

Original Paper

Cochlear NMDA Receptors as a Therapeutic Target of Noise-Induced Tinnitus

Dan Bing^a Sze Chim Lee^b Dario Campanelli^b Hao Xiong^{b,d} Masahiro Matsumoto^b
Rama Panford-Walsh^{b,e} Stephan Wolpert^b Mark Praetorius^c Ulrike Zimmermann^b
Hanqi Chu^a Marlies Knipper^b Lukas Rüttiger^b Wibke Singer^b

^aHuazhong University of Science & Technology, Tongji Medical College, Tongji Hospital, Department of Otorhinolaryngology, Head & Neck Surgery, Wuhan, P. R. China; ^bUniversity of Tübingen, Department of Otorhinolaryngology, Hearing Research Centre Tübingen, Molecular Physiology of Hearing, Tübingen, Germany; ^cUniversity of Heidelberg, Department of Otorhinolaryngology, Head and Neck Surgery, Otology and Neuro-otology, Heidelberg, Germany; ^dDepartment of Otolaryngology, Sun Yat-sen Memorial Hospital, and Institute of Hearing and Speech-Language Science, Sun Yat-sen University, Guangzhou, China (present address HX); ^eDNA Genotek Inc., Ottawa, ON, Canada (present address RPW)

Key Words

NMDA receptor • Local application • Tinnitus • Inner ear • IHC ribbon synapse

Abstract

Background: Accumulating evidence suggests that tinnitus may occur despite normal auditory sensitivity, probably linked to partial degeneration of the cochlear nerve and damage of the inner hair cell (IHC) synapse. Damage to the IHC synapses and deafferentation may occur even after moderate noise exposure. For both salicylate- and noise-induced tinnitus, aberrant N-methyl-d-aspartate (NMDA) receptor activation and related auditory nerve excitation have been suggested as origin of cochlear tinnitus. Accordingly, NMDA receptor inhibition has been proposed as a pharmacologic approach for treatment of synaptopathic tinnitus.

Methods: Round-window application of the NMDA receptor antagonist AM-101 (Esketamine hydrochloride gel; Auris Medical AG, Basel, Switzerland) was tested in an animal model of tinnitus induced by acute traumatic noise. The study included the quantification of IHC ribbon synapses as a correlate for deafferentation as well as the measurement of the auditory brainstem response (ABR) to close-threshold sensation level stimuli as an indication of sound-induced auditory nerve activity. **Results:** We have shown that AM-101 reduced the trauma-induced loss of IHC ribbons and counteracted the decline of ABR wave I amplitude generated in the cochlea/auditory nerve. **Conclusion:** Local round-window application of AM-101 may be a promising therapeutic intervention for the treatment of synaptopathic tinnitus.

Copyright © 2015 S. Karger AG, Basel

Dr. Wibke Singer
and Dr. Lukas Rüttiger

HNO-Klinik, Universität Tübingen, Elfriede-Aulhorn-Straße 5,
D-72076 Tübingen (Germany)
Tel. +49 7071 2988243, Fax +49 7071 294950,
E-Mail wibke.singer@uni-tuebingen.de and E-Mail lukas.ruttiger@uni-tuebingen.de

Introduction

Tinnitus, the perception of sound in the absence of external acoustic stimulation, is a widespread pathology and affects around 10-20% of the adult population [1] with increased prevalence as individuals age [2]. Tinnitus frequently occurs in conjunction with a loss of auditory sensitivity [3], but neither threshold shift nor outer hair cell (OHC) loss seem to be a prerequisite for developing tinnitus (reviewed in [4]). Thus, clinically normal auditory thresholds (i.e. less than 20 dB (decibel) hearing threshold loss for frequencies up to 8 kHz [5]) can be found in tinnitus patients (for review see [6, 7]) in spite of IHC deafferentation, which has been proposed to be causally involved in tinnitus generation [8-11].

Over the past decades, clinical studies have consistently reported noise overexposure as the main cause of tinnitus in humans [12, 13], whereas exposure to ototoxic drugs (e.g. salicylate) has been reported much less frequently. While salicylate- and noise-induced tinnitus seems to be rather different, there is strong evidence that they share a common mechanistic pathway, converging at the level of the IHC synapse. The synapse between IHCs and primary afferent neurons is glutamatergic (reviewed in [14]); AMPA as well as NMDA receptors (NMDA-Rs) are expressed [15, 16]. AMPA receptors mediate the fast excitatory synaptic transmission and therefore maintain the temporal fidelity [17] essential for hearing, while NMDA-Rs are not involved in fast excitatory neurotransmission as they are blocked at resting potential by Mg^{2+} . NMDA-Rs are instead activated by excessive release of neurotransmitter from IHCs, which then becomes excitotoxic to the primary auditory neuron [18]. These excitotoxic events may result from salicylate overexposure, noise trauma, or other events related to sudden deafness [1, 16]. This can then lead to an imbalance of activity in NMDA- vs. non-NMDA receptors and create significant discharge aberrations leading to altered activity of the auditory nerve, which may provide a starting point for tinnitus in the auditory periphery [16, 19-21]. To compensate for an ongoing alteration of auditory nerve activity, it is assumed that subcortical projection neurons in the dorsal cochlear nucleus (DCN), become hyperactive [22]. This is the earliest point where changes of auditory nerve fibers are compensated. However, this compensating hyperactivity can only occur when a critical number of auditory fibers continue to increase their discharge rate sufficiently after cochlear injury [22, 23].

The prevention of deafferentation may therefore be a crucial target for a tinnitus therapy [10]. Protecting the postsynaptic target by glutamate receptor antagonists may lead to a significant reduction in functional impairment. There have been various attempts to use NMDA receptor inhibition for the treatment of tinnitus, so far with mixed outcomes (for a review see [24]). Since NMDA receptor antagonists, when systemically given, tend to have a number of negative central nervous system side effects such as hallucinations or anesthesia (for which e.g. Ketamine was originally developed), their clinical potential has been limited. This limitation is further aggravated by the fact that high systemic doses are usually required to achieve therapeutic concentrations in the cochlea. Conversely, local/cochlear application of glutamate receptor antagonists has previously been shown to reduce salicylate- or noise-induced tinnitus in animal models [25-27]. Specific NMDA receptor subunits have been suggested to be involved in changing the kinetics of the discharge behavior of auditory fibers in response to salicylate-induced tinnitus [16]. Local round window application of ifenprodil, a selective NR2B subunit antagonist, was shown to attenuate behavioral correlates of tinnitus in an animal model with salicylate- or noise-induced tinnitus within the first 4 days post insult [27]. Whether the NR1 subunit also has a function in noise-induced tinnitus, similar to the NR2B subunit, is so far unknown.

Aiming to better understand the therapeutic potential of NMDA receptor inhibition in tinnitus therapy, we tested a non-competitive small molecule NMDA receptor antagonist, AM-101 (Esketamine hydrochloride gel; Auris Medical AG, Basel, Switzerland), for its effects on tinnitus in a noise trauma model. AM-101 aims to treat tinnitus in the acute stage before it becomes centralized or memorized within higher structures of the auditory system and/or brain. It is delivered locally in the form of a hyaluronic acid gel applied to the round window

membrane. This approach was previously tested in two clinical studies [28, 29] and provides for a highly targeted cochlear therapy with only minimal systemic exposure [30, 31]. This method has proven to be effective in patients suffering from acute tinnitus triggered by noise trauma or otitis media [29].

We investigated the number of individual synaptic contacts (presynaptic immunopositive CtBP2/RIBEYE ribbons) at the IHC synapse [32] as a correlate for afferent innervation [33, 34]. These synapses (referred to as ribbon synapses), are electron-dense presynaptic specializations that tether synaptic vesicles for exocytosis at the active zone (for review: [35-37]). Ribbon synapses support an extremely high rate of exocytosis through maintenance of a large ready releasable pool of synaptic vesicles, thereby enabling fine intensity discrimination over a wide dynamic range [38, 39]. Since the release of neurotransmitters from ribbon synapses determines the generation of action potentials (spikes) in afferent auditory fibers [40] and as the reliability and precision of spikes determines the amplitude of the propagating sound-induced activity in the ascending auditory pathway [41], we used the size of suprathreshold auditory brainstem responses as a correlate for the transmission of afferent auditory information at the IHC synapse.

Materials and Methods

Animals

Adult female Wistar rats (Charles River Laboratories, Sulzfeld, Germany) weighing between 200 and 300 grams were used for this study (Refer to Table 1 for the number of animals used in each experimental group).

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.

Anesthesia

Animals were anesthetized with ketamine hydrochloride 75 mg/kg body weight (Ketamine 50 Curamed, CuraMED Pharma, Karlsruhe, Germany) and xylazine hydrochloride 5 mg/kg body weight (Rompun 290, Bayer, Leverkusen, Germany) for pre hearing measurements (6 d before noise exposure application and 8 d before drug application) and final measurements (15 d post noise exposure). To avoid any interference of ketamine with AM-101 during drug application and noise exposure, intraperitoneal injection of medetomidine hydrochloride, 0.33 mg/kg (Sedator 1 mg/ml, Eurovet Animal Health Care B.V., Bladel, Netherlands) was used. Additional doses of anesthetics were administered subcutaneously every hour; a single dose of fentanylcitrat (0.075 mg/kg, Fentanyl, Ratiopharm, Ulm, Germany) was administered subcutaneously prior to surgery.

Noise exposure

Animals were exposed to pure tones while under anesthesia (Sedator). Supplemental doses of anesthetics were administered subcutaneously if the toe reflex recovered. Calibrated loudspeakers (Visaton DR 45 N, Haan, Germany, and SC Piezo Horn 335835 Conrad Elektronik, Tübingen, Germany) inside a reverberating chamber (a chamber of approximately 50 x 50 x 50 cm with tilted, non-parallel walls and central top speaker to avoid standing waves and to achieve a mostly homogeneous sound field) delivered the sound. The sound consisted of a continuous 10 kHz pure-tone presented at 120 dB SPL (sound pressure level). Acoustic stimuli were calibrated at the head level of the animal. Following the treatment protocols of Kaltenbach et al. [42], the rats were exposed for 2 h and sacrificed 10-15 d following noise exposure (acoustic trauma, AT). This exposure paradigm has been reported to induce tinnitus in more than 90% of rats (29 out of 31 rats with tinnitus; [11]). Sham-exposed animals were anesthetized and placed in the reverberating chamber, but not exposed to acoustic stimulus (i.e., the speaker remained turned off). These animals had completely normal hearing and will be referred to as "sham exp." within this paper.

Auditory brainstem response (ABR) measurements

Hearing thresholds were determined by recordings of ABR. ABR measurements were performed as described [10, 43]. In short, electrical brainstem responses to free field click (100 μ s), noise burst (1 ms) and pure tone (3 ms, 1 ms ramp) acoustic stimuli were recorded with subdermal silver wire electrodes at the ear, the vertex and the back of the animal. After amplification (100,000 times) the signals were averaged for 64-256 repetitions at each sound pressure level presented (ca. 0-100 dB SPL in steps of 5 dB). The auditory threshold was determined by the lowest sound pressure that produced potentials visually distinct from noise level.

ABR waveform analysis

In order to estimate the recovery of ABR waves after noise exposure as well as the possible effect of AM-101 treatment, a method similar to the one described in Rüttiger et al. [10] was used to mathematically correlate the ABR waveforms before noise exposure (pre AT) and 15 d after noise exposure (post AT). A correlation factor (CorF) was calculated for each individual rat as follows:

$$\text{CorF} = \text{Cov}(A_{\text{pre}}, A_{\text{post}}) / \text{Var}_{\text{pre}}$$

where

Cov=covariance of the click-ABR waveforms pre AT and 15 d post AT

A_{pre} =amplitudes at point 1 to 200 (0 to 10 ms) pre AT

A_{post} =amplitudes at point 1 to 200 (0 to 10 ms) 15 d post AT

Var_{pre} =variance of the click-ABR wave pre AT

The CorF normalizes the covariance by the variance of the measurement before noise exposure. As outlined by Rüttiger et al. [10], this analysis can both reflect the changes in waveform resemblance and amplitude of any two ABR waveforms as opposed to the conventional Pearson correlation coefficient, where signal energies of the two waveforms are normalized by their own variances [10]. Typically, high values of CorF (e.g., approaching 1.0) indicate high similarity in waveform and amplitude, while low values of CorF (e.g., close to zero) reflect loss of both waveform similarity and amplitude. Thus, a high value of CorF between pre AT and 15 d post AT reflects better recovery in general, and a low value reflects worse recovery after noise exposure for a particular rat.

CorFs for all noise-exposed and all sham-exposed rats in each treatment group were averaged and group means compared. The polarity of the CorF reflects the overall synchrony between two ABR waveforms with a positive CorF indicating consistent timing of the occurrence of peaks in the two waveforms and a negative CorF showing desynchronized peaks.

ABR waveform amplitude analysis

The ABR waveforms for noise burst stimuli were analyzed for consecutive amplitude deflections (waves), each wave consisting of a starting negative (n) peak and the following positive (p) peak. Peak amplitudes of ABR waves I and IV were extracted in the present study and defined as follows: wave I, $I_n - I_p$ (0.9-1.4 ms), wave IV, $IV_n - IV_p$ (3.5-5.8 ms). A customized computer program was used for the extraction of ABR peaks based on the definitions given above. For estimating the positions (latencies in reference to stimulus onset and corresponding amplitudes) of the peaks in the ABR, peaks were approximated for individual measurements based on a template. Averages from the responses to the three highest stimulus levels in each of the time ranges given above for wave I and IV were taken. Adjustments to these estimations were performed by changing the width of the time ranges and the distances between time ranges until negative and positive peaks corresponding to each prominent wave were identified. After adjustments of the peak positions for the template, the peak positions for all stimulation levels were selected using a fixed set of parameters for all analyzed data. Peak data were included only if the corresponding ABRs were above the threshold and if the peak latencies for adjacent stimulation intensities were close. ABR peak-to-peak wave amplitude growth functions were constructed for individual ears for increasing stimulus levels. All ABR wave amplitude growth functions were calculated for increasing stimulus levels with reference to the corresponding wave thresholds.

Drug preparation

For these experiments, the small molecule non-competitive NMDA receptor antagonist AM-101 (Esketamine hydrochloride, Auris Medical AG, Basel, Switzerland) was used. AM-101 contains the S-(+)-

Enantiomer of ketamine as active substance. AM-101 is being developed for the intratympanic treatment of acute inner ear tinnitus and has been previously tested with some promise in a placebo-controlled randomized trial in humans [28]. For the preparation of AM-101 (under permission from Auris Medical AG, Basel, Switzerland), artificial perilymph (AP) was prepared according to Guitton et al. and stored at -20°C [26]. A stock solution of Esketamine hydrochloride (Chemie Uetikon, Lahr, Germany) was prepared at 5 mM in AP. The 5 mM stock solution was diluted in AP to final concentrations of 200 µM. AP was used as placebo.

For repeated administration of AM-101, a 100 mM phosphate-buffered stock solution of Esketamine hydrochloride was prepared. The stock solution was diluted to the final concentration of 200 µM. For the final formulation (AM-101), sodium hyaluronic acid (Contipro Biotech s.r.o., Dolni Dobrouc, Czech Republic) was dissolved under continuous mixing in the Esketamine solution and stirred for at least 16 h at room temperature to obtain a clear gel, which was stored at 2–8°C. For the placebo group, the sodium hyaluronic acid was dissolved in phosphate buffered solution as described before.

Surgery and drug administration

Animals of all treatment groups went through the same surgery and application process of either AM-101 or placebo. For single local drug treatment, animals were anesthetized by an intraperitoneal injection of Sedator. Fifteen minutes prior to surgery, an additional dose of Fentanyl was administered. The fur behind the ears was removed and the bulla exposed in a retro-auricular approach. A small hole was carefully drilled into the bony bulla just above the round-window niche (0.6–1 mm in diameter). The mucosa was opened and the region around the round-window carefully dried of fluid. Through the hole, a small gelfoam pellet was deposited into the round-window niche. 5–8 µl of AM-101 or placebo was applied on the gelfoam by means of a precision pipette with gel loader tips, in order to avoid trapping air bubbles under the gel. The niche was visually inspected to ensure that it was completely filled and covered with the gel. The hole in the bulla was then covered from the outside with muscular tissue and the wound was sutured with surgical thread (4-0 Vicryl, ETHICON Products, Norderstedt, Germany). Both ears were treated in the same manner. Postoperatively, Rimadyl (5 mg/kg Carprofen, Pfizer, Karlsruhe, Germany) was administered subcutaneously as an analgesic. The animals were kept warm and their body temperature monitored until they woke up.

For repeated application of AM-101, a port system (Micro-Renathane tubing, silicone port, 60 mm bulb distance, S-ISC 60S, Braintree Scientific, Braintree, MA, USA) was used. Because of the more invasive surgery, animals were anesthetized using ketamine/xylazine (see ABR measurements). In this case, only one ear was operated on. Two holes were drilled into the bulla. A small hole was made slightly higher than the midpoint of the bulla in order to observe the round-window niche. A second hole with a slightly larger diameter was drilled below the midpoint of the bulla for the insertion of the ear cannula. The middle ear was examined for signs of infection. A sterilized ear cannula with catheter was placed through the larger opening into the middle ear. A small drop of tissue glue (Histoacryl, B. Braun, Bad Melsungen, Germany) was used to hold the catheter in place against the bone. Carboxylate cement (Paladur, Heraeus Dental, Hanau, Germany) was used to secure the catheter and cover the opening. The catheter with injection port (S-ISC 60S, Braintree Scientific, Braintree, MA, USA) was placed subcutaneously on the midline of the upper part of the back. Finally, the ear cannula was connected to the port through silicon tubes and steel tube adapters. The incision was closed with Vicryl (ETHICON Products, Norderstedt, Germany). The ports were rinsed regularly in rats under light midazolam-anesthesia (1.5 mg/kg, Dormicum, Roche, Grenzach-Wyhlen, Germany) using NaCl 0.9% (Fresenius, Bad Homburg, Germany) to prevent plugging.

For drug administration, the animals were anesthetized with Sedator. AM-101 or placebo was applied via the port system either (i) 2 days post sham or noise-exposure, (ii) 2 and 4 d post exposure, or (iii) 2, 4, and 8 d post exposure. Animals receiving AM-101 only one or two times, received placebo on the subsequent treatment day(s).

Behavioral animal model

We developed an animal model for the observation of induced tinnitus in rats using the administration of salicylate [44]. Rats were conditioned in a customized Skinner-box system to perform a specific motor task (foraging behavior for sugar water) when an external noise sound was played (reward during periods of sound) and to cease this motor activity in periods of silence (electrical foot shock during periods of silence). After the training period, properly trained rats would perform this behavior in experimental

situations when no reward or foot shocks are given. After tinnitus induction, these animals actively execute the motor task even when the external noise sound is switched off, a measure for tinnitus behavior (for further details see [10, 43, 44]). The improvement of tinnitus perception was analyzed by calculating the delta of the tinnitus behavior as relative activity during silence (silence activity, SA) 3 d and 10 d after exposure.

Tissue preparation

Animals were euthanized with carbon dioxide, decapitated, and cochleae were dissected followed by immersion fixation in 2% paraformaldehyde, 125 mM sucrose in 100 mM phosphate buffered saline (PBS), pH 7.4, for 2 h. Cochleae were decalcified in Rapid Bone Decalcifier (Eurobio, Les Ulis Cedex, France) followed by an overnight incubation in Hanks buffered saline with 25% sucrose. Cochleae were embedded in O.C.T. compound (Miles Laboratories, Elkhart, IA, USA) and cryosectioned at 10 μ m thickness, mounted on SuperFrost®/plus microscope slides and stored at -20°C .

Immunohistochemistry and quantification of IHC synaptic contacts

Immunohistochemistry was carried out as previously described [45]. Slides from cochlear tissues were dried at room temperature for 30 minutes. Afterward, they were permeabilized for 3 minutes with 0.1% Triton X-100/PBS, washed with PBS and blocked with 1% bovine serum albumin (BSA)/PBS. Slides were incubated over night with primary antibodies (mouse monoclonal anti-CtBP2/RIBEYE; BD Transduction Laboratories, San Jose, CA, USA, 612044; 1:50 and rabbit otoferlin 1:8000, generated as described previously [46]) diluted in 0.5% BSA/PBS. On the next day, slides were washed with PBS and incubated with a secondary antibody for 1 h (Alexa Fluor 488-conjugated antibodies, Molecular Probes, Bar Harbor, ME, USA). Slides were washed again and mounted with Vectashield containing DAPI (Vector Laboratories, Burlingame, CA, USA). Ribbon synapses of IHCs (Fig. 1) were counted as previously described [11, 47]. Briefly, cross sections of the cochleae containing all three cochlear turns (apical/medial, midbasal and basal) were used for immunohistochemistry. Ribbons were counted for each cochlear turn in at least three independent slices per cochlea. Mean values per turn were calculated for each animal. The mean values from the single animals are used to calculate the average ribbon number per cochlear turn and treatment. Sections were viewed using an Olympus AX70 microscope equipped with epifluorescence illumination (100x objective, NA = 1.35) and a motorized z-axis. Images were acquired using a CCD camera and the imaging software Cell[^]F (OSIS GmbH, Münster, Germany). CtBP2/RIBEYE-immunopositive spot counting on cryosectioned cochleae was performed through imaging over a distance of 8 μ m with the complete coverage of the IHC nucleus and beyond in an image-stack along the z-axis (z-stack). Typically, z-stacks consist of 30 layers with a z-increment of 0.276 μ m, for each layer one image per fluorochrome was acquired. Z-stacks were 3-dimensionally deconvoluted using Cell[^]F's RIDE module with the Nearest Neighbour algorithm (OSIS GmbH, Münster, Germany). Ribbon synapses were counted for each animal from several cochlear sections in at least three experiments for all cochlear turns.

Statistical analysis

Unless otherwise stated, all data were presented as group mean with standard deviation (S.D.) or with standard error of the mean (S.E.M.). N gives the number of animals per experimental group. Differences of the means were compared for statistical significance either by Student's *t*-test, 1-way, or 2-way ANOVA tests. All ANOVA tests were followed by multiple *t*-tests with Bonferroni adjustment of α -level. The resulting *P* values are reported in the legends. **P* < 0.05; ***P* < 0.01; ****P* < 0.001; *n.s.* = not significant.

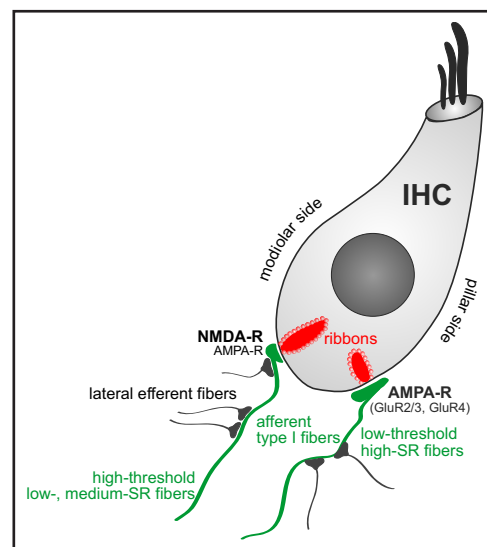
Results

To test the NMDA receptor antagonist AM-101 for possible neurotoxic effects, AM-101 (200 μ M) was applied locally to the round window niche of sham-exposed (no noise exposure) rats. Hearing function and the number of inner hair cell (IHC) synaptic ribbon number was analyzed 15 d following local drug application. As shown in Figure 2, the hearing threshold measured by ABR with either click stimulus or pure tones in AM-101 treated animals remained unchanged as compared to animals treated with placebo (Fig. 2A).

Table 1. Number of animals for each experimental approach. *three sham-exposed sub-groups were pooled into a single group with n = 8 animals for data analyses

Group	Treatment	Legend	Number of animals
single application	1 Sham exp. + placebo 2 d	sham exp.	6
	2 AT + placebo 2 d	AT	6
	3 AT + AM-101 2 d (gelfoam)	AT + AM-101 (gelfoam)	6
repeated applications	4* Sham exp. + placebo 2 d, 4 d, 8 d	sham exp.	2
	4* Sham exp.+ AM-101 2 d; placebo 4 d, 8 d	sham exp.	2
	4* Sham exp. + AM-101 2 d, 4 d; placebo 8 d	sham exp.	2
	4* Sham exp. + AM-101 2 d, 4 d, 8 d	sham exp.	2
	5 AT + placebo 2 d, 4 d, 8 d	AT	4
	6 AT + AM-101 2 d; placebo 4 d, 8 d	AT + AM-101 1x (port)	4
	7 AT + AM-101 2 d, 4 d; placebo 8 d	AT + AM-101 2x (port)	4
	8 AT + AM-101 2 d, 4 d, 8 d	AT + AM-101 3x (port)	4

Fig. 1. Predicted subcellular positions of high- and low-spike rate (SR) fibers at the inner hair cell (IHC). IHC ribbon synapses have synaptic active zones that are characterized by a presynaptic electron-dense structure known as synaptic ribbon to which synaptic vesicles are tethered. Ribbon synapses are glutamatergic, wherein glutamate is released at high and sustained rates. Glutamate released by the IHC synapses in response to the receptor potential drives the firing pattern of the primary auditory neurons upon binding to the AMPA- or NMDA receptors. Afferent auditory nerve fibers of IHCs are classified according to their spontaneous action potential discharge rate (spike rate). High-threshold, low- and medium-SR fibers are presumably preferentially located at the modiolar side of the IHC, where larger ribbons are associated with smaller patches of NMDA-R and AMPA-R. Low-threshold, high-SR fibers are presumably preferentially located at the pillar side of the IHC, where smaller ribbons oppose larger AMPA-R patches. Modified after Knipper et al. [4].



To get a more detailed insight into the effect of AM-101 for the stabilization of IHC synaptic contacts to auditory nerve fibers, we counted the ribbons of IHCs as a metric for IHC afferent innervation [48] using a ribbon synapse marking antibody against CtBP2/RIBEYE (Fig. 2B). No difference in the number of IHC ribbons was observed between animals treated with placebo or AM-101 (Fig. 2B), indicating that AM-101 did not deteriorate the stability of the IHC synapse per se.

We then tested multiple applications of AM-101 (200 μ M) in sham-exposed animals treated either 2 d (once, 1x), 2 d and 4 d (twice, 2x) or 2 d, 4 d and 8 d (three times, 3x) following sham exposure (Fig. 2C, D). For this application paradigm a port system was implanted as described in the Methods section, which allowed successive drug application on multiple days. No significant difference in hearing threshold was observed between sham-exposed animals treated with placebo and animals treated with AM-101 (Fig. 2C). Likewise, no significant difference in IHC ribbon number was observed between the different treatment groups (Fig. 2D). We therefore conclude that AM-101 was not detrimental to the auditory system in terms of hearing threshold and the integrity of IHC ribbon synapse even after multiple applications.

To investigate the therapeutic potential of NMDA receptor antagonists, the hearing function in sham-exposed and noise-exposed rats treated either with placebo or AM-101 (200 μ M) 2 d after acoustical induction of trauma was analyzed (Fig. 3). Animals were

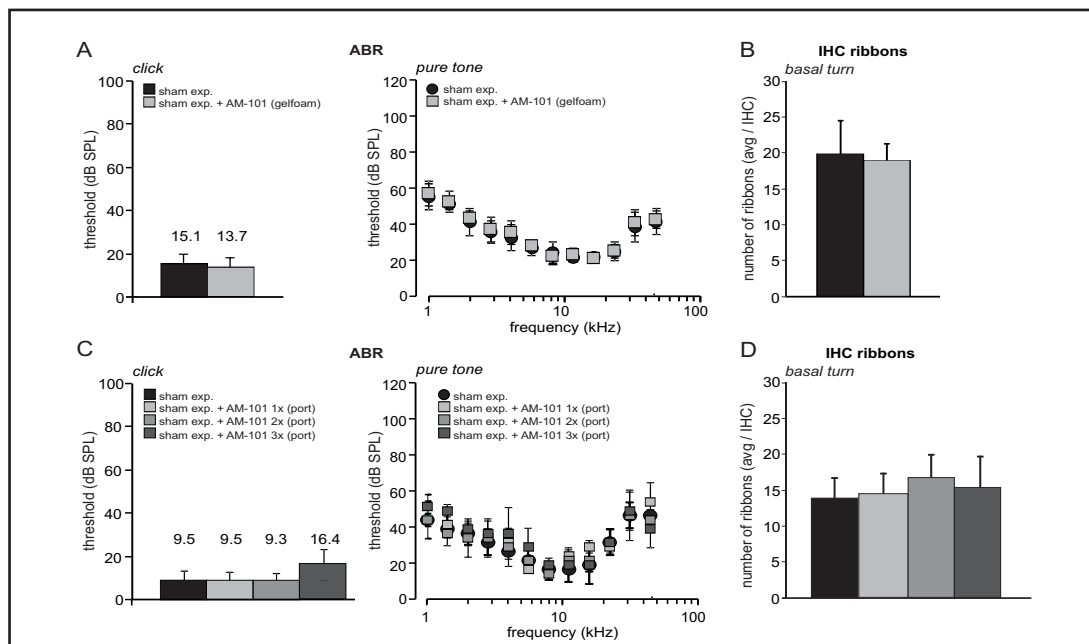
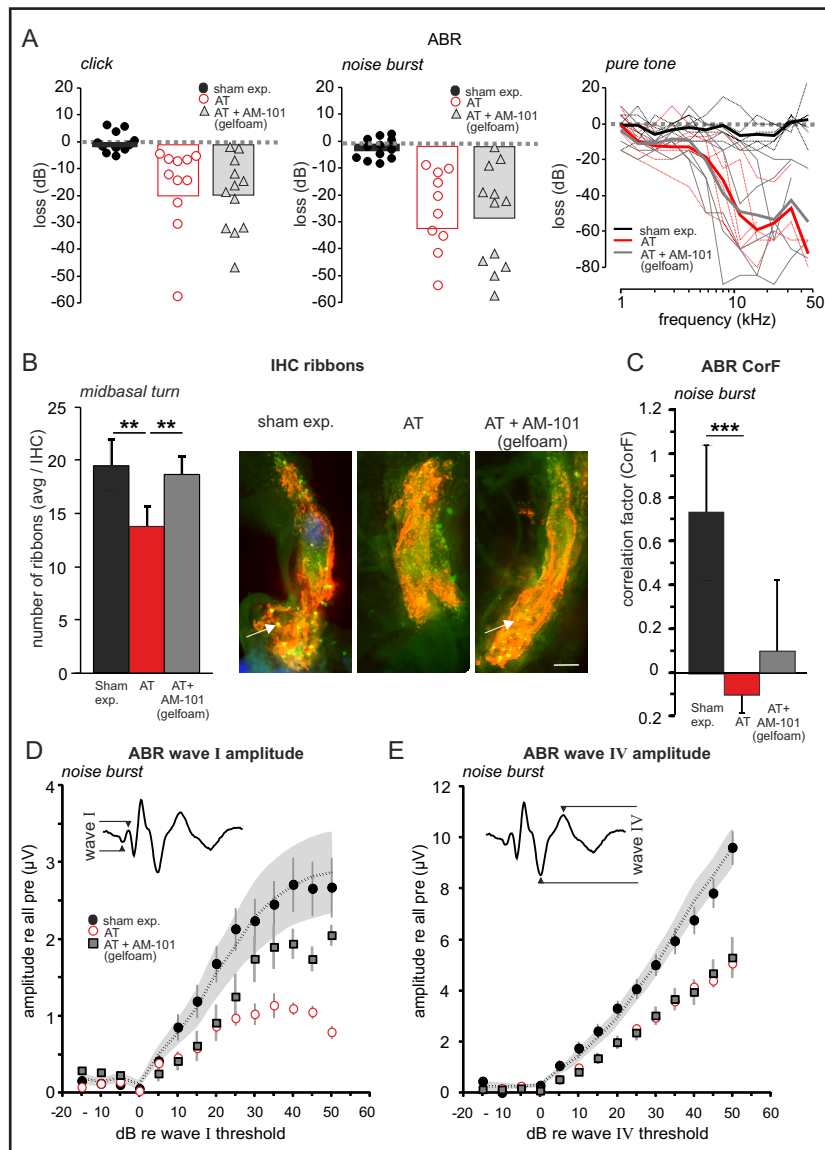


Fig. 2. Audiometric hearing threshold (A, C) and quantification of immunopositive IHC synaptic ribbons (B, D) in sham-exposed animals with and without treatment of 200 μ M AM-101 via gelfoam (A, B), as well as sham-exposed animals treated with 200 μ M AM-101 in a single (1x), double (2x), or triple (3x) application through port system 15 d after sham exposure. (A) Mean \pm S.D. audiometric thresholds determined by click and pure tone stimulus evoked ABR in sham-exposed animals and sham-exposed animals treated locally with 200 μ M AM-101 via gelfoam. No significant difference was observed between the two animal groups (click: 2-sided Student's *t*-test, $P = 0.413$; pure tone: 2-way ANOVA, $P = 0.238$ for effects of treatment). (B) Mean \pm S.D. number of IHC ribbons for basal cochlear turn in animals measured in (A) (sham-exposed animals: $n = 14$ ears of 7 animals; sham-exposed + AM-101: $n = 12$ ears of 6 animals). No significant difference was observed between the two animal groups (apical turn: $P = 0.650$; midbasal turn: $P = 0.312$; basal turn: $P = 0.381$, 2-sided Student's *t*-test). (C) Mean \pm S.D. audiometric thresholds determined by click and pure tone stimulus evoked ABR in sham-exposed animals and animals treated locally with 200 μ M AM-101 in a single (1x), double (2x), or triple (3x) application (AT + AM-101) 15 d following sham exposure. No significant difference was observed between the animal groups (click: 1-way ANOVA, $P = 0.461$; pure tone stimulus: 2-way ANOVA, $P = 0.266$ for effects of treatment; $n = 4$ ears of 2 animals for each group). (D) Number of IHC ribbons in animals measured in (C). No significant difference was observed between the different groups as shown for the basal cochlear turn. (apical turn: $P = 0.729$; midbasal turn: $P = 0.614$; basal turn: $P = 0.730$, 1-way ANOVA).

exposed to a traumatizing sound (AT) of 10 kHz, 120 dB SPL for 2 h, which is reported to induce tinnitus in more than 90% of the rats (29 out of 31 rats with tinnitus; [11]). AM-101 was applied locally to the round-window niche 48 h following noise exposure using a gelfoam carrier. Noise-exposed animals showed a significant hearing loss measured by click stimulus evoked ABR, which was expectedly more pronounced when noise burst stimulus containing more energy at higher frequency range (>8-10 kHz)(noise-ABR) was utilized. This high-frequency hearing loss was confirmed by the measurement of frequency-specific pure tone evoked ABRs depicting a hearing loss of noise-exposed rats at 8 kHz and higher (Fig. 3A; Table 2). AM-101 did not alter hearing function in addition to the trauma induced hearing loss.

Due to the hearing loss in the higher frequencies, IHC ribbon numbers were analyzed in detail in the high-frequency cochlear turns (Fig. 3B). The number of IHC ribbons in the midbasal cochlear turns was significantly reduced in noise-exposed placebo treated rats, in comparison to sham-exposed rats and noise-exposed rats treated with AM-101

Fig. 3. Loss of audiometric hearing threshold (A), quantification of immunopositive IHC synaptic ribbon structures (B), and ABR waveform analysis (C, D, E) in sham-exposed animals (sham exp.), noise-exposed placebo treated animals (AT) and noise-exposed animals treated with 200 μ M AM-101 (AT + AM-101) 15 d after noise exposure. Trauma was induced by a 10 kHz pure tone at 120 dB SPL for 2 h; treatment was effected 2 d post noise exposure. (A) Mean (bar) and individual ear loss of audiometric threshold (dB) from baseline determined from click, noise burst and frequency-specific pure-tone evoked ABRs. The threshold shift measured by click and noise burst stimulus was significantly larger for noise-exposed animals (1-way ANOVA, $P = 0.001$, $n = 12$ ears of 6 animals, respectively), but not statistically different between noise-exposed placebo treated and noise-exposed AM-101 treated animals (*post hoc* Student's *t*-test, $P > 0.999$). A similar significant high frequency hearing loss was observed in both noise-exposed groups (2-way ANOVA, $P < 0.001$, $n = 6$ ears of 6 animals, see also Table 2). (B) Mean \pm S.D. number of IHC ribbons in the midbasal cochlear turn, noise-exposed animals showed significant reduction of ribbons in comparison to sham-exposed animals (1-way ANOVA, midbasal turn, $P < 0.001$). The noise-exposed animals treated with AM-101 had a similar number of ribbons as the sham-exposed animals. Example of an immunohistochemistry with fluorescent antibody specific for otoferlin (red) and CtBP2/RIBEYE (green, white arrows) shown for the midbasal cochlear turn of animals analyzed in (A). Otoferlin is an IHC marker. A reduced IHC basal pole with massive loss of IHC ribbons (yellow dots, white arrow) was observed in noise-exposed placebo treated animals (AT) in comparison to sham-exposed and noise-exposed AM-101 treated animals (AT+AM-101). Scale bar, 10 μ m. Nuclear staining DAPI (blue). (C) Mean \pm S.D. ABR wave correlation (CorF) to noise burst evoked ABR before and 15 d after noise exposure analyzed 20 dB above threshold. CorF was significantly lower in both noise-exposed groups than in the sham-exposed group (1-way ANOVA, $P < 0.001$; *post hoc* Student's *t*-test between sham-exposed and noise-exposed placebo groups, $P < 0.001$, between sham-exposed and noise-exposed AM-101 treated groups, $P = 0.003$, and between noise-exposed placebo treated and noise-exposed AM-101 treated groups, $P = 0.736$). $n = 12$ ears of 6 animals for each group. (D) Mean \pm S.E.M. noise-ABR wave I peak-to-peak amplitude growth function. Wave I amplitudes were reduced for all noise-exposed rats compared with the sham-exposed rats (2-way ANOVA, $P < 0.001$, see also Table



2). (B) Mean \pm S.D. number of IHC ribbons in the midbasal cochlear turn, noise-exposed animals showed significant reduction of ribbons in comparison to sham-exposed animals (1-way ANOVA, midbasal turn, $P < 0.001$). The noise-exposed animals treated with AM-101 had a similar number of ribbons as the sham-exposed animals. Example of an immunohistochemistry with fluorescent antibody specific for otoferlin (red) and CtBP2/RIBEYE (green, white arrows) shown for the midbasal cochlear turn of animals analyzed in (A). Otoferlin is an IHC marker. A reduced IHC basal pole with massive loss of IHC ribbons (yellow dots, white arrow) was observed in noise-exposed placebo treated animals (AT) in comparison to sham-exposed and noise-exposed AM-101 treated animals (AT+AM-101). Scale bar, 10 μ m. Nuclear staining DAPI (blue). (C) Mean \pm S.D. ABR wave correlation (CorF) to noise burst evoked ABR before and 15 d after noise exposure analyzed 20 dB above threshold. CorF was significantly lower in both noise-exposed groups than in the sham-exposed group (1-way ANOVA, $P < 0.001$; *post hoc* Student's *t*-test between sham-exposed and noise-exposed placebo groups, $P < 0.001$, between sham-exposed and noise-exposed AM-101 treated groups, $P = 0.003$, and between noise-exposed placebo treated and noise-exposed AM-101 treated groups, $P = 0.736$). $n = 12$ ears of 6 animals for each group. (D) Mean \pm S.E.M. noise-ABR wave I peak-to-peak amplitude growth function. Wave I amplitudes were reduced for all noise-exposed rats compared with the sham-exposed rats (2-way ANOVA, $P < 0.001$, see also Table

2); however, they were less reduced for noise-exposed rats treated with AM-101 than for noise-exposed placebo treated rats. The growth function with shaded area represents the grand mean \pm 95% confidence interval (CI) for rats in all three experimental groups before noise exposure. Growth functions of individual treatment groups are shifted relative to the grand mean. Inset: example of ABR waveform stimulated at the level of 20 dB above individual hearing threshold; denoting peak-to-peak wave I amplitude. (E) Mean \pm S.E.M. noise-ABR wave IV peak-to-peak amplitude growth function for the same rats as in (D). Exposed rats had significantly reduced wave IV amplitudes compared with the amplitudes of the sham exposed rats (2-way ANOVA, $P < 0.001$, see also Table 2). Rats treated or not treated with AM-101 showed similar extent of reduction of wave IV amplitudes when compared to the sham-exposed rats (Table 2). Inset: example of ABR waveform stimulated at the level of 20 dB above individual hearing threshold; denoting peak-to-peak wave IV amplitude.

Table 2. *Post hoc* comparisons (Student's *t*-tests with Bonferroni adjustment) of ABR threshold shift for pure tone stimuli, ABR wave I and IV amplitudes evoked by noise burst stimuli. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; n.s.: not significant. sham exp.: sham-exposed; AT: sound-exposed placebo; AT +AM101 (gelfoam): sound-exposed AM-101 treated animals

Statistical significance	Pure tone frequency (kHz)											
	1	1.4	2	2.8	4	5.7	8	11.3	16	22.6	32	45.3
sham exp. vs. AT	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	*	**	***	***	**	***
sham exp. vs. AT + AM-101 (gelfoam)	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	**	**	***	***	***	***
AT + AM-101 (gelfoam) vs. AT	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
	Intensity (dB re wave I threshold)											
	0	5	10	15	20	25	30	35	40	45	50	
sham exp. vs. AT	n.s.	n.s.	n.s.	n.s.	*	***	***	***	***	***	***	
sham exp. vs. AT + AM-101 (gelfoam)	n.s.	n.s.	n.s.	n.s.	*	**	n.s.	n.s.	*	**	n.s.	
AT + AM-101 (gelfoam) vs. AT	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	*	*	*	n.s.	**	
	Intensity (dB re wave IV threshold)											
	0	5	10	15	20	25	30	35	40	45	50	
sham exp. vs. AT	n.s.	n.s.	n.s.	n.s.	**	**	***	***	***	***	***	
sham exp. vs. AT + AM-101 (gelfoam)	n.s.	n.s.	n.s.	n.s.	*	***	***	***	***	***	***	
AT + AM-101 (gelfoam) vs. AT	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	

(Fig. 3B). Noise-exposed rats treated with AM-101 had similar ribbon numbers as sham-exposed animals (Fig. 3B). In Fig. 3B, immunohistochemistry of the midbasal cochlear turn for otoferlin (red) as IHC marker and CtBP2/RIBEYE (yellow dots, white arrow) as ribbon marker is shown exemplarily for each treatment group. In noise-exposed placebo treated rats, a reduced IHC basal pole as well as a loss of ribbons (yellow dots) was seen when compared to IHCs of sham-exposed and noise-exposed rats treated with AM-101. Since a similar amount of hearing loss was observed between the two noise-exposed groups but a significant difference in IHC ribbon numbers was found, hearing function was examined at sensation level (suprathreshold) by analyzing the correlation of the ABR waves for noise burst stimulus before and 15 d after noise exposure (Fig. 3C). This correlation revealed a significant loss in ABR wave similarity for noise-exposed placebo treated animals in comparison to sham-exposed rats (Fig. 3C). However, ABR waveform equally lost similarity

Fig. 4. The auditory signal along the auditory pathway can be measured by ABRs providing information regarding auditory function and hearing sensitivity. The normal ABR consists of five prominent waves that occur during the first 10 ms after presentation of a transient sound. These ABR waves are labeled by Roman numerals (I-V). The different peaks of the waves can be assigned to different parts of the ascending auditory pathway. Wave I is generated exclusively by the auditory nerve, whereas waves II, III, IV, and V have contributions from more than one anatomical structure of the ascending auditory pathways. Wave II is mainly generated by the CN, wave III by the superior olivary complex (SOC), wave IV by the lateral lemniscus (LL), and wave V by the LL and its termination in the IC. After noise exposure (post exposure), all amplitudes of ABR waves are reduced compared with non-traumatized control animals (pre exposure). Modified after Knipper et al. [4].

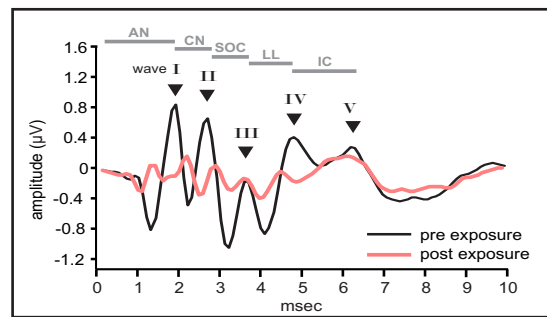


Table 3. *Post hoc* comparisons[†] of ABR threshold loss for pure tone stimuli. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; n.s.: not significant. [†] *Post hoc* comparison between sound-exposed (AT) groups were not significant over all listed frequencies and were omitted from the table for clarity. sham exp.: sham-exposed, AT: sound-exposed placebo, AT + AM101 1x (port): sound-exposed AM-101-treated animals (one time treated), AT + AM101 2x (port): sound-exposed AM-101-treated animals (two times treated), and AT + AM101 3x (port): sound-exposed AM-101-treated animals (three times treated)

Statistical Significance	Pure tone frequency (kHz)											
	1	1.4	2	2.8	4	5.7	8	11.3	16	22.6	32	45.3
sham exp. vs. AT	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	**	n.s.	n.s.	n.s.	***
sham exp. vs. AT + AM-101 1x (port)	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	***
sham exp. vs. AT + AM-101 2x (port)	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	*	**	*	***
sham exp. vs. AT + AM-101 3x (port)	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	*	**	**	***

for the noise-exposed placebo group and the noise-exposed AM-101 group for both click (data not shown) and noise burst evoked responses (Fig. 3C). Since the correlation factor (CorF) addresses only the overall similarity of waveforms between the two noise-exposed animal groups and does not consider individual wave amplitude differences, amplitudes of wave I and IV (Fig. 4) of the noise burst evoked-ABR measurements were analyzed (Fig. 3D, E). The amplitude of ABR wave I, reflecting the synchronous summated neural activity of the auditory nerve, was reduced in noise-exposed animals (Table 2). The noise-exposed placebo treated rats (Fig. 3D, red circles) had a significantly more pronounced reduction in ABR wave I amplitude than the noise-exposed rats treated with AM-101 (Fig. 3D, grey squares; Table 2). For noise-exposed placebo treated animals, the drop in amplitude mirrored the reduced number of ribbons in the midbasal cochlear turn (Fig. 3B). The amplitudes of wave IV (predominantly reflecting the synchronous summated neural activity of the IC) in both noise-exposed animal groups were concertedly reduced (Fig. 3E, Table 2).

In conclusion, when AM-101 was applied 48 h after traumatic noise exposure, the number of IHC ribbons in the midbasal (high-frequency) cochlear turn and the morphology of the ABR wave were partially conserved, suggesting a protective effect of AM-101 with respect to IHC ribbon loss and neural degeneration.

Based on these results and the suggested time window for cochlear NMDA receptor inhibition [27], we studied the effect of multiple successive AM-101 applications two days after noise trauma using a port system. Following intensive sound exposure (120 dB SPL, 10 kHz,

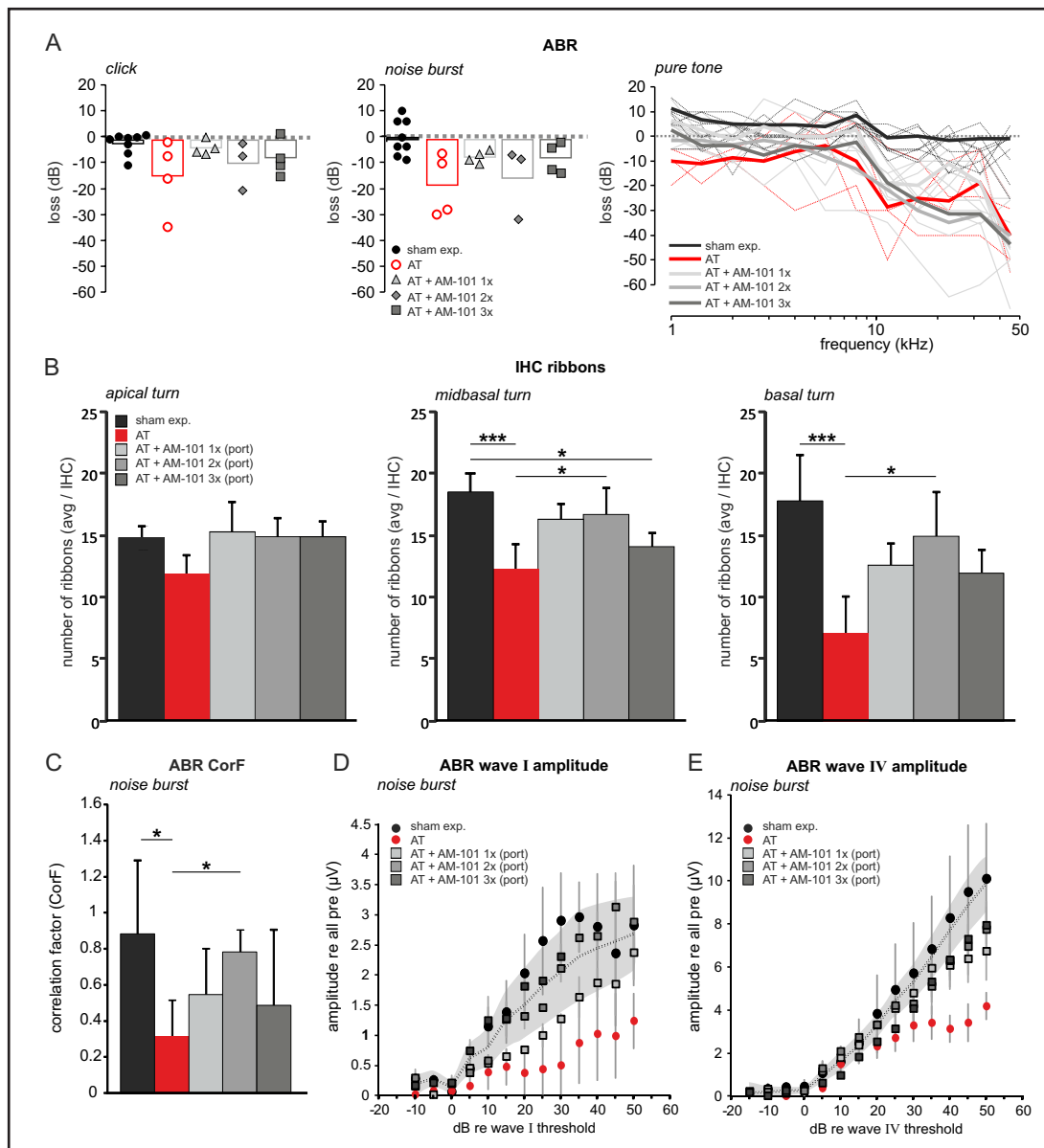


Fig. 5. Audiometric threshold loss (A), number of immunopositive IHC synaptic ribbon structures (B), and ABR waveform analysis (C, D, E) of sham-exposed (sham exp.), noise-exposed (AT) and noise-exposed animals treated with 200 μ M AM-101 in a single (1x), double (2x), or triple (3x) application (AT + AM-101) 15 d after noise exposure. (A) Mean (bar) and individual ear audiometric threshold loss (dB) determined from ABRs for click and noise burst stimuli were not statistically significantly different (click: 1-way ANOVA, $P = 0.135$; noise burst: 1-way ANOVA, $P = 0.092$). Significant threshold losses at frequencies above 11.3 kHz were determined by pure tone evoked ABR for noise-exposed rats as compared with sham-exposed rats (2-way ANOVA, $P < 0.001$, $n = 8$ animals and 4 animals for sham and AT group, respectively). No significant difference was found between the noise-exposed vehicle group and any of the noise-exposed AM-101 treated groups (Table 3). (B) Mean \pm S.D. number of IHC ribbons. No significantly difference was detected among groups in apical turn. In the midbasal turn, noise-exposed placebo treated animals showed a significant reduction in ribbon numbers in comparison to sham-exposed animals and to noise-exposed AM-101 2x animals (1-way ANOVA; $P = 0.002$). A significantly reduced ribbon number was also observed for noise-exposed AM-101 3x treated animals in the midbasal turn. In the basal turn, noise-exposed placebo treated animals showed a significant reduction in ribbon numbers in comparison to sham-exposed animals and to noise-exposed AM-101 2x treated animals (1-way ANOVA; $P = 0.003$). (C) ABR wave correlation (CorF) for

noise burst stimuli before and 15 d after noise exposure analyzed 20 dB above threshold. A significantly reduced correlation was observed in noise-exposed placebo treated animals in comparison to sham-exposed animals and the noise-exposed AM-101 2x treated group (1-way ANOVA, $P = 0.046$). (D) Mean \pm S.E.M. noise-ABR wave I peak-to-peak amplitude growth function 15 d after noise exposure. Reductions of wave I amplitudes were observed for all noise-exposed animals (2-way ANOVA, $P < 0.001$, Table 4). The growth function with shaded area represents the grand mean \pm 95% confidence interval (CI) for rats in all three experimental groups before noise exposure. Normalization is performed as described in Figure 2. (E) Mean \pm S.E.M. noise-ABR wave IV peak-to-peak growth function after exposure. Wave IV amplitudes were reduced for noise-exposed animals without AM-101 treatment (2-way ANOVA, $P < 0.001$, Table 4).

Table 4. *Post hoc* comparisons[†] of ABR wave I and wave IV amplitudes evoked by noise stimuli. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; n.s.: not significant. [†] Comparisons that gave non-significant results in *post hoc* tests over all listed intensities were omitted from the table for clarity, the omitted comparisons are: sham exp + AM-101 1x (port) vs. AT for wave IV amplitudes only; sham exp + AM-101 2x (port) vs. AT for both wave I and wave IV amplitudes; sham exp + AM-101 3x (port) vs. AT for both wave I and wave IV amplitudes; AT + AM-101 1x (port) vs. AT for both wave I and wave IV amplitudes

Statistical Significance	Intensity (dB re wave I threshold)						
	0	5	10	15	20	25	30
sham exp. vs. AT	n.s.	n.s.	n.s.	n.s.	**	***	***
sham exp + AM-101 1x (port) vs. AT	n.s.	n.s.	n.s.	n.s.	*	**	**
AT + AM-101 2x (port) vs. AT	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	**
AT + AM-101 3x (port) vs. AT	n.s.	n.s.	n.s.	n.s.	**	**	***

Statistical Significance	Intensity (dB re wave IV threshold)										
	0	5	10	15	20	25	30	35	40	45	50
sham exp. vs. AT	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	**	***	***
AT + AM-101 2x (port) vs. AT	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	*	*
AT + AM-101 3x (port) vs. AT	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	**	*

2 h) animals were treated 2 d (once), 2 d and 4 d (twice), or 2 d, 4 d and 8 d (three times). After noise exposure, a consistent hearing loss in noise-exposed animals was observed (Fig. 5A) in comparison to sham-exposed animals. Noise-exposed animals treated with AM-101 showed no significant difference for ABR thresholds (1-way ANOVA, $P = 0.092$) among different dose regimens. A significant hearing loss for high frequencies was observed in noise-exposed animals (Fig. 5A). Noise-exposed animals treated with AM-101 were not significantly different in thresholds when compared to the noise-exposed placebo treated animals (Table 3).

The number of ribbon synapses in high frequency cochlear turns (midbasal and basal turn) was significantly reduced in noise-exposed placebo treated animals in comparison to sham-exposed rats and noise-exposed animals treated twice with AM-101 (Fig. 5B). IHC ribbon loss was consistently lower in noise-exposed AM-101-treated animals than in noise-exposed placebo treated animals. No statistically significant difference between the single, double and triple AM-101 treatment groups was observed.

Next, ABR waves for the noise burst evoked-ABR were analyzed (Fig. 5C). Although all noise-exposed animals showed a reduced correlation of the ABR waves, the reduction

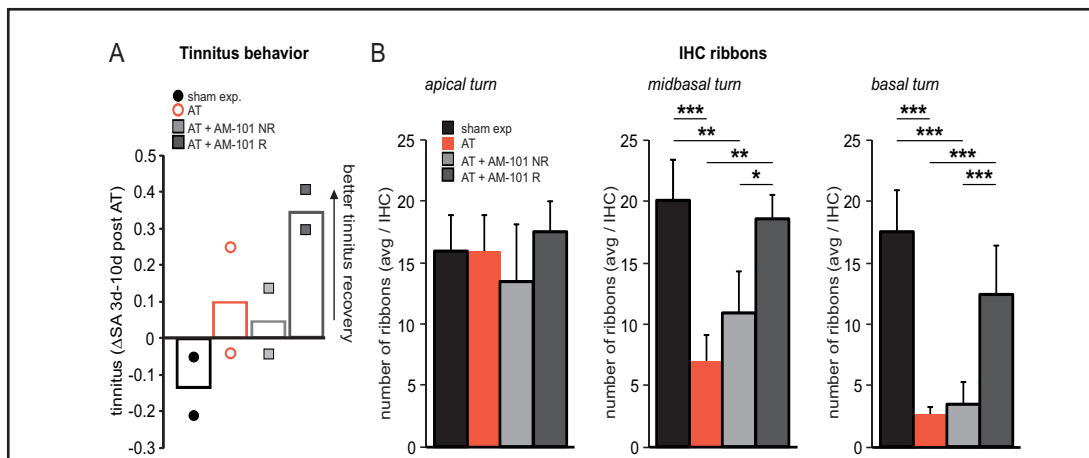


Fig. 6. Improvement of tinnitus behavior and quantification of immunopositive IHC synaptic ribbons in sham-exposed animals (sham exp.), noise-exposed animals treated with placebo (AT) and 200 μ M AM-101 treated noise-exposed animals with or without improvement of tinnitus behavior (non-responder, NR; responder, R) determined by activity during silence (silence activity, SA) through a behavioral approach described in Methods between 3 d and 10 d after noise exposure (SA3d-SA10d). (A) Mean (bar) and individual improvement of tinnitus. The negative values for sham-exposed animals are due to a generally increased activity 10 d following sham exposure probably indicating unlearning. Placebo treated exposed animals show a slight improvement in tinnitus behavior. Exposed animals treated with AM-101 were divided into two groups, one group with very little improvement (non-responder, NR) and another group with a strong recovery (responder, R). (B) Mean \pm S.D. number of IHC ribbons in animals behaviorally tested in (A). In the apical cochlear turn, no significant reduction in ribbon number was observed in the exposed non-responder animals (1-way ANOVA, $P = 0.441$). In the midbasal cochlear turn, placebo treated AT animals and non-responders showed a significant reduction in ribbon number in comparison to sham-exposed animals and responders (1-way ANOVA, $P < 0.001$). Exposed rats responding to AM-101 have no significant ribbon loss compared with the sham exposed animals. In the basal turn placebo treated AT animals and non-responders showed a significant reduction in ribbon number in comparison to sham-exposed animals and to responders (1-way ANOVA, $P < 0.001$). Exposed responders had a slightly but non-significant loss of ribbon compared with the sham exposed animals.

reached significance only in the noise-exposed placebo treated rats (Fig. 5C). Noise-exposed animals treated twice with AM-101 had significantly better waveform similarity than noise-exposed placebo treated animals (Fig. 5C). This positive effect of AM-101 was confirmed by analysis of the individual ABR wave amplitudes (Fig. 5D, E). All noise-exposed groups treated with AM-101 showed less reduced wave I and wave IV amplitudes (Fig. 5D, E, squares; Table 4) in comparison to noise-exposed animals treated with placebo (Fig. 5D, E, red circles), indicating stronger sound evoked neuronal activity at the level of both auditory nerve and IC.

In conclusion, a successive application of AM-101 through a port-tube-ear cannula system to the round-window 2 and 4 d following noise exposure reduced the trauma-induced damage of the IHC synapse (IHC ribbon loss) in high-frequency cochlear regions with a better conservation of central neuronal activity.

Previous studies suggested IHC ribbon loss and ABR wave I amplitude loss as a risk factor for tinnitus [10, 11]. We thus aimed to study the effect of AM-101 in acoustically traumatized animals behaviorally trained to show tinnitus [10]. Eight rats were trained and sound-exposed as described [10], the change of behavioral activity during periods of silence (silence activity, SA as indicator for tinnitus percept) was calculated as a measure for tinnitus 3 d and 10 d after exposure in the presence and absence of AM-101 in comparison to sham exposed animals (Fig. 6). We retrieved two groups of exposed animals treated with AM-101, one group ($n = 2$) showing a clear improvement in tinnitus behavior (responder) between 3

d and 10 d after exposure, and a second AM-101 treated group ($n = 2$) without improvement (non-responder) (Fig. 6A). When number of IHC ribbons was analyzed (Fig. 6B) in the apical cochlear turn no significant difference was observed (Fig. 6B). However, in the midbasal and basal cochlear turn, animals behaviorally responsive for AM-101 lost significantly less IHC ribbons than animals treated with placebo or animals treated with AM-101 but behaviorally non-responsive (Fig. 6B). In comparison to sham-exposed rats, noise-exposed rats responsive to AM-101 showed only a slight but non-significant reduction in IHC ribbon number. Also, revisiting the ribbon data depicted in Figure 5B about 50% of the AM-101 treated animals would be classified as responders, though not behaviorally tested, using the 95% confidence interval of the ribbon numbers from noise-exposed placebo treated rats (basal cochlear turn).

In conclusion, we found groups of AM-101 responders and non-responders. This is similar to a published clinical study [28] only responders experienced an amelioration of tinnitus. Our behavioral tinnitus data presented here are in line with the molecular data of the IHC synapses. Animals with reduced tinnitus showed a less severe reduction of the numbers of IHC synaptic contacts. However, non-responders were not significantly different as compared to placebo treated noise-exposed animals.

Discussion

During the past decades, various studies have consistently reported sound overexposure as the main cause of tinnitus in humans [13]. There is strong evidence that acoustic overstimulation induces functional IHC (synapse) deafferentation and alters auditory nerve activity [20, 21]. This might be due to glutamate-induced excitotoxicity leading to NMDA receptor activation, dendritic swelling, and the production of reactive oxygen species [16]. In the present study we examined whether non-competitive NMDA inhibition with round window applied AM-101 may be beneficial in counteracting the trauma-related deterioration of the IHC synapse and the maladaptive auditory patterns in the ascending auditory pathway during tinnitus [4, 49]. It was already shown that the competitive NMDA receptor antagonist D-AP5 (D(-)-2-amino-5-phosphonopentanoic acid), when infused into non-auditory regions (paraflocculus) of rats months following noise exposure had a long-term relieving effect for tinnitus perception [50]. In case of synaptopathic tinnitus, the therapeutic time window of NMDA receptor inhibition not exactly known. Here, we show that local application of AM-101 in the consolidation window (several days) as it was previously suggested for ifenprodil [27], has beneficial effects on ribbon synapses and ABR waveform. We could also show that at least in rodents, several AM-101 applications during a time period of 8 days did not affect hearing sensitivity and signal transmission in the auditory nerve. No neurotoxic side effects were observed. Considering the cochlear maturation in rats and humans [51] this treatment time would likely correspond to a significantly longer time period in humans. While clinical trials have already demonstrated that single doses of intra-tympanic AM-101 are safe for use in tinnitus patients [28], the safety of repetitive applications still needs to be verified. It is however likely that successive intra-tympanic applications over months in humans may not be deleterious, due to the limited contribution of NMDA receptors to synaptic transmission during physiological hearing conditions.

After noise exposure the application of AM-101 had a beneficial effect on trauma-induced auditory injury. While AM-101 treatment obviously conserved the IHC ribbon synapses and the signal transmission in the auditory nerve, no effect on hearing thresholds was observed. This is probably due to the fact that NMDA receptors are only expressed in low-spontaneous rate (low-SR), high-threshold auditory fibers, as suggested by Pujol et al. [52]. These fibers make up approximately 40% of the unbranched radial afferent fibers connecting IHCs [53]. Additionally, low-SR high threshold fibres are the most vulnerable to acoustic overstimulation [54, 55] and are suggested to trigger loudness pathologies as hyperacusis [11, 56, 57], and

IHC synaptopathies contributing to tinnitus [10, 11]; reviewed in [4, 57]. The effect of the loss of this subgroup of auditory fibers would not necessarily manifest as an elevation of the ABR threshold, but would rather be seen as a reduced growth in the first ABR wave which represents the sound-evoked activity within the auditory nerve [33, 58]. Audiometric thresholds are therefore not a suitable parameter to judge the integrity of IHC synapse/afferent fiber contacts [55]. This is in line with the results obtained in the present study and leads to the hypothesis that the protective effect of AM-101 on synaptic ribbons and the signal transmission in the auditory nerve after noise trauma may be predominantly related to a conservation of low-SR high-threshold fibers.

The conservation of IHC synaptic ribbons also maintains the central auditory responses. This is indicated by the nearly complete restoration of the more centrally generated ABR wave IV amplitudes after noise exposure, shown for the repetitive application of AM-101 via the ear cannula and the port application system. However, for the single application the wave IV amplitudes and also ABR waveform similarity remained reduced. This may be explained by the variance in effectiveness of the traumatizing sound. Inflammatory responses and swelling within the middle ear may reduce the sound level reaching the inner ear due to the more invasive surgical procedure: the cannula had been implanted unilaterally six days before noise exposure while the gelfoam was applied only two days after noise exposure. Indeed the hearing sensitivity and ABR waveform similarity was not as dramatically reduced in the ears with pre-implanted ear cannula when compared to the non-operated ear in the same animals and those animals treated with single application.

Thus the difference in the central response that either leads to restoration of ABR wave IV amplitudes after noise exposure (as shown in the multiple application tests) or a failure in wave IV restoration (as shown in single application paradigm) can be traced back to a difference in the degree of deafferentation. A critical level (60%) of active afferent fibers and stabilized IHC synaptic contacts must be preserved to maintain the capability to generate compensating central gain [10, 11].

Administration of AM-101 led to the prevention of trauma-induced degeneration of afferent fibers and a loss of the centrally generated ABR wave amplitudes. This in turn reduced the risk of tinnitus specifically in a subgroup of affected animals that had a particularly good conservation of synaptic ribbons. The observation that AM-101 in a subgroup of behaviorally trained animals had the potential to reduce the risk of tinnitus onset, may indicate that AM-101 cannot rescue every afferent fiber loss per se, but at least contributes to a critical preservation of auditory nerve activity at sensation levels. Due to the limited number of behaviorally trained animals, the data on tinnitus relief upon AM-101 treatment in the present study are preliminary but nevertheless may support the value of local tinnitus treatment with NMDA receptor antagonists.

Conclusion

In patients the heterogeneity of tinnitus seems to be the key reason for the large variability in the results obtained with NMDA receptor antagonists although a common pathophysiological pathway seems to exist for certain types of tinnitus. Types based on noise trauma or vascular disruptions or glutamate excitotoxicity can probably not be generalized to the entire tinnitus population. In addition to etiology, the duration of the disorder and the potential centralization processes need to be taken into account. In focusing on traumatic origins and the acute stage as well as targeted delivery of the compound to the cochlea, AM-101 has been evaluated under relatively well defined conditions. The results reported here and in phase II clinical trials [29] show that NMDA receptor antagonists have the potential of being used for local/cochlear treatment of tinnitus in patients.

Financial support

This work was supported by Auris Medical, by the Deutsche Forschungsgemeinschaft DFG (Kni 316/3-2 and Kni 316/3-3), by a grant from the Medical Faculty of the University of Tübingen (fortune), and by the European Commission, Marie Curie Training Site, HEARING (QLG3-CT-2001-60009).

Abbreviations

ABR (auditory brainstem response); AP (artificial perilymph); AT (noise exposure (acoustic trauma)); BSA (bovine serum albumin); CI (confidence interval); CorF (correlation factor); D-AP5 (D(-)-2-amino-5-phosphonopentanoic acid); dB SPL (decibel sound pressure level); DCN (dorsal cochlear nucleus); IC (inferior colliculus); IHC (inner hair cell); NMDA (N-methyl-D-aspartate); NMDA-R (NMDA receptor); OHC (outer hair cell); PBS (phosphate buffered saline); post AT (after noise exposure); pre AT (before noise exposure); SA (silence activity); S.D. (standard deviation); S.E.M. (standard error of the mean); SPL (sound pressure level); SR (spike (discharge) rate).

Disclosure Statement

The authors declare no conflict of interest

References

- 1 Lockwood AH, Salvi RJ, Burkard RF: Tinnitus. *N Engl J Med* 2002;347:904-910.
- 2 Ahmad N, Seidman M: Tinnitus in the older adult: Epidemiology, pathophysiology and treatment options. *Drugs Aging* 2004;21:297-305.
- 3 Dauman R, Bouscau-Faure F: Assessment and amelioration of hyperacusis in tinnitus patients. *Acta Otolaryngol* 2005;125:503-509.
- 4 Knipper M, Van Dijk P, Nunes I, Rüttiger L, Zimmermann U: Advances in the neurobiology of hearing disorders: Recent developments regarding the basis of tinnitus and hyperacusis. *Prog Neurobiol* 2013;111:17-33.
- 5 Sanchez TG, Medeiros IR, Levy CP, Ramalho Jda R, Bento RF: Tinnitus in normally hearing patients: Clinical aspects and repercussions. *Braz J Otorhinolaryngol* 2005;71:427-431.
- 6 Gu JW, Halpin CF, Nam EC, Levine RA, Melcher JR: Tinnitus, diminished sound-level tolerance, and elevated auditory activity in humans with clinically normal hearing sensitivity. *J Neurophysiol* 2010;104:3361-3370.
- 7 Schaette R: Tinnitus in men, mice (as well as other rodents), and machines. *Hear Res* 2013
- 8 Weisz N, Hartmann T, Dohrmann K, Schlee W, Norena A: High-frequency tinnitus without hearing loss does not mean absence of deafferentation. *Hear Res* 2006;222:108-114.
- 9 Bauer CA, Brozoski TJ, Myers K: Primary afferent dendrite degeneration as a cause of tinnitus. *J Neurosci Res* 2007;85:1489-1498.
- 10 Rüttiger L, Singer W, Panford-Walsh R, Matsumoto M, Lee SC, Zuccotti A, Zimmermann U, Jaumann M, Rohbock K, Xiong H, Knipper M: The reduced cochlear output and the failure to adapt the central auditory response causes tinnitus in noise exposed rats. *PLoS One* 2013;8:e57247.
- 11 Singer W, Zuccotti A, Jaumann M, Lee SC, Panford-Walsh R, Xiong H, Zimmermann U, Franz C, Geisler HS, Köpfschall I, Rohbock K, Varakina K, Verpoorten S, Reinbothe T, Schimmang T, Rüttiger L, Knipper M: Noise-induced inner hair cell ribbon loss disturbs central arc mobilization: A novel molecular paradigm for understanding tinnitus. *Mol Neurobiol* 2013;47:261-279.

- 12 Coles RR: Epidemiology of tinnitus: (1) prevalence. *J Laryngol Otol Suppl* 1984;9:7-15.
- 13 Nicolas-Puel C, Faulconbridge RL, Guitton M, Puel JL, Mondain M, Uziel A: Characteristics of tinnitus and etiology of associated hearing loss: A study of 123 patients. *Int Tinnitus J* 2002;8:37-44.
- 14 Puel JL, Saffiedine S, Gervais d'Aldin C, Eybalin M, Pujol R: Synaptic regeneration and functional recovery after excitotoxic injury in the guinea pig cochlea. *C R Acad Sci III* 1995;318:67-75.
- 15 Nordang L, Cestreicher E, Arnold W, Anniko M: Glutamate is the afferent neurotransmitter in the human cochlea. *Acta Otolaryngol* 2000;120:359-362.
- 16 Ruel J, Chabbert C, Nouvian R, Bendris R, Eybalin M, Leger CL, Bourien J, Mersel M, Puel JL: Salicylate enables cochlear arachidonic-acid-sensitive nmda receptor responses. *J Neurosci* 2008;28:7313-7323.
- 17 Traynelis SF, Wollmuth LP, McBain CJ, Menniti FS, Vance KM, Ogden KK, Hansen KB, Yuan H, Myers SJ, Dingledine R: Glutamate receptor ion channels: Structure, regulation, and function. *Pharmacol Rev* 2010;62:405-496.
- 18 Pujol R, Puel JL, Gervais d'Aldin C, Eybalin M: Pathophysiology of the glutamatergic synapses in the cochlea. *Acta Otolaryngol* 1993;113:330-334.
- 19 Cazals Y: Auditory sensori-neural alterations induced by salicylate. *Prog Neurobiol* 2000;62:583-631.
- 20 Salvi RJ, Wang J, Ding D: Auditory plasticity and hyperactivity following cochlear damage. *Hear Res* 2000;147:261-274.
- 21 Eggermont JJ, Roberts LE: The neuroscience of tinnitus. *Trends Neurosci* 2004;27:676-682.
- 22 Schaette R, Kempster R: Computational models of neurophysiological correlates of tinnitus. *Front Syst Neurosci* 2012;6:34.
- 23 Schaette R, Kempster R: Predicting tinnitus pitch from patients' audiograms with a computational model for the development of neuronal hyperactivity. *J Neurophysiol* 2009;101:3042-3052.
- 24 Langguth B, Elgoyhen AB: Current pharmacological treatments for tinnitus. *Expert Opin Pharmacother* 2012;13:2495-2509.
- 25 Puel JL, Ruel J, Gervais d'Aldin C, Pujol R: Excitotoxicity and repair of cochlear synapses after noise-trauma induced hearing loss. *Neuroreport* 1998;9:2109-2114.
- 26 Guitton MJ, Caston J, Ruel J, Johnson RM, Pujol R, Puel JL: Salicylate induces tinnitus through activation of cochlear nmda receptors. *J Neurosci* 2003;23:3944-3952.
- 27 Guitton MJ, Dudai Y: Blockade of cochlear nmda receptors prevents long-term tinnitus during a brief consolidation window after acoustic trauma. *Neural Plast* 2007;2007:80904.
- 28 Muehlmeier G, Biesinger E, Maier H: Safety of intratympanic injection of am-101 in patients with acute inner ear tinnitus. *Audiol Neurootol* 2011;16:388-397.
- 29 van de Heyning P, Muehlmeier G, Cox T, Lisowska G, Maier H, Morawski K, Meyer T: Efficacy and safety of am-101 in the treatment of acute inner ear tinnitus--a double-blind, randomized, placebo-controlled phase ii study. *Otol Neurotol* 2014;35:589-597.
- 30 Lustig LR: The history of intratympanic drug therapy in otology. *Otolaryngol Clin North Am* 2004;37:1001-1017.
- 31 Meyer T: Intratympanic treatment for tinnitus: A review. *Noise Health* 2013;15:83-90.
- 32 Matthews G, Fuchs P: The diverse roles of ribbon synapses in sensory neurotransmission. *Nat Rev Neurosci* 2010;11:812-822.
- 33 Kujawa SG, Liberman MC: Adding insult to injury: Cochlear nerve degeneration after „temporary“ noise-induced hearing loss. *J Neurosci* 2009;29:14077-14085.
- 34 Lin HW, Furman AC, Kujawa SG, Liberman MC: Primary neural degeneration in the guinea pig cochlea after reversible noise-induced threshold shift. *J Assoc Res Otolaryngol* 2011;12:605-616.
- 35 Fuchs PA: Time and intensity coding at the hair cell's ribbon synapse. *J Physiol* 2005;566:7-12.
- 36 Moser T, Brandt A, Lysakowski A: Hair cell ribbon synapses. *Cell Tissue Res* 2006;326:347-359.
- 37 Schmitz F: The making of synaptic ribbons: How they are built and what they do. *Neuroscientist* 2009;15:611-624.
- 38 Johnson SL, Marcotti W, Kros CJ: Increase in efficiency and reduction in ca2+ dependence of exocytosis during development of mouse inner hair cells. *J Physiol* 2005;563:177-191.
- 39 Khimich D, Nouvian R, Pujol R, Tom Dieck S, Egner A, Gundelfinger ED, Moser T: Hair cell synaptic ribbons are essential for synchronous auditory signalling. *Nature* 2005;434:889-894.

- 40 Buran BN, Strenzke N, Neef A, Gundelfinger ED, Moser T, Liberman MC: Onset coding is degraded in auditory nerve fibers from mutant mice lacking synaptic ribbons. *J Neurosci* 2010;30:7587-7597.
- 41 Johnson DH, Kiang NY: Analysis of discharges recorded simultaneously from pairs of auditory nerve fibers. *Biophys J* 1976;16:719-734.
- 42 Kaltenbach JA, Afman CE: Hyperactivity in the dorsal cochlear nucleus after intense sound exposure and its resemblance to tone-evoked activity: A physiological model for tinnitus. *Hear Res* 2000;140:165-172.
- 43 Knipper M, Zinn C, Maier H, Praetorius M, Rohbock K, Köpschall I, Zimmermann U: Thyroid hormone deficiency before the onset of hearing causes irreversible damage to peripheral and central auditory systems. *J Neurophysiol* 2000;83:3101-3112.
- 44 Rüttiger L, Ciuffani J, Zenner HP, Knipper M: A behavioral paradigm to judge acute sodium salicylate-induced sound experience in rats: A new approach for an animal model on tinnitus. *Hear Res* 2003;180:39-50.
- 45 Engel J, Braig C, Rüttiger L, Kuhn S, Zimmermann U, Blin N, Sausbier M, Kalbacher H, Münkner S, Rohbock K, Ruth P, Winter H, Knipper M: Two classes of outer hair cells along the tonotopic axis of the cochlea. *Neuroscience* 2006;143:837-849.
- 46 Schug N, Braig C, Zimmermann U, Engel J, Winter H, Ruth P, Blin N, Pfister M, Kalbacher H, Knipper M: Differential expression of otoferlin in brain, vestibular system, immature and mature cochlea of the rat. *Eur J Neurosci* 2006;24:3372-3380.
- 47 Heidrych P, Zimmermann U, Kuhn S, Franz C, Engel J, Duncker SV, Hirt B, Pusch CM, Ruth P, Pfister M, Marcotti W, Blin N, Knipper M: Otoferlin interacts with myosin vi: Implications for maintenance of the basolateral synaptic structure of the inner hair cell. *Hum Mol Genet* 2009;18:2779-2790.
- 48 Liberman MC, Dodds LW, Pierce S: Afferent and efferent innervation of the cat cochlea: Quantitative analysis with light and electron microscopy. *J Comp Neurol* 1990;301:443-460.
- 49 Knipper M, Müller M, Zimmermann U: Molecular mechanism of tinnitus; in Eggermont JJ, Zeng FG, Popper AN, Fay RR (eds): *Tinnitus*. New York, Springer Science + Business Media, 2010.
- 50 Brozoski TJ, Wisner KW, Odintsov B, Bauer CA: Local nmda receptor blockade attenuates chronic tinnitus and associated brain activity in an animal model. *PLoS One* 2013;8:e77674.
- 51 Jewett DL, Romano MN: Neonatal development of auditory system potentials averaged from the scalp of rat and cat. *Brain Res* 1972;36:101-115.
- 52 Pujol R, Puel JL, Eybalin M: Implication of non-nmda and nmda receptors in cochlear ischemia. *Neuroreport* 1992;3:299-302.
- 53 Yates GK: Auditory-nerve spontaneous rates vary predictably with threshold. *Hear Res* 1991;57:57-62.
- 54 Heinz MG, Young ED: Response growth with sound level in auditory-nerve fibers after noise-induced hearing loss. *J Neurophysiol* 2004;91:784-795.
- 55 Furman AC, Kujawa SG, Liberman MC: Noise-induced cochlear neuropathy is selective for fibers with low spontaneous rates. *J Neurophysiol* 2013;110:577-586.
- 56 Hickox AE, Liberman MC: Is noise-induced cochlear neuropathy key to the generation of hyperacusis or tinnitus? *J Neurophysiol* 2013
- 57 Zeng FG: An active loudness model suggesting tinnitus as increased central noise and hyperacusis as increased nonlinear gain. *Hear Res* 2013;295:172-179.
- 58 Melcher JR, Kiang NY: Generators of the brainstem auditory evoked potential in cat. Iii: Identified cell populations. *Hear Res* 1996;93:52-71.