Tinnitus development is associated with synaptopathy of inner hair cells in Mongolian gerbils

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Abstract

Human hearing loss is often accompanied by comorbidities like tinnitus. This perception of phantom sounds without external stimulation is affecting up to 15% of the adult population and can lead to psychological disorders like depression. Animal studies in rodents could show that tinnitus may not only be a result of hearing loss due to cochlear hair cell damage but can also be a consequence of synaptopathy at the inner hair cells already induced by moderate sound traumata (hidden hearing loss).

In this study we investigate the synaptopathy previously shown in mice in our animal model, the Mongolian gerbil, and relate it to behavioral signs of tinnitus in this species. Tinnitus was induced by a mild monaural acoustic trauma leading to a monaural noise induced hearing loss in the animals quantified by auditory brainstem response audiometry. Behavioral signs of tinnitus percepts were detected by measurement of pre-pulse inhibition of the acoustic startle response in a gap-noise paradigm in about two-thirds of the animals. 14 days after trauma the cochleae of traumatized and non-traumatized ears were isolated and inner hair cell synapses were counted within several spectral regions of the cochlea. In the here presented preliminary data inner hair cell synaptopathy was only found in animals with behavioral signs of tinnitus, independent of type of hearing loss. On the other hand, animals with apparent hearing loss but without behavioral signs of tinnitus showed a reduction in amplitudes of auditory brainstem response waves I/II (cochlear nerve and dorsal cochlear nucleus) but no significant changes in the number of synapses at the inner hair cells. We conclude – in line with the literature – that hearing loss is caused by damage to the inner and / or outer hair cells but that the development of tinnitus, at least in our animal model is closely linked to synaptopathy at the inner hair cells.

Introduction

Around 10 to 15% of the general adult population suffer from unremitting central tinnitus of some kind [1]. This phantom sound condition can lead to insomnia, psychological disorders or, for the most severe cases, even suicide [2-4]. The exact mechanism and the origin of this maladaptive process within the auditory pathway are still under debate [5-11], but most researchers consider malfunction of the inner ear – caused by presbyacusis, injuries or noise traumata – to be the primary cause for the development of central tinnitus. On the other hand, also patients and animals without a measurable hearing loss can perceive such an auditory mispercept, indicating also a role of neuropathic deafferentation in its development [12-14]

Currently, it is still unknown why some patients with or without hearing loss do develop chronic tinnitus while others with comparable hearing impairments do not, although first results and models have been published trying to solve this riddle [8-10, 15, 16]. In mice it has been shown that a relatively mild acoustic trauma (8–16 kHz octave-band noise at 98 dB SPL for 2 h) may lead to a massive loss of synapses (= synaptopathy) at the inner hair cells (IHC) forming the afferent output of the cochlea, and subsequently to a deafferentation (= neuropathy) of the receptor epithelium [17]. This synaptic loss recently has been termed hidden hearing loss [10], and has been investigated in the context of tinnitus in mice [14] and rats [9]. In their study, although conducted with a relatively low spectral resolution comprising just the two frequencies 11.3 kHz and 32 kHz, Hickox and Libermann found tinnitus percepts – based on the change of the prepulse inhibition (PPI) of the acoustic startle response – only in normal hearing but neuropathic animals but not in those without neuronal loss. Consistently, Rüttiger and colleagues were able to show a significant reduction of IHC synapses after a mild mid-frequency trauma (120 dB SPL at 10 kHz for 1 h) in high-frequency regions of the cochlea only in animals that subsequently developed a tinnitus percept (based on foraging behavior for sugar water during sound presentation) but did not report a hearing loss in these animals.

In this preliminary report we investigated mild trauma (115 dB SPL at 2 kHz for 75 min) induced synaptopathy at IHCs and auditory brainstem response (ABR) wave changes with high spectral resolution (1 to 16 kHz in octave steps) in Mongolian gerbils with or without behavioral signs of tinnitus, as assessed by PPI of the acoustic startle response [15, 18, 19], and with or without permanent noise induced hearing loss. In our animal model we found a clear link between tinnitus development and synaptopathy at the IHCs but not between tinnitus development and the hearing loss that in turn can be related to IHC and / or outer hair cell (OHC) damage [20].

Material and Methods

Ethical statement

Mongolian gerbils (*Meriones unguiculatus*) were housed in standard animal racks (Bio A.S. Vent Light, Ehret Labor- und Pharmatechnik, Emmendingen, Germany) in groups of 2 to 3 animals with free access to water and food at 20 to 24°C room temperature under 12/12 h dark/light cycle. The use and care of animals was approved by the state of Bavaria (Regierungspräsidium Mittelfranken, Ansbach, Germany, No. 54-2532.1-02/13). A total of 11 ten to twelve weeks old male gerbils from Charles River Laboratories Inc. (Sulzfeld, Germany) were used in this study.

Behavioral testing and auditory brainstem responses

All animals were handled before the beginning of the experiments and accustomed to the setup environment to minimize stress. All behavioral and electrophysiological methods used have previously been described in detail [15, 16, 21] and therefore are only briefly summarized here: During the first week, animals were tested first, with a GAP-NOGAP paradigm of PPI of the acoustic startle response [19] to acquire baseline data for later tinnitus percept assessment. Band-pass filtered background noise (60 dB SPL) with center frequencies of 1 kHz, 2 kHz, 4 kHz, 8 kHz and 16 kHz and a half octave width were presented with or without a 20 ms gap 100 ms before a startle stimulus (white noise burst, 20 ms duration, 2 ms $\sin^2$-ramp, 105 dB SPL). Second, frequency-specific ABR

measurements were performed under ketamine-xylazine anesthesia to obtain individual baseline audiograms for stimulation frequencies between 1 and 16 kHz in octave steps for stimulation intensities ranging from 0 to 90 dB SPL in 5 dB steps. The three silver electrodes were placed subcutaneously retroaural above the bulla of the tested ear, central between both ears and at the basis of the tail. The signal was recorded and filtered (bandpass filter 400 to 2000 Hz) via a Neuroamp 401 amplifier (JHM, Mainaschaff, Germany). One week after these measurements a mild acoustic trauma (2 kHz, 115 dB SPL, 75 min) was induced monaurally under deep ketamine-xylacin anesthesia via a Canton Plus X Series 2 placed directly in front of the traumatized ear; the non-traumatized control ear was protected using earplug foam (3M™ earplugs 1110, 3M, Neuss, Germany) adding at least 20 dB attenuation [22]. The extent of the trauma was evaluated by a second ABR measurement 3 to 5 days after the trauma and used to group the animals into those with a significant, permanent hearing loss of at least 10 dB at the traumatized ear (8/11 animals; *apparent hearing loss group*) and those without (3/11 animals; as it turned out that all these 3 animals developed synaptopathy and behavioral signs of tinnitus, this group was classified as *hidden hearing loss group*). In all animals a possible persistent tinnitus percept was identified by a second PPI of acoustic startle response measurement and calculation of the PPI change [16] 13 days after the trauma.

Histology

14 days post trauma, after obtaining all behavioral and electro-physiological data, the animals were sacrificed, and the cochleae of the traumatized and non-traumatized ears were extracted and fixed in 4% formaldehyde for 1 hour. After de-calcification in 0.1 M EDTA for 1-2 days, the cochlear turns were usually cut into 3 pieces and immunostained for synaptic ribbon protein carboxy-terminal binding protein 2 [CTBP2; 23]. Cochlear whole mounts were immunostained overnight at 4°C with primary antibody against CTBP2 (mouse anti-CTBP2 at 1:200; BD Transduction Labs, Heidelberg, Germany). Secondary antibody (donkey anti mouse conjugated with Cy3, 1:400, Dianova, Hamburg, Germany) was applied for 1h at room temperature.

Data evaluation and statistical analysis

The obtained data of the PPI and ABR measurements were evaluated objectively and automatically by custom made Matlab programs (MathWorks, Natick, MA, USA). Tinnitus development was tested for each animal and frequency individually by Kolmogorov-Smirnov tests: A significant decrease ($p<0.05$; Bonferroni corrected) of the PPI- induced change of the response to the acoustic startle response after the trauma (PPI_{post}) relative to before the trauma (PPI_{pre}) in that test was rated as an indication of a tinnitus percept in that specific frequency. With this behavioral approach we are able to group animals independent of any other measurement into those with behavioral signs of a tinnitus percept and those without.

The threshold determination was performed by an automated objective approach using the root mean square (RMS) values of the ABR amplitudes fitted with a sigmoid function utilizing the background activity as offset. The median threshold was set at the level of 5% above offset of this sigmoid fit independent for each frequency and for each time point. For further statistical analysis, the interquartile range of each threshold was estimated by subsampling of the data. Hearing loss was rated based on the pre and post thresholds and individually tested by Mann-Whitney U-tests and Wilcoxon

matched pair tests (Suppl. Fig. 1A) independent of any other measurement.

Peak-to-peak amplitudes of the ABR waves I/II, III, IV and V – corresponding to the activity in the cochlear nerve/dorsal cochlear nucleus, inferior olivary complex/trapezoid body, lateral lemniscus and inferior colliculus [24], respectively – were extracted from measurements at 90 dB SPL, ensuring a valid measurement at all tested frequencies, and analyzed by Kruskal-Wallis ANOVAs and Mann-Whitney U-tests [cf. 16]. RMS values were calculated within a time window from 1 to 7 ms after stimulus onset from the mean ABR signal of each tested frequency and attenuation and analyzed by 2-factorial ANOVAs (as normal distribution can be assumed for this type of data). To rule out any group effects not found in the individual the difference between pre and post trauma data was calculated for each animal and analyzed by 1-factorial ANOVA (Suppl. Fig. 1B).

The number of synapses per IHC was counted by eye, by one investigator without knowledge of the status of the cochlea (traumatized/non-traumatized ear) or any other attribute of the animal: The frequency map of the cochlea from 500 Hz to 16 kHz in half octave steps (Figure 1A, left panel) was created with the software Keyence BZ-II-Analyzer by measuring the relative distances beginning from the apex of the cochlea along the spiral, according to the published cochlear frequency map for the Mongolian gerbil [25]. Full-focus microscopic images in the range of the synapses of the IHCs of the resulting 11 regions from the subjacent inner spiral bundle to the nerve terminal in the supra-nuclear region including all visible synaptic ribbons were obtained using a fluorescent microscope BZ9000 (Keyence, Neu-Isenburg, Germany) with a 40x objective (0.6 numerical aperture) and analyzed using a ImageJ plugin. Labeled synapses of the IHC region were counted for groups of 15 IHC (roughly equivalent 200 Hz frequency range; Figure 1A, right panel), according to the matching regions of the frequency map. The obtained number of synapses was evaluated separately for tinnitus and non-tinnitus groups, as well as for trauma and control side counts (Figure 1B). Subsequently, all statistical analyses of the synapse counts were performed by Kruskal-Wallis ANOVAs in Statistica (StatSoft, Hamburg, Germany).

Results – Preliminary data

Development of NIHL and tinnitus

11 animals were traumatized monaurally (cf. Methods). Development of a significant noise induced hearing loss (in the following termed “apparent hearing loss”) was checked by ABR measurements 3 to 5 days after the trauma on the trauma side, and development of behavioral signs of a tinnitus percept (tinnitus group) was tested behaviorally 13 days after trauma (GAP-NOGAP paradigm of PPI of the acoustic startle response, cf. Methods) and is indicated by a negative PPI change in at least one of the 5 tested frequencies. Out of the 11 animals, 3 developed apparent HL but no signs of tinnitus (no-tinnitus group), 3 developed signs of tinnitus but no apparent hearing loss and 5 animals developed both.

The tinnitus animals with apparent hearing loss (5/8, 62.5%) showed 1 affected frequency in 4 cases and 2 affected frequencies in 1 case. All of the 3 tinnitus animals with no apparent hearing loss also showed 1 affected frequency only. An overview of the median PPI change (± 25% to 75% quantile) for the 8 tinnitus (red, together with the number of significant tinnitus tests as a function of test frequency, orange bars) and 3 no-tinnitus animals (blue) is given in Figure 2.

The quantification of the noise induced hearing loss was based on comparisons of (3 to 5 days) post trauma versus pre trauma, ABR based threshold estimation. An example of such a measurement at 2 kHz with sound intensity levels between 0 and 90 dB SPL in 5 dB steps is given in Figure 3A. The ABR thresholds of both ears pre trauma as well as of post trauma control ears of all 11 animals did not show any significant differences (Fig. 3B, black, blue, and grey symbols). Post trauma (Fig. 3B, red symbols) we found a tendency for hearing loss in the traumatized ears audiograms in the multiple comparisons of mean ranks tests (p=0.07), and a significant mean noise induced hearing loss across when averaging across all frequencies by means of Kruskal-Wallis ANOVA.

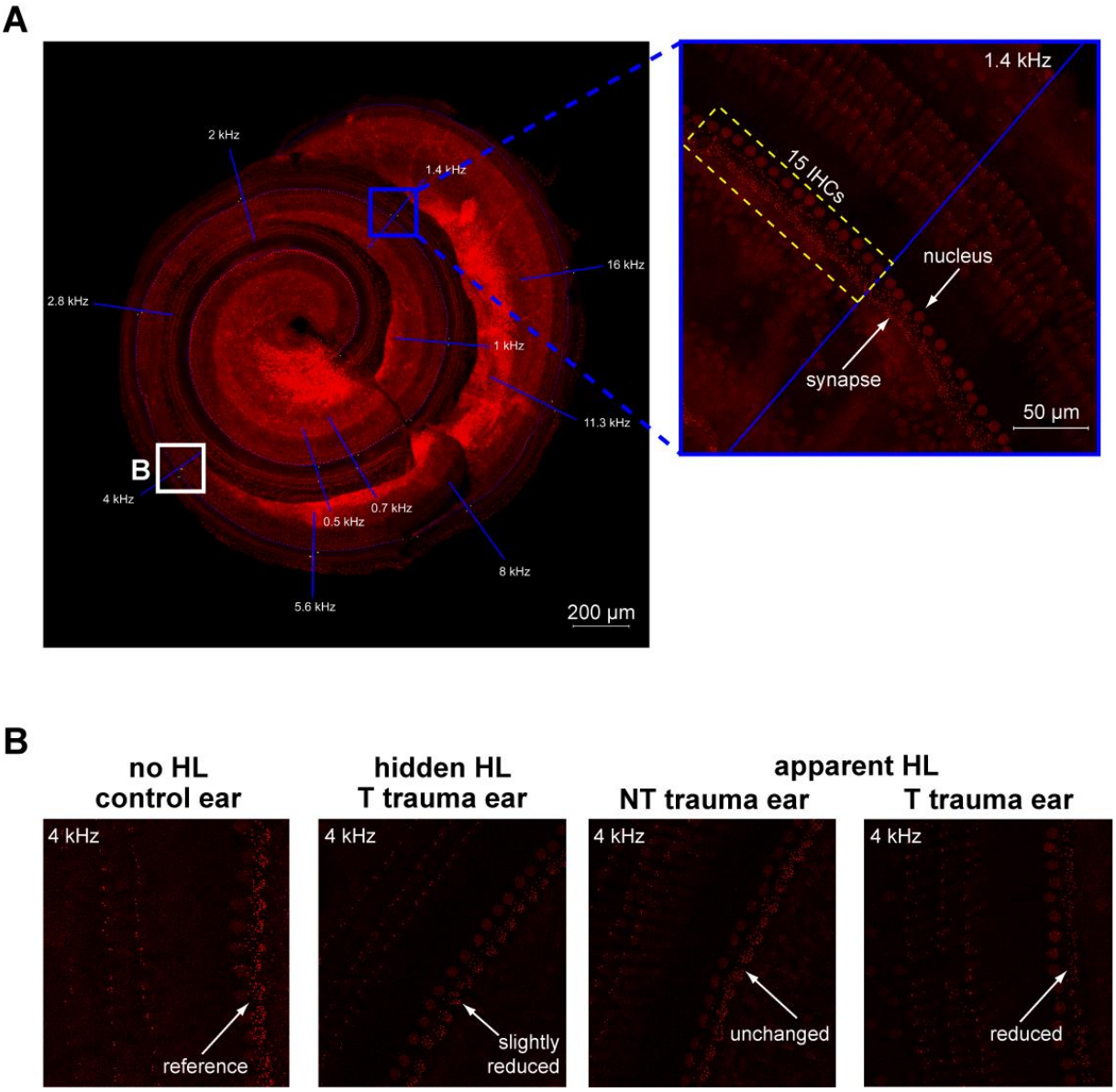

Figure 1

Immunohistological stainings of the Mongolian gerbil cochlea. **A**: Left: Overview of an isolated cochlea immunostained by CTBP2. Blue lines indicate the borders of the different half octave regions indicated. Right: Magnified image depicting the synapses of the 15 IHCs counted (yellow rectangle) directly above a frequency border. **B**: Exemplary images depicting the 4 kHz region of the cochlea (from left to right) of a control ear and the trauma sides of an animal with hidden hearing loss (HL) with behavioral signs of tinnitus, an animal with apparent hearing loss without behavioral signs of tinnitus (NT) and an animal with apparent hearing loss and tinnitus (T).

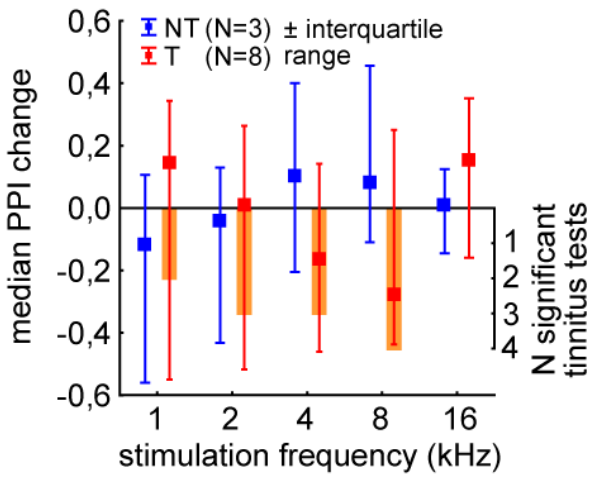

Figure 2

Overview of the median PPI changes in the behavioral test for tinnitus across all 11 animals for the 5 frequencies tested. Blue symbols depict the median PPI changes (whiskers give the interquartile range) of the no-tinnitus animals (no significant change), red symbol depict the changes in tinnitus animals (at least 1 frequency significantly affected). The orange bars depict the number of significant PPI changes indicating a possible tinnitus percept for each frequency.

Based on the individual ABR data we classified the animals into groups with and without a significant hearing loss (i.e. apparent and hidden hearing loss, respectively; Fig. 3C. Note that all animals in our sample with no significant noise induced hearing loss had synaptopathy (cf. below) and therefore are termed “hidden hearing loss”.). In apparent hearing loss animals (Fig. 3C left panel) the significant Kruskal-Wallis ANOVA averaged across all frequencies indicated a specific trauma effect, which was further evaluated by multiple comparisons of mean ranks. This test revealed that in contrast to the control side, the trauma side showed increased hearing thresholds compared to pre trauma conditions (Fig. 3C left panel inset, $p<0.001$). Per definition no significant threshold changes were found in hidden hearing loss animals (Fig. 3C, right panel).

From the 8 animals with apparent hearing loss 5 developed behavioral signs of tinnitus while 3 did not. In both groups, significant threshold elevations were found on the traumatized side. In addition, the control side in the no-tinnitus group was also slightly affected (Fig. 3D). More importantly, no significant thresholds differences between tinnitus and no-tinnitus animals could be detected, indicating that the amount of threshold shift may not be the exclusive cause for the development of a tinnitus percept.

Effects of noise trauma on ABR and their relation to tinnitus development

The peak-to-peak amplitudes of ABR waves I/II, III, IV and V obtained at 90 dB SPL were analyzed in detail by Kruskal-Wallis ANOVAs and multiple comparison of mean ranks tests. We compared the pre trauma data of both ears with the post trauma data, independently for tinnitus animals with apparent and hidden hearing loss and no-tinnitus animals. Generally, the ABR amplitude data of the animals’ left and right ears were not significantly different. For the apparent hearing loss group, the analyses revealed decreases in no-tinnitus animals’ wave I/II peak-to-peak amplitudes in both ears (Fig. 4A) and a decrease of tinnitus animals’ wave V on the control side. In animals with hidden hearing loss only tinnitus animals were found in our sample. We compared the four different ABR wave amplitudes between the non-traumatized control ear and the trauma-tized ear at 90 dB SPL by Mann-Whitney U-tests and found no differences between the two ears or changes over time (always $p>0.05$). Note that amplitudes of no-tinnitus animals – in line with our earlier results [15, 16] – are larger than tinnitus animals’ amplitudes by a factor of roughly 10 while the two tinnitus animal groups show similar amplitudes.

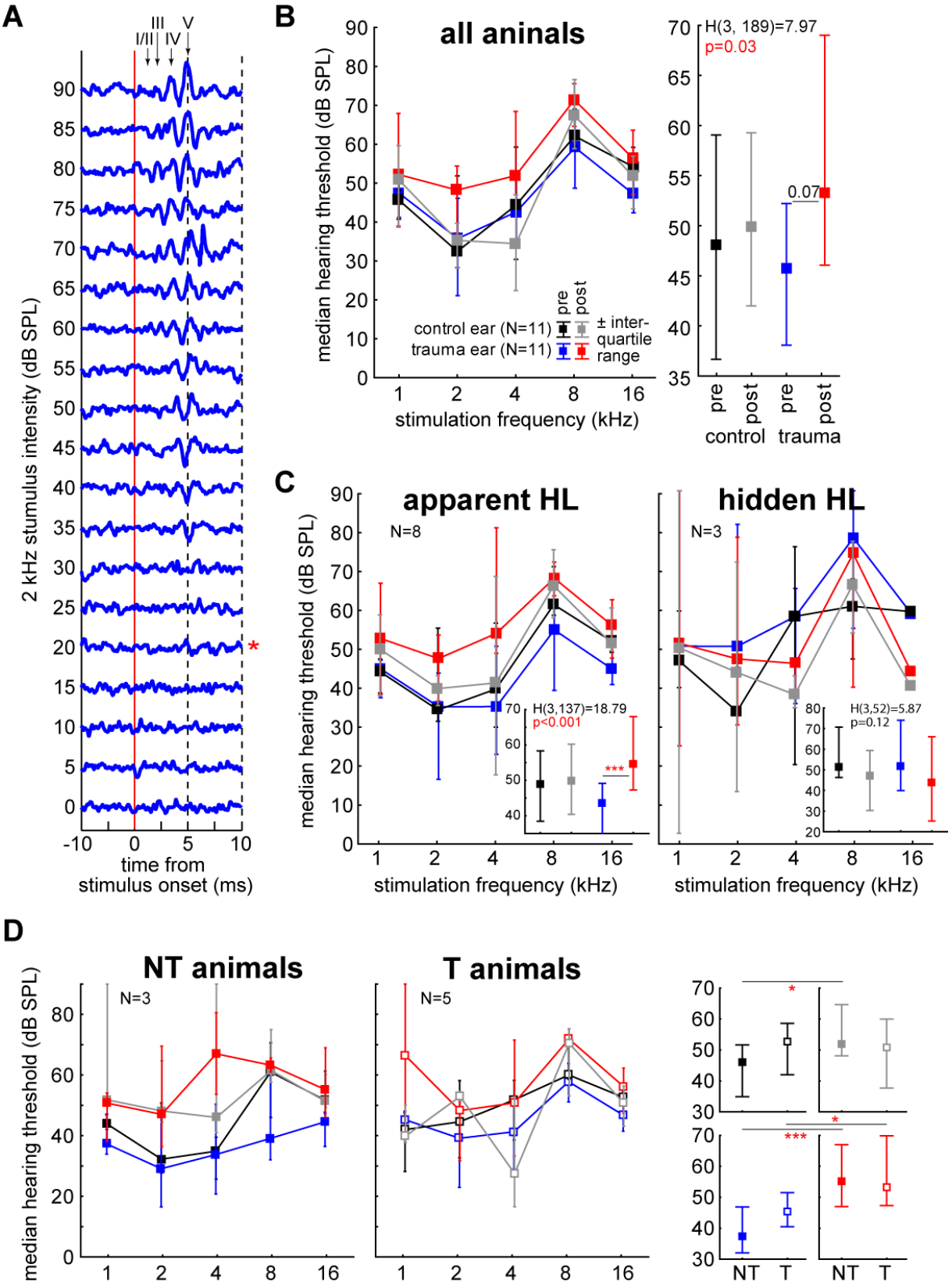

Figure 3

Analysis of hearing thresholds by Kruskal-Wallis ANOVAs. **A**: Exemplary ABR data measured with a 2 kHz pure tone stimulation ranging from 0 to 90 dB SPL with 5 dB attenuation steps. Asterisk indicates the automatically detected threshold level. **B**: Median hearing thresholds of all animals' (N=11) traumatized (blue and red symbols) and non-traumatized control ears (black and grey symbols) before and after trauma. Given are the median thresholds as a function of stimulation frequency (left) and the averaged thresholds across all stimulation frequencies for the different ears (right). Whiskers give the

interquartile range. **C**: Left: Overview of median hearing thresholds of both ears of the apparent hearing loss animals as a function of stimulation frequency. Inset: Kruskal-Wallis ANOVA for the averaged threshold data. Right: Overview of median hearing thresholds of both ears of the hidden hearing loss animals as a function of stimulation frequency. Inset: Kruskal-Wallis ANOVA for the averaged threshold data. **D**: Left: Median thresholds of no-tinnitus animals with apparent hearing loss control and trauma ears as a function of stimulation frequency. Center: Median thresholds of tinnitus animals with apparent hearing loss control and trauma ears as a function of stimulation frequency. Right: Comparison of averaged thresholds of no-tinnitus and tinnitus animals with apparent hearing loss. Asterisks give the significance values of Mann-Whitney U-tests. * $p<0.05$, *** $p<0.001$.

In a second step of analysis (Fig. 4B), we pooled the data from both ears of each tinnitus animal (apparent hearing loss with tinnitus and hidden hearing loss with tinnitus) as nearly no difference was found between ears and tinnitus animal groups (cf. above). We compared *pre and post trauma* ABR RMS data aligned to the lowest tinnitus frequency determined with the behavioral paradigm. The reasoning behind this approach was that due to the differences in perceived tinnitus frequencies (cf. Fig. 2) any possible specific tinnitus related amplitude effect may be blurred. By such re-aligning to the individual tinnitus frequency we found a significant general effect of a trauma induced decrease of overall ABR RMS values (Fig. 4B, left panel). Interestingly, an ‘edge’ effect was observed for responses below the tinnitus frequency compared to the ABR RMS values at and above the tinnitus frequency (Fig. 4B, center panel). In the significant interaction of both factors (*distance from tinnitus frequency* and *time point*) we found a significant drop of response after trauma at the tinnitus frequency only (Tukey post-hoc tests, Fig. 4B, right panel) which indicated a specific trauma effect on the ABR at that frequency in tinnitus animals.

Synapse counts of IHCs

We now compared the IHC synapse counts of the control ears and the traumatized ears separately for the tinnitus and no-tinnitus group with apparent hearing loss and for the tinnitus group with hidden hearing loss (Fig. 5). Our main finding was that all tinnitus animals – independent of the type of hearing loss - showed a significant loss of synapses in the traumatized ear only. First of all, we investigated the effect of the trauma on the number of synapses per IHC and found no difference between trauma and control side (Fig. 5A, left panel), neither at specific frequency ranges nor averaged across all frequencies (Mann-Whitney U-tests, always $p>0.05$). We then separated the animals for the type of hearing loss (Fig. 5A, center panel) and again did not find any significant differences between trauma and control sides of the two groups (Kruskal-Wallis ANOVAs, always $p>0.05$). When separating the tinnitus animals – with or without detectable hearing loss – from the no-tinnitus animals (Fig. 5A, right panel) the Kruskal-Wallis ANOVA at 4 kHz became significant ($H(3, 22)=8.68$, $p=0.04$) and multiple comparison of mean ranks tests revealed a significant loss of synapses/IHC on the trauma side of tinnitus animals compared to the control side ($p=0.03$) as well as in relation to both sides of no-tinnitus animals (both $p=0.01$). The Kruskal-Wallis ANOVA averaged across all frequencies did only show a tendency ($p=0.07$) for a general difference between the trauma and the control sides.

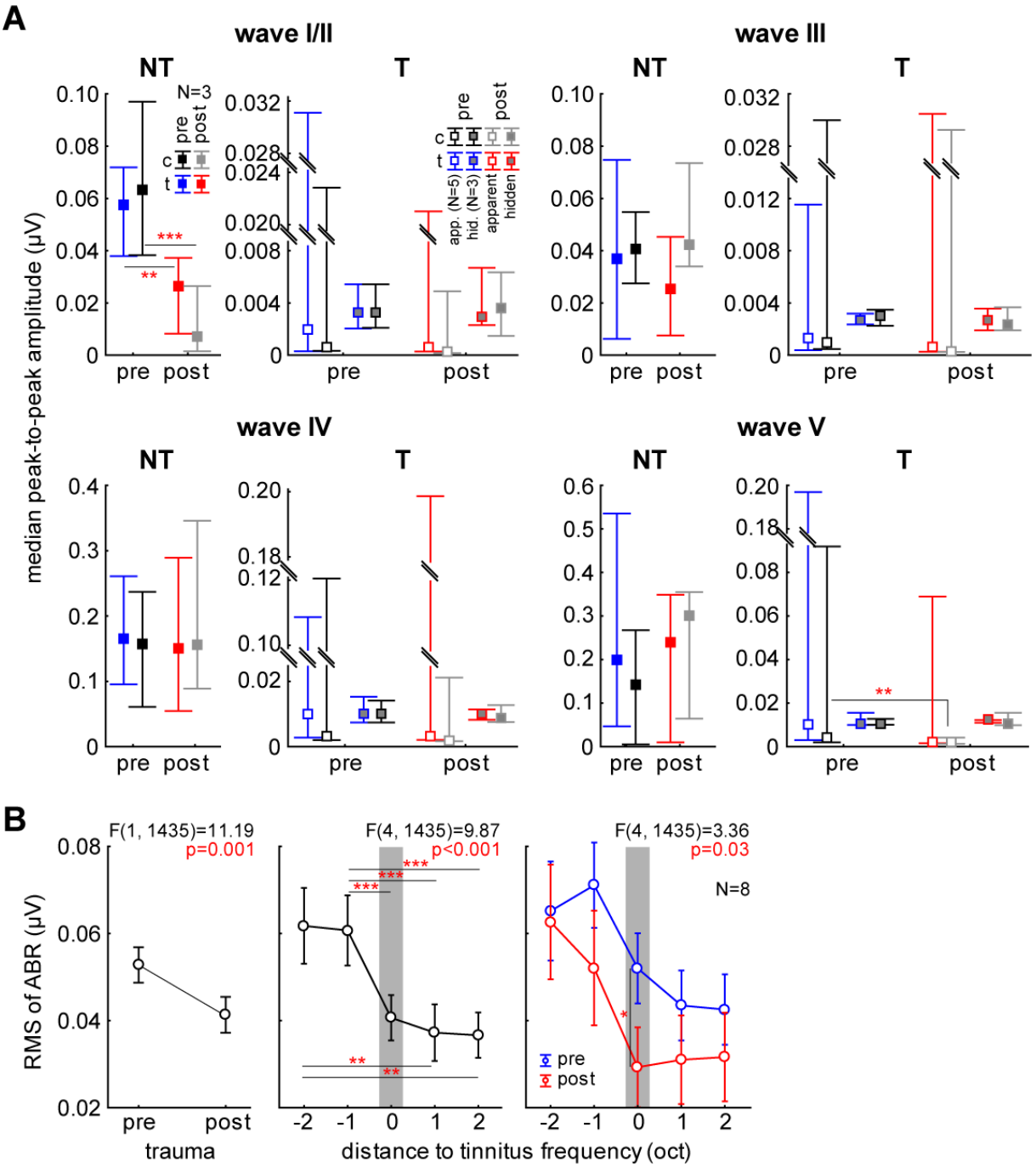

Figure 4

Overview of ABR wave analyses performed with Kruskal-Wallis ANOVAs and ABR RMS 2-factorial ANOVA analysis. **A** Median peak-to-peak amplitudes of single ABR waves in animals with apparent hearing loss of both ears with a stimulation intensity of 90 dB SPL averaged across all stimulation frequencies **B** RMS values of the ABR signals of all tinnitus animals averaged across all frequencies, intensities and both ears as a function of distance from the individual *tinnitus frequency* and *time point* of measurement. The grey bar indicates the tinnitus frequency the data are aligned to. Asterisks indicate significance values of the Tukey post-hoc tests; * p<0.05, ** p<0.01, *** p<0.001.

We next compared the synapse counts of both types of tinnitus animals (apparent and hidden hearing loss, Fig. 5B, left panel) and again found a significant effect at 4 kHz (Kruskal-Wallis ANOVA, H(3, 14)=9.05, p=0.04), namely a significant synapse loss on the trauma side of the tinnitus animals with apparent hearing loss compared to the control side (p=0.007). No difference was seen

between the two trauma sides, but the synapse loss at the trauma side of the tinnitus animals with hidden hearing loss compared to their control side showed a tendency of being stronger (p=0.09). Nevertheless, the Kruskal-Wallis ANOVA averaged across all frequencies did show a significant difference between the groups with multiple comparison of mean ranks revealing a significant loss of synapses on the trauma side of the apparent hearing loss tinnitus animals compared to the control side (p=0.02). We finally compared the two groups with apparent hearing loss with and without a tinnitus percept (Fig. 5B, right panel). Again the Kruskal-Wallis ANOVA showed a significant loss of synapses at 4 kHz (H(3, 16)=12.92, p=0.008) on the trauma side of the tinnitus animals compared to control and no-tinnitus trauma side (both p<0.001), while in no-tinnitus animals synapse counts on the trauma side were not different from their respective control side. Also the multiple comparison of mean ranks testing of the averaged counts across all frequencies (Fig 5B insets) showed a significant loss of synapses on tinnitus animals' trauma side compared to the control side (p=0.04).

To rule out erroneous results based on outliers we calculate the individual synapse per IHC difference between the traumatized and non-traumatized sides (Fig. 5C) and analyzed the effects of animal groups as a function of cochlear frequency as well as averaged across all frequency ranges by means of Kruskal-Wallis ANOVAs. We again found a significant effect at the 4 kHz cochlear location (H(2, 11) =12.60, p=0.03) with a significant drop of synapse counts in both tinnitus animal groups compared to the no-tinnitus animals (both p<0.05). For the averaged data again only the tinnitus animals with apparent hearing loss showed a significant synapse loss compared to no-tinnitus group.

Discussion

In this study we investigated the synapse loss at IHCs after mild acoustic trauma that has previously been shown in mice [17] in the Mongolian gerbil and relate it to possible behavioral signs of tinnitus [15, 18]. Further, we compared tinnitus in animals with hidden and apparent hearing loss and for the first time investigated, if synaptic loss depends on the type of hearing loss. For noise trauma induction we chose a paradigm previously used by our lab, consisting of a 2 kHz pure tone and thereby targeting only a specific region of the cochlea. A specific goal was to evaluate, if the extend of synapse loss within the cochlea is correlated to maladaptive processes along the auditory pathway as described earlier [9, 14]. The main finding reported here – even though preliminary – is a frequency specific reduction in IHC synapse counts, most prominently around one octave above the trauma frequency, only in animals with behavioral signs of tinnitus and independent of the kind of hearing loss, hidden or apparent.

Methodological considerations of the behavioral paradigm

For identification of behavioral signs of tinnitus in our animal model (the Mongolian gerbil) we use a gap-startle paradigm as introduced by Turner and colleagues (2006). The measurement of the PPI of the acoustic startle response 13 days post trauma relative to pre trauma conditions is analyzed [21]. This approach has the advantage of being independent of training [e.g., 9, 26, 27] but is considered not to be failsafe [for review see 28]. To overcome this problem we always relate the behaviorally determined classification of animals in tinnitus and no-tinnitus with additional data, like electro-physiological recordings [15, 16, 29] or in this report

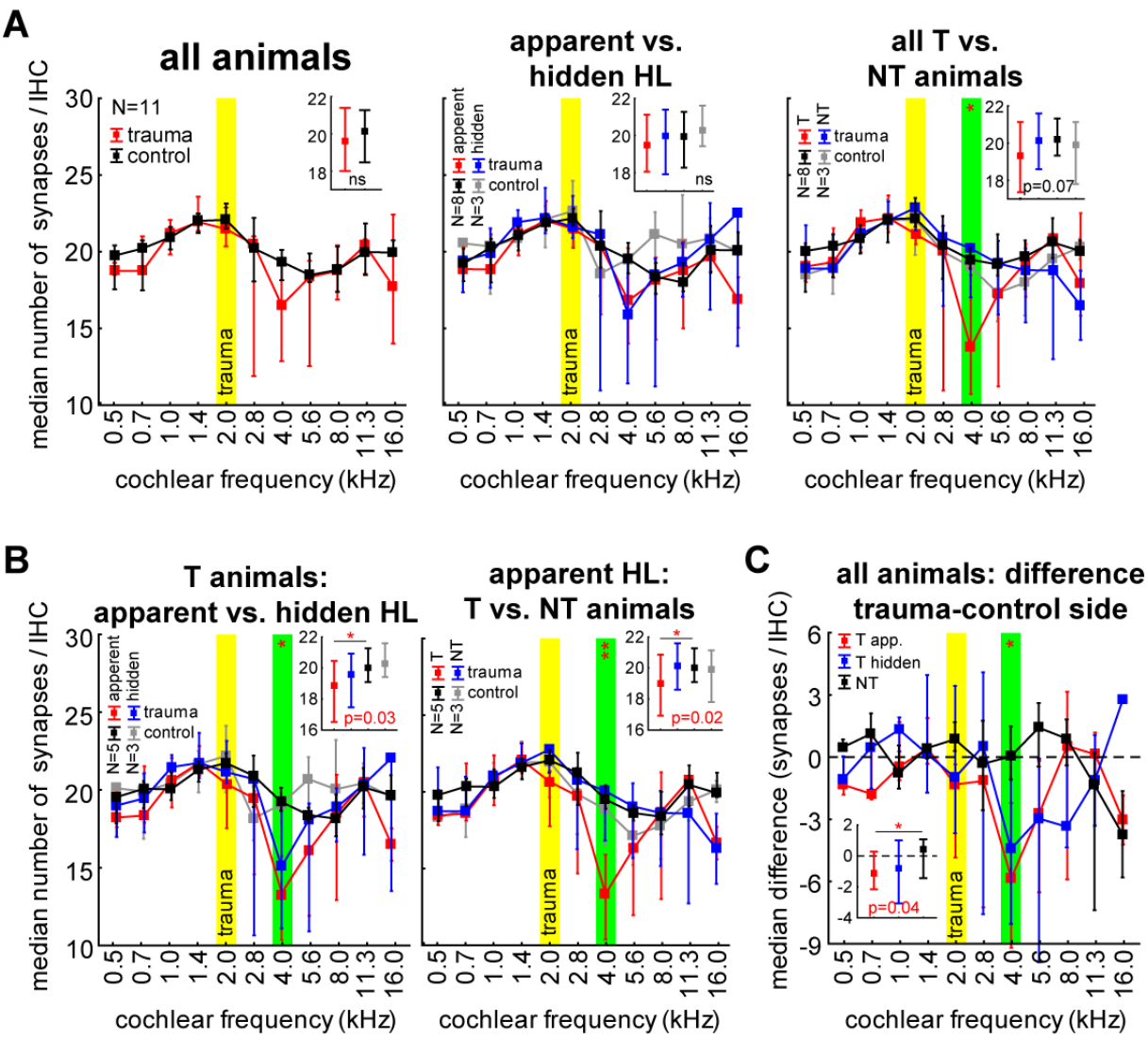

Figure 5

IHC synapse loss in animals with and without behavioral signs of tinnitus assessed by Mann-Whitney U-tests or Kruskal-Wallis ANOVAs. **A:** Median synapse/IHC counts of trauma and control cochleae of all animals subdivided into different subgroups. Panels give the overview across all investigated frequencies, insets depict the averaged median synapse/IHC counts with significance of the Kruskal-Wallis ANOVA and asterisks depicting the significance levels of the multiple comparison of mean ranks tests. Yellow bars depict the trauma frequency, green bars depict significant Kruskal-Wallis ANOVAs (asterisks give significance values, * $p<0.05$, ** $p<0.01$). **B**: Subgroups of 8 different animals. Symbols and statistics as above. **C**: Individual difference of trauma vs control side counts across all 11 animals. Symbols and statistics as above.

electro physiological (ABR) and anatomical data. Using this approach in overall 11 animals,we found a tinnitus percept in hidden (N=3) or apparent hearing loss (N=5) animals (8/11, 72.7%) and no behavioral signs of tinnitus in 3 animals (27.3%). This is well in the range of our previous results with a tinnitus developing in 65% and 80% of animals with NIHL even though the major differences to our usual approach was the use of monaural rather than binaural traumata for obtaining intra-individual controls.

Noise trauma induced tinnitus – predisposition and development

The differences in ABR amplitudes between tinnitus and no-tinnitus animals, with no-tinnitus animals showing generally

larger amplitudes than tinnitus animals even before induction of noise trauma, are consistent with our earlier results [16]. We were able to reproduce the generally larger peak-to-peak ABR amplitudes in all identified wave complexes in no-tinnitus compared to tinnitus animals. In our earlier studies we compared ABR before and immediately after noise trauma and found no differences between pre and post waves with the exception of a general increase of wave V amplitudes (inferior colliculus) which was usually stable up to 7 days after the trauma. Here we compared the pre trauma ABR with those 3 to 5 days after trauma, that is, probably within time frame of the consolidation of a tinnitus percept (cf. Ahlf et al., 2012) which we here measured 13 days after the trauma. As the response levels of tinnitus animals are roughly 10 times smaller than those of no-tinnitus animals, a potential difference between the very small pre and post trauma data is hardly detectable, even though we found a reduction of wave V amplitudes on the control side of the animals with apparent hearing loss and behavioral signs of tinnitus. This could indicate that the plugging of the ear did not completely protect the control ear and by that activity in the inferior colliculus is reduced. Alternatively it could also be a response within the auditory system to level out both sides and could demonstrate that the development or prevention of development of subjective tinnitus involves complex changes throughout the auditory pathway that take place consecutively in a certain temporal order rather than simultaneously. This view is supported by the lack of threshold increase on the control side of the tinnitus animals (cf. Fig. 3C, D) and the reduced activity in the ABR RMS values of the tinnitus animals which was clearly related to the minimal tinnitus frequency determined by the behavioral paradigm (Fig. 4B). On the other hand, we found a binaural decrease of peak-to-peak amplitudes measured at 90 dB SPL in ABR wave complex I/II in no-tinnitus animals only, indicating either a decrease of neuronal activity [e.g., 30] or a loss of synchronization [cf. 31] corresponding to the elevated thresholds on both sides in these animals (cf. Fig 3D). Again it is possible that the control ear was not completely protected by the plugging so that a mild hearing loss has been induced.. Nevertheless, a specific loss of synapses one octave above the trauma – in line with data reported before [32] – is only related to tinnitus and not to hearing loss in our animal sample (cf. below).

Synaptopathy and tinnitus

We found significant synaptopathic effects at the IHC of the cochlea in all animals with behavioral signs of tinnitus, irrespective of the type of hearing loss, hidden or apparent. Interestingly, the strongest reduction of synapses (roughly 25%) was not located at the trauma frequency but one octave above (cf. Fig. 5), consistent with effects reported in mice [14, 17] and rats [9], although these studies used a lower spectral resolution in their analysis of cochlear regions (2 frequencies: 11.3 kHz and 32 kHz). In addition, this frequency range has frequently been reported to reflect the perceived tinnitus frequency in Mongolian gerbils [e.g., 33]. No such loss of IHC synapses was found in no-tinnitus animals even though the apparent noise induced hearing loss in that group was not different from the one in tinnitus animals with hearing loss (cf. Fig. 3). It is notable that the mild hearing loss in our study is not accompanied by a loss of IHCs in the range of the trauma, which is in line with an earlier study demonstrating that apoptosis is only found after strong traumata with threshold losses of at least 50 dB [e.g., 34].

An unexpected finding of this study was that in animals that did not develop tinnitus (no tinnitus group), no significant

reductions in IHC synapse counts could be detected, although they did show apparent hearing loss after noise trauma on both sides. The exact cause of this finding remains elusive, but a number of possibilities are conceivable, like stereocilia damage [35, 36] to high threshold hair cells [e.g., 37] or the animals' noise protection efferent system [e.g., 38, 39-41] being more efficient. The possibility that such synapse reductions may have been overlooked can be excluded as we scanned the cochlea with high spectral resolution, i.e. in half octave steps (cf. Methods). Therefore, the apparent hearing loss, that is increased hearing thresholds, in our no-tinnitus group do not seem to be based on IHC synapse damage but probably rather outer hair cell (OHC) damage. Interestingly, mild traumata lead to a higher percentage of animals with behavioral signs of tinnitus than more severe traumata [42, 43], which may correlate with the variable amount of damage to IHC and OHC, respectively.

Taken together, we conclude that at least in our animal model, a hearing threshold increase induced by mild acoustic trauma is associated with synaptopathy at the IHC and/or OHC damage [44-46] while tinnitus seems to be associated with IHC synaptopathy only, independent of a threshold increase.

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