The vulnerability of the auditory sense to noise exposure, demanding environmental conditions, and pharmacological cGMP cascade stimulation in a model for age-related hearing loss in the rat and the gerbil

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Summary

Age-related hearing loss (ARHL) is a complex degenerative disease commonly seen in elderly people. It is considered as most often sensory impairment in the elderly. ARHL is a rapidly growing healthcare issue due to the aging of the population. Up to now our knowledge on the pathology of ARHL was restricted to the loss or dysfunction of outer hair cells that normally increase the amplitude and frequency selectivity of sound vibrations by electromechanical feedback. Recently however it was shown that neurodegeneration of afferent neural fibers in mice can progress over age independent of outer hair cells (OHCs) loss in mice. In the present study we were able to confirm this finding in the rat and gerbil animal model. Shown in detail for the rat, an age dependent loss of inner hair cells (IHCs) ribbons that was used as a correlate of auditory fiber loss was observed together with a moderate high-frequency hearing loss independent of OHCs dysfunction in the first period of a rat's life. The high frequency hearing loss and IHCs ribbon loss corresponded to a loss of summed auditory nerve activity shown with auditory brainstem response (ABR) wave I amplitude loss progressed further with age. Only in the second period of life OHCs dysfunction could be detected in addition to IHCs synapse deterioration. Interestingly the progressive decline of auditory nerve fibers over age could not be centrally compensated. In contrast, we found that young rats could centrally compensate sensory deprivation was induced by moderate noise exposure. Elderly rats were unable to compensate damage but remained less sensitive for noise exposure. It appeared that the failure to compensate the damage and the reduced sensitivity for noise exposure was caused by the loss of auditory fibers over age and therefore neither fibers nor reduced amplitudes could be reduced further. In addition over age the brain may have lost the capacity to compensate peripheral damage, what may go hand in hand with a loss of a capacity for central homeostatic adaptation. We questioned whether this neurodegenerative effect in the cochlea over age after trauma could be compensated by environmental enrichment of the housing conditions. In the present study we could show that environmental enrichment as well as a stimulation of the cGMP cascade through activation of the soluble guanylate cyclase could counteract the age-related high-frequency hearing loss. Further studies are essential to analyse to what extend this positive effects are the result of direct impact on IHCs synapse afferent contacts. The findings are discussed on the assumptions that a vulnerable part of auditory fibers can be lost over age or noise. Young animals can compensate an auditory deprivation and only in young animals environmental enrichment or pharmacological intervention may have beneficial effects.

Zusammenfassung

Altersbedingter Hörverlust (ARHL) ist eine komplexe degenerative Krankheit, die als die häufigste sensorische Störung in der älteren Bevölkerung festgestellt wird. ARHL ist ein rasch zunehmendes Gesundheitsproblem, bedingt durch die immer älter werdende Gesellschaft. Bis jetzt waren unsere Kenntnisse über die ARHL-Pathologie beschränkt auf Verlust oder Fehlfunktion der äusseren Haarzellen (ÄHZ), welche normalerweise die Bandbreite und die Frequenzselektivität der Schallschwingungen durch elektromechanische Rückkopplung erhöhen. Kürzlich wurde bei Mäusen gezeigt, dass die Neurodegeneration von afferenten Nervenfasern während des Alterungsprozesses unabhängig vom Verlust der ÄHZs zunimmt. In der gegenwärtigen Studie waren wir in der Lage, diesen Befund im Ratten- und Wüstenrennmaus-Tiermodell zu bestätigen. Im Detail wurde in der Ratte gezeigt, dass ein altersabhängiger Verlust der Bändersynpasen (Ribbonsynpasen) der inneren Haarzellen (IHZ), welcher als Korrelat für den Verlust von auditorischen Fasern verwendet wurde, zusammen mit einem moderaten hochfrequenten Hörverlust auftritt, der unabhängig von einer beobachteten ÄHZ-Fehlfunktion im ersten Lebensabschnitt einer Ratte ist. Der hochfrequente Hörverlust und der Verlust der Bändersynpasen spiegeln sich in einem Verlust der Summenaktivität des auditorischen Nervs wieder, was durch einen fortschreitenden Verlust der Amplitude von Welle I der Hirnstammaudiometrie (ABR) während des Alterns gezeigt werden konnte. Erst im zweiten Lebensabschnitt de Ratte konnten ÄHZ-Fehlfunktionen zusätzlich zur IHZ-Synapsenrückbildung entdeckt werden. Interessanterweise konnte der allmähliche Rückgang von Hörnervenfasern während des Alterns nicht zentral ausgeglichen werden. Hingegen fanden wir heraus, dass junge Ratten sensorische Deprivation, verursacht durch moderate Lärmbelastung, zentral ausgleichen konnten. Ältere Ratten waren unfähig Defekte auszugleichen, jedoch waren sie weniger empfindlich gegen Lärmbelastung. Es scheint, dass das Versagen den Defekt auszugleichen und die reduzierte Empfindlichkeit gegen Lärmbelastung, durch den anhaltenden Hörfaserverlust verursacht wurden und deshalb weder Fasern noch reduzierte Bandbreiten weiter reduziert werden konnten. Zusätzlich könnte das Gehirn während des Alterns die Kapazität verloren haben periphere Schäden zu kompensieren, was Hand in Hand mit einem Verlust der zentralen homöostatischen Adaption geht. Wir fragten uns, ob dieser neurodegenerative Effekt in der Innenohrschnecke während des Alterns nach einem Trauma durch Haltung in angereicherter Umgebung ausgeglichen werden kann. In der gegenwärtigen Studie konnten wir zeigen, dass sowohl ein angereichertes Umfeld als auch eine pharmakologische Stimulation der cGMP (zyklisches

Guanosinmonophosphat) -Kaskade durch Aktivierung der löslichen Guanylatcyclase dem altersbedingten hochfrequenten Hörverlust entgegenwirken können. Weitere Studien sind essentiell, um zu analysieren in wie weit diese positiven Effekte direkten Einfluss auf die Kontakte zwischen IHZ-Synapsen und afferent Fasern haben. Die Ergebnisse werden in der Annahme diskutiert, dass ein empfindlicherer Teil der Hörnervenfasern während des Alterungsprozesses oder nach akustischem Trauma verloren gehen kann. Junge Tiere können auditorische Deprivation kompensieren und nur bei jungen Tieren kann eine angereicherte Umgebung oder pharmakologische Behandlung positive Effekte haben.

1. Introduction

Age-related hearing loss (ARHL) is a complex degenerative disease commonly seen in elderly people. Hearing acuity declines with age and the rate of decline accelerates with age even without a history of noise exposures or diseases that affect the ear (ISO 1999). ARHL is a rapidly growing healthcare issue due to the demographic change resulting from higher numbers of elderly people.

It is well known that both genetical and environmental factors contribute to age-related hearing loss, called presbyacusis (Fetoni et al., 2011). ARHL can be categorized according to the pathophysiological background: sensory (loss of hair cell), neural (loss of spiral ganglion neurons, SGNs), metabolic (atrophy of stria vascularis), and mechanical (thickening and stiffening of the basilar membrane) (Schuknecht, 1964). In the clinic many patients suffering from ARHL show a combination of these pathologies which may lead to additive hearing loss (Schuknecht and Gacek, 1993). Not a lot known about the molecular basis of presbyacusis.

Recent findings showed that even auditory exposure, that in normal situation would lead to a complete recovery of hearing thresholds, lead to slow degeneration of the auditory nerve fibres (AN) and progressive hearing loss in mice and guinea pigs (Kujawa and Liberman, 2009). In this case hearing loss initially would not become apparent in the clinical treshold testing (Kujawa and Liberman, 2011), but only later during lifespan. Therefore investigating a relation of the deafferentation of IHCs during acoustic trauma and ageing is an important first step for the development of strategies for the prevention of these pathological conditions.

1.1. Physiology of the hearing

Prior to description of the processes that occur along the auditory pathway during aging, the basic anatomy and organization of the auditory system would be introduced.

1.1.1. Inner ear

The inner ear (**Fig. 1A**) contains the sensory organs for hearing (cochlea) and balance.

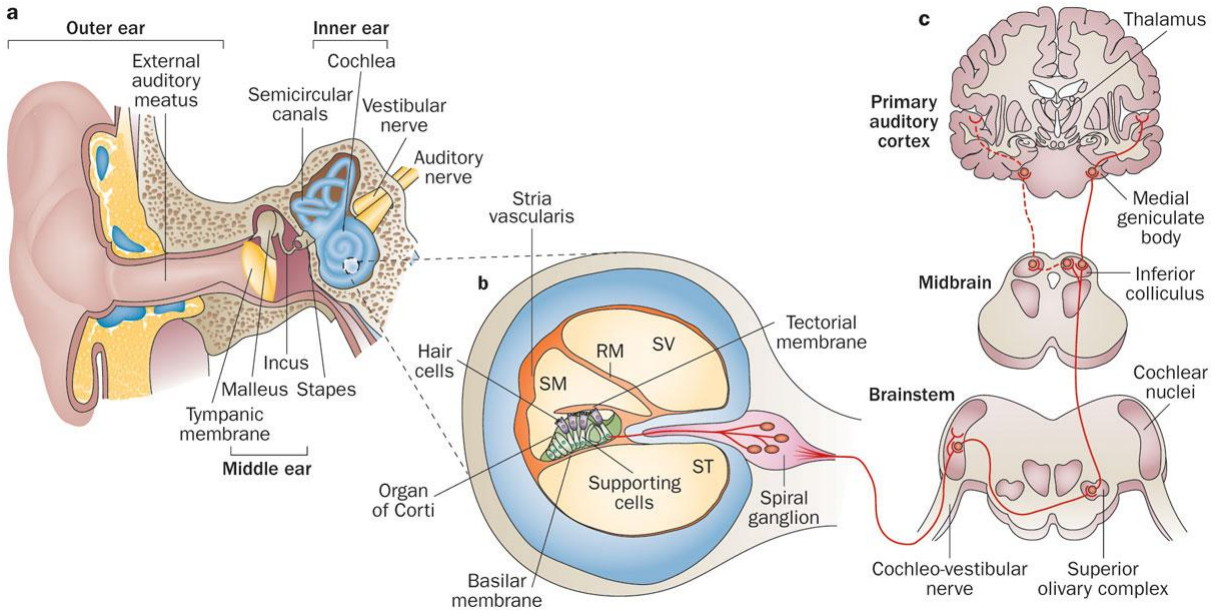

Figure 1. The inner ear and central auditory pathway (from the review of Ng L. et al, 2013).
A: Cut-away view of the human inner ear. B: Section of a turn of the cochlear spiral showing the organ of Corti, which contains the hair cells. Afferent neurons within the spiral ganglion act as the primary relay neurons between the hair cells and the brain. C: Spiral ganglion neurons project to the cochlear nucleus in the brainstem, which connects to higher auditory centers in the brain. A simplified representation of the primary (contralateral) afferent pathway is illustrated; however, extensive crossing occurs within the ascending auditory system. Efferent innervation of the cochlea is not indicated here. Abbreviations: RM, Reissner's membrane; SM, scala media; ST, scala tympani; SV, scala vestibule.

The cochlea (**Fig. 1B**) is a small coiled structure that is filled with two types of fluids: endolymph and perilymph. The oval window and the round window are situated at the basal end of this tube. The basilar membrane is a main structural element that separates the scala media from the scala tympani and determines the mechanical wave propagation properties of the cochlear partition. Another function of cochlear partition is to support tectorial membrane that extends along the cochlea parallel to the basilar membrane. There are fluid-filled spaces on each side of the cochlear partition, named the scala vestibuli and the scala tympani; a distinct channel, the scala media, runs within the cochlear partition. The cochlear partition forms an opening, helicotrema that connects with the scala vestibuli and the scala tympani. As a result of this structural arrangement, inward movement of the oval window displaces the fluid of the inner ear, which causes the round window to bulge out slightly and deforms the basilar membrane (Neuroscience, 2nd edition, 2001).

Cochlear nerve fibers synapse ends in dorsal and ventral cochlear nuclei. The cochlear nuclei (**Fig. 1C**) receive an input from the ipsilateral cochlear nerve. Part of the axons of the neurons from the cochlear nuclei on both sides of the brain go into the brainstem and make

synaptic connections in a region of the medulla that is called the superior olivary complex (SOC), when the other part forms the connections with the olivary cells of the same side (Casseday, Neff, 1975). Trapezoid body is formed here by these fibers. Going from dorsal cochlear nucleus, fibers partially cross the midline and connect with the cells of the nuclei of the lateral lemniscus (Glendenning et al., 1981). Neuronal fibers from both sides of the ventral cochlear nuclei and the olivary complex also end up connecting the nuclei of the lateral lemniscus. The lateral lemniscus is a major axonal tract. Most of the fibers that form this tract go to the midbrain and end in the inferior colliculus (IC). Few fibers go through the IC and end in the medial geniculate body, where also fibers going from IC end up (FitzPatrick, 1975; Osen, 1972). From the medial geniculate body there is a projection of fibres to a portion of the cortex of the temporal lobe.

1.1.2. Organ of Corti

The cochlea consists of three liquid filled tubes, which are the scala tympani, the scala media and the scala vestibuli. The organ of Corti is situated in the scala media. It rests upon the basilar membrane and represents the sensory epithelium (Fig. 1B). The sensory epithelium consists of several types of supporting cells and two types of sensory cells (Fig. 2A): inner hair cells (IHCs) and outer hair cells (OHCs). An arrangement of the hair cells is not equal: the IHCs are arranged in a single row, the OHCs in three parallel rows (Fig. 2A) (Lim, 1986; Slepecky, 1996). The oragan of Corti is situated on the tectorial membrane and is indirectly connected to the osseus spiral lamina through the spiral limbus. Only the stereocilia of the OHCs are in contact with the tectorial membrane.

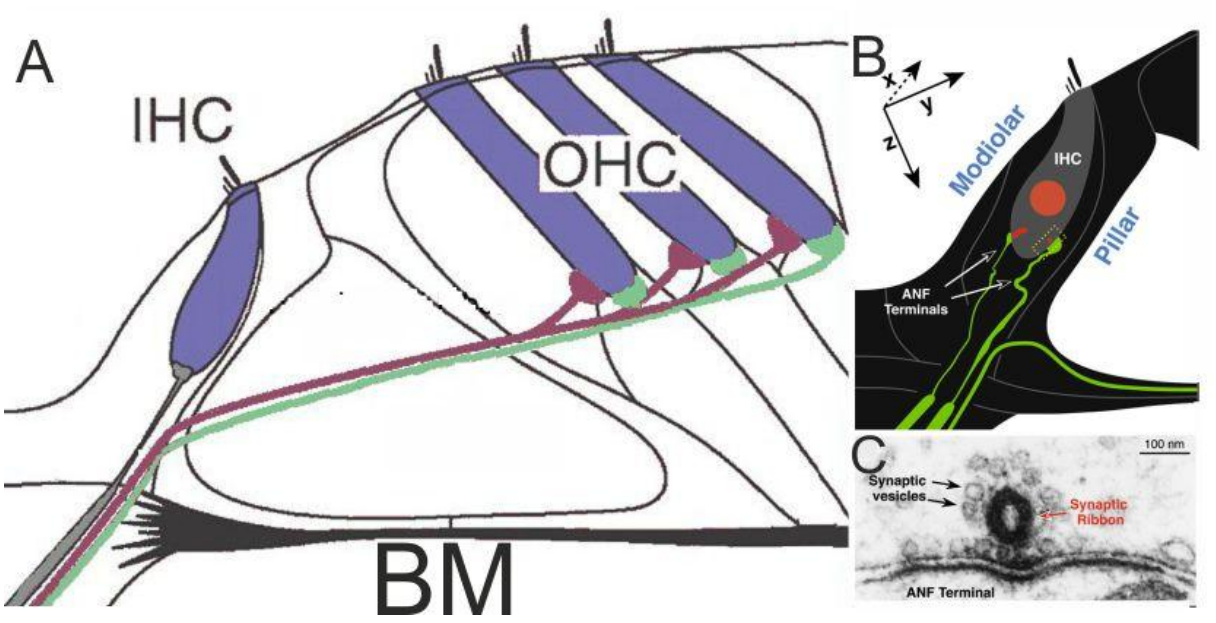

Figure 2. The normal afferent innervation of the inner hair cell (IHC).
A: Schematic crossection of the cochlea shows the organ of Corti (Fu et al., 2010). **B:** Schematic illustration of two unmyelinated cochlear nerve terminals (green) making synaptic contact with a single IHC. At each synapse, a pre-synaptic ribbon (small red dots) is present within the IHC. **C:** An electron micrograph of a synaptic complex illustrating the pre-synaptic ribbon and its halo of synaptic vesicles within the IHC (Lin et al., 2011).

Movements between the basilar membrane with the sensory epithelium, on the one hand, and the tectorial membrane, on the other hand, cause receptor potentials to be produced in the hair cells (Lim, 1986; Russell et al., 1986; Nobili et al., 1998).

The cochlear hair cells together and supporting cells are organized in the pattern that is a geometrically regular (Keithley et al., 1992). The rat has about 3800–4000 OHCs (364, average total density of OHC/mm of the length of the organ of Corti) and 1000–1300 IHCs (121, average total density of IHC/mm of the length of the organ of Corti). However, the density of hair cells is not uniform.

75–80% of all hair cells are the OHCs, that are situated on the supporting cells called Deiter's cells and are organized in three parallel rows (Fig. 2A). The OHCs have a cylinder shape, and their size and number is gradually varied along the basilar membrane. The number of stereocilia per OHCs is larger at the base than at the apex (75 vs 62) (Burda et al., 1988). In contrast to the IHCs, OHCs receive only 5–10% of the afferent innervation from the cochlear nerve but receive much more of efferent innervation by contacting a large number of efferent nerve terminals starting in the olivocochlear bundle (Warr, 1992).

Sensory transduction in the cochlea has been largely studied during recent decades. The connection of the hair-cell stereocilia through tip-links and displacement of the cilia toward the longer stereocilia form the openings of ion channels located near the tips of the stereocilia (Hudspeth et al., 2000; Müller, 2008). Depolarization of the hair cells and release of neurotransmitters are resulted by the inflow of potassium (K+) ions. Thus the hair cells in the inner ear transform vibrations into action potentials with the deviation of the stereocilia.

Functions of the IHCs and OHCs are different. The IHCs play a role of the primary sensory cell (Russell, 1983; Markin and Hudspeth, 1995), while the OHCs act as the motor cells converting membrane potential into a mechanical force (Nobili et al., 1998). OHCs are able to contract and elongate, therefore generating mechanical forces that are capable to alter the mechanics of the cochlear duct. These processes increase frequency selectivity and sensitivity (Neely and Kim, 1983; Dallos and Evans, 1995; Dallos, 1997; Nobili et al., 1998). Regarding functions described, the cochlea could be characterized as an active nonlinear filter allowing transmission of the auditory signals to the auditory nerve by the IHCs. This frequency selectivity becomes possible because of the suppression of contiguous frequencies, which is an equivalent to the concept of lateral inhibition in the central nervous system (Nobili et al., 1998).

1.2. Age-related hearing loss

ARHL or presbyacusis is one of the most common age-related impairments and the most often sensory impairment in elderly people. ARHL is considered to be the result of physiological degeneration and the accumulation of the negative effects with age like acoustic overexposure, medical disorders, experiencing treatment with ototoxic medicaments, as well as hereditary susceptibility (CHABA, 1988). The definition of ARHL was not updated for a long time. Gates and Mills in 2005 showed that the term presbyacusis refers to hearing loss in the elderly and therefore represents the contributions of a lifespan to the auditory system. It is characterized by reduced hearing sensitivity and speech understanding in noisy environments, delay in central processing of acoustic input, and difficulties with localization of sound sources (Gates GA and Mills JH, 2005).

1.2.1. Animal models of age-related hearing loss

To study ARHL several animal models were used. Every animal model has advantages and disadvantages, and could be used dependently on the final goal of the experiment.

Mouse animal models of ARHL

For several mouse strains spiral ganglion neuron (SGN) degeneration was reported as a source of hearing dysfunction in elderly animals (Saitoh et al., 1994; Dazert et al., 1996; McFadden et al., 2001; Bao et al., 2005; Kujawa and Liberman, 2006; Lang et al., 2006). These studies did not highlight whether this effect is primary to ARHL, or whether it follows the loss of OHC. For example, for 129S6/SvEv mice it was shown that SGN loss starts while number of inner hair cells (IHCs) is unaffected (Ohlemiller and Gagnon, 2004). Also mouse models allow to characterize strial ARHL (Ohlemiller et al., 2006;Ohlemiller et al., 2009).

CBA/Ca mouse and C57BL/6 mouse strains are the most commonly used to model sensory ARHL. Hearing function of CBA/Ca mouse is intact up to 18 months, after this time period progressive high-frequency hearing loss starts which progresses gradually to the low frequencies (Li and Borg, 1991; Li and Hultcrantz, 1994; Spongr et al., 1997). In contrast, the C57BL/6 mouse represents a rapid onset of hearing deficit and rapid decline (Li and Borg, 1991). Hair cells degeneration also progresses dramatically over age and by one year

complete loss of hair cells could be observed (Li and Hultcrantz, 1994; Sponger et al., 1997). Similarly the DBA/2J mouse is a model of accelerated ARHL, that develops hearing loss by three weeks after birth (Willott, 1981; Willott et al., 1982). Hearing over all frequency range becomes severely impaired by 2 months (Willott et al., 2005). Underlying pathophysiology is characterized with progressive loss of cochlear sensory cells.

Mongolian gerbil model of ARHL

The Mongolian gerbil model is characterized with an ARHL correlates with a pattern of strial ARHL. By 36 months, Mongolian gerbil shows a 15–35 dB threshold shift. This hearing dysfunction is more prominent in the high frequencies (Mills et al., 1990). This damage is associated with the cochlear blood supply failure (Thomopoulos et al., 1997; Gratton and Schulte, 1995). The damage of stria vascularis is associated with a putative reduction in the supply of mitochondrial adenosine triphosphate (ATP) in the strial marginal cells (Spicer and Schulte, 2005), that leads to a loss of Na, K-ATPase activity and a decrease in the endocochlear potential (EP) (Schulte and Schmiedt, 1992; Gratton et al., 1997).

Fischer 344 rat model of ARHL

The Fischer 344 (F344) is an albino inbred rat strain (Chesky and Rockstein, 1976; Rao and Boorman, 1990) that develops a progressive hearing loss starting in the high frequencies that shifts gradually to lower frequencies as the animal ages. Recent studies on the F344/DuCrl substrain found that the source of ARHL are changes in the middle ear impedance (Popelar et al., 2006) and strial degeneration (Buckiova et al., 2006; Buckiova et al., 2007), as well as OHC loss. The F344/NHsd rat substrain develops a progressive high-frequency hearing loss very similar to one of the F344/DuCrl substrain. In the F344/NHsd rat loss of OHCs followed by loss of SGNs in the basal, midbasal, and medial turns of the cochlea has been reported (Keithley et al., 1992). The EP was found to be intact in old F344/NHsd rat (Bielefeld et al., 2008).

1.3. Effects of noise stimulation on hearing

Both age-related and noise-induced hearing losses in humans could be caused by several factors. Gates and co-workers were able to show in 2000 in a clinical study that the noise-damaged ear does not "age" at the same rate as the non-noise damaged ear (Gates et al., 2000). Kujawa and Liberman showed in their study in 2006 that after non-traumatizing sound stimulation substantial, ongoing deterioration of cochlear neural responses without additional change in preneural responses, could be observed (Kujawa and Liberman, 2006).

1.3.1. Cochlear damage after mild noise exposure

Previously it was hypothesized that in healthy animals full regeneration of cochlear nerve terminals occurs after neural degeneration induced with acoustic overexposure (Puel et al., 1998; Pujol and Puel, 1999). In contrast recent findings showed that acoustic overexposure causes a permanent loss of peripheral nerve terminals on IHC (Kujawa and Liberman, 2009; Lin et al., 2011). Ability of the IHCs to transmit sound information over a large intensity range (Robles and Ruggero, 2001) can be achieved only through numerous afferent contacts. Innervation of the IHCs is performed by unbranched spiral ganglion dendrites (Fig. 2). IHCs contain presynaptic structures that are called ribbons (Glowatzki and Fuchs, 2002), that maintain a releasable pool of neurotransmitters. By this type of signaling temporal characteristics can be coded in reliable way (Buran et al., 2010). The afferent fibers that innervate IHCs are classified based on their response threshold and spontaneous rate (SR). Low threshold high-SR fibers are sensitive to low sound pressure levels, with thresholds between 0 and 20 dB SPL. High threshold low-SR and medium-SR fibers have thresholds between 20 and 40 dB (Heinz and Young, 2004; Liberman, 1978; Merchan-Perez and Liberman, 1996; Müller and Robertson, 1991; Sachs and Abbas, 1974; Schroeder et al., 2001; Spoendlin and Schrott, 1989; Yates, 1991).

IHC damage would change cochlear transduction and lower the firing rates of auditory nerve fibers (Liberman and Kiang, 1984; Sewell, 1984). Acoustic exposure causes neurodegeneration of auditory nerve (Kujawa and Liberman, 2009; Lin et al., 2011) that was found to correlate with a reduced number of IHCs ribbon synapses (Jaumann et al., 2012; Kujawa and Liberman, 2009; Lin et al., 2011; Rüttiger et al., 2013; Zuccotti et al., 2012).

1.3.2. Alterations in the central neural system caused by ambient noise exposure

In the central auditory system, nerve signals are transduced from the auditory nerve (AN) (Fig. 3A, 1) through the cochlear nucleus (CN) (Fig. 3A, 2) to higher brain regions. The ABR wave I (Fig. 3B, "I") shows the activity of the auditory nerve (AN), while later ABR waves are produced by synchronous neural activity in the auditory brainstem (Melcher and Kiang, 1996). The auditory nerve bifurcates in the brainstem and continues to dorsal and ventral parts of the cochlear nucleus. In the dorsal cochlear nucleus (DCN) the signal is transduced through projection neurons (Kaltenbach, 2007) to the contralateral side (Fig. 3A, 5, red) to the inferior colliculus (IC) and then to the thalamus. From ventral cochlear nucleus (VCN) signal (Fig. 3B, "II") goes further either ipsilaterally (Fig. 3A, 6, blue) or contralaterally (Fig. 3A, 7, red) crossing the superior olivary complex (SOC) the lateral lemniscus (LL) (Fig. 3A and B, "III") (Coleman and Clerici, 1987) and then to the IC (Fig. 3B, "IV"). From IC neural fibers project to the ipsi- (Fig. 3A, 8, blue) and contralateral (Fig. 3A, 9, red) medial geniculate body (MGB) in the thalamus. From the MGB, signals are finally transduced (Fig. 3A, 10, blue) to the auditory cortex (AC) (Malmierca and Merchan, 2004).

The central auditory system is able to compensate the decline of hearing function by upregulating response in central circuitries (Salvi et al., 2000). Central compensation occurs initially in the auditory brainstem, and could spread to ascending auditory nuclei (Manzoor et al., 2013; Mulders and Robertson, 2013). In humans (Gu et al., 2012) and animals (Rüttiger et al., 2013; Singer et al., 2013), suprathreshold effects of ABR waves could be analyzed to study central activity following cochlear damage.

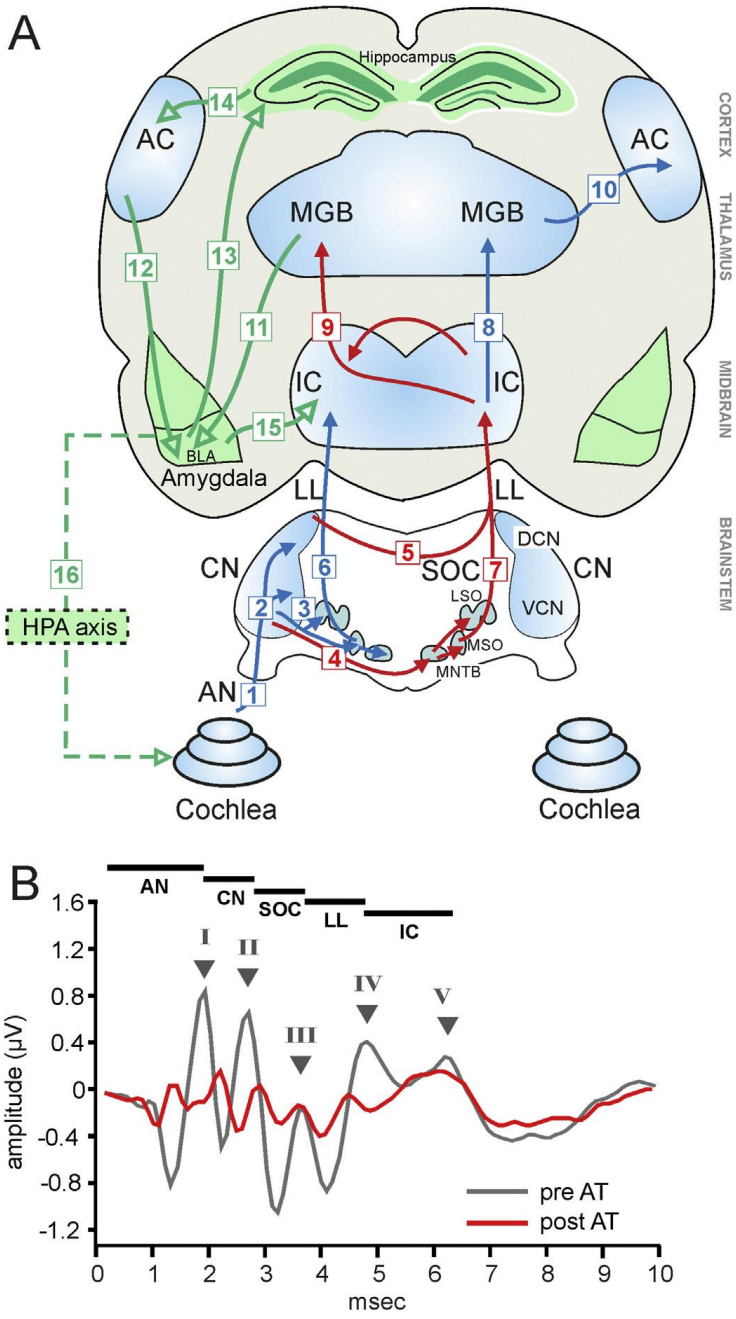

Figure 3. Central auditory circuits and auditory brainstem responses.

A: The sound transduction pathway through the central auditory circuits. See description in text. **B:** The electrical signal measured from the ABRs. The normal ABR consists of five waves that occur during the first 10 ms after presentation of sound. These ABR waves are labeled by Roman numerals (I-V) (taken from Knipper et al., 2013)

1.4. Environmental enrichment

It was already shown that conditioning with a sound of a moderate sound pressure level before a traumatizing noise exposure partially protects from noise-induced hearing loss (Kujawa and Liberman, 1999). The mechanism of this effect is still unclear. Also enriched acoustic environment after acoustic overexposure has been shown to be protective against noise-induced pathological effects as it caused reorganization of the tonotopic map and an increase in spontaneous firing rate correlated with increased neuronal synchrony (Norena and Eggermont, 2005). Furthermore, the hearing of acoustically overexposed cats that were kept in the acoustically enriched environment after noise exposure appeared to be comparable with that of unexposed animals (Norena and Eggermont, 2006). Even though the effect of enriched environment was studied in details in motor (Marques et al., 2014) and sensory systems other than auditory (Wang et al., 2013), an effect of environmental enrichment on presbyacusis so far has not been studied neither for humans nor for rodents. However, a beneficial effect of enriched environment on presbyopia was shown recently (Polat et al., 2012).

1.5. cGMP-cGK$_I$ signaling pathway

The role of the NO-sGC-cGMP signaling pathway in the cochlea is not studied in details. However, an important role of this cascade in the large number of cellular processes is described (Denninger and Marletta, 1999). Nitrogen monoxide (NO) is synthesized from L-Arginin with the help of nitrogen monoxide synthase (NOS). Three isoforms of NOS have been described: neuronal (nNOS), endothelial (eNOS) and inducible (iNOS) NOS (Ghalayini, 2004).

Only recently the research on NO function in the cochlea was initiated and the first overview was given in 2004 by Takumida and Anniko (2002). While the role of NO in the sensory epithelium is a matter of debates, there are no doubts that NO activated signal pathways play an important role in physiological and pathophysiological conditions in the cochlea (Takumida, 2002). It was already shown to be protective (Ruan, 2002) as well as damaging (Chen and Tseng, 2008). The most recent study shows a protective effect of cGMP-cGK$_I$ signaling pathway stimulation in rats and mice (Jaumann et al., 2012).

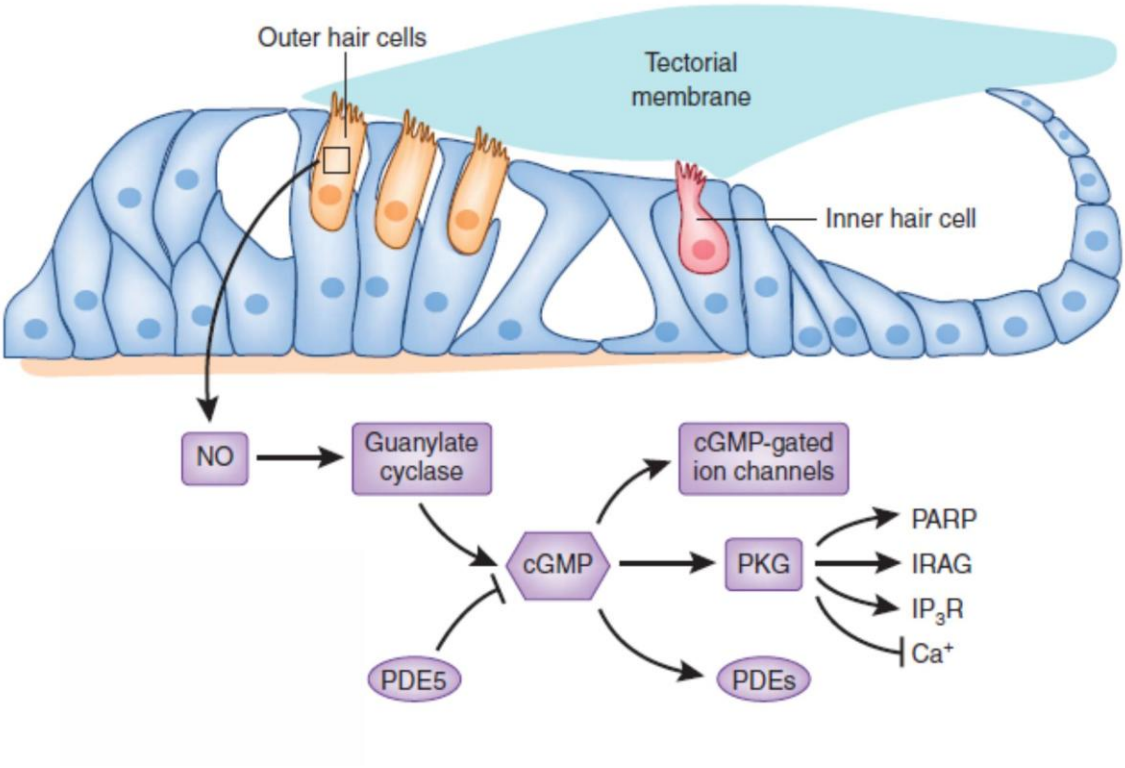

Figure 4. NO-sGC-cGMP signaling pathway.
The top image shows a cross section of the cochlear sensory epithelium, with the inner and outer hair cells and the surrounding supporting structures. The diagram below shows the cGMP-PKG signaling pathway in outer hair cells (adapted from Layman W and Zuo J, 2012).

Released NO activates the soluble guanilat cyclase (sGC), that enzymatically catalyses cyclic guanosine-3 '-5 '-monophosphate (cGMP) synthesis from guanosine-5 '-triphosphate (GTP). cGMP activates cGMP dependent proteinkinases (cGK). The presence of these enzymes was described in numerous tissues (Hofman et al., 2009). In the ear phosphodiesterase-5 (PDE5) modulates cGMP signal by hydrolyzing cGMP. The signaling pathway is shown schematically in the Figure 4.

In the context of the protective role of PDE inhibitors for noise induced hearing loss (Jaumann et al., 2012), this study investigated the possible protective role of sGC stimulators, that are a component of drugs used in therapy of pulmonary hypertension (Stasch and Evgenov, 2013).

1.5.1. Cyclic guanosine monophosphate (cGMP)

cGMP is a "second messenger", that was first described in 1961 by Smith (Smith et al., 1961). His discovery became a basis for a large number of works researching the role of cGMP in blood pressure regulation, coagulation, signal transduction in the eye, and long term potentiation (Schmidt et al., 2009).

1.5.2. Soluble guanylate cyclase (sGC)

The NO-sGC-cGMP-cGK signaling pathway can be also modulated by interacting with the guanylate cyclase (GC). GC is a heterodimer, composed from one alpha and one heme-binding beta subunits. The mammalian enzyme contains one heme subunit per dimer. In its Fe (II) - form heme binds nitrogen monooxide (Poulos, 2006). Besides soluble GC (sGC) there are membrane-based particulate GCs (pGC) that can be activated by natriuretic peptides (D'Souza et al., 2004) and thus could activate cGMP synthesis independent of the NO presence.

In comparison with PDE inhibition that acts only on endogenously available cGMP, sGC-stimulation affects cGMP concentrations directly and increases heme concentration independently. A disadvantage of the sGC stimulation is that cGMP in cells with high PDE concentration could be eliminated more efficiently by PDE, while sGC regulation of cGMP levels is an enzymatic process that requires GTP (Kass, 2007).

1.6. Aim of the study

We aimed to research the source of an auditory fiber loss over age and used CtBP2 as a marker of an afferent fiber structure in comparison with ABR method as a measure of hearing function. By using rat and gerbil animal model we regarded differences between young, elderly and old animals as previous studies show that an age-related decline of hearing function is observed in the late period of life (Rüttiger et al., 2007). We focused on the changes on the neuronal level independent of OHCs loss. In this context we also were interested whether vulnerability of hearing function for the noise exposure is diverse for different age groups (young, elderly and old). Another goal was to look for the possibilities to counteract neural degeneration caused by aging and mild noise exposure. In order to prevent these negative effects, we performed environmental enrichment of the housing condition as it was previously shown that long-term conditioning in acoustically enriched environment prior to noise exposure could protect animals from hearing loss (Kujawa and Liberman, 1999), while the protective mechanism was not investigated yet. Also we questioned whether we could counteract this neurodegenerative effect by stimulation of the cGMP cascade, which previously was shown to protect from noise induced hearing loss (Jaumann et al., 2012).

2. Materials and Methods

2.1. Animal models

In this study 2 animal model were used to study effects of stimulated cGMP cascade and environmental enrichment on the vulnerability for the hearing loss after noise exposure during aging.

2.1.1. Wistar rats

Female Wistar rats (Rattus norvegicus) weighting between 200 and 250 were purchased from Charles River Laboratories, Research Models and Services, Germany GmbH, Sulzfeld). Rats were housed in the groups of 4 in the standard-sized cages (0.28 x 0.44 x 0.16 m) with the wooden bedding for up to 2 years in an animal care facility of ENT-clinic in Tübingen. The day/night regimen was 12 hours with the light turned on at 6:00 o'clock and off at 18:00 o'clock. Animals had unlimited access to the food and water.

The care and use of the animals were carried out in accordance with the ethical guidelines prescribed by the University of Tübingen Veterinary Care Unit. The experimental protocol were reviewed and approved by the animal welfare commissioner and the regional board for scientific animal experiments in Tübingen.

2.1.2. Mongolian gerbils

Male and female gerbils (Meriones unguiculatus) aged between 2 and 42 months and weighting between 55 and 120 gram were breed in the animal facility of the ENT-clinic in Tübingen from the ancestors previously bought from Interfauna (Tuttlingen, Germany) in 2003. Gerbils were housed for up to 3,5 years in an animal care facility. As for the rat the care and use of the animals during the course of this study were carried out in accordance with the ethical guidelines prescribed by the University of Tübingen Veterinary Care Unit.

Enriched environment protocol

Gerbils were divided in 2 groups: the first was kept in the enriched environment, the second in the deprived. Enriched environment had an aim to stimulate animals' auditory,

olfactory and visual systems and provide possibilities for extra motor activity. In contrast the deprived environment provides sound protected surroundings.

The animals in the enriched environment were kept apart from the animals in the deprived environment in a separate room of the ENT-clinic animal care facility. In this room no animals from the other experiments were held. Animals were held in the standard cages with up to 4 animals in the cage. To establish enriched environment every cage was equipped with a running wheel (diameter 17 cm) with fine-meshed tread to prevent possible injuries from the metal frame of the wheel that was hanged up to a cage grid, clay labyrinth and sand bath. To train the balance system of the animals, big branches were placed in the cages to allow animals to climb. Also smaller branches, dry grass and small pieces of paper for nibbling were placed in the cage. Food was provided inside the cage mixed with the linen bedding to activate food searching behavior.

Auditory stimulation

The auditory stimulation was performed in two ways. Firstly, a radio was placed on the average distance of 110 cm from the shelf with the animal cages, due to the difference in the cage positions on the rack distance was from 100 to 120 cm. Radio program played a sequence of short episodes of speech and music 16 hours per day. As a conventional radio covers not all the frequencies of the gerbils' hearing range (only frequencies from approximately 100 Hz to 12 kHz), a sound generator (Generator DS345 Synthesized Function, Stanford Research System, Sannyvale, USA)that generated sweeps and bursts between 125 Hz and 40 kHz between 22:00 and 6:00 o'clock was also placed in the room. With the sound of the radio and sound generator loudness of 75 dB SPL was not exceeded. The sound level was recorded with a preamplifier (Mikrosystems) and microphone (Brüehl & Kjær; Nr. 4191 2113985, Nærum, Denmark).

Visual stimulation

For the visual enrichment a light reflection ball hanged under the reck was illuminated with a projector (Eurolite LED PAR64 RGB with multicolored LEDs, Steinigke Showtechnic, Waldbüttelbrunn, Germany) was installed in front of the shelf. As a result light points of the different colors were projected on the cages. With the help of the computer program with suitable interface (DMX Controller, IMG Stage Line, Buckinghamshire, UK) the color of the

light projected on the cages was changed. In the dark phase (19:00 – 6:00 o'clock, which is the prevailing activity time of the gerbils that are mainly night and dusk active animals according to Stutz AM, 1974) weak blue light was projected on the cages. From 6:00 o'clock the light was changed to the orange-red, from 8:00 o'clock started to change gradually adding blue color. From 10:00 o'clock only blue light was on. In the remaining time (7 hours from 12:00 to 19:00) the standard light conditions were present. In this time the room was illuminated by the ceiling light with a light intensity of approximately 100 Lux.

The animals were taken from the cages 3 times per week. The weight of the animals was controlled every week to control whether food self-administration was appropriate and enough to maintain the health of animals. The cages were cleaned every week. While cleaning, bedding was changed and food was added. Besides this, small branches, dry grass and paper were exchanged weekly. As an additional olfactory stimulus every week some drops of the fragrance oil (patchouli, orange, ilang-ilang) were added to the cage (on the grass, small benches and paper).

Standard housing conditions

In contrast to the gerbils in the enriched environment, gerbils in the deprived environment were held in the standard conditions. Besides the gerbils that were in the experiment, breeding cages and offspring reserved for other experiments were also kept in the same room of the facility. They were housed in the groups of maximally 4 animals in the standard-sized cages (0,28 x 0,44 x 0,16 m) with wooden bedding for up to 3,5 years in the animal care facility of ENT-clinic of Tübingen. The day/night regimen was 12 hours with the light turned on at 6:00 and off at 18:00. Animals had unlimited access to the food and water that was in abundance. The cages of these gerbils were equipped only with the wooden house. Besides the noises made by the other animals and animal care staff during cleaning no other acoustic stimuli were presented.

Designing experimental groups

Each of these two groups contained initially 6 gerbils with the similar age (according to age groups) and gender distribution (ratio of 1:1 was kept when possible). The total number of animals in both groups was 78 animals (42 animals were held in enriched and 36 animals in deprived environment).

2.2. Hearing measurements

The hearing function of Wistar rats and Mongolian gerbils was studied by the Auditory Brainstem Response (ABR) and the Distortion Product of the Otoacoustic emission (DPOAE). These measurements were performed on young, elderly and old Wistar rats and young and old Mongolian gerbils with mature hearing. Hearing function was monitored before, directly after (15 to 30 min), 1, 3, 7, 28 days and up to 6 months after noise exposure.

2.2.1. Anaesthesia

For the hearing measurements, Wistar rats were anaesthetized by intraperitoneal injection of 75mg/kg body weight (b.w.) ketamin hydrochloride (Ketavet, Pharmacia/Pfizer, Karlsruhe, Germany) and 5 mg/kg b.w. xylazin hydrochloride (Rompun 290, Bayer, Leverkusen, Germany).

Gerbils were anaesthetized by intraperitoneal injection of the mixture of 2 mg/kg b.w. midazolam (Dormicum, Roche Pharma AG, Grenzach-Wyhlen), 0,15 mg/kg b.w. medetomidin (Sedator, Eurovet Animal Health, Bladel, Netherlands), and 0,05 mg/kg b.w. fentanyl (Fentanyl, Ratiopharm, Ulm, Germany). Supplemental doses of anaesthetics were administered subcutaneously as needed.

To antagonize anesthesia in gerbils was performed with subcutaneous injection of the mixture of 0,03 mg/kg b.w. naloxonhydrochloride (Naloxon, Hameln Pharma Plus GmbH, Hameln), 0,01 mg/kg b.w. flumazenil (Flumazenil, Fresenius Kabi Deutschland GmbH, Bad Homburg), and 1 mg/kg b.w. atipamezolhydrochloride (Antisedan, Elanco Animal Health, Bad Homburg).

2.2.2. ABR-measurements

Discovered nearly 40 years ago (Jewett and Williston, 1971; Jewett, 1970; Moushegian et al., 1973), ABRs can be measured using subcutaneous electrodes that pick up electrical potentials generated by the synchronous activity of populations of neurons in the brainstem. Because these aggregated neural responses can be recorded objectively and passively, they are an excellent technique to assess the auditory function in a clinical setting. ABRs are particularly suitable also for the animal studies, because the protocol allows performing the measurements on anaesthetized subjects.

Experimental set up

Auditory evoked brainstem responses to click and pure tone auditory stimuli were recorded in a sound-attenuating chamber (IAC 400-A, Industrial Acoustics Company, Niederkruechten, Germany) with a customized electrophysiology setup using a Multi IO Card (National Instruments PCI-6052E, Austin, TX, USA) for stimulus generation and recording of evoked potentials, a free field loudspeaker (Fostex, FT28D, Tokyo, Japan) at 3 cm distance from the pinna, and a high precision microphone (Bruel and Kjaer, type 4191,Naerum, Denmark),preamplifier (Bruel and Kjaer 2670, 1/4", 5 mV/Pa,Naerum, Denmark) and measurement amplifier (Bruel and Kjaer 2610,Naerum, Denmark) for stimulus control (Fig. 6). Signals were amplified 50,100 times (94 dB), and bandpass was filtered with the between 0,2 kHz and 5 kHz (6-pole Butterworth hardware filter, Wulf Elektronik, Frankfurt). Three silver electrodes (diameter: 0.25 mm, Goodfellow, Cambridge, UK) connected to the set up from one side, were put with the help of a needle subcutaneous retroauricular (positive, active electrode), on the vertex above the eyes (negative, reference electrode), and on the back (ground electrode).

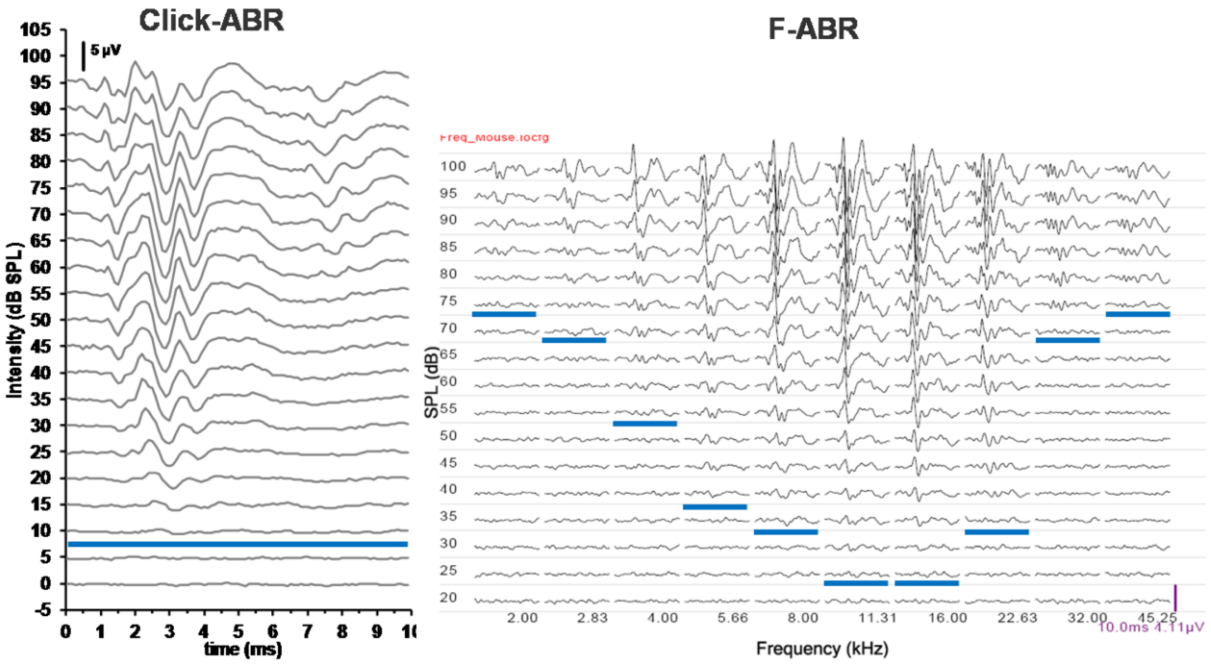

Figure 5. Click- and frequency ABR of a normal hearing rat.
Shown as a function of time (10 ms) with increasing loudness. On the left panel a result of click-ABR measurement could be seen with the threshold underlined with the blue line. On the right panel the result of frequency ABR measurement of a normal hearing rat is presented with the thresholds underlined with the blue line at the each specific frequency. Thresholds were defined as the lowest sound pressure that produced potentials visually distinct from noise level.

Types of stimulation

The generation of the stimuli as well as an attenuation or amplification of the signals was performed with a custom-made computer program (CAP.exe, L. Rüttiger, 2008, based on C++). ABR signals were evoked with click (duration 100 µs), noise burst (1 ms) or pure tone stimuli (3 ms, including 1 ms cosine squared rise and fall envelope, 2- 45 kHz for rats and 1 - 32 kHz for gerbils) presented with increasing stimulus levels (0-100 dB Sound Pressure Level, SPL). Typical responses observed could be seen at the Figure 5. Thresholds were defined as the lowest sound pressure that produced potentials visually distinct from noise level. The click stimulus has a defined timing and therefore evokes a very well synchronized neural population response. Peak latencies and amplitudes of ABR signals evoked by clicks are affected by the place of generation along the cochlea, the stimulus level and the degree of hearing loss. The noise burst stimulus contains more energy at higher frequencies (>10 kHz) than the click stimulus. Frequency-specific pure tone stimuli were used to correlate the hearing function with the tonotopical organization of the cochlea. High frequency sounds are processed by the base of the cochlea, whereas low frequency sounds are apically processed.

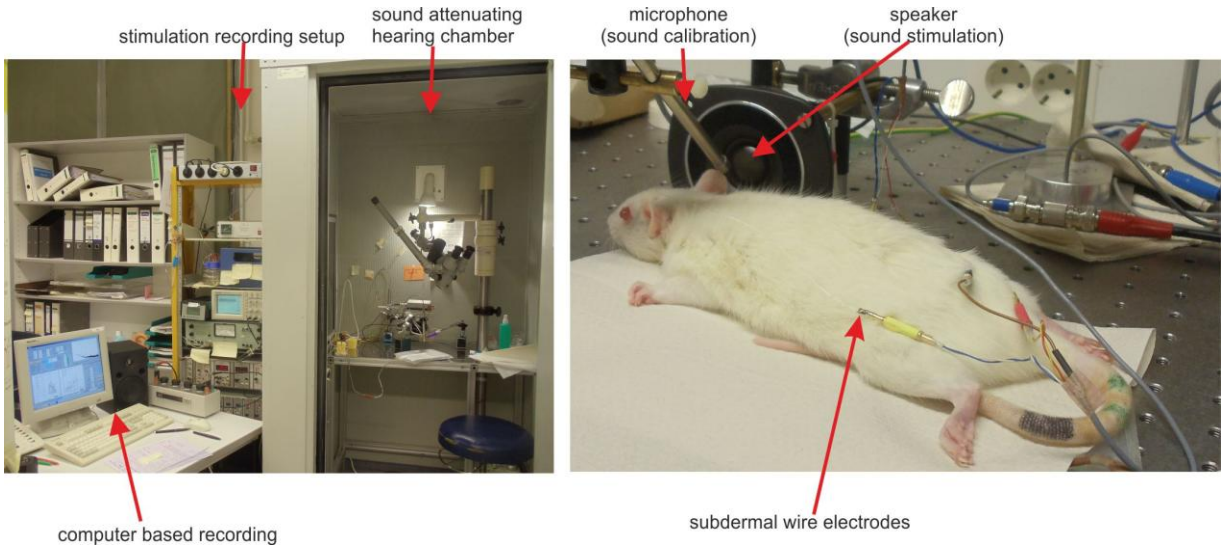

Figure 6. ABR measurements in rodents.
The experimental set up is displayed on the left panel. Positioning of the animal relative to speaker and microphone is depicted on the right panel.

2.2.3. DPOAE-measurements

OHCs function was assessed by the growth function and the maximum response in the DP-gram of the cubic DPOAEs as described (Knipper et al., 1998). DPOAEs depend on the functionality of the cochlear amplifier and represent a measure of the amplification of sound operated by OHCs.

For every experimental animal DPOAE measurement was performed. In a sound-attenuating chamber the anaesthetised animals were placed on a warm mat on the side, so that the ear pointed upwards. Before the measurement all animals were anaesthetized as described above (see 2.2.1.) and the eyes were covered with an ocular gel (Visidic, Berlin, Germany). Under visual control with an operation microscope (Zeiss OPMI1-104937, Germany) a coupler by which at the same time acoustic signals can be sent and be received was placed in the auditory canal before the eardrum (Fig. 7).

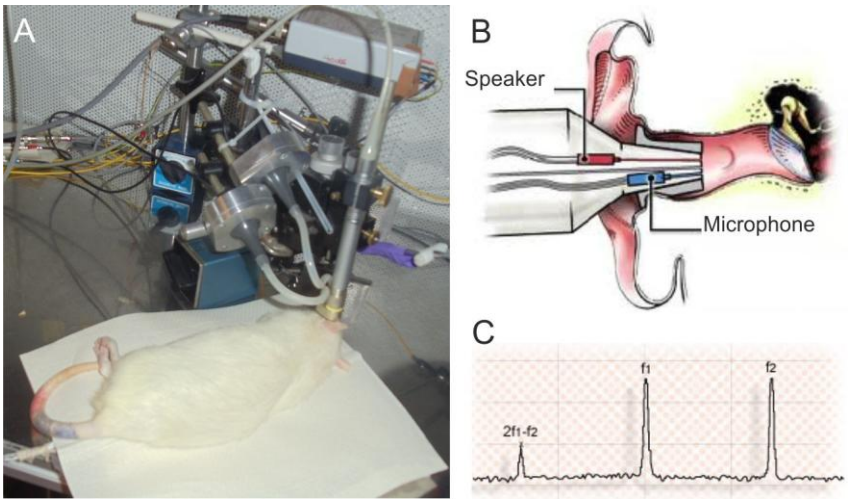

Figure 7. Technique of the DPOAE measurements in rodents
A: Animal is placed in the way that coupler would be positioned before the eardrum of the tested ear. B: The coupler combined the free field loudspeaker and a microphone that were placed before the eardrum. C: Pair of primary tones (f1 and f2) with particular intensity is used to evoke DPOAEs. The cubic distortion tone is represented by the frequency: $f_{DP}=2f_1-f_2$

Computer-controlled (computer program:DPOAE.exe, 2008, M. Müller, L. Rüttiger, based on C ++) sinus tones through an attenuator (2669C, Brüel & Kjaer, Denmark) and afterwards amplifier (Nexus, Brüel & Kjaer, Denmark) were sent to the coupler. The coupler combined sound from the two loudspeakers in the closed field (Beyer DT-911, Germany) to the ear canal. A microphone (1/2“, MG 231, Microtech, Gefell, Germany) that was connected to the closed sound field was used for a calibration. Frequency pairs of tones (ratio f2/f1 = 1.25, sound pressure levels L1 = L2+10 dB) between f1 = 4 kHz and f2 = 32 kHz for Wistar rats and f1 = 1 kHz and f2 = 32 kHz for gerbils were presented. The threshold of the DP (2f1-f2) was defined as the sound pressure L1 that elicited a DP-signal clearly above noise level that is normally at -20 dB SPL.

2.3. Noise Exposure

Animals were exposed to a non-traumatic (100 dB SPL) broad band (8 – 16 kHz with the peak at 11 kHz) noise for 2 hours causing temporary threshold shift in rats and gerbils (Singer et al., 2013; Kujawa and Liberman, 2006; Kujawa and Liberman, 2009; Lin et al., 2011). Anaesthetized animals were placed in a pre-warmed reverberating chamber (a chamber of ca. 50 x 50 x 50 cm with tilted, non-parallel walls to avoid standing waves and to achieve a mostly homogeneous sound field) with six small loudspeakers (Soundcraft piezo, Conrad Electronic, Hirschau, Deutschland) mounted inside to deliver sound, on a turntable slowly continuously moving the animals through the sound field. Control animals were anesthetized and treated the same way, but not exposed to the acoustic stimulus (i.e., the speaker remained turned off). They will be referred to as “sham exposed” within this study.

2.4. Morphological analysis

To get the tissues for the morphological analysis animals were deeply anaesthetized with carbon dioxide and immediately killed by decapitation with a large surgical scissor.

2.4.1. Tissue preparation

For immunohistochemistry cochleae were isolated and dissected as previously described (Knipper et al., 2000). Cochleae were fixed for 2 hours by immersion in 2% paraformaldehyde (PFA, Merck, Darmstadt) containing 125 mM sucrose in 100 mM phosphate buffered saline (PBS, pH 7.4), for 2 hours at 4^0C on over-head shaker while constantly moving. It was decalcified after fixation for 1 hour to 2 hours (depending on the age of the animal) in Rapid Bone Decalcifier (Eurobio, Fischer-Scientific, Nidderau, Deutschland), followed by overnight incubation in 25% sucrose (Merck, Darmstadt, Germany) in Hanks buffered saline (HBS) at 4°C. After overnight incubation, cochleae were cryoembedded in O.C.T. compound (Miles Laboratories, Elkhart, Ind., USA) and stored at -80^0C. Tissues were then cryosectioned at 10 μm thickness, mounted on SuperFrost*/plus microscope slides and stored at -20°C before use.

Table 1. Substances used

Name	Manufacturer
Antisedan® (Atipamezol)	Elanco Animal Health, Bad Homburg, Germany
BAY 41-8543	Bayer, Wuppertal, Germany
Dormicum® (Midazolam)	Roche Pharma AG, Grenzach-Wyhlen, Germany
Flumazenil	Fresenius Kabi Deutschland GmbH, Bad Homburg, Germany
Ketavet® (Ketamin)	Pharmacia/Pfizer, Karlsruhe, Germany
Naloxon	Hameln Pharma Plus GmbH, Hameln, Germany
O.C.T. compound	Miles Laboratories, Elkhart, IN, USA
Rapid Bone Decalcifier	Eurobio, Fischer-Scientific, Nidderau, Germany
RNAlater	Self made (40mL 0,5M EDTA, 25mL 1M Sodium citrate, 700g Ammonium sulfate in 935mL of ultrapure water)
Rompun® (Xylazine)	Bayer Pharma AG, Leverkusen, Germany
Sedator® (Medetomidine hydrochloride)	Eurovet Animal Health, Bladel, Netherlands
Vectashield®	Vector Laboratories, Burlingame, CA, USA
Vidisic®	Dr. Mann Pharma, Berlin, Germany

2.4.2. Immunohistochemistry

For immunohistochemistry, rat cochlear sections were stained and imaged as described (Knipper et al., 1998; Knipper et al., 2000). Briefly, cochlear sections were thawed and permeabilized with 0,5% Triton X-100 for 10 min at room temperature, pre-blocked with 4% normal goat serum (NGS) in PBS, and incubated overnight at 4°C with primary antibodies in the recommended concentration (see Table 1). Anti-Otoferlin (1:8000; Shug et al., 2006) and anti-CtBP2 (1:50; BD-Transduction Laboratories, CA, USA) were simultaneously incubated for identical time periods. Antibodies were diluted in PBS containing 2% NaCl, 0,1% Triton

X-100 and 1% NGS. Primary antibodies were visualized with either Cy3-conjugated goat anti-rabbit IgG (1:1500; Jackson Immuno Research Laboratories, PA, USA) or with Alexa488 conjugated goat-anti-mouse (1:500; Molecular Probes, Leiden, The Netherlands), or with Alexa488 conjugated goat-anti-rabbit (1:750; Molecular Probes) antibodies. Sections were rinsed, mounted in Vectashield containing the nuclear stain DAPI (Vector Laboratories, Burlingame, CA, USA).

Table 2. Antibodies used in this study

Primary Antibody	Species	Dilution	Manufacturer
Anti-Otoferlin	rabbit (polyclonal)	1:8000	Schug et al., 2006
Anti-CtBP2	mouse (monoclonal)	1:50	BD-Transduction Laboratories, CA, USA
Secondary Antibody			
Cy3	goat (anti-rabbit)	1:1500	Jackson ImmunoResearch Laboratories Suffolk, UK
Alexa 488	goat (anti-mouse)	1:500	Molecular Probes Darmstadt, Germany

All histological examinations and documentations were done using an Olympus BX61 microscope equipped with epifluorescence illumination. Images were acquired with a CCD camera and analyzed with cellSens software (OSISGmbH, Germany). The region of interest (IHC, OHC) was imaged over a distance of several µm with the coverage of the IHC synaptic region in an image stack along the z-axis (z-stack) and three-dimensionally deconvoluted using cell^F's RIDE module with the cellSens ADVMLE algorithm (OSIS, Germany). Typically z-stacks consisted of 30 layers with a z-increment of 0.49 µm, for each layer one image per fluorochrome was acquired.

2.4.3. Wholemount immunohistochemistry

On the first day cochleae tissue were washed in PBS for 10 minutes at room temperature (RT), permeabilized for 10 minutes in 0,2% Triton X-100 (Sigma-Aldrich, Steinheim, Germany) in PBS at RT, blocked for 30 minutes in 1% Bovine Serum Albumine

(BSA, Sigma-Aldrich, Steinheim, Germany) in PBS and afterwards incubated over night at 4 °C with primary antibodies in the recommended concentration (see Table 1) in 0,5% BSA, 0,1% Triton X-100 in PBS.

On the second day cochleae were washed for 15 minutes in 0,1 %Triton X-100 in PBS two more times and then incubated with secondary antibodies in the recommended concentration in 0,5% BSA, 0,1% Triton X-100 in PBS for one hour at RT. After two more subsequent washes in 0,1%Triton X-100 in PBS at RT, cochleae were covered with Vectashield and cover glass.

2.5. Drug application

BAY 41-8543 (2-[1-[(2-fluorophenyl)methyl]-1H-pyrazolo[3,4-b]pyridin-3-yl]-5(4-morpholinyl)-4,6-pyrimidinediamine) was obtained from Bayer (Bayer Pharma AG, Leverkusen, Germany). BAY 41-8543 is a heme-dependent stimulator of sGC, increasing the activity of recombinant sGC dose-dependently (Stasch et al., 2002).

As a chronic daily application of the substance during the time for up to the 6 months is difficult, substance was pressed into the pellets of standard rat/gerbil diet by Ssniff company (Soest, Germany). To determine which concentration of substance should be pressed into 1 kilo of food, quantity of food consumed by 100 g of gerbil/rat per day was calculated by weighting the food in the beginning and the end of the day and dividing the weight by number of animals in the cage. By this experiment it was determined that to reach the planned concentration of 55 mg/kg/day concentration of the experimental substance should be 29,2 mg/kg of gerbil food and 125 mg/kg of rat food. Control groups received an equal quantity of regular diet. Administration of the sGC-stimulator containing diet (sGC diet) started on the day 3 after noise or sham exposure.

An action of the sGC diet was tested in two experimental rounds in rats. In short-term experiment treatment with sGC diet was started on the 3rd day after noise exposure and continued for 25 days. In long-term experiment treatment with sGC diet was started on the 3rd day after noise exposure and continued for 5-6 months. After the treatment preparation was performed.

Long-term treatment was also performed for the gerbils, but due to the age-related health problems had to be finished earlier. Gerbils were euthanized after 3-4 months treatment with sGC food.

2.6. ABR fine structure analysis

In order to extract and quantify the information of ABR wave components that reflects the summated neural activity along the ascending auditory pathway, ABR waves that consist of starting negative peaks and the consecutive positive peaks were analyzed. Firstly, the latencies and amplitudes of both positive and negative peaks of the ABR waveforms were extracted by a custom-made computer (Peak program) program. Individual ABR peak amplitudes and peak latencies from 2-16 ears were grouped in clusters of similar peak latencies to construct the average ABR wave amplitude growth functions, i.e., computing the wave amplitudes for increasing stimulus intensities.

2.6.1. ABR waveform peak detection

ABR data were processed by a Peak program to estimate latencies reference to stimulus and corresponding amplitudes of the peaks of ABR waves. In this program, waveforms from individual ears were firstly displayed from highest to lowest sound stimulation intensities (dB in relative scale).

As ABR threshold was one of the essential criteria in the peak detection algorithm implemented by the program, for the reason that ABR waves could only be well-defined when they are evoked by suprathreshold stimuli, before peaks could be estimated, thresholds of the ABRs needed to be estimated by the computer program. Determination of the thresholds was based on certain criteria. These criteria are related to the absolute waveform amplitude, energy, waveform signal-to-noise ratio (SNR), and waveform waviness, quantified by the cumulative sum of signal derivative along the 10 ms recording interval. Parameters related to each threshold estimation criteria for this study are presented in Table 3. To evaluate the ABR waveform evoked stimuli from highest to lowest sound intensities, each of these criteria was applied. Threshold corresponding to each criterion was estimated at a level where the parameter associated with the criterion of the corresponding waveform could not exceed the defined values. Thresholds estimated by Peak program for all ABR recordings were compared with the estimates obtained by visual inspection during the hearing measurement as described in chapter 2.5.1. Maximum difference between these two estimates was 5 dB for all recordings.

After threshold estimation, initial guesses of peaks were performed for user-selected representative waveform, known as “Template”. Extracted peak should be an extremum point of the “Template” waveform in time ranges denoted by two vertical lines with the same color as for the extracted peak. By changing the width of the time windows, the distances between time ranges, or adding and excluding peaks from the analysis manually until the peaks corresponding to the prominent waves were identified upon visual inspections over all sound pressure levels, adjustments of the initial guesses of the peaks of the “Template” waveform were performed.

After all peaks are found, peak positions (latencies and the corresponding amplitude) were extracted and evaluated. Peaks of the stacked ABR waveforms were extracted from the highest to the lowest sound intensities.

These peaks are the local extrema within the time range corresponded the ranges determined for the “Template” waveforms. Each identified peak with the same peak number (marked by the same color) was evaluated from the highest to the lowest sound intensity. These peaks were determined as “valid” if the corresponding waveforms were above thresholds and if the latencies of the peaks were close for adjacent stimulation intensities. Adjustments were made by changing the time ranges at lower sound intensities by visual inspection of the peaks of all ABR waveforms in the way that more “valid” peaks could be obtained. Data about all extracted peak positions for both “valid” and “non-valid” peaks were saved to a data file for later processing. Results of evaluation of all peaks were also saved to the same data file so that “non-valid” peaks could be differentiated from the “valid” peaks for later analyses. The main function of this computer program was to extract peaks of the ABR waveform from the recordings in the way that later these peaks could be clustered by the latencies corresponding to the ABR wave components and the wave amplitude growth function were computed. As far as the acquired ABR data contain some variation the criteria mentioned above were implemented by the computer program to increase the rate of the valid peak detection and reducing the rate of invalid peak detection.

2.6.2. Construction of peak-to-peak growth function

Peaks extracted from the computer program as described in chapter 2.7.1 were grouped according to latencies corresponding to ABR wave I, II and IV. ABR waves defined as the voltage deflection staring from the negative peak to the following positive peak and

were marked with the Roman numbers and either with “n” (negative peak) or with “p” (positive peak). The latencies for wave I, wave II and wave IV were: wave I: I_n-I_p (latency, 0,9 – 2,3 ms), wave II: II_n-II_p (latency, 1,4 – 3 ms), and wave IV: IV_n-IV_p (latency, 3,3 – 5,5 ms).

All peaks that were evaluated as “non-valid” were discarded for further analyses. For the selected peaks, amplitude growth functions were derived by calculating the peak-to-peak amplitudes for increasing stimulus levels. In case if both the negative and the following positive peak of the wave were extracted and evaluated as “valid” peak-to-peak amplitude of an ABR wave was calculated.

To study the suprathreshold shape of ABRs without being distracted by threshold differences, all ABR wave amplitude growth functions were automatically adjusted by Peak program in relation to the ABR thresholds of individual ears as described in chapter 2.7.1.

Table 3. Parameters of estimating ABR threshold by the Peak program

	Program values
Critical Signal/Noise	1,3
Critical Signal Energy	1,4
Critical Signal Derivative	1,3
Critical Peak Amplitude (%)	5

2.7. Statistical analysis

Threshold differences between experimental groups were tested using the Student's t-test with alpha-level correction for multiple testing (pre-AT/spontaneous hearing functions) and using two-way analysis of variance (hearing loss after noise exposure) on the $\alpha = 0,05$ level. Data are presented as mean of n ears ± standard deviation (SD). Statistical analysis was performed using the two-tailed Student's t-test, with alpha = 0,05. If statistical calculations were performed on dependent data, as in the case of the frequency specific ABR measurements, variance analyses (analysis of variance, ANOVA) were calculated in GraphPrism V6.

For statistical analyses of quantification of immunopositive spots in IHC of apical and medial cochlear turns (low-frequency region) and IHC of midbasal and basal cochlear turns (high-frequency region) of Wistar rats, data were compared and displayed as a mean ± SD. For statistical analyses, an F-test was performed to compare variances of the data obtained from counting and unpaired Student's t-test was performed accordingly, with n representing the number of analyzed hair cells. Differences in the number of immunopositive spots and cells were considered statistically significant for $p<0,05$.

3. Results

3.1. Effects of aging on the auditory system

Previously age-related hearing loss was considered to happen due to the loss or dysfunction of outer hair cells (Keithley and Feldman, 1982; Milbrandt et al., 2000; Guang-Di Chen et al., 2009). Recently it was shown that afferent neural fiber loss in mice may progress over age (Sergeyenko et al., 2013), causing hearing deficit independently of the OHCs function loss. The present study investigates whether this holds for rat and gerbil animal models.

3.1.1. Characterization of aging effects in the rat cochlea

Initially changes of hearing function over age were investigated in Wistar rat animal model.

Effects of aging in the early period of lifespan in the rat animal model

To investigate whether hearing function decline could be observed in the initial period of lifespan of rats hearing of young (2 – 3 months) and elderly (8 – 10 months) were characterized.

A tendency for the high-frequency hearing loss occurred in the first year of life in the rat

Auditory brainstem response could be measured non-invasively using intra-scalp electrodes through which electrical potentials generated by the activity of the neuron populations in the brainstem can be detected. This method allows recording these neural responses objectively; therefore it is a suitable way to characterize the hearing function in a clinical setting, where active, subjective reactions of patients cannot be obtained or are unwanted because of possible interference with the results.

ABR thresholds on click auditory stimuli (click-ABR, Fig. 8A) were not significantly different (1-sided Student's *t*-test: n.s.) between 2 – 3 months old Young rats (n = 10/5 ears/rats) and 8 – 10 months old Elderly rats (n = 10/5 ears/rats). Frequency-specific ABR (f-ABR, Fig. 8B) revealed significant differences between Young and Elderly rats for mean threshold at 1 – 2 kHz and 22,63 – 45,25 kHz (Young: n = 10/5 ears/rats; Elderly: n = 10/5 ears/rats, $P < 0{,}0001$ by two-way ANOVA with Bonferroni multiple comparison test at 1 – 2 kHz and 22,63 – 45,25 kHz, between 2 kHz and 22,63 kHz two-way ANOVA: n.s.). DPOAE

thresholds of Young rats were insignificantly elevated in comparison with the Elderly, while still being in the normal range. This effect could be explained by the natural variation and anatomical differences in the diameter of the ear canal, that make access to the ear of young animals more complicated (Fig. 8C, 8D; Young: *n* = 10/5 ears/rats; Elderly: *n* = 10/5 ears/rats, *t*-test: n.s.).

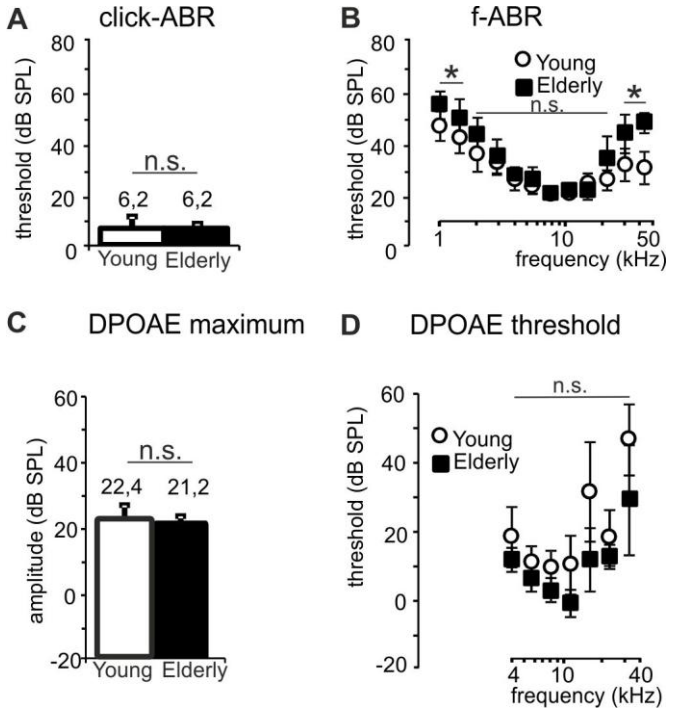

Figure 8. Functional difference in hearing of Young and Elderly Wistar rats: ABRs and DPOAEs
A: ABR thresholds (click-ABR) presented as mean±SD for Young (2 – 3 months old; white bars) and Elderly (9,5 – 10 months old; black bars) Wistar rats. **B:** Frequency specific pure tone thresholds (mean±SD) for Young (white circles) and Elderly (black squares) Wistar rats. **C:** DPOAE maximum amplitude at f2=4,8–22,6 kHz, giving a rating for the force and integrity of OHC function of Young (white bar) and Elderly (black bar) Wistar rats. **D:** DPOAE thresholds (dB SPL f1) of young (white circles) and elderly (black squares) Wistar rats.

Summarizing data shown, I can conclude that after the first period of their life span Wistar rats exhibit a low and high frequency hearing loss, while middle frequencies hearing is safe. Click-ABR and DPOAE maximum amplitude are not different from those of young animals. Difference in DPOAE thresholds could be explained by natural variation.

Synaptopathy caused by aging could be detected in the first year of life in the rat

The number of ribbon structures in IHCs that are known to be responsible for the vesicle delivery to the active zone of the IHC synapse (Khimich et al., 2005) was investigated. As in the mammalian cochlea most of the cochlear nerve fibers, contact a single IHC via one terminal with a single active zone (Liberman, 1990), ribbon counts allow to characterize the

IHC afferent innervation. By immunohistochemistry for the expression and distribution of C-terminal-binding-protein 2 (CtBP2) as marker of IHC synaptic ribbons was analyzed in IHC of Young (2 – 3 months old) and Elderly (8 – 10 months old) Wistar rats (Fig. 9). Otoferlin staining (red) in the midbasal (Fig. 9A-B) and basal (Fig. 9C-D) turns of the cochlea of Young (Fig. 9A, C) and Elderly (Fig. 9B, D) Wistar rats highlights IHCs. By visual inspection the reduction of CtBP2-positive dots (green) could be quantified in the midbasal turn as well as in basal turn of the Elderly Wistar rat cochlea (Fig. 9B, D) as compared to the midbasal and basal turns of the Young Wistar rat cochlea (Fig. 9A, C). The quantification of IHC synaptic ribbon contacts (Fig. 10B) was done in three cochlear regions (from apex to base), covering all frequency regions according to the tonotopic cochlea map of the rats (Müller M., 1991).

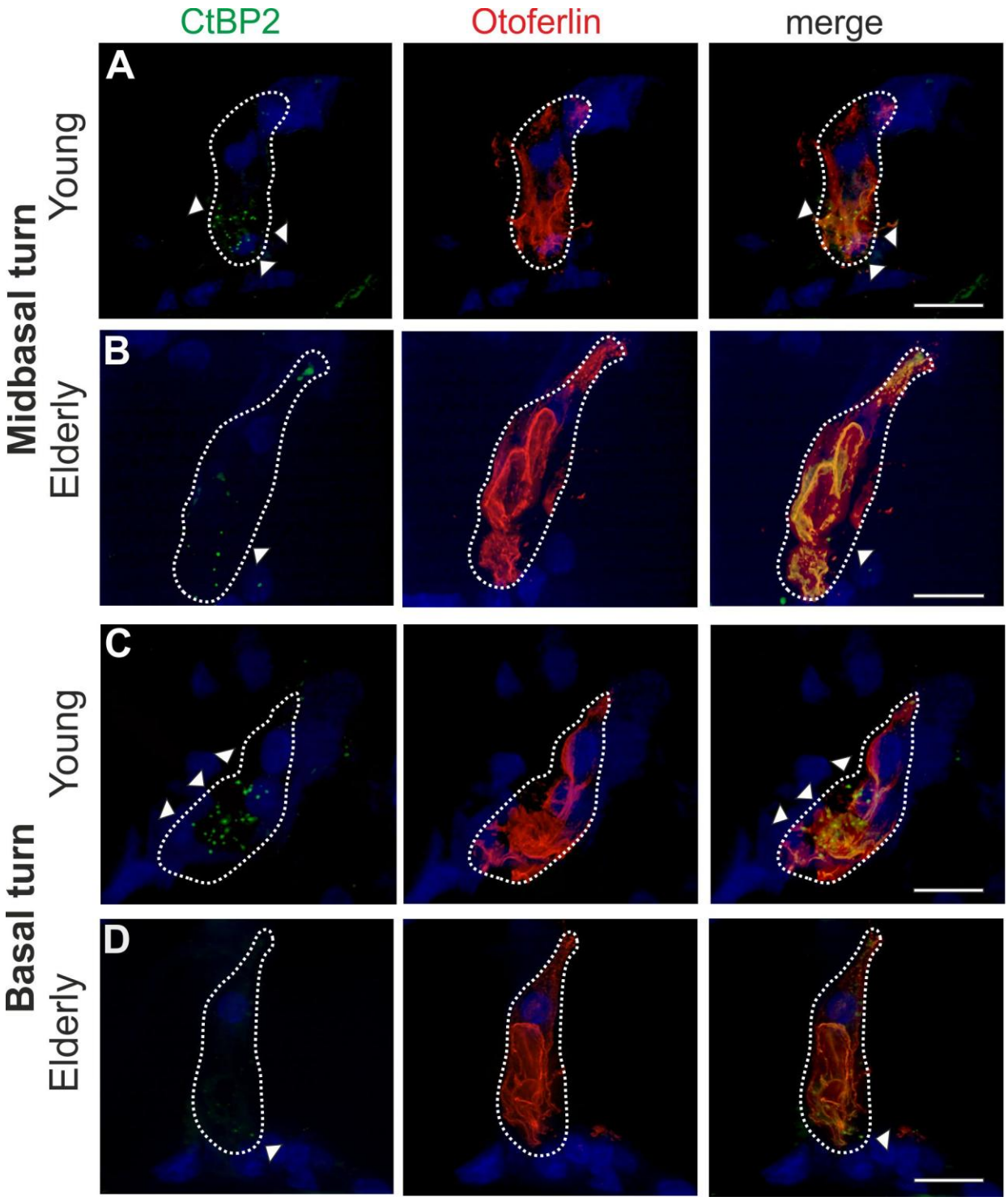

Figure 9. IHCs ribbon synapses were reduced in the basal and midbasal turns of the cochlea of young and elderly Wistar rats

The loss of the synaptic contacts in the IHC of elderly rats could be observed. Images of the IHC of midbasal (A, B) and basal (C, D) turns of young (A, C) and elderly (B, D) animals immunostained for synaptic ribbons (CtBP2, green, marked with arrowheads) and IHC (Otoferlin, red, encircled with line). Scale bars = 10 μm. Nuclear marker: DAPI (blue).

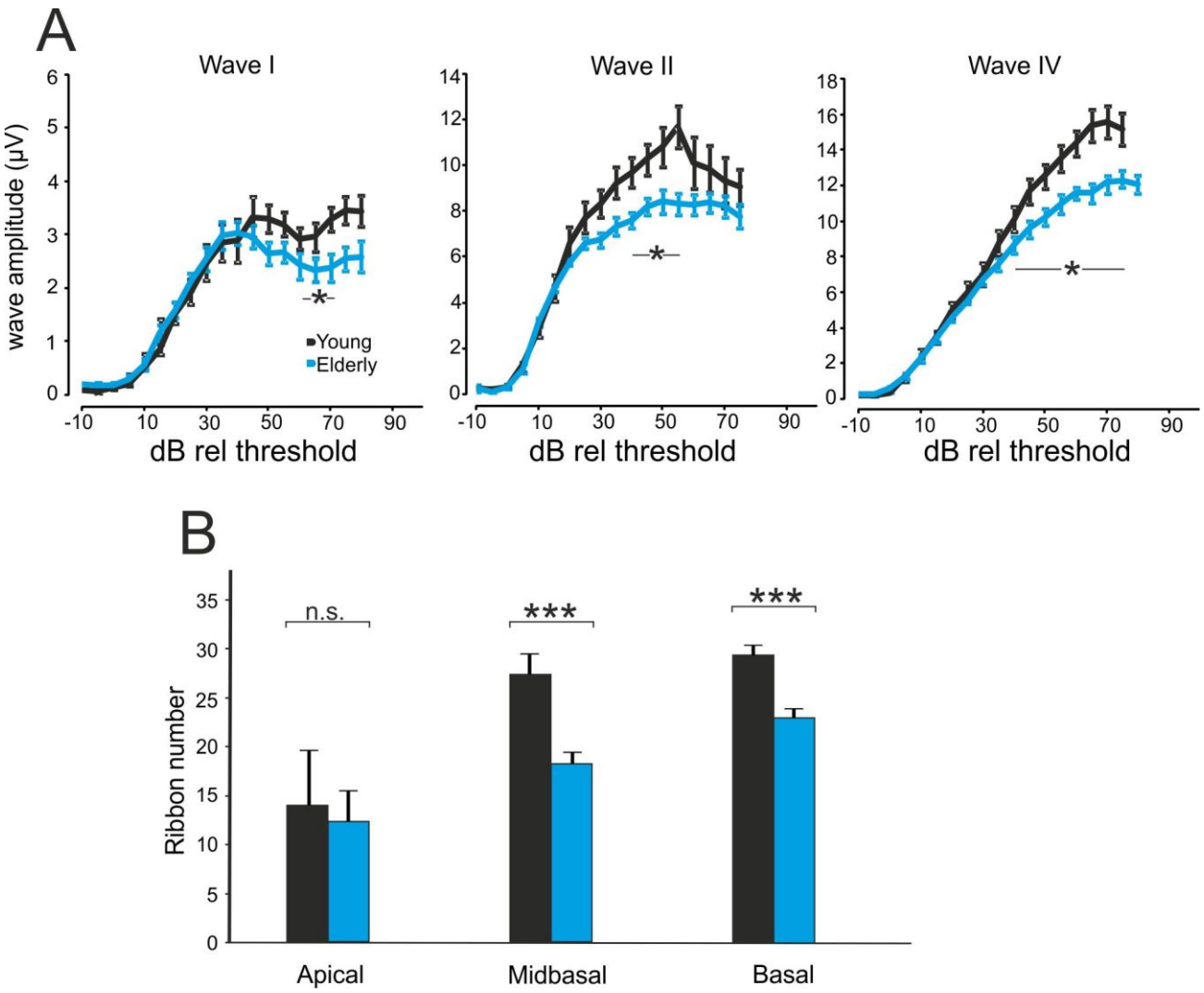

Figure 10. Reduction of the IHCs ribbon synapses in basal and midbasal turns of the cochlea correlated with the loss of the ABR amplitudes

A: Mean ± SEM click-evoked ABR wave I, wave II and wave IV amplitudes growth functions for Young (black line, *n* = 10/5 ears/rats) and Elderly (blue line, *n* = 10/5 ears/rats). **B:** Ribbon counting from Young (black) and Elderly (blue) Wistar rats, expressed in average number per IHC (±SEM), in different cochlear turns.

CtBP2-positive points in single IHCs were counted in Elderly (8 – 10 months old) Wistar rats in comparison to Young Wistar rats (2 – 3 months old) and a significant reduction of ribbons became evident in the midbasal and basal turns (Fig. 3B). No significant differences in the number of ribbons between Young and Elderly Wistar rats were noted in the apical turn (Fig. 10B). IHC ribbon synapses number was reduced by 17% in the basal and 31% in the midbasal turns of the cochlea of Elderly Wistar rats.

Table 3. ***P*-values of pair wise comparisons of amplitude data in Figure 10**

Young vs. Elderly	dB above threshold										
	30	35	40	45	50	55	60	65	70	75	80
Wave I	0,8607	0,7591	0,7633	0,411	0,0655	0,0966	0,0693	0,0432 (*)	0,0181 (*)	0,0174 (*)	0,1127
Wave II	0,3579	0,0566	0,0427 (*)	0,008 (**)	0,003 (**)	0,0027 (**)	0,0011 (**)	0,0006 (**)	0,0027 (**)	0,0037 (**)	0,0217 (*)
Wave IV	0,0167 (*)	0,0115 (*)	0,0057 (**)	0,009 (**)	0,0152 (*)	0,0017 (**)	0,0765	0,0976	0,1682	0,1002	0,0723

1-sided Student's *t*-test: *(*) p<0,05;(**) p<0,01*

In Elderly Wistar rats ABR wave I amplitude was smaller than in Young Wistar rats starting from 50 dB above threshold and above (Fig. 10A). This difference was found significant at 65 dB above threshold up to 75 dB above threshold (1-sided Student's *t*-test: see table 4 for p-values), compared with the young animals. Also a difference in wave II amplitude was found between Young and Elderly Wistar rats at 40 dB above threshold and above (Fig. 10A). This difference was significant at 30 dB above threshold up to 55 dB above threshold (1-sided Student's *t*-test: see table 4 for p-values, no Bonferroni-Holms correction), compared with the Young animals. The amplitude of ABR wave IV was declined starting from 45 dB above threshold (Fig. 10A). That difference was found significant at 45 dB above threshold and up to 80 dB above threshold as compared to young animals (1-sided Student's *t*-test: see table 4 for p-values, no Bonferroni-Holms correction). These data show that Elderly animals have reduced ABR wave I, wave II and wave IV amplitudes at high sound pressure levels compared with Young animals.

Summarizing data shown above, amplitudes of central ABR waves (II and IV) as well as the amplitude of the ABR wave I that reflects the auditory nerve input, were reduced in Elderly Wistar rats in comparison to those of Young Wistar rats. The immunohistochemical analysis of the rat cochlea revealed a loss of synaptic ribbons throughout the basal and midbasal parts of the cochlea of Elderly Wistar rats. The quantitative analysis showed that 28 days after exposure ribbon counts are reduced by up to 30% in the midbasal part of the cochlea. Therefore a threshold shift found at high frequencies in Elderly Wistar rats could be

a result of reduction in afferent fiber density and ribbons at the active zones of the midbasal and basal IHCs.

Effects of aging in the late period of lifespan in the rat animal model

To investigate whether hearing function decline progresses in the late period of lifespan of rats hearing of Elderly (8 – 10 months) and Old (21 – 26 months) Wistar rats were characterized.

Age-related hearing loss could be detected in rats 1,5 years of age and older

Hearing function of the Elderly and Old Wistar rats was tested by ABR and DPOAE recordings. All the recordings were performed as described above (see 2.2.).

ABR thresholds on click auditory stimuli (click-ABR, Fig. 11A) were significantly different (1-sided Student's *t*-test: p = 0,0009) between 8 – 9 months Elderly rats (*n* = 12/6 ears/rats) and 21 – 26 months Old rats (*n* = 10/5 ears/rats). Frequency-specific ABR (f-ABR, Fig. 11B) revealed significant differences between Elderly and Old rats at 1-2 kHz, 4kHz, 8kHz, 32 – 45,25 kHz (Elderly rats: *n* = 12/6 ears/rats; Old rats: *n* = 10/5 ears/rats; P < 0,0001 by two-way ANOVA with Bonferroni multiple comparison test at 1-2 kHz, 4kHz, 8kHz, 32 – 45,25 kHz). In accordance with the elevated ABR thresholds in Elderly and Old rats, similar growth functions of DPOAEs could be measured in Elderly and Old Wistar rats, with similarly elevated maximum amplitudes and thresholds (Fig. 11C, Elderly: *n* = 10/5 ears/rats; Old: *n* = 10/5 ears/rats, *t*-test: *p* = 0,002; Fig. 4D; P < 0,0001 by two-way ANOVA with Bonferroni multiple comparison test 8kHz, no significance by two-way ANOVA at other measured frequencies). This difference was significant only at 8 kHz.

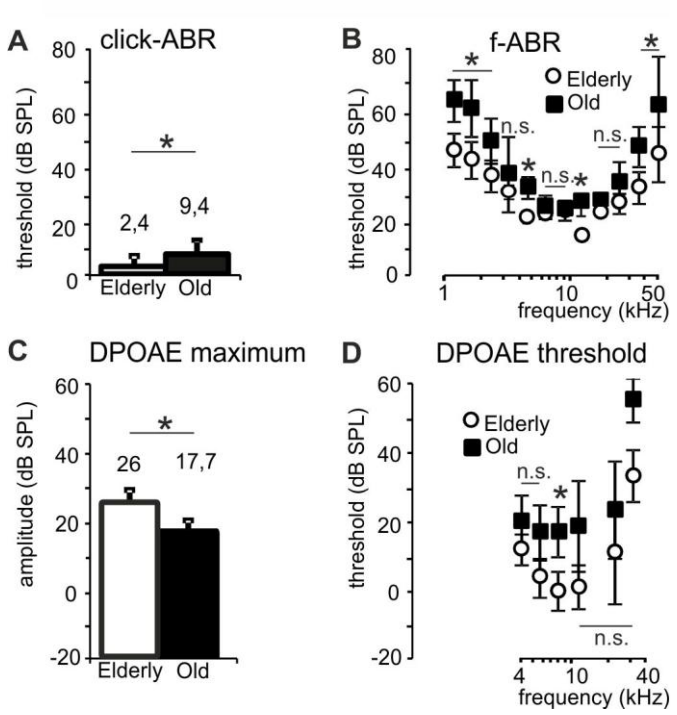

Figure 11. Functional difference in hearing of Elderly and Old Wistar rats: ABRs and DPOAEs
A: Average ABR thresholds (click-ABR) ± SD for Elderly (8 – 9 months old; white bar) and Old (21 – 26 months old; black bar) Wistar rats. **B:** Average ABR thresholds (frequency ABR) ± SD for Elderly (white circles) and Old (black squares) Wistar rats. **C:** DPOAE maximum amplitude at f2=4,8–28 kHz of Elderly (white bar) and Old (black bar) Wistar rats. **D:** DPOAE thresholds (dB SPL f1) of Elderly (white circles) and Old (black squares) Wistar rats.

Concluding data presented above, I can say that Wistar rats showed age-related hearing decline that could be characterized by every functional study performed (click-ABR, frequency ABR, DPOAE maximum amplitude and DPOAE thresholds).

Progression of age-related synaptopathy in the late period of a rats' lifespan

The number of ribbon structures in IHCs was investigated. By immunohistochemistry the expression and distribution of C-terminal-binding-protein 2 (CtBP2) as marker of IHC synaptic ribbons was analyzed in IHC of Elderly (8 – 10 months old) and Old (21 – 26 months old) Wistar rats (Fig. 12).

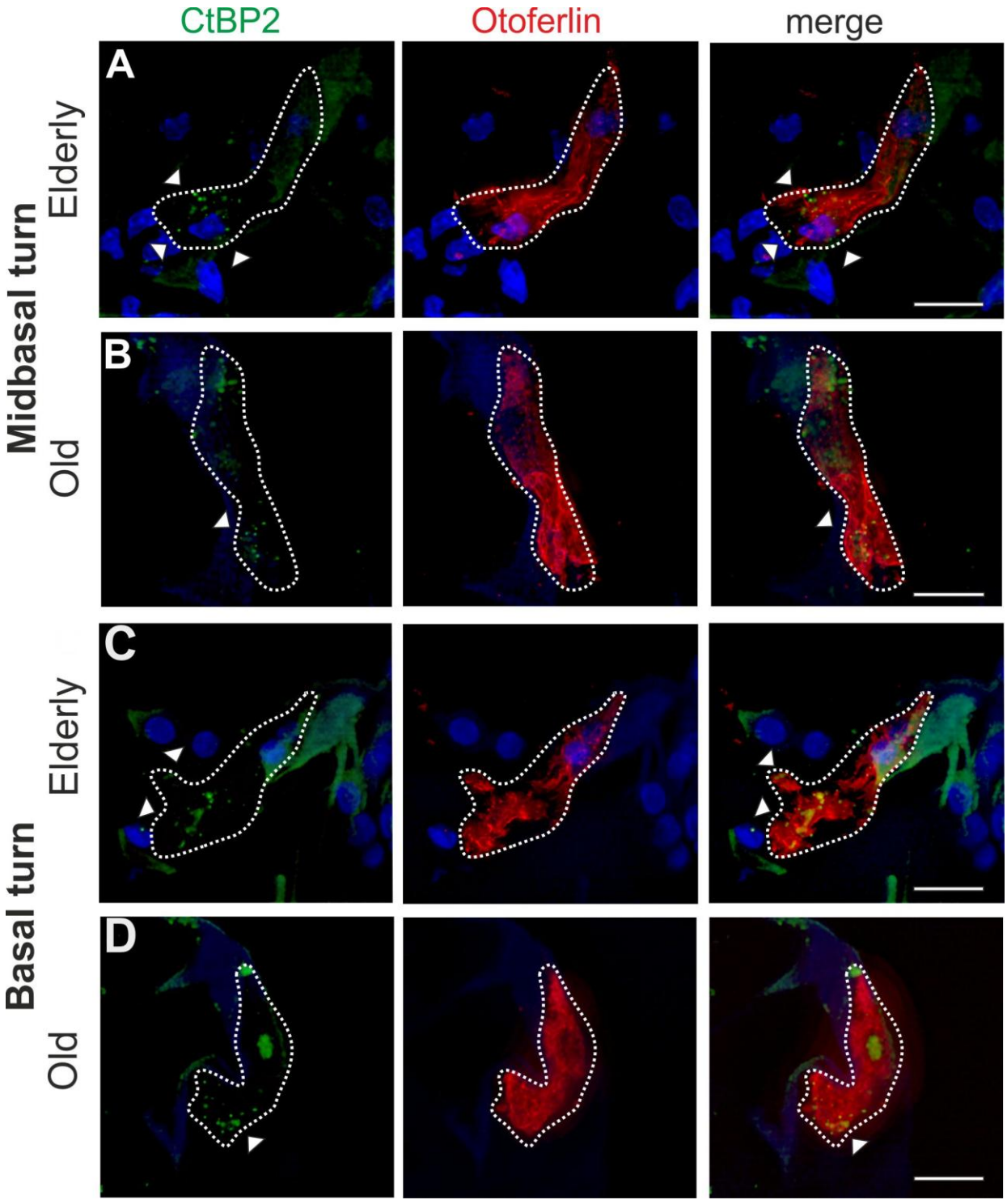

Figure 12. Number of IHCs ribbon synapses was reduced in the basal and midbasal turns of the cochlea of Elderly and Old Wistar rats

The loss of the synaptic contacts in the IHC of old rats could be observed. Images of the IHC of midbasal (A, B) and basal (C, D) turns of Elderly (A, C) and Old (B, D) animals immunostained for synaptic ribbons (CtBP2, green, marked with arrowheads) and IHC (Otoferlin, red, encircled with line). Scale bars = 10 μm. Nuclear marker: DAPI (blue).

Otoferlin staining (red) in the midbasal (Fig. 12A-B) and basal (Fig. 12C-D) turns of the cochlea of Elderly (Fig. 12A, C) and Old (Fig. 12B, D) Wistar rats highlights IHC

position. By visual inspection the reduction of CtBP2-positive dots (green) could be seen in the midbasal turn as well as in basal turn of the Old Wistar rat cochlea (Fig. 12B, D) as compared to the midbasal and basal turns of the Elderly Wistar rat cochlea (Fig. 12A, C). The quantification of IHC synaptic ribbon contacts is shown in the Fig. 13B. It was performed in three cochlear regions (from apex to base).

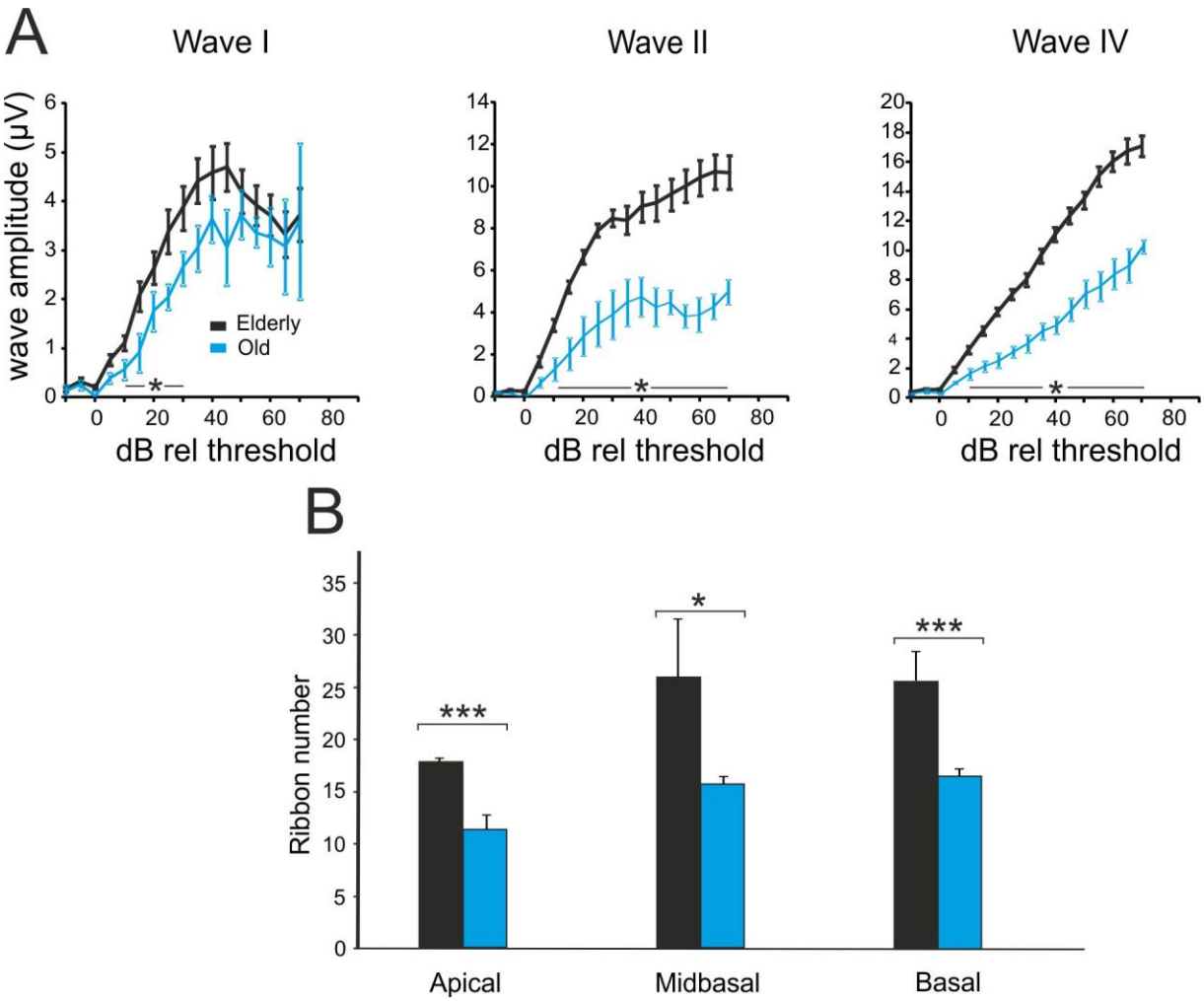

Figure 13. Reduction of the IHC ribbon synapses in basal and midbasal turns of the cochlea correlated with the loss of the ABR waves amplitudes

A: Mean ± SEM click-evoked ABR wave I, wave II and wave IV amplitudes growth functions for Elderly (black line, *n* = 10/5 ears/rats) and Old (blue line, *n* = 10/5 ears/rats). **B:** Ribbon counting from Elderly (black) and Old (blue) Wistar rats, expressed in average number per IHC (±SEM), in different cochlear turns (*n*=3).

CtBP2-positive points in single IHCs were counted in Elderly Wistar rats in comparison to Old Wistar rats, and reduction of ribbons became evident in all cochlear turns of Old animals (Fig. 13B). IHC ribbon synapses number was reduced by 35% in the basal and 45% in the midbasal turns of the cochlea of Old Wistar rats.

Table 4. *P*-values of pair wise comparisons of amplitude data in Figure 13

Elderly vs. Old	dB above threshold												
	10	15	20	25	30	35	40	55	60	65	70	75	80
Wave I	0,003 (**)	0,003 (**)	0,02 (*)	0,034 (*)	0,05 (*)	0,09	0,114	0,147	0,168	0,07	0,337	0,467	0,38
Wave II	0,00012 (**)	$2*10^{-6}$ (**)	$1,5*10^{-6}$ (**)	$2,8*10^{-6}$ (**)	$1*10^{-5}$ (**)	0,0015 (**)	0,0016 (**)	0,00034 (**)	0,00023 (**)	$6*10^{-5}$ (**)	$6*10^{-5}$ (**)	0,0002 (**)	$7*10^{-5}$ (**)
Wave IV	$9,5*10^{-7}$ (**)	$2*10^{-5}$ (**)	$6*10^{-7}$ (**)	$3*10^{-7}$ (**)	$2*10^{-7}$ (**)	$2*10^{-7}$ (**)	$2*10^{-7}$ (**)	$5*10^{-6}$ (**)	$3*10^{-6}$ (**)	$1*10^{-6}$ (**)	$8*10^{-6}$ (**)	$2*10^{-6}$ (**)	$1,5*10^{-5}$ (**)

1-sided Student's *t*-test: *(*) p<0,05;(**) p<0,01*

In Old Wistar rats ABR wave I amplitude was decreased in comparison with Elderly group at 10 dB above threshold and above (Fig. 13A). Difference found significant at 10 dB above threshold up to 30 dB above threshold (1-sided Student's *t*-test: see table 5 for p-values, no Bonferroni-Holms correction), compared with the Elderly animals. Also the difference in wave II amplitude had a statistically significant decrease in old Wistar rats at 10 dB above threshold and above in comparison with elderly rats (Fig. 13A; 1-sided Student's *t*-test: see table 5 for p-values, no Bonferroni-Holms correction). The amplitude of ABR wave IV is declined starting from 10 dB above threshold in Old rats (Fig. 13A). This decline was found significant at all sound pressure levels as compared to Elderly animals (1-sided Student's *t*-test: see table 5 for p-values, no Bonferroni-Holms correction). These data show that Old animals showed reduced ABR waves I, II and IV amplitudes compared with Elderly animals.

Summarizing data shown above, amplitudes of central ABR waves (II and IV) as well as the amplitude of the ABR wave I that reflects the auditory nerve input, were reduced in Old Wistar rats as compared with Elderly Wistar rats. The immunohistochemical analysis of the rat cochlea reveals a loss of synaptic ribbons throughout the basal and midbasal parts of the cochlea of Old Wistar rats in comparison with Elderly Wistar rats. The quantitative analysis showed that 28 days after exposure ribbon counts were reduced by up to 45% in the midbasal part of the cochlea of Old Wistar rats.

3.1.2. Characterization of aging effects in the gerbil cochlea

To investigate whether hearing function decline could be also observed in the late period of lifespan of gerbils hearing of Young (10 – 13 months) and Elderly (19 – 28 months) Mongolian gerbils were characterized.

Effects of aging in the early period of lifespan in the gerbil animal model

Hearing function of the Young and Elderly Mongolian gerbils was tested by ABR and DPOAE recordings. All the recordings were performed as described above (see 2.2.).

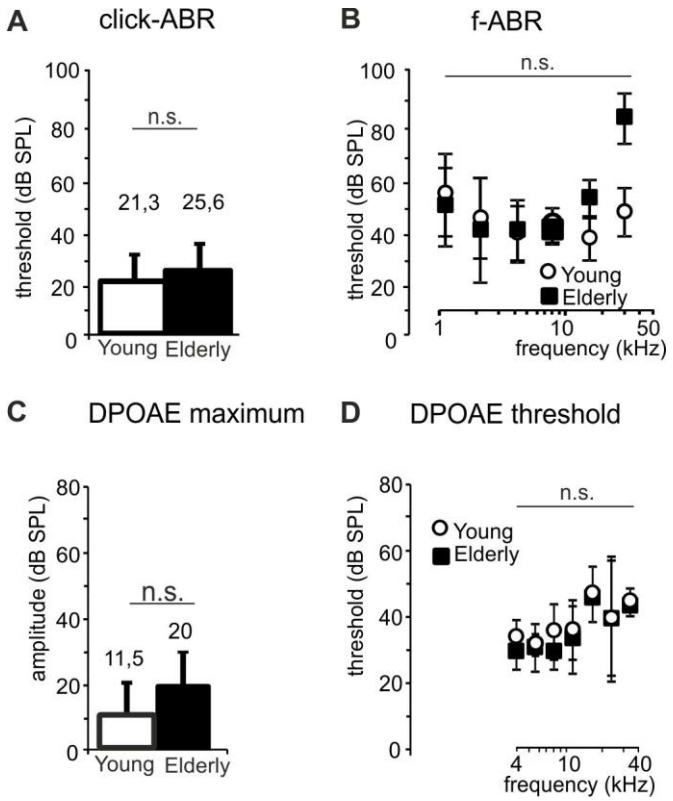

Figure 14. Functional difference in hearing of Young and Elderly Mongolian gerbils: ABRs and DPOAEs

A: ABR thresholds (click-ABR) presented as mean±SD for Young (10 – 12 months old; white bar) and Elderly (27,5 – 30 months old; black bar) Mongolian gerbils. **B:** Frequency specific pure tone thresholds (mean±SD) for Young (white circles) and Elderly (black squares) Mongolian gerbils. **C:** DPOAE maximum amplitude at f2=2,8–28 kHz of Young (white bar) and Elderly (black bar) Mongolian gerbils. **D:** DPOAE thresholds (dB SPL f1) of Young (white circles) and Elderly (black squares) Mongolian gerbils.

ABR thresholds on click auditory stimuli (click-ABR, Fig. 14A) were not significantly different (1-sided Student's *t*-test: p = 0,23) between Young gerbils (*n* = 10/5 ears/gerbils) and Elderly gerbils (*n* = 4/2 ears/gerbils). Frequency-specific ABR (f-ABR, Fig. 14B) did revealed small, but not significant differences between Young and Elderly gerbils for mean

threshold at all measured frequencies (Young gerbils: n = 10/5 ears/rats; Elderly gerbils: n = 4/2 ears/gerbils), but there was a tendency to the elevation of the ABR thresholds in the high frequency range. Interestingly, maximum amplitudes of growth functions of DPOAEs of Elderly gerbils had a trend to be elevated (Fig. 14C, Young: n = 8/5 ears/gerbils; Elderly: n = 4/2 ears/gerbils, 1-sided Student's t-test: n.s.). Due to the small number of ears that could be measured, this tendency is not statistically significant. DPOAE thresholds (Fig. 14D, two-way ANOVA: n.s.) didn't reflect the same tendency.

Summarizing, a trend to age-related high frequency loss could be observed in Elderly gerbils in comparison with Young gerbils. This hearing deficit was not linked to the OHCs dysfunction as no difference in DPOAEs was observed.

3.2. Effects of mild noise exposure on the auditory system

Previously it was suggested that in healthy animals full regeneration of cochlear nerve occurs after caused by acoustic overstimulation neural degeneration (Puel et al., 1998; Pujol and Puel, 1999). Recent findings showed that acoustic overexposure causes a permanent loss of peripheral nerve terminals on IHC (Kujawa and Liberman, 2009; Lin et al., 2011). In the present study we investigated the difference between vulnerability to noise in Young Elderly and Old Wistar rats, and Young and Elderly Mongolian gerbils.

3.2.1. Characterization of mild noise exposure effects in the rat cochlea

Vulnerability to mild noise exposure was investigated short time (4 weeks) and long time (6 months) after acoustic stimulation in Wistar rats of different age groups (Young, Elderly and Old).

Effects of mild noise exposure in short-term prospective in the rat animal model

Initially we tested effects of mild noise exposure 4 weeks after acoustic stimulation in Young and Elderly Wistar rats.

Vulnerability of hearing function of young and elderly rats for noise 28 days after mild noise exposure

Researching the question if after noise exposure Young (2 – 3 months old) and Elderly (8 – 10 months old) would exhibit differences in hearing threshold recovery, Young and Elderly Wistar rats were acoustically stimulated as described above (see2.3.). Hearing function was monitored as described earlier (Fig. 15A).

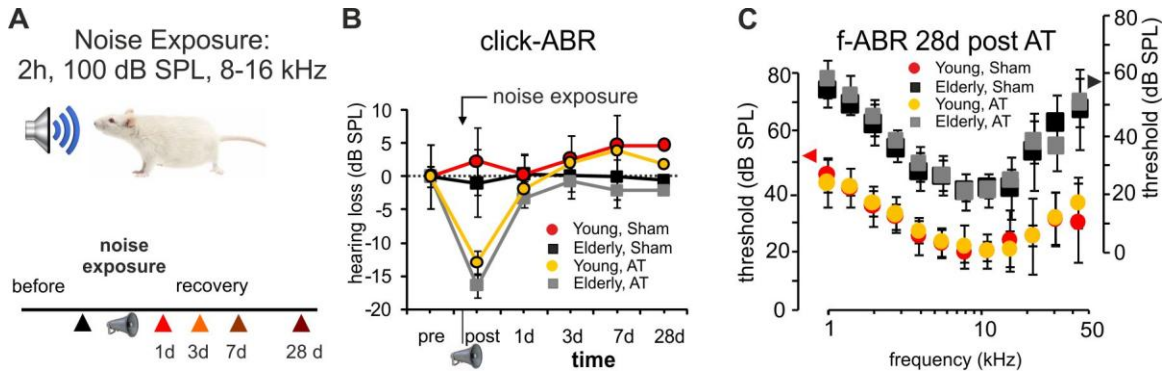

Figure 15. The vulnerability of hearing function of young and elderly rats for noise 28 days after mild noise exposure

A: Schematic illustration for the noise exposure paradigm chosen to test the vulnerability and recovery of Young compared with Elderly Wistar rats. **B:** After noise exposure (AT, 8-16 kHz broad band noise stimulus of 100 dB SPL for 2 hours; unexposed controls defined as Sham), Young Wistar rats exhibit a noise-induced hearing loss of approximately 15 dB for click stimuli, similar to the loss of Elderly Wistar rats. The recovery from noise-induced hearing loss was complete after 3 days and there was no permanent threshold shift (PTS) after this time period; the recovery was similar in Young and Elderly Wistar rats. **C:** At day 28 the f-ABR thresholds recovered completely at all frequencies in both age groups. For better representation of the data y-axis and the graph for aged group is shifted up. Each group contained of *n* = 10/5 ears/animals.

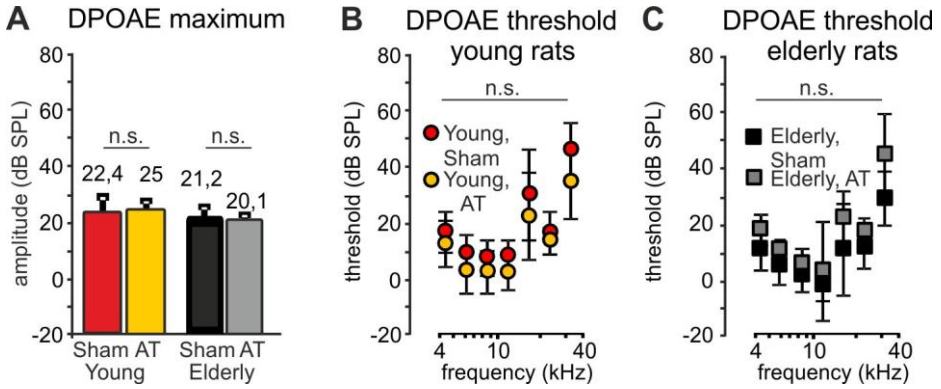

Figure 16. OHC function in Young and Elderly Wistar rats 28 days after exposure

A: Amplitudes of distortion products of otoacoustic emissions (DPOAE) of unexposed (Sham) and exposed to noise (AT) Young (Sham: red, AT: yellow) and Elderly (Sham: black, AT: grey) Wistar rats. Amplitudes were not reduced in both aged groups after noise exposure (AT) compared to control unexposed (Sham) age-matched rats. **B:** DPOAE thresholds of noise exposed (yellow circles) Young rats revealed no reduction of DPOAE thresholds in comparison with unexposed (red circles) Young rats over the whole studied frequency range. **C:** DPOAE thresholds of unexposed (black squares) and noise exposed (grey squares) Elderly rats revealed no reduction of DPOAE thresholds in Elderly noise exposed animals over all frequency range. Young rats: unexposed *n* = 10/5 ears/rats, noise exposed *n* = 12/6 ears/rats; Elderly rats: unexposed *n* = 10/5 ears/rats, noise exposed *n* = 10/5 ears/rats.

Noise exposure led to a temporary hearing loss of approximately 15 dB SPL in both Young and Elderly group of Wistar rats (Fig. 15B). 28 days after trauma, hearing recovered completely, indicating no noise induced permanent threshold shift (PTS). Already 1 day after exposure the temporal threshold shift (TTS) was much less pronounced in both groups verified through click-ABR. 3 days after exposure hearing function of both age groups recovered completely and stayed unchanged until the day 28 (Fig. 15B). The frequency-

specific ABR thresholds showed no hearing impairment neither in Young nor in Elderly Wistar rats over the whole frequency range 28 days after exposure (Fig. 15C; two-way ANOVA: n.s.).

The function of OHCs was analyzed in Young and Elderly Wistar rats by measuring the DPOAE (Fig. 16) before and 28 days after exposure. OHC function in Young and Elderly noise exposed rats in respect to maximal amplitudes of DPOAE showed no reduction (Fig. 16A; Young: yellow, *n* = 12/6 ears/rats; Elderly: grey, *n* = 10/5 ears/rats, *t*-test: n.s) when compared to their unexposed age-mates (Fig. 16A; Young: red, *n* = 10/5 ears/rats; Elderly: black, *n* = 10/5 ears/rats, *t*-test: n.s). Regarding the DPOAE thresholds of young (Fig. 16B) and aged (Fig. 16C) animals before and after exposure no differences were detected between groups (two-way ANOVA: n.s.).

Summarizing the data shown, I could conclude that both Young and Elderly Wistar rats have normal hearing function and equal vulnerability for mild noise exposure, characterized by a TTS that was completely recovered after 3 days and did not change up to day 28 after mild noise exposure, and unchanged maximum amplitudes and thresholds of DPOAEs.

Synaptopathy occurred 28 days after mild noise exposure

By immunohistochemistry the expression and distribution of CtBP2 as marker for IHC synaptic ribbons was analyzed in IHC of Young (2 – 3 months old) and Elderly (8 – 10 months old) noise exposed and Sham exposed Wistar rats (Fig. 17, Fig. 19). Otoferlin staining (red) in the midbasal (Fig. 17A-B, Fig. 19A-B) and basal (Fig. 17C-D, Fig. 19C-D) turns of the cochlea of Young Sham exposed (Fig. 17A, C) and noise exposed (Fig. 17B, D), and Elderly Sham exposed (Fig. 19A, C) and Elderly noise exposed (Fig. 19B, D) Wistar rats highlights IHC position. By visual inspection the reduction of CtBP2-positive dots (green) could be seen in the midbasal turn as well as in basal turn of the Young noised exposed Wistar rat cochlea (Fig. 17B, D) as compared to the midbasal and basal turns of the Young unexposed Wistar rat cochlea (Fig. 17A, C). Same tendency could be observed in Elderly animals in basal (Fig. 19C-D), but not in midbasal (Fig. 19A-B) cochlear turns. The quantification of IHC synaptic ribbon contacts was done in three cochlear regions (from apex to base).

CtBP2-positive points in single IHCs were counted in Young Sham exposed Wistar rats in comparison to Young noise exposed Wistar rats, and a significant reduction of ribbons

became evident in the midbasal and basal turns (Fig. 18B). No significant differences in the number of ribbons between Young Sham exposed and Young noise exposed Wistar rats were noted in the apical turn (Fig. 18B). IHC ribbon synapses number was reduced by 37% in the basal and 31% in the midbasal turns of the cochlea of Young noise exposed Wistar rats in comparison with Sham exposed.

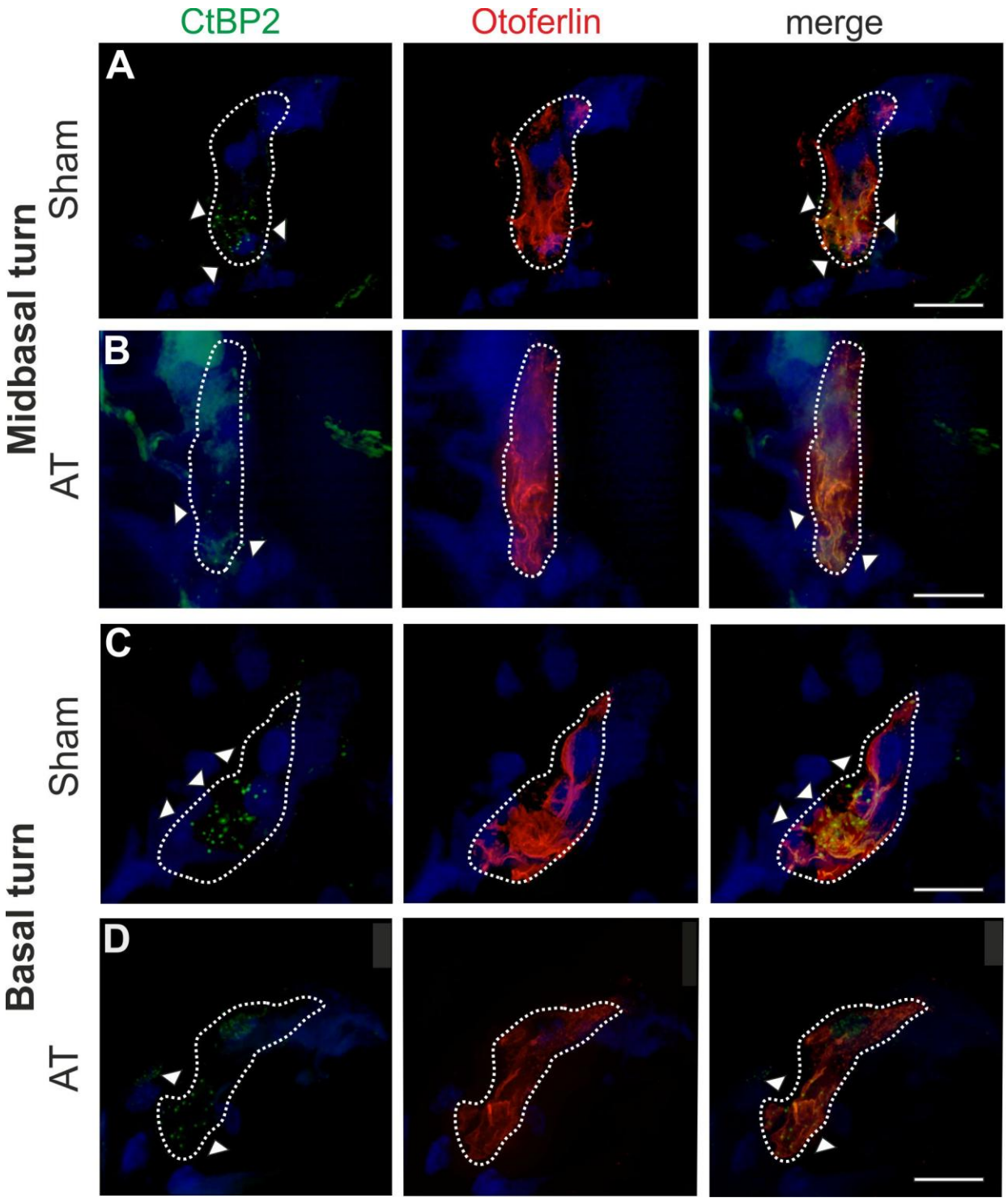

Figure 17. Fewer ribbon synapses in the basal and midbasal turns of cochlea IHCs of Young noise exposed Wistar rats

The loss of the synaptic contacts in the IHC of noise exposed rats could be observed. Images of the IHC of midbasal (A, B) and basal (C, D) turns of unexposed (A, C) and exposed (B, D) animals immunostained for synaptic ribbons (CtBP2, green, marked with arrowheads) and IHC (Otoferlin, red, encircled with line). Scale bars = 10 μm. Nuclear marker: DAPI (blue).

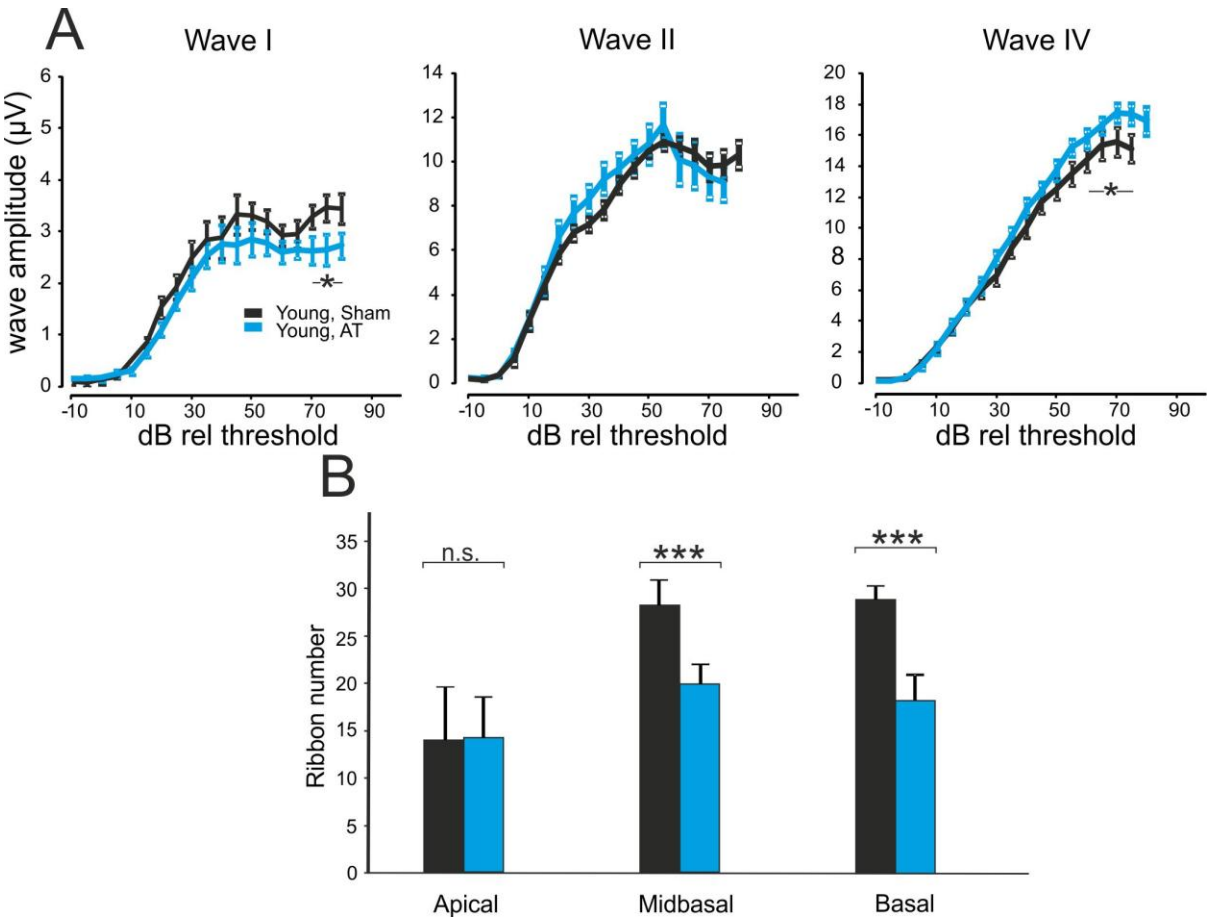

Figure 18. Reduction of the IHC ribbon synapses in basal and midbasal turns of the cochlea of Young noise exposed rats correlated with the loss of the ABR wave I amplitude

A: Mean ± SEM click-evoked ABR wave I, wave II and wave IV amplitudes growth function for Young unexposed (black line, *n* = 10/5 ears/rats) and Young noise exposed (blue line, *n* = 10/5 ears/rats). **B:** Ribbon counting from Young unexposed (black) and Young noise exposed (blue) Wistar rats, expressed in average number per IHC (±SD), in different cochlear turns (*n*=3).

Table 5. *P*-values of pair wise comparisons of amplitude data in Figure 18

Young AT vs. Young Sham	dB above threshold										
	30	35	40	45	50	55	60	65	70	75	80
Wave I	0,109	0,135	0,2771	0,0738	0,077	0,0618	0,0843	0,0947	0,0229 (*)	0,0159 (*)	0,0344 (*)
Wave II	0,2909	0,3379	0,4783	0,4344	0,3151	0,2347	0,1978	0,1695	0,1821	0,202	0,2177
Wave IV	0,177	0,322	0,31	0,234	0,1534	0,1264	0,0178 (*)	0,005 (**)	0,0016 (**)	0,00087 (**)	0,138

1-sided Student's *t*-test: *(*) p<0,05;(**) p<0,01*

As a correlate of auditory nerve fiber loss, in Young noise exposed Wistar rats ABR wave I amplitude was reduced in comparison with Young unexposed Wistar rats at 25 dB above threshold and above (Fig.18A). This difference was found significant at 70 dB above threshold and above (1-sided Student's *t*-test: see table 6 for p-values, no Bonferroni-Holms correction). There was no difference in wave II amplitude between Young unexposed and Young noise exposed Wistar rats (Fig. 18A; 1-sided Student's *t*-test: see table 6 for p-values, no Bonferroni-Holms correction). The amplitude of ABR wave IV is increased in Young noise exposed Wistar rats starting from 45 dB above threshold (Fig. 18A). That difference found significant at 60 dB above threshold and up to 75 dB above threshold as compared to young animals (1-sided Student's *t*-test: see table 6 for p-values, no Bonferroni-Holms correction). These data show that noise exposed animals showed a reduced ABR wave I amplitude, while wave II is unchanged, and wave IV amplitude was increased at high sound pressure levels when compared with wave amplitudes of Young unexposed Wistar rats.

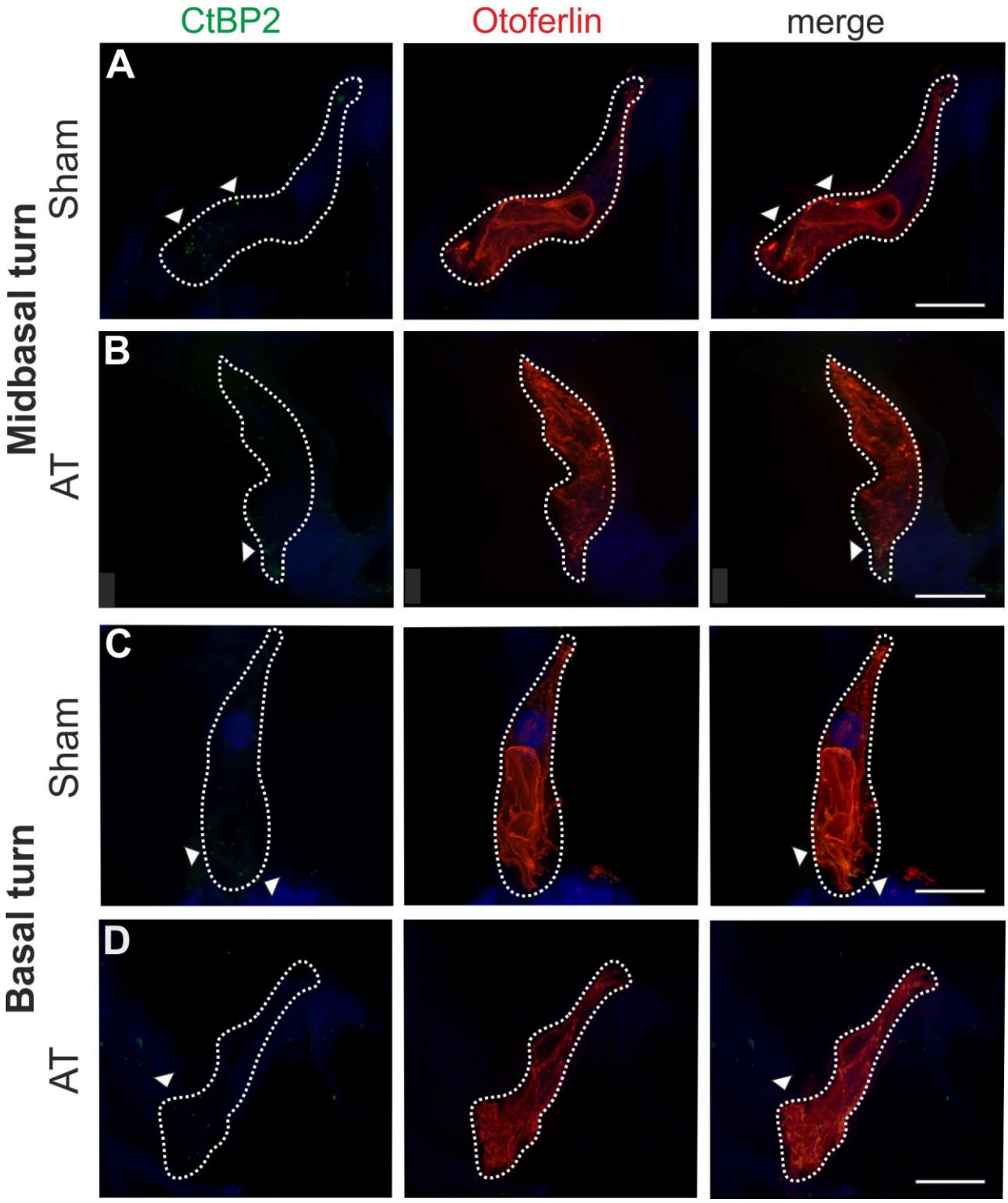

Figure 19. Ribbon synapses of the IHC were reduced in the basal and midbasal turns of the cochlea of Elderly noise exposed Wistar rats

The loss of the synaptic contacts in the IHC of Elderly noise exposed rats could be observed. Images of the IHC of midbasal (A, B) and basal (C, D) turns of unexposed (A, C) and exposed (B, D) animals immunostained for synaptic ribbons (CtBP2, green, marked with arrowheads) and IHC (Otoferlin, red, encircled with line). Scale bars = 10 μm. Nuclear marker: DAPI (blue).

CtBP2-positive points in single IHCs were also counted in Elderly unexposed Wistar rats in comparison to elderly noise exposed Wistar rats, and a significant reduction of ribbons

became evident in basal turn, but not in midbasal cochlear turns (Fig. 20B). No significant differences in the number of ribbons between Young and Elderly Wistar rats were noted in the apical turn (Fig. 20B). IHC ribbon synapses number was reduced by 14% in the basal turns of the cochlea of noise exposed Elderly Wistar rats in comparison with unexposed animals.

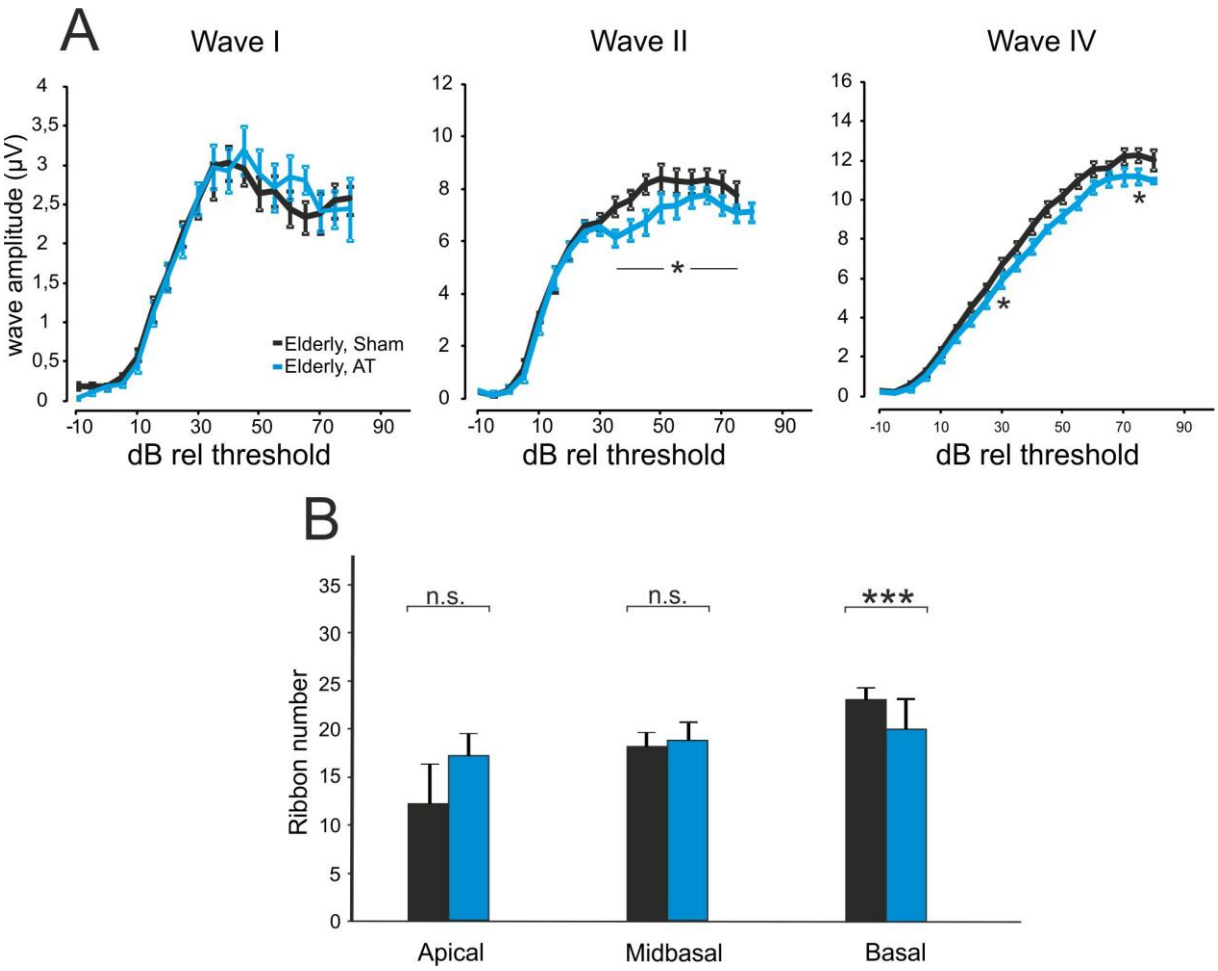

Figure 20. Number of IHC ribbon synapses in Elderly noise exposed and unexposed rats cochlea and ABR waveforms

A: Mean ± SEM click-evoked ABR wave I, wave II and wave IV amplitudes growth function for Elderly unexposed (black line, *n* = 10/5 ears/rats) and Elderly noise exposed (blue line, *n* = 10/5 ears/rats). **B:** Ribbon counting from Elderly unexposed (black) and Elderly noise exposed (blue) Wistar rats, expressed in average number per IHC (±SD), in different cochlear turns (*n*=3).

Table 6. ***P*-values of pair wise comparisons of amplitude data in Figure 20**

Elderly Sham vs. Elderly AT	dB above threshold										
	30	35	40	45	50	55	60	65	70	75	80
Wave I	0,38	0,42	0,465	0,3276	0,2949	0,3928	0,2464	0,262	0,38	0,4517	0,383
Wave II	0,16	$5*10^{-3}$ (**)	$9*10^{-4}$ (**)	$3*10^{-4}$ (**)	$4*10^{-4}$ (**)	$4*10^{-5}$ (**)	$2*10^{-5}$ (**)	$2*10^{-5}$ (**)	$2*10^{-5}$ (**)	$4*10^{-6}$ (**)	0,005 (**)
Wave IV	0,037 (*)	0,078	0,234	0,322	0,4844	0,269	0,1066	0,075	0,062	0,0442 (*)	0,282

1-sided Student's *t*-test: *(*) p<0,05;(**) p<0,01*

In elderly noise exposed Wistar rats ABR wave I amplitudes were not different in comparison with elderly unexposed Wistar rats (Fig. 20A; 1-sided Student's *t*-test: see table 7 for p-values, no Bonferroni-Holms correction). Decrease in wave II amplitudes were observed in elderly noise exposed Wistar rats in comparison with unexposed elderly Wistar rats (Fig. 20A; 1-sided Student's *t*-test: see table 7 for p-values, no Bonferroni-Holms correction). The amplitudes of ABR wave IV were decreased in elderly noise exposed Wistar rats starting from 30 dB above threshold (Fig. 20A). That difference was found to be significant at 30 dB and 75 dB above threshold as compared to Elderly unexposed animals (1-sided Student's *t*-test: see table 7 for p-values, no Bonferroni-Holms correction). These data showed that noise exposed animals showed reduced ABR wave I amplitudes, while wave II amplitudes was unaffected, and wave IV amplitude was increased at high sound levels compared with young unexposed Wistar rats.

Summarizing data shown, a synaptopathy characterized with the IHCs ribbon synapses degeneration could be observed in Young Wistar rats. Similar, but much less prominent effects could be observed in Elderly Wistar rats.

Long-term effects of mild noise exposure in the rat animal model

The vulnerability to noise was investigated with physiological and molecular methods in Elderly (8 – 10 months) and Old (21 – 26 months) Wistar rats 6 months after acoustic stimulation.

Effects of mild noise exposure in long-term prospective in the rat animal model

The hearing threshold recovery 6 months after acoustic stimulation was investigated between Elderly and Old Wistar rats. Anaesthetized Elderly and Old Wistar rats were exposed to noise as described above (see 2.3.), and hearing function was monitored before, shortly after, and 1, 3, 7 days and 6 months after noise exposure (Fig. 21A). Noise exposure led to a hearing loss in both Elderly and Old group of Wistar rats (Fig. 21B). 6 months after trauma, hearing of elderly rats recovered completely, indicating no noise induced PTS, while old rats developed a PTS of approximately 5 dB. Already 1 day after exposure the TTS was reduced in both groups verified through click-ABR. 3 days after hearing function of both age groups recovered completely and hearing function remained stable until 6 months later for Elderly, but not for Old Wistar rats (Fig. 21B). The frequency-specific ABR thresholds showed no hearing impairment in Elderly Wistar rats, but a tendency to the elevation of thresholds in Old Wistar rats over all frequency range, that was significant at middle frequencies (5,66 kHz – 11,3 kHz; $P < 0,0001$ by two-way ANOVA with Bonferroni multiple comparison test; other frequencies: n.s. by two-way ANOVA; Fig. 21C).

Function of OHC were analyzed in Elderly and Old Wistar rats by measuring the DPOAE (Fig. 22) before and 6 months after exposure to noise stimulation as described above. OHC function of Elderly noise exposed rats (Fig. 22A; yellow) in respect to maximal amplitudes of DPOAE showed no reduction (*t*-test: n.s) when compared to Shem exposed rats of the same age (Fig. 22A; red). OHC function of Old noise exposed rats (Fig. 22A; grey) in respect to maximal amplitudes of DPOAE showed a tendency to the reduction by 5,7 dB when compared to their unexposed age-matched Wistar rats (Fig 22A; black; 1-sided Student's *t*-test: n.s.), that was not statistically significant. After AT the DPOAE thresholds of Elderly rats were not reduced over the whole studied frequency range (Fig. 22B), while a clear tendency to the elevation of thresholds in low frequencies (4 – 8 kHz) was observed in Old rats (Fig. 22C, two-way ANOVA: n.s.).

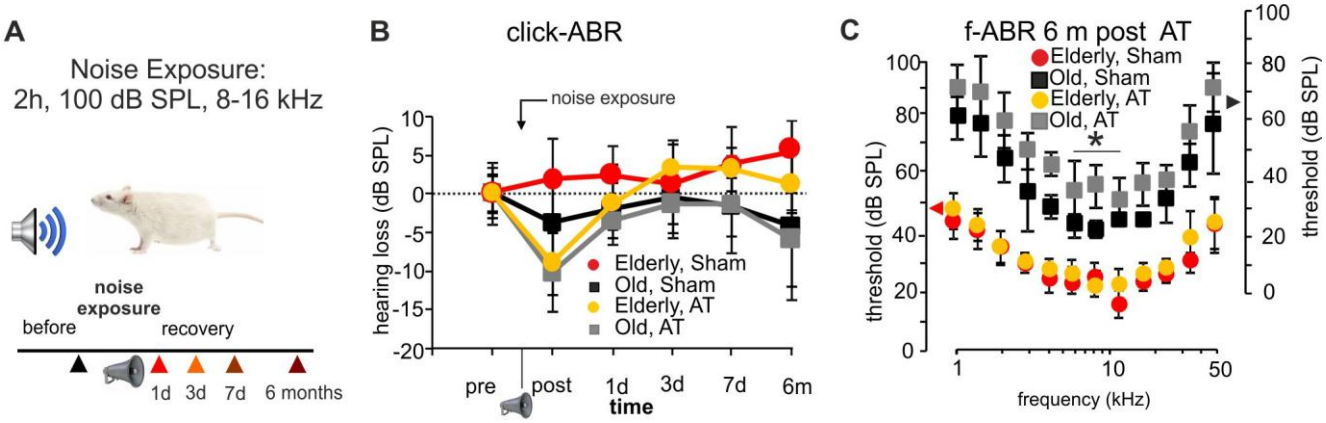

Figure 21. Vulnerability of the hearing function to noise of Elderly and Old Wistar rats up to 6 months after noise exposure

A: Schematic illustration for the noise exposure paradigm chosen to test the vulnerability and recovery of Elderly (2 – 3 months old before exposure) in comparison with Old (15 – 20 months old before exposure) Wistar rats. **B:** After noise exposure, Elderly Wistar rats exhibited a noise-induced hearing loss of approximately 10 dB for click stimuli, similar to the loss of Old Wistar rats. **C:** After 6 months the f-ABR thresholds recovered completely at all frequencies in aged (unexposed: red circles, *n* = 12/6 ears/rats; noise exposed: yellow circles, *n* = 10/5 ears/rats), but not in old (unexposed: black squares, *n* = 6/3 ears/rats; grey squares: orange circles, *n* = 6/3 ears/rats) Wistar rats. For better representation of the data y-axis and the graph for Elderly group is shifted up.

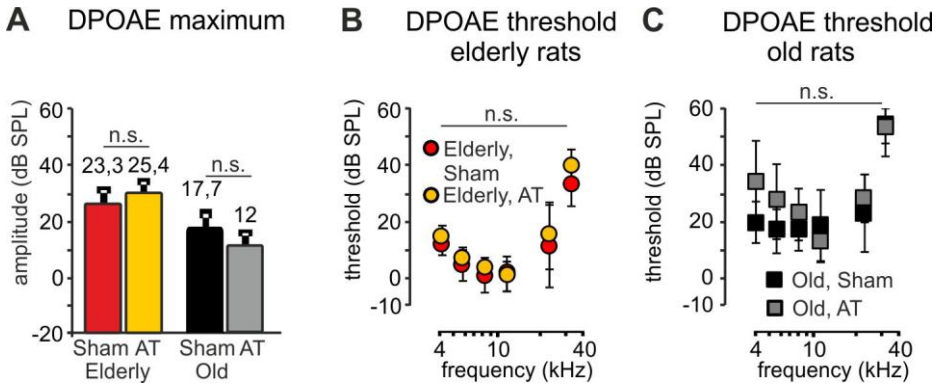

Figure 22. OHC function in Elderly and Old Wistar rats 6 months after exposure

A: Amplitudes of distortion products of otoacoustic emissions (DPOAE) of unexposed (Sham) and exposed to noise (AT) Elderly (Sham: red, AT: yellow) and Old (Sham: black, AT: grey) Wistar rats. Amplitudes were not significantly reduced in both age groups 6 months after noise exposure compared to unexposed age-matched rats (Sham). Old Wistar rats showed a clear tendency to the decrease of maximum amplitude of DPOAE. **B:** DPOAE thresholds of unexposed (red circles) and noise exposed (yellow circles) Elderly rats revealed no elevation of DPOAE thresholds after noise exposure over the studied frequency range. **C:** DPOAE thresholds of unexposed (black squares) and noise exposed (grey squares) Old rats revealed a tendency to the elevation of DPOAE thresholds in Old noise exposed animals in low frequencies (4 kHz – 8 kHz). These changes were not significantly different. DPOAE thresholds higher than 8 kHz showed no difference in DPOAE thresholds between unexposed and noise exposed animals. Elderly: unexposed *n* = 10/5 ears/rats, noise exposed *n* = 10/5 ears/rats; Old rats: unexposed *n* = 6/3 ears/rats, noise exposed *n* = 6/3 ears/rats.

Concluding, Elderly and Old Wistar rats have different hearing function and vulnerability to mild noise exposure. Old Wistar rats had a PTS of approximately 5 dB 6 months after noise exposure, elevated thresholds over the whole frequency range in frequency

ABR measurement, loss of the maximum amplitudes of DPOAEs and elevated low frequency (4 – 8 kHz) DPOAE thresholds. The hearing of the elderly Wistar rats recovered completely.

Synaptopathy occurred 6 months after mild noise exposure

By immunohistochemistry the expression and distribution of CtBP2 as marker of IHC synaptic ribbons was analyzed in IHC of Elderly (8 – 10 months old) and Old (21 – 26 months old) noise exposed and Sham exposed Wistar rats (Fig. 23, Fig. 25). Otoferlin staining (red) in the midbasal (Fig. 23A-B, Fig. 25A-B) and basal (Fig. 23C-D, Fig. 25C-D) turns of the cochlea of Elderly Sham exposed (Fig. 23A, C) and Elderly noise exposed (Fig. 25B, D), and Old unexposed (Fig. 23A, C) and Old noise exposed (Fig. 25B, D) Wistar rats highlights IHC position. By visual inspection the reduction of CtBP2-positive dots (green) could be seen in the midbasal turn as well as in basal turn of the Elderly noised exposed Wistar rat cochlea (Fig. 23B, D) as compared to the midbasal and basal turns of the Elderly unexposed Wistar rat cochlea (Fig. 23A, C). The same tendency could be observed in Old animals in basal (Fig. 25C-D), but not in midbasal (Fig. 25A-B) cochlear turns. The counting of IHC synaptic ribbon contacts was done in three cochlear regions (from apex to base; more counts needed).

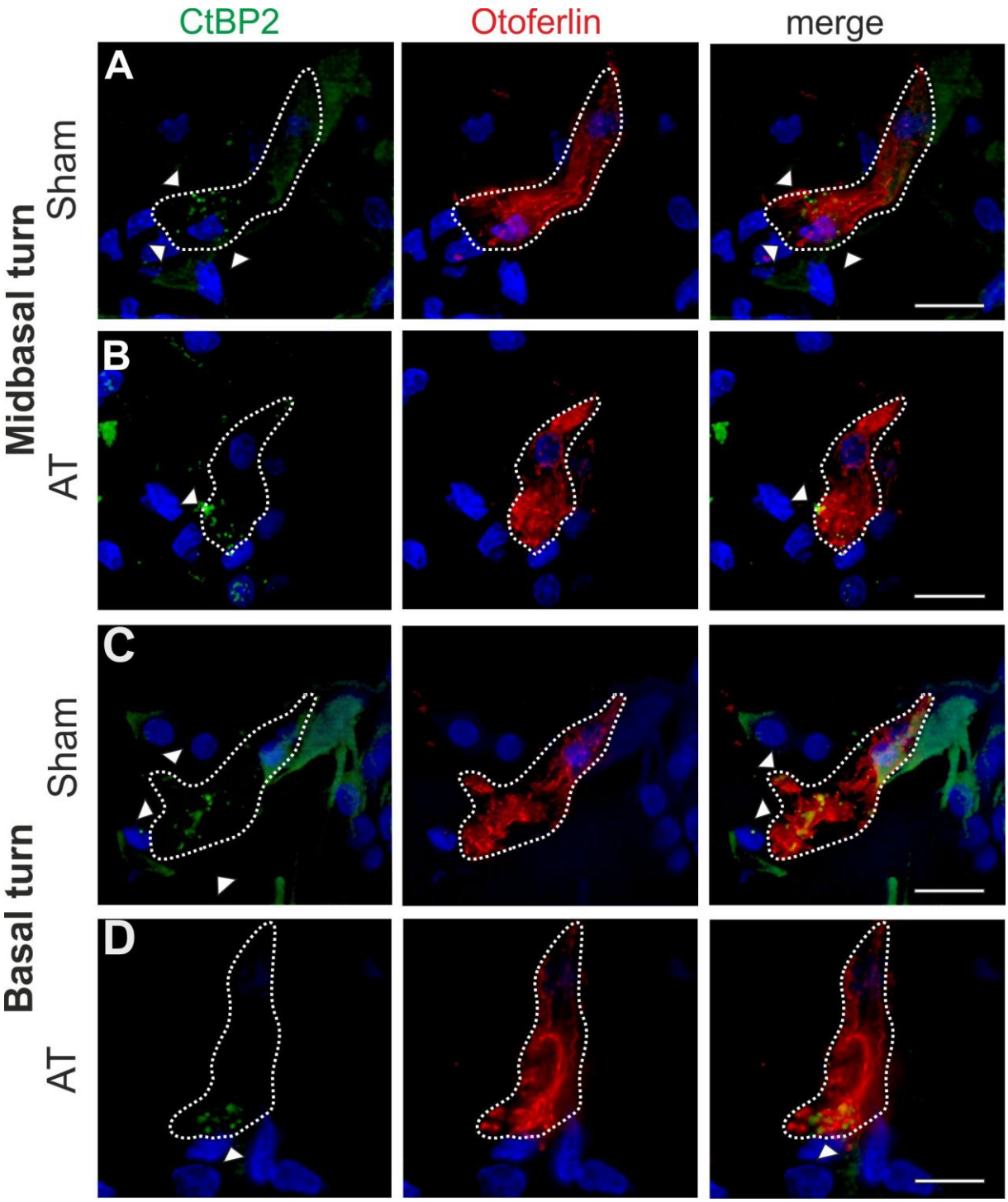

Figure 23. Ribbon synapses of the IHCs were reduced in the basal and midbasal turns of the cochlea of elderly noise exposed Wistar rats 6 months after exposure

The loss of the synaptic contacts in the IHC of noise exposed rats could be observed. Images of the IHC of midbasal (A, B) and basal (C, D) turns of unexposed (A, C) and exposed (B, D) animals immunostained for synaptic ribbons (CtBP2, green, marked with arrowheads) and IHC (otoferlin, red, encircled with line). Scale bars = 10 μm. Nuclear marker: DAPI (blue).

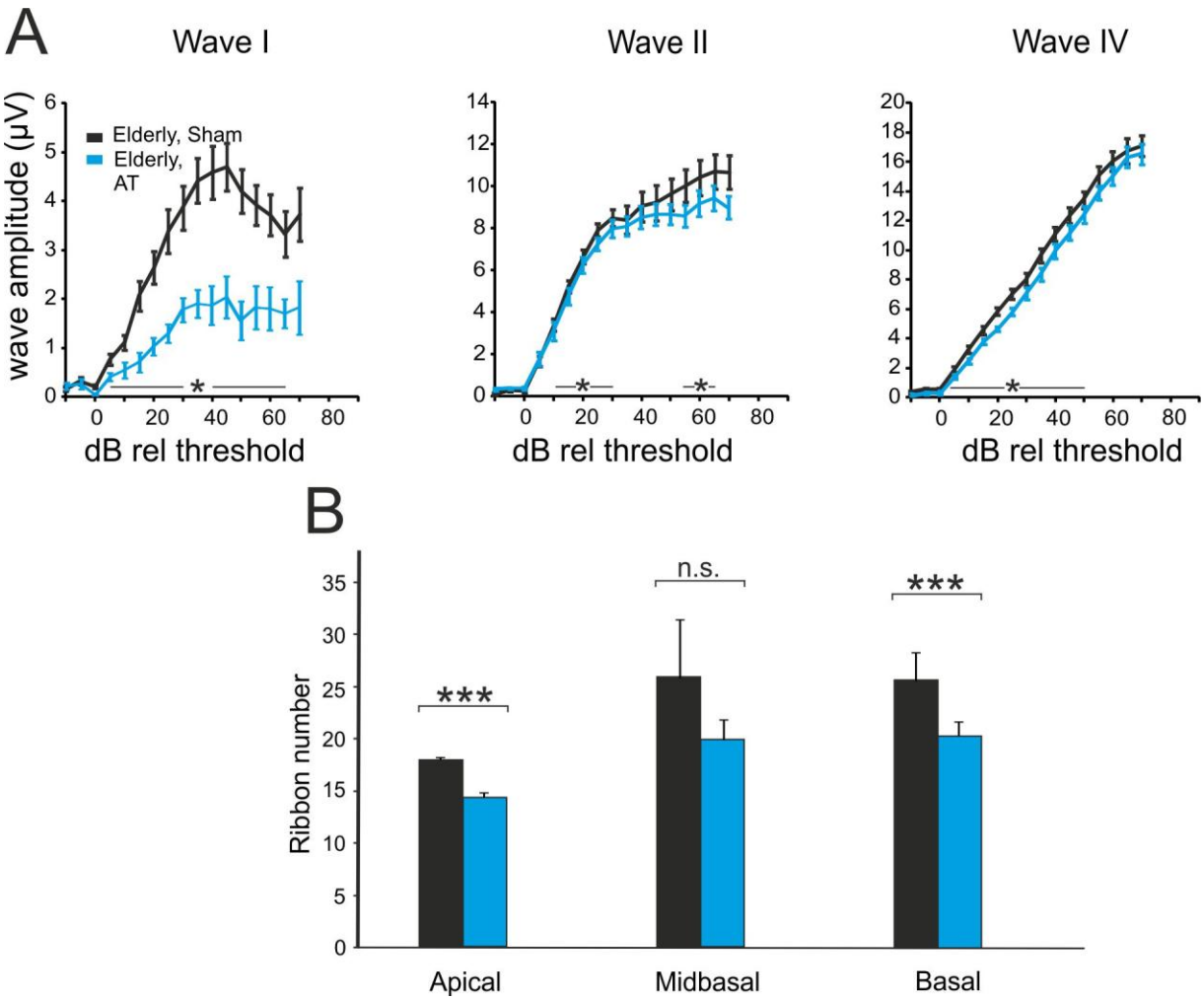

Figure 24. Reduction of IHC synaptic ribbons in basal and midbasal turns of the cochlea of elderly noise exposed rats correlated with the loss of the ABR wave I amplitude

A: Mean ± SEM click-evoked ABR wave I, wave II and wave IV amplitudes growth function for Elderly unexposed (black line, *n* = 10/5 ears/rats) and Elderly noise exposed (blue line, *n* = 10/5 ears/rats). **B:** Ribbon quantification from Elderly unexposed (black) and Elderly noise exposed (blue) Wistar rats, expressed in average number per IHC (±SD), in different cochlear turns (*n*=3).

CtBP2-positive points in single IHCs were counted in Elderly unexposed Wistar rats in comparison to Elderly noise exposed Wistar rats, and a significant reduction of ribbons became evident in the midbasal and basal turns (Fig. 24B). No differences in the number of ribbons between elderly unexposed and elderly noise exposed Wistar rats were noted in the apical turn (Fig. 24B). IHC synaptic ribbon number is reduced by 20% in the basal, 24% in the midbasal (tendency, 1-sided Student's t-test: n.s.), and 20% in the apical turns of the cochlea of noise exposed Elderly Wistar rats in comparison with unexposed.

Table 7. *P*-values of pair wise comparisons of amplitude data in Figure 24

Elderly Sham vs. Elderly AT	dB above threshold														
	0	5	10	15	20	25	30	35	40	45	50	55	60	65	70
Wave I	0,48	0,04 (*)	0,005 (**)	0,011 (*)	0,011 (*)	0,01 (*)	0,013 (*)	0,03 (*)	0,018 (*)	0,03 (*)	0,028 (*)	0,013 (*)	0,03 (*)	0,028 (*)	0,145
Wave II	0,05	0,09	0,001 (**)	$8*10^{-4}$ (**)	$7,7*10^{-4}$ (**)	0,004 (**)	0,015 (*)	0,3	0,117	0,08	0,105	0,044 (*)	0,027 (*)	0,043 (*)	0,084
Wave IV	0,02 (*)	0,001 (**)	0,002 (**)	0,02 (*)	$5,8*10^{-4}$ (**)	0,002 (**)	0,02 (*)	0,01 (*)	0,012 (*)	0,02 (*)	0,017 (*)	0,18	0,09	0.214	0,317

1-sided Student's *t*-test: *(*) p<0,05;(**) p<0,005*

In Elderly noise exposed Wistar rats ABR wave I amplitude was reduced in 6 months after noise exposure in comparison with Elderly unexposed Wistar rats at 5 dB above threshold and above (Fig. 24A). This difference was found significant from 5 dB above threshold and up to 65 dB above threshold (1-sided Student's *t*-test: see table 8 for p-values, no Bonferroni-Holms correction). ABR wave II amplitude was also reduced in elderly noise exposed Wistar rats 6 months after noise exposure (Fig. 24A; 1-sided Student's *t*-test: look table 8 for p-values, no Bonferroni-Holms correction). This difference was found statistically significant from 10 dB above threshold up to 30 dB above threshold, and from 55 dB above threshold up to 65 dB above threshold. The amplitude of ABR wave IV was decreased in Elderly noise exposed Wistar rats starting from 0 dB above threshold (Fig. 24A). That difference was found to be significant up to 40 dB above threshold as compared to Elderly unexposed animals (1-sided Student's *t*-test: see table 8 for p-values, no Bonferroni-Holms correction). These data showed that Elderly noise exposed rats show reduced ABR wave I, while wave II is unaffected, and wave IV amplitude is also reduced at low sound levels compared with Elderly unexposed Wistar rats.

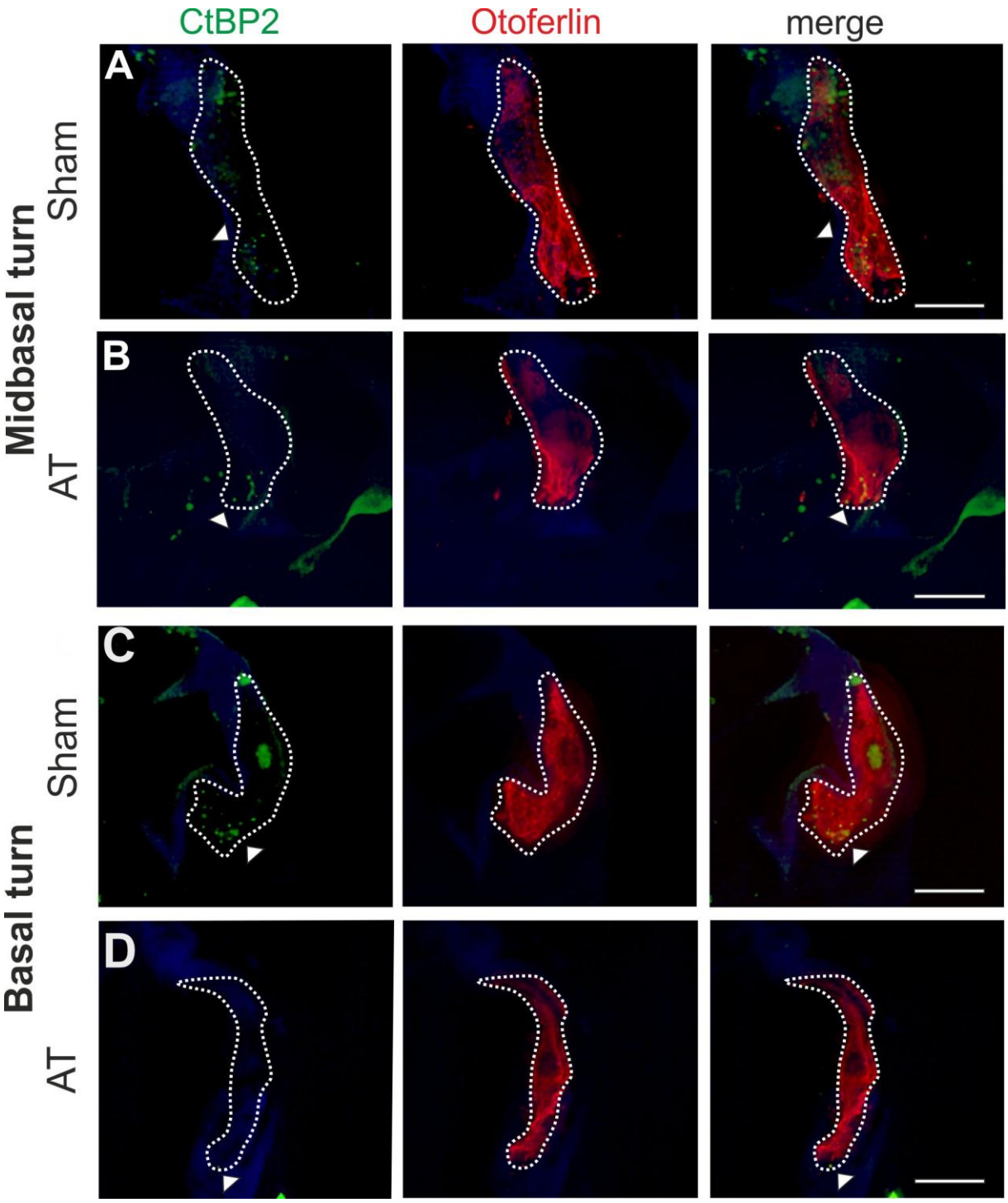

Figure 25. Ribbon synapses of the IHCs were reduced in the basal and midbasal turns of the cochlea of old noise exposed Wistar rats 6 months after exposure

The loss of the synaptic contacts in the IHC of noise exposed rats could be observed. Images of the IHC of midbasal (A, B) and basal (C, D) turns of unexposed (A, C) and exposed (B, D) animals immunostained for synaptic ribbons (CtBP2, green, marked with arrowheads) and IHC (otoferlin, red, encircled with line). Scale bars = 10 μm. Nuclear marker: DAPI (blue).

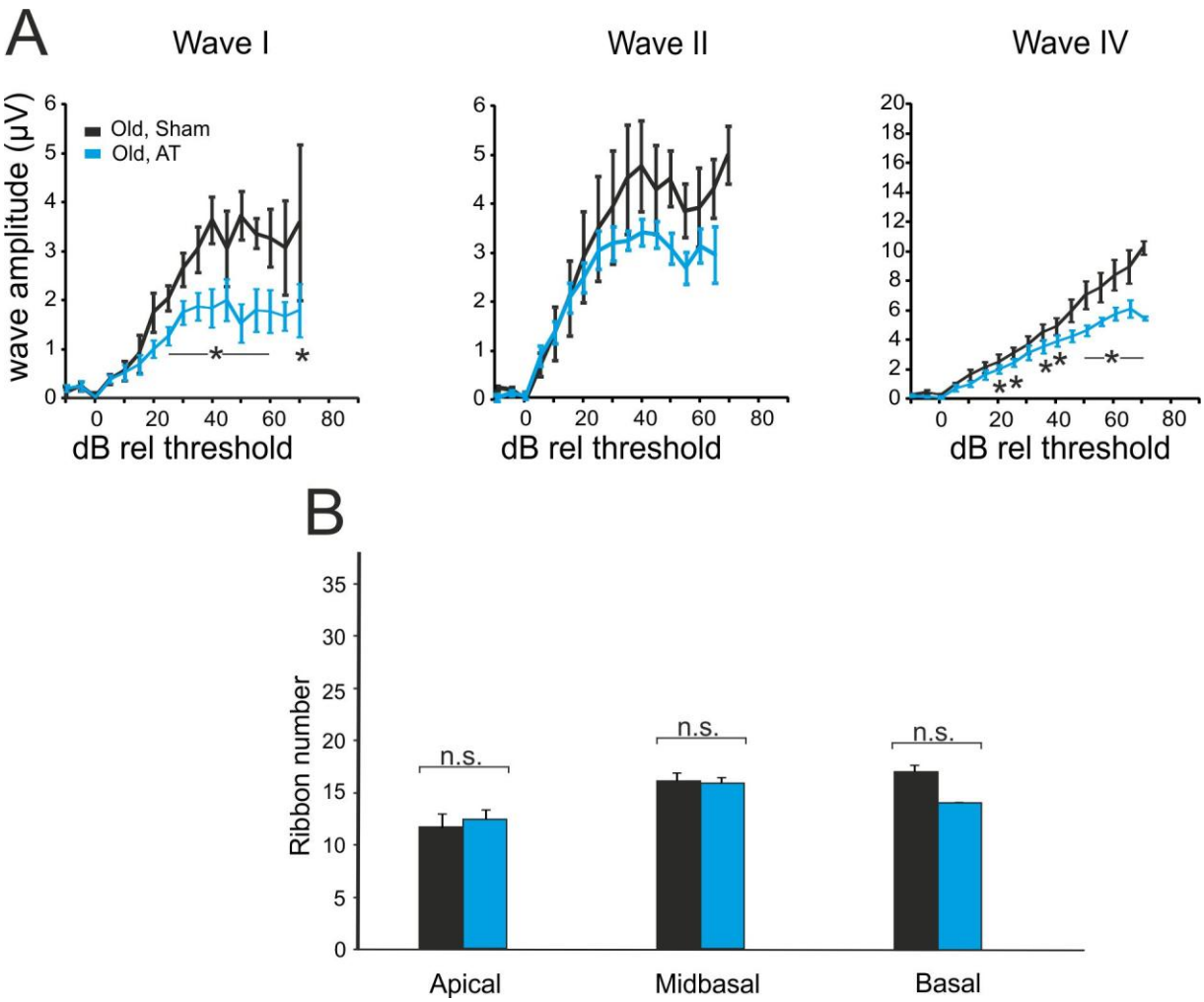

Figure 26. Reduction of IHC synaptic ribbons in basal turn of the cochlea of old noise exposed rats correlated with the loss of the ABR wave I amplitude

A: Mean ± SEM click-evoked ABR wave I, wave II and wave IV amplitudes growth function for Old unexposed (black line, *n* = 6/3 ears/rats) and Old noise exposed (blue line, *n* = 6/3 ears/rats). **B:** Ribbon counting from Old unexposed (black) and Old noise exposed (blue) Wistar rats, expressed in average number per IHC (±SD), in different cochlear turns (*n*=3, experiments were done in duplicate).

CtBP2-positive points in single IHCs were also counted in old unexposed Wistar rats in comparison to old noise exposed Wistar rats, and a reduction of ribbons became evident in basal turn, but not in midbasal cochlear turn (Fig. 26B). No differences in the number of ribbons between Old unexposed and Old noise-exposed Wistar rats were noted in the apical turn (Fig. 26B). IHC ribbon synapses number was reduced by 18% in the basal turns of the cochlea of noise exposed elderly Wistar rats in comparison with unexposed animals. This difference was not found statistically significant, so we could talk only about tendency.

Table 8. *P*-values of pair wise comparisons of amplitude data in Figure 26

Old Shamvs. Old AT	dB above threshold										
	20	25	30	35	40	45	50	55	60	65	70
Wave I	0,0709	0,006 (*)	0,0078 (*)	0,008 (*)	0,0003 (**)	0,011 (*)	0,005 (**)	0,0137 (**)	0,0055 (*)	0,06	0,028 (*)
Wave II	0,055	0,125	0,084	0,067	0,1389	0,151	0,34	0,1107	0,125	0,077	0,18
Wave IV	0,02 (*)	0,027 (*)	0,0888	0,0467 (*)	0,0324 (*)	0,087	0,038 (*)	0.022 (*)	0,0182 (*)	0,0152 (*)	0,014 (*)

1-sided Student's *t*-test: *(*) p<0,05;(**) p<0,005*

In Old noise exposed Wistar rats ABR wave I amplitudes were reduced in comparison with Old unexposed Wistar (Fig. 26A; 1-sided Student's *t*-test: see table 9 for p-values, no Bonferroni-Holms correction) starting from 15 dB above threshold (statistically significant from 25 dB above threshold to 60 dB above threshold, and at 70 dB above threshold). Decrease in wave II amplitudes were also observed in Old noise exposed Wistar rats in comparison with unexposed Old Wistar rats (Fig. 26A; 1-sided Student's *t*-test: look table 9 for p-values, no Bonferroni-Holms correction), but this trend was not statistically significant over the whole frequency range. The amplitudes of ABR wave IV were decreased in old noise exposed Wistar rats starting from 10 dB above threshold (Fig. 26A). That difference found significant at 20 – 25 dB, 35 – 40 dB, and 50 – 70 dB above threshold as compared to Old unexposed animals (1-sided Student's *t*-test: see table 9 for p-values, no Bonferroni-Holms correction). These data show that noise exposed animals show reduction in every out of analyzed ABR waves amplitudes (wave I, wave II and wave IV) compared with old unexposed Wistar rats.

Summarizing data shown above, amplitudes of all ABR waves' amplitudes of elderly noise exposed rats were decreased, but the most profound loss of the amplitude was found in ABR wave I amplitudes. In old noise exposed animals amplitudes of all ABR waves were significantly decreased. The immunohistochemical analysis of the rat cochlea reveals a loss of synaptic ribbons throughout the basal and midbasal parts of the cochlea in elderly

noise exposed rats in comparison with unexposed one. The quantitative analysis showed that 6 months after exposure ribbon numbers are in both groups for 20%.

3.2.2. Characterization of mild noise exposure effects in the gerbil cochlea

In order to establish the protocol of mild non-traumatizing noise exposure in gerbils, Young (10 – 13 months old) and Elderly (23 – 29 months old before exposure) were acoustically stimulated.

Vulnerability of hearing function of young and elderly gerbils for noise 4 months after mild noise exposure

Anaesthetized Young and Elderly Mongolian gerbils were exposed to the mild noise as described above (see 2.3.). Hearing function was monitored before, shortly after and 1, 3, 7, and 12 months after noise exposure (Fig. 20A). Noise exposure led to a hearing loss of approximately 10 dB in Young and 20 dB in Elderly group of Mongolian gerbils (Fig. 27B, D). 1 day after exposure the TTS was already much less pronounced in both groups verified through click-ABR. 4 months after trauma, hearing recovered completely only in elderly unexposed group, indicating no PTS. Young unexposed animals exhibited a PTS of 10 dB, while young noise exposed gerbils exhibited PTS up to 20 dB. PTS of elderly noise exposed animals was even bigger: up to 35 dB loss.

The frequency-specific ABR thresholds showed no hearing impairment in Young (Fig. 27C), but a pronounced hearing loss in low frequency range in Elderly Mongolian gerbils (Fig. 27E) in 4 months after exposure. This difference appeared to be significant at the range of 1 – 2 kHz ($P < 0,0001$ by two-way ANOVA with Bonferroni multiple comparison test for 1 – 2 kHz; other frequencies: n.s. by two-way ANOVA).

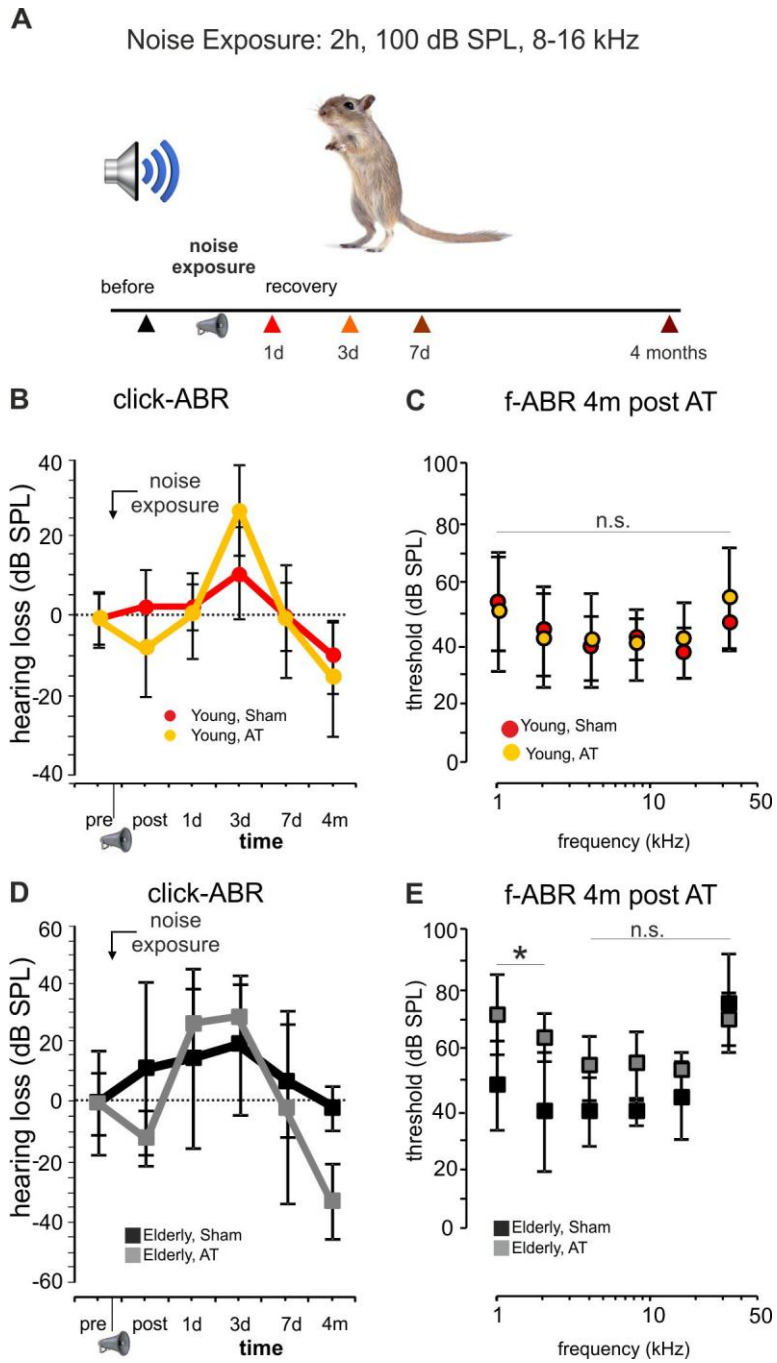

Figure 27. The vulnerability of hearing function of young and elderly gerbils to noise 4 months after mild noise exposure

A: Schematic illustration for the noise exposure paradigm chosen to test the vulnerability and recovery of Young compared with Elderly Mongolian gerbils. **B:** After induction of acoustic trauma, Young Mongolian gerbils exhibit a noise-induced hearing loss of approximately 10 dB for click stimuli. **C:** After 12 months the f-ABR thresholds recovered completely at all frequencies in Young gerbils (*n* = 10/5 ears/animals). **D:** After induction of acoustic, Elderly Mongolian gerbils exhibit a noise-induced hearing loss of 15 dB for click stimuli. The recovery from noise-induced hearing loss was not complete after 4 months and there was a permanent threshold shift (PTS) after this time period in elderly noise exposed gerbils. **E:** After 12 months the f-ABR thresholds recovered completely at all frequencies in Elderly gerbils (*n* = 10/5 ears/animals).

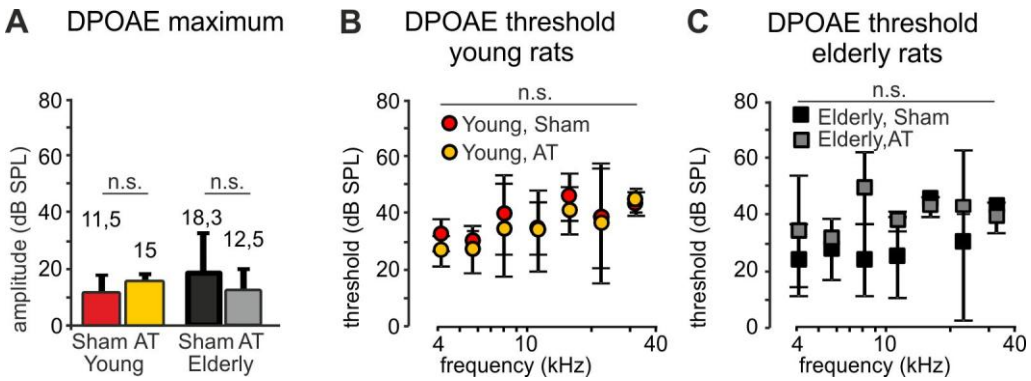

Figure 28. OHC function in young and elderly Mongolian gerbils 4 months after exposure

A: Amplitudes of distortion products of otoacoustic emissions (DPOAE) of unexposed (Sham) and exposed to noise (AT) Young (Sham: red, AT: yellow) and Elderly (Sham: black, AT: grey) Mongolian gerbils. Amplitudes were not significantly reduced in both age groups after noise exposure (AT) compared to control unexposed age-mated gerbils (Sham), but only age group showed a clear tendency to the decrease of maximum amplitude of DPOAE. **B:** DPOAE thresholds of unexposed (red circles) and noise exposed (yellow circles) Young gerbils revealed no difference in DPOAE thresholds after noise exposure over the whole frequency range. **C:** DPOAE thresholds of unexposed (black squares) and noise exposed (grey squares) Elderly gerbils no difference in DPOAE thresholds after noise exposure over the whole frequency range. Young gerbils: unexposed *n* = 10/5 ears/gerbils, noise exposed *n* = 10/5 ears/gerbils; elderly gerbils: unexposed *n* = 2/1 ears/gerbils, noise exposed *n* = 10/5 ears/gerbils.

The function of OHC was analyzed in Young and Elderly Mongolian gerbils by measuring the DPOAE (Fig. 28) before and 4 months after noise exposure. OHC function in Young and Elderly noise exposed gerbils in respect to maximal amplitudes of DPOAE showed a trend to reduction (Fig. 28A; Young: yellow, *n* = 10/5 ears/gerbils; Elderly: grey, *n* = 2/1 ears/gerbils, 1-sided Student's *t*-test: n.s) when compared to their unexposed age-mates (Fig. 28A; Young: red, *n* = 10/5 ears/rats; Elderly: black, *n* = 8/5 ears/gerbils, 1-sided Student's *t*-test: n.s). DPOAE thresholds of Young animals before and after exposure revealed no differences between groups (Fig. 28B) (two-way ANOVA: n.s.). DPOAE thresholds of noise exposed (grey squares). Elderly rats also showed no changes of DPOAE thresholds in comparison to unexposed (black squares) animals over the whole frequency range (Fig. 28C) (two-way ANOVA: n.s.).

In summary, a trend to high frequency hearing loss could be observed in elderly noise exposed animals, which is similar to one of rats 6 months after noise exposure. These changes could not be linked to the alterations in the OHCs, therefore we could suggest that underlying mechanism is connected with neurodegeneration.

3.3. Effects of environmental enrichment on the auditory system

It was shown that long-term sound conditioning prior to a traumatizing noise exposure protects animals from the noise-induced hearing loss, while the mechanism of this effect could not be fully understood (Kujawa and Liberman, 1999). We questioned whether environmental enrichment could counteract age-related and noise-induced neurodegeneration.

3.3.1. Environmental enrichment effects on aged-related processes in auditory system of gerbils

Pointing the question if environmental enrichment could affect hearing of Young (10 – 13 months) and Elderly (23 – 29 months) gerbils, animals were separated in equal groups that were kept in normal environment (NE) and enriched environment (EE) that was enriched with olfactory, acoustical, visual and tactile stimuli.

Keeping gerbils in enriched environment reduces high-frequency hearing loss over age

Hearing function was monitored as described above (Fig. 29). ABR thresholds (click-ABR) are significantly different between young and elderly gerbils in both environmental groups (Fig. 22A). Trend to age-related high-frequency loss that could be observed for elderly animals kept in normal environment, that was significant only at 32 kHz (Fig. 29B; $P < 0,0001$ by two-way ANOVA with Bonferroni multiple comparison test; other frequencies: n.s. by two-way ANOVA). This loss is rescued in elderly animals kept in enriched environment (Fig. 29C; two-way ANOVA: n.s.).

OHC function in Young and Elderly gerbils kept in normal environment showed no reduction, when characterized with DPOAE measurements (Fig. 30A; Young, NE: red, n = 10/5 ears/gerbils; Elderly: black, n = 4/2 ears/gerbils, 1-sided Student's t-test: n.s) when compared to their age-mates kept in enriched environment (Fig. 30A; Young: yellow, n = 10/5 ears/rats; Elderly: grey, n = 6/3 ears/rats, 1-sided Student's t-test: n.s). Regarding the DPOAE thresholds of young and elderly gerbils kept in normal environment (Fig. 30B; two-way ANOVA: n.s.) and young and elderly gerbils kept in enriched environment (Fig. 30C; two-way ANOVA: n.s.) animals before and after exposure no differences were detected between groups.

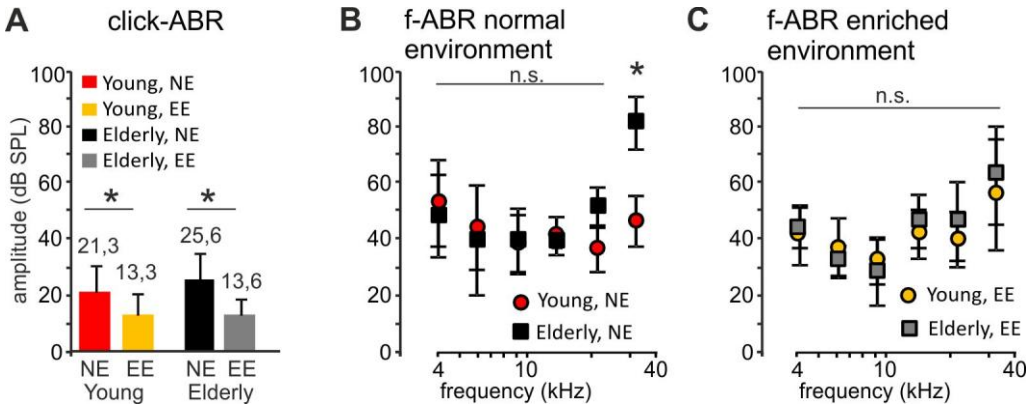

Figure 29. Functional difference in hearing of young and elderly gerbils in enriched and normal environment: ABRs

A: ABR thresholds (click-ABR) presented as mean±SD for Young (Normal Environment (NE): red bar, Enriched environment (EE): yellow bar) and Elderly (NE: black bar, EE: grey bar) gerbils. **B:** Frequency specific pure tone thresholds (mean±SD) for Young (red circles) and Elderly (black squares) gerbils kept in normal environment showed a trend to high-frequency hearing loss. **C:** Frequency specific pure tone thresholds (mean±SD) for Young (yellow circles) and Elderly (grey squares) gerbils kept in enriched environment shows rescue of age-dependent high-frequency hearing loss.

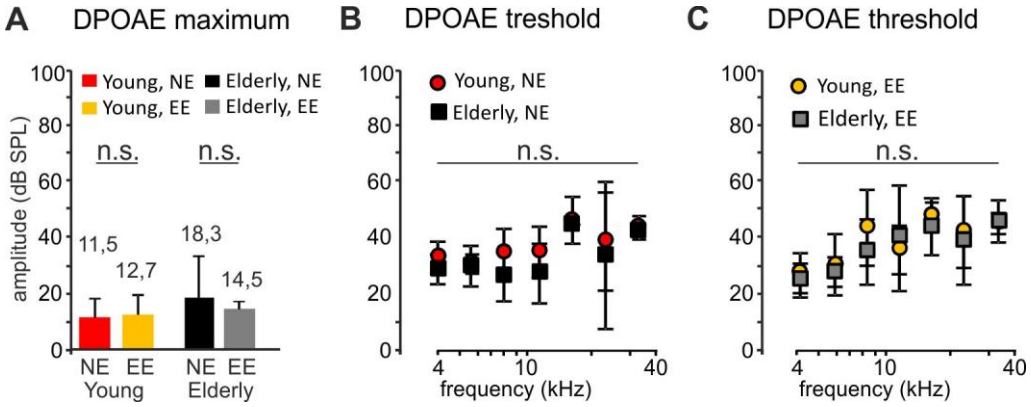

Figure 30. OHC function in young and elderly Mongolian gerbils that were kept under the different environmental conditions

A: Amplitudes of distortion products of otoacoustic emissions (DPOAE) of kept in normal environment (NE) and enriched environment (EE) Young (NE: red, EE: yellow) and Elderly (NE: black, EE: grey) gerbils. Amplitudes were not different between groups kept in NE and EE. **B:** DPOAE thresholds of Young (red circles) and Elderly (black squares) gerbils kept in normal environment revealed no differences of DPOAE thresholds between both age groups kept in normal environment. **C:** DPOAE thresholds of Young (yellow circles) and Elderly (grey squares) gerbils kept in enriched environment revealed no changes in DPOAE thresholds between age groups. Young gerbils: NE *n* = 11/6 ears/gerbils, EE *n* = 9/5 ears/gerbils; Elderly gerbils: NE *n* = 4/2 ears/gerbils, EE *n* = 6/3 ears/gerbils.

Therefore I could conclude that housing gerbils in the enriched environment reduces age-related high-frequency loss in Elderly Mongolian gerbils.

Better low-frequency hearing in animals kept in enriched environment

Trying to evaluate if we could observe any differences in hearing of gerbils from the same age group kept in different environmental conditions, Young (10 – 13 months old) and Elderly (23 – 29 months old) gerbils were kept in normal (deprived) environment (NE) and

enriched environment (EE) that was enriched with olfactory, acoustical and visual stimuli data shown above was analyzed in the way that the hearing of the age-matched animals kept under the different environmental conditions could be analyzed. Trend to better low-frequency hearing could be observed in young gerbils kept in enriched environment in comparison with age-mates kept in normal environment (Fig. 31A, P = 0,0001 by two-way ANOVA with Bonferroni multiple comparison test at 1kHz, other frequencies: n.s. by two-way ANOVA) 4 months after exposure. Elderly gerbils kept in enriched environment also have better low-frequency in comparison with the same age group rats kept in normal environment, but the effect was less pronounced (Fig. 31B; two-way ANOVA: n.s.).

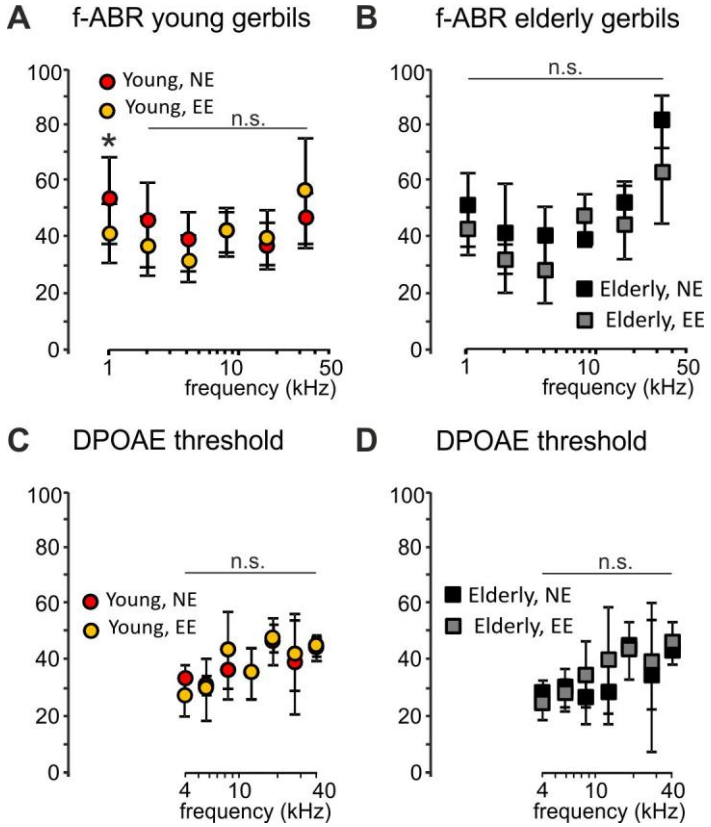

Figure 31. Functional difference in hearing of young and elderly gerbils in enriched and regular environment: ABRs and DPOAEs

A: Frequency specific pure tone thresholds (mean±SD) for Young gerbils kept in NE (red circles) and Young gerbils kept in EE (yellow circles) showed a trend to be beneficial for low-frequency hearing thresholds. **B:** Frequency specific pure tone thresholds (mean±SD) for Elderly gerbils kept in NE (black squares) and Elderly gerbils kept in EE (grey squares) showed a trend to be beneficial for low-frequency hearing thresholds. **B:** DPOAE thresholds of Young kept in normal environment (red circles) and Young kept on enriched environment (yellow circles) gerbils revealed no differences of DPOAE thresholds between groups. **C:** DPOAE thresholds of Elderly gerbils kept in normal environment (black squares) and Elderly gerbils kept in enriched environment (grey squares) revealed no changes in DPOAE thresholds between age groups.

The function of OHC was analyzed in Young and Elderly Mongolian gerbils by measuring the DPOAE. This data show that mechanism of protection was not connected to the OHC function as no differences between animals kept in different environments was

observed neither in young (Fig. 31C; two-way ANOVA: n.s.) nor in elderly gerbils (Fig. 31D: two-way ANOVA: n.s.).

Concluding, in Young Mongolian gerbils better lower frequency was observed. The same trend was true also for Elderly Mongolian gerbils, but it was less pronounced.

3.3.2. Characterization of environmental enrichment effects on vulnerability for noise in young and aged gerbils

Investigating the question whether there is a difference in vulnerability to noise in gerbils kept under different housing conditions, Young (10 – 13 months old) and Elderly (23 – 29 months old) gerbils kept in enriched and normal environment were exposed to mild noise using a protocol described above (see 2.3.).

Mild noise exposure conditions were traumatizing for young gerbils kept in enriched environment, but not in regular environment

Click-ABR thresholds of Young noise-exposed gerbils kept in enriched environment was significantly higher than those of age-matched non-exposed controls (Fig. 32A; Young, Sham, EE: black bar, n = 18/9 ears/gerbils; Young, AT, EE: grey bar, n = 10/5 ears/gerbils, 1-sided Student's t-test: p = 0,04), while thresholds of young noise exposed gerbils kept in normal environment were not different from those of age-matched controls (Fig. 32A; Young, Sham, NE: red bar, n = 10/5 ears/gerbils; Young, AT, NE: yellow bar, n = 14/7 ears/gerbils, 1-sided Student's t-test: n.s.). Trend to the hearing loss over the whole frequency range could be observed in Young noise-exposed gerbils kept in enriched environment in comparison with unexposed age-mates kept under the same conditions (Fig. 32D, two-way ANOVA: n.s.), while hearing of young noise exposed gerbils kept in normal environment was not different in comparison with unexposed age-mates kept under the same conditions (Fig. 32B; two-way: n.s.).

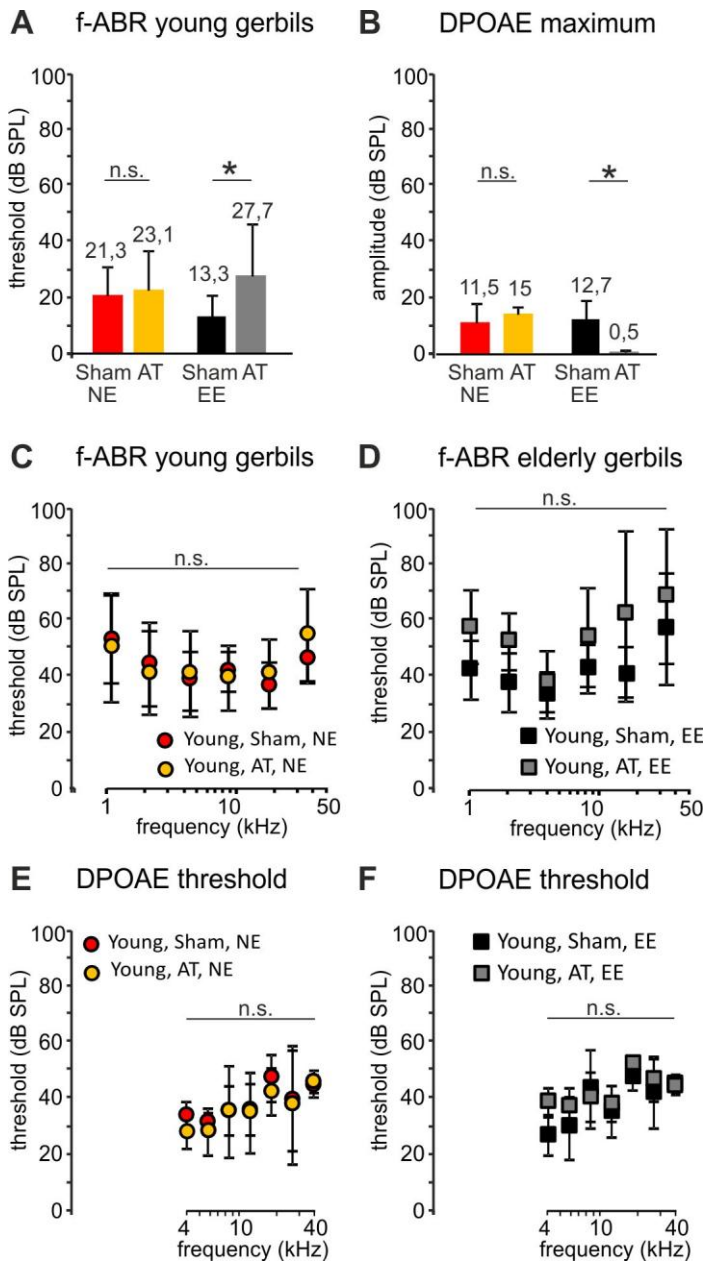

Figure 32. Functional difference in vulnerability to noise of young gerbils kept under different environmental conditions: ABRs and DPOAEs

A: ABR thresholds (click-ABR) presented as mean±SD for young (10 – 13 months old) noise exposed (AT) and unexposed (Sham) gerbils kept in normal environment (NE) and enriched environment (EE). **B:** Amplitudes of DPOAE of young (10 – 13 months old) noise exposed (AT) and unexposed (Sham) gerbils kept in normal environment (NE) and enriched environment (EE). **C:** Frequency specific pure tone thresholds (mean±SD) for Young unexposed gerbils kept in NE (red circles) and Young noise exposed gerbils kept in NE (yellow circles). **D:** Frequency specific pure tone thresholds (mean±SD) for Young unexposed gerbils kept in EE (black squares) and Young noise exposed gerbils kept in EE (grey squares). **E:** DPOAE thresholds of Young unexposed gerbils kept in normal environment (red circles) and Young noise exposed gerbils kept in normal environment (yellow circles). **F:** DPOAE thresholds of Young unexposed gerbils kept in enriched environment (black squares) and Young unexposed gerbils kept in enriched environment (grey squares).

The function of OHC was analyzed in Young unexposed and noise exposed gerbils kept in normal and enriched environment by measuring the DPOAE. Even thought there was a significant difference in maximum DPOAE amplitudes between noise exposed gerbils kept in enriched environment and unexposed gerbils kept under the same conditions (Fig. 32B;

Young, Sham, EE: black bar, *n* = 12/6 ears/gerbils; Young, AT, EE: grey bar, *n* = 4/2 ears/gerbils, 1-sided Student's *t*-test: *p* = 0,01), DPOAE thresholds did not reveal any differences over all frequency range (Fig. 32 F, two-way ANOVA: n.s.). No significant differences in maximum DPOAE amplitudes between young noise exposed gerbils kept in normal environment and unexposed gerbils kept under the same conditions were found (Fig. 32B; Young, Sham, NE: red bar, *n* = 8/5 ears/gerbils; Young, AT, NE: yellow bar, *n* = 6/4 ears/gerbils, 1-sided Student's *t*-test: n.s.). No DPOAE thresholds of young noise exposed gerbils kept in normal environment were also changed in comparison with age-mate unexposed controls kept under the same conditions (Fig, 32E, two-way ANOVA: n.s.).

Environmental conditions did not affect vulnerability for noise in elderly gerbils

Elderly gerbils kept in enriched and normal environment were exposed to mild noise using a protocol described above (see 2.3.). Hearing function was monitored as described above. Click-ABR thresholds of Elderly noise-exposed gerbils kept in enriched environment was significantly higher than those of non-exposed controls of the same age (Fig. 33A; Elderly, Sham, EE: black bar, *n* = 6/3 ears/gerbils; Elderly, AT, EE: grey bar, *n* = 6/3 ears/gerbils, 1-sided Student's *t*-test: *p* = 0,003). Thresholds of Elderly noise exposed gerbils kept in normal environment were also elevated in comparison with unexposed controls of the same age kept under the same conditions (Fig. 33A; Elderly, Sham, NE: red bar, *n* = 4/2 ears/gerbils; Elderly, AT, NE: yellow bar, *n* = 4/2 ears/gerbils, 1-sided Student's *t*-test: n.s.). Hearing loss over the whole frequency range could be observed in Elderly noise-exposed gerbils kept in enriched environment in comparison with unexposed gerbils of the same age kept under the same conditions (Fig. 33D, P = 0,0012 by two-way ANOVA with Bonferroni multiple comparison test at 1 – 2 kHz; n.s. by two-way ANOVA at other frequencies of the tested frequency range). In Elderly noise exposed gerbils kept in the normal environmental conditions in comparison with Elderly unexposed gerbils kept under the same conditions the same effect could be observed (Fig. 33C, P < 0,0001 by two-way ANOVA with Bonferroni multiple comparison test at 1- 2 kHz; n.s. by two-way ANOVA at other frequencies of the tested frequency range).

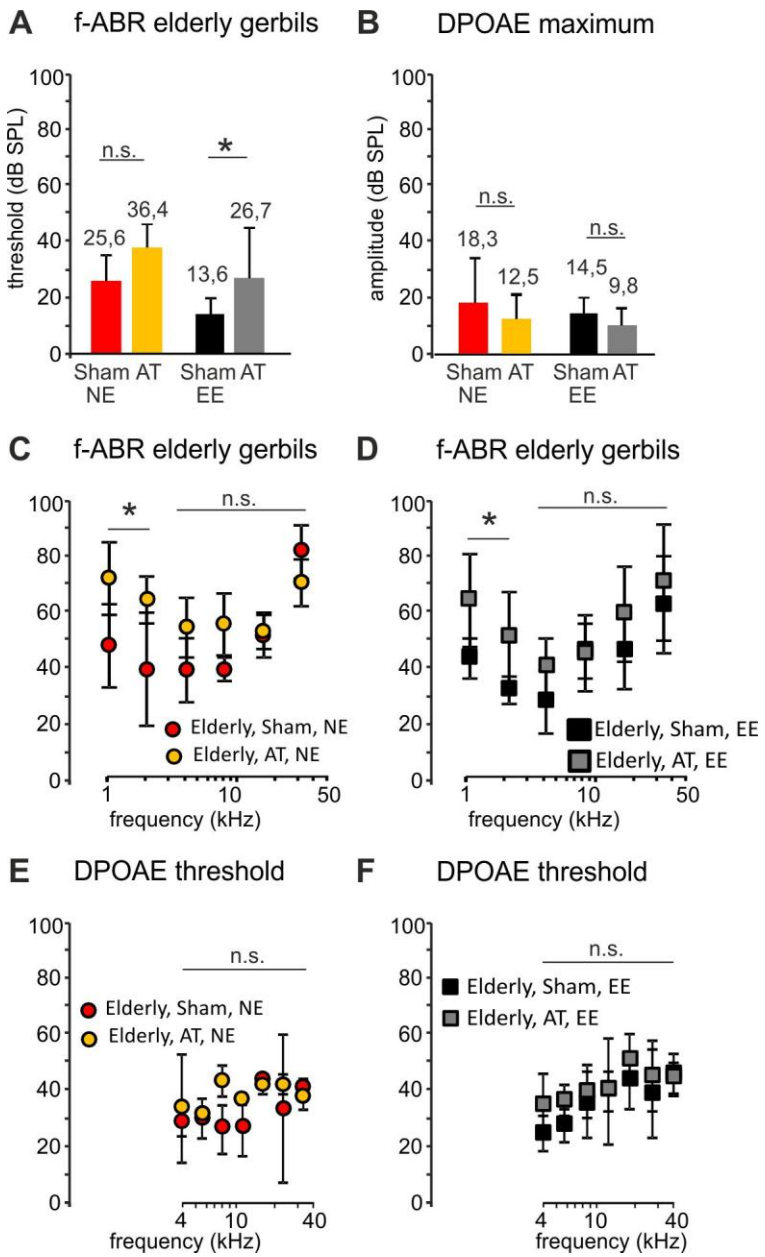

Figure 33. Functional difference in vulnerability to noise of elderly gerbils kept under different environmental conditions: ABRs and DPOAEs

A: ABR thresholds (click-ABR) presented as mean±SD for Elderly (23 – 29 months old) noise exposed (AT) and unexposed (Sham) gerbils kept in normal environment (NE) and enriched environment (EE). **B:** Amplitudes of DPOAE of Elderly noise exposed (AT) and unexposed (Sham) gerbils kept in normal environment (NE) and enriched environment (EE). **C:** Frequency specific pure tone thresholds (mean±SD) for Elderly unexposed gerbils kept in NE (red circles) and Elderly noise exposed gerbils kept in EE (yellow circles). **D:** Frequency specific pure tone thresholds (mean±SD) for Elderly unexposed gerbils kept in EE (black squares) and Elderly noise exposed gerbils kept in EE (grey squares). **E:** DPOAE thresholds of Elderly unexposed gerbils kept in normal environment (red circles) and Elderly noise exposed gerbils kept in normal environment (yellow circles). **F:** DPOAE thresholds of Elderly unexposed gerbils kept in enriched environment (black squares) and Elderly unexposed gerbils kept in enriched environment (grey squares).

The function of OHC was analyzed in Elderly unexposed and noise exposed gerbils kept in normal and enriched environment by measuring the DPOAE. Even thought there was a significant difference in maximum DPOAE amplitudes between noise exposed gerbils kept in enriched environment and unexposed gerbils kept under the same conditions (Fig. 33B;

Elderly, Sham, EE: black bar, *n* = 6/3 ears/gerbils; Elderly, AT, EE: grey bar, *n* = 6/3 ears/gerbils, 1-sided Student's *t*-test: n.s.), DPOAE thresholds did not reveal any differences over all frequency range (Fig. 33F, two-way ANOVA: n.s.). No significant differences in maximum DPOAE amplitudes between young noise exposed gerbils kept in normal environment and unexposed gerbils kept under the same conditions were found (Fig. 33B; Elderly, Sham, NE: red bar, *n* = 3/2 ears/gerbils; Elderly, AT, NE: yellow bar, *n* = 2/1 ears/gerbils, *t*-test could not be performed due to the lack of data). DPOAE thresholds of Elderly noise exposed gerbils kept in normal environment were changed in comparison with age-mate unexposed controls kept under the same conditions (Fig. 33E, two-way ANOVA: n.s.).

3.4. Effects of cGMP cascade activation on auditory system

Looking for the possibilities to counteract the neurodegeneration occurred with age and after noise exposure we considered the stimulation of the cGMP cascade, which previously was shown to protect from noise induced hearing loss (Jaumann et al., 2012).

3.4.1. Effects of treatment with sGC-stimulator on age-related processes in the auditory system

Prior we could question whether activation of cGMP cascade is beneficial for age-related and induced by mild noise exposure hearing loss, we tested whether administering sGC stimulator itself has an effect on the auditory system.

Effects of cGMP cascade activation on age-related processes in auditory system of rats

In order to find out whether cGMP cascade activation by administering sGC stimulator affects hearing of young Wistar rats, the hearing of Young (8 – 10 months old) and Elderly (8 – 10 months old) Wistar rats was tested after 4 weeks and after 6 months of treatment.

4 weeks long cGMP cascade activation did not cause alterations in the auditory system of the rat

ABR thresholds for click auditory stimuli (click-ABR, Fig. 34A) were not significantly different (*t*-test: n.s.) between young untreated Wistar rats (n = 10/5 ears/rats) and young Wistar rats treated with sGC stimulator (n = 12/6 ears/rats). Frequency-specific ABR (f-ABR, Fig. 34B) revealed no significant hearing loss in young Wistar rats treated with sGC stimulator (two-way ANOVA: n.s.). Maximum DPOAE amplitudes of young Wistar rats treated with normal food were not different from those of young Wistar rats treated with sGC stimulator (Fig. 34C, young treated with normal food: n = 10/5 ears/rats; young treated with sGC stimulator: n = 12/6 ears/rats, 1-sided Student's *t*-test: n.s.).

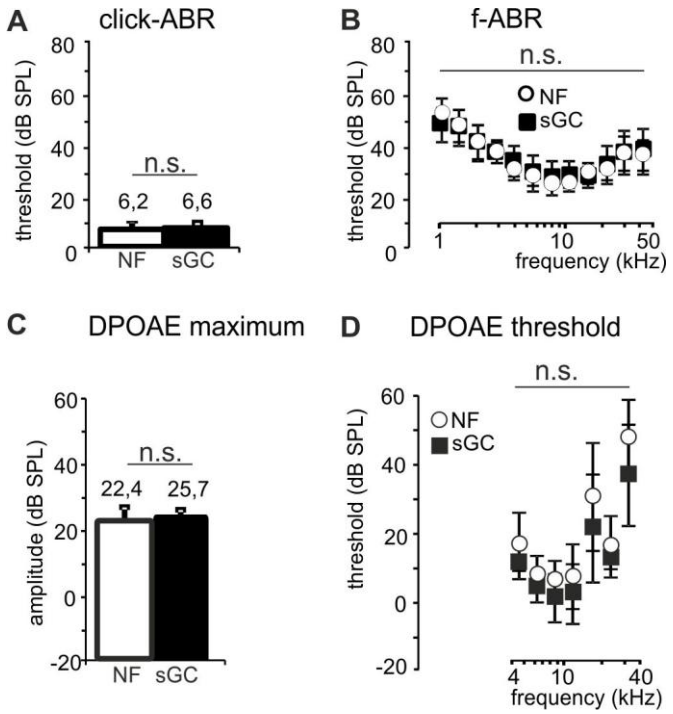

Figure 34. Hearing function of young Wistar rats treated for 4 weeks with sGC simulator Wistar rats in comparison with untreated Wistar rats of the same age: ABRs and DPOAEs

A: ABR thresholds (click-ABR) presented as mean±SD for Young Wistar rats treated with normal food (NF: white bar) and Young Wistar rats treated with food containing sGC stimulator (sGC: black bar). **B:** Frequency specific pure tone thresholds (mean±SD) for Young Wistar rats treated with normal food (white circles) and Young Wistar rats treated with sGC stimulator (black squares). **C:** DPOAE maximum amplitude at f2 = 4,8–5,8 kHz of Young Wistar rats treated with NF (white bar) and Young Wistar rats treated with sGC stimulator (black bar). **D:** DPOAE thresholds (dB SPL f1) of Young Wistar rats treated with normal food (white circles) and Young Wistar rats treated with sGC stimulator (black squares) Wistar rats.

No differences in DPOAE thresholds of Young Wistar rats treated with normal food were observed in comparison with Young Wistar rats treated with sGC stimulator (Fig. 34D; two-way ANOVA: n.s.).

To find out whether cGMP cascade activation during 4 weeks affects hearing of Elderly Wistar rats, the hearing was tested 4 weeks after beginning of the treatment with sGC stimulator. ABR thresholds on click auditory stimuli (click-ABR, Fig. 35A) were not significantly different between Elderly treated with normal food Wistar rats (*n* = 10/5 ears/rats) and Elderly treated with sGC stimulator Wistar rats (*n* = 10/5 ears/rats). Frequency-specific ABR (f-ABR, Fig. 35B) revealed no difference in hearing thresholds of Elderly treated with sGC stimulator Wistar rats in comparison with Elderly treated with normal food Wistar rats (Fig. 35B; two-way ANOVA: n.s.). Maximum DPOAE amplitudes of Elderly treated with normal food Wistar rats were similar to ones of Elderly treated with sGC stimulator Wistar rats (Fig. 35C, Elderly Wistar rats treated with normal food: *n* = 10/5

ears/rats; Elderly Wistar rats treated with sGC stimulator: *n* = 10/5 ears/rats; 1-sided Student's *t*-test: n.s.).

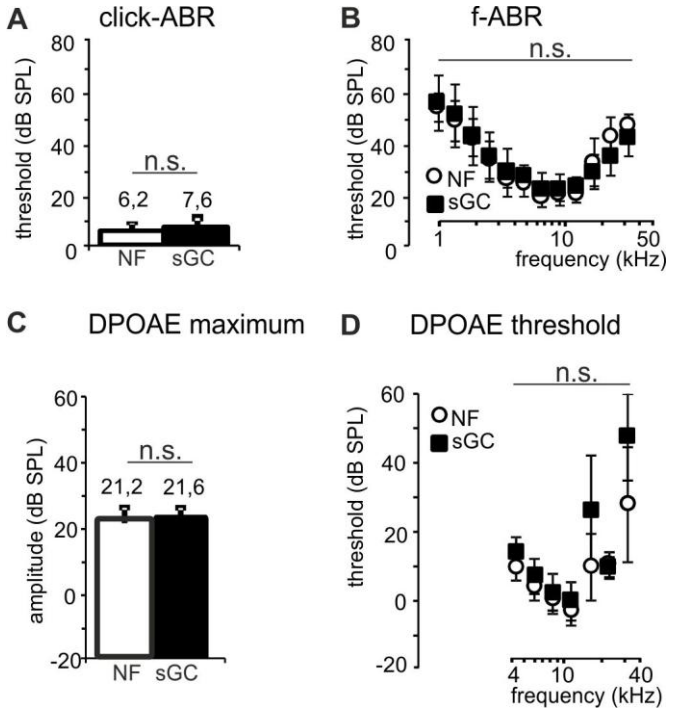

Figure 35. Hearing function of elderly Wistar rats treated for 4 weeks with sGC simulator in comparison with untreated age-matched Wistar rats: ABRs and DPOAEs

A: ABR thresholds (click-ABR) presented as mean±SD for Elderly Wistar rats treated with normal food (NF: white bar) and Elderly Wistar rats treated with sGC stimulator substance (sGC: black bar). **B:** Frequency specific pure tone thresholds (mean±SD) for Elderly treated with normal food Wistar rats (white circles) and Elderly treated with sGC stimulator Wistar rats (black squares). **C:** DPOAE maximum amplitude at f2 = 8–22 kHz of elderly Wistar rats treated with NF (white bar) and elderly treated with sGC stimulator Wistar rats (black bar). **D:** DPOAE thresholds (dB SPL f1) of Elderly treated with normal food (white circles) and Elderly treated with sGC stimulator (black squares) Wistar rats.

No differences in DPOAE thresholds of Elderly treated with normal food Wistar rats were observed in comparison with those of Elderly treated with sGC stimulator Wistar rats (Fig. 35D; two-way ANOVA with Bonferroni multiple comparison test: n.s.).

Summarizing data shown above, I could conclude that 4 weeks long cGMP cascade activation by sGC stimulation affected hearing of neither Young nor Elderly Wistar rats.

6 months long cGMP cascade activation did not cause changes in the auditory system of the rat

Questioning whether long-term cGMP cascade activation affects hearing of Elderly (8 – 10 months) Wistar rats, hearing tests were performed 6 months after beginning of the treatment with sGC stimulator. ABRs thresholds on click auditory stimuli (click-ABR, Fig. 36A) did not reveal differences (1-sided Student's *t*-test: n.s.) between Elderly Wistar rats

treated with regular diet (*n* = 10/5 ears/rats) and Elderly sGC stimulator treated Wistar rats (*n* = 14/7 ears/rats). Frequency-specific ABR (f-ABR, Fig. 36B) revealed no significant hearing loss in Elderly sGC stimulator treated Wistar rats (two-way ANOVA: n.s.). Maximum DPOAE amplitudes were not significantly different between Elderly Wistar rats treated with normal food and Elderly sGC stimulator treated Wistar rats (Fig. 36C, Elderly treated with normal food: *n* = 10/5 ears/rats; Elderly treated with sGC stimulator: *n* = 12/6 ears/rats, 1-sided Student's *t*-test: n.s.). No differences in DPOAE thresholds of Elderly treated with normal food Wistar rats were observed in comparison with Elderly sGC treated Wistar rats (Fig. 36D; two-way ANOVA: n.s.).

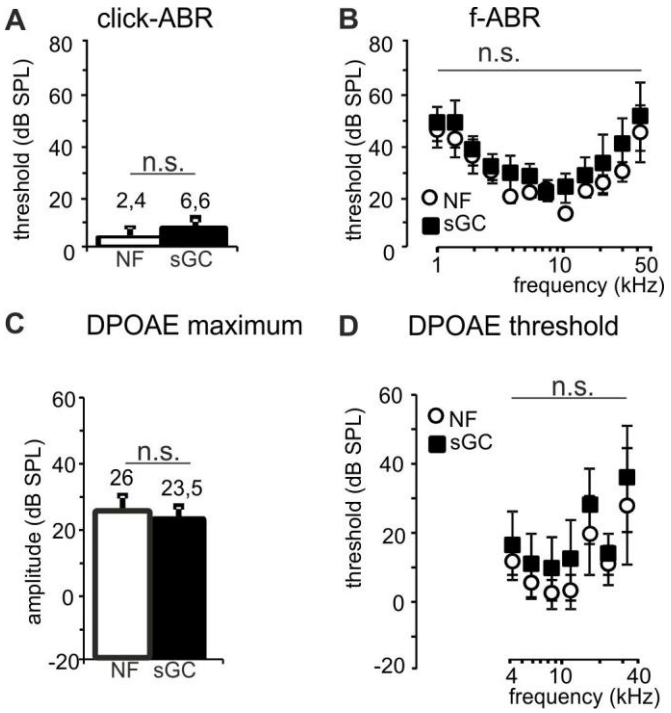

Figure 36. Hearing function of elderly Wistar rats treated with sGC simulator for 6 months in comparison with untreated Wistar rats of the same age: ABRs and DPOAEs

A: ABR thresholds (click-ABR) presented as mean±SD for Elderly treated with normal food Wistar rats (NF: white bar) and Elderly treated with sGC stimulator Wistar rats (sGC: black bar). **B:** Frequency specific pure tone thresholds (mean±SD) for Elderly treated with normal food Wistar rats (white circles) and Elderly sGC stimulator treated Wistar rats (black squares). **C:** DPOAE maximum amplitude at f2 = 8–22 kHz of Elderly treated with normal food Wistar rats (white bar) and Elderly sGC stimulator treated Wistar rats (black bar). **D:** DPOAE thresholds (dB SPL f1) of Elderly treated with normal food Wistar rats (white circles) and Elderly sGC stimulator treated Wistar rats (black squares).

To find out whether long-term cGMP cascade activation affects hearing of Old Wistar rats, the hearing of Old (21 – 26 months old) Wistar rats were tested 6 months after beginning of the treatment with sGC stimulator. ABRs thresholds on click auditory stimuli (click-ABR, Fig. 37A; 1-sided Student's *t*-test: n.s.) were not different between Old normal food treated Wistar rats (*n* = 6/3 ears/rats) and Old sGC stimulator treated Wistar rats (*n* = 12/6 ears/rats).

Frequency-specific ABR (f-ABR, Fig. 37B) revealed no difference in hearing thresholds of Old sGC stimulator treated Wistar rats in comparison with Old Wistar rats treated with normal food (Fig. 37B; two-way ANOVA: n.s.).

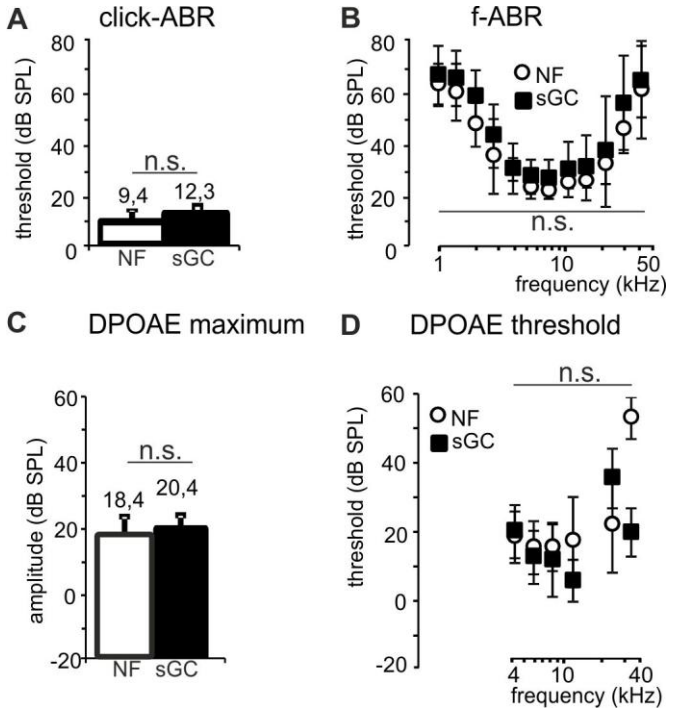

Figure 37. Hearing function of old Wistar rats treated with sGC simulator for 6 months in comparison with untreated Wistar rats of the same age: ABRs and DPOAEs

A: ABR thresholds (click-ABR) presented as mean±SD for Old treated with normal food Wistar rats (NF: white bar) and Old treated with sGC stimulator Wistar rats (sGC: black bar). **B:** Frequency specific pure tone thresholds (mean±SD) for Old treated with NF Wistar rats (white circles) and Old treated with sGC stimulator Wistar rats (black squares). **C:** DPOAE maximum amplitude at f2 = 8–22 kHz of Old treated with normal food Wistar rats (white bar) and Old treated with sGC stimulator Wistar rats (black bar). **D:** DPOAE thresholds (dB SPL f1) of Old treated with normal food Wistar rats (white circles) and Old treated with sGC stimulator Wistar rats (black squares).

Maximum DPOAE amplitudes were not different between Old Wistar rats treated with normal food and Old sGC stimulator treated Wistar rats (Fig. 37C; *t*-test: n.s.; Old normal food treated Wistar rats: n = 6/3 ears/rats; Old sGC stimulator treated Wistar rats: n = 8/4 ears/rats). Also no significant differences in DPOAE thresholds of old Wistar rats treated with regular diet can be observed in comparison with Old sGC stimulator treated (Fig. 37D; two-way ANOVA with Bonferroni multiple comparison test: n.s.).

Therefore we can conclude that administering sGC stimulator itself did not affect the hearing function neither in 4 weeks nor in 6 months after the start of the treatment.

Effects of cGMP cascade activation on age-related processes in auditory system of gerbils

In order to find out whether 4 months long cGMP cascade activation affects hearing of young and elderly gerbils kept in normal environment, the hearing of Young (10 – 13 months old) and Elderly (23 – 29 months old) gerbils were tested 4 months after beginning of the treatment with sGC stimulator .

6 months long cGMP cascade activation did not cause alterations in the auditory system of the young gerbils

ABRs thresholds on click auditory stimuli (click-ABR, Fig. 38A) were not significantly different (1-sided Student's *t*-test: n.s.) between Young normal food treated Mongolian gerbils kept in normal environment (n = 10/5 ears/gerbils) and Young sGC stimulator treated Mongolian gerbils kept in normal environment (n = 10/5 ears/gerbils). Frequency-specific ABR (f-ABR, Fig. 38B) revealed significant hearing loss in Young sGC stimulator treated gerbils at 32 kHz with no differences in other frequencies ($P < 0{,}0001$ by two-way ANOVA with Bonferroni multiple comparison test; other frequencies: n.s. by two-way ANOVA).

Maximum DPOAE amplitudes were not significantly different between Young normal food treated gerbils and Young sGC treated gerbils (Fig. 38C, Young treated with normal food: n = 8/5 ears/gerbils; Young treated with sGC stimulator: n = 9/5 ears/gerbils, 1-sided Student's *t*-test: n.s.). No differences in DPOAE thresholds of Young normal food treated gerbils kept in normal environment were observed in comparison with Young sGC stimulator treated gerbils kept under the same conditions (Fig. 38D; two-way ANOVA: n.s.).

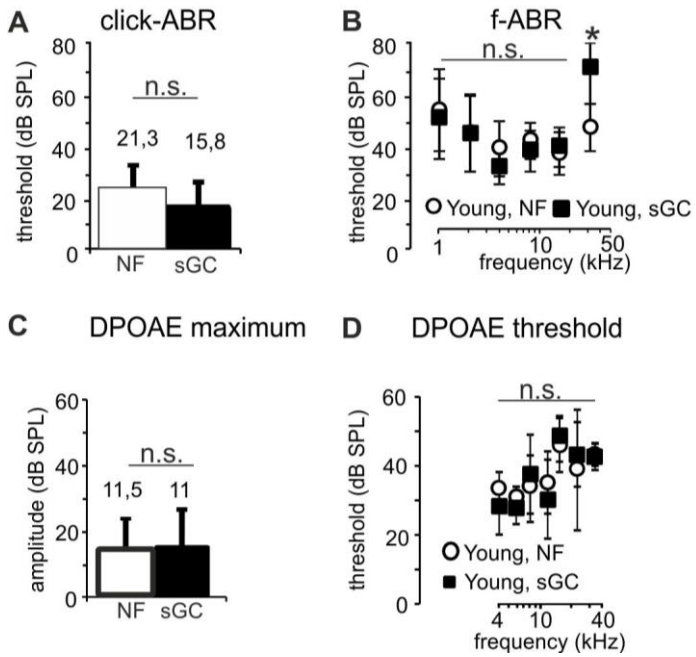

Figure 38. Hearing function of young gerbils treated with sGC simulator in comparison with untreated gerbils of the same age: ABRs and DPOAEs

A: ABR thresholds (click-ABR) presented as mean±SD for Young normal food treated gerbils (NF: white bar) and Young sGC stimulator treated gerbils (sGC: black bar). **B:** Frequency specific pure tone thresholds (mean±SD) for Young normal food treated gerbils (white circles) and Young sGC stimulator treated gerbils (black squares) kept in normal environment. **C:** DPOAE maximum amplitude at f2 = 4,8–5,8 kHz of Young normal food treated gerbils (white bar) and young sGC stimulator treated gerbils (black bar) kept in normal environment. **D:** DPOAE thresholds (dB SPL f1) of Young normal food treated gerbils (white circles) and Young sGC stimulator treated gerbils (black squares) kept in normal environment.

Therefore we could observe a deficit in high-frequency hearing of Young Mongolian gerbils after 4 months treatment with sGC stimulator.

Activation of cGMP cascade causes a hearing loss in elderly gerbils

Investigating effects of cGMP cascade activation on hearing in Elderly gerbils (23 – 29 months old) kept in normal environment, hearing of animals was tested 4 months after beginning of the treatment with sGC stimulator.

ABR thresholds on click auditory stimuli (click-ABR, Fig. 39A) were significantly different (1-sided Student's *t*-test: $p = 0{,}011$) between Elderly normal food treated gerbils kept in normal environment ($n = 4/2$ ears/gerbils) and Elderly sGC stimulator treated gerbils kept in normal environment ($n = 6/3$ ears/gerbils). Frequency-specific ABR (f-ABR, Fig. 39B) revealed an increase in hearing thresholds of Elderly sGC stimulator treated gerbils in comparison with Elderly normal food treated gerbils that was significant at low frequencies (Fig. 39B; P <0,0001 by two-way ANOVA with Bonferroni multiple comparison test at 1 – 2 kHz; other measured frequencies: n.s. by two-way ANOVA).

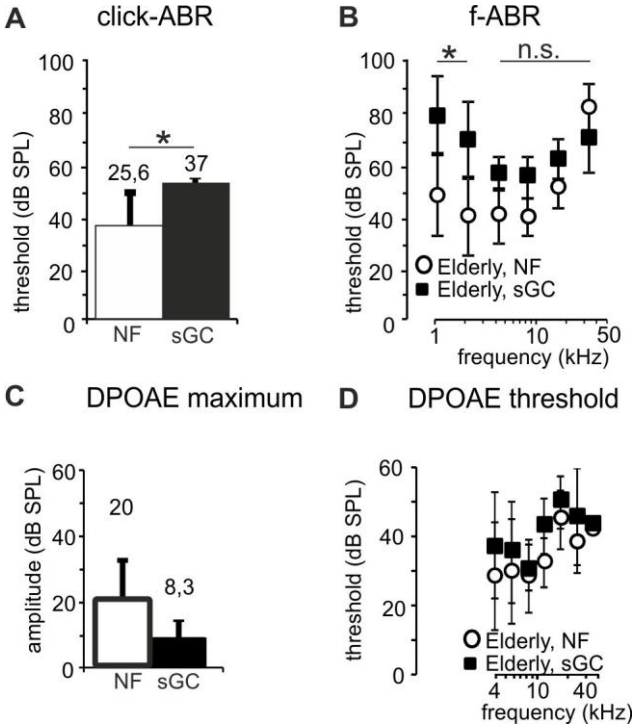

Figure 39. Hearing function of elderly gerbils treated with sGC simulator in comparison with untreated gerbils of the same age: ABRs and DPOAEs

A: ABR thresholds (click-ABR) presented as mean±SD for Elderly normal food treated gerbils (NF: white bar) and Elderly sGC stimulator treated gerbils (sGC: black bar). **B:** Frequency specific pure tone thresholds (mean±SD) for Elderly normal food treated gerbils (white circles) and Elderly sGC stimulator treated gerbils (black squares) kept in normal environment. **C:** DPOAE maximum amplitude at f2 = 4,8–5,8 kHz of Elderly normal food treated (white bar) and Elderly sGC stimulator treated (black bar) gerbils kept in normal environment. **D:** DPOAE thresholds (dB SPL f1) of Elderly normal food treated gerbils (white circles) and Elderly sGC stimulator treated gerbils (black squares) kept in normal environment.

Maximum DPOAE amplitudes were not significantly different between Elderly normal food treated gerbils and Elderly sGC stimulator treated gerbils (both groups kept in normal environment; Fig. 39C, Elderly gerbils treated with normal food: n = 2/2 ears/gerbils; Elderly gerbils treated with sGC stimulator: n = 4/2 ears/gerbils). Here and further no statistical analysis is performed, when n < 3. No differences in DPOAE thresholds of Elderly treated with normal food gerbils kept in normal environment were observed in comparison with Elderly treated with sGC stimulator gerbils kept under the same conditions (Fig. 39D).

Therefore we can conclude that administering sGC stimulator during 4 months period did not affect hearing in Young gerbils, while low frequency hearing loss was observed in Elderly gerbils after the same time of the treatment with sGC stimulator.

Effects of cGMP cascade activation and environmental enrichment on the hearing of young and elderly gerbils

Trying to investigate the effect of cGMP cascade activation by administering sGC stimulator in Young (10 – 13 months old) and Elderly (23 – 29 months old) gerbils kept in enriched environment, the hearing function was tested 4 months after beginning of the treatment.

ABR thresholds on click auditory stimuli (click-ABR, Fig. 40A) were significantly different (1-sided Student's *t*-test: $p < 0{,}0001$) between Young gerbils kept in normal environment and treated with sGC stimulator (n = 10/5 ears/gerbils) and Elderly gerbils kept in normal environment and treated with sGC stimulator (n = 6/3 ears/gerbils). ABR thresholds on click auditory stimuli (click-ABR, Fig. 40A) were not different (1-sided Student's *t*-test: n.s.) between Young gerbils kept in enriched environment and treated with sGC stimulator (n = 8/4 ears/gerbils) and Elderly gerbils kept in normal environment and treated with sGC stimulator (n = 4/2 ears/gerbils). Frequency-specific ABR (f-ABR, Fig. 40B) revealed an increase in hearing thresholds of Elderly gerbils kept in normal environment and treated with sGC stimulator in comparison with Young gerbils kept in normal environment and treated with sGC stimulator that was significant (Fig. 40B; $P < 0{,}0001$ by two-way ANOVA with Bonferroni multiple comparison test at 1 – 16 kHz; other measured frequencies: n.s. by two-way ANOVA). No difference between frequency-specific ABRs of Young gerbils kept in enriched environment and treated with sGC stimulator containing food and Elderly gerbils kept in enriched environment and treated with sGC stimulator containing food was found (Fig. 40C; two-way ANOVA: n.s.).

Maximum DPOAE amplitudes were not significantly different between Young sGC stimulator treated gerbils kept in normal environment and Elderly sGC stimulator treated gerbils kept in normal environment (Fig. 41A, Young sGC stimulator treated gerbils kept in normal environment, n = 9/5 ears/gerbils; Elderly sGC stimulator treated gerbils kept in normal environment: n = 4/2 ears/gerbils, 1-sided Student's *t*-test: n.s.). Maximum DPOAE amplitudes of Young sGC stimulator treated gerbils kept in enriched environment were lower than those of Elderly sGC stimulator treated gerbils kept in enriched environment (Fig. 41A, Young sGC stimulator treated gerbils kept in enriched environment: n = 6/3 ears/gerbils; Elderly sGC stimulator treated gerbils kept in enriched environment: n = 2/1 ears/gerbil).

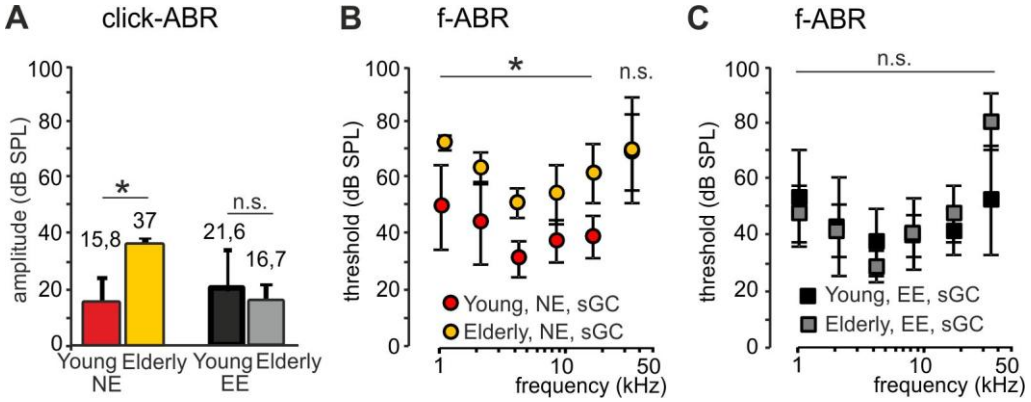

Figure 40. Hearing function of Young and Elderly gerbils kept in normal and enriched environment and treated with sGC simulator: ABRs

A: ABR thresholds (click-ABR) presented as mean±SD for Young gerbils kept in normal environment (red bar) and in enriched environment (black bar) and Elderly animals kept in normal environment (yellow bar) and in enriched environment (grey bar). All groups were treated with sGC stimulator. **B:** Frequency specific pure tone thresholds (mean±SD) for Young gerbils (red circles) and Elderly gerbils (yellow circles) treated with sGC stimulator and kept in normal environment. **C:** Frequency specific pure tone thresholds (mean±SD) for Young gerbils (black squares) and Elderly gerbils (grey squares) treated with sGC stimulator and kept in enriched environment.

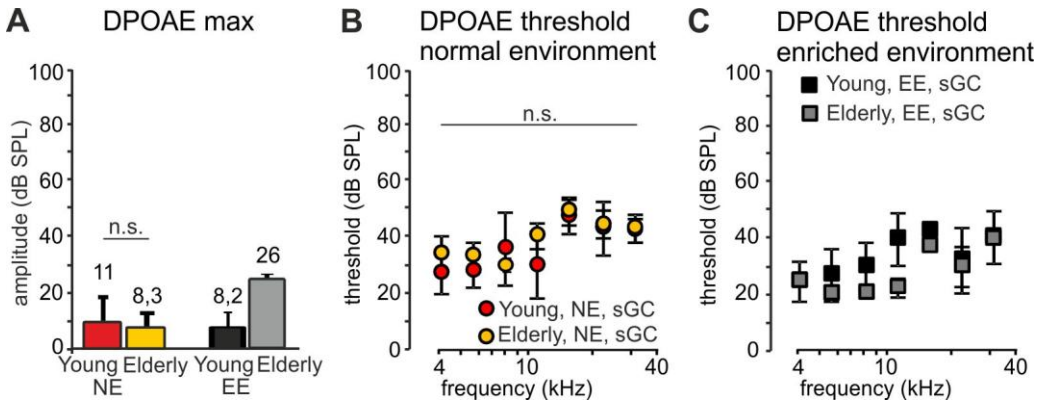

Figure 41. Hearing function of Young and Elderly gerbils kept in normal and enriched environment and treated with sGC simulator: DPOAEs

A: DPOAE maximum amplitude at f2 = 4,8–5,8 kHz of Young animals kept in normal environment (red bar) and in enriched environment (black bar) and Elderly animals kept in normal environment (yellow bar) and in enriched environment (grey bar). All groups were treated with sGC stimulator (sGC). **B:** DPOAE thresholds (dB SPL f1) of Young gerbils kept in normal environment and treated with sGC stimulator (red circles) and Elderly gerbils kept in normal environment and treated with sGC stimulator (yellow circles). **C:** DPOAE thresholds (dB SPL f1) of Young gerbils (black squares) and Elderly gerbils (grey squares) treated with sGC stimulator and kept in enriched environment.

No differences in DPOAE thresholds of Young gerbils kept in normal environment and treated with sGC stimulator containing substance were observed in comparison with Elderly gerbils treated with sGC stimulator kept under the same conditions (Fig. 41B; two-way ANOVA: n.s.). Also a difference in DPOAE thresholds of Young gerbils kept in enriched environment and treated with sGC stimulator in comparison with Elderly gerbils kept in enriched environment and treated with sGC stimulator could not be found (Fig. 41C).

Questioning whether keeping gerbils in enriched environment in combination with sGC stimulation affects the hearing range of Young (10 – 13 months old) Mongolian gerbils, hearing function was monitored after 6 months of keeping animals in enriched environment and treatment with sGC stimulator.

ABR thresholds on click auditory stimuli (click-ABR, Fig. 35A) were not significantly different (*t*-test: n.s.) between Young gerbils kept in normal environment (*n* = 10/5 ears/gerbils) and Young gerbils kept in enriched environment (*n* = 10/5 ears/gerbils). Both groups were treated with sGC stimulator. Frequency-specific ABR (f-ABR, Fig. 42B) revealed no significant difference in hearing between groups (Fig. 42B; two-way ANOVA: n.s.).

Maximum DPOAE amplitudes were not significantly different for Young gerbils kept in normal environment and treated with sGC stimulator containing food, and Young gerbils kept in enriched environment and treated with sGC stimulator (Fig. 42C, Young gerbils kept in normal environment: *n* = 9/5 ears/gerbils; Young gerbils kept in enriched environment: *n* = 6/3 ears/gerbils, 1-sided Student's *t*-test: n.s.). No differences in DPOAE thresholds of Young gerbils kept in normal environment and treated with sGC stimulator were observed in comparison with Young gerbils kept in enriched environment and treated with sGC stimulator (Fig. 42D; two-way ANOVA: n.s.).

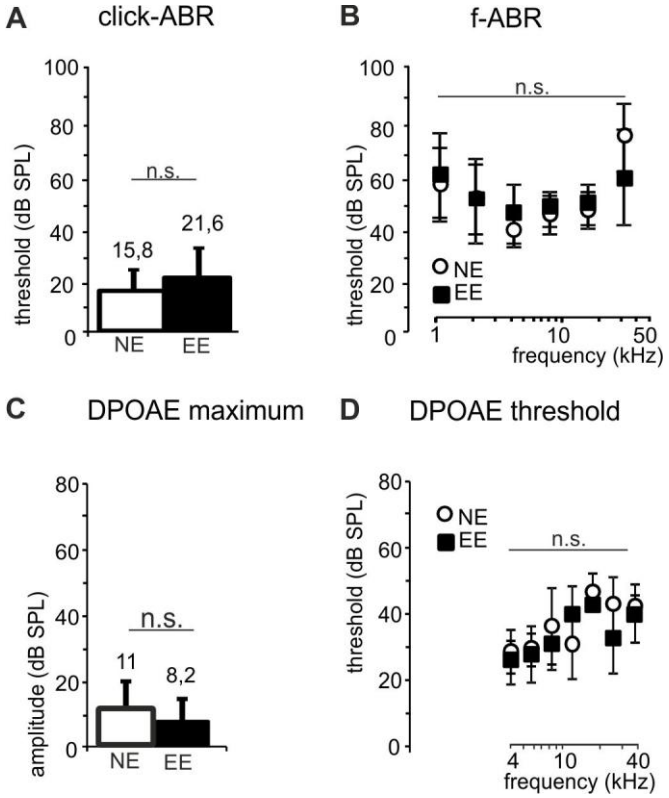

Figure 42. Hearing function of Young gerbils kept in normal environment in comparison with gerbils of the same age kept in enriched environment: ABRs and DPOAEs

A: ABR thresholds (click-ABR) presented as mean±SD for Young gerbils kept in normal environment (NE: white bar) and same age gerbils kept in enriched environment (EE: black bar). **B:** Frequency specific pure tone thresholds (mean±SD) for Young gerbils kept in NE (white circles) and same age gerbils kept in EE (black squares). **C:** DPOAE maximum amplitude at f2 = 4,8–5,8 kHz of Young gerbils kept in NE (white bar) and Young gerbils kept in EE (black bar). **D:** DPOAE thresholds (dB SPL f1) of Young gerbils kept in NE (white circles) and Young gerbils kept in EE (black squares).

Trying to find out whether the effect is different in Elderly (23 – 29 months old) gerbils, hearing function was monitored after 6 months of keeping animals to enriched environment, and treatment with sGC stimulator containing food.

ABR thresholds on click auditory stimuli (click-ABR, Fig. 43A) were significantly different (1-sided Student's *t*-test: $p < 0{,}0001$) between Elderly gerbils kept in normal environment (n = 4/2 ears/gerbils) and Elderly gerbils kept in enriched environment (n = 6/3 ears/gerbils). Both groups were treated with sGC stimulator containing food. Frequency-specific ABR (f-ABR, Fig. 43B) revealed a significant decrease in hearing thresholds in Elderly gerbils kept in enriched environment in comparison with age-mates kept in normal environment (Fig. 43B; P <0,0001 by two-way ANOVA with Bonferroni multiple comparison test at 1 – 16 kHz: other measured frequencies: n.s. by two-way ANOVA). Maximum DPOAE amplitudes were different between Elderly gerbils kept in normal environment and Elderly gerbils kept in enriched environment (Fig. 43C, Elderly gerbils kept

in normal environment: *n* = 2/1 ears/gerbils; Elderly gerbils kept in enriched environment: *n* = 4/2 ears/gerbils). A trend to better DPOAE thresholds of Elderly gerbils kept in enriched environment was observed in comparison with Elderly gerbils kept in normal environment (Fig. 43D).

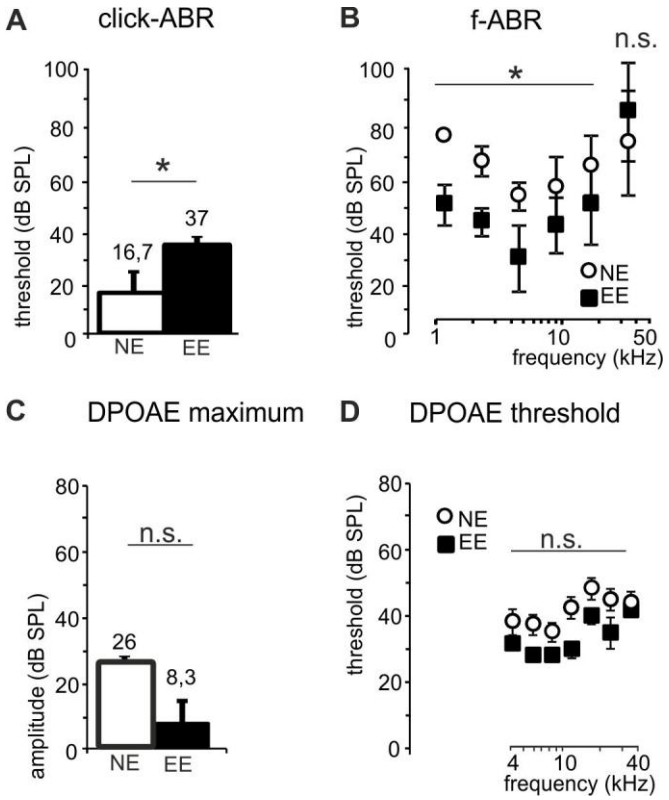

Figure 43. Hearing function of elderly gerbils kept in normal environment and treated with sGC stimulator containing food in comparison with age-matched gerbils kept in enriched environment and treated with sGC stimulator containing food: ABRs and DPOAEs

A: ABR thresholds (click-ABR) presented as mean±SD for Elderly gerbils kept in normal environment (NE: white bar) and gerbils of the same age kept in enriched environment (EE: black bar). **B:** Frequency specific pure tone thresholds (mean±SD) for Elderly gerbils kept in NE (white circles) and gerbils of the same age kept in EE (black squares). **C:** DPOAE maximum amplitude at f2 = 4,8–5,8 kHz of Elderly kept in NE (white bar) and Elderly kept in EE (black bar) gerbils. **D:** DPOAE thresholds (dB SPL f1) of Elderly kept in NE (white circles) and Elderly kept in EE (black squares) gerbils.

Therefore, when combining cGMP cascade activation by cGC stimulating and environmental enrichment, beneficial effect of environmental enrichment on low frequency hearing vanishes. But sGC stimulation does not affect protective effect of environmental enrichment on age-related high-frequency loss.

3.4.2. Effects of cGMP cascade activation in noise exposed animals

Knowing that cGMP cascade stimulation rescues hearing from acute noise-induced hearing loss (Jaumann et al., 2012) we questioned whether the same cascade could also be protective against progressive neurodegeneration caused by mild noise exposure.

Effects of cGMP cascade activation in noise-exposed rats

Short-term (4 weeks) and long-term (6 months) effects of cGMP cascade activation by sGC stimulation on noise exposed Wistar rats and Sham exposed Wistar rats as a control were tested.

Effects of 4 weeks long cGMP cascade activation in young and elderly noise exposed rats

Investigating if administering the sGC-stimulator after noise exposure to Young (2 – 3 months old) and Elderly (8 – 10 months) Wistar rats would exhibit differences in hearing threshold recovery, rats were acoustically stimulated and hearing function was measured as described above (see 2.2. and 2.3.; Fig. 44A). In 4 weeks morphological analysis was performed to characterize the level of neurodegeneration caused by mild noise exposure.

Vulnerability of hearing function of young and elderly sGC stimulator treated for 4 weeks rats for noise exposure

Noise exposure led to a hearing loss of approximately 15 dB SPL in both Young and Elderly group of Wistar rats treated with the sGC-stimulator containing food for 25 days (Fig. 44B). 28 days after noise exposure, hearing recovered completely, indicating no noise induced PTS. 1 day after exposure the TTS was already much less pronounced in both groups verified through click-ABR. 3 days after exposure hearing function of both age groups recovered completely and did not exhibit further changes until the day 28 (Fig. 44B). The frequency-specific ABR thresholds showed no hearing impairment neither in Young nor in Elderly Wistar rats in any frequency 28 days after exposure (Fig. 44C; two-way ANOVA with Bonferroni multiple comparison test: n.s.), though a trend to the elevation of thresholds could be observed in Elderly sGC stimulator treated rats.

The function of OHC was analyzed in Young and Elderly Wistar rats by measuring the DPOAE (Fig. 45) before and 28 days after noise exposure. OHC function in Young and Elderly noise exposed rats in respect to maximal amplitudes of DPOAE showed no reduction (Fig. 45A; Young: yellow, n = 12/6 ears/rats; Elderly: grey, n = 14/7 ears/rats, 1-sided Student's t-test: n.s) when compared to their unexposed age-mates (Fig. 45A; Young: red, n = 12/6 ears/rats; Elderly: black, n = 10/5 ears/rats, 1-sided Student's t-test: n.s). DPOAE thresholds of Young animals before and after exposure revealed no differences between groups (Fig. 45B; two-way ANOVA: n.s.). DPOAE thresholds of noise exposed (grey squares) Elderly rats revealed a tendency to the elevation of DPOAE thresholds in comparison

to unexposed (black squares) animals in middle frequencies (8 kHz – 16 kHz), that was not significant (Fig. 45C; two-way ANOVA: n.s.).

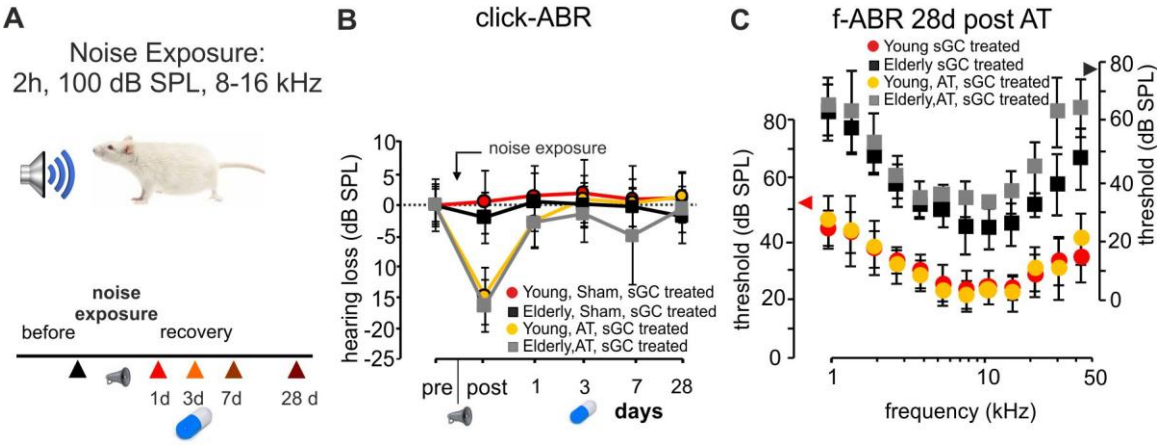

Figure 44. Hearing vulnerability of Young and Elderly Wistar rats treated with sGC stimulator containing food for 25 days after noise exposure

A: Schematic illustration for the noise exposure paradigm chosen to test the vulnerability and recovery of Young compared with Elderly Wistar rats. **B:** After noise exposure Young Wistar rats exhibited a noise-induced hearing loss of approximately 15 dB for click stimuli, similar to the loss of Elderly Wistar rats. The recovery from noise-induced hearing loss was complete after 3 days with no permanent threshold shift (PTS); after this time period treatment with sGC-stimulator was started. The recovery was similar in Young and Elderly Wistar rats. **C:** At day 28 the f-ABR thresholds of Elderly noise exposed animals, especially in the middle and high frequencies, were slightly elevated, but this difference was not significantly different. For better representation of the data y-axis and the graph for aged group is shifted up. Young rats: unexposed *n* = 12/6 ears/rats, noise exposed *n* = 12/6 ears/rats; aged rats: unexposed *n* = 10/5 ears/rats, noise exposed *n* = 14/7 ears/rats.

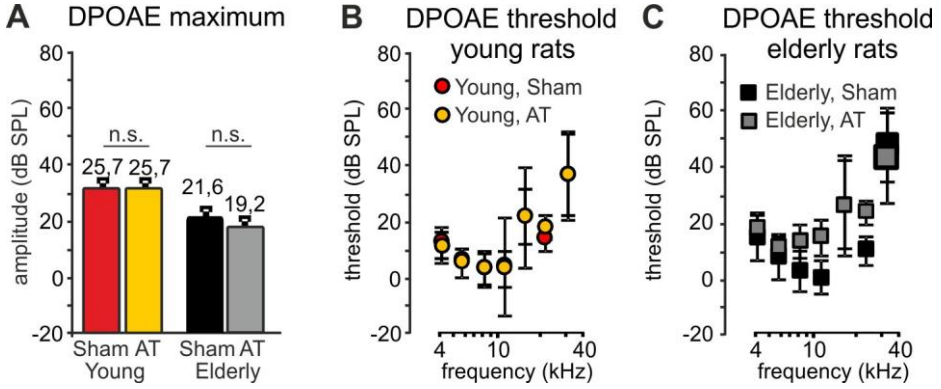

Figure 45. OHC function in Young and Elderly Wistar rats, treated with sGC-stimulating diet for 25 days, 28 days after exposure

A: Amplitudes of distortion products of otoacoustic emissions (DPOAE) of unexposed (Sham) and exposed to noise (AT) Young (Sham: red, AT: orange) and Elderly (Sham: black, AT: grey) Wistar rats. Amplitudes were not significantly reduced in both age groups after noise exposure compared to control unexposed age-matched rats. **B:** DPOAE thresholds of unexposed (red circles) and noise exposed (orange circles) Young rats revealed no reduction of DPOAE thresholds in Young noise exposed animals over the whole frequency range. **C:** DPOAE thresholds of unexposed (black squares) and noise exposed (grey squares) Elderly rats revealed a tendency to the elevation of DPOAE thresholds in Elderly noise exposed animals in middle frequencies (8 kHz – 16 kHz), that was not significantly different. Young rats: unexposed *n* = 12/6 ears/rats, noise exposed *n* = 12/6 ears/rats; elderly rats: unexposed *n* = 10/5 ears/rats, noise exposed *n* = 14/7 ears/rats.

In summary, administering of sGC stimulator to Young noise exposed rats does not affect hearing function, while Elderly animals exhibit a trend to the high frequency loss in frequency ABR measurement. A DPOAE threshold increase could also be observed in the middle frequencies (8 kHz – 16 kHz). This does not correlate with the effect of noise itself (see 3.2.1.1.1.), which mean that this might be an effect of sGC stimulator.

4 weeks long activation of cGMP cascade rescues synaptopathy

Immunohistochemically the expression and distribution of CtBP2 as marker for IHC synaptic ribbons was analyzed in IHCs of Young (2 – 3 months old; Fig. 46) and Elderly (8 – 10 months old; Fig. 48) noise exposed and unexposed Wistar rats (Fig. 46, Fig. 48) sGC-stimulator treated. Otoferlin staining (red) in the midbasal (Fig. 46A-B, Fig. 48A-B) and basal (Fig. 46C-D, Fig. 48C-D) turns of the cochlea of sGC-stimulator treated Young unexposed (Fig. 46A, C) and noise exposed (Fig. 48B, D), and Elderly unexposed (Fig. 46A, C) and noise exposed (Fig. 48B, D) Wistar rats highlights IHC position. By visual inspection no reduction of CtBP2-positive dots (green) could be seen in the midbasal turn as well as in the basal turn of the sGC stimulator treated Young noised exposed Wistar rat cochlea (Fig. 46B, D) as compared to the midbasal and basal turns of the Young unexposed sGC treated Wistar rat cochlea (Fig. 46A, C). Same tendency could be observed in sGC stimulator treated Elderly animals in midbasal (Fig. 48C-D), but not in basal (Fig. 48A-B) cochlear turns.

CtBP2-positive points in single IHCs were counted in Young unexposed Wistar rats treated with sGC simulator Wistar rats in comparison to Young noise exposed Wistar rats treated with sGC stimulator. Reduction of ribbons was detected in the midbasal, but not basal cochlear turn (Fig. 47B). No significant differences in the number of ribbons between Young unexposed and Young noise exposed Wistar rats were noted in the apical turn (Fig. 47B). IHC ribbon synapses number was not reduced in the basal and was reduced only by 15% in the midbasal cochlear turn of noise exposed young Wistar rats treated with sGC stimulator for 25 days in comparison with unexposed sGC stimulator treated Wistar rats.

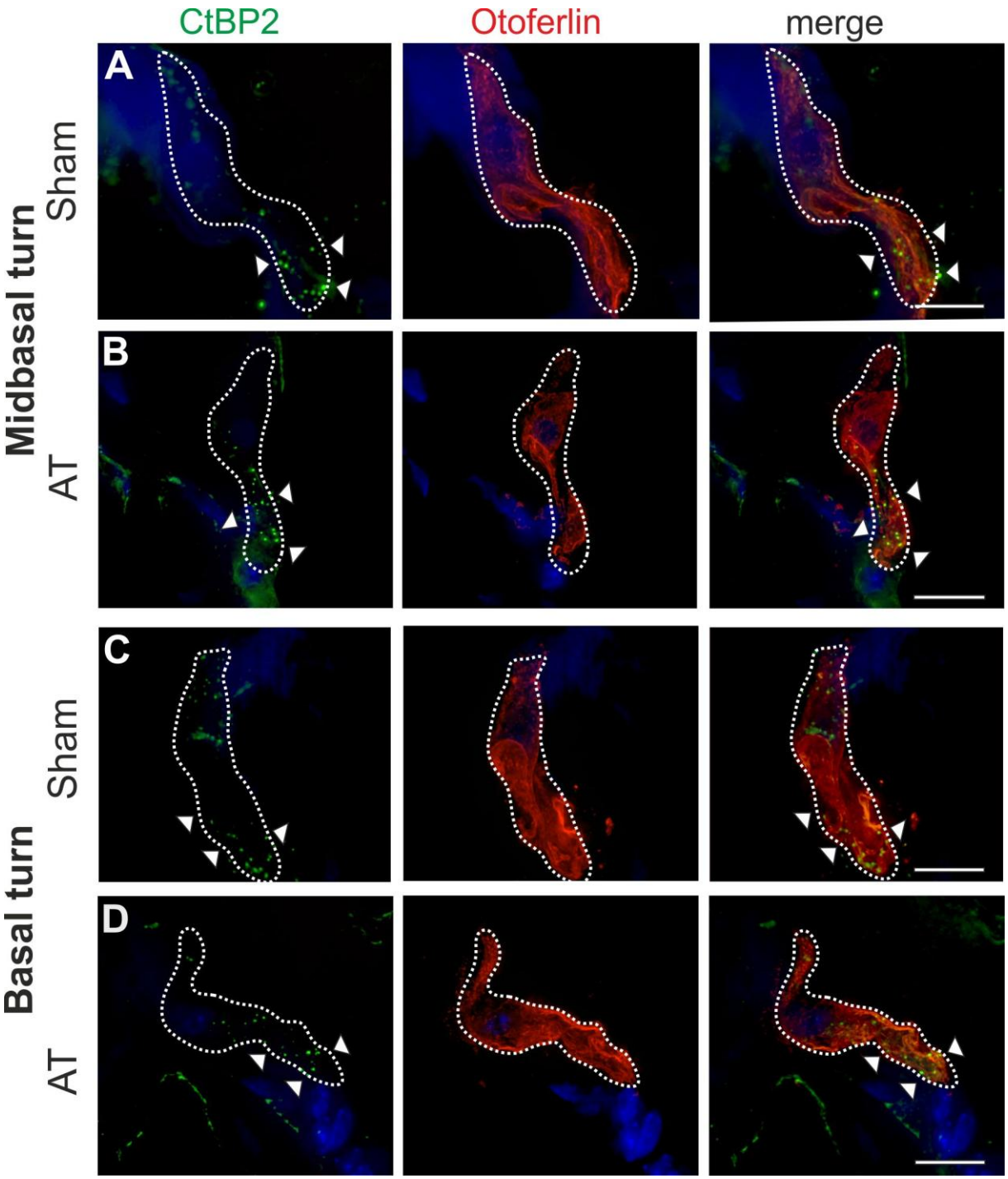

Figure 46. IHCs ribbon synapses were rescued in the basal, but not midbasal turns of the young noise exposed rats cochlea by treatment with sGC stimulator

The recovery of the synaptic contacts in the IHC of young noise exposed rats could be observed after 25 days treatment with sGC stimulator. Images of the IHC of midbasal (A, B) and basal (C, D) turns of unexposed (Sham: A, C) and noise-exposed (AT: B, D) and treated with sGC stimulator for 25 days animals immunostained for synaptic ribbons (CtBP2, green, marked with arrowheads) and IHC (otoferlin, red, encircled with dotted line). Scale bars = 10 μm. Nuclear marker: DAPI (blue).

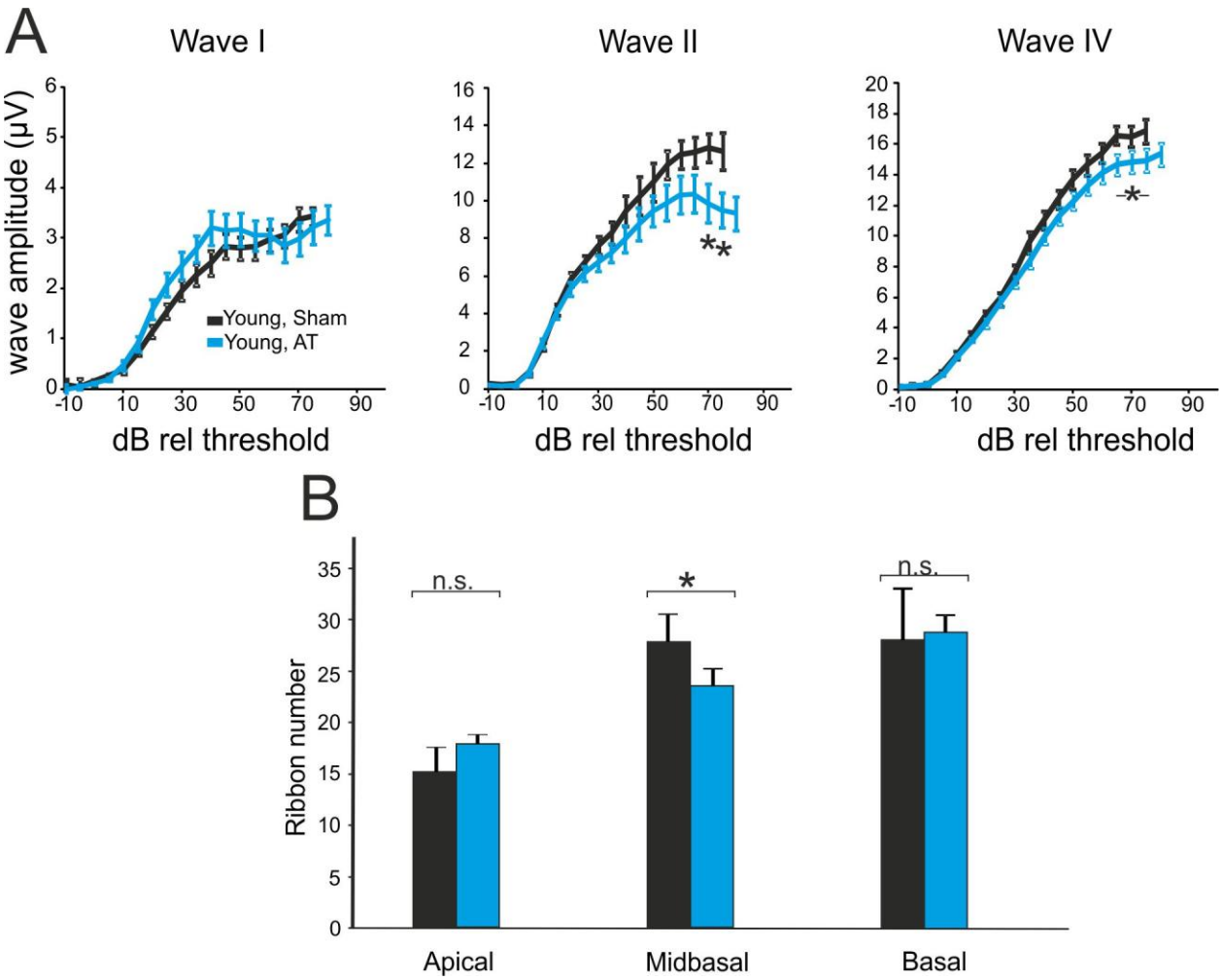

Figure 47. Less reduction of the IHC ribbon synapses of young noise exposed sGC stimulator treated rats correlated with no loss of the ABR wave I amplitude

A: Mean ± SEM click-evoked ABR wave I, wave II and wave IV amplitudes growth function for Young unexposed treated with sGC stimulator rats (black line, *n* = 10/5 ears/rats) and Young noise exposed treated with sGC stimulator rats (blue line, *n* = 10/5 ears/rats). **B:** Ribbon counting from Young unexposed (black) and young noise exposed (blue) Wistar rats, expressed in average number per IHC (±SD), in different cochlear turns (*n*=3).

In Young noise exposed sGC stimulator treated Wistar rats wave I amplitude was not reduced in comparison with Young unexposed sGC stimulator treated Wistar rats (Fig. 47A; 1-sided Student's *t*-test: see table 10 for p-values, no Bonferroni-Holms correction). There was a reduction in ABR wave II amplitudes between Young noise exposed sGC stimulator treated Wistar rats in comparison with Young sGC stimulator treated unexposed Wistar rats that was significant at the level of 70 – 75 dB above threshold (Fig. 47A; 1-sided Student's *t*-test: see table 10 for p-values, no Bonferroni-Holms correction). The amplitude of ABR wave IV was also decreased in Young noise exposed sGC stimulator treated Wistar rats starting from 65 dB above threshold up to 75 dB above threshold (Fig. 40A; 1-sided Student's *t*-test: see table 7 for p-values, no Bonferroni-Holms correction). That difference was found significant at 60 dB above threshold and up to 75 dB above threshold as compared to Young

animals (1-sided Student's *t*-test: see table 10 for p-values, no Bonferroni-Holms correction). Noise exposed animals showed no reduction in ABR wave I, while wave II and wave IV amplitudes were reduced at high sound levels compared with Young unexposed Wistar rats.

Table 9. *P*-values of pair wise comparisons of amplitude data in Figure 47

Young Sham vs. Young AT	dB above threshold										
	30	35	40	45	50	55	60	65	70	75	80
Wave I	0,138	0,146	0,125	0,351	0,307	0,4128	0,468	0,3494	0,132	0,2169	0,2257
Wave II	0,132	0,1333	0,136	0,2019	0,195	0,0911	0,0744	0,0783	0,0312 (*)	0,0315 (*)	0,2159
Wave IV	0,211	0,0887	0,098	0,091	0,053	0,0854	0,0863	0,022 (*)	0,05 (*)	0,045 (*)	0,194

1-sided Student's *t*-test: *(*) p<0,05*

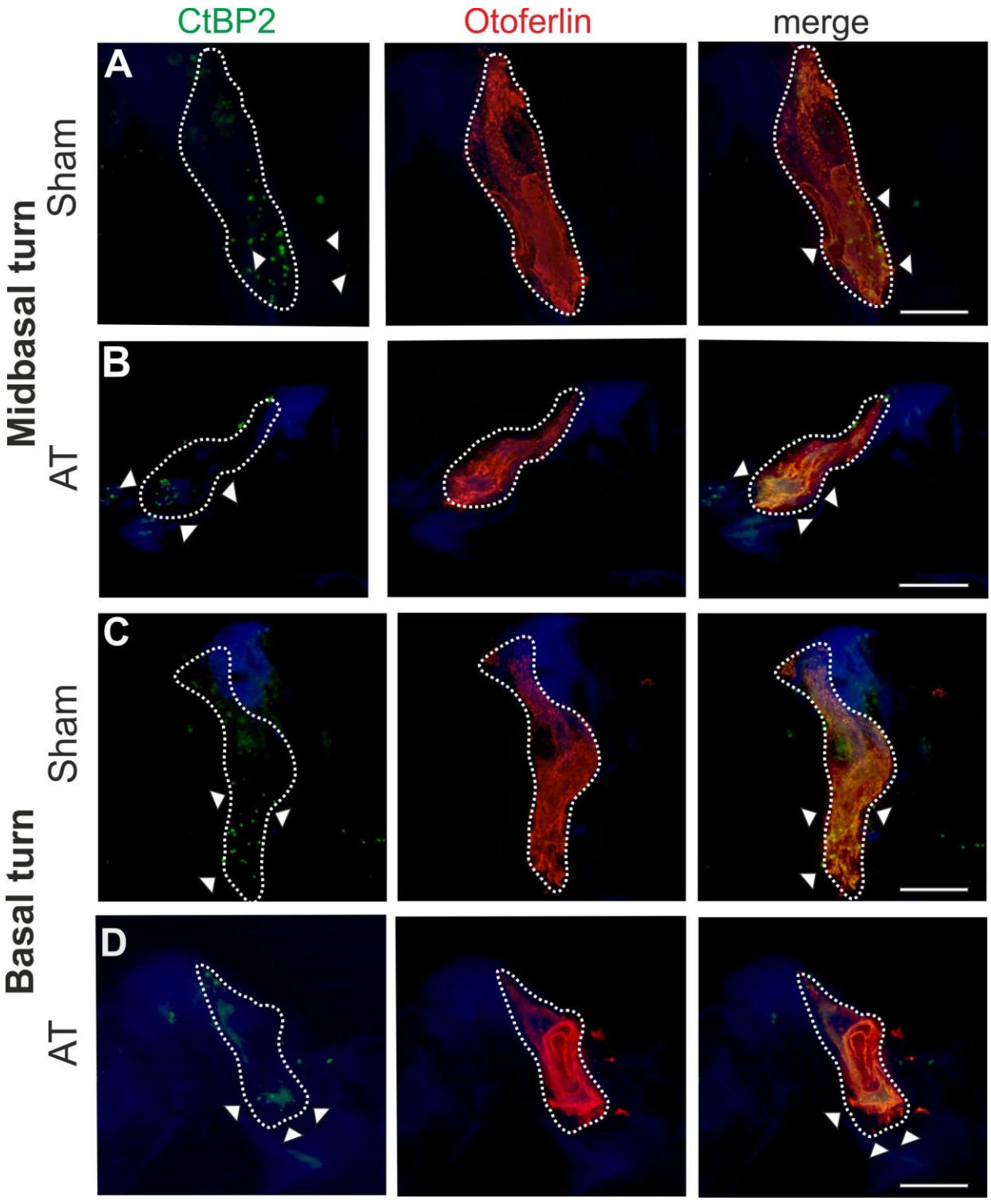

Figure 48. IHCs ribbon synapses could not be completely rescued in basal and midbasal turns of the cochlea of elderly noise exposed sGC stimulator treated Wistar rats

No recovery of the synaptic contacts in the IHC of noise exposed elderly rats could be observed after 25 days treatment with sGC stimulator. Images of the IHC of midbasal (A, B) and basal (C, D) turns of unexposed (Sham: A, C) and noise-exposed (AT: B, D) and treated with sGC stimulator for 25 days animals immunostained for synaptic ribbons (CtBP2, green, marked with arrowheads) and IHC (otoferlin, red, encircled with line). Scale bars = 10 μm. Nuclear marker: DAPI (blue).

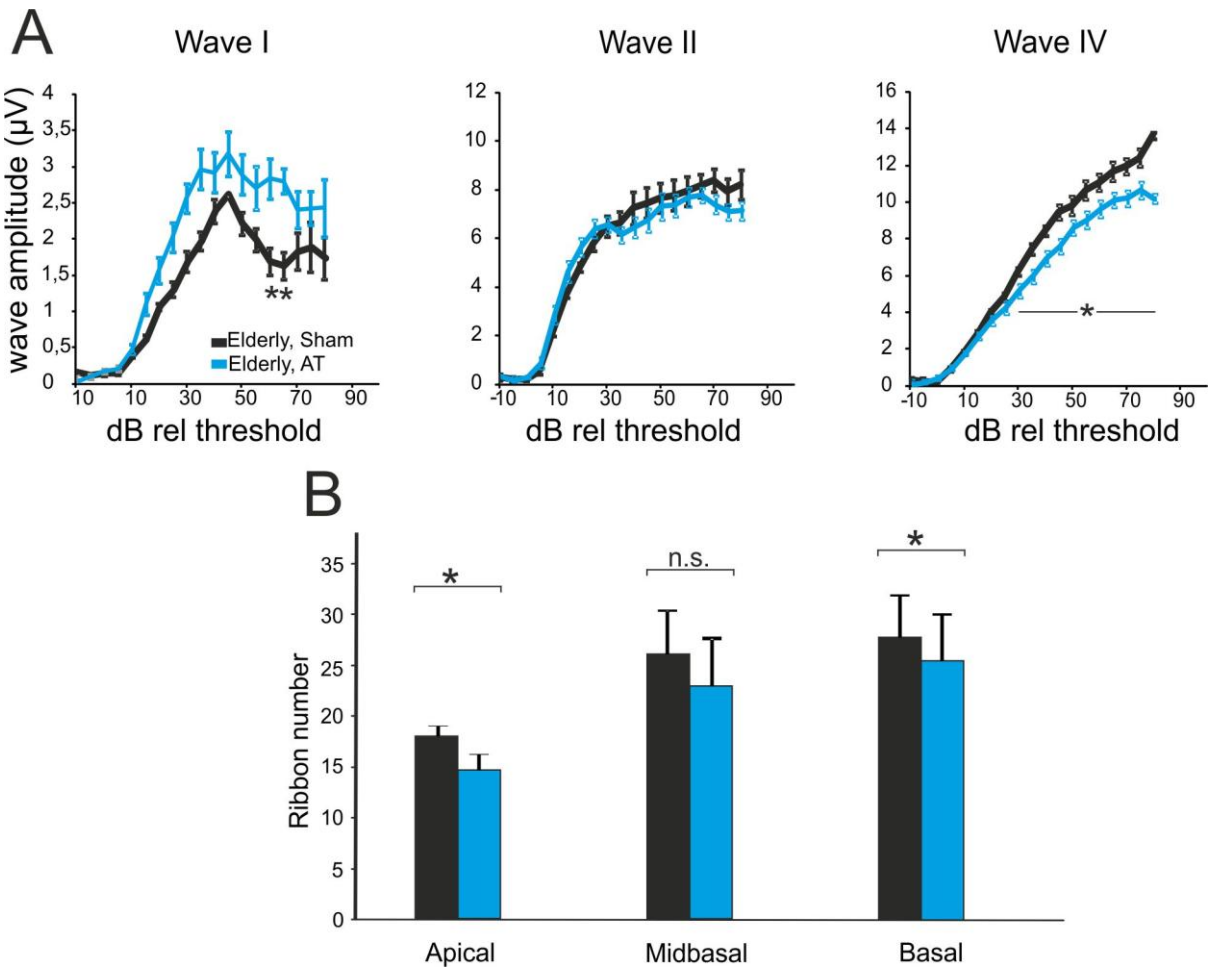

Figure 49. Reduction of the IHCs ribbon synapses could not be saved by sGC stimulator in elderly animals

A: Mean ± SEM click-evoked ABR wave I, wave II and wave IV amplitudes growth function for Elderly unexposed (black line, *n* = 10/5 ears/rats) and Elderly noise exposed (blue line, *n* = 10/5 ears/rats) rats treated with sGC stimulator containing food. **B:** Ribbon counting from Elderly unexposed (black) and Elderly noise exposed (blue) Wistar rats, expressed in average number per IHC (±SD), in different cochlear turns (*n*=3).

CtBP2-positive points in single IHCs were also counted in Elderly unexposed Wistar rats in comparison to Elderly noise exposed Wistar rats (both groups treated with sGC stimulator), and a significant reduction of ribbons became evident in basal turn, but not in midbasal cochlear turn of Elderly noise exposed sGC stimulator treated Wistar rats, where only a tendency could be seen (Fig. 49B). A statistically significant difference in number of ribbons between unexposed and noise exposed Elderly Wistar rats treated with sGC stimulator was also noted in the apical turn (Fig. 49B). IHC ribbon synapses number was reduced by 14% in all cochlear turns of noise exposed Elderly Wistar rats in comparison with unexposed animals.

In Elderly noise exposed sGC stimulator treated Wistar rats ABR wave I amplitudes were increased in comparison with Elderly unexposed sGC stimulator treated Wistar rats (Fig. 49A; 1-sided Student's *t*-test: see table 11 for p-values, no Bonferroni-Holms correction). No difference in ABR wave II amplitudes were observed in Elderly noise exposed sGC stimulator treated Wistar rats in comparison with unexposed sGC stimulator treated Elderly Wistar rats (Fig. 49A; 1-sided Student's *t*-test: see table 11 for p-values, no Bonferroni-Holms correction). The amplitudes of ABR wave IV were decreased in Elderly noise exposed Wistar rats starting from 30 dB above threshold (Fig. 49A). ABR wave IV amplitudes of noise exposed Elderly rats treated with sGC stimulator were decreased in comparison with unexposed Elderly rats treated with sGC stimulator.

Table 10. *P*-values of pair wise comparisons of amplitude data in Figure 42

Elderly Sham vs. Elderly AT	dB above threshold										
	30	35	40	45	50	55	60	65	70	75	80
Wave I	0,303	0,4	0,331	0,113	0,342	0,1814	0,05 (*)	0,013 (*)	0,908	0,239	0,08
Wave II	0,072	0,12	0,158	0,17	0,214	0,2171	0,207	0,144	0,146	0,2512	0,24
Wave IV	$8*10^{-5}$ (**)	$2*10^{-5}$ (**)	$9*10^{-5}$ (**)	0,0009 (**)	0,002 (**)	0,002 (**)	0,004 (*)	0,002 (**)	0,001 (**)	0, 006 (*)	-

1-sided Student's *t*-test: *(*) p<0,05;(**) p<0,005*

Noise exposed animals showed reduced ABR wave I, while wave II was intact, and wave IV amplitude was increased at high sound levels compared with Elderly unexposed Wistar rats.

Summarizing data shown above, neurodegeneration caused by mild noise exposure could be partially rescued in Young, but not in Elderly Wistar rats treated with sGC stimulator.

Effects of 6 months long cGMP cascade activation in young and elderly noise exposed rats

As 4 weeks treatment was shown to have a protective effect on synapthpathy caused by mild noise exposure (see 3.4.2.1.1.), we decided to test whether long-term administering of the sGC-stimulator after noise exposure to Elderly (8 – 10 months old) and Old (21 – 24 months) Wistar rats would have the same effect. Noise exposure and hearing measurements protocol was the same as described above. Treatment with sGC stimulator was started 3 days after noise exposure and continued for 6 months (Fig. 50A).

Vulnerability of hearing function of young and elderly sGC stimulator treated during 6 months Wistar rats for noise exposure

Noise exposure led to a hearing loss of approximately 10 dB in both the Elderly and the Old group of Wistar rats treated with the sGC stimulator for 6 months (Fig. 50B). 6 months after noise exposure, hearing recovered completely, indicating no noise induced PTS. One day after exposure TTS was already completely recovered in both groups verified through click-ABR and stayed unchanged until the last measurement 6 months after exposure (Fig. 50B). The frequency-specific ABR thresholds showed no hearing impairment in Elderly Wistar rats over the whole frequency range, while ABR thresholds had a trend to elevation that was not statistically significant in low and high frequencies in Old Wistar rats 6 months after noise exposure (Fig. 50C).

The function of OHC was analyzed in Elderly and Old Wistar rats by measuring the DPOAE (Fig. 51) before and 6 months after noise exposure. OHC function in Elderly and Old noise exposed rats in respect to maximal amplitudes of DPOAE showed no reduction (Fig. 51A; Elderly: yellow, n = 14/7 ears/rats; Old: grey, n = 2/1 ears/rats, 1-sided Student's t-test: n.s) when compared to their unexposed age rats of the same age (Fig. 51A; young: red, n = 14/7 ears/rats; aged: black, n = 8/4 ears/rats, 1-sided Student's t-test: n.s). DPOAE thresholds of Elderly animals before and after exposure revealed no differences between groups (Fig. 51B; two-way ANOVA: n.s.). DPOAE thresholds of noise exposed Old rats revealed a tendency to the decrease of DPOAE thresholds in comparison to unexposed animals in middle frequencies (8 kHz – 16 kHz), that was not significantly different (two-way ANOVA: n.s.).

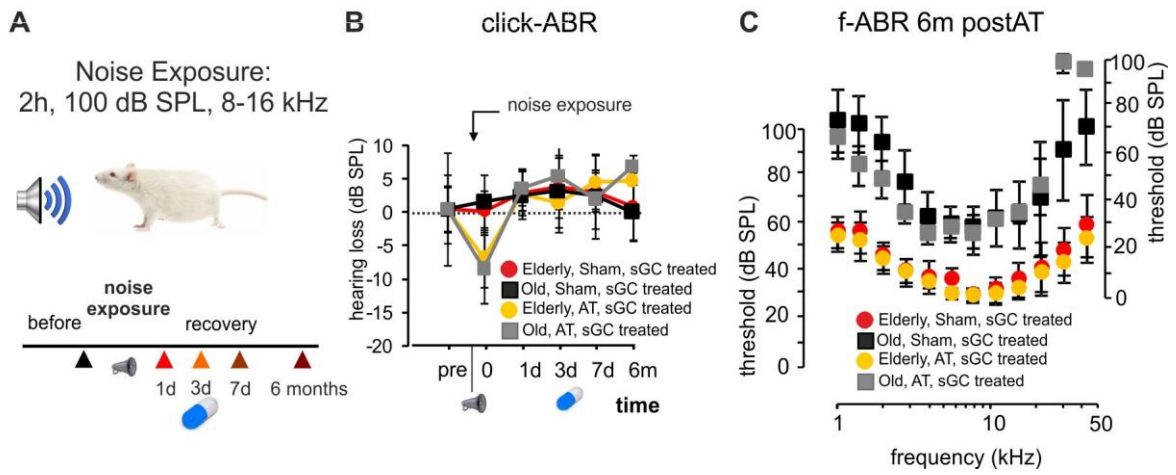

Figure 50. Vulnerability of hearing function for noise of Elderly and Old Wistar rats, that were treated with sGC stimulator for 6 months

A: Schematic illustration for the noise exposure paradigm chosen to test the vulnerability and recovery of Elderly compared with Old Wistar rats. Treatment with sGC-stimulator was started on the 3rd day after noise exposure. **B:** After noise exposure, Elderly Wistar rats exhibit a noise-induced hearing loss of approximately 10 dB for click stimuli, similar to the loss of Old Wistar rats. The recovery from noise-induced hearing loss was complete after 3 days with no PTS. After this time period treatment with sGC-stimulator was started. The recovery was similar in Elderly and Old Wistar rats. **C:** After 6 months the f-ABR thresholds of Elderly noise exposed animals, especially in the low and high frequencies, were slightly elevated, but this difference was not significant. For better representation of the data y-axis and the graph for aged group is shifted up. Elderly rats: unexposed *n* = 14/7 ears/rats, noise exposed *n* = 14/7 ears/rats; Old rats: unexposed *n* = 10/5 ears/rats, noise exposed *n* = 2/1 ears/rats.

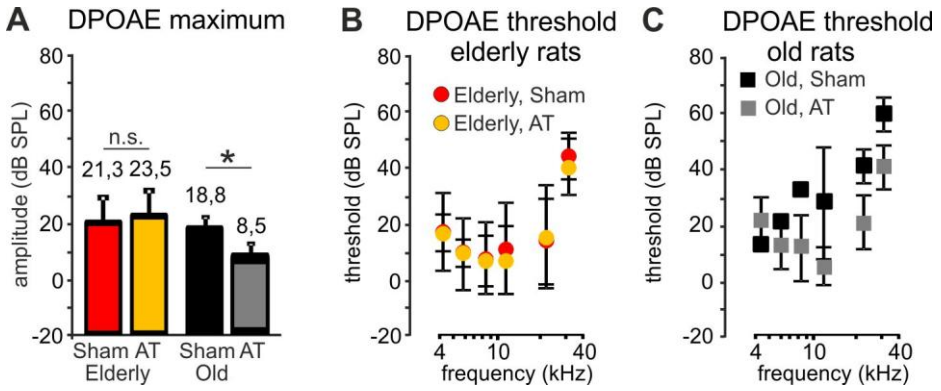

Figure 51. OHC function in Elderly and Old Wistar rats, treated with sGC-stimulating diet for 6 months, 6 months after exposure

A: Amplitudes of distortion products of otoacoustic emissions (DPOAE) of unexposed (Sham) and exposed to noise (AT) Elderly (Sham: red, AT: yellow) and Old (Sham: black, AT: grey) Wistar rats. Amplitudes were not significantly reduced in Elderly group after noise exposure (yellow) compared to control unexposed (red) Wistar rats of the same age. There was a significant difference between Old animals after noise exposure (grey) compared to control unexposed (black) age-matched rats. **B:** DPOAE thresholds of unexposed (red circles) and noise exposed (yellow circles) Elderly rats revealed no difference in DPOAE thresholds in the whole frequency range. **C:** DPOAE thresholds of unexposed (black squares) and noise exposed (grey squares) Old rats revealed a tendency to the reduction of the DPOAE thresholds in Old unexposed animals in middle frequencies that was not significantly different. Elderly rats: unexposed *n* = 14/7 ears/rats, noise exposed *n* = 14/7 ears/rats; Old rats: unexposed *n* = 10/5 ears/rats, noise exposed *n* = 2/1 ears/rats.

Summarizing data shown, I could conclude that there was no effect of sGC stimulator observed in Elderly animals. But in the both ears of a single Old animal that survived till the end of the experiment hearing loss caused by mild noise exposure (see 3.2.1.2.1.) was rescued.

6 months long activation of cGMP cascade rescues synaptopathy

By immunohistochemistry the expression and distribution of CtBP2 as marker of IHC synaptic ribbons was analyzed in IHC of Elderly noise exposed and unexposed (8 – 10 months old), and Old (21 – 24 months old) noise exposed and unexposed Wistar rats (Fig. 52, Fig. 54). All groups were treated with sGC stimulator. Otoferlin staining (red) in the midbasal (Fig. 52A-B, Fig. 54A-B) and basal (Fig. 52C-D, Fig. 54C-D) turns of the cochlea of Elderly unexposed (Fig. 52A, C) and Elderly noise exposed (Fig. 54B, D), and Old unexposed (Fig. 52A, C) and Old noise exposed (Fig. 54B, D) Wistar rats highlights IHC position. By visual inspection the slight reduction of CtBP2-positive dots (green) could be seen in the midbasal turn as well as in basal turn of the Elderly noised exposed Wistar rat cochlea (Fig. 52B, D) as compared to the midbasal and basal turns of the Elderly unexposed Wistar rat cochlea (Fig. 52A, C) treated with sGC stimulator. Unfortunately, only one Old noise exposed and treated with sGC stimulator survived till the end of the experiment. But for this exact animal treatment with sGC stimulator seemed to be beneficial, and IHC were restored in basal (Fig. 54C-D), as well as in midbasal (Fig. 54A-B) cochlear turns.

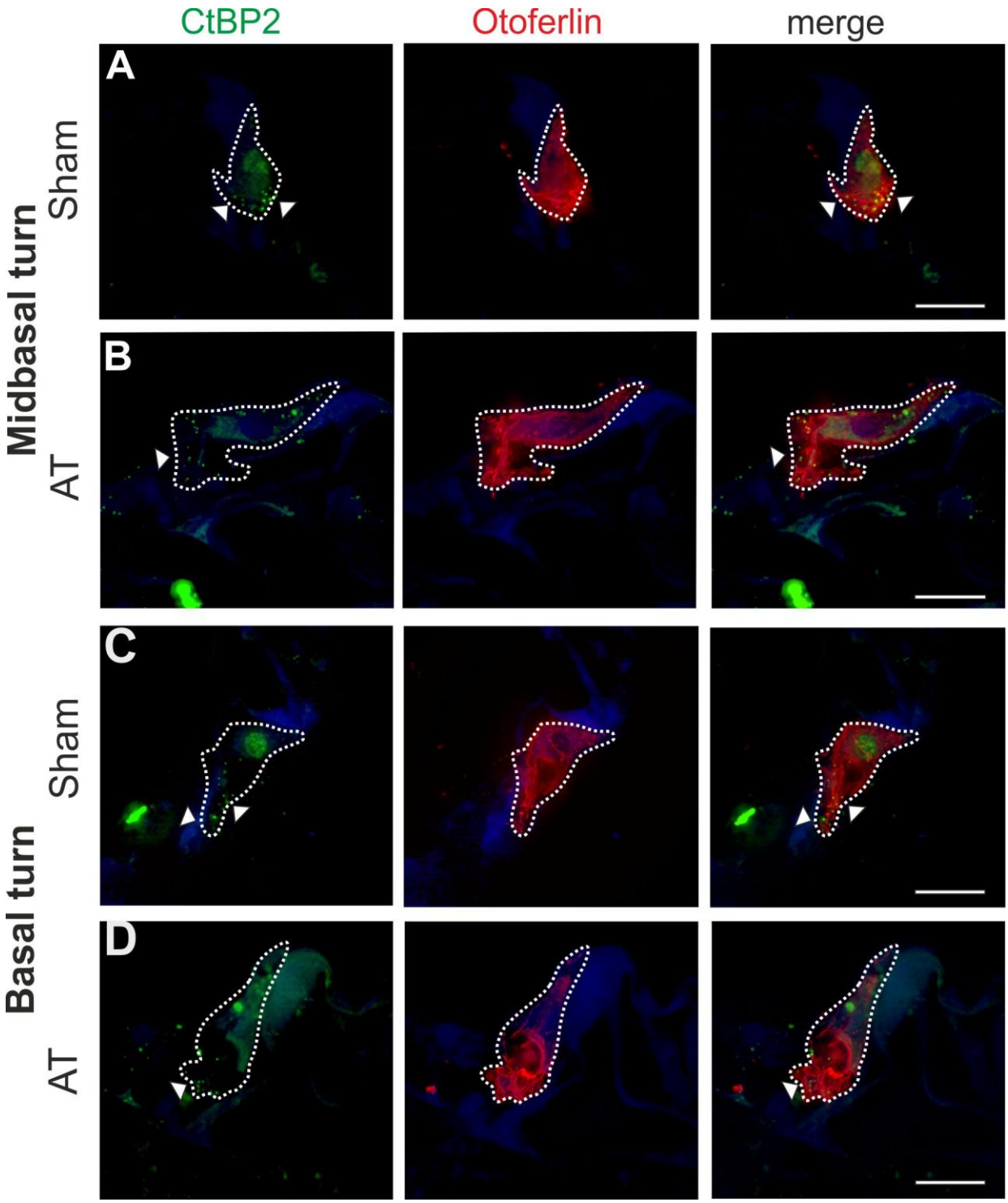

Figure 52. IHCs ribbon synapses were partially restored in the basal and midbasal turns of the cochlea of Elderly noise exposed Wistar rats sGC stimulator treated for 6 months after exposure

The restoration of the synaptic contacts in the IHCs of noise exposed rats could be observed after 6 months treatment with sGC stimulator. Images of the IHC of midbasal (A, B) and basal (C, D) turns of Sham exposed (A, C) and noise exposed (B, D) animals immunostained for synaptic ribbons (CtBP2, green, marked with arrowheads) and IHC (otoferlin, red, encircled with line). Scale bars = 10 μm. Nuclear marker: DAPI (blue).

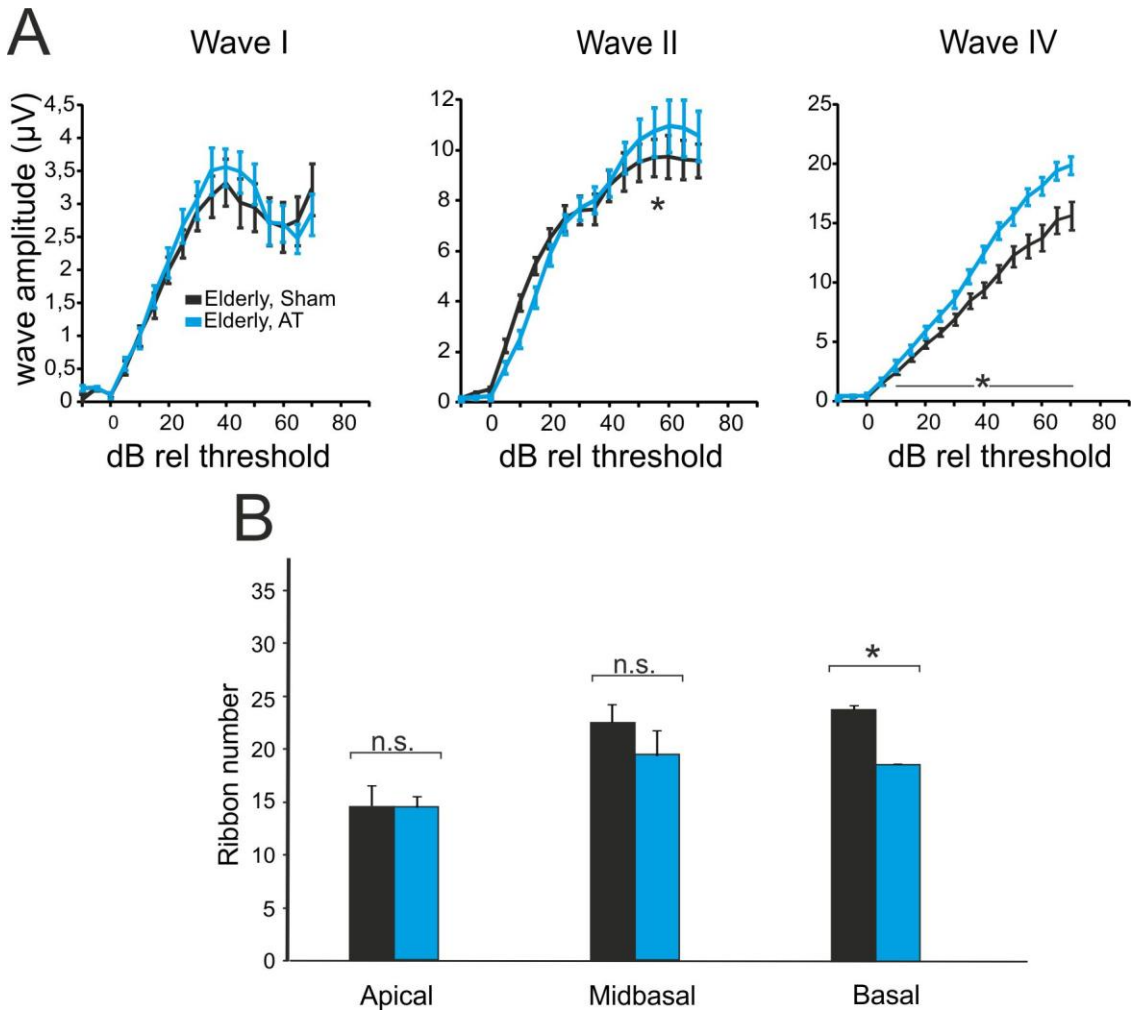

Figure 53. Minor reduction of the IHCs ribbon synapses in basal and midbasal turns of the cochlea of elderly noise exposed rats correlated with the conserved ABR wave I amplitude after 6 months treatment with sGC stimulator

A: Mean ± SEM click-evoked ABR wave I, wave II and wave IV amplitudes growth function for Elderly unexposed (black line, *n* = 14/7 ears/rats) and Elderly noise exposed (blue line, *n* = 14/7 ears/rats). **B:** Ribbon counting from Elderly unexposed (black) and Elderly noise exposed (blue) Wistar rats, expressed in average number per IHC (±SD), in different cochlear turns (*n*=3).

CtBP2-positive points in single IHCs were counted in Elderly unexposed Wistar rats in comparison to Elderly noise exposed Wistar rats treated with sGC stimulator during 6 months, and small reduction of ribbons became evident in the midbasal and basal turns (Fig. 53B). No differences in the number of ribbons between Elderly unexposed and Elderly noise exposed Wistar rats were noted in the apical turn (Fig. 53B) after 6 months of treatment with sGC stimulator containing substance. IHC ribbon synapses number was reduced by 18% in the basal and 15% in the midbasal turns of the cochlea of noise exposed Elderly Wistar rats treated with sGC stimulator for 6 months in comparison with unexposed rats.

Table 11. *P*-values of pair wise comparisons of amplitude data in Figure 53

Elderly Sham vs. Elderly AT	dB above threshold														
	10	15	20	25	30	35	40	45	50	55	60	65	70	75	80
Wave I	0,18	0,22	0,411	0,296	0,235	0,2257	0,49	0,394	0,166	0,3777	0,275	0,451	0,302	0,1606	0,386
Wave II	0,181	0,288	0,306	0,45	0,473	0,373	0,1427	0,1996	0,0933	0,0344 (*)	0,064	0,18	0,286	0,2288	0,268
Wave IV	0,0145 (*)	0,008 (*)	0,0089 (*)	0,0057 (*)	0,0032 (**)	0,0024 (**)	0,0042 (**)	0,0005 (**)	0,0022 (**)	0,0008 (**)	0,0024 (**)	0,0009 (**)	0,0009 (**)	0,0025 (**)	0,002 (**)

1-sided Student's *t*-test: *(*) p<0,05;(**) p<0,005*

In Elderly noise exposed Wistar rats ABR wave I amplitude 6 months after noise exposure was not different from the ABR wave I amplitude of Elderly unexposed Wistar rats (Fig. 46A). ABR wave II amplitude was increased in high SPL in Elderly noise exposed Wistar rats treated with sGC stimulator 6 months after noise exposure; this difference was significant at 55 dB above threshold (Fig. 53A; 1-sided Student's *t*-test: see table 12 for p-values, no Bonferroni-Holms correction). The amplitude of ABR wave IV was increased in Elderly noise exposed sGC stimulator treated Wistar rats starting from 10 dB above threshold (Fig. 53A; 1-sided Student's *t*-test: look table 12 for p-values, no Bonferroni-Holms correction). These data show that Elderly noise exposed animals treated with sGC stimulator showed intact ABR wave I, while ABR wave II was moderately increased at high SPL, and ABR wave IV amplitude is increased 6 months after noise exposure as compared with Elderly unexposed Wistar rats.

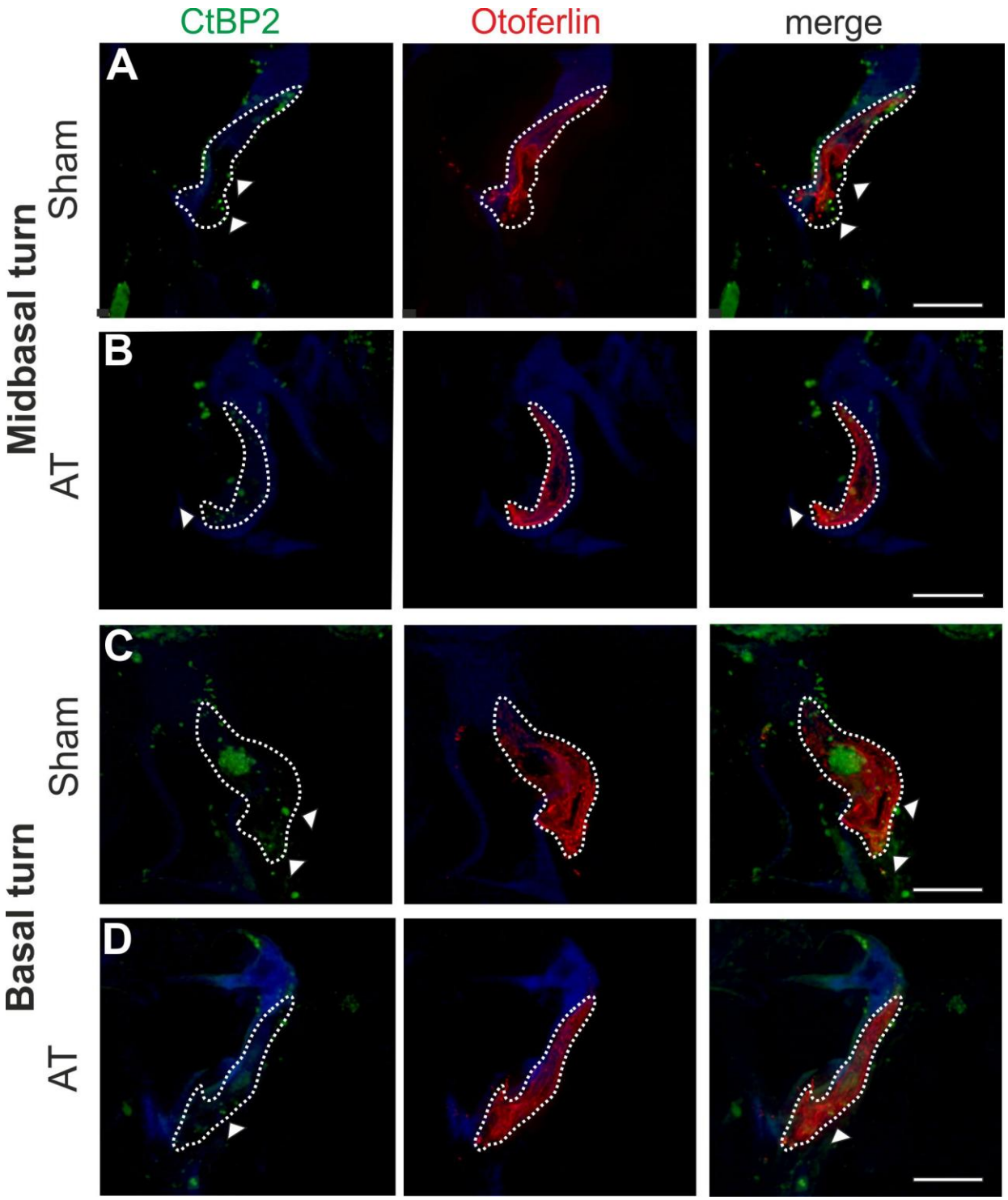

Figure 54. Treatment with sGC stimulator rescued IHCs ribbon synapses in the basal and midbasal turns of the cochlea of Old noise exposed Wistar rat that survived 6 months after exposure

Number of the synaptic contacts in the IHC of noise exposed Old rat did not change after exposure in comparison with unexposed rats. Images of the IHC of midbasal (A, B) and basal (C, D) turns of unexposed (A, C) and exposed (B, D) animals immunostained for synaptic ribbons (CtBP2, green, marked with arrowheads) and IHC (otoferlin, red, encircled with line). Scale bars = 10 μm. Nuclear marker: DAPI (blue).

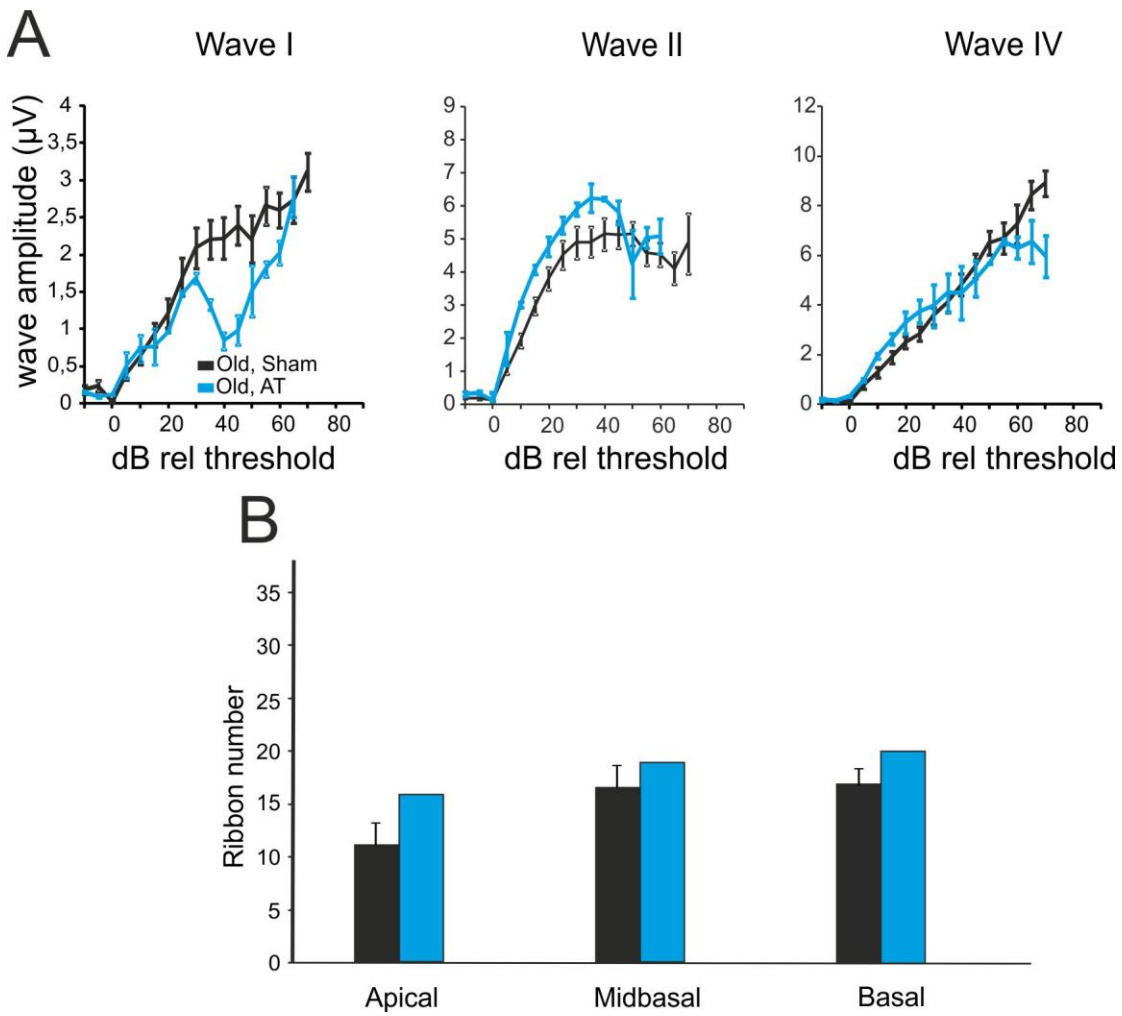

Figure 55. Reduction of the IHCs ribbon synapses in basal turn of the cochlea of old noise exposed rats correlated with the loss of the ABR wave I amplitude

A: Mean ± SEM click-evoked ABR wave I, wave II and wave IV amplitudes growth function for Old unexposed (black line, *n* = 12/6 ears/rats) and Old noise exposed (blue line, *n* = 2/1 ears/rats). **B:** Ribbon counting from Old unexposed (black) and Old noise exposed (blue) Wistar rats, expressed in average number per IHC (±SD), in different cochlear turns (*n*=3 in unexposed group, n=1 in noise exposed group).

CtBP2-positive points in single IHCs were also counted in Old unexposed sGC stimulator treated Wistar rats in comparison to Old noise exposed sGC stimulator treated Wistar rats, and a reduction of ribbons became evident in basal turn, but not in midbasal cochlear turns (Fig. 55B). No differences in the number of ribbons between Old unexposed sGC stimulator treated and Old noise exposed sGC stimulator treated Wistar rats were noted in the apical turn (Fig. 55B). IHC ribbon synapses number was reduced by 25% in the basal turns of the cochleae of Old noise exposed sGC stimulator treated Wistar rats in comparison with unexposed animals.

In Old noise exposed Wistar rats ABR wave I amplitudes were reduced from 30 dB above threshold up to 70 dB above threshold in comparison with Old unexposed Wistar (Fig. 55A). Increase in wave II amplitudes were observed in old noise exposed Wistar rats from 10

dB up to 50 dB above threshold in comparison with unexposed Old Wistar rats (Fig. 55A). The amplitudes of ABR wave IV were decreased in Old noise exposed Wistar rats starting from 60 dB above threshold (Fig. 55A). Noise exposed sGC treated animals showed the reduction of ABR wave I amplitude, elevation of ABR wave II amplitude, and minor reduction of ABR wave IV amplitude at high SPL as compared with Old unexposed sGC treated Wistar rats.

Summarizing data shown above, synaptopathy could be partially rescued in Elderly noise exposed Wistar rats by the long term treatment with sGC stimulator in 6 months after noise exposure. Unfortunately, only one Old animal treated with sGC stimulator survived during 6 months after noise exposure. However this animal showed positive effect of the treatment: the IHC ribbon synapses were rescued. Data from more animals are needed to confirm this trend.

Effects of cGMP cascade activation in noise-exposed gerbils

Long-term (4 months) effects of cGMP cascade activation by sGC stimulation on noise exposed Mongolian gerbils and Sham exposed Mongolian gerbils as a control were tested.

Effects of 4 months long cGMP cascade activation in young and elderly noise exposed gerbils kept in normal environment

In order to find out whether long-term cGMP cascade activation by administering sGC stimulator affects vulnerability for noise exposure in Young (10 – 13 months old) and Elderly (23 – 29 months old) animals kept in normal environment, animals were acoustically stimulated. Noise exposure and hearing measurements were performed as described above (see 2.2. and 2.3.). Treatment with sGC stimulator was started on the 3rd day after noise exposure.

Effects of 4 months long cGMP cascade activation on vulnerability for noise exposure in young gerbils kept in normal environment

ABRs thresholds for click auditory stimuli (click-ABR, Fig. 56A) were significantly different (1-sided Student's *t*-test: p = 0,015) between Young unexposed sGC stimulator treated gerbils kept in normal environment (*n* = 10/5 ears/gerbils) and Young noise exposed sGC stimulator treated gerbils kept in normal environment (*n* = 12/6 ears/gerbils). Frequency-specific ABR (f-ABR, Fig. 56B) revealed significant hearing loss at 32 kHz (P < 0,0001 by

two-way ANOVA with Bonferroni multiple comparison test; other measured frequencies: n.s. by two-way ANOVA). No differences in other frequencies were observed between noise exposed and Sham exposed sGC stimulator treated gerbils kept in normal environment.

Maximum DPOAE amplitudes were not significantly different between Young unexposed sGC stimulator treated gerbils and Young noise exposed sGC stimulator treated animals (both groups kept in the normal environment; Fig. 49C, Young unexposed: n = 9/5 ears/gerbils; Young noise exposed: n = 10/5 ears/gerbils, 1-sided Student's t-test: n.s.). No differences in DPOAE thresholds of Young noise exposed treated with sGC stimulator gerbils kept in the normal environment were observed in comparison with Young unexposed sGC stimulator treated gerbils kept under the same conditions (Fig. 56D; two-way ANOVA with Bonferroni multiple comparison test: n.s.).

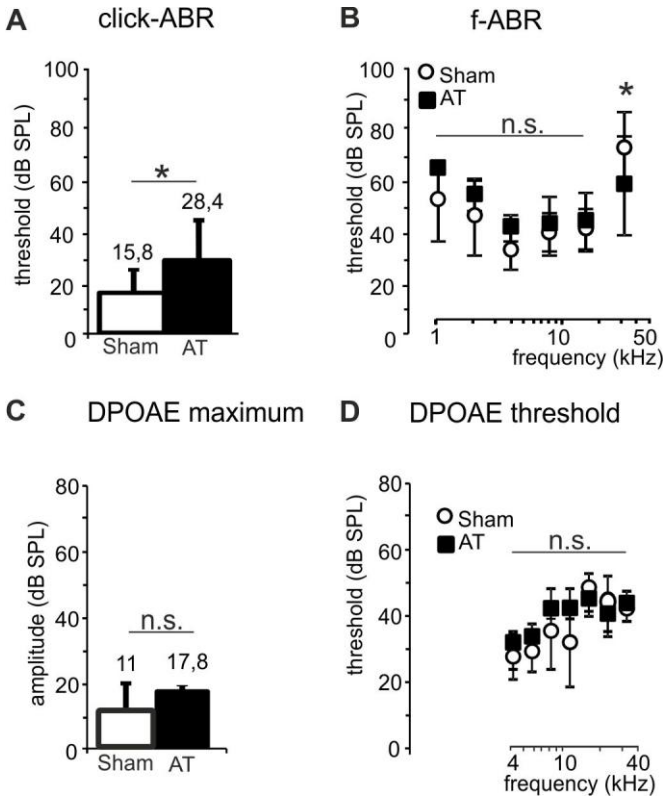

Figure 56. Vulnerability to noise of young treated with sGC simulator gerbils kept in normal environment: ABRs and DPOAEs

A: ABR thresholds (click-ABR) presented as mean±SD for Young unexposed (white bar) and noise exposed (black bar) gerbils treated with sGC stimulator (sGC). **B:** Frequency specific pure tone thresholds (mean±SD) for young unexposed (white circles) and Young noise exposed (black squares) gerbils treated with sGC stimulator. **C:** DPOAE maximum amplitude at f2 = 4,8–5,8 kHz of Young unexposed (white bar) and Young noise exposed (black bar) gerbils treated with sGC stimulator. **D:** DPOAE thresholds (dB SPL f1) of Young unexposed (white circles) and Young noise exposed (black squares) gerbils treated with sGC stimulator.

In conclusion I could tell that cGMP cascade activation by cGMP stimulation during 6 months did not cause changes in hearing of Young sGC stimulator treated gerbils. Difference in ABR thresholds at 32kHz could be explained by natural variation.

4 months long cGMP cascade activation prevents hearing loss after mild noise exposure in elderly gerbils

ABRs thresholds on click auditory stimuli (click-ABR, Fig. 57A) were not significantly different (1-sided Student's *t*-test: n.s.) between Elderly unexposed sGC stimulator treated gerbils (n = 6/3 ears/gerbils) and Elderly noise exposed sGC stimulator treated gerbils (n = 10/5 ears/gerbils). Frequency-specific ABR (f-ABR, Fig. 57B) revealed no significant difference in hearing between groups over all frequency range (Fig. 57B; two-way ANOVA: n.s.).

Maximum DPOAE amplitudes were also not significantly different between Elderly unexposed sGC stimulator treated gerbils and Elderly noise exposed sGC stimulator treated animals (both groups kept in the normal environment; Fig. 57C, Elderly unexposed: n = 4/2 ears/gerbils; Elderly noise exposed: n = 8/5 ears/gerbils, 1-sided Student's *t*-test: n.s.). No differences in DPOAE thresholds of Elderly noise exposed treated with sGC stimulator gerbils kept in the normal environment were observed in comparison with Elderly unexposed gerbils treated with sGC stimulator kept under the same conditions (Fig. 57D; two-way ANOVA: n.s.).

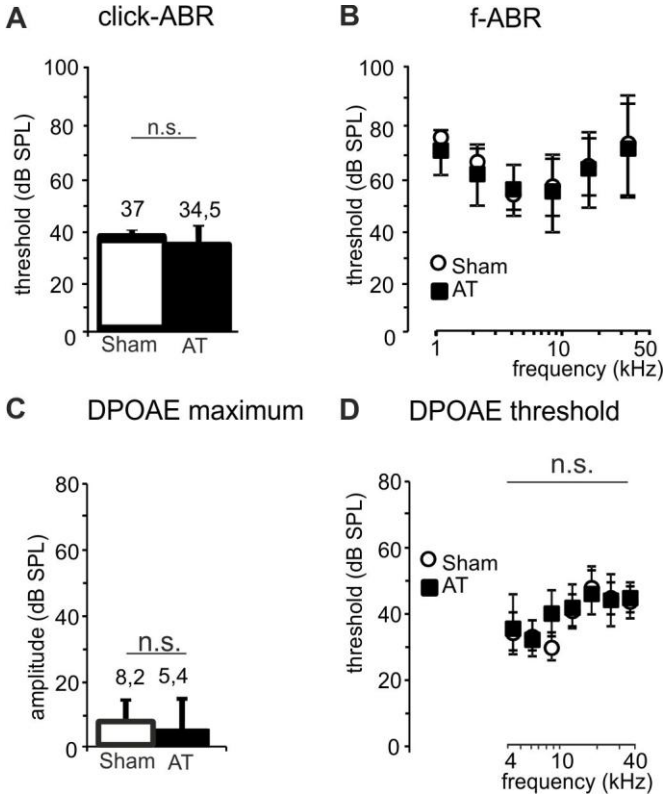

Figure 57. Vulnerability for noise exposure of elderly gerbils treated with sGC simulator kept in normal environment: ABRs and DPOAEs

A: ABR thresholds (click-ABR) presented as mean±SD for Elderly unexposed (white bar) and Elderly noise exposed (black bar) gerbils treated with sGC stimulator. **B:** Frequency specific pure tone thresholds (mean±SD) for Elderly unexposed (white circles) and Elderly noise exposed (black squares) gerbils treated with sGC stimulator. **C:** DPOAE maximum amplitude at f2 = 4,8–5,8 kHz of Elderly unexposed (white bar) and Elderly noise exposed (black bar) gerbils kept in normal environment and treated with sGC stimulator. **D:** DPOAE thresholds (dB SPL f1) of Elderly unexposed (white circles) and Elderly noise exposed (black squares) gerbils treated with sGC stimulator.

Summarizing, cGMP cascade activation by sGC stimulation during 6 months prevents hearing loss after mild noise exposure in Elderly gerbils.

Effects of 4 months long cGMP cascade activation in young and elderly noise exposed gerbils kept in enriched environment

Questioning whether 6 months cGMP cascade activation affects vulnerability to noise exposure in young gerbils kept in the enriched environment, Young (10 – 13 months old) and Elderly gerbils (23 – 29 months old) were acoustically stimulated as described above (see 2.3.) and treated with sGC stimulator. Hearing measurements were performed as previously described in 2.2. Treatment with sGC stimulator was started on the 3rd day after noise exposure.

4 months long cGMP cascade activation decrease vulnerability for noise exposure in young gerbils kept in enriched environment

ABR thresholds for click auditory stimuli (click-ABR, Fig. 58A) were not significantly different (1-sided Student's *t*-test: n.s.) between Young unexposed gerbils kept in enriched environment (*n* = 8/4 ears/gerbils) and Young noise exposed gerbils kept in enriched environment (*n* = 10/5 ears/gerbils). Frequency-specific ABR (f-ABR, Fig. 58B) revealed a significant difference in the hearing between groups in high frequency range (Fig. 58B; P < 0,0001 by two-way ANOVA with Bonferroni multiple comparison test at 8 – 32 kHz; n.s. below 8 kHz by two-way ANOVA), indicating a high-frequency hearing loss in Young noise exposed gerbils treated with sGC stimulator.

Maximum DPOAE amplitudes were not significantly different between Young unexposed gerbils treated with sGC stimulator and Young noise exposed animals treated with sGC stimulator (both groups kept in enriched environment; Fig. 58C, Young unexposed: *n* = 6/3 ears/gerbils; Young noise exposed: *n* = 8/5 ears/gerbils, 1-sided Student's t-test *t*-test: n.s.). No differences in DPOAE thresholds of Young noise exposed treated with sGC stimulator gerbils kept in the enriched environment were observed in comparison with Young unexposed sGC stimulator treated gerbils kept under the same conditions (Fig. 58D; two-way ANOVA: n.s.).

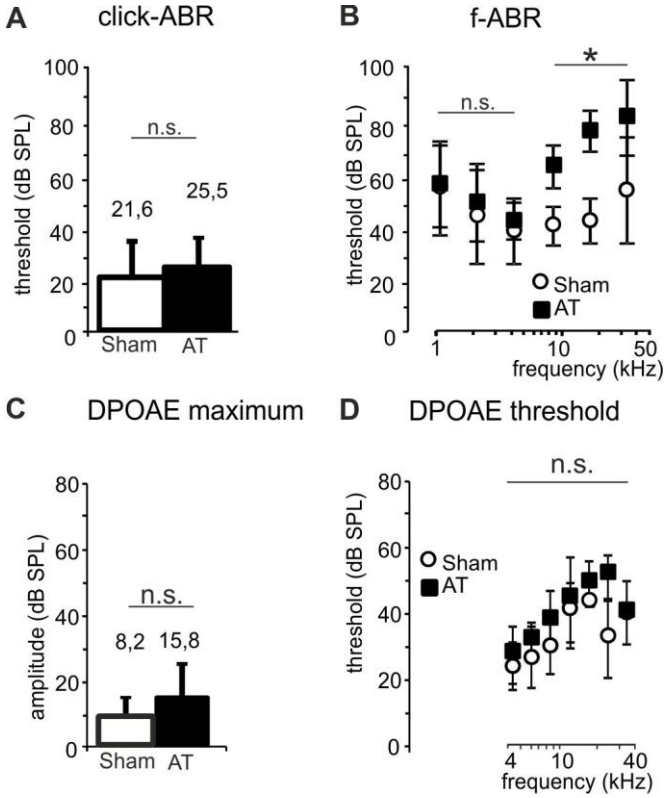

Figure 58. Vulnerability for noise of young sGC simulator treated gerbils kept in enriched environment: ABRs and DPOAEs

A: ABR thresholds (click-ABR) presented as mean±SD for Young unexposed (white bar) and Young noise exposed (black bar) gerbils kept in enriched environment (EE) and treated with sGC stimulator (sGC). **B:** Frequency specific pure tone thresholds (mean±SD) for Young unexposed (white circles) and Young noise exposed (black squares) sGC stimulator treated gerbils kept in EE. **C:** DPOAE maximum amplitude at f2 = 4,8–5,8 kHz of Young unexposed (white bar) and Young noise exposed (black bar) gerbils kept in EE and treated with sGC stimulator. **D:** DPOAE thresholds (dB SPL f1) of Young unexposed (white circles) and Young noise exposed (black squares) sGC stimulator treated gerbils kept in EE.

In conclusion, 4 months cGMP cascade activation by sGC stimulation decreases vulnerability of young gerbils kept in enriched environment for noise.

4 months long cGMP cascade activation decreases vulnerability for noise exposure in elderly gerbils kept in enriched environment

Investigating whether long-term cGMP cascade activation by administering sGC stimulator affects vulnerability to noise exposure in elderly animals kept in enriched environment, elderly (23 – 29 months old) gerbils were acoustically stimulated. Noise exposure and hearing measurements were performed as described above (see 2.2. and 2.3.) ABRs thresholds for click auditory stimuli (click-ABR, Fig. 59A) were significantly different (1-sided Student's *t*-test: p = 0,0267) between Elderly unexposed gerbils kept in enriched environment (n = 4/2 ears/gerbils) and Elderly noise exposed gerbils kept in enriched environment (n = 6/3 ears/gerbils). Frequency-specific ABR (f-ABR, Fig. 59B) revealed no significant difference in hearing between groups (Fig. 59B; two-way ANOVA: n.s.), but a

tendency to the hearing loss over the whole frequency range was observed in Elderly noise exposed gerbils kept in enriched environment.

Maximum DPOAE amplitudes were not significantly different between Elderly unexposed sGC stimulator treated gerbils and Elderly noise exposed sGC stimulator treated gerbils (both groups kept in enriched environment; Fig. 59C, Elderly unexposed: n = 2/1 ears/gerbils; Elderly noise exposed: n = 2/1 ears/gerbils, 1-sided Student's t-test: n.s.). Elevated DPOAE thresholds of Elderly noise exposed sGC stimulator treated gerbil kept in the enriched environment were observed in comparison with Elderly unexposed sGC stimulator treated gerbil kept under the same conditions (Fig. 59D).

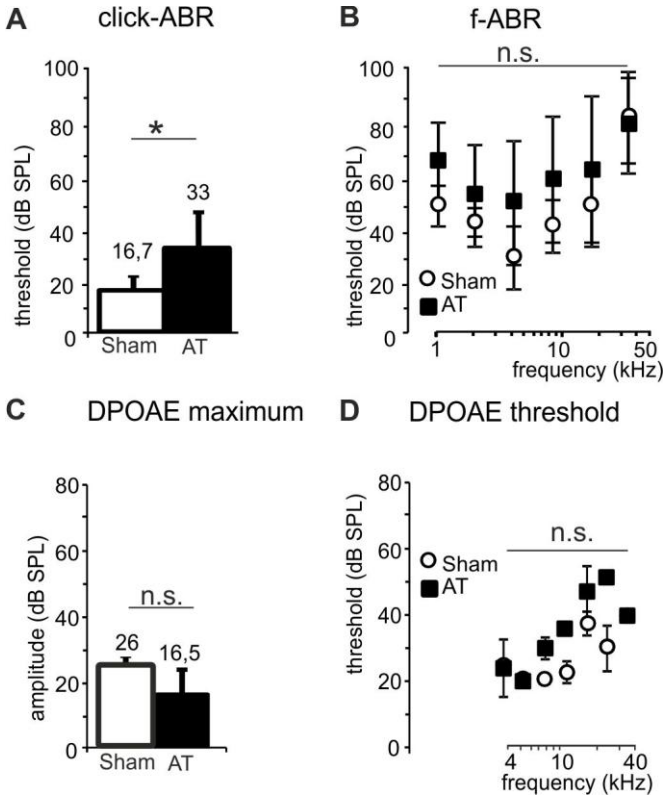

Figure 59. Vulnerability for noise of elderly gerbils treated with sGC simulator and kept in enriched environment: ABRs and DPOAEs

A: ABR thresholds (click-ABR) presented as mean±SD for Elderly unexposed (white bar) and Elderly noise exposed (black bar) gerbils kept in enriched environment and treated with sGC stimulator. **B:** Frequency specific pure tone thresholds (mean±SD) for Elderly unexposed (white circles) and Elderly noise exposed (black squares) sGC stimulator treated gerbils kept in EE. **C:** DPOAE maximum amplitude at f2 = 4,8–5,8 kHz of Elderly unexposed (white bar) and Elderly noise exposed (black bar) sGC stimulator treated gerbils kept in EE. **D:** DPOAE thresholds (dB SPL f1) of Elderly unexposed (white circles) and Elderly noise exposed (black squares) gerbils treated with sGC stimulator and kept in EE.

Therefore in Elderly gerbils kept in enriched environment we can also observe a trend to hearing loss after 4 months treatment with sGC stimulator, which goes hand in hand with the effect observed in Young gerbils after the same treatment.

4. Discussion

In the present study presumptive properties of auditory nerve degeneration over age in the rat and gerbil animal model and possible approaches for pharmacological therapeutic intervention was investigated. We found evidence that a limited population of auditory nerve fibers exist that can be lost over age and with auditory trauma. This population of fibers may be responsive for a rescue through environmental enrichment or drug therapy upon cGMP cascade stimulation in young, but less in elderly animals. The data are discussed in the context of a decline of auditory nerve fiber loss and loss of central plasticity over age.

4.1. Aging itself is a cause of progressive neural degeneration

Up to now age-related hearing loss was assumed to be restricted to the loss or dysfunction of outer hair cells (OHCs) (Keithley and Feldman, 1982; Milbrandt et al., 2000; Guang-Di Chen et al., 2009). Only recently it was shown that afferent neural fiber loss in mice may progress over age and cause a hearing deficit independent from OHCs loss (Sergeyenko et al., 2013). The present study can confirm the study of Sergeyenko also for the rat and the gerbil animal models (Sergeyenko et al., 2013). Both animal models exhibit a moderate high-frequency hearing loss in the first period of their life that was 10 months in rats, and 20 months in gerbils, that progressed further with age. In the initial period of life a moderate hearing loss was found independent of OHC dysfunction in the present study. Accordingly, a decline in active cochlear mechanics, described upon DPOAE measurements, could not be observed. This allows to predict that at least at the initial period of life the OHC function was safe, in contrast with the late period of life, when OHC dysfunction progressed.

To evaluate the source of high-frequency hearing deficit that occurred in the initial period of life suprathreshold sound responses were measured, such as ABR wave amplitudes that are expected to change proportionally to the size of discharge rates and number of synchronously firing auditory fibers (Johnson and Kiang, 1976). The slight high frequency hearing loss that animals (rats and gerbils) exhibit in the initial period of life could be linked to the reduction of ABR wave I (shown for rats), indicating that the summed activity within the auditory nerve is reduced.

The amplitude of the ABR wave critically depends on the discharge rate of auditory fibers and the synchronicity with the fibers spike (Johnson and Kiang, 1976). The precision of

discharge rate of auditory fibers is defined by synaptic specialization within the first inner hair cell synapse of the auditory pathway, the so called ribbon synapse (Buran et al., 2010). We therefore considered that the reduction of ABR wave amplitude that can be observed over age and that is correlated with a high frequency hearing loss was the result of an altered number of ribbon synapses in the base of IHCs. Indeed for the rat a loss of the IHC ribbon synapses was found in basal and midbasal cochlear turns that according to the tonotopic map of the rat cochlea (Müller M., 1991) correspond to high frequency regions.

While the hearing deficit in the initial period of life occurred independent of OHC related hearing loss, in the late period of life hearing loss progresses also through OHC dysfunction as shown in the rat model. Similar to what was shown by Sergeyenko et al. (2013) in mice, the present study revealed also an accelerating deterioration of IHC ribbon synapses and ABR wave I loss after 18 months in rats. Thus the IHC ribbon loss progressed in rat animal model in the late period of life in addition to OHC function loss that indicates that in the late period of life OHC and IHC both exhibit an age-dependent damage. Thus different from previous studies that suggested age-dependent hearing loss is due to OHC deficits (Keithley and Feldman, 1982) newer studies (Sergeyenko et al., 2013) and the present study point to progressive neurodegeneration preceding OHCs loss.

The auditory fibers have two distinct functions: one subpopulation (60%) exhibits high spontaneous spiking rates and is very sensitive for low sound pressure levels. A subpopulation with high thresholds has low spontaneous spiking rates and elevate their spike rates with growing sound intensity only when sound pressure levels are already elevated (Heinz and Young, 2004; Liberman, 1978; Merchan-Perez and Liberman, 1996; Müller and Robertson, 1991; Sachs and Abbas, 1974; Schroeder et al., 2001; Spoendlin and Schrott, 1989; Yates, 1991). According to findings of Furman et al. (2013), loss of auditory fibers after noise exposure is mostly restricted to high threshold low spontaneous rate fibers. If this holds we would expect a reduced dynamic range to increasing sound intensities. Indeed a typical sign of presbyacusis in humans is a reduced dynamic loudness range (Frisina RD, 2009). This may indicate that at least in aging brains the loss of dynamic range cannot be compensated centrally. The reason for the loss of dynamic range in humans over age is currently not understood. In particular indicate that our brain has a capacity to compensate sensory deprivation, e.g. following noise exposure, as shown in various studies. After auditory trauma homeostatic compensatory processes triggered by desihnibition enable

neurons respond to a reduced auditory input with a higher spike rate. Accordingly a homeostatic process after auditory trauma based on desinhibition has been shown in the cochlea nucleus (CN), inferior colliculus (IC), and auditory cortex (AC) (Melcher and Kiang, 1996; Cai et al., 2009; Gu et al., 2010; Jakawich et al., 2010, Lindskog et al., 2010; Tyagarajan and Fritschy, 2010). This compensatory process after noise exposure goes hand in hand with a restoration of ABR waves (Rüttiger et al., 2013; Singer et al., 2013).

In the present study we were able to show that the age-dependent loss of the auditory fibers (reduced amplitude of ABR wave I) could not be compensated centrally at the level of the IC (persistent reduction of the amplitude of the ABR wave IV). Such a mechanism may explain the reduced dynamic loudness range of sound perception over age (Frisina, 2009). A failure to compensate loss of auditory fibers over age centrally is an important observation, that confirms previous studies that describe that sensory deprivation after noise exposure (also causing tinnitus) is characterized through a failure to compensate the peripheral loss centrally (Singer et al., 2013). The risk of tinnitus may therefore increase with age (Wang et al., 2011).

4.2. **Vulnerability of elderly animals to noise is reduced**

Questioning why central compensation of fiber loss is reduced over age while central compensation of fiber loss after acoustic overstimulation in young animals at least after mild acoustic exposure is successful (Singer et al., 2013), we considered differences in the vulnerability upon noise and central compensation in young and old animals.

Previous reports suggested that in healthy animals full regeneration of cochlear nerve terminals can occur after acute noise-induced neural degeneration (Puel et al., 1998; Pujol and Puel, 1999). In contrast recent findings showed that acoustic overexposure causes a permanent loss of peripheral nerve terminals on IHC (Kujawa and Liberman, 2009; Lin et al., 2011). Also in the present study we observed that noise exposure induced an auditory fiber loss in young, but not in elderly animals. ABR wave I amplitude and IHC synaptic ribbons number were reduced after noise exposure in young, but not in elderly animals.

The most likely explanation for the different vulnerability of auditory fibers after noise exposure could be that fibers are already lost over age and cannot be lost anymore by noise exposure. In accordance with this hypothesis is the observation that IHC ribbon numbers were already reduced in elderly animals. Importantly, also a compensatory elevation of ABR wave

IV was observed in young, but not in aged animals. The data support that the loss of synaptic plasticity impairs capacity for central homeostatic adaptation over age.

4.3. **The beneficial effect of environmental enrichment on hearing**

It was shown that long-term sound conditioning prior to a traumatizing noise exposure protects animals from the noise-induced hearing loss (Kujawa and Liberman, 1999). The mechanism of this effect is not fully understood and was suggested to be due to the contralateral feedback on OHC through efferent neural fibers. Norena and Eggermont showed in 2005 that enriched acoustic environment after noise trauma prevents the reorganization of the tonotopic map and triggers an increase in spontaneous firing correlated with an increased neuronal synchrony. The hearing of acoustically traumatized cats that were kept in the acoustically enriched environment after noise exposure finally revealed to a hearing level similar to that of unexposed animals (Norena and Eggermont, 2005; Norena and Eggermont, 2006). An effect of environmental enrichment on presbyacusis so far has not been studied neither for humans nor for rodents. In the visual system however a beneficial effect of enriched environment on presbyopia was shown (Polat et al, 2012). In the present study we could show that environmental enrichment could counteract the age-related high-frequency hearing loss. The positive effect of environmental enrichment on age-dependent hearing loss was likely due to an effect on the IHC synapse or the auditory fibers. Studies are ongoing to dissect the morphological changes in IHC ribbon synapses and to analyze the ABR waves' amplitudes in these animals to validate this hypothesis.

A trend for an improvement of low-frequency hearing could be seen in both young and elderly gerbils kept in the enriched environment, which could be due to the fact that gerbils use mostly low-frequency hearing in natural environment. Furthermore, vulnerability to noise was increased in young gerbils kept in the enriched environment, but not in elderly gerbils. If we hypothesize that environmental enrichment may protect auditory fibers that are most sensible for loss after noise exposure, we may consider that environmental enrichment stabilizes IHCs synaptic contacts through an increase of a metabolic reservoir that is also needed for stabilizing IHCs synaptic contacts during noise exposure. Therefore, we may hypothesize that environmental enrichment and noise exposure may use the similar signaling pathways.

4.4. **Activating the cGMP cascade in the cochlea is beneficial for young, but not for elderly animals**

Looking for possibilities to counteract neural degeneration caused by aging and mild acoustic overstimulation we considered the stimulation of the cGMP cascade, which previously was shown to protect from noise induced hearing loss (Jaumann et al., 2012). After short-term treatment with sGC stimulator of animals that were exposed to a mild noise, the resulting synaptopathy of IHCs in high frequency areas of the cochlea could be partially rescued in young rats.

In the present study it was shown that administering sGC stimulator itself causes a slight high frequency hearing loss in elderly rats and gerbils. As the sGC stimulator used was shown to have vasodilatory effects (Stasch et al., 2002), this high frequency loss could be possibly explained by the manifested hypotension. An alteration of the vasomotor system associated with a hypotensive condition was already shown to be a possible factor in the origin of a reversible cochlear damage in the prospective study (Pirodda et al., 1999).

The weakness of the effect that the experimental substance chosen here had on the elderly rats could be explained by the fact, that as discussed above, aged animals may have already experienced age-related neural degeneration. Thus the administering sGC stimulating substance does not help to restore connections that are already lost, but can only prevent the progression of further neurodegeneration. Accordingly a slight therapeutical effect was observed after long-term treatment with sGC stimulator that however was much less pronounced than the effect observed in young noise exposed animals. Furthermore, the sGC stimulator seems to counteract the beneficial effect of the enriched environment on the hearing of gerbils. On the basis of the results from the current study, this effect cannot be explained. The molecular mechanisms of should be investigated in a future study.

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