucial to know whether the primary target site is the cochlear or the central nervous system.

* Corresponding author at: Department of Otolaryngology, Tübingen Hearing Research Centre, Molecular Physiology of Hearing, University of Tübingen, Elfriede-Aulhorn-Straße 5, 72076 Tübingen, Germany. Tel.: +49 (0)7071 2988194; fax: +49 (0)7071 294950.

E-mail address: lukas.ruettiger@uni-tuebingen.de (L. Rüttiger).
[1] These authors contributed equally to the manuscript.

We previously observed in rats that cochlear deafferentation, depending on the degree of inner hair cell (IHC) synaptopathy, can nevertheless lead to subsequently normal or even enhanced auditory midbrain responses (wave IV of the auditory brainstem response) (Rüttiger et al., 2013; Singer et al., 2013).

We here compared young, middle-aged, and old rats for differences in central responsiveness to age-related or noise-induced synaptopathy. We measured the auditory brainstem response (ABR) wave amplitudes corresponding to the auditory nerve (wave I) and lateral lemniscus and inferior colliculus (IC) (wave IV) (Melcher and Kiang, 1996) and examined the correlation with altered numbers of IHC ribbon synapses before and after noise exposure in rats of different ages. To study the temporal sensitivity linked to sound in noise responsiveness, we analyzed auditory steady state responses (ASSRs), which are periodic electrical brain oscillations evoked by acoustic stimuli, sinusoidally modulated in amplitude and frequency (Picton et al., 2003). As ASSR are thought to reflect synchronous discharge of auditory neurons phase-locked to the modulation frequency of tonal stimulation (Brenner et al., 2009; Dolphin and Mountain, 1992; Kuwada et al., 2002; Parthasarathy and Bartlett, 2012), they are used as a metric of temporal resolution of sound processing.

Groups of young (2–3-month-old), middle-aged (6–10-month-old), or old rats (19–22-month-old) were analyzed before and after moderate acoustic trauma. Exposed young but also middle-aged animals showed a surprisingly robust increase in neuronal gain that maintained normal auditory brainstem responses, in contrast to a severe decay of neuronal gain in old animals. In middle-aged animals, with age-related cochlear neuropathy, responses to fast amplitude-modulated stimuli were normal, whereas in old animals, temporal sensitivity and response strength were reduced. These findings may support the idea that deficits in neuronal gain mobilization with age rather than cochlear neuropathy may promote temporal coding deficits, possibly crucial knowledge for future therapeutic intervention strategies for presbycusis.

2. Methods

2.1. Animals

Female Wistar rats were purchased from Charles River Laboratories (Research Models and Services, Germany GmbH) and were housed for up to 2 years in the animal care facility of the institute, where noise levels did not exceed 50–60 dB sound pressure level (SPL). The care and use of animals was approved by the University of Tübingen, Veterinary Care Unit and the Animal Care and Ethics Committee of the regional board of the Federal State Government of Baden-Württemberg, Germany, and followed the guidelines of the EU Directive 2010/63/EU for animal experiments.

2.2. Hearing measurements: auditory brainstem response (ABR), distortion product otoacoustic emission (DPOAE), noise exposure, and ABR waveform analysis

The recordings were performed in a soundproof chamber (IAC, Niederkrüchten, Germany) as described (Rüttiger et al., 2013). OHC function was assessed by the growth function and the DP-gram of the $2 \times f1 - f2$ DPOAE (Engel et al., 2006; Knipper et al., 2000).

Animals were exposed to moderate broadband sound (8–16 kHz, 100 dB SPLrms for 2 hours) as described (Singer et al., 2013; Tan et al., 2007). Hearing function was monitored before, directly after (15–30 minutes), and 2 or 4 weeks after noise exposure. For each individual ear, the ABR wave amplitudes and latencies were analyzed as described in (Rüttiger et al., 2013). Growth functions of latencies and amplitudes were constructed

for stimulus levels over a range up to 100 dB. The latency dynamic response range was determined by an iterative procedure based on the 85% CI of consecutive data points starting at the minimum latency within a defined range of 10–80 dB. The slope of ABR wave latency growth function was calculated by linear regression.

Auditory steady-state responses were measured with amplitude-modulated sinusoidal stimuli (carrier frequency 8 kHz). The stimuli were presented at 20 dB above threshold (dB sensation level). Stimuli were amplitude-modulated at 100% between 128 and 1024 Hz in half-octave steps. A modulation index was calculated for individual animals by building the ratio between the maximal signal and the baseline (defined as the average of all points except the maximum and the neighboring points). At a fixed modulation frequency of 512 Hz, responses to modulation depths of 100%, 70%, 50%, 35%, 25%, 17%, 12%, 8%, 6%, 4%, and 0% (unmodulated) were recorded. Response thresholds to modulated stimuli were defined as the lowest modulation depth where the signal exceeded the 85% CI of the noise level. The slope of the amplitude modulation growth function was determined by linear regression for above-threshold responses.

Unless otherwise stated, all data were presented as group mean with standard deviation or with standard error of the mean for n animals per experimental group. Differences of the means were compared for statistical significance either by Student t test, 1-way, 2-way, or repeated measures analysis of variance (ANOVA). ANOVA tests were followed by multiple t-tests with the α-level of 0.05 and correction for type 1 error after Bonferroni-Holms. Differences of the slopes built by linear regression were compared by analysis of covariance (ANCOVA). The resulting p values are reported in the legends $^*p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$; $^{****}p < 0.0001$; and n.s., not significant.

2.3. Tissue preparation and immunohistochemistry

Cochleae were isolated, prepared, and cryosectioned as described (Knipper et al., 2000; Tan et al., 2007). At least 3 animals for either group were used. For cochlear whole-mount preparations, the temporal bone was dissected on ice and immediately fixed for 2 hours using 2% paraformaldehyde in 100-mM phosphate-buffered saline by perfusing the cochlea through the round and oval window. For immunohistochemistry, rat cochlear sections or whole-mount preparation were stained with antibodies against CtBP2/RIBEYE, NF200, KCNQ4, and prestin as described (Duncker et al., 2013; Weber et al., 2002). Figures are shown as a composite image, which represents the maximum-intensity projection over all layers of a z-stack [see (Zampini et al., 2010) for details]. The number of CtBP2/RIBEYE-immunopositive dots was quantified as indicators for ribbon synapses of IHCs with dendrites from spiral ganglion neurons (SGNs) (Khimich et al., 2005) to estimate deafferentation (Kujawa and Liberman, 2009). Image acquisition and CtBP2/RIBEYE-immunopositive spot counting were carried out as previously described (Heidrych et al., 2009).

3. Results

3.1. Age-related ABR threshold elevation in the last third of life

Aiming to follow central changes in activation by auditory stimulation (central responsiveness) to age- or noise-induced IHC synaptopathy, we analyzed hearing in Wistar rats of young (2- to 3-month-old), middle (6.5–10-month-old), and old (19–21-month-old) age. Hearing function during aging was examined by click stimulus-evoked ABR threshold (Fig. 1A). ABR to click stimuli confirmed a significantly elevated average auditory threshold in
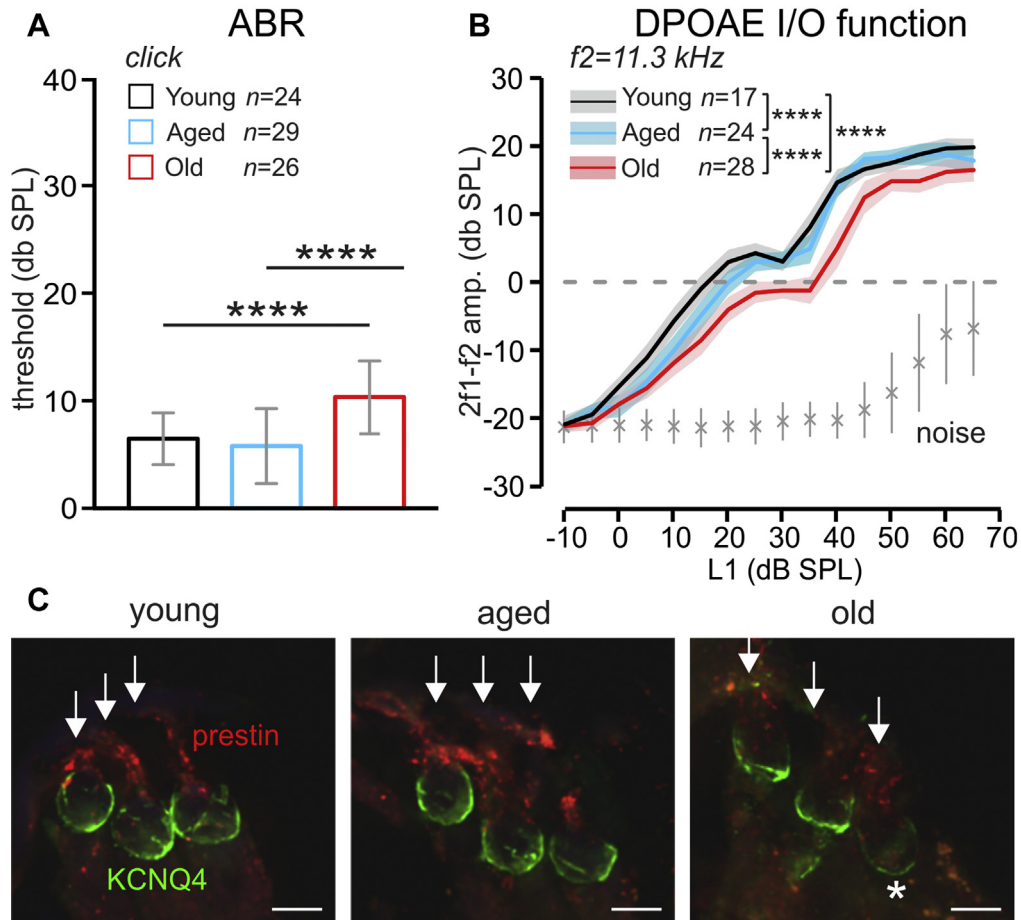
Fig. 1. Click stimulus—evoked ABR threshold and OHC function and phenotype slightly worsened over age. (A) Mean ± standard deviation click-evoked ABR thresholds for young (black), middle-aged ("aged", blue), and old rats (red). Old rats showed significantly elevated thresholds for click-ABRs compared with young and middle-aged rats. (B) Mean ± 95% CI DPOAE amplitude I/O function evoked by stimulus f2 = 11.3 kHz for young (black line and gray area), middle-aged ("aged", blue line and blue area), and old rats (red line and red area) showing significant differences between young, middle-aged, and old rats. (C) Immunohistochemical staining for KCNQ4 (green) and prestin (red) in young, middle-aged (aged), and old rats revealed deteriorated hair cell structure in old rats. Arrows indicate OHCs. Asterisks indicate partial loss of OHCs. Scale bars, 10 μm. Abbreviations: ABR, auditory brainstem response; DPOAE, distortion product otoacoustic emission; I/O, input-output; OHC, outer hair cell.

the last third of the lifespan (10.3 ± 3.4 dB, n = 26/51 rats/ears, 19.5—21-month-old) compared to young (6.5 ± 2.4 dB, n = 24/48 rats/ears, 2—3-month-old, 2-tailed Student t test, $p < 0.0001$) and middle-aged rats (5.8 ± 3.5 dB, n = 29/58 rats/ears, 6.5—10-month-old, 2-tailed Student t test, $p < 0.0001$). The elevation of hearing threshold in old animals was largely caused by a loss of OHC responses as examined by DPOAE input-output (I/O) function of emission amplitudes evoked by f2 = 11.3 kHz (Fig. 1B), the frequency of the highest hearing sensitivity for rats. DPOAE I/O functions were significantly lower in old (n = 28/56 rats/ears, 2-way ANOVA, $p < 0.0001$) and middle-aged rats (n = 24/48 rats/ears, 2-way ANOVA, $p < 0.0001$) compared with young rats (n = 17/34 rats/ears). The loss of OHC function in old animals may be linked to a partial loss of OHCs in the midbasal cochlear turn, exemplarily shown in serial cochlear sections in an old (22-month-old) rat (Fig. 1C, right panel) but not a young (2-month-old) or middle-aged (10-month-old) rat, suggesting a first structural link to DPOAE deficits in this region (Müller, 1991). To visualize OHCs, sections were stained with antibodies against the voltage-dependent K$^+$ channel KCNQ4, important for maintaining the viability of OHCs (Marcotti and Kros, 1999) and the OHC motor protein prestin encoded by the gene Slc26a5 (Zheng et al., 2000) (Fig. 1C, left and middle panel).

Thus, consistent with previous studies (Frisina and Frisina, 2013; Rüttiger et al., 2007), in rodents, OHC loss and elevated hearing thresholds are characteristic features of the last third of the lifespan only.

3.2. Age-related cochlear synaptopathy can be centrally compensated in middle-aged but not old rats

To assess cochlear neuronal degeneration preceding late-onset elevation of cochlear thresholds, we analyzed ABR wave I amplitudes. ABR wave I reflects the summed response to sound stimulation in afferent fibers of the SGNs innervating the IHCs of the cochlea. ABR wave I responses to click stimuli cover potentials generated in the midbasal cochlear turns [25th and 75th percentiles at 2.2 and 13.8 kHz, respectively, (Müller, 1991)]. Response amplitudes were significantly lower both in middle-aged rats (Fig. 2A; young: n = 10/19 rats/ears; middle-aged, n = 24/48 rats/ears; 2-way ANOVA, $p < 0.0001$) and in old rats (Fig. 2A; young, n = 10/19 rats/ears; old, n = 12/23 rats/ears; 2-way ANOVA, $p < 0.0001$) compared to young animals across a broad stimulus range (20—80 dB) above threshold (dB re threshold; Fig. 2A, Table 1, 2-tailed Student t test). We quantified the number of CtBP2/RIBEYE-immunopositive dots as indicators for ribbon synapses with
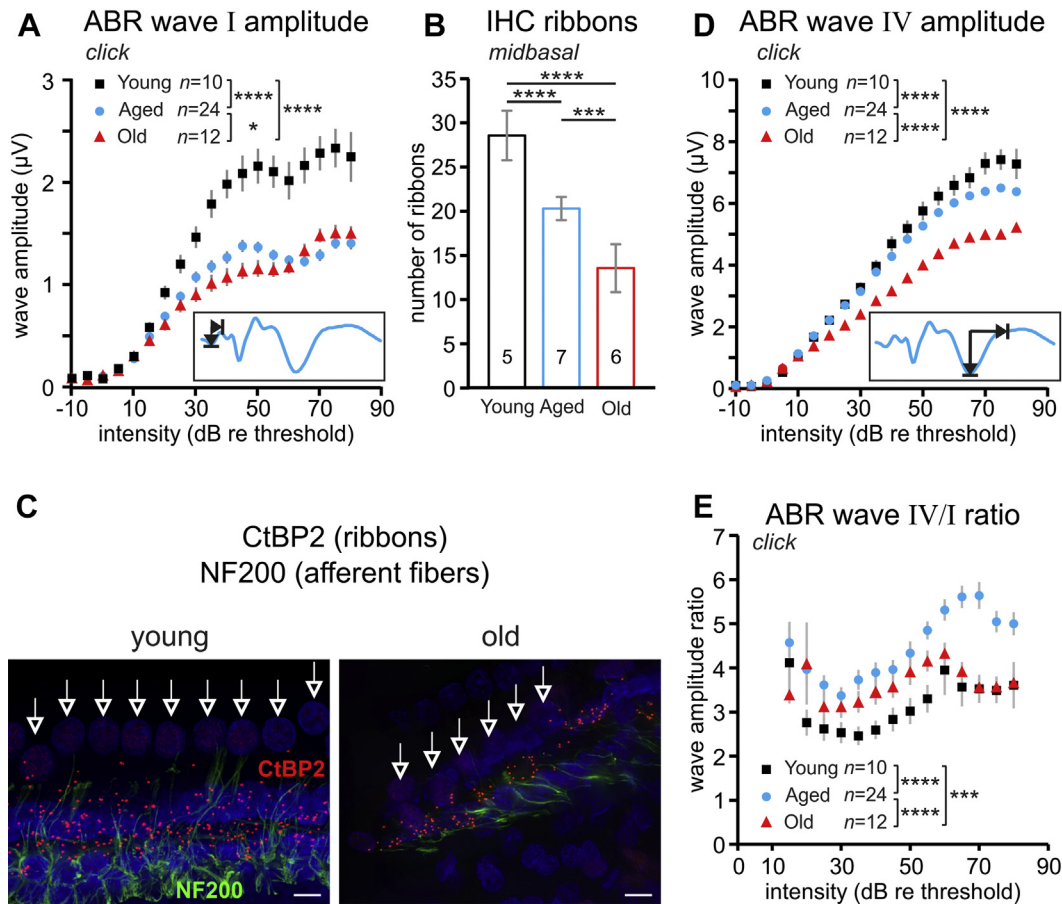
Fig. 2. Age-related cochlear synaptopathy can be centrally compensated in middle-aged but not old rats. (A) Mean ± standard error of the mean (SEM) click-evoked ABR wave I amplitude growth functions in young (black squares), middle-aged ("aged", blue circles), and old rats (red triangles). ABR wave I amplitudes were decreased in middle-aged and old rats. Inset: ABR waveform, indicating wave I peak-to-peak amplitude 40 dB above the hearing threshold. (B) Mean ± standard deviation ribbon numbers in midbasal cochlear turns are reduced with age (2-tailed Student t test, middle-aged vs. old $p = 0.0001$). Numbers of animals per group are given in the bars. (C) Whole-mount preparation of the midbasal cochlear turn in young, middle-aged, and old rats labeled with antibodies against CtBP2/RIBEYE (red) and NF200 (green). Cell nuclei were counterstained with 4′,6-Diamidin-2-phenylindol (DAPI) (arrows). Scale bars, 5 μm. (D) Mean ± SEM click-evoked ABR wave IV amplitude growth functions in young (black squares), middle-aged ("aged", blue circles), and old rats (red triangles). ABR wave IV amplitudes were significantly decreased in old rats over a wide stimulus range compared with young and to middle-aged. Inset: ABR waveform, indicating wave IV peak-to-peak amplitude (E) Mean ± SEM ABR wave IV/I ratios in young (black squares), middle-aged ("aged", blue circles), and old rats (red triangles) were most increased in middle-aged rats for stimulus levels greater than 65 dB. Asterisks denote p values: $^*p < 0.05$; $^{***}p < 0.001$; $^{****}p < 0.0001$. Abbreviation: ABR, auditory brainstem response.

dendrites from SGNs of the IHC in young, middle-aged, and old animals to estimate deafferentation. As shown for the midbasal cochlear regions (Fig. 2B), numbers of ribbons in IHCs from young rats (28.50 ± 2.87, n = 5/5 rats/ears, 2-month-old) were significantly higher than those in middle-aged (20.26 ± 1.32, n = 7/7 rats/ears, 2-tailed Student t test, $p < 0.0001$) and old rats (13.5 ± 2.72, n = 6/6 rats/ears, 2-tailed Student t test, $p < 0.0001$). By 19–20 months, nearly 53% loss of IHC ribbons and by 6–10 months, nearly 29% loss of IHC ribbons were observed in midbasal turns (Fig. 2B). The reduction of IHC ribbons with age is shown, for example, in a midbasal cochlear turn in a whole-mount preparation, costained for CtBP2 (Fig. 2C, red) and neurofilament NF200 (Fig. 2C, green) as a marker for afferent auditory fibers. As shown in Fig. 2C, a decline in CtBP2-positive dots in old animals coincides with a severe reduction in afferent fibers (Fig. 2B and C). This means that despite normal ABR thresholds, middle-aged and old animals exhibit a reduction of ABR wave I amplitude compared to young rats.

The postulated central compensation of the peripheral deafferentation was analyzed from the amplitudes of the ABR wave IV (Fig. 2D). In contrast to the reduced ABR wave I, amplitudes of ABR wave IV of middle-aged rats remained close to control levels, only reduced for 70–75 dB above threshold (Table 2, 2-tailed Student t test) but not for lower or near threshold stimulus intensities (0–65 dB above threshold, Table 2, 2-tailed Student t test). In contrast, ABR wave IV amplitudes of old rats remained significantly lower over the stimulus range from 20 to 80 dB above threshold compared with young rats (Fig. 2D, Table 2, 2-tailed Student t test) and 15–80 dB above threshold compared with middle-aged rats (Fig. 2D, young: n = 10/19 rats/ears; middle-aged, n = 24/48 rats/ears; old, n = 12/23 rats/ears; 2-way ANOVA, young vs. middle-aged $p < 0.0001$; young vs. old $p < 0.0001$; middle-aged vs. old $p < 0.0001$, Table 2, 2-tailed Student t test). To further explore the central compensation of neuronal responsiveness after reduced peripheral input, we calculated the ratio of ABR wave IV to I amplitude for individual ears (Fig. 2E). It became evident that compared to young and old rats, middle-aged animals had disproportionately higher ABR wave IV amplitudes relative to the reduced ABR wave I, especially at higher stimulus levels (greater 65 dB, Table 3, 2-tailed Student t test). For lower stimulus levels (until 35 dB), ABR wave IV/I ratio was increased in middle-aged rats compared with young rats and was similar to old animals

Table 1
Auditory brainstem response wave I amplitudes for young, middle-aged, and old rats evoked by click stimuli

dB	−10	−5	0	5	10	15	20	25	30	35	40	45	50	55	60
Young versus aged	0.9306	1.8227	0.7998	2.2283	1.7421	0.8878	0.0184	0.0138	0.0052	0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	*	*	**	***	***	***	***	***	***
Young versus old	1.5637	0.1017	0.5252	1.8579	0.8867	0.2641	0.0129	0.0119	0.0006	0.0001	<0.0001	<0.0001	<0.0001	<0.0001	0.0003
	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	*	*	***	***	***	***	***	***	***
Aged versus old	1.2657	0.7310	1.5069	0.9799	2.2505	1.9802	2.7410	2.4812	1.0366	1.4144	1.1067	0.4130	0.6616	1.5256	2.6419
	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.

Bonferroni-Holms corrected p values, ***$p < 0.001$, **$p < 0.01$, and *$p < 0.05$ (2-sided t test).
Key: n.s. not significant.

Table 2
Auditory brainstem response wave IV amplitudes for young, middle-aged and old rats evoked by click stimuli

dB	−10	−5	0	5	10	15	20	25	30	35	40	45	50	55	60
Young versus aged	2.1197	1.0091	0.0589	0.4413	1.7658	2.5176	0.9710	1.5098	1.9367	1.7626	0.5396	1.0286	0.5136	0.3754	0.4213
	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
Young versus old	1.1131	0.2427	0.2695	0.3481	0.7574	0.1746	0.0047	0.0003	0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	**	***	***	***	***	***	***	***	***
Aged versus old	0.9330	1.3757	1.1828	0.9448	1.0924	0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
	n.s.	n.s.	n.s.	n.s.	n.s.	***	***	***	***	***	***	***	***	***	***

Bonferroni-Holms corrected p values, ***$p < 0.001$, **$p < 0.01$, and *$p < 0.05$ (2-sided t test).
Key: n.s. not significant.

Table 3
Auditory brainstem response wave IV/I ratio for young, middle-aged and old rats evoked by click stimuli

dB	15	20	25	30	35	40	45	50	55	60	65	70	75	80
Young versus aged	0.5875	0.0631	0.0637	0.0606	0.0139	0.0143	0.0364	0.0532	0.0020	0.0716	0.0013	0.0027	0.0100	0.0712
	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	*	*	n.s.	n.s.
Young versus old	2.2393	1.7119	1.5367	0.9180	0.3284	0.3423	0.5230	0.4819	0.3931	2.5480	2.5605	0.9060	2.4441	1.7803
	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
Aged versus old	0.7983	0.8653	1.2205	0.9059	1.2538	1.2943	1.1302	1.0364	0.3950	0.1440	0.0005	0.0003	0.0030	0.0126
	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	***	***	**	*

Bonferroni-Holms corrected p values, ***$p < 0.001$, **$p < 0.01$, and *$p < 0.05$ (2-sided t test).
Key: n.s. not significant.

(Fig. 2E; 2-way ANOVA, young vs. middle-aged $p < 0.0001$; young vs. old $p = 0.0006$; middle-aged vs. old $p < 0.0001$, Table 3, 2-tailed Student t test).

This indicates that middle-aged but not old animals are able to centrally compensate for reduced cochlear input through increased central neuronal gain.

3.3. Cochlear synaptopathy in young animals after acoustic trauma is comparable to age-dependent cochlear synaptopathy

We next aimed to test the consequences of acute synaptopathy on the hearing function of rats at different ages. Young, middle-aged, and old animals were exposed to acoustic overstimulation paradigms expected to induce acute synaptopathy with a "reversible" threshold shift (8–16 kHz, 100 dB SPL, 2 hours). Accordingly, 2 and 4 weeks after acoustic exposure, DPOAEs amplitude I/O function evoked by stimulus f2 = 11.3 kHz was not reduced (data not shown). After exposure, in young rats, cochlear damage was indicated by reduced ABR wave I amplitudes (Fig. 3A, repeated measures ANOVA, $p = 0.0394$) and reduced numbers of IHC ribbons in midbasal (Fig. 3D, 2-tailed Student t test, $p = 0.00068$) and basal (not shown) cochlear turns. In contrast, in middle-aged (Fig. 3B, repeated measures ANOVA, $p = 0.6679$) and old animals (Fig. 3C, repeated measures ANOVA, $p = 0.0733$), the ABR wave I amplitude was not further reduced through acoustic trauma, indicating that the aging process had already reduced those fibers that are damaged through noise in young animals. In line with this, synaptopathy in middle-aged and old animals, as indicated by reduced IHC ribbons in midbasal cochlear turns, was not different for sham-exposed and noise-exposed animals (Fig. 3D, 2-tailed Student t test, middle-aged $p = 0.2094$; old $p = 0.0566$). In the midbasal cochlear turn, the number of IHC ribbons in noise-exposed young animals was reduced to the level of sham-exposed middle-aged animals (Fig. 3D, 2-tailed Student t test, $p = 0.7695$) but was still different from IHC ribbon number in old animals (Fig. 3D, 2-tailed Student t test, $p = 0.0020$). Consistent with this, a reduction of IHC ribbons after noise, shown for a midbasal cochlear turn in a whole-mount preparation, costained for CtBP2 (Fig. 3E, red) and NF200 (Fig. 3E, green), resembled that of a middle-aged animal (compare Figs. 2C and 3E).

Overall, these results may indicate that acute synaptopathy weeks after moderate acoustic trauma in young animals leads to a decline of auditory fibers in cochlear regions that are also preferentially sensitive for auditory fiber loss over age.

Amplitudes of ABR wave IV were analyzed in the same exposed young, middle–aged, and old animal groups to assess the central brainstem responses (Fig. 3F–H). The significant reduction of ABR wave I amplitudes in noise-exposed young rats elicited a central response of significantly increased midbrain activity (Fig. 3F). Here, for high stimulus intensity (65 dB re threshold), the ABR wave IV amplitudes after noise exposure even exceeded the ABR wave IV amplitudes before exposure (Fig. 3F, repeated measures ANOVA,

$p = 0.0139$, 2-tailed paired Student t test, $p = 0.0088$). This suggests that in young animals, a robust neuronal gain can enhance brainstem responses after moderate acoustic trauma. In contrast, in middle-aged and old animals (Fig. 3G and H), the acoustic trauma that did not reduce cochlear neuronal response (Fig. 3B) did not further influence ABR wave IV amplitudes (middle age: Fig. 3G, repeated measures ANOVA, $p = 0.0580$; old age: Fig. 3H, repeated measures ANOVA, $p = 0.7114$).

This indicates that moderate acoustic trauma will not further reduce ABR wave I or ABR wave IV amplitudes when the aging process alone has already largely diminished ABR wave I, and the central compensation has already reached its maximum magnitude.

We used early or late ABR wave intensity-latency function to predict auditory nerve or central brainstem deficits. Peak latencies for ABR wave I in middle-aged animals were slightly shortened (Fig. 4A, 2-way ANOVA, $p < 0.0001$), whereas regression analysis of the ABR wave I latency dynamic range did not show age-related change in the slope (Fig. 4A, young: -8.8 μs/dB, middle-aged: -8.4 μs/dB, ANCOVA, young vs. middle-aged $p = 0.222$). Moreover, latencies for wave IV were profoundly shortened in middle-aged rats at all stimulus levels compared with young (Fig. 4B, 2-way ANOVA, $p < 0.0001$, Table 4, 2-tailed Student t test) and at stimulus levels above 10 dB compared to old (Fig. 4B, 2-way ANOVA, $p < 0.0001$, Table 4, 2-tailed Student t test). This suggests that auditory fiber loss with age does not necessarily lead to prolonged central ABR peak latencies.

In old animals, peak latency for ABR wave I was rather prolonged (Fig. 4A, 2-way ANOVA, young vs. old $p = 0.0177$; middle-aged vs. old $p < 0.0001$), whereas regression analysis of the latency dynamic range did not show a change in slope for wave I latency growth functions (Fig. 4A, young: -8.8 μs/dB, old: -8.5 μs/dB, ANCOVA, young vs. old $p = 0.371$; middle-aged vs. old $p = 0.7946$). The slope of wave IV latency in old animals was significantly different than that in young and middle-aged rats (Fig. 4B, young: -10.6 μs/dB, middle-aged: -9.8 μs/dB, and old: -6.6 μs/dB, ANCOVA, young vs. middle-aged $p = 0.5943$; young vs. old $p = 0.01947$; middle-aged vs. old $p = 0.04997$). This suggests a central, temporal, and processing deficit in old animals.

Auditory steady state responses were used to investigate the temporal capacity of auditory neurons in the auditory pathways up to the cortex. For stimulation, a pure tone carrier of 8 kHz was amplitude-modulated with modulation frequencies between 128 Hz and 1024 Hz. The best modulation frequency eliciting maximal responses at 20 dB sensation level was similar in young, middle-aged, and old rats, that is, 362 Hz. However, the modulation index at 362 Hz, as a measure for response gain, showed a slight, yet insignificant reduction in old rats compared with young and middle-aged (Fig. 4C, 1-way ANOVA, $p = 0.4267$). To test the sensitivity for amplitude-modulated tones at a fixed modulation frequency of 512 Hz at 20 dB above threshold, responses to modulation depths of 100%–0% (unmodulated) were recorded.
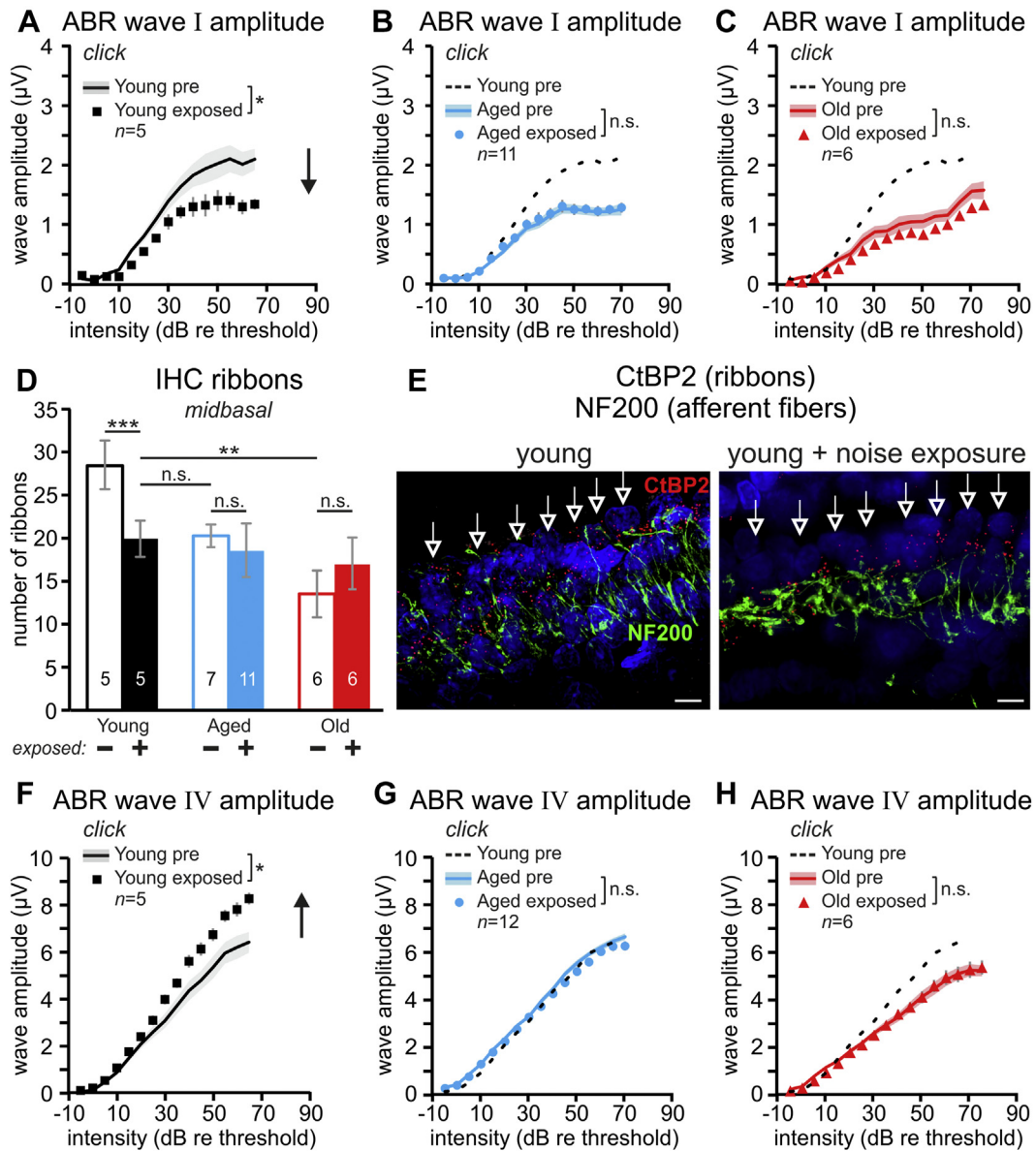
Fig. 3. Cochlear synaptopathy in young rats after acoustic trauma is related to age-dependent cochlear synaptopathy; central gain after noise-induced cochlear synaptopathy is lost with age. (A) Mean ± standard error of the mean (SEM) click-evoked ABR wave I amplitudes are significantly reduced in young rats after noise exposure (black squares) compared with preexposure (black line and gray area). (B) Amplitudes of middle-aged rats postexposure ("aged", blue circles) were not different from amplitudes preexposure (blue line and area). (C) Amplitudes of old rats preexposure (red line and area) were not different from amplitudes postexposure (red triangles). (D) Mean ± standard deviation ribbon counts from midbasal cochlear regions in sham- (open bars) or noise-exposed (filled bars) rats of young, middle-aged, and old rats. Numbers of animals per group are given in the bars. (E) Whole-mount preparation of the midbasal cochlear turn in young and noise-exposed young rats labeled with antibodies for CtBP2/RIBEYE (red) and NF200 (green). Cell nuclei were counterstained with 4′,6-Diamidin-2-phenylindol (DAPI) (arrows). Scale bars, 5 μm. (F) Mean ± SEM click-evoked ABR wave IV amplitudes in young rats preexposure (black line and gray area) were significantly enhanced after noise exposure (black squares). (G) Amplitudes of middle-aged rats before exposure ("aged pre", blue line and area) were not significantly different after noise exposure ("aged exposed", blue dots) or from young rats preexposure (black dots; 2-way analysis of variance [ANOVA], $p = 0.3571$). (H) Amplitudes of old rats after noise exposure (red triangles) were not significantly different from before exposure (red line and area), but both were significantly different from amplitudes of young rats preexposure (black dots; 2-way ANOVA, $p < 0.0001$). *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; n.s. not significant. Abbreviations: ABR, auditory brainstem response; IHC, inner hair cell; n.s., not significant.

Response thresholds to modulated stimuli were defined as the lowest modulation depth where the signal exceeded the 85% CI of the noise level. We found a tendency for higher thresholds with age although this difference cannot be statistically evaluated (Fig. 4D, young: 17%, middle-aged: 25%, old: 35%). The response strength increased with modulation depth in all age groups, but linear regression showed that the slope significantly decreased only in the last third of life (Fig. 4D, young: 0.9119, middle-aged: 0.8951, old: 0.5877, ANCOVA, young vs. middle-aged $p = 0.8957$; young vs. old $p = 0.04242$; middle-aged vs. old $p = 0.06355$).

Our data indicate that in middle-aged animals with otherwise normal-hearing thresholds, the capacity to generate synchronous discharge phase-locked to the modulation frequency of tonal stimulation is as good as in young animals, despite significantly reduced ABR wave I amplitudes linked to IHC ribbon loss in middle age. Responses to temporally modulated stimuli of old animals can still follow fast amplitude-modulated stimuli, but in contrast to middle-aged animals, sensitivity is partly lost and response strength is reduced. This coincides with a change in response latency of centrally generated ABR wave IV.
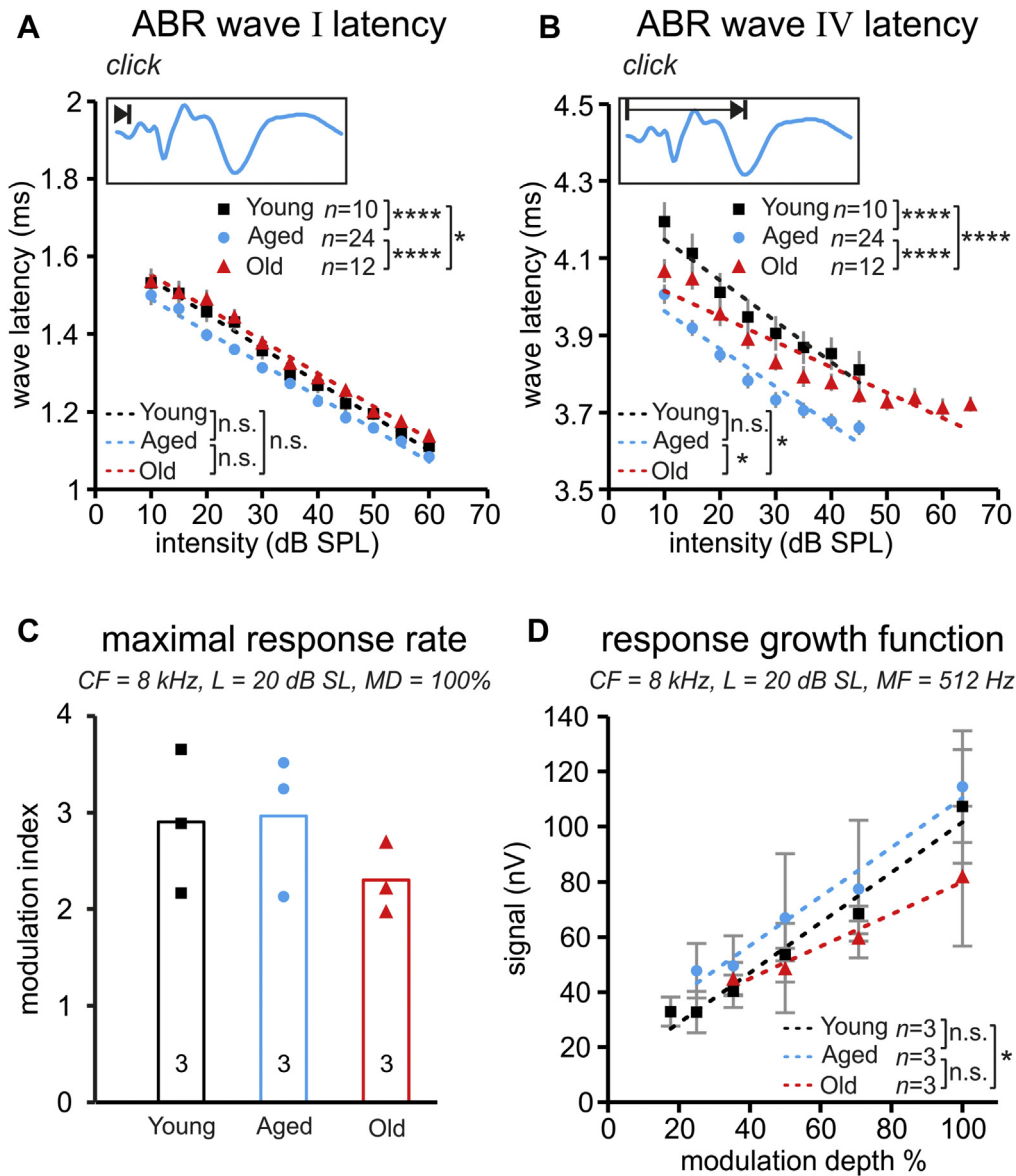
Fig. 4. Age-related decline in temporal resolution mainly occurs in the last third of life. (A) Mean ± standard error of the mean (SEM) ABR wave I latency growth functions in young (black squares), middle-aged ("aged", blue circles), and old rats (red triangles). ABR wave I latencies were decreased in middle-aged and increased in old rats compared to young. The slopes of the linear regression (dotted lines) showed no age-related differences (young: $Y = -0.0088 \times X + 1.584$, $r^2 = 0.992$; middle aged: $Y = -0.0084 \times X + 1.532$, $r^2 = 0.994$; and old: $Y = -0.0085 \times X + 1.595$, $r^2 = 0.991$). Inset: ABR waveform, indicating the leading negative peak of wave I 40 dB above the hearing threshold. (B) Mean ± SEM ABR wave IV latencies were decreased in middle-aged ("aged", blue circles) and old rats (red triangles) compared with young (black squares). The slope of the linear regression (dotted lines) was less steep with age (young: $Y = -0.0106 \times X + 4.202$, $r^2 = 0.937$; middle-aged: $Y = -0.0098 \times X + 4.012$, $r^2 = 0.941$; and old: $Y = -0.0066 \times X + 4.049$, $r^2 = 0.870$). Inset: ABR waveform, indicating the leading negative peak of wave IV 40 dB above the hearing threshold. (C) Modulation index (ratio of maximal signal and the baseline) of individual young (black squares), middle-aged ("aged", blue circles), and old rats (red triangles). n = 3 rats. Amplitude-modulated stimuli were presented with carrier frequency (CF) 8 kHz at 100% modulation depth (MD) between 128 and 1024 Hz, in half-octave steps at a sensation level (L) of 20 dB SL. Mean modulation index (open bars) was slightly reduced in old rats. (D) Mean ± standard deviation response growth functions to stimuli with increasing modulation depths in young rats (black squares), middle-aged ("aged", blue circles), and old rats (red triangles). Amplitude-modulated stimuli were presented at 512 Hz modulation frequency (MF). Response thresholds to modulated stimuli increased with age, whereas the slope of the amplitude modulation growth function determined by linear regression for above-threshold responses (dotted lines) was decreased in old rats (young: $Y = 0.9119 \times X + 10.46$, $r^2 = 0.969$; middle-aged: $Y = 0.8951 \times X + 20.89$, $r^2 = 0.968$; and old: $Y = 0.5877 \times X + 21.22$, $r^2 = 0.970$). $*p < 0.05$; $****p < 0.0001$; n.s. not significant. Abbreviations: ABR, auditory brainstem response; SL, sensation level.

3.4. Conclusion

In young animals, acoustic trauma reduces ABR wave I amplitudes (Fig. 5C, wave I), likely through a preferential loss of low-SR, high-threshold cochlear nerve fibers (Furman et al., 2013). This enables a robust enhancement of brainstem responses through neuronal gain, resulting in restored or overamplified midbrain responses (Fig. 5C, wave IV). In the second third of the lifespan (middle-aged animals), the neuronal responses of IHCs are already reduced (Fig. 5B, wave I) and thereby become insensitive to further damage through moderate acoustic trauma (Fig. 5D, wave I). Nevertheless, in these middle-aged animals, reduced cochlear input can still produce nearly normal sound-induced midbrain activities through neuronal gain (Fig. 5D, wave IV). In the last third of life (old animals), the neuronal responses of IHCs are slightly more reduced than those in middle-aged animals (Fig. 5B, wave I), confirming progressive neuronal degeneration during aging (Sergeyenko et al., 2013). Similar to the middle-aged group, in the

Table 4
Auditory brainstem response wave IV latencies for young, middle-aged and old rats evoked by click stimuli

dB	10	15	20	25	30	35	40	45
Young versus aged	0.0013 **	0.0007 ***	0.0010 **	0.0012 ***	0.0009 ***	0.0012 ***	0.0005 ***	0.0008 ***
Young versus old	0.2444 n.s.	0.7504 n.s.	0.3523 n.s.	0.5749 n.s.	0.7315 n.s.	0.6637 n.s.	0.7837 n.s.	0.8135 n.s.
Aged versus old	0.1519 n.s.	0.0054 **	0.0205 *	0.0172 *	0.0175 *	0.0278 *	0.0182 *	0.0243 *

Bonferroni-Holms corrected p values, ***$p < 0.001$, **$p < 0.01$, and *$p < 0.05$ (2-sided t test).
Key: n.s. not significant.

old group, an insensitivity of ABR wave I (Fig. 5E, wave I) to moderate acoustic trauma points to a previous loss of noise-vulnerable auditory fibers by that age. In contrast to the middle-aged group, however, the old group has lost the capability to compensate for the reduced cochlear input to regain brainstem responses (Fig. 5E, wave IV). Note that in middle-aged animals, normal sound-induced midbrain activities (Fig. 5B, D, wave IV) are associated with normal responses to fast amplitude-modulated stimuli, whereas in old animals, reduced sound-induced midbrain activities (Fig. 5B, E, wave IV) result in reduced temporal sensitivity.

4. Discussion

Age-related hearing loss is particularly accompanied by poor speech discrimination, specifically in background noise [see (Ouda et al., 2015) for a review]. This cognitive limitation has been thought to reflect auditory processing deficits at the level of the central auditory pathway (Herman et al., 1977; Konkle et al., 1977). However, recent studies suggest that cochlear neuropathy is also causal of poor speech discrimination by reduced encoding precision of suprathreshold sound (Bharadwaj et al., 2014). This hypothesis was based on the findings that in humans and animals, the temporal precision of the early auditory representation can be poor even when hearing thresholds seem normal (Bharadwaj et al., 2014). Here, we demonstrate in an animal model that middle-aged rats, in contrast to old rats, with normal hearing thresholds, have the auditory neuron capacity to generate synchronous activity and therefore normal temporal precision (as determined by ASSRs), despite progressing auditory cochlear neuropathy (as determined by number of IHC ribbons and ABR wave I amplitudes). This cochlear neuropathy particularly occurred in the midbasal cochlear turn, the region of maximal sound sensitivity in the rodent cochlea (Meyer et al., 2009) that conveys detection thresholds (Heil et al., 2008) and defines cortical best-frequency regions (Montgomery and Wehr, 2010). We thus may suggest that as long as the central neuronal gain can compensate for the deprived auditory input from behaviorally relevant frequency regions, temporal resolution deficits are marginal.

4.1. Old but not middle-aged animals develop OHC dysfunction

In the present study, we observed that in rats, the DPOAE I/O function emission amplitudes changed little over the first two-thirds of life, with largely similar function in young and middle-aged animals for behaviorally relevant stimuli (Hage and Ehret, 2003). A significant rightward shift in DPOAE I/O emission amplitude function was observed mainly toward the last third of life (>19 months) pointing to OHC dysfunction, which is also
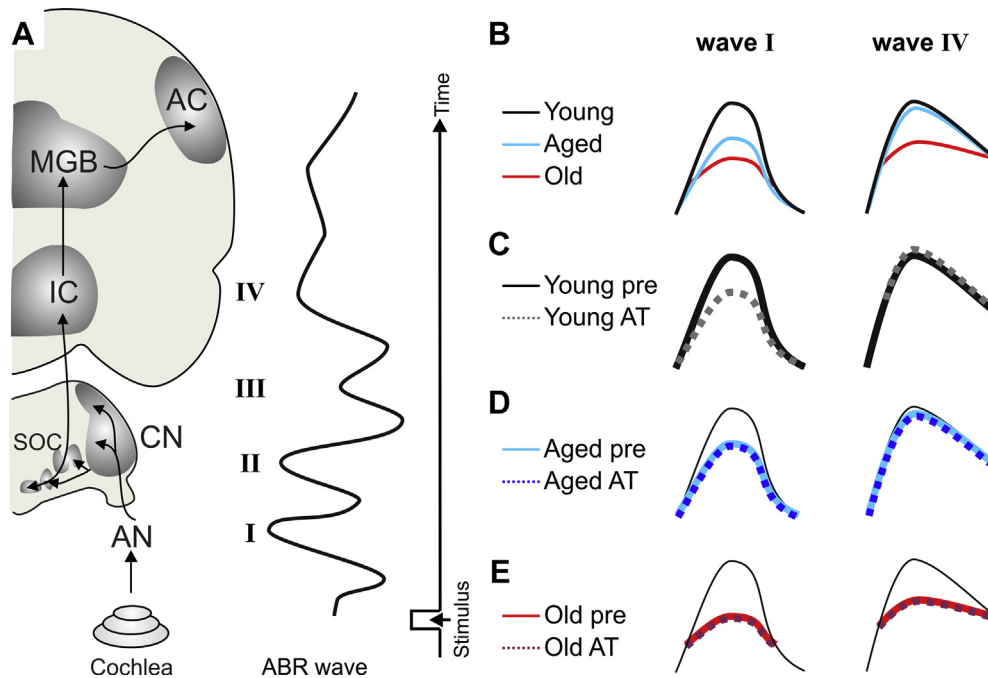

Fig. 5. Auditory brainstem responses in young, middle-aged, and old animals before and after acoustic trauma. (A) The auditory signal along the auditory pathway can be measured by auditory brainstem responses (ABRs), providing information regarding auditory function and hearing sensitivity. The different peaks of the ABR wave can be assigned to different parts of the ascending auditory pathway. Wave I is generated by the auditory nerve (AN), wave II by the cochlear nucleus (CN), wave III by the superior olivary complex (SOC), and wave IV by the lateral lemniscus and inferior colliculus (IC). (B) Middle-aged and old animals have reduced ABR waves I compared with young animals, whereas ABR wave IV is reduced in old but not in middle-aged animals. (C) Although ABR wave I is reduced in young animals after acoustic trauma (AT), ABR wave IV is not reduced. (D) ABR wave I in middle-aged animals is already reduced before AT (comparable with ABR wave I of young animals after AT) and is insensitive to further damage by acoustic trauma. ABR wave IV is normal before AT and also not reduced after AT (comparable with young animals before AT). (E) In old animals, before AT, ABR wave I is slightly more reduced than that in middle-aged animals, but ABR wave IV is strongly reduced. Comparable with the middle-aged group, both ABR waves are insensitive to further damage through acoustic trauma. Abbreviations: AC, auditory cortex; MGB, medial geniculate body.

responsible for a shift in click stimulus–evoked ABR threshold. This is in line with previous studies that describe OHC function loss in the last third of life in rodents (Altschuler et al., 2015; Fernandez et al., 2015; Rüttiger et al., 2013; Sergeyenko et al., 2013). Also in humans, the most profound elevation of hearing thresholds occurs in the last third of the lifespan above 65 years of age (Frisina, 2009; Gordon-Salant, 2005; WHO, 2012).

4.2. Middle-aged and old animals exhibit cochlear neuropathy

A pronounced cochlear neuropathy, here defined as loss of IHC ribbons in higher frequency cochlear turns, and a reduction of the summed response of the auditory nerve (ABR wave I amplitude) was found in middle-aged and old rats. Unlike old animals, middle-aged animals still had normal hearing thresholds. This shows that the cochlear neuropathy develops far before the deterioration of OHC function, confirming findings of normal hearing thresholds with reduced auditory input in aged mice (Sergeyenko et al., 2013) and gerbils (Schmiedt et al., 1996). A comparable degree of cochlear neuropathy and IHC synaptopathy was achieved in young rats by exposing them to moderate acoustic trauma (Fig. 3), without reducing their OHC responses, reinforcing previous findings of auditory neuropathy independent of auditory threshold shift after moderate trauma (Furman et al., 2013; Kujawa and Liberman, 2009). Swelling of NF200 fibers in young animals after moderate acoustic trauma (Fig. 3E) mirrored the observed morphologic correlate of acute excitotoxic events (Ruel et al., 2007).

Neurophysiological studies on age-related hearing loss in gerbils (Schmiedt et al., 1996) or mice (Sergeyenko et al., 2013), but also of normal hearing noise-exposed mice (Furman et al., 2013), suggest that the auditory neurodegeneration is selective for low-SR, high-threshold fibers. These low-SR, high-threshold fibers (~40%) are known to have a higher vulnerability to noise-induced trauma (Furman et al., 2013; Heinz and Young, 2004; Heinz et al., 2005; Ruel et al., 2008) and, unlike the loss of high-SR, low-threshold fibers, their decline would leave auditory thresholds intact (Furman et al., 2013; Kujawa and Liberman, 2009; Sergeyenko et al., 2013). Regarding these aspects, IHC synaptopathy in combination with a normal hearing threshold as observed in middle-aged animals might already support the preferential loss of low-SR, high-threshold fibers. This hypothesis is further corroborated by a failure of moderate acoustic trauma to extend the ABR wave I amplitude reduction in middle-aged animals. Here, the noise-vulnerable auditory fibers have obviously already been lost through the aging process. In addition, also in old animals, moderate acoustic trauma does not further diminish the number of IHC ribbons or ABR wave I amplitude, which might suggest that noise vulnerability of auditory fibers is reduced with aging or has already reached saturation point.

4.3. Middle-aged but not old animals can centrally compensate for cochlear neuropathy

Despite only small differences in reduced ABR wave I amplitudes for click stimuli between middle-aged and old animals, central ABR wave IV amplitudes were strongly different. In middle-aged animals only, ABR wave IV amplitudes were nearly completely restored despite the reduced ABR wave I amplitudes. This may result from renormalization of neuronal response magnitude within the brainstem after reduced neuronal output from the cochlea in middle-aged animals. Clearly, this process is no longer effective in old animals. Compensating central activity after cochlear damage has been observed in humans (Gu et al., 2012; Schaette and McAlpine, 2011) and animals (Heeringa and van Dijk, 2014; Rüttiger et al., 2013; Singer et al., 2013). The earliest site at which the central compensation of reduced mean auditory nerve activity can occur is the target

cells of the auditory nerve fibers in the ventral cochlear nucleus (VCN) or the dorsal cochlear nucleus (DCN) in the brainstem (Brigande and Heller, 2009). Various studies have reported increased spontaneous and evoked activity in both brainstem areas after auditory trauma (Cai et al., 2009; Dehmel et al., 2012; Gröschel et al., 2011, 2014; Kaltenbach, 2007; Middleton et al., 2011; Salvi et al., 2000; Vogler et al., 2011; Wang et al., 2009).

The generation of ABR waves is dominated by the response of spherical or globular bushy cells in the VCN (wave II). From there, the response propagates to the ascending auditory nuclei (Melcher and Kiang, 1996). Therefore, altered VCN activity after acoustic trauma is expected to modify ABR wave IV amplitudes. Following this idea, previous studies used auditory nerve and brainstem responses to assess compensating central activity after cochlear damage in young animals (Chumak et al., 2015; Rüttiger et al., 2013; Singer et al., 2013; Zuccotti et al., 2012, 2013). The elevated ABR wave IV amplitude in middle-aged animals (Fig. 2) likely mirrors VCN output activities. From a computational model, it was suggested that the enhanced neuronal activity after auditory input deprivation critically depends on the response characteristics of the remaining afferent fibers. These are the fibers with a higher discharge rate, which is important, because otherwise the generation of a sufficient increase in discharge rate to compensate for deprived auditory input may be hampered (Schaette and Kempter, 2009, 2012). Taking this into account, we propose a preferential loss of low-SR, high-threshold fibers in middle-aged rats, which still allows central neuronal gain generation. In further support, the disproportionately elevated ABR wave IV amplitude in middle-aged rats that coincided with shorter evoked response latencies (Fig. 4) can be taken as evidence of central gain. Shortened response latencies, which might be associated with enhanced firing rates, were also observed in previous studies in aged gerbils with normal thresholds most pronounced for ABR wave IV latencies (Boettcher et al., 1993). A reduced evoked response latency may be a direct consequence of neuronal gain and higher firing rates in neurons due to the decrease in inhibition (Caspary et al., 1995; Heeringa and van Dijk, 2014; Levakova et al., 2015).

Despite minor differences between middle-aged and old animals in their degree of reduced auditory nerve response (wave I amplitude), old animals had a comparably larger decline in ABR wave IV amplitude response. In addition, in contrast to middle-aged rats, old rats showed rather prolonged ABR wave IV latencies (Fig. 3). Indeed, a delay in evoked ABR wave responses is a typical feature of old rats (Backoff and Caspary, 1994), see (Frisina, 2001) for a review. An increase of late ABR wave latency could either be due to a critical loss of high-SR, low-threshold fibers in the periphery (Chumak et al., 2015; Knipper et al., 2015) or be due to a generally lower capacity of the brain to increase neuronal gain in response to the reduced cochlear output.

4.4. Old but not middle-aged animals exhibit temporal precision deficits despite cochlear neuropathy occurring at both ages

Damage to lower-SR auditory fibers is thought to be particularly detrimental to suprathreshold coding of sound envelopes, as high-SR fibers cannot robustly encode envelope timing cues in sounds at comfortable listening levels (Bharadwaj et al., 2014). As indicated above, middle-aged animals mainly lose low-SR, high-threshold fibers. Surprisingly, their responses to amplitude-modulated stimuli were similar to the responses of young animals. This may indicate that suprathreshold temporal coding may be undisturbed with age as long as sufficient central neuronal gain is generated, even with a remarkable loss of low-SR, high-threshold fibers.

Only in old animals, but not middle-aged, the modulation index as a measure for response gain was slightly reduced, along with a

significantly shallower slope of the amplitude modulation growth function to stimuli with increasing modulation. This age-related decrease in the detection of amplitude-modulated tones likely indicates a severe disturbance in encoding envelope timing, as also suggested for older human listeners who exhibit significant age-related deficits in the detection of sinusoidal amplitude modulation imposed on pure-tone (He et al., 2008) or noise carriers (Kumar and Sangamanatha, 2011; Takahashi and Bacon, 1992). However, further studies are indicated to investigate why changes in temporal processing in the aging human auditory system already start at middle age (Snell and Frisina, 2000). Dependent on the animal species and its lifespan, remarkable differences in the capacity of the brain to maintain adaptive neuronal gain are to be expected. A behavioral approach with a rodent animal model may be appropriate to measure temporal resolution of auditory processing and study the mechanisms of poor speech recognition in aged human listeners.

In conclusion, the present study suggests that age-dependent cochlear synaptopathy may not per se lead to the loss of central responsiveness and impaired temporal coding. This supports previous suggestions that age-related immunohistochemical changes in central (subcortical) structures, which are probably associated with age-related deterioration in the processing of the temporal parameters of acoustic stimuli, do not exclusively depend on peripheral deafferentation, but are rather of central origin (Ouda and Syka, 2012; Šuta et al., 2011). As long as central homeostatic processes successfully drive compensatory network activity, central auditory brainstem response and temporal encoding may be restored. Because encoding envelope timing cues are essential for maintaining speech intelligibility in noise (Bharadwaj et al., 2014; Costalupes et al., 1984; Lorenzi and Moore, 2008), speech in noise deficits in the elderly may be linked to a decline in the capacity to generate central compensatory gain.

Therapeutic interventions for maintaining speech intelligibility in noise over age may therefore focus on maintaining key players essential for homeostatic scaling and neuronal gain. For example, an age-dependent reduction in the activation pattern of key regulators of homeostatic adaptation processes, e.g., brain-derived nerve growth factor, has been found in brains of elderly people (Navarro-Martinez et al., 2015). In this context, one of the rare genes that have achieved genome-wide significance for presbyacusis and speech detection in older adults is the gene encoding the metabotropic glutamate receptor 7 that was shown to mediate excitatory synaptic neurotransmitter signaling and plasticity in the mammalian brain (Newman et al., 2012). The generation of compensating central gain may also be different between males and females due to predicted sex hormone–dependent auditory plasticity [see (Canlon and Frisina, 2009) for review]. Therefore, future research is essential to explore possible gender-related differences in age-related hearing loss and central auditory plasticity that were not regarded in the present study.

A prerequisite for successful treatment to preserve hearing ability in the aging population is early and accurate diagnosis [see (Steel, 2000) for a review]. Regarding the findings in the present study, there is the need to establish and refine diagnostic tools for early detection of cochlear synaptopathy, deficits in central neuronal gain, as well as temporal processing deficits—independent of elevated hearing thresholds in humans.

Disclosure statement

The authors have no actual or potential conflicts of interest.

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