Aus dem Zentrum für angewandte Medizintechnik der HNO-Klinik

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DISSERTATION

Investigation of Protective Mechanisms to Prevent Sensori-neural Impairments Due to Traumatic Interventions of the Cochlea

Untersuchung von Schutzmechanismen gegen sensorisch-neurale Störungen durch traumatische Eingriffe in die Cochlea

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Science is but an image of the truth.

Francis Bacon

(1561 – 1626; philosopher and statesman)

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1. Abstract

Noise-induced hearing loss (NIHL) and loss of residual hearing after cochlear implantation are particularly associated with cochlear hair cell loss. There are several approaches to inhibiting hair cell loss. These include the use of near-infrared radiation (NIR). Previous studies have already applied NIR after noise trauma was delivered and observed significantly reduced amounts of hearing loss. However, the amount of hearing protection could possibly be higher if a single NIR treatment was made before an inner ear trauma. This is because the NIR activates the cytochrome C oxidase and thus more ATP is produced in advance, hence should be better able to protect and stabilize cellular structures.

The first part of the project examined the effect of a single NIR pre-treatment to prevent noise induced hearing loss (NIHL). Cochleae of one group of adult mice were pre-treated via the external auditory canal with NIR (808 nm, 120 mW for 5, 10, 20, 30, or 40 minutes). These mice were then noise-exposed with broadband noise (5-20 kHz) for 30 minutes at 115 dB SPL (sound pressure level). Another experimental group was exclusively noise-exposed with the same noise dose specified above. A further group of mice served as untreated control. Frequency-specific auditory brainstem response (ABR) recordings were performed before all treatments and two weeks later to determine the threshold shift. Furthermore, the amplitude increase of wave IV was determined. Compared to the non-NIR irradiated controls, hearing thresholds of 5-minute NIR-irradiated animals were significantly lower after noise exposure for three frequencies. The entire frequency range tested was significantly lower in all other NIR pre-treatment groups compared with the non-NIR irradiated controls. For more than 10 minutes pre-treatment, saturation occurred in hearing protection. Due to NIR light, the amplitude of wave IV deteriorated significantly less after noise exposure than in non-NIR irradiated controls.

The second part of the project investigated the effectiveness of NIR pre-treatment on electrode array insertion into the cochleae of Dunkin-Hartley guinea pigs. Conically shaped, cochlear implant electrode arrays were implanted bilaterally into cochleae of adult guinea pigs. Only one side, randomly selected, was irradiated with NIR for 15 minutes. The other side served as an intra-individual control. Shortly before and four weeks after implantation, frequency-specific auditory brainstem thresholds were

measured as to be able to determine the implantation related hearing loss. Furthermore, the cochlea's hair-cell loss was analyzed.

Hearing thresholds of NIR-pre-treated ears were significantly lower compared to the untreated side. The amount of missing outer hair cells was significantly reduced for the single NIR pre-treatment to about one-third compared to the untreated ears. Both sides also showed that the cochlea lacked fewer cells in basal regions than apically, potentially due to the unavoidable pressure wave produced during insertion of the electrode array. In both parts of this project, no loss of inner hair cells was found.

The present results indicate that a single NIR pre-treatment of approximately 15 minutes is effective for the reduction of NIHL, as well as for the protection of hearing in otoneurosurgery.

2. Zusammenfassung

Lärminduzierter Hörverlust (NIHL) und Restgehörverlust nach Cochlea-Implantation sind besonders mit dem Verlust der Cochlea-Haarzellen verbunden. Es gibt mehrere Ansätze, um Haarzellverlust zu hemmen wie die Verwendung von Nahinfrarotlicht (NIR). In früheren Studien wurde NIR-Licht schon nach einem Lärmtrauma effektiv eingesetzt. Trotzdem könnte der Schutz bei einer Einzel-Behandlung vor einem Innenohrtrauma eventuell höher ausfallen, da die Bestrahlung die Cytochrom-C-Oxidase aktiviert und somit vorab mehr ATP gebildet wird (Stabilisation und Schutz von Zellstrukturen).

Der erste Projektabschnitt untersucht die Wirkung einer Einzel-NIR-Vorbehandlung zur NIHL-Vorbeugung. Cochleae adulter Mäuse wurden über den äußeren Gehörgang mit NIR-Licht (808 nm, 120 mW für 5, 10, 20, 30 oder 40 Minuten) vorbehandelt und mit Breitband-Rauschen (5-20 kHz) für 30 Minuten bei 115 dB SPL (Schalldruckpegel) beschallt. Eine Versuchsgruppe wurde nur beschallt. Eine weitere Gruppe diente als unbehandelte Kontrolle. Frequenzspezifische ABR (auditive Hirnstammreaktion)-Aufzeichnungen wurden vor allen Behandlungen und zwei Wochen nach den NIR- und Lärmbehandlungen durchgeführt (Bestimmung der Schwellenverschiebung). Auch der Amplitudenanstieg der Welle IV wurde bestimmt. Die Hörschwellen der mit NIR-Licht vorbehandelten Tiere waren nach Lärmexposition für drei Frequenzen der 5-minütigen Vorbehandlung und für den gesamten, in allen anderen Behandlungsgruppen getesteten Frequenzbereich im Vergleich zu den Kontrollen signifikant niedriger. Bei mehr als 10 Minuten Vorbehandlung trat eine Sättigung auf. Durch das NIR-Licht verringerte sich die Amplitude der Welle IV nach der Lärmexposition signifikant als bei der Kontrolle.

Der zweite Projektabschnitt untersucht die Effektivität der NIR-Vorbehandlung an einer Cochlea-Elektrodeninsertion bei Dunkin-Hartley-Meerschweinchen. Konisch geformte Cochlea-Implantat-Elektroden-Arrays wurden bilateral in Cochleae adulter Meerschweinchen implantiert. Nur eine Seite wurde randomisiert mit NIR-Licht für 15 Minuten bestrahlt. Die andere Seite diente als intraindividuelle Kontrolle. Kurz vor und vier Wochen nach der Implantation wurden die frequenzspezifischen Hörschwellen bestimmt (Bestimmung des implantationsbedingten Hörverlusts). Auch der cochleäre Haarzellverlust wurde analysiert.

Die Hörschwellen der mit NIR vorbehandelten Ohren waren verglichen zur unbehandelten Seite signifikant niedriger. Die Anzahl der fehlenden äußeren Haarzellen

war nach einer einzelnen NIR-Vorbehandlung gegenüber den unbehandelten Ohren signifikant um ca. einem Drittel reduziert. Beide Seiten zeigten auch, dass basal in der Cochlea weniger Haarzellen fehlten als apikal resultierend aus einer nicht zu unterbindenden Insertionsdruckwelle. Bei beiden Projektabschnitten fehlten keine inneren Haarzellen.

Die vorliegenden Ergebnisse zeigen, dass eine Einzel-NIR-Vorbehandlung von ca. 15 Minuten zur NIHL-Verringerung und zum Gehörschutz in der Otoneurochirurgie wirksam ist.

Partial results of the present work were published in:

- Basta, D., Gröschel, M., Strübing, I., Boyle, P., Fröhlich, F., Ernst, A. and Seidl, R. 2020. Near-infrared-light pre-treatment attenuates noise-induced hearing loss in mice. *PeerJ.* 8: e9384. [36]
- Strübing, I., Gröschel, M., Schwitzer, S., Ernst, A., Fröhlich, F., Jiang, D., Boyle, P. and Basta, D. 2020. Neuroprotective effect of near-infrared light in an animal model of CI surgery. Audiol Neurootol. 26: 95–101. [39]

3. Introduction

The number of different diseases afflicting human beings has been increasing over many decades. After cardiovascular disease, hearing loss is the third most common disease reported in the adult population. It is estimated that of approximately all 7.8 billion people in the world population, up to 500 million people (6.4%) are at risk or suffer from hearing loss from ototoxic drugs, aging and infections, as well as noise-induced hearing loss (NIHL). In addition to impairing quality of life, NIHL has now become one of the most common health hazards in the workplace. Symptoms of NIHL are especially common when working with noisy machines and / or technological processes in industrial environments. This correlation has been known from clinical observations of NIHL for over 100 years. Thus, NIHL not only leads to sharply rising social costs in workers' compensation and pensions, but also leads to considerable economic losses in the form of lost productivity. In the private sphere, too, there is a risk of hearing loss due to environmental influences such as fireworks, shooting and listening to loud music [1].

3.1. Background and treatment options of noise induced hearing loss

Several studies show that NIHL is often associated with a variety of consequences: increased hearing thresholds, reduced speech understanding, or the sequelae of tinnitus, but also associated with sleep disorders, cardiovascular diseases or cognitive decline. Direct damage in the middle and inner ear are particularly serious. The damage caused by noise via intensive sound pressure waves affects not only the eardrum and middle ear but also the particularly delicate structures that are housed in the cochlea, such as the organ of Corti, with its large numbers of sensory cells. The organ of Corti is an interface

between acoustic mechanical vibrations and resulting nerve signals. It is located in the scala media and is the carrier of the sensor cells in the inner ear for all mammals. The scala media is separated from the scala tympani by the basilar membrane and from the scala vestibuli by Reissner membrane. The organ of Corti contains three rows of outer and one row of inner hair cells (hearing sensor cells). These hair cells contain stereocilia that protrude into a gap that is filled with endolymph. An individual cell can have up to one hundred stereocilia. The tectorial membrane, a gelatinous mass, is located above the gap but within the scala media. The longest stereocilia of the outer hair cells are in contact with the tectorial membrane. The deflection of the stereocilia of the inner hair cells triggers the stimulus transduction and thus the hearing sensation [2, 3]. Sound pressure waves lead to an up and down vibration of the entire organ of Corti. The incoming pressure waves vibrate the perilymph of scala vestibuli by moving the oval window by the stapes footplate. These vibrations move along the organ of Corti to the apex and travel back down the scala tympani where a corresponding movement of the round window occurs. Differential movements between the tectorial membrane and basilar membrane deflect outer hair cell stereocilia bundles with their typical regular V-shaped arrangement. The movement of the fluid in between deflects inner hair cell stereocilia bundles. The stereocilia bundles of the outer hair cells are in direct contact with the tectorial membrane and are deflected during vibration. This opens K^+ channels in the cilia and, due to the high endocochlear potential, K^+ flows into the outer hair cells and depolarizes them. When swinging back, the K^+ channels close and the cell repolarizes to generate force and amplify the sound pressure wave [2]. Thus, loss of the outer hair cells means loss of the cochlea's amplification mechanism for the acoustic sensing function. Up to a thousand times gain of the traveling wave is due to the extremely fast movement of the outer hair cells, which move up to twenty thousand times per second (20,000 Hz). This movement is initiated by a special motor protein, prestin. Also, the inner hair cells, that act as sensory cells in the organ of Corti, can die due to high levels of noise. Such damage impacts signal transmission through the spiral ganglion cells in the direction of the brain. In the inner hair cells, the acoustic stimulus generally triggers an electrical signal (mechano-electrical transduction). These signals send a chemical signal to an auditory nerve fiber (transformation) through the synaptic transmitter glutamate, whereby each fiber passes on the frequency selection of the inner hair cell with which it synapses. The auditory nerve fibers respond electrically via action potentials and extend to the cochlear nucleus of the brainstem where the spiral ganglion cells medial processes synapse. When exposed to

high noise levels, not only the structures of the peripheral auditory pathway just described, but also all other structures and connections of the central auditory pathway are permanently damaged. These structures include the cochlear nucleus, the olivary complex, the lateral lemniscus, the inferior colliculus, the medial genicular body and the auditory cortex [4, 5].

This damage, in the form of cell loss, is associated with apoptotic and necrotic processes via a complex cascade with proteolytic enzymes and caspases from the mitochondria of the cells. In most cases it is not reversible [6, 7, 8].

Persistent or intense noise may result in a transient threshold shift (TTS) or a persistent threshold shift (PTS). This is also reflected in changes in the growth functions of the auditory nerve's output. While TTS is associated with predominantly reversible damage to hair cell stereocilia or hair cell synapses, PTS results in permanent damage or loss of hair cells and synapses [9, 10, 11]. So far, there is no curative solution for hearing loss bordering on deafness. The available treatments are sound or vibration amplifying acoustic hearing aids (mild and moderate hearing loss) and cochlear implants (severe hearing loss or deafness).

There are several medical approaches under study to treat or prevent noise-induced hearing loss. These include a variety of pharmaceutical substances such as anti-inflammatory therapies, use of antioxidants as inhibitors of intracellular stress pathways, neurotrophic factors, inhibition of programmed cell death pathways, and neurotransmission blockers [8, 12]. Especially within anti-inflammatory therapies, steroids, such as dexamethasone and dehydroepiandrosterone, have mostly been investigated as treatments against noise-induced traumata in guinea pigs and mice [13–16]. The antioxidants, with reactive oxygen species inhibitor/scavenger activity, contain a large number of substances that are currently being used, or are still in their clinical trial phase, such as N-acetyl-L-cystein (NAC), acetyl-L-carnitine, ebselen, coenzyme Q10, resveratrol, glutathione, ginseng, D-methionine, and vitamins A, C, E and B12 [8, 17]. A single dose of neurotrophin-3 (NT3) and brain-derived neurotrophic factor (BDNF) as neurotrophic factors are well known to protect the hair cells and lessen synaptopathy after a noise-induced trauma in guinea pigs [18]. The same result is reported after treatment with the glutamatergic neurotransmission blocker riluzole and the glutamate receptor antagonist caroverine, applied as a hearing preservation measure [19, 20]. The programmed cell death pathway (caspase-independent apoptotic pathway) also plays a subordinate role in the loss of hair cells, as the apoptosis-inducing factors (AIFs), as well

as the endonuclease G (endoG), have been transferred to the nuclei and are involved in the death of the hair cells [21]. In addition, several reports indicate other substances with protective effects for hair cells like the basicfibroblast growth factor [22]. Most of the concepts discussed are still under study, and it is difficult to predict which approach will finally enter clinical practice. Table 1 shows the most interesting approaches with their positive effects and limitations against NIHL.

Table 1: Overview of the currently most important therapeutic approaches against NIHL.

		effects on noise-induced hearing loss	
		positive effects	**limitations**
approaches	**basicfibroblast growth factor [22]**	bFGF protects SGNs against glutamate neurotoxicity and protects hair cells from acoustic trauma	only SGN protection in vitro, intramuscularly injection needed
	NT3 and BDNF [18]	reduced synaptopathy and recovered high-frequency hearing in ears exposed to 95 dB SPL noise → reduced hidden hearing loss	medication at round window needed, no threshold changes, only reduced amplitude growth
	AIF and endoG translocation [21]	translocation of endoG from mitochondria into the nucleus was also found in apoptotic outer hair cells → potential medication approach	only discovery of AIF and endoG as factors in a noise induced hair cell death pathway, no clinical implementation
	NAC and HPN-07 [17]	reduces temporary and permanent threshold shifts, reduce aberrant	NAC and HPN-07 only intraperitoneally in medical combination

approaches		activation of neurons in the central auditory regions	possible, drug delivery as a long-lasting post-treatment
	glutamatergic neurotransmission blocker riluzole [19]	clear dose-dependent reduction (ED_{50}=16.8 µM) of permanent hearing loss and complete protection at 100 µM	perfusion into the cochlea via an osmotic minipump needed, intraperitoneal injection less efficient than intracochlear perfusion
	glutamate receptor antagonist caroverine [20]	provide significant dose dependent protection against noise with local administration either immediately prior or up to 1 h post-exposure through blocking excitotoxic pathways	applied onto the round window membrane with gelfoam → complicated medication, therapeutic window is narrow, and application at 24 h failed to provide functional protection
	steroid dexamethasone [13, 15, 16]	dose-dependent reduction in noise-induced outer hair cell loss, attenuation of the noise-induced ABR threshold shifts	mini-osmotic pump directly into scala tympani or intra-tympanic application needed
	neurosteroide dehydroepiandrosterone sulfate (DHEAS) [14]	improvement in the compound action potential threshold shifts and in amplitude reduction of distortion-product otoacoustic emissions	intravenous administration of DHEAS

In the early 1990s, near-infrared (NIR) light therapies were approved by the US Food and Drug Administration (FDA). As a result, the first successful applications, including those in stroke treatment, have emerged due to near-infrared light being able to penetrate tissue to depths greater than light of other wavelengths and also being able to penetrate the skull bones [23]. Later, the field for NIR light treatment became larger, including applications related to hearing loss. For application in hearing, the light must pass through the tympanic membrane to reach the cochlea and affect the organ of Corti. One important consideration is that the status of the tympanic membrane must not be affected. Moon et al. (2016) examined the eardrum in an animal model using an 830 nm laser at different powers. NIR was emitted daily for 30 minutes through the tympanic membrane for 14 consecutive days. With a laser power of 250 mW, the histological changes in the tympanic membrane included: edema, vascular blockages and inflammation. At an output of 200 mW, however, only a few lymphocytes were observed [24]. Thus, producing additional damage to the cochlea beyond any existing hearing loss can be excluded with the use of a power level of only 120 mW [25]. As the laser power is low, tissue temperature does not rise more than 1 °C, even for relatively long exposure times.

A NIR treatment by infrared (IR) laser or IR LEDs is based on the physical force of photobiomodulation triggered by light in the red-to-near-infrared area (630 to 1000 nm) [26]. Photobiomodulation has a direct influence on cytochrome C oxidase activity in the respiratory chain of the mitochondria. Through the oxidation of cytochrome C and the reduction of molecular oxygen to water and the resulting protons, the ATP of the respiratory chain is increasingly produced, resulting in a protective mechanism for the cells. This increased ATP level activates kinases, leading to the formation of cAMP (cyclic adenosine monophosphate) levels and calcium release, also as an increased gene expression, resulting in lower rates of inflammation and apoptosis [27]. So far, there are only scientific studies on animal models that confirm the positive effect of the NIR delivered as a post-trial application. There was a sustained improvement in hearing loss reported after daily 12-day post-traumatic NIR treatment [28]. In the present work, the effect of using the near-infrared light as a pre-treatment, before a noise trauma, will be examined to reduce the cochlea damage.

The hypothesis of the work's first goal is that the prophylactically increased ATP (produced by the near-infrared pre-treatment) protects the hair cells more effectively against cell death than a near-infrared application after a noise trauma. The results from

a single pre-conditioning of cochlear hair cells in-vitro [29] are very promising for the success of the present in-vivo histological and functional study.

3.2. Preservation of residual hearing in cochlea implantees

One of the most common treatment methods for severe to profound hearing loss and deafness is the implantation of an inner ear prosthesis, such as a cochlea implant [30]. Treatment with a cochlea implant (CI) is indicated when the best conventional sound-amplifying hearing aids can no longer be used to achieve adequate speech understanding. The sound signal is picked up by a microphone, is amplified, compressed in its dynamic range, and digitized. The core of every CI system is the signal processing realized by an externally worn sound processor. The algorithm is intended to mimic the time and place coding of how the healthy inner ear normally treats sound and which is optimized for the perception of speech for the cochlear implant recipient. The implanted part of the system generally consists of a hermetically sealed electronic part, a receiver coil, a magnet and an intra-cochlear electrode array. Cochlear implants try to replicate the frequency differentiation of sound in the inner ear that takes place along the basilar membrane and is called tonotopy. The processed high frequencies are directed to electrode contacts at the base of the cochlea, low frequencies to contacts located closer to the apex, although typically only part of the cochlea is addressed by the electrode array. The auditory nerve, with its remaining intact nerve fibers, is stimulated by up to 22 individual electrode contacts arranged along the scala tympani, stimulating being delivered by means of charge-balanced biphasic electrical pulses. With such multi-channel electrode systems, it is possible to electrically stimulate relatively narrow regions of the cochlea and thereby trigger auditory impressions of different pitches [31]. Within the last decade the medical indication for a cochlea implant has been enlarged to include patients with a reasonable amount of residual low-frequency hearing. Those patients are stimulated by specific electro-acoustic cochlear implant sound processors. Unfortunately, the residual hearing is often diminished or disappears completely postoperatively. A lot of research has already been conducted to apply the promising techniques described earlier for the prevention of NIHL to CI-patients with residual hearing. In addition, perioperative interventions such as cooling of the cochlea are currently under study, again with the intention of preserving residual hearing following cochlear implantation [32].

Intracochlear pressure changes during the electrode insertion are proposed as one possible reason for the loss of residual hearing. The pressure wave in the scala tympani

can damage remaining hair cells along the organ of Corti in the same manner as sound pressure waves generated by noise exposure. It is, therefore, particularly important to insert the electrode array slowly in order to produce as little pressure change as possible. This slow insertion can only be optimized by various techniques, such as arm rests and moistening of the electrode surface [33–35].

After the verification of the hypothesis of a more effective pre-treatment than post-treatment with NIR to protect hair cells before a noise trauma the second goal of the present work is to investigate whether a short pre-treatment with light in the near-infrared range is helpful for the protection of the residual hearing during cochlea implantation. Important information for a possible effective dosage should be delivered by the first part of the present work.

4. Methods

4.1. NIR pre-treatment and noise-induced hearing loss [36]

To assess the influence of a near-infrared light pre-treatment on hearing thresholds following noise exposure, the experiment starts with the measurement of auditory brainstem response (ABR)-thresholds. ABR recordings were made via subdermal needle electrodes for acoustic stimulation of between 5 and 40 kHz. These recordings were made two days before a noise exposure in all animals (78 adult female NMRI-mice aged 10 to 11 weeks, Figure 1A). All ABR measurements were recorded under anesthesia (60 mg/kg ketamine, 6 mg/kg xylazine). Afterward ABR measurement, the mice were randomized into seven experimental groups. One group received only a 30-minute noise exposure (broadband noise 5 to 20 kHz at 115 dB SPL binaurally from a loudspeaker above the animal's head). Five experimental groups received a single NIR pre-treatment of varying durations: 5, 10, 20, 30 or 40 minutes. The pre-treatment always had the same NIR characteristics, 808 nm wavelength with a power of 120 mW, after which the same noise exposure specified above was given. NIR pre-exposure was produced using an adjustable isolated point laser module at the outer ear canal with a total coverage of the cochlea with the laser beam of approximately seven mm diameter (Figure 1C). The remaining experimental group served as untreated control. Two weeks after the light and noise treatment, the ABR derived hearing thresholds in free-field mode were again

measured in all animals. The hearing threshold shift calculated from the ABR measurements and the differences in the amplitude increases of ABR wave IV, the most robust wave, were expected to provide information about the influence of near-infrared pre-treatment on the excitability of the lower central auditory pathway. A visual inspection of the ABR recording per frequency was used, with descending acoustic stimulation level until the wave IV was no longer visible (Figure 1B). It is believed that in ABR measurements, the auditory system generally generates the following responses [37, 38]:

Wave I - spiral ganglion and auditory nerve
Wave II - ipsilateral cochlear nucleus
Wave III - contralateral superiorolivary complex
Wave IV - lateral lemniscus and inferior colliculus
Wave V - inferior colliculus

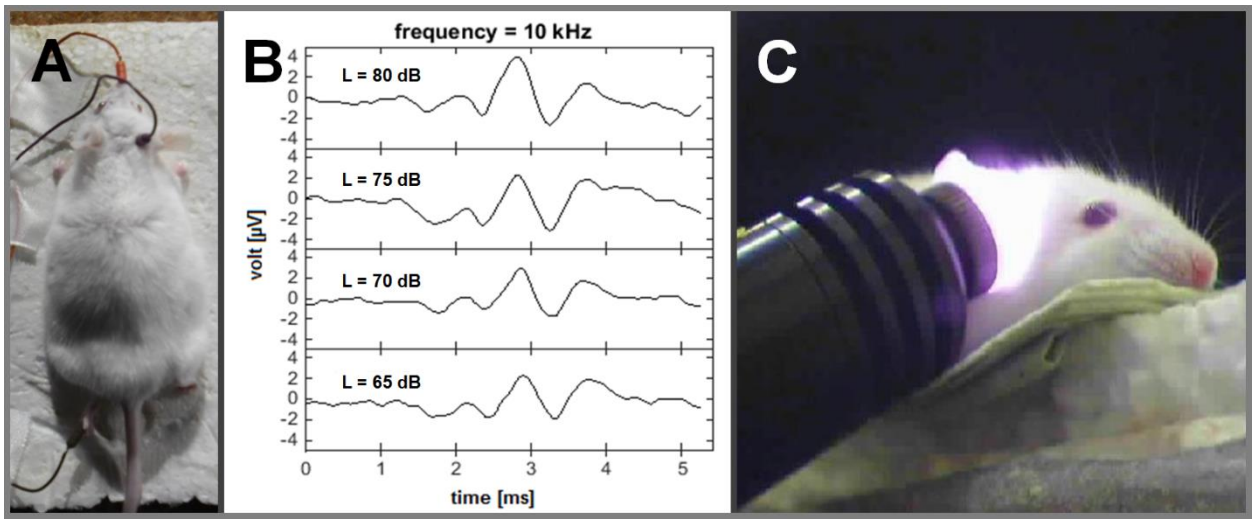


Figure 1: Auditory brainstem recordings. Frequency specific ABR-recordings before and after NIR-light treatment (electrode positions: mastoid = active; nose = reference; foot = ground; A); ABR-waves II, III and IV at a frequency of 10 kHz and different sound levels (65–80 dB SPL; B); NIR-light beam fully covers the mouse cochlea via the outer ear canal with specific angle for a NIR-light pre-treatment (visualized by a NIR-camera; C).

After the second ABR measurement, the animals were perfused with 4% paraformaldehyde via the left heart ventricle. The fixed cochleae of the mice were decalcified with EDTA (ethylenediaminetetraacetic acid) and prepared to obtain maximum half-turns of the organs of Corti. These were then stained with Alexa-fluoro-488-phalloidin (visualization of portions of the cytoskeleton via the high affinity for filamentous F-actin) and DAPI (4',6-diamidin-2-phenylindole, for labeling nuclear DNA) and visualized and photographed under the microscope. The staining was used to visualize hair cells that are strongly enriched in F-actin. The superimposed channels for DAPI (340 nm) and phalloidin (480 nm) were used to count the missing inner and outer hair cells of the organs of Corti of all experimental animals and these counts were statistically evaluated.

4.2. NIR pre-treatment and cochlear implantation [39]

Based on the results of the mouse experiments, a series of experiments focusing on electrode insertion into the cochleae of eight 28 to 30 week old Dunkin Hartley guinea pigs was prepared. Guinea pigs have been a well-established model in hearing research for many years, having been used for electrode insertion research as a precursor to cochlear implantation in humans. In the guinea pigs, as with the mouse test series, the thresholds of both ears per animal are determined by means of ABR measurement. Unilateral, frequency-specific ABRs were recorded via subdermal needle electrodes for acoustic stimulation ranging between 4 and 32 kHz, the recordings being made under anesthesia (fentanyl, midazolam and medetomidine) two weeks before cochlear implant surgery that implanted electrode arrays into both ears. A cochleostomy was performed bilaterally and used to insert a conically shaped cochlea implant electrode array specifically designed for the guinea pig cochlea. An insertion speed of one mm/s was used, for an insertion depth of six mm into the open bulla. The electrode array was designed to fill the scala tympany volume in a comparable manner to the situation in human cochlear implant recipients. A randomized left or right ear per animal was pre-treated with a 15 minute NIR treatment using a point laser module producing a seven mm diameter NIR beam with a total coverage of the cochlea (808 nm, 120 mW; Figure 2). The pre-treatment was made immediately before electrode array insertion.

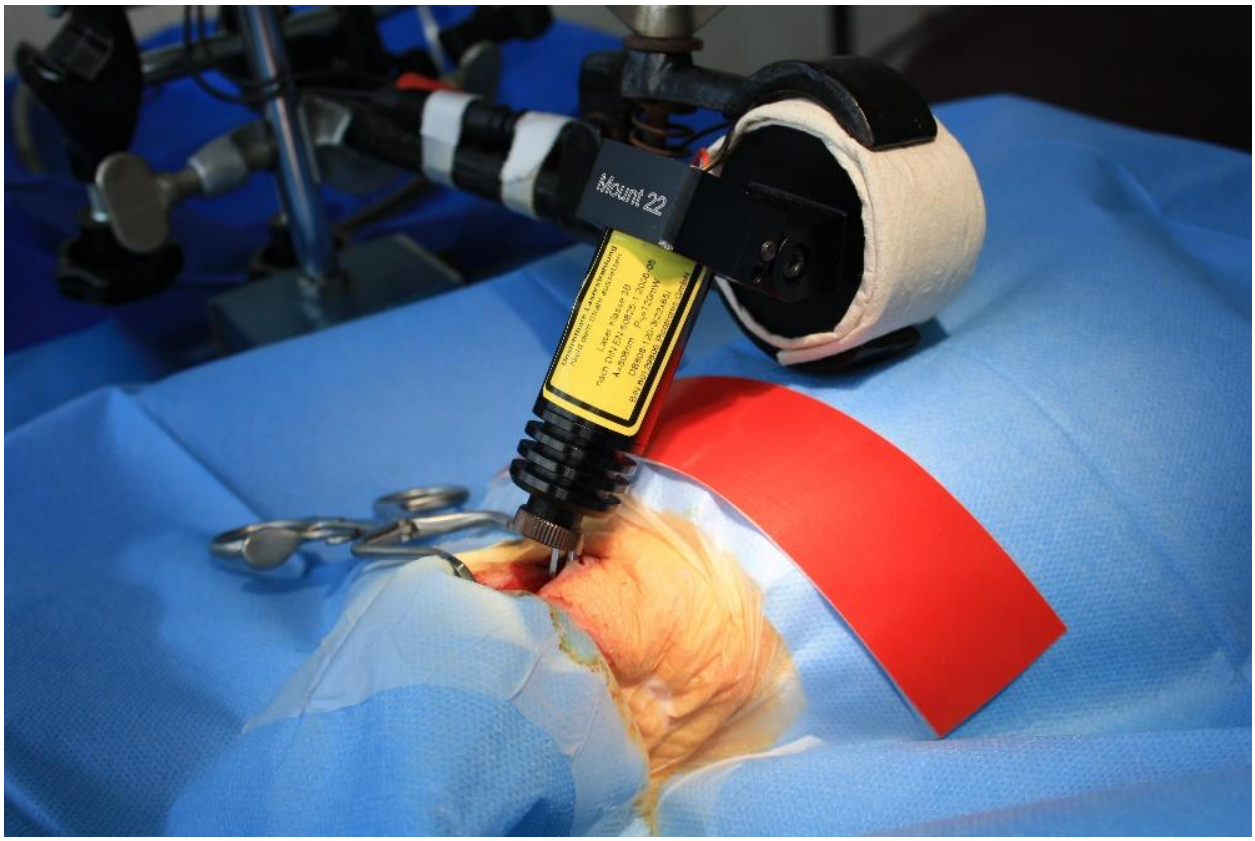


Figure 2: Animal experimental setup with NIR light irradiation.

The non-pre-treated ear served as an intra-individual, contralateral control (sham-exposed). Four weeks after the electrode array implantation, the ABR thresholds were measured again, on both ears separately. The contra-lateral ear was masked by a broadband noise (2 to 32 kHz) while the ipsi-lateral ABR was being recorded. As in the mouse experiments, the post-experimental ABR measurement was followed by perfusion with 4% paraformaldehyde to fixate the animal tissue. The cochleae were decalcified with EDTA, as in the previous NIR mouse experiment. A stain was applied to the prepared organs of Corti with DAPI and Alexa-fluor-488-phalloidin to enable a visualization of F-actin compartments in the cytoskeleton. Based on this staining, the missing outer and inner hair cells were counted and statistically evaluated (Figure 3).
Furthermore, as an additional check, the temperature inside the four cochleae of two additional guinea pigs, aged 28 to 30 weeks, was determined during the NIR irradiation by the insertion of an electrode sized temperature sensor. The temperature during the NIR irradiation ranged between 35 and 36 °C (increase of 0.95 ± 0.12 °C).

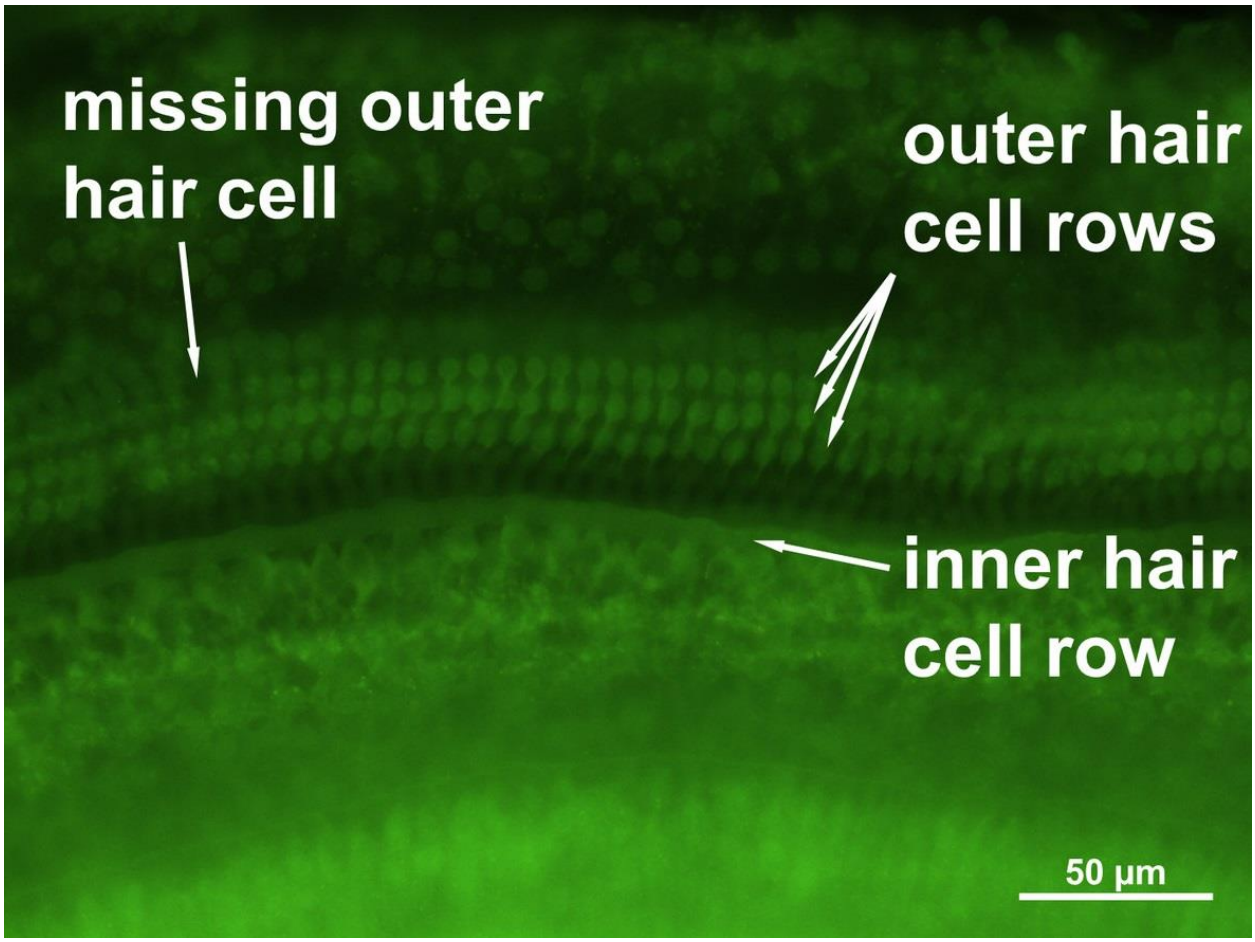


Figure 3: Cochlear hair cell preparation. Sample microphotograph with scale bar of the mouse cochlear whole mount immunofluorescence staining (400x magnification).

5. Results

5.1. Effect of NIR on hearing threshold shift [36]

Compared to the noise-only group without NIR pre-treatment, all NIR pre-treated groups achieved a lower hearing loss. The hearing loss of the noise-only group ranged on average between 35.77 dB and 46.77 dB, which was also the highest hearing loss value for any group. All pre-treated groups showed a range of hearing loss between the lowest value of 11.88 dB and 35.56 dB on average. The hearing loss across frequency for the experimental group with 5 min NIR pre-treatment ranged from 23.38 dB to 35.56 dB, whereas the remaining experimental groups showed less hearing loss, ranging from 11.88 dB and 22.75 dB. Hearing losses for the 5-minute pre-treatment group were

significantly less elevated ($p < 0.05$) compared to the noise-only group for frequencies of 5, 35, and 40 kHz. The entire tested frequency range tested in all other treatment groups (10 to 40 minute NIR pre-treatment) was significantly less elevated ($p < 0.05$) compare to the noise exposure only group. Between the 10, 20, 30 and 40 min pre-treatment groups, no statistically significant differences with respect to hearing loss were found.

The hearing loss of the control group without noise and light pre-treatment are negligible. Here, the values range between 0 dB at 10 kHz and 30 kHz up to 7.14 dB at 5 kHz. Table 2 illustrates the results of all the investigated groups.

Figure 4 shows the hearing loss and Figure 5 shows the hearing protection of all experimental groups. Hearing protection results from the difference between the measured hearing loss and the values of the noise-only group. The hearing loss values of the control group (without noise and light pre-treatment) ranged between 35.3 dB at 5 kHz and 42.5 dB at 40 kHz. In summary, a NIR pre-treatment of between 10 and 40 minutes resulted in a hearing protection between 20.2 dB (at 10 kHz) and 34.1 dB (at 40 kHz). A 5-minute NIR pre-treatment reduced the noise induced hearing loss to a range of 5.4 dB (at 25 kHz) to 23.4 dB (at 40 kHz).

Table 2: Mean results in dB of the hearing threshold shifts of all experimental groups and the controls with associated standard error (SEM).

		Frequency (kHz)							
		5	**10**	**15**	**20**	**25**	**30**	**35**	**40**
NIR light treatment time (min)	**5**	24.94	24.31	29.63	28.06	35.56	29.63	24.00	23.38
	SEM	2.38	3.24	2.62	2.42	2.88	2.92	3.68	2.70
	10	18.38	14.31	20.88	19.94	20.56	20.56	22.75	17.44
	SEM	3.16	3.37	3.09	3.77	3.52	4.25	3.75	3.83
	20	17.00	15.57	19.00	17.71	17.57	17.00	18.29	12.71
	SEM	4.33	3.88	4.73	5.56	4.06	5.06	4.90	4.27
	30	18.60	15.10	19.60	21.60	19.60	14.10	19.60	18.10
	SEM	4.14	4.28	3.53	6.59	4.74	5.86	6.01	6.16
	40	14.38	11.88	18.75	20.00	20.63	15.00	18.13	17.50
	SEM	4.38	2.30	4.41	3.90	3.33	2.50	3.40	3.27
	noise only	42.43	35.77	42.93	42.10	40.93	40.93	43.93	46.77
	SEM	5.14	5.10	4.58	5.17	4.98	4.89	4.44	4.55
	control	7.14	0.00	2.14	1.43	0.71	0.00	3.57	4.29
	SEM	3.43	3.62	3.60	3.03	3.17	3.09	4.46	4.42

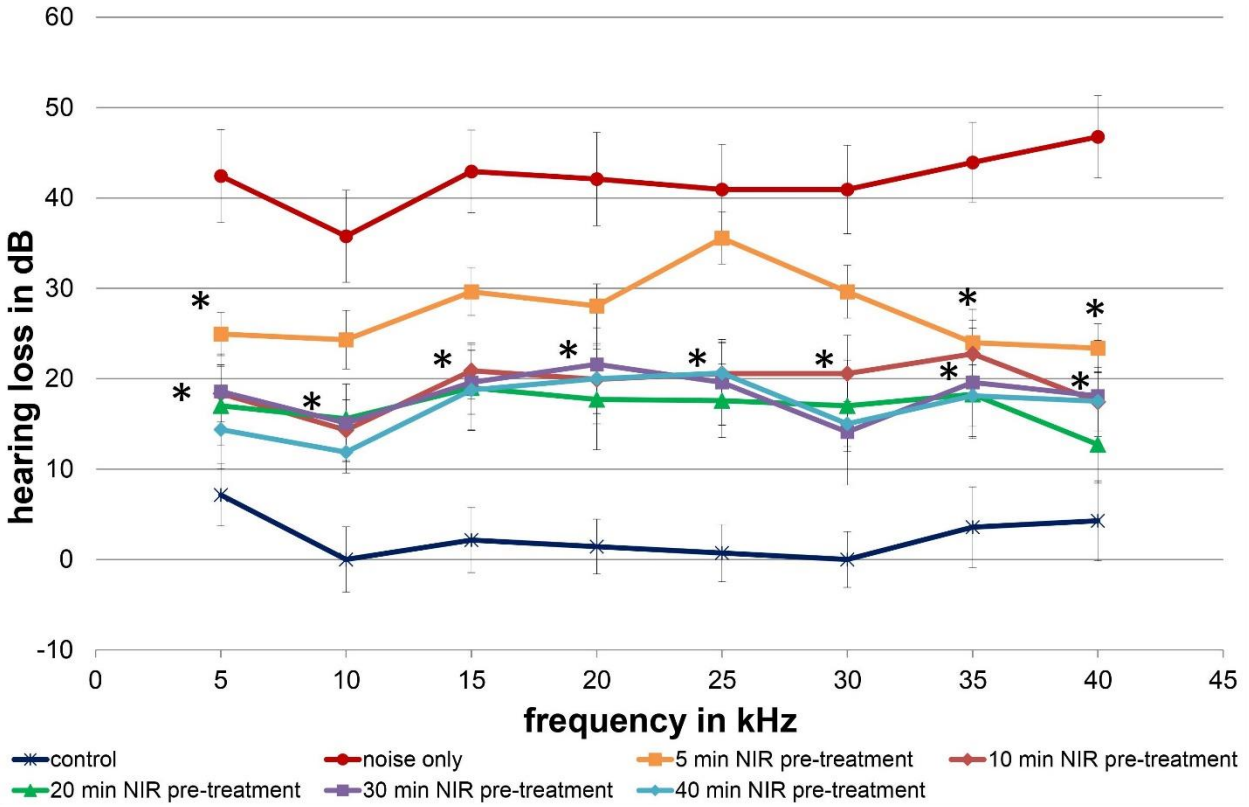


*Figure 4: Means (±SEM) in dB of noise-induced hearing loss for different NIR-light pre-treatments of the cochlea. The 5-minute pre-treatment group shows statistically significant differences at 5, 35 and 40 kHz. All other pre-treated groups show statistically significant differences at all frequencies. The control group without noise and NIR pre-treatment showed only a negligible hearing loss. Filled asterisks indicate statistically significant differences between the pre-treatment groups and the "noise only"-group (significance level *: $p < 0.05$).*

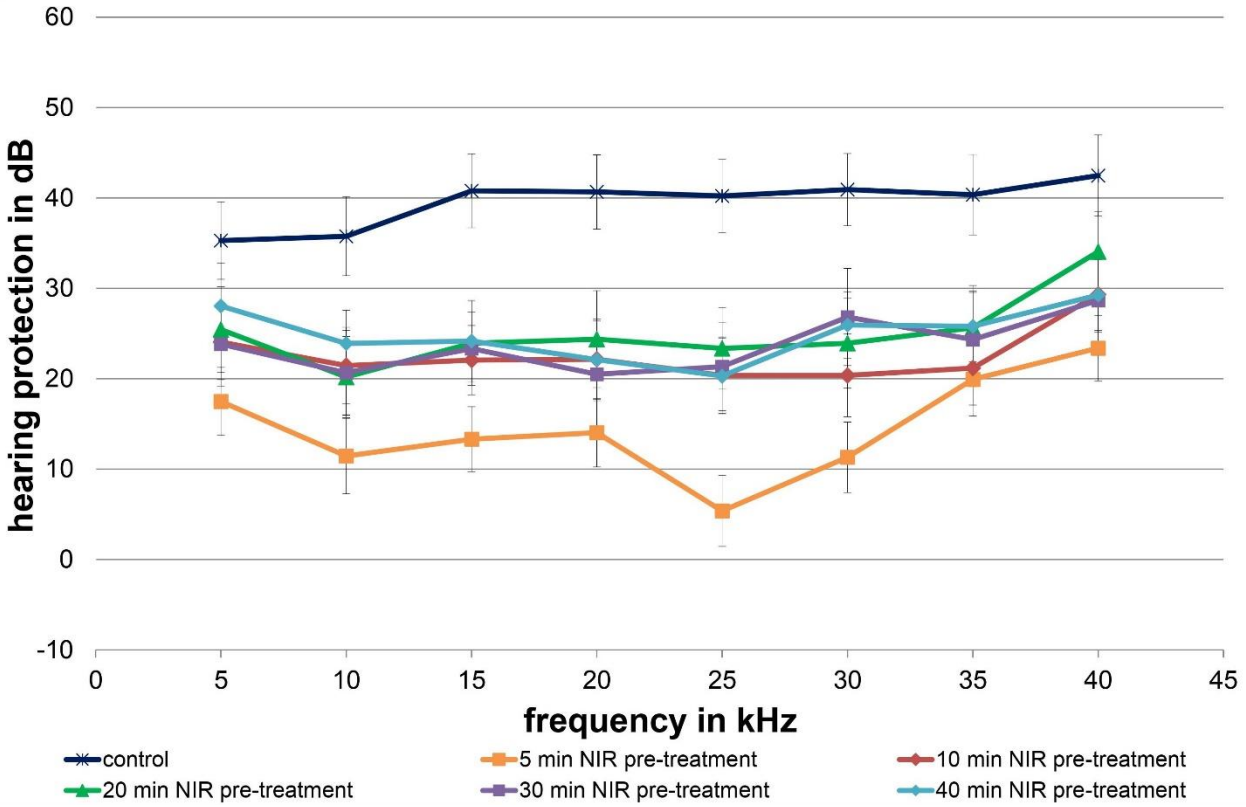


Figure 5: Means (±SEM) in dB of noise-induced hearing protection for different NIR-light pre-treatments of the cochlea as the difference between the "noise-only"-group and all other groups.

5.2. Effect of NIR on ABR wave IV slope

The aim of an additional wave IV analysis was to identify to what extent the central suprathreshold processing has changed due to noise exposure, with or without NIR pre-treatment. Wave IV reflects important areas of the auditory brainstem (such as superior olivary complex and inferior colliculus). Changes in stimulus-related responses suggest modified central auditory processing. The slope of the linear portion within the wave-IV amplitude growth function was calculated, then the post noise exposure slope was subtracted from the pre-exposure slope. In these evaluations, a larger difference in the slope of the amplitude growth function indicates that a lower slope was detected post-experimentally. In the noise-only group, on average, the slope difference had a value of 0.053 ± 0.005 µV / dB and was more than ten times higher than the "10 min NIR" group (steepness difference 0.004 ± 0.005 µV / dB) across all frequencies tested. This experimental group had the lowest slope difference of all five NIR-pre-treated test groups.

A smaller difference in slope indicates a higher post-noise exposure slope. Except for the “5 min NIR" group (0.047 ± 0.009 µV / dB), all test groups showed significantly smaller slope differences than the noise-only group up to a maximum value of 0.025 µV / dB (Figure 6).

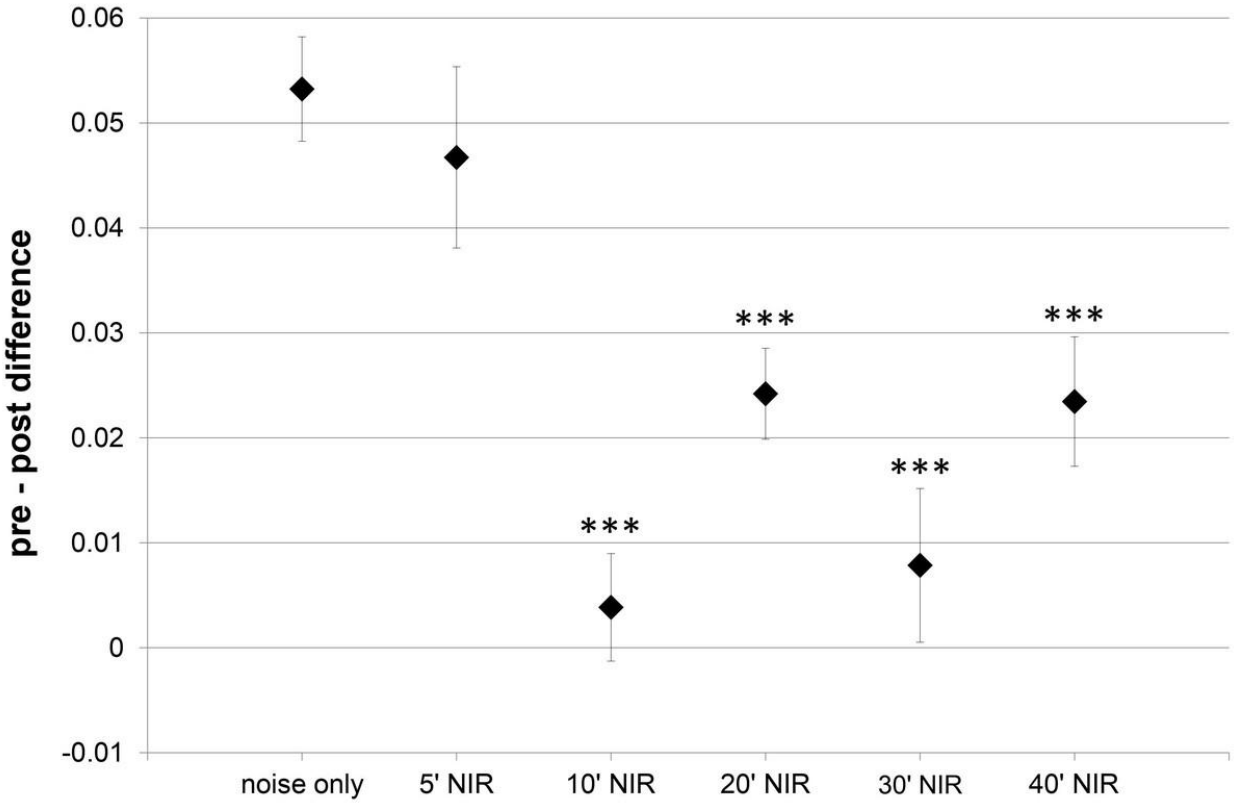


*Figure 6: Slope of the amplitude growth function of ABR wave IV (±SEM). Difference of the slope of the linear part within the amplitude growth function of ABR wave IV (averaged over all tested frequencies) between pre- and post-exposure measurements (post-noise exposure values are subtracted from pre-exposure values). A higher slope difference indicates a smaller post-treatment slope. Asterisks mark statistically significant differences between the NIR-treated groups and the “noise only”-group (significance level ***: p < 0.001).*

5.3. Effect of NIR on cochlea hair cells

It could be shown from the stained cochlear half turns that, in general, for all experimental groups noise expoposure results in a significant decrease of the outer hair cell numbers on both ears (Figure 7 and Figure 8).

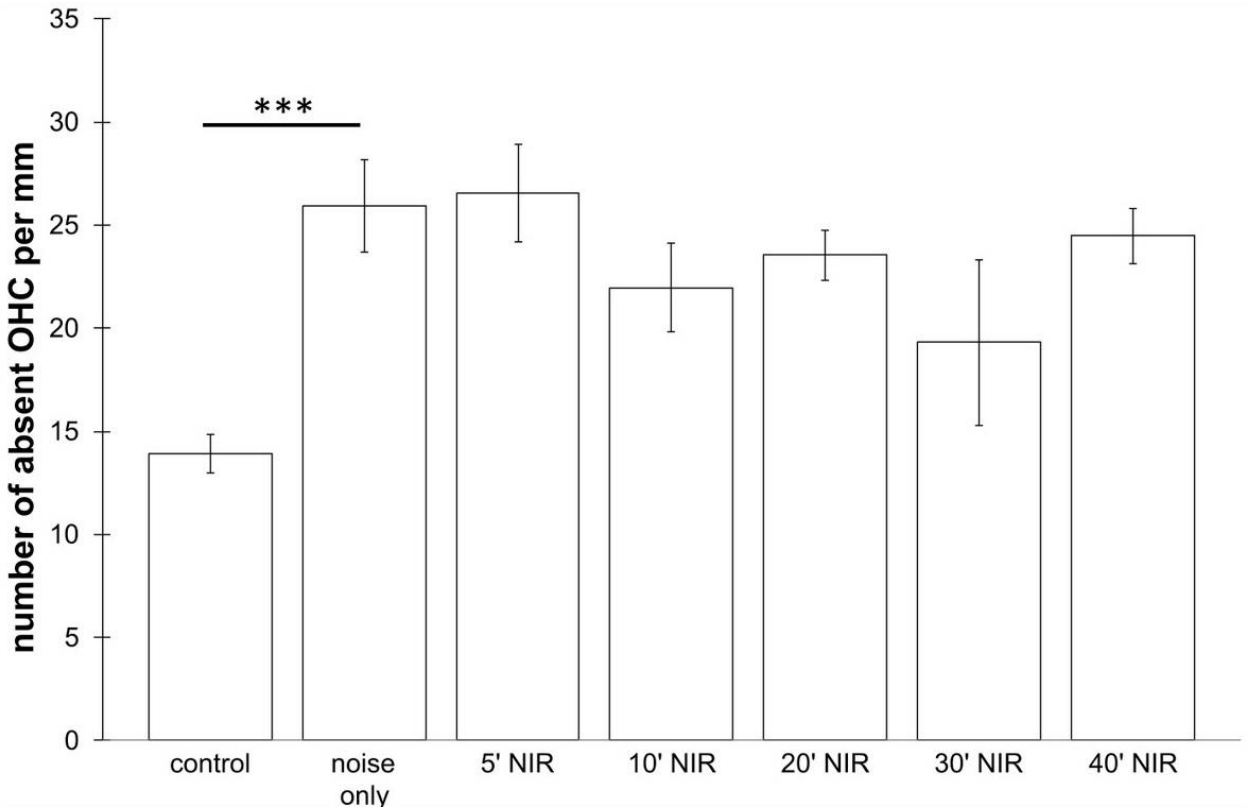


*Figure 7: Outer hair cell counting. Means (±SEM) of absent outer hair cells per mm on the pre-treated side following different durations of NIR-light exposure. Asterisks indicate statistically significant differences (significance level ***: p < 0.001). The "control"-group was not noise exposed and received no NIR pre-treatment. The "noise only"-group received no NIR pre-treatment.*

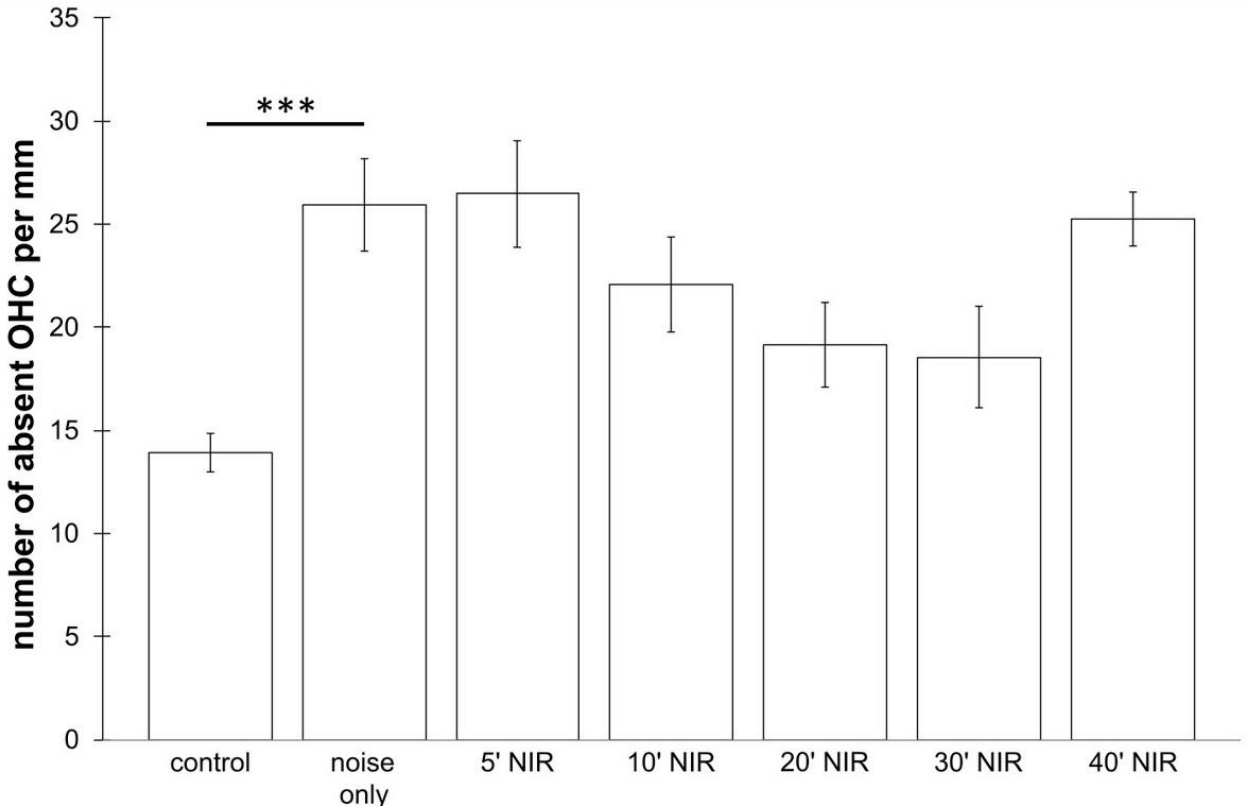


*Figure 8: Outer hair cell counting. Means (±SEM) of absent outer hair cells per mm on the not pre-treated side following different duration of contra-lateral NIR-light exposure. Asterisks indicate statistically significant differences (significance level ***: $p < 0.001$). The "control"-group was not noise exposed and received no NIR pre-treatment. The "noise only"-group received no NIR pre-treatment.*

However, comparing these values with the number of outer hair cells of an intact healthy cochlea shows that the amount of missing outer hair cells is only about 6.5%, which is rather low [40]. No missing inner hair cells were found bilaterally in any of the groups. Furthermore, the mean number of missing outer hair cells over the entire length of the organ of Corti was determined. Outer hair cell loss decreased from base to apex without any specific correlation to the functional analysis (ABR-thresholds). This was found for all experimental and control groups (Figure 9). It is of note that the count differences of the outer hair cells between the pre-treatment groups and between ipsi- and contralateral sides of each experimental group failed to reach statistical significance.

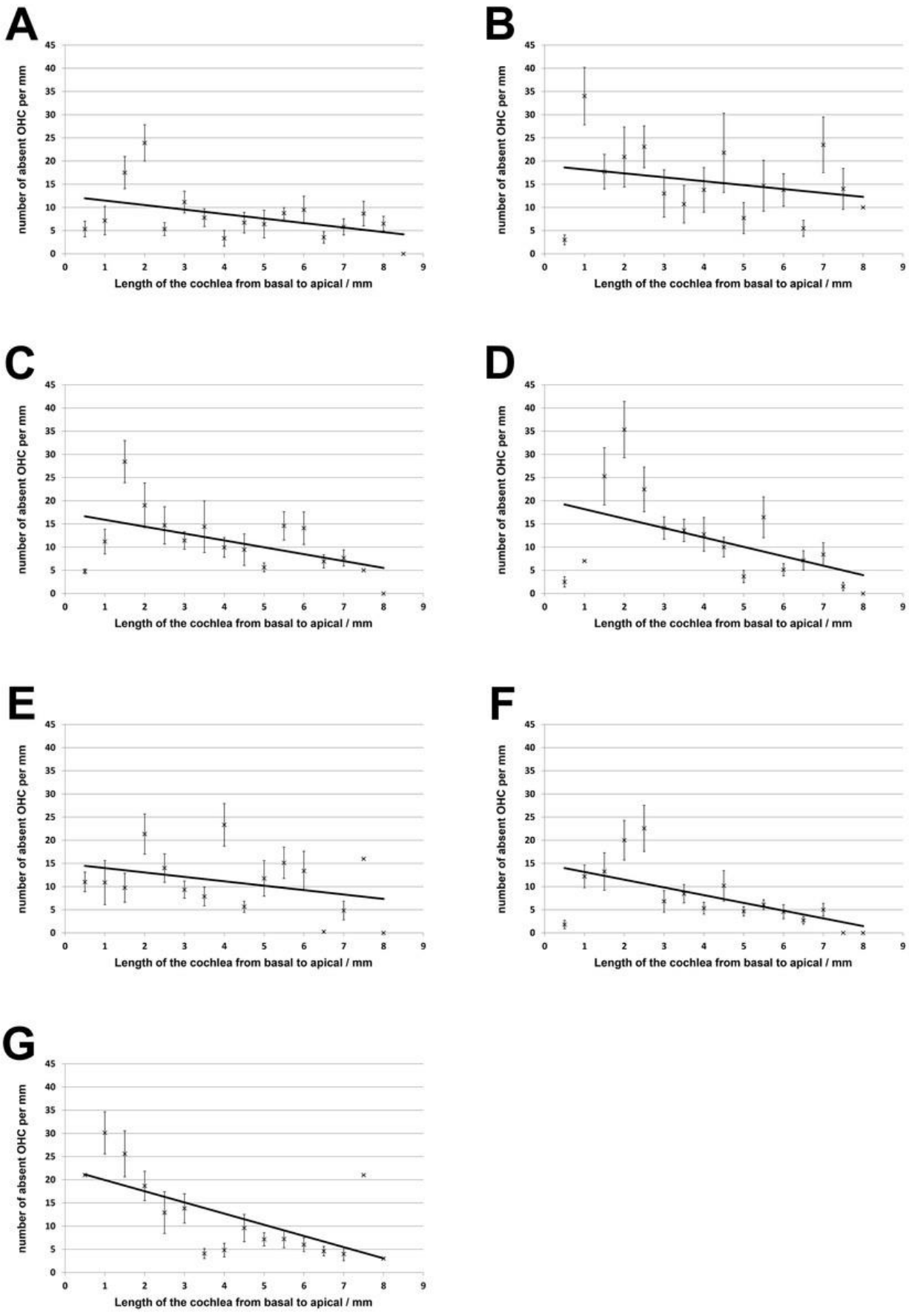

A
B
C
D
E
F
G
number of absent OHC per mm
Length of the cochlea from basal to apical / mm

Figure 9: Distribution of cochlear outer hair cell loss. Distribution of outer hair cell loss along the organ of Corti for all experimental and control groups (A, untreated controls; B, noise only; C, 5 min NIR-pretreatment; D, 10 min NIR-pretreatment; E, 20 min NIR-pretreatment; F, 30 min NIR-pretreatment; G, 40 min NIR-pretreatment). Error bars represent standard error (SEM).

5.4. Effect of NIR on the residual hearing upon cochlear implant electrode array insertion [39]

Our results demonstrated a lower hearing loss four weeks after cochlear implantation for implanted cochleae that received a 15 min NIR treatment preoperatively. The differences between the sham- and NIR-exposed ABR-threshold shifts were significant from 8 kHz onward ($p < 0.05$; Figure 10).

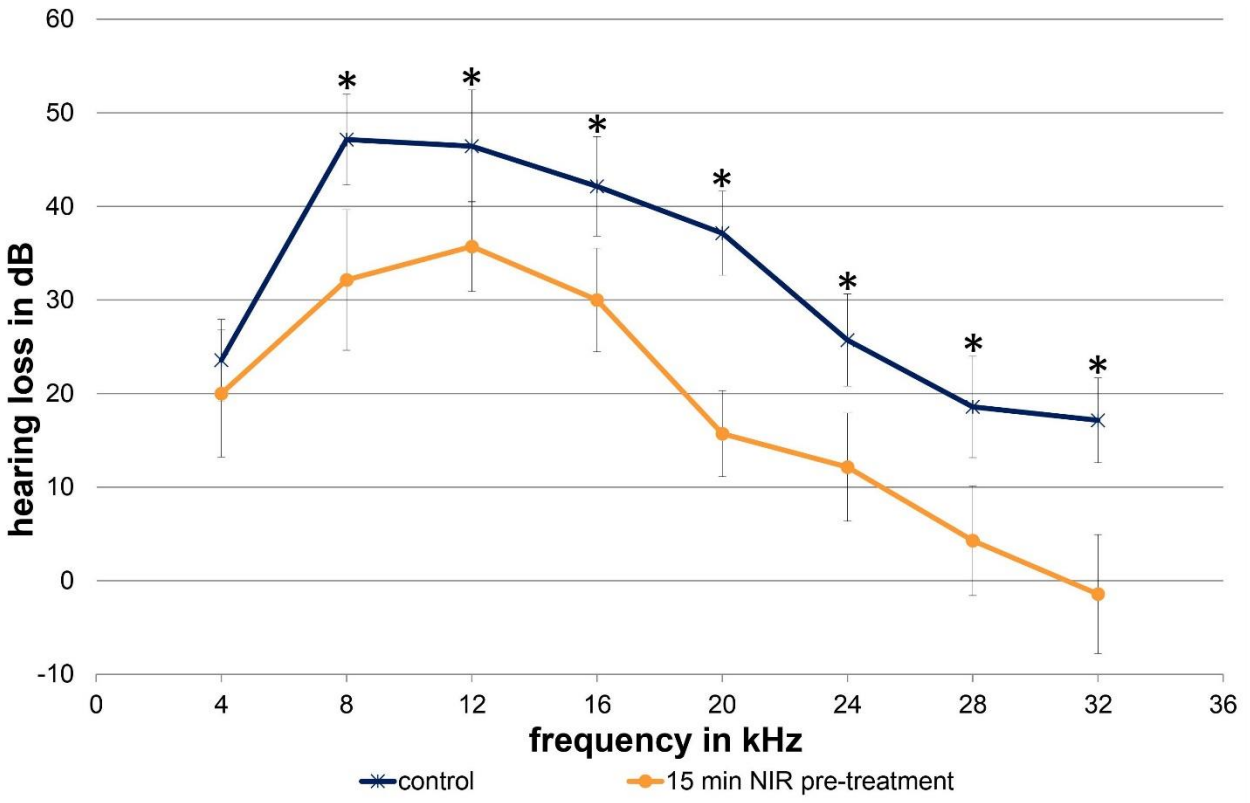


*Figure 10: Threshold shift of ABR following cochlear implantation in NIR pre-treated ears and controls. Error bars represent standard error (SEM). Asterisks indicate significant differences between the groups (significance level *: $p < 0.05$).*

The hearing loss of the sham-exposed and implanted control group ranged between 17.14 dB and 47.14 dB, the pre-treated and implanted groups ranged between −1.43 dB and 35.71 dB on average (Table 3). After the electrode array was inserted, the highest hearing loss was observed in the low-frequency range and the lowest hearing loss was observed in the high-frequency range. The mean hearing losses and standard deviations were 32.2 ± 5.0 dB for the control ears and 18.6 ± 6.0 dB for the NIR pre-treated ears. The protection of the NIR pre-treatment is shown in Figure 11. The values with a significant protection ranged between 10.7 and 21.4 dB, across frequency.

Table 3: Mean values in dB for the hearing threshold shifts after 15 minutes NIR light pre-treatment and without NIR light pre-treatment (contra-lateral side) with associated standard error (SEM).

		Frequency (kHz)							
		4	**8**	**12**	**16**	**20**	**24**	**28**	**32**
NIR light treatment time (min)	**15**	20.00	32.14	35.71	30.00	15.71	12.14	4.29	−1.43
	SEM	6.79	7.52	4.80	5.54	4.58	5.77	5.86	6.36
	0 (control)	23.57	47.14	46.43	42.14	37.14	25.71	18.57	17.14
	SEM	4.35	4.84	6.02	5.31	4.49	4.92	5.42	4.53

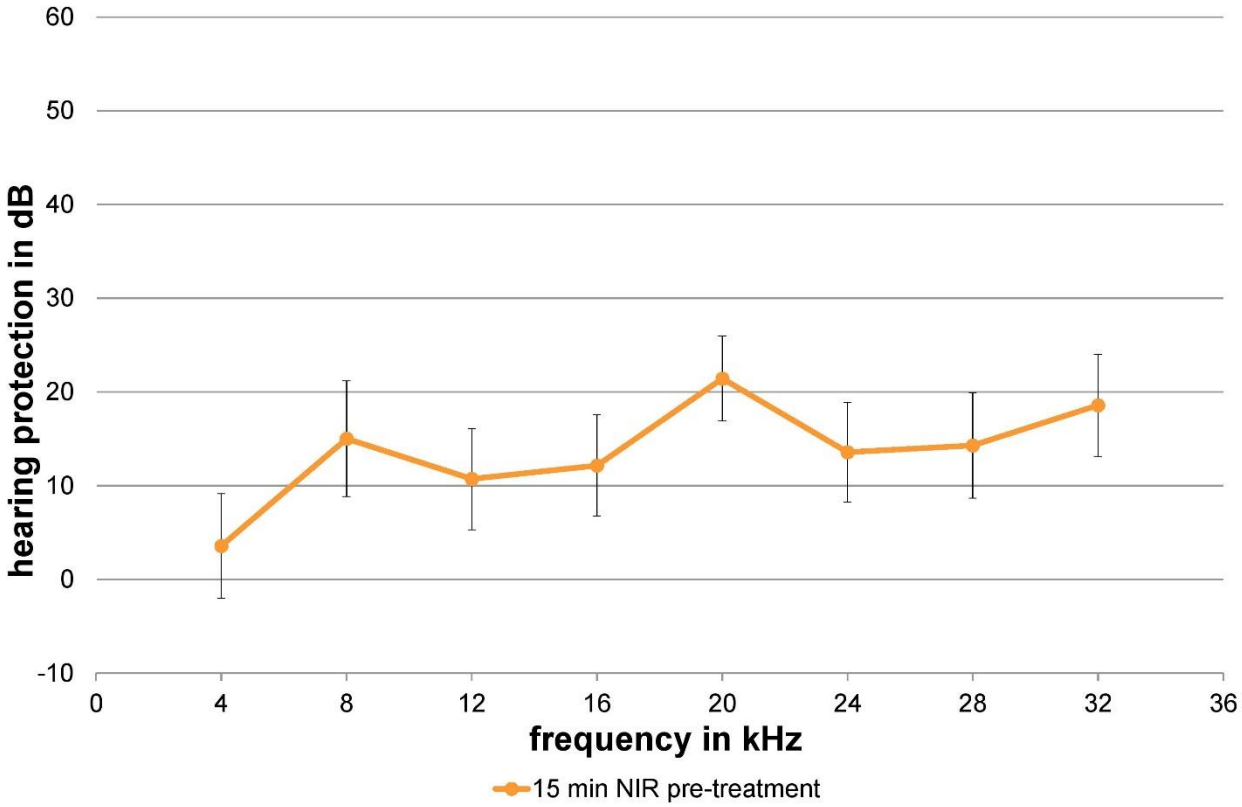


Figure 11: Means (±SEM) in dB of hearing protection for a 15 min NIR-light pre-treatment before cochlear implantation.

The analysis of the cochlear hair cells showed no loss of inner hair cells on either the NIR light-treated or untreated sides. In the sham-exposed and implanted ears, the contralateral side, an increased number of missing outer hair cells was found on average compared to the ipsilateral NIR-exposed and implanted side. This difference was statistically significant ($p < 0.05$). Implanting without a NIR pre-treatment induced an outer hair cell loss which ranged from 103 to 164 cells (mean 129.29 ± SEM 7.77) along the entire length of the cochlea. The NIR-pre-treated group showed a range of outer hair cell loss from 68 cells up to 124 cells (mean 88.75 ± SEM 7.33) cells along the entire length of the cochlea. This represents a 39.8% reduction in outer hair cell loss due to the NIR light pre-treatment (Figure 12).

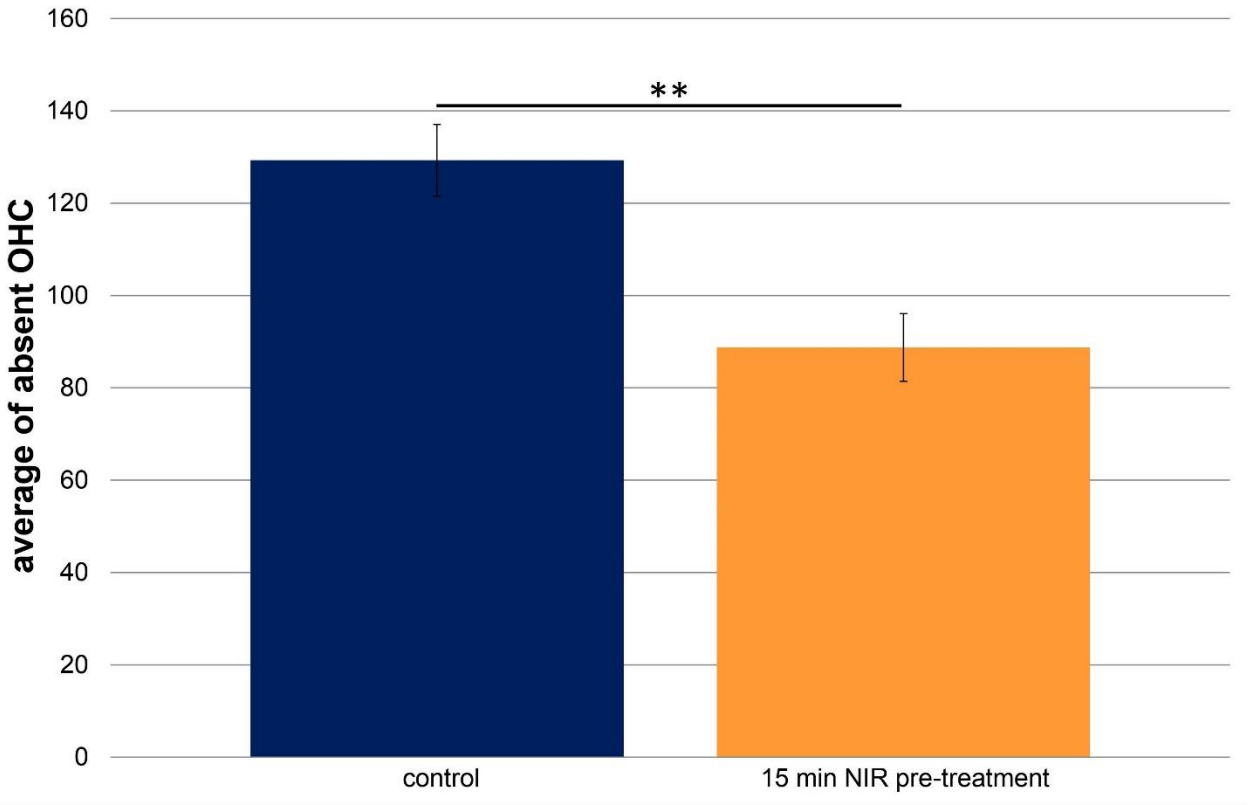
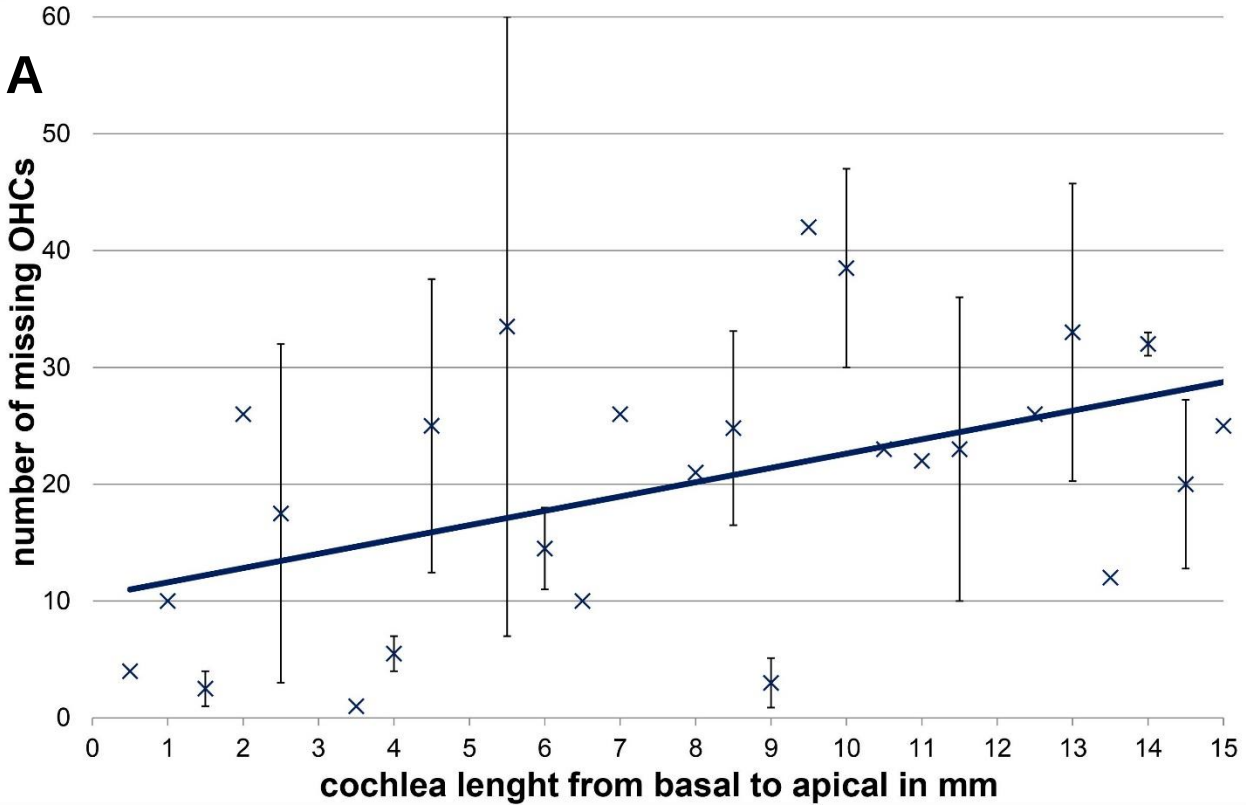


*Figure 12: Total number of missing OHCs following cochlear implantation in pretreated (NIR) ears and controls. Asterisks indicate highly significant differences between the groups (significance level **: $p < 0.01$). Error bars represent standard error (SEM).*

The missing cells were counted per mm along the cochlea and plotted graphically (Figure 13). An analysis of hair cell loss along the cochlea showed that there was a tendency for more cells to be missing at the apex of the cochlea than at the basal level, starting at the round window. The comparison of the number of missing basal with apical hair cells was not statistically significant. This finding applies to both, the untreated contralateral side (basal at 0.5 mm: 4 cells to apical at 15 mm: 25 cells) and the NIR-pre-treated ipsilateral side (basal at 0.5 mm: 0 cells to apical at 15 mm: 14 cells), with the linear trend line showing an identical slope. However, the trend also indicates that the NIR pre-treatment was similarly effective for all cochlear areas.


A
number of missing OHCs
0
10
20
30
40
50
60
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
cochlea lenght from basal to apical in mm

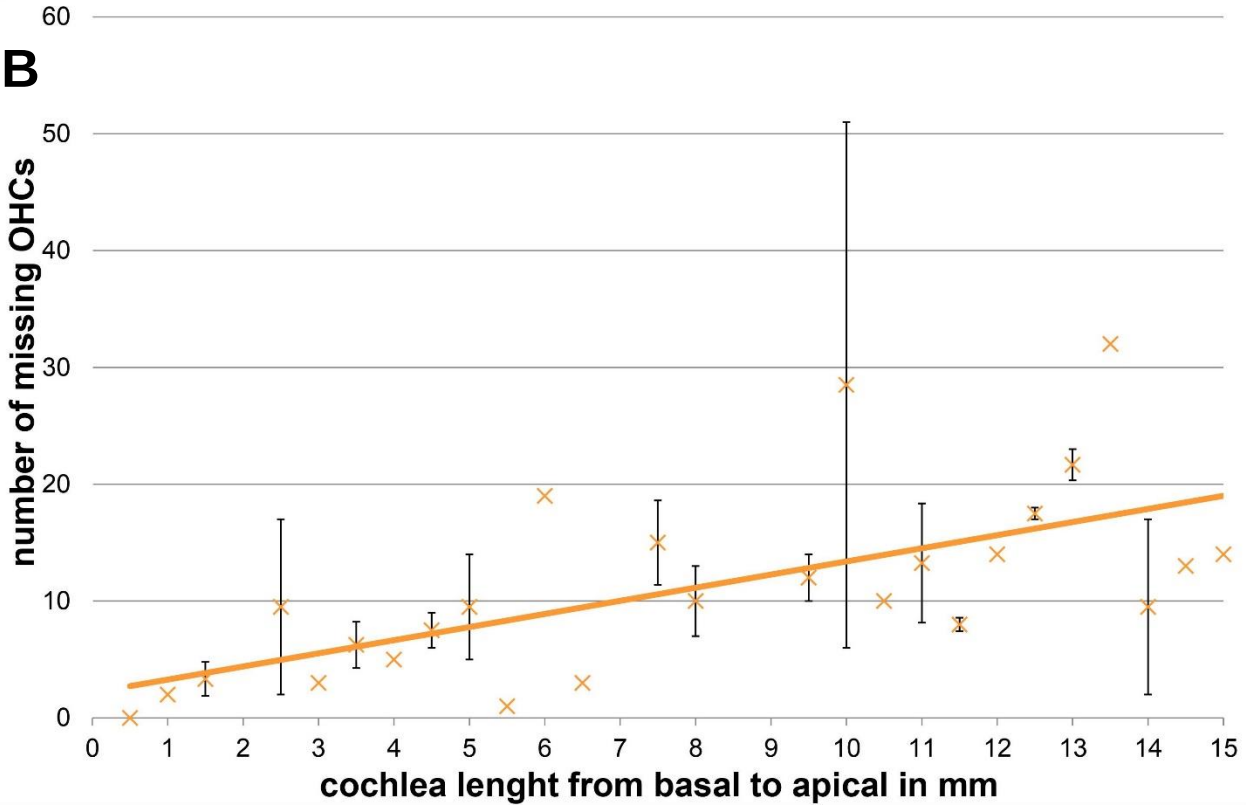

B
number of missing OHCs
0
10
20
30
40
50
60
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
cochlea lenght from basal to apical in mm

Figure 13: Missing OHC distribution along the basilar membrane (basal to apical) for controls (A) and NIR light-pre-treated ears (B) after cochlear implantation. Error bars represent SEM.

6. Discussion

Data from the present NIR study suggest that a pre-treatment with NIR has a protective effect on hearing thresholds after noise exposure, or cochlea implant electrode array insertion. The ABR results indicate, that the selected pre-treatment with 10 to 15 minutes NIR light leads to a significant reduction of hearing loss over the entire frequency range investigated. The efficacy of a single short duration cochlea pre-treatment with NIR light was similar compared with multiple prolonged post-treatments at 60 minutes per day for 12 days [28].

6.1. Effect of NIR on noise induced hearing loss

In the first study of this thesis [36], it was shown that hearing loss decreased significantly for one NIR exposure period compared to the unirradiated but noise-exposed group. Surprisingly, this effect does not improve beyond the 10 minutes treatment time, but leads instead to saturation. Cochleae pre-treated with 5 minutes NIR light show statistically significant differences from noise exposed only cochleae only at low (5 kHz) and high (35 and 40 kHz) frequencies. The area in between these frequencies is overlapped by the broad-band white noise trauma from 5 to 20 kHz. The resulting damage in cochlear regions outside the trauma frequencies seems to be better protected by the short NIR light application than within the trauma frequencies due to the lower amount of pressure on this area.

Within the NMRI mice experiment [36], it seems to be contradictory that the NIR pre-treatment causes no significant changes in the loss of the inner and outer hair cells of mice cochleae. This indicates that the cause for the observed effect on hearing thresholds has to be located within another structure of the auditory pathway, such as the spiral ganglion cells as a connection between the inner hair cells and the auditory brain structures, the lateral wall, or the ribbon synapses. In order to investigate this effect in more depth, the relationship between the stimulus intensity and the amplitude of a

summating potential, generated in the auditory brainstem, is used. This is the distinct ABR wave IV in the present case. Our investigations have shown that there is a significant difference between the slope of this relationship in animals with and without NIR pre-treatment. The observed difference, determined from the pre- and post-experimental wave IV amplitude growth slopes, is an indication of near-normal sensitivity to increased stimulus intensity and, at the same time, almost normal loudness growth due to the NIR pre-treatment.

Even if the target of the NIR is unclear until now, the postulated mechanism for the observed hearing protection is the activation of cytochrome C oxidase in the target structure. This enhances ATP synthesis and thus increases ATP production in advance. The mitochondrially-produced ATP is strongly suspected of overcoming harmful damage [41–43].

6.2. Effect of NIR on cochlear implant electrode insertion trauma

The results of the outer hair cell evaluation after cochlear implantation [39] correlate well with the observed hearing preservation. When counting the missing outer hair cells, it was found that on the pre-treated side the loss of those hair cells was reduced by almost 40%, compared to the intra-individual contralateral control. This result of preoperative irradiation with the NIR can be seen both in the average and in the absolute values. The real effect of the treatment could be possibly much larger, since recent studies have shown that the contra-lateral side could also have received some amount of radiation [44]. The 808 nm NIR, applied in the present studies, penetrates the much bigger human skull with a significant therapeutic effect in stroke patients [45]. Nevertheless, the damage to the cochlear hair cells due to implantation turned out to be much less than expected. Only approximately 130 outer hair cells (OHCs) were missing out of almost 6,300 in the guinea pig cochlea. The same holds true for the protective effect. It is very unlikely that the rescue of approximately 40 OHCs can explain protection of 20 dB on average for the hearing threshold. As discussed above, for the experiments in noise induced hearing loss, other or additional targets appear to be mainly responsible for the hearing protection by NIR. The investigation of these targets is of particular interest, since only a minor amount of outer hair cells contribute to the residual hearing in cochlea implant patients.

When looking closely at the outer hair cell loss along the cochlea, in the guinea pig [39] it can be seen that as we look along the cochlea that loss increases towards the apex: more outer hair cells are missing due to implantation. This result is found in both ipsilateral

NIR-pre-treated ears and in the contralateral control cochleae. This corresponds well with the higher hearing loss in the low frequency range. According to first considerations, this actually contradicts the insertion trauma, because during the electrode insertion, purely mechanically, the first basal turn and thus the outer hair cells located there were directly affected by the electrode. Therefore, this result suggests that the insertion of the conical electrode triggered a pressure wave that was so strong that even in the apical area of the cochlea significant hair cell damage could be caused. In this case, it would be of great interest for future experiments whether a reduced pressure during the insertion can reduce the hair cell loss.

Another promising approach for the protection of residual hearing during CI-surgery is based on the use of a thermal probe for cooling the cochlea before and during electrode insertion [32]. A mild to moderate therapeutic hypothermia of 4 to 6 °C when applied to the cochlea shows a reduction in residual hearing loss associated with electrode insertion trauma. The local hypothermia was delivered to the central turn of the cochlea in the rat animal model for a 20 minute period before and after implantation of a cochlea electrode, using a probe specially perfused with chilled fluorocarbon. In the group of normothermic implants, a significant loss of residual hearing was observed by ABR measurements. Residual hearing was significantly preserved in the cochleae receiving therapeutic hypothermia. Histology confirmed a significant loss of outer hair cells in normothermic cochleae receiving the surgical trauma compared to the hypothermic group. This result shows a similar innovative effect as the pre-treatment with NIR of this work, especially for the preservation of the residual hearing in the course of a cochlea implantation. However, the use of the NIR is technically much easier to implement clinically.

The present work shows in particular that the use of near-infrared light as a therapeutic approach is very innovative and promising. Not only the pecuniary advantages of a one-time purchase, but also the potential importance in everyday clinical practice, highlight the unique advantages of near-infrared light as a pre-treatment method e. g. in cochlear implant surgery.

The surgeon benefits from a simple structure of the near-infrared light apparatus and the required duration for the pre-treatment of approximately 15 minutes could be implemented in the surgical procedure.

Further investigations should strongly focus on targets for the observed NIR effects on hearing preservation. This could possibly further increase the efficacy of the method.

7. List of abbreviations

°C – degree Celsius
% – percent
ABR – auditory brainstem response
AIF – apoptosis-inducing factor
ATP – adenosine triphosphate
BDNF – brain-derived neurotrophic factor
bFGF – basicfibroblast growth factor
cAMP – cyclic adenosine monophosphate
CI – cochlea implant
DAPI – 4',6-diamidin-2-phenylindole
dB – decibel
DHEAS – dehydroepiandrosterone sulfate
DNA – deoxyribonucleic acid
ED_{50} – effective dose for 50% of the population
EDTA – ethylenediaminetetraacetic acid
endoG – endonuclease G
FDA – US Food and Drug Administration
h – hour
HPN-07 – 2,4-disulfophenyl-N-tert-butylnitrone, a nitrone-based free radical trap
Hz – hertz
IR – infrared
K^+ – potassium ion
kHz – kilohertz
LED – light-emitting diode
min – minute
mm – millimeter
mW – milliwatt
µm – micrometer
µM – micromolar
µV – microvolt
NAC – N-acetyl-L-cystein

NIHL – noise-induced hearing loss
NIR – near-infrared light
nm – nanometer
NMRI – Naval Medical Research Institute, NMRI mouse is a general purpose model in different studies
NT3 – neurotrophin-3
OHC – outer hair cell
p – significance level
PTS – persistent threshold shift
SEM – standard error
SGN – spiral ganglion neuron
SPL – sound pressure level
TTS – transient threshold shift

significance levels:

***** – $p < 0.05$

****** – $p < 0.01$

******* – $p < 0.001$

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9. Eidesstattliche Erklärung

Ich, Ira Margitta Strübing, versichere an Eides statt durch meine eigenhändige Unterschrift, dass ich die vorgelegte Dissertation mit dem Thema: „Investigation of Protective Mechanisms to Prevent Sensori-neural Impairments Due to Traumatic Interventions of the Cochlea / Untersuchung von Schutzmechanismen gegen sensorisch-neurale Störungen durch traumatische Eingriffe in die Cochlea“ selbstständig und ohne nicht offengelegte Hilfe Dritter verfasst und keine anderen als die angegebenen Quellen und Hilfsmittel genutzt habe.

Alle Stellen, die wörtlich oder dem Sinne nach auf Publikationen oder Vorträgen anderer Autoren/innen beruhen, sind als solche in korrekter Zitierung kenntlich gemacht. Die Abschnitte zu Methodik (insbesondere praktische Arbeiten, Laborbestimmungen, statistische Aufarbeitung) und Resultaten (insbesondere Abbildungen, Graphiken und Tabellen) werden von mir verantwortet.

Ich versichere ferner, dass ich die in Zusammenarbeit mit anderen Personen generierten Daten, Datenauswertungen und Schlussfolgerungen korrekt gekennzeichnet und meinen eigenen Beitrag sowie die Beiträge anderer Personen korrekt kenntlich gemacht habe (siehe Anteilserklärung). Texte oder Textteile, die gemeinsam mit anderen erstellt oder verwendet wurden, habe ich korrekt kenntlich gemacht.

Meine Anteile an etwaigen Publikationen zu dieser Dissertation entsprechen denen, die in der untenstehenden gemeinsamen Erklärung mit dem Erstbetreuer, angegeben sind. Für sämtliche im Rahmen der Dissertation entstandenen Publikationen wurden die Richtlinien des ICMJE (International Committee of Medical Journal Editors; www.icmje.og) zur Autorenschaft eingehalten. Ich erkläre ferner, dass ich mich zur Einhaltung der Satzung der Charité – Universitätsmedizin Berlin zur Sicherung Guter Wissenschaftlicher Praxis verpflichte.

Weiterhin versichere ich, dass ich diese Dissertation weder in gleicher noch in ähnlicher Form bereits an einer anderen Fakultät eingereicht habe.

Die Bedeutung dieser eidesstattlichen Versicherung und die strafrechtlichen Folgen einer unwahren eidesstattlichen Versicherung (§§156, 161 des Strafgesetzbuches) sind mir bekannt und bewusst.

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Datum Unterschrift

10. Anteilserklärung an den erfolgten Publikationen

Ira Margitta Strübing hatte folgenden Anteil an den folgenden Publikationen:

Publikation 1:

Strübing I, Gröschel M, Schwitzer S, Ernst A, Fröhlich F, Jiang D, Boyle P, Basta D. Neuroprotective effect of near-infrared light in an animal model of CI surgery. *Audiol Neurotol.* 2020: Vol. 26: 95–101.

- Journal Impact Factor: 1.791 (2018).
- Beitrag: Studiendesign der Versuche in Zusammenarbeit mit PD Dr. D. Basta und Dr. M. Gröschel; Durchführung der Lärm- und otochirurgischen Experimente der Meerschweinchen und der Präparationen und Färbungen der Corti-Organe in Zusammenarbeit mit Dr. M. Gröschel; Erhebung, Auswertung und Interpretation der Daten in Zusammenarbeit mit PD Dr. D. Basta und Dr. M. Gröschel; Verfassung der Manuskripterstellung in Zusammenarbeit mit PD Dr. D. Basta und Dr. M. Gröschel; Erstellung aller sechs Abbildungen und Überarbeitung des Manuskripts in Zusammenarbeit mit allen Autoren kritisch.

Publikation 2:

Basta D, Gröschel M, **Strübing I**, Boyle P, Fröhlich F, Ernst A, Seidl R. Near-infrared-light pre-treatment attenuates noise-induced hearing loss in mice. *PeerJ.* 2020: Vol. 8: e9384.

- Journal Impact Factor: 2.353 (2018).
- Beitrag: Studiendesign der Versuche in Zusammenarbeit mit PD Dr. D. Basta und Dr. M. Gröschel; Durchführung der Lärm- und Laserexperimente der Mäuse und der Präparationen und Färbungen der Corti-Organe; Erhebung, Analyse und Interpretation der Daten; Erstellung des Manuskripts in Zusammenarbeit mit PD Dr. D. Basta und Dr. M. Gröschel; Erstellung aller sieben Abbildungen und Überarbeitung des Manuskripts in Zusammenarbeit mit allen Autoren kritisch.

Publikation 3:

Fröhlich F, Gröschel M, **Strübing I**, Ernst A, Basta D. Apoptosis in the cochlear nucleus and inferior colliculus upon repeated noise exposure. *Noise Health.* 2018: Vol. 20: 223–231.

- Journal Impact Factor: 1.798 (2016).
- Beitrag: Auswertung der TUNNEL-Färbungen auf den Hirnschnitten.

Publikation 4:

Fröhlich F, Basta D, **Strübing I**, Ernst A, Gröschel M. Time course of cell death due to acoustic overstimulation in the mouse medial geniculate body and primary auditory cortex. *Noise Health.* 2017. Vol. 19: 133–139.

- Journal Impact Factor: 1.798 (2016).
- Beitrag: Auswertung der TUNNEL-Färbungen auf den Hirnschnitten.

Publikation 5:

Fröhlich F, Ernst A, **Strübing I**, Basta D, Gröschel M. Apoptotic mechanisms after repeated noise trauma in the mouse medial geniculate body and primary auditory cortex. *Exp Brain Res.* 2017: Vol. 235: 3673–3682.

- Journal Impact Factor: 1.917 (2016).
- Beitrag: Auswertung der TUNNEL-Färbungen auf den Hirnschnitten.

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Datum Unterschrift

11. Selected publications

11.1. - Publication 1:

Strübing I, Gröschel M, Schwitzer S, Ernst A, Fröhlich F, Jiang D, Boyle P, Basta D. Neuroprotective effect of near-infrared light in an animal model of CI surgery. *Audiol Neurotol.* 2020: Vol. 26: 95–101.

Journal Data Filtered By: **Selected JCR Year: 2018** Selected Editions: SCIE,SSCI
Selected Categories: **"AUDIOLOGY and SPEECH-LANGUAGE PATHOLOGY"**
Selected Category Scheme: WoS
Gesamtanzahl: 25 Journale

Rank	Full Journal Title	Total Cites	Journal Impact Factor	Eigenfactor Score
1	Trends in Hearing	139	3.024	0.000660
2	HEARING RESEARCH	8,833	2.906	0.011270
3	EAR AND HEARING	4,791	2.842	0.007950
4	JOURNAL OF FLUENCY DISORDERS	968	2.714	0.001010
5	BRAIN AND LANGUAGE	6,186	2.439	0.009710
6	Augmentative and Alternative Communication	806	2.388	0.000860
7	INTERNATIONAL JOURNAL OF LANGUAGE & COMMUNICATION DISORDERS	1,745	2.195	0.003210
8	Language Cognition and Neuroscience	413	1.852	0.001940
9	Noise & Health	1,147	1.798	0.001920
10	AUDIOLOGY AND NEURO-OTOLOGY	1,659	1.791	0.002780
11	JOURNAL OF SPEECH LANGUAGE AND HEARING RESEARCH	6,675	1.771	0.008890
12	INTERNATIONAL JOURNAL OF AUDIOLOGY	2,940	1.733	0.005150
13	JOURNAL OF THE ACOUSTICAL SOCIETY OF AMERICA	43,266	1.547	0.031740
14	LANGUAGE AND SPEECH	1,242	1.471	0.001280
15	JOURNAL OF COMMUNICATION DISORDERS	1,674	1.348	0.002200
16	Journal of the American Academy of Audiology	1,893	1.208	0.002840
17	LANGUAGE SPEECH AND HEARING SERVICES IN SCHOOLS	1,305	1.193	0.001530
18	International Journal of Speech-Language Pathology	675	1.179	0.001960

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Neuroprotective Effect of Near-Infrared Light in an Animal Model of CI Surgery

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Keywords
Near-infrared light · Cochlear implant · Residual hearing

Abstract
Introduction: The preservation of residual hearing has become an important consideration in cochlear implant (CI) recipients in recent years. It was the aim of the present animal experimental study to investigate the influence of a pretreatment with near-infrared (NIR) light on preservation of sensory hair cells and residual hearing after cochlear implantation. ***Methods:*** NIR was applied unilaterally (15 min, 808 nm, 120 mW) to 8 guinea pigs, immediately before a bilateral scala tympani CI electrode insertion was performed. The nonirradiated (contralateral) side served as control. Twenty-eight days postoperatively, auditory brainstem responses (ABRs) were registered from both ears to screen for hearing loss. Thereafter, the animals were sacrificed and inner hair cells (IHCs) and outer hair cells (OHCs) were counted and compared between NIR-pretreated and control (contralateral) cochleae. ***Results:*** There was no IHC loss upon cochlear implantation. OHC loss was most prominent on both sides at the apical part of the cochlea. NIR pretreatment led to a statistically significant reduction in OHC loss (by 39.8%). ABR recordings (across the frequencies 4–32 kHz) showed a statistically significant difference between the 2 groups and corresponds well with the apical structural damage. Hearing loss was reduced by about 20 dB on average for the NIR-pretreated group ($p \leq 0.05$). ***Discussion/Conclusion:*** A single NIR pretreatment in this animal model of CI surgery appears to be neuroprotective for residual hearing. This is in line with other studies where several NIR posttreatments have protected cochlear and other neural tissues. NIR pretreatment is an inexpensive, effective, and noninvasive approach that can complement other ways of preserving residual hearing and, hence, should deserve further clinical evaluation in CI patients.

Introduction

Near-infrared (NIR) light has different effects when applied to mammalian tissue, usually mediated through modulation of the mitochondrial respiratory chain (Fig. 1) and the cytochrome C oxidase in particular [Tsai and Hamblin, 2017]. Its neurotherapeutic efficiency has been widely tested in animal models of Parkinson's [Reinhart et al., 2017], retinal degeneration [Albarracin et al., 2011], in patients following stroke, head trauma or neurodegenerative disorders [Johnstone et al., 2015], and in

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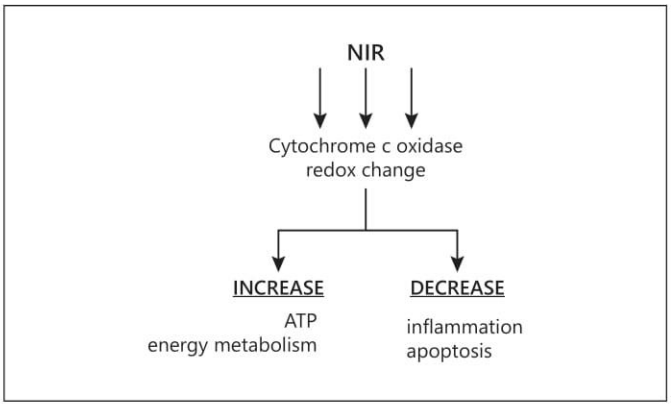

Fig. 1. Effect of NIR light stimulation on the respiratory chain. NIR, near-infrared light.

noise-induced hearing loss [Rhee et al., 2012a]. Animal experiments using NIR to treat noise exposure have shown a decreased cochlear hair cell loss and a significantly lower hearing threshold shift for a daily post-traumatic NIR-treatment over 12 days [Rhee et al., 2012a]. Cell cultures of cochlear outer hair cells (OHCs) showed significantly better recovery from gentamicin exposure if given a daily NIR treatment for 6 days [Rhee et al., 2012b]. Also, a single NIR-preconditioning exposure reduced inflammatory cytokines and markers of oxidative stress upon gentamicin or lipopolysaccharide application [Bartos et al., 2016]. The effects described above are based on photo-biomodulation, that is, a stimulation of mitochondria which in turn increases intracellular ATP production and, thus, reduces apoptosis [Tsai and Hamblin, 2017]. The preservation of residual hearing is becoming more and more important in cochlear implant (CI) surgery as today's CI candidates present with useful amounts of low-frequency residual hearing. Unfortunately, a significant amount of residual hearing frequently disappears postoperatively, even if the electrode array insertion was performed slowly using soft surgery approaches. The main contributors to hearing loss are the cochlear hair cells which can be mechanically overstimulated by the pressure applied during electrode array insertion [Bas et al., 2012]. Based on the previous findings that an NIR treatment seems to increase the robustness of cochlear hair cells, our aim was to apply this tool in an animal model of CI surgery. Usually, the insertion trauma associated with electrode array insertion in guinea pigs leads to a programmed cell death and a significant loss of the OHC [Eshraghi et al., 2015]. The NIR would best be applied as a pretreatment, to allow the cochlea to better withstand the surgical approach. The results from a single preconditioning of cochlear hair cells in vitro [Bartos et al., 2016] are very promising for the success of this in vivo histological and functional study. As an additional contribution in the host of international attempts to preserve residual hearing, the noninvasive, inexpensive application of NIR light appears to be both appropriate and interesting.

Materials and Methods

Eight guinea pigs (Dunkin Hartley, aged 28–30 weeks) were included in this study. Two weeks before surgery, frequency-specific auditory brainstem responses (ABRs) (4 dB increments) were recorded under anesthesia (fentanyl, midazolam, and medetomidine) for the frequencies 4, 8, 12, 16, 20, 24, 28, and 32 kHz from both ears (for methodology see Basta et al., 2015). Subdermal needle electrodes were placed at the vertex (reference), mastoid (active), and leg (ground). Tone burst stimuli of 3-ms duration and using a Blackman envelope (1.5 ms raise/fall time) were delivered through in-ear high-frequency transducers. Masking of the contralateral ear was achieved using a broadband noise (2–32 kHz) at a level 30 dB below that of the stimulus intensity. Recording and data acquisition parameters used a 31.1 bursts per second stimulus rate having an alternating phase. There were 1,024 sweeps made per frequency and level. The recording parameters included an amplifier gain of 100k, recording via a bandpass filter of 100–3000 Hz. The sampling rate used to collect electrophysiological data was 32 kHz. Stimulation and recording were controlled using the SmartEP software: version 5.33 (Intelligent Hearing Systems, Miami, FL, USA). For each recorded frequency, the peak-to-peak ABR amplitudes, specifically the maximum deflection of waves III/IV were determined for each different stimulus intensity. The hearing threshold was estimated by a stepwise decrease of the stimulus intensity until no electrophysiological response was visually detectable.

Immediately before implantation via the open bulla, each animal had the entire cochlea of 1 randomly selected side pretreated with NIR. The NIR dosage was applied for 15 min, using a wavelength of 808 nm and a power of 120 mW. Delivery was from a point laser module (Picotronic GmbH, Koblenz, Germany), that, from its output lens that produced a 7-mm diameter beam of collimated NIR. The contralateral side was not pretreated and, hence, served as a control. The laser beam was directed toward the cochlea (Fig. 2), its direction being controlled through a web camera having sufficient bandwidth to image the NIR.

Following NIR pretreatment, all 8 animals were implanted on both sides under anesthesia (fentanyl, midazolam, and medetomidine) with a conically shaped CI electrode array designed for the guinea pig cochlea (Advanced Bionics GmbH, Hanover, Germany). The electrode array diameter was tapered, ranging from 0.4 mm at the tip to 0.6 mm at the proximal end. Insertion was performed using a speed of 1 mm/s, for an insertion depth of 6 mm. After the insertion was complete, the area around the cochlea was closed using muscle tissue, secured using fibrin glue.

Four weeks after surgery, the same frequency-specific ABR recordings described above were repeated. Mean hearing threshold shift was calculated for each individual ear from these results, and

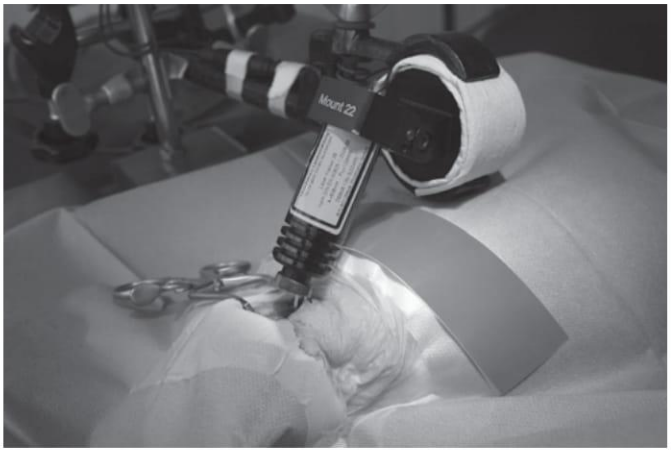

Fig. 2. Animal experimental setup with NIR light irradiation. NIR, near-infrared light.

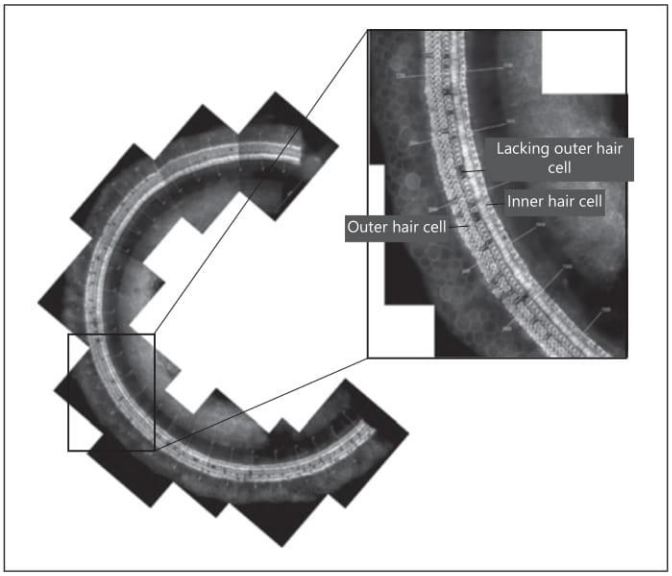

Fig. 3. Whole-mount preparations of the phalloidin-stained guinea pig cochlea.

the results obtained 2 weeks before surgery, with results presented as the mean relative hearing loss.

After the postoperative ABR measurement, the animals were sacrificed, being fixated using a perfusion with 4% paraformaldehyde delivered via the left heart chamber. The cochleae were carefully taken out. After decalcification overnight (replaced after 8 h) with 0.4 M EDTA (pH 8.0; Roth, Germany), the cochleae were stained by using the Alexa fluor-488-phalloidin staining to visualize F-actin compartments in the cytoskeleton as follows. Cochleae were washed in PBS (Gaithersburg, MD, Gibco, USA) and incubated for 30 min with 0.2% Triton-X (Sigma-Aldrich, Darmstadt, Germany) in PBS. Afterward the tissue was incubated for 60 min with Alexa Fluor-488-phalloidin (Molecular Probes, Eugene, OR, USA) solution (1:40 with PBS) on a shaker in the dark and finally washed with PBS. Each organ of Corti was dissected from the modiolus of the cochlea by a micro-scissor to obtain large fragments of half turns. All cochlear fragments were embedded with Roti-Mount Fluorocare and diamidino phenylindole (DAPI; Roth, Germany) on microscope slides with coverslips. The stained cochleae were magnified microscopically (20×) with fluorescence channels for phalloidin (470 nm) and DAPI (365 nm). The images were digitized with the AxioCam ICc1 digital camera (Zeiss, Oberkochen, Germany) using the ZEN 2.3 camera software (Zeiss, Oberkochen, Germany). The guinea pig organ of corti represents a clear architecture of 1 row of inner hair cells (IHCs) and 3 rows of OHCs [Wang et al., 2002]. So, it is usual to count only the missing hair cells, which are clearly detectable by the spaces within a row. Figure 3 is a microphotograph showing IHCs and OHCs within the guinea pig cochlea. The number of missing hair cells was counted manually on the basis of the DAPI staining with permanent comparison of the phalloidin staining. The basilar membrane length was measured using the ImageJ 1.47d software (National Institutes of Health, USA).

Data were compared between the 2 ears of each animal, that is, with or without NIR pretreatment using either the Wilcoxon test (for not normally distributed data) or *t* test for paired samples (for normally distributed data). Data distribution was tested with the Kolmogoroff-Smirnoff test. Data from ABR recordings were compared using the *t* test for paired samples (normally distributed data) with a Bonferoni alpha-correction for multiple comparisons. The total number of missing OHCs following cochlear implantation was compared between sides (pretreated/not pretreated) by the *t* test for paired samples (normally distributed data). The SPSS software (IBM SPSS Statistics Version 25, IBM Corp., New York, NY, USA) was used for all statistical analyses. The level of significance for all statistical tests was set at $p < 0.05$.

As an additional control, temperature measurements of the cochlea were performed during NIR exposure in 2 additional animals (4 ears). The surgery was very similar to that used for the 16 electrode array implantations described above. A thermistor probe (Pt 100 type K, diameter 0.5 mm) was inserted into the cochleostomy. Temperature was measured with a calibrated high-resolution temperature measurement system (T955, Dostmann Electronic GmbH, Wertheim, Germany) before NIR exposure and then after 5, 10, and 15 min of NIR light application.

Results

Frequency Specific ABR Recordings

The CI electrode array insertion induced the highest amount of hearing loss in the low-frequency range and the lowest amount of hearing loss in the high-frequency range (Fig. 4). There was a statistically significant difference between the pretreated ears and the control ears. The level of significance was reached for frequencies of 8 kHz and above (Fig. 4). The average hearing loss and standard deviations of the control and pretreated ears were 33.5 ± 5.1 dB and 18.4 ± 5.8 dB, respectively. The protective ef-

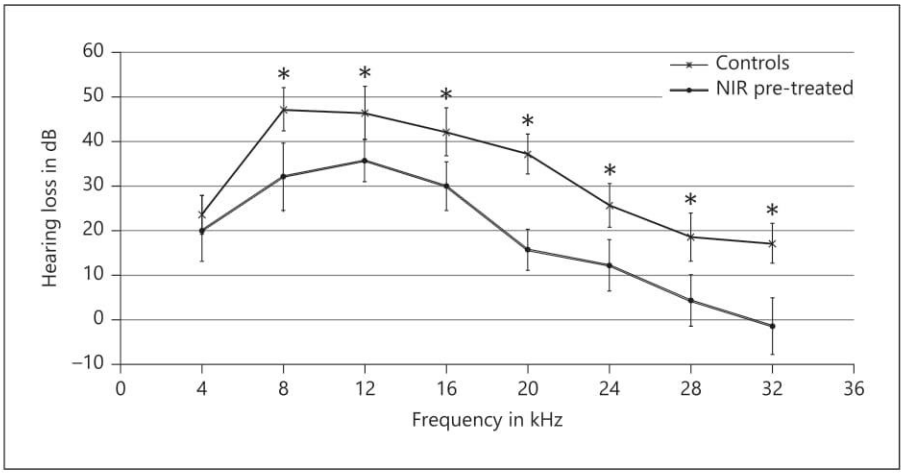

Fig. 4. Threshold shift of ABR following cochlear implantation in pretreated (NIR light) ears and controls. Error bars represent standard error. Asterisks indicate significant differences between the groups ($p < 0.05$). ABR, auditory brainstem response; NIR, near-infrared light.

fect of the NIR pretreatment amounted to between 10.7 and 21.4 dB depending on the individual frequency being compared.

Temperature Measurements

The average local intra-cochlear temperature increase after 15 min of NIR pretreatment was 0.95 ± 0.12°C. The increase from the baseline (34.3 ± 0.15°C) was nearly linear, with values of 34.6 ± 0.11°C, 34.8 ± 0.06°C, and 35.25 ± 0.2°C after 5, 10, or 15 min, respectively.

Hair Cell Counts

When analyzing the cochleae after sacrifice, no loss of IHCs was detected bilaterally. Cochlear implantation induced a higher OHC loss in the apical part of the cochlea than the basal part (Fig. 5). All parts of the cochlea were similarly affected by the NIR pretreatment in that the differences in OHC counts between pretreated cochleae and control cochleae were uniformly distributed along the cochleae.

The OHC counts showed statistically significant differences between the pretreated and the control ears ($p = 0.006$; Fig. 3). This results in a reduction of OHC loss by 39.8% for the pretreated group. Along the whole length of the basilar membrane, the controls showed an average OHC loss of 129.3 ± 7.8 cells, in contrast to 88.7 ± 7.3 cells in the pretreated group (Fig. 6).

Discussion

Our results confirm the neuroprotective effect of NIR in our animal model of CI surgery. As demonstrated earlier, NIR can penetrate the bony shell of the skull or – in our case – the temporal bone and in particular the cochlea [Jagdeo et al., 2012]. This ability makes NIR with an 808-nm wavelength an interesting tool for sensory organs (retina and cochlea), and also for application in the brain. Apart from those acute lesions as induced with a CI electrode array, chronic neurodegenerative disorders (e.g., Parkinson's [Reinhart et al., 2017]) respond to this type of irradiation in animal models and human applications as well.

Our findings demonstrated neuroprotective effects not only audiological by ABR recordings but also by microstructural analysis of the cochlear OHCs. Interestingly, the preservation of residual hearing by NIR could be much higher than we observed. The intra-individual comparison of the pretreated and control sides might have masked the so-called remote effects of NIR treatment [Ganeshan et al., 2019]. This new concept of a systemic NIR action shows a protective effect on neuronal tissue independent of the place of NIR treatment. Even if the effect on the area directly exposed to NIR is significantly higher than elsewhere, the remaining tissue of the same animal may still be influenced.

Surprisingly, the apical OHCs were more affected than the basal ones, where the electrode array was located and some insertion trauma might have been expected [Roland and Wright, 2006]. This observation corresponds well with the higher hearing loss recorded in the low-frequency region. It appears that intracochlear pressure changes applied to the apical part of the cochlea during the insertion process influence OHC loss more than the electrode array touching cochlear structures in the basal turn [Robertson and Johnstone, 1979; Todt et al., 2014]. These pressure changes were minimized as far as possible since the electrode was inserted very slowly and the arm of the surgeon was supported

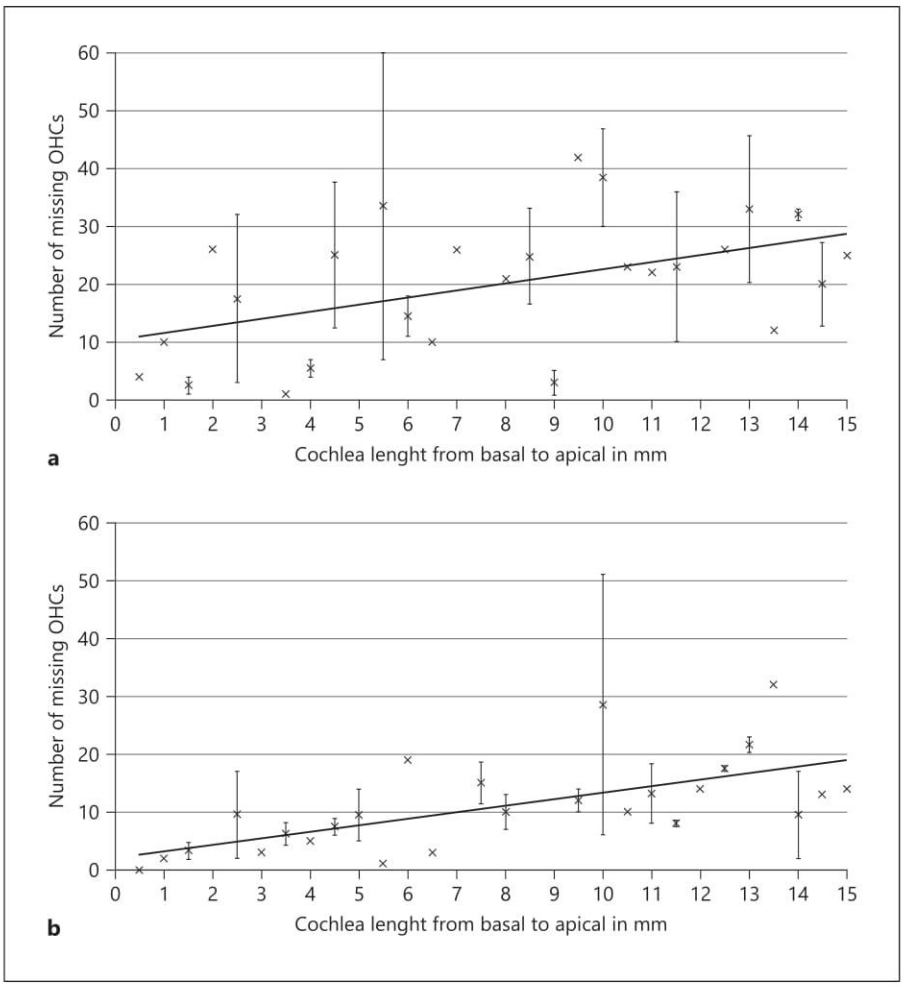

Fig. 5. OHC distribution along the basilar membrane (basal to apical) of controls (**a**) and NIR light-pretreated ears (**b**) following cochlear implantation. Error bars represent standard error. OHC, outer hair cell; NIR, near-infrared light.

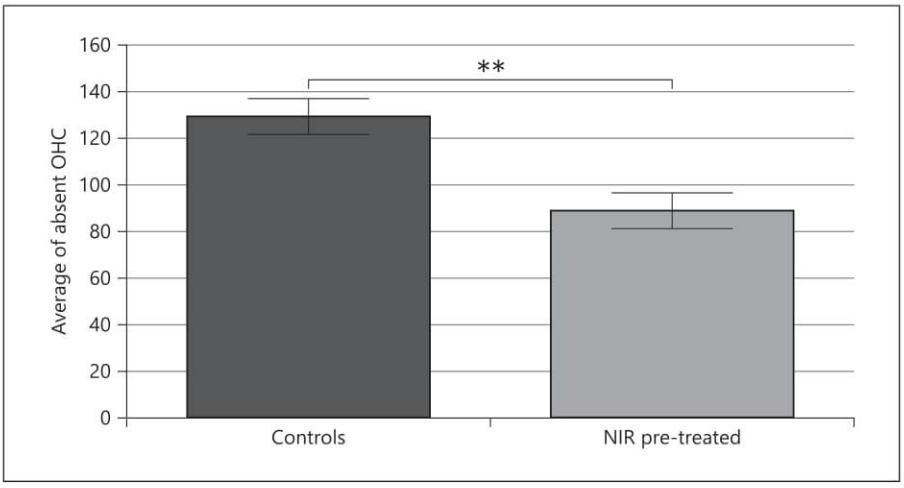

Fig. 6. Total number of missing OHCs following cochlear implantation in pretreated (NIR light) ears and controls. Asterisks indicate highly significant differences between the groups ($p < 0.01$). Error bars represent standard error. OHC, outer hair cell; NIR, near-infrared light.

during insertion [Todt et al., 2016]. It is possible this is why the IHCs were all perfectly preserved. In addition, the insertion of the electrode array could possibly induce neuroprotective mechanisms, responding to the mechanical injury. Such effects have been previously described in studies of retinal injury [Faktorovich et al., 1992]. Thus, the region where the electrode array was located could be more protected from neurodegeneration than apical regions which

receive pressure changes only. Even if the threshold shift of ABR responses corresponds well with the region of highest OHC loss, the insertion-induced threshold shift could not be explained alone by approximately 130 missing OHC out of almost 6,300 in the guinea pig cochlea. The same holds true for the protective effect. It is very unlikely that the rescue of approximately 40 OHCs can explain protection of 20 dB on average for the hearing threshold. Besides the effects observed for cochlear OHCs, there must be other pathological mechanisms arising from electrode array insertion (e.g., lateral wall pathologies and synaptic changes [Wright and Roland, 2013; Reiss et al., 2015]). Fortunately, the NIR pretreatment seems to also have a protective effect on these uncovered mechanisms. Future research should investigate these additional processes in more detail.

Surprisingly, a single short NIR pretreatment was similarly effective in our CI experiments to several longer posttreatments (60 min per day over 12 days), particularly considering a comparable wavelength and power [Rhee et al., 2012a]. This could be related to different pathologies induced by noise and our mechanical intervention. Possibly, the efficacy of a pretreatment is higher due to protective mechanisms being activated before any structural damage is induced. It could be more complicated to reverse structural changes (e.g., apoptotic mechanisms) than to prevent them occurring in the first place. More research is also necessary to clarify this hypothesis.

Several groups have proposed various steps to preserve residual hearing following cochlear implantation (e.g., drug application, surgical technique modifications, and electrode design variations). However, the long-term results are not yet very promising [Snels et al., 2019].

NIR light with its intracellular effects on the mitochondrial respiratory chain increases the proton transport out of mitochondria and, thus, increases the production of ATP, so that inflammatory and apoptotic mechanisms accompanying any neurotrauma (e.g., insertion of a CI electrode) are downregulated [Tsai and Hamblin, 2017]. As a result, this rather inexpensive, noninvasive, one-time (before surgery) NIR light application appears to be an another promising method in the set of tools available to help preserve residual hearing.

Conclusion

A single NIR pretreatment in this animal model of CI surgery appears equally neuroprotective to several previously demonstrated posttreatments, in cochlear and in other neural tissues. NIR pretreatment is an inexpensive, effective, noninvasive approach that can be combined with other approaches to help preserve residual hearing and, hence, deserves further clinical application and evaluation in CI patients.

Statement of Ethics

The Board for Granting Permission to Perform Animal Experiments (Land Berlin) approved the study (approval number: G 0146/14).

Conflict of Interest Statement

A.E. is a member of the Surgical Advisory Board (Europe) for Advanced Bionics GmbH. P.B. is employed in a scientific role by Advanced Bionics GmbH, Hanover, Germany. All other authors disclose no conflicts of interest.

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Author Contributions

Ira Strübing – acquisition, analysis of data, drafting the work, and final approval of the version to be published. Moritz Gröschel – acquisition, analysis of data and interpretation, and final approval of the version to be published. Arne Ernst – conception of the work, revising it critically for important intellectual content, and final approval of the version to be published. Susanne Schwitzer – acquisition, revising it critically for important intellectual content, and final approval of the version to be published. Felix Fröhlich – revising it critically for important intellectual content and final approval of the version to be published. Dan Jian – conception of the work, revising it critically for important intellectual content, and final approval of the version to be published. Patrick Boyle – conception of the work, revising it critically for important intellectual content, and final approval of the version to be published. Dietmar Basta – conception of the work, analysis of data, drafting the work, revising it critically for important intellectual content, and final approval of the version to be published. All authors were accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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11.2. - Publication 2:

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Gesamtanzahl: 69 Journale

Rank	Full Journal Title	Total Cites	Journal Impact Factor	Eigenfactor Score
1	NATURE	745,692	43.070	1.285010
2	SCIENCE	680,994	41.037	1.070190
3	National Science Review	1,842	13.222	0.006500
4	Science Advances	21,901	12.804	0.110010
5	Nature Communications	243,793	11.878	1.103290
6	Nature Human Behaviour	1,230	10.575	0.006550
7	PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF THE UNITED STATES OF AMERICA	661,118	9.580	1.022190
8	Science Bulletin	3,569	6.277	0.009840
9	Scientific Data	3,240	5.929	0.015610
10	Frontiers in Bioengineering and Biotechnology	1,994	5.122	0.006540
11	Journal of Advanced Research	2,691	5.045	0.004780
12	Research Synthesis Methods	1,932	5.043	0.005420
13	GigaScience	2,674	4.688	0.012510
14	Annals of the New York Academy of Sciences	46,385	4.295	0.025840
15	Scientific Reports	302,086	4.011	1.061540
16	Journal of the Royal Society Interface	12,933	3.224	0.029190
17	NPJ Microgravity	203	3.111	0.000670
18	PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY A-MATHEMATICAL PHYSICAL AND ENGINEERING SCIENCES	19,227	3.093	0.028200

Rank	Full Journal Title	Total Cites	Journal Impact Factor	Eigenfactor Score
19	FRACTALS-COMPLEX GEOMETRY PATTERNS AND SCALING IN NATURE AND SOCIETY	1,429	2.971	0.001120
20	Journal of Radiation Research and Applied Sciences	860	2.963	0.001860
21	MIT Technology Review	929	2.893	0.001910
22	JOURNAL OF KING SAUD UNIVERSITY SCIENCE	1,120	2.835	0.001670
23	PROCEEDINGS OF THE ROYAL SOCIETY A-MATHEMATICAL PHYSICAL AND ENGINEERING SCIENCES	18,683	2.818	0.018940
24	PLoS One	650,727	2.776	1.706770
25	COMPLEXITY	2,753	2.591	0.003890
26	Royal Society Open Science	4,118	2.515	0.017150
27	PeerJ	11,911	2.353	0.045900
28	SCIENCE AND ENGINEERING ETHICS	1,719	2.275	0.003450
29	INTERNATIONAL JOURNAL OF BIFURCATION AND CHAOS	7,008	2.145	0.007390
30	Symmetry-Basel	2,097	2.143	0.002590
31	SCIENTIFIC AMERICAN	6,609	1.946	0.003540
32	Science of Nature	508	1.839	0.002000
33	PROCEEDINGS OF THE JAPAN ACADEMY SERIES B-PHYSICAL AND BIOLOGICAL SCIENCES	1,532	1.833	0.001960
34	Journal of Taibah University for Science	779	1.640	0.001240
35	Frontiers in Life Science	241	1.622	0.000500
36	ARABIAN JOURNAL FOR SCIENCE AND ENGINEERING	3,838	1.518	0.005840
37	SCIENCE PROGRESS	521	1.500	0.000400

Near-infrared-light pre-treatment attenuates noise-induced hearing loss in mice

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ABSTRACT

Noise induced hearing loss (NIHL) is accompanied by a reduction of cochlear hair cells and spiral ganglion neurons. Different approaches have been applied to prevent noise induced apoptosis / necrosis. Physical intervention is one technique currently under investigation. Specific wavelengths within the near-infrared light (NIR)-spectrum are known to influence cytochrome-c-oxidase activity, which leads in turn to a decrease in apoptotic mechanisms. It has been shown recently that NIR can significantly decrease the cochlear hair cell loss if applied daily for 12 days after a noise exposure. However, it is still unclear if a single NIR-treatment, just before a noise exposure, could induce similar protective effects. Therefore, the present study was conducted to investigate the effect of a single NIR-pre-treatment aimed at preventing or limiting NIHL. The cochleae of adult NMRI-mice were pre-treated with NIR-light (808 nm, 120 mW) for 5, 10, 20, 30 or 40 minutes via the external ear canal. All animals were noised exposed immediately after the pre-treatment by broad band noise (5–20 kHz) for 30 minutes at 115 dB SPL. Frequency specific ABR-recordings to determine auditory threshold shift were carried out before the pre-treatment and two weeks after the noise exposure. The amplitude increase for wave IV and cochlear hair cell loss were determined. A further group of similar mice was noise exposed only and served as a control for the NIR pre-exposed groups. Two weeks after noise exposure, the ABR threshold shifts of NIR-treated animals were significantly lower ($p < 0.05$) than those of the control animals. The significance was at three frequencies for the 5-minute pre-treatment group and across the entire frequency range for all other treatment groups. Due to NIR light, the amplitude of wave four deteriorates significantly less after noise exposure than in controls. The NIR pre-treatment had no effect on the loss of outer hair cells, which was just as high with or without NIR-light pre-exposure. Relative to the entire number of outer hair cells across the whole cochlea, outer hair cell loss was rather negligible. No inner hair cell loss whatever was detected. Our results suggest that a single NIR pre-treatment induces a very effective protection of cochlear structures from noise exposure. Pre-exposure of 10 min seems to emerge as the optimal dosage for our experimental setup. A saturated effect occurred with higher dosage-treatments. These results are relevant for protection of residual hearing in otoneurosurgery such as cochlear implantation.

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Keywords Near infrared light, Noise-induced hearing loss, Auditory brainstem response, Cochlear hair cells

INTRODUCTION

Noise affects the morphology and function of peripheral and central auditory structures. Different mechanisms lead to damage to inner and outer cochlear hair cells, e.g., mechanical intervention, ischemia (*Ohgami et al., 2012*; *Gyo, 2013*; *Moussavi Najarkola et al., 2013*; *Perny et al., 2017*), oxidative stress (*Evans & Halliwell, 1999*; *Henderson et al., 2006*) or calcium dysregulation (*Le Prell et al., 2006*). Microscopic signs for outer hair cells' damage include: uncoupling from the tectorial membrane and mechanical disruption of their stereociliary arrays (*Liberman & Beil, 1979*; *Slepecky, 1986*; *Patuzzi, Yates & Johnstone, 1989*; *Nordmann, Bohne & Harding, 2000*; *Kurabi et al., 2017*). Such processes are initiated very rapidly following noise exposure (*Lin et al., 2011*; *Bullen et al., 2019*) and can lead in turn to irreversible loss of outer hair cells and their mechanical amplifying abilities (*Wagner et al., 2005*; *Henderson et al., 2006*; *Salvi et al., 2017*). Severe to profound hearing loss is largely the result of an inner hair cell loss. However, frequently a reduced innervation of inner hair cells appears to be responsible for the extent of the noise-induced hearing loss, evidenced by spiral ganglion cells dying after noise exposure, even when the structure of the inner hair cells obviously survives (*Kujawa & Liberman, 2009*; *Choi & Choi, 2015*).

Various pharmacological approaches have been undertaken to prevent noise-induced hearing loss. The most promising strategies appear to be: anti-inflammatory therapies, use of antioxidants as inhibitors of intracellular stress pathways, neurotrophic factors, inhibition of programmed cell death pathways, and neurotransmission blockers. For an overview see *Waqas et al. (2018)*. Unfortunately, most of these interventions carry undesired side effects. A quite successful neuroprotective approach, without serious side-effects, appears to be the use of near-infrared (NIR) light. NIR-light has already been applied in various fields of medicine (*Hashmi et al., 2010*). It has regulatory approval for the treatment humans having skin diseases (630 nm, *Eells et al., 2004*; *Martins, Trindade & Leite, 2008*), pain (*Chow et al., 2009*) and stroke (808 nm, *Lampl et al., 2007*).

Several in vitro and in vivo studies have investigated the effects of NIR-treatment in the auditory system in response to cochlear injury. In vitro studies have demonstrated protection of cochlear or vestibular hair cell cultures after pharmacological insult: e.g., gentamycin-induced damage (*Rhee et al., 2012b*; *Chang et al., 2019*). Additionally, in vivo studies were able to show protective effects, mediated by near-infrared light, for both aminoglycoside ototoxicity (*Rhee et al., 2013*; *Zhang et al., 2015*; *Lee et al., 2017*) and noise-induced hearing loss (*Tamura et al., 2015*; *Tamura et al., 2016*; *Rhee et al., 2012a*; *Lee et al., 2016a*).

Animal experiments into noise exposure have shown a decreased cochlear hair cell loss and a significantly lower hearing threshold shift for a daily NIR-treatment over 12 days following a trauma induced by noise exposure (*Rhee et al., 2012a*). The underlying physiological mechanism of NIR-light effects is called "photo-biomodulation", triggered

by light in the red to near-infrared range (630 to 1,000 nm). Specific wavelengths within the spectrum of NIR-light are known to influence cytochrome-C-oxidase activity via photon absorption, which leads in turn to a decrease of apoptotic mechanisms (*Wong-Riley et al., 2005*). Cytochrome-C-oxidase is part of the mitochondrial respiratory chain with absorption maxima of 680 and 830 nm, where cytochrome C is oxidized and molecular oxygen is reduced to water. This chain transports protons directly from mitochondria, maintaining the membrane potential for an activation of the adjusted ATP synthase, hence leading to a highly significant increase in ATP. High levels of ATP are known to reduce cell apoptosis (*Oron et al., 2007*). The ATP increase leads primarily to an enhancement of the cAMP level, calcium release and gene expression (*Wong-Riley et al., 2001*; *Byrnes et al., 2005b*; *Liang et al., 2006*). The numerous important outcomes of this triggered cascade are: decreased neuroinflammation (*Byrnes et al., 2005a*), reduced expression of the proapoptotic protein Bax, increased expression of the anti-apoptotic protein Bcl-2 (*Liang et al., 2006*), decreased amounts of superoxide radicals and nitric oxide production (*Liang et al., 2008*).

From in vitro studies in the auditory periphery, a strong correlation has been found between NIR-treatment, following gentamicin application, and mitochondrial function, accompanied by elevated ATP levels and an increased mitochondrial membrane potential (*Chang et al., 2019*). This idea is also supported by in vivo NIR experiments showing the activation of superoxide dismuthase-1 (SOD-1), a mitochondria-related enzyme with antioxidative properties, in the vestibular sensory epithelium (*Zhang et al., 2015*). Although most studies in the inner ear focused on the protective properties of NIR-treatment on sensory tissue, that is cochlear and vestibular hair cells, recent investigations have also observed neuroprotective effects in the cochlea. Studies by Lee and colleagues observed NIR-related rescue of neural structures, including inner hair cell postsynaptic puncta, neurofilaments and spiral ganglion cells, when applied after ouabain treatment, a drug leading to auditory neuropathy by selectively damaging spiral ganglion cells (*Lee et al., 2016b*). In another study, the same group was also able to demonstrate a protection from inner hair cell synaptopathy through NIR treatment following moderate noise exposure (*Lee et al., 2019*; *Chang et al., 2019*).

A decreased amount of superoxide radicals and nitric oxide (NO) production was observed upon a single 100 s NIR pre-treatment of cultured outer hair cells before gentamicin application, compared to gentamicin application alone (*Bartos et al., 2016*). Superoxide radicals react with NO and form peroxynitrite, which blocks cellular respiration and diminishes ATP production (*Srinivasan & Avadhani, 2012*). To our knowledge no in vivo studies have yet been conducted to assess the impact of NIR-treatment applied before auditory insult (i.e., a pre-treatment to induce protective effects). Our hypothesis is that an increase of the intracellular ATP-level by an NIR-pre-treatment would pre-condition for cochlear neuroprotection during a subsequent trauma. Therefore, the present study's rationale is to investigate the effect of a single NIR-pre-treatment on hearing and hair cell loss following noise exposure made immediately after the pre-treatment.

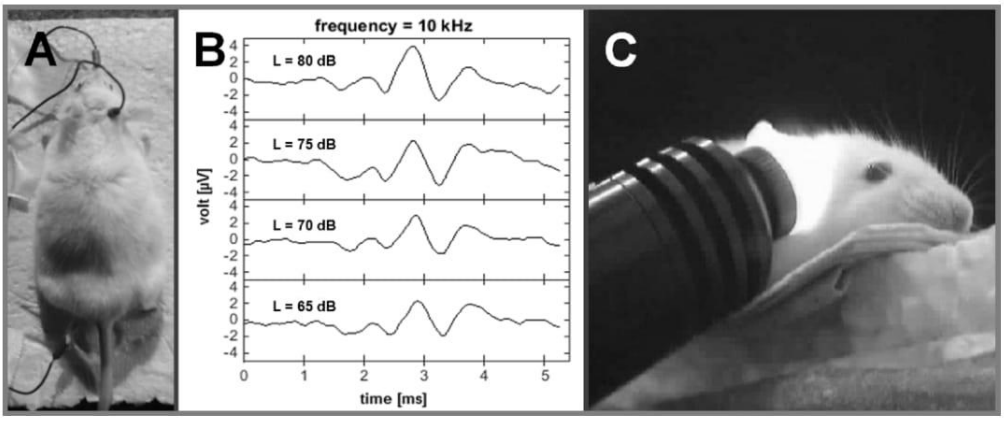

Figure 1 Auditory brainstem recordings. Frequency specific ABR-recordings before and after NIR-light-treatment (electrode positions: mastoid = recording; nose = reference; foot = ground; A); ABR-waves II, III and IV at a frequency of 10 kHz and different sound levels (65–80 dB SPL; B); NIR-light beam fully cover the mouse cochlea via the outer ear canal with specific angle for a NIR-light pre-treatment (visualized by a NIR-camera; C).

Full-size DOI: 10.7717/peerj.9384/fig-1

MATERIAL AND METHODS

Auditory brainstem responses

Frequency-specific auditory brainstem responses (ABR) were recorded for 5 kHz spaced frequencies, between 5 and 40 kHz inclusive, in adult female mice of the NMRI strain (Charles River Laboratories, Erkrath, Germany). The mice were aged 10 to 11 weeks. All mice were caged in groups of 6 animals. In addition to a resting house, each cage contained enrichment features (plastic tunnels and nesting material). The animals had permanent access to food and water and were kept in a 12/12 h dark/light regime. All mice were divided among the experimental groups so that no animal survived. The study protocol stipulated that animals with a weight loss of more than 20% should be euthanatized by a single injection of 180 mg/kg ketamine and 18 mg/kg xylazine. No animal reached this criterion during the study.

ABR recordings were made under anaesthesia (60 mg/kg ketamine, 6 mg/kg xylazine) two days before and two weeks after a noise exposure. Tone stimuli were presented binaurally at different sound pressure levels (SPL) using a sine-wave generator (FG 250 D, H-Tronic, Germany) and were adjusted with an audio amplifier (AMP-50, Tangent, Denmark). ABR waveforms were collected from the output of a recording amplifier (USB-ME16-FAI-System, Multi-Channel Systems, Germany). Subdermal needle electrodes were placed (Fig. 1A) on the forehead (reference), mastoid (active) and at one foot (ground). For each individual frequency, the peak-to-peak amplitudes within the maximum deflection of wave III/IV were measured for different sound intensities (Fig. 1B). The ABR threshold per frequency was estimated by visual inspection of the series of recordings for each frequency and determining when the wave III/IV was no longer visible.

From these data, threshold differences for each frequency (mean threshold shifts) were calculated between the controls and the noise-exposed animals using the group

average values. Results are represented as mean relative hearing loss (±S.E.) in dB for each experimental group compared to the control group.

NIR pre-treatment

Two control groups were formed, one without any noise exposure or NIR pre-treatment (control; $n = 7$) and one with noise-exposure but without NIR-treatment (noise only; $n = 16$). The remaining animals were unilaterally treated with NIR-light immediately before noise exposure of different durations, depending on the experimental group: 5 min NIR-treatment (5′ NIR; $n = 16$), 10 min NIR-treatment (10 ′NIR; $n = 16$), 20 min NIR-treatment (20 ′NIR; $n = 7$), 30 min NIR-treatment (30 ′NIR; $n = 8$), 40 min NIR-treatment (40 ′NIR; $n = 8$). An adjustable isolated point laser module (DB808-120-3(22 ×65), Picotronic, Germany) was used for the application of near-infrared light having a wavelength of 808nm and a power of 120mW. The laser module was placed at the outer ear canal at exactly the angle that allowed a total coverage of the cochlea with the laser beam of approximately seven mm diameter (Fig. 1C). The power density of the NIR-light was 312 mW/cm^2. Safety in terms of tissue damage in general, and to the tympanic membrane in particular, has been verified by several other studies using higher power densities. For example *Rhee et al. (2013)* used a power density of 900 mW/cm^2, delivered for 60 min/day over10 days). Moreover, *Moon et al. (2016)* demonstrated tympanic membrane damage resulting from NIR application for power densities above at least 1,137 mW/cm^2 applied for 30 minutes/day over 14 consecutive days. This previous work dispelled any safety concerns for the NIR power density applied in the present study (*Moon et al., 2016*).

Noise exposure

Just after receiving its NIR pre-treatment, each animal was exposed for 30 min to noise in a soundproof chamber measuring 0.8 m × 0.8 m × 0.8 m, having a minimal sound attenuation of 60 dB). Noise exposure was made using a broad-band white noise with corner frequencies of 5 and 20 kHz and having a level of 115 dB SPL. Noise was delivered binaurally from a loudspeaker (HTC 11.19, Visaton, Germany) placed above the animal's head. The speaker was connected to an audio amplifier, which provided the broad-band noise from a DVD source (DK DVD-438, DK, Germany). The noise level was calibrated by using a sound level meter (Voltcraft 329, Voltcraft, Germany) placed close to the animal's ear. Anaesthesia was controlled through observation via a video camera inside of the lighted chamber. Body temperature was maintained at a constant level of 37 °C using a heating pad.

Histological analysis

After its post-noise exposure ABR recording, each animal was perfused via the left heart chamber with 4% paraformaldehyde (PFA, pH 7.4, Sigma, Germany) in order to fixate the neural tissue. Inner ears were carefully removed from the skull and stored in PFA at 4 °C until further processing. Both cochleae of each animal were prepared for staining. After decalcification of the cochleae for 8 h with the solution renewed after 4 h, using 0.4 M ethylenediaminetetraacetic acid (EDTA, pH 8.0, Roth, Germany), cochleae were dissected twice horizontally to obtain large segments of half turns. Half turns were stained

by using the Alexa-fluor-488-phalloidin staining to visualise F-actin compartments in the cytoskeleton. Cochleae were placed in a 24-well plate and washed with 2 ml phosphate buffered saline (PBS, Gibco, USA). Two ml of 0.2% Triton-X (Sigma, Germany) in PBS was added to the cochleae as permeabilization solution and incubated for 30 min on a shaker at room temperature. After washing with 2 ml PBS, 80 µl Alexa-Fluor-488-phalloidin dilution (1:40 in PBS, Molecular Probes, USA) was added and incubated for 60 min on a shaker at room temperature in the dark. All cochlear segments were embedded with RotiMount Fluorocare and diamidino phenylindole (DAPI, Roth, Germany) on microscope slides with coverslips over night at room temperature in the dark.

The stained cochleae were magnified microscopically with fluorescence channels for phalloidin (480 nm) and DAPI (340 nm). The images were digitized with the AxioCam ICc1 (Zeiss, Germany) and the ZEN 2.3 camera software (Zeiss, Germany). The number of missing hair cells was counted by hand on printouts of overlaid DAPI and phalloidin staining pictures. The gaps between cells were marked using a pen so that the number of cells that were missing could be counted.

The basilar membrane's length was estimated using the ImageJ 1.47d software (National Institutes of Health, USA).

Statistical procedures

The hearing threshold data and cochlear hair cell counts were analysed and compared between the experimental and the control groups by comparing the results using the *U*-test (not normally distributed data) or the *t*-test (normally distributed data). Data distribution was tested using the Kolmogoroff-Smirnoff-test. The SPSS software (IBM SPSS Statistics Version 25, IBM Corp., USA) was used for all statistical analyses. The level of significance for all statistical tests was set at $p < 0.05$. A Bonferroni alpha correction was applied for the use of multiple comparisons.

The Landesamt für Gesundheit und Soziales, Berlin, Germany approved the study protocol (approval number LAGeSo-G 0146/14).

RESULTS

Effect on ABR-threshold shift

All NIR pre-treated groups showed a lower group mean hearing loss following the noise exposure compared to the "noise only"-control group (Fig. 2). A frequency-specific analysis showed significant changes between the controls and the 5′NIR-group for the 5; 35 and 40 kHz frequencies. All other pre-treated groups differed significantly from the noise only controls for every frequency tested (Fig. 2). No statistically significant differences between ABR-thresholds were found for the 10′; 20′; 30′and 40′ NIR-groups. The "control"-group showed only a small, negligible hearing loss.

Effect on amplitude increase of ABR wave IV

Due to the ABR recording electrode positions, ABR-wave IV was the only wave that was robust and prominent in all animals and experimental groups. This is why more detailed ABR-analysis was limited to this wave. Additional wave IV analysis aimed to identify

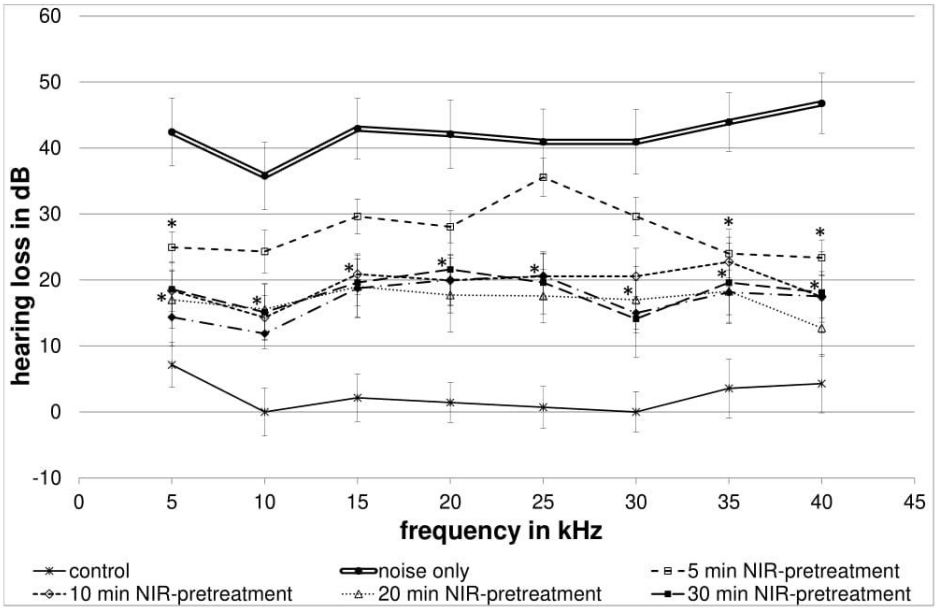

Figure 2 Noise induced hearing loss. Means (±SE) of noise-induced hearing loss for different NIR-light pre-treatments of the cochlea. The 5-minute pre-treatment group shows statistically significant differences at 5, 35 and 40 kHz. All other pre-treated groups show statistically significant differences at all frequencies. The control group without noise and NIR pre-treatment showed only a negligible hearing loss. Filled asterisks indicate statistically significant differences between the pre-treatment groups and the "noise only"-group.

Full-size DOI: 10.7717/peerj.9384/fig-2

any alteration in central suprathreshold processing resulting from noise exposure, with or without NIR pre-treatment. Wave IV represents bilateral activation of the auditory midbrain level, hence any changes in stimulus-related responses could point to modified central auditory processing. The slope of the wave IV amplitude growth function's linear part was calculated and the post-exposure slope was subtracted from the pre-exposure slope (Fig. 3). The average slope difference over all frequencies tested for the "10′-NIR"-group (slope difference 0.004 ± 0.005 μV/dB) was more than 10 times lower than for the "noise only"-group (slope difference 0.053 ± 0.005 μV/dB). A higher slope difference indicates a smaller post-noise exposure slope for the amplitude growth function.

Apart from the values of the "5′-NIR"-group (0.047 ± 0.009 μV/dB) all experimental groups showed significantly lower slope differences compared to the "noise-only"-group (Fig. 3).

Effect on cochlear hair cells

No loss of inner hair cells could be detected bilaterally for any of the groups. Figure 4 shows a sample image of the whole cochlear immunofluorescence staining mount. For the noise only control group, a significant decrease in outer hair cell counts was found for both ears (Figs. 5 and 6), compared to the control group where no noise exposure was given. However, the percentage of missing outer hair cells was only 6.5% compared to a normal cochlea (*Burda, Ballast & Bruns, 1988*). No significant differences were found between the

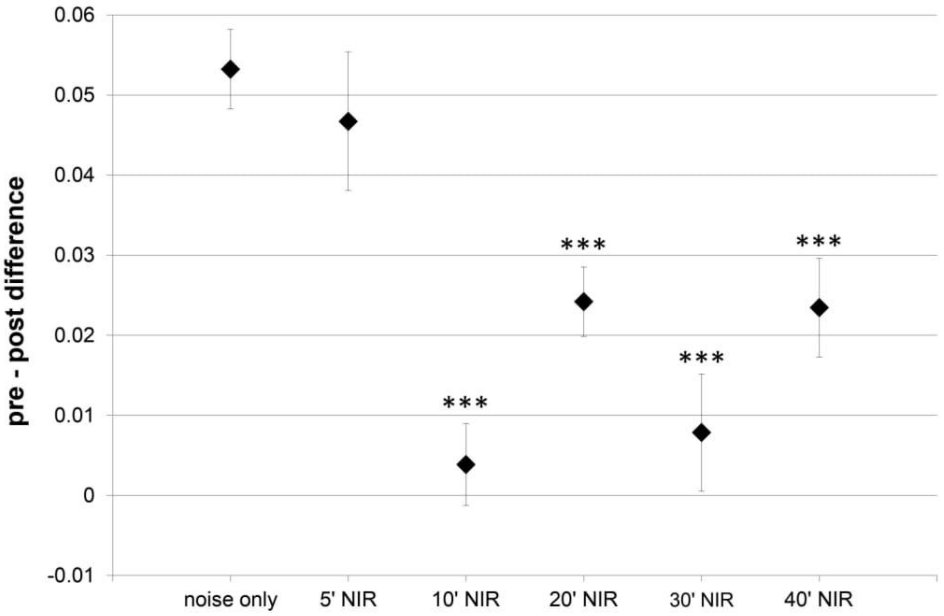

Figure 3 Slope within the amplitude growth function of ABR wave IV. Difference of the slope of the linear part within the amplitude growth function of ABR wave IV (averaged over all tested frequencies) between pre- and post-exposure measurements (post-noise exposure values are subtracted from pre-exposure values). A higher slope difference indicates a smaller post-treatment slope. Asterisks mark statistically significant differences between the NIR-treated groups and the "noise only" -group.
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number of absent outer hair cells in any of the NIR pre-treated experimental groups and the "noise only"-group (Fig. 5). This also holds true for the non-pre-treated side (ear) of the animals (Fig. 6). In addition, the mean number of absent outer hair cells was plotted for the entire length of the organ of corti (Fig. 7). These graphs indicate that hair cell loss decreased from base to apex without any specific correlation to the functional analysis (ABR-thresholds). This was found for all experimental and control groups.

DISCUSSION

The present results show that a single NIR pre-treatment of at least 10 min produces a significantly protective effect on the entire frequency range that we investigated: 5 to 40 kHz. The protective effect plateaued when a longer NIR pre-treatment was applied. It is hypothesized that this is related to a saturation effect for ATP generation. A pre-treatment of 5 min showed a statistically significant protection only at low (5 kHz) and high (35 and 40 kHz) frequencies. The area in between these frequencies corresponds well with the broad-band noise trauma applied. It appears that the activation of protective mechanisms produced by the shorter pre-treatment was too weak for the main noise-affected frequency range, but sufficient for the surrounding areas.

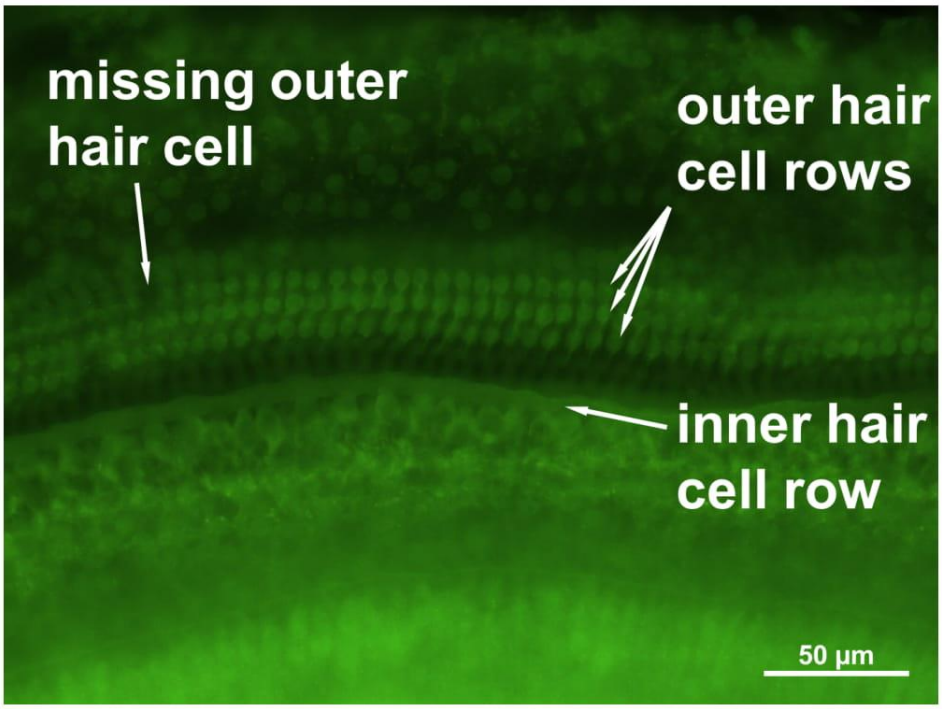

Figure 4 Cochlear hair cell preparation. Sample microphotograph with scale bar of the mouse cochlear whole mount immunofluorescence staining (400× magnification).
Full-size DOI: 10.7717/peerj.9384/fig-4

Efficiency of NIR pre-treatment

The noise-induced elevation of hearing thresholds, as determined by ABR-measurements, was reduced on average by 24 dB in the NIR-pre-treated groups when calculated across all effective treatment groups and the whole frequency range investigated. This shows that a single pre-treatment has a similar effect to a daily post-traumatic NIR-treatment delivered over 12 consecutive days (*Rhee et al., 2012a*). However, the energy density of the NIR-treatment in the present study was twice as that used by *Rhee et al. (2012a)*. It should also worth considering that the exposure time in the earlier investigation was 12 × 60 min (total of 720 min), instead of a single 10 min treatment in the present study. Thus, the total NIR-dosage applied in the post-treatment experiments was much higher than for this pre-treatment study. Another consideration is that the noise exposure differed greatly between the two studies: 115 dB SPL broad-band white noise (5–20 kHz) for 30 min in this study, compared to a 116 dB SPL narrow band noise (centred at 16 kHz (bandwidth 1 kHz) for 6 h in the post-treatment study. However, the effect on hearing thresholds was quite similar for both studies, showing a threshold shift of around 40 dB. This could be related to the different species used in both studies (rats and mice) and their possibly different susceptibility to noise.

Thus, a pre-treatment regime appears to be much more effective than several post-treatments. The reason for this is largely unknown. Earlier studies have shown that pre-conditioning using a very low NIR dose induced a significant decrease in superoxide

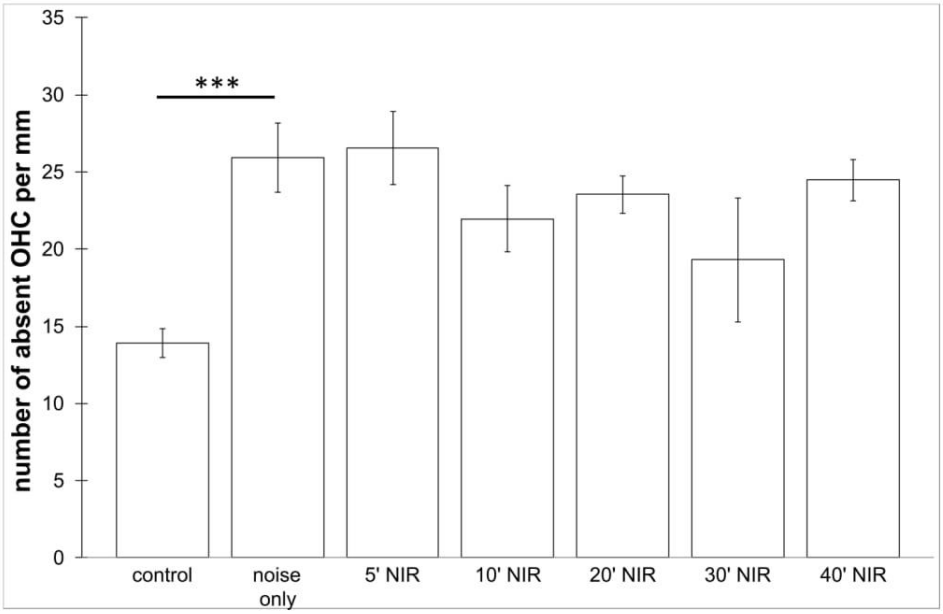

Figure 5 Outer hair cell counting. Means (±SE) of absent outer hair cells per mm on the pre-treated side following different duration of NIR-light exposure. Asterisks indicate statistically significant differences. The "control" -group was not noise exposed and received no NIR pre-treatment. The "noise only" -group received no NIR pre-treatment.

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radicals and NO production for cultured outer hair cells (*Bartos et al., 2016*). This could have resulted in an enhanced intracellular ATP level compared to the non-pre-treated cells, since superoxide radicals react with NO to form peroxynitrite, which blocks cellular respiration (*Srinivasan & Avadhani, 2012*). Furthermore, recent studies have revealed that the nuclear factor NF- κB is activated by NIR-treatment. This leads to protection against inducible NO synthase-triggered oxidative stress and, subsequently, to a reduced caspase-3-mediated apoptosis (*Tamura et al., 2016*). The time point of NIR-treatment plays an important role in the activation or inactivation of NF- κB. The difference being the presence or absence of inflammation in the cells at the time of the treatment (*Chen et al., 2011*). Pro inflammatory cytokines are activated during a noise exposure (*Nakamoto et al., 2012*). Thus, the effects of NIR pre-treatment are not affected by these inflammatory processes. This could explain why NIR pre-treatment is much more efficient than post-traumatic treatment.

Effect on cochlear hair cells

Apart from the quite similar outcome found for hearing preservation in our study, there are some differences in outer hair cell counts compared to earlier investigations. Former studies described a significant reduction of outer hair cells and no loss of inner hair cells due to noise exposure (*Rhee et al., 2012a*; *Tamura et al., 2015*). These reports are in line with our present results. However, NIR post-treatment led to a significant reduction of outer hair cell loss in the medial part of the cochlea. The basal and apical parts showed

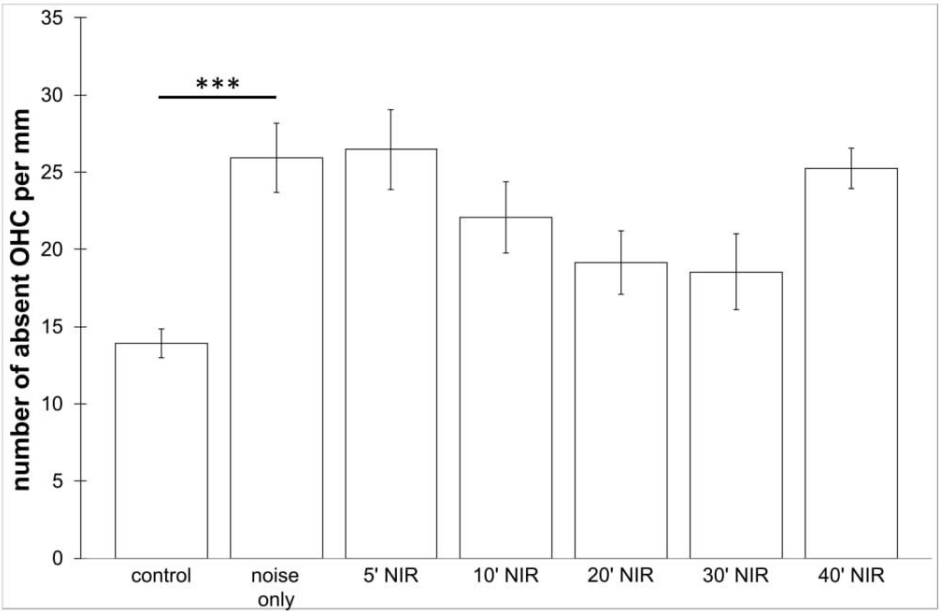

Figure 6 Outer hair cell counting. Means (±SE) of absent outer hair cells per mm on the not pre-treated side following different duration of contra-lateral NIR-light exposure. Asterisks indicate statistically significant differences. The "control" -group was not noise exposed and received no NIR pre-treatment. The "noise only" -group received no NIR pre-treatment.

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no statistically significant protective effect from NIR-light on hair cell loss (*Rhee et al., 2012a*). Another study which applied the NIR treatment after noise exposure found a protection of outer hair cells in all parts of the cochlea (*Tamura et al., 2015*). However, both studies applied a more intense noise trauma than in the present investigation. The relative loss of outer hair cells was very small in the present study compared to the earlier work: 6.5% vs approx. 30% (*Tamura et al., 2015*). Hence, a significant rescue of outer hair cells was harder to achieve in the present study. The present results show a trend for hair cell protection, even if no correlation between hair cell loss along the cochlea and frequency specific ABR-thresholds could be found. The bilateral effect is not contradictory for this hypothesis since both sides (left and right cochleae) are likely simultaneously treated due to the surprisingly good penetration of NIR-light of 808 nm through the small mouse skull (*Lee et al., 2016a*). Additionally, the discrepancy between hair cell counts and hearing thresholds, as found in the present study, is also well known for both animals and humans (*Le Calvez et al., 1998*; *Landegger, Psaltis & Stankovic, 2016*). The correlation coefficient between hearing threshold shift and cochlear hair cell loss was described as only around 0.5 with 1.0 being a perfect correlation (*Landegger, Psaltis & Stankovic, 2016*).

It is possible that functional damage to hair cells occurred in the present experiments (e.g., stereocilia *Wang, Hirose & Liberman, 2002*), or that other cochlear structures are responsible for the hearing loss and the NIR-related preservation observed. Suitable candidates which are known to be influenced by NIR-light are spiral ganglion cells, pre- and postsynaptic structures at the inner hair cells (ribbon synapse), cochlear neurofilaments and

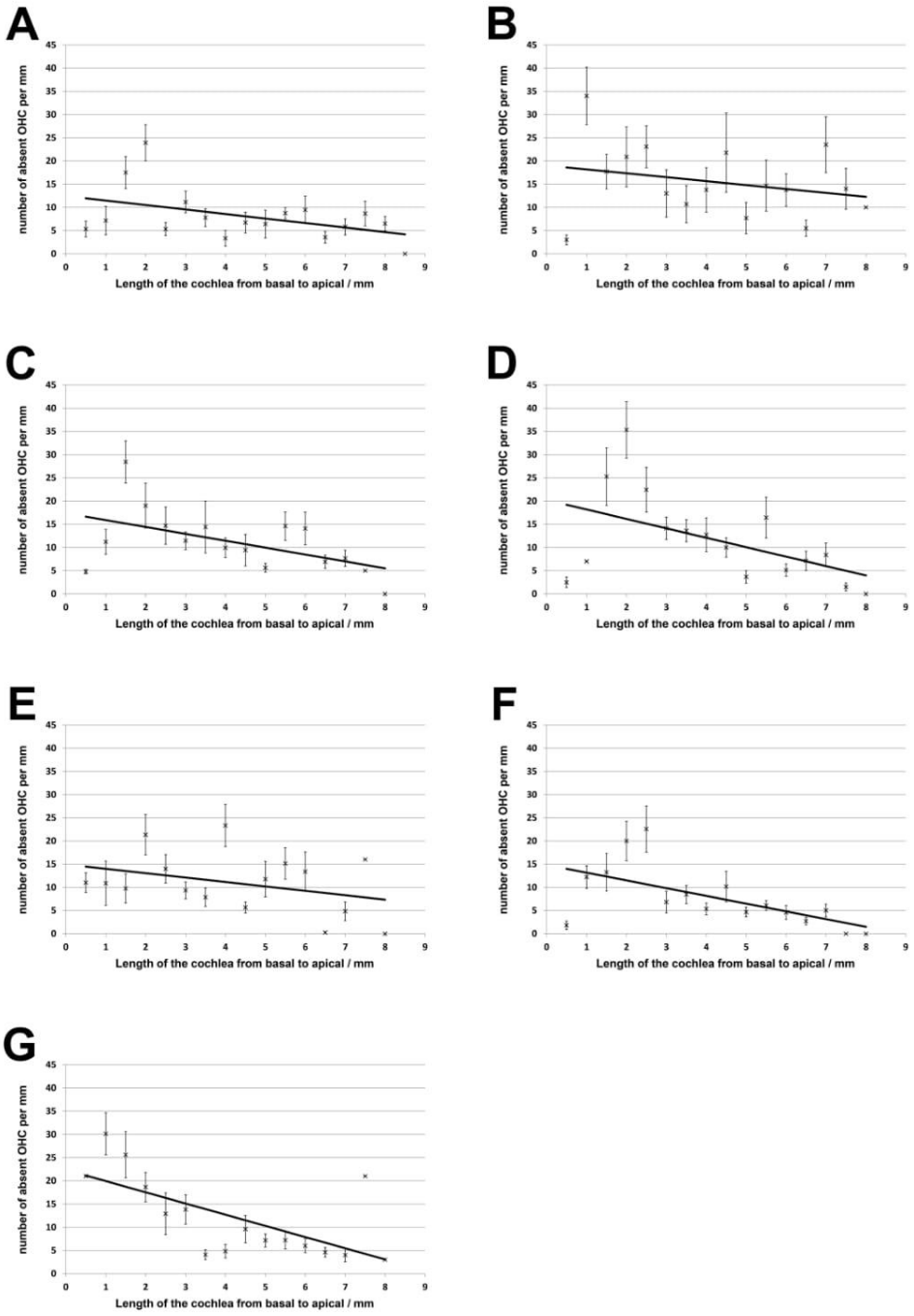

Figure 7 **Distribution of cochlear outer hair cell loss.** Distribution of outer hair cell loss along the organ of corti for all experimental and control groups (A, untreated controls; B, noise only; C, 5 min NIR-pretreatment; D, 10 min NIR-pretreatment; E, 20 min NIR-pretreatment; F, 30 min NIR-pretreatment; G, 40 min NIR-pretreatment). Error bars represent standard error.

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fibrocytes of the lateral wall (*Tamura et al., 2016*; *Lee et al., 2016b*; *Lee et al., 2019*; *Chang et al., 2019*). A first sign of such effects could be the relationship between the amplitude of a summating potential, which is generated within the auditory brainstem/midbrain (e.g., ABR wave IV), and the stimulus intensity. The significant differences that we describe between the slopes of this relationship in animals with and without a NIR-pre-treatment supports this hypothesis. The lower slope difference in NIR pre-treated animals indicates a near-normal sensitivity to an increased stimulus intensity and thus a near-normal loudness growth. This could be related to a preservation of cochlear structures by NIR-light.

CONCLUSIONS

In essence, the present results show that a single NIR pre-treatment of at least 10 min produces a significant hearing protection upon noise exposure. The observed effect was not based on the protection of cochlear hair cell density.

Further investigations are necessary to identify the anatomical correlates for the functional preservation of hearing resulting from a single NIR pre-treatment.

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Competing Interests

Arne Ernst is a member of the Surgical Advisory Board (Europe) for Advanced Bionics GmbH. Patrick Boyle is employed in a scientific role by Advanced Bionics GmbH, Hanover, Germany. All other authors declare that they have no competing interests.

Author Contributions

- Dietmar Basta conceived and designed the experiments, performed the experiments, analyzed the data, prepared figures and/or tables, authored or reviewed drafts of the paper, and approved the final draft.
- Moritz Gröschel conceived and designed the experiments, performed the experiments, analyzed the data, authored or reviewed drafts of the paper, and approved the final draft.
- Ira Strübing performed the experiments, analyzed the data, prepared figures and/or tables, authored or reviewed drafts of the paper, and approved the final draft.
- Patrick Boyle conceived and designed the experiments, authored or reviewed drafts of the paper, and approved the final draft.

- Felix Fröhlich performed the experiments, authored or reviewed drafts of the paper, and approved the final draft.
- Arne Ernst conceived and designed the experiments, authored or reviewed drafts of the paper, and approved the final draft.
- Rainer Seidl conceived and designed the experiments, analyzed the data, authored or reviewed drafts of the paper, and approved the final draft.

Animal Ethics

The following information was supplied relating to ethical approvals (i.e., approving body and any reference numbers):

The Landesamt für Gesundheit und Soziales in Berlin, Germany approved this research (LAGeSo-G 0146/14).

Data Availability

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All raw measurements are available in the Supplementary Files.

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18	International Journal of Speech-Language Pathology	675	1.179	0.001960

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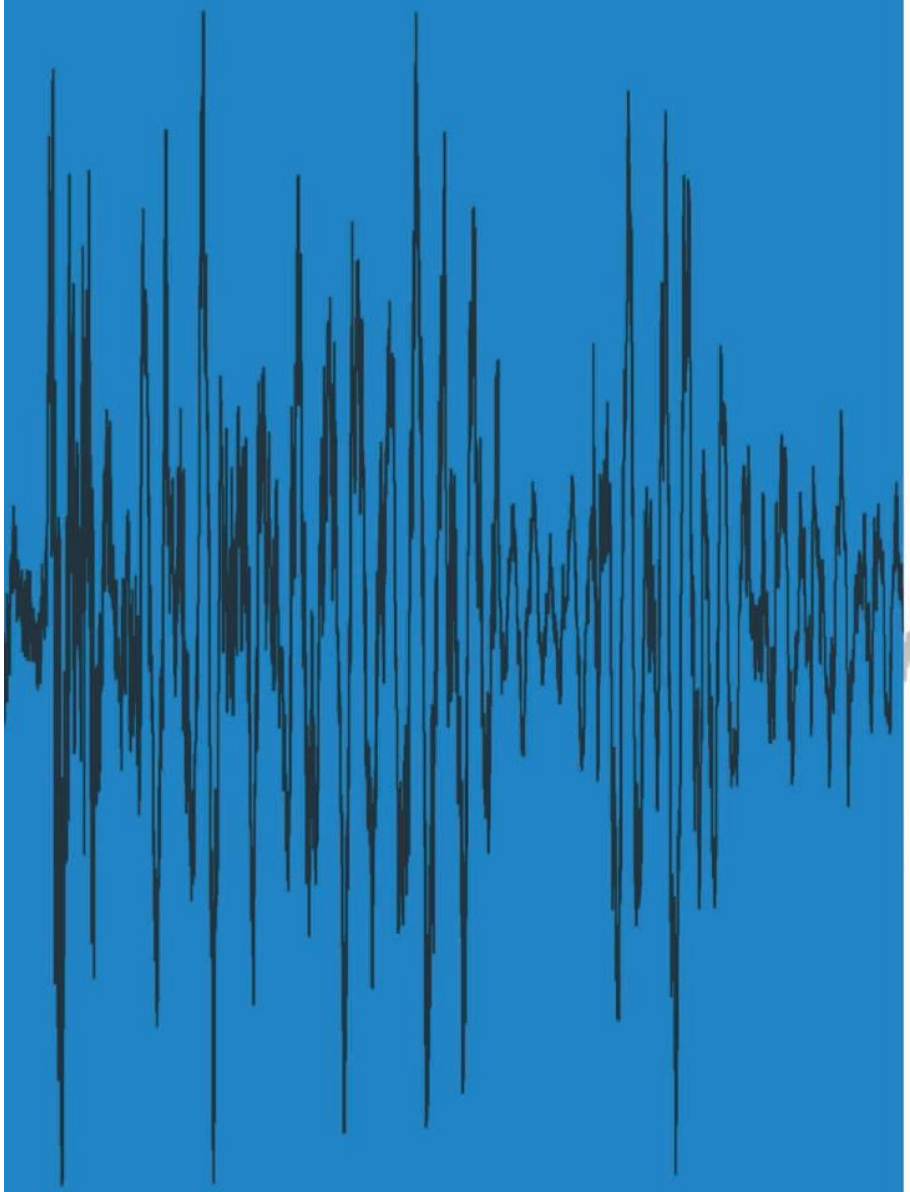

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Original Article

Apoptosis in the Cochlear Nucleus and Inferior Colliculus Upon Repeated Noise Exposure

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Abstract

The time course of apoptosis and the corresponding neuronal loss was previously shown in central auditory pathway of mice after a single noise exposure. However, repeated acoustic exposure is a major risk factor for noise-induced hearing loss. The present study investigated apoptosis by terminal deoxynucleotidyl transferase deoxyuridine triphosphate nick end labeling (TUNEL) assay after a second noise trauma in the ventral and dorsal cochlear nucleus and central nucleus of the inferior colliculus. Mice [Naval Medical Research Institute (NMRI) strain] were noise exposed [115 dB sound pressure level, 5–20 kHz, 3 h) at day 0. A double group received the identical noise exposure a second time at day 7 post-exposure and apoptosis was either analyzed immediately (7-day group-*double*) or 1 week later (14-day group-*double*). Corresponding single exposure groups were chosen as controls.

No differences in TUNEL were seen between 7-day or 14-day single and double-trauma groups. Interestingly, independent of the second noise exposure, apoptosis increased significantly in the 14-day groups compared to the 7-day groups in all investigated areas.

It seems that the first noise trauma has a long-lasting effect on apoptotic mechanisms in the central auditory pathway that were not largely influenced by a second trauma. Homeostatic mechanisms induced by the first trauma might protect the central auditory pathway from further damage during a specific time slot. These results might help to understand the underlying mechanisms of different psychoacoustic phenomena in noise-induced hearing loss.

Keywords: Noise-induced apoptosis, noise-induced hearing loss, repeated noise exposure, TUNEL-staining

Introduction

Noise is well known as an important environmental factor for hearing disorders. Repeated noise exposure is a major risk factor for noise-induced hearing loss (NIHL), the most common consequence of noise overexposure.[1]

Previous studies have shown the influence of acoustic overexposure on the middle (e.g., deluxation of ossicles) and inner ear (e.g., hair cell loss).[2-6] This has been investigated for decades and is still the subject of research. Change in the central auditory pathway because of acoustic overstimulation is another important topic that has been addressed during the last few years. Deafferentation because of acoustic overexposure as well as after cochlea ablation was shown in the cochlear nucleus (CN).[7-9] NIHL leads to axon degeneration in the CN and superior olivary complex.[10,11] Besides the effect of deafferentation, acoustic overstimulation has a direct impact on central mechanisms and leads to an intense metabolic activity that can result in the formation of free radicals and reactive oxygen species or lipid peroxidation.[12-15] Reactive oxygen species and mitochondrial dysfunction are major causes of cell pathologies that accelerate cell death mechanisms such as apoptosis.[16] As a consequence, apoptosis is supposed to be directly stimulated by noise and hypothesized as one underlying pathophysiological mechanism for noise-induced neuronal loss in the central auditory pathway.[11,17-20] Noise-induced apoptosis was recently described in the auditory pathway.[19-21] In humans, for example, factory workers, nightclub visitors, and musicians, repeated acoustic exposure to traumatizing noise is the most common situation.[1] Little is known

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about central changes after multiple noise exposure. Besides NIHL, a phenomenon called "toughening" and an upregulation of activity-dependent calcium-binding protein in the spiral ganglion and CN were found after repeated noise exposure.[22] Also, an increase in Ca^{2+}-dependent activity in key structures of the central auditory pathway after a second noise trauma was described.[23] The effects on hearing thresholds depend on the noise exposure paradigm and threshold shift amplitudes differ between single and repeated noise trauma in most of the studies.[21-23]

Our working group recently described the time course of apoptosis in the ventral CN (VCN), dorsal CN (DCN), central nucleus of the inferior colliculus (ICC), medial geniculate body, and primary auditory cortex (AI) after a single noise exposure.[19,20] Apoptosis after repeated noise trauma was already seen in the medial geniculate body and auditory cortex.[21,24] The present study aimed at investigating the incidence of cell death after a second noise exposure in the VCN, DCN, and ICC. This might help to understand hearing pathologies in NIHL such as tinnitus or changed speech intelligibility.[25-27]

Material and Methods

Eighteen young adult (30–40 days of age) female normal hearing mice [Naval Medical Research Institute (NMRI) strain] with fully developed auditory systems were used in the present study. Recent studies showed sex-specific results in mice after NIHL.[28,29] To keep variance of the data low, only female mice were used in the present experiments. The experimental protocol was approved by the government commission for animal studies (LaGeSo, Berlin, Germany; PI: Dr. Dietmar Basta, approval number: G0416/10). Experiments were carried out in accordance with the European Union (EU) Directive 2010/63/EU on the protection of animals used for scientific purposes. All efforts were made to minimize pain and discomfort in the animals.

Noise exposure

The noise exposure paradigm has been described in recent publications of our group.[18-20,23,24] Briefly, animals were exposed to a broadband flat spectrum noise (5–20 kHz) for 3 hours at 115 dB sound pressure level under anesthesia (6 mg/kg xylazine and 60 mg/kg ketamine) in a soundproof chamber (80 cm × 80 cm × 80 cm, minimal attenuation 60 dB). An amplifier (Tangent AMP-50; Eltax, Aulum, Denmark) and a DVD player were connected to loudspeakers (HTC 11.19; Visaton, Haan, Germany) placed above the animals' heads. A sound level meter (Voltcraft 329; Conrad Electronic, Wernberg-Köblitz, Germany) was placed next to the animal's ear to calibrate sound pressure level. A heating pad (Thermolux CM 15W; Acculux, Murrhardt, Germany) was placed under the animals to keep the body temperature constant at 37°C during video camera-controlled anesthesia.

Schedule of treatment

Different groups of animals were investigated at various point of time after single or double noise exposure [Figure 1] analogous to earlier investigations.[24,23] Eighteen animals of the experimental groups were noise exposed at day 0 and randomly assigned to the experimental groups. The single exposure group was exposed to the initial trauma only and either investigated at day 7 post-exposure ("7-day group-single"; $n = 4$ animals) or at day 14 post-exposure ("14-day group-single"; $n = 5$ animals). The remaining nine animals were exposed to noise for a second time 1 week after the first noise trauma ("double trauma group"). Five of these mice were investigated immediately after the second exposure ("7-day group-double"), and the other four animals were left in their cages for 1 week and analyzed on day 14 after the first exposure ("14-day group-double").

TUNEL staining

Terminal deoxynucleotidyl transferase deoxyuridine triphosphate nick end labeling (TUNEL) was performed

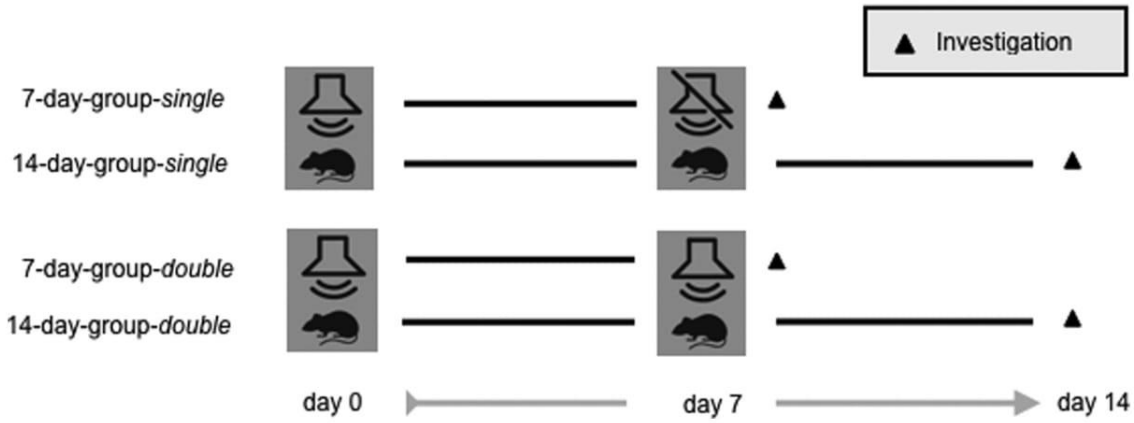

Figure 1: Schematic illustration of the experimental groups. All mice were noise exposed at day 0 and either analyzed on day 7 (7-day groups) or on day 14 (14-day groups) post-exposure. Double groups were noise exposed for second time at day 7 and either investigated immediately after this trauma (7-day group-double) or 1 week later (14-day group-double) and compared to the corresponding single group (7-day group-single, 14-day group-single).

according to earlier investigations.[20,24] Briefly, on the day of investigation, animals were perfused via the left heart chamber with a fixative solution (4% paraformaldehyde). The skull was carefully opened to remove the brain. After embedding in paraffin, 10-μm-thick slices in the frontal plane were cut using a rotation microtome (Euromex Präzisions Minot Rotations Mikrotom MT.5505; Euromex, Arnhem, The Netherlands). The frontal plane and the sagittal axis were defined according to the mouse brain atlas of Paxinos and Franklin.[30] Comparable sections of the respective investigated structure from each animal were used for the analysis. The slices were stained by using the TUNEL method (In Situ Cell Death Detection Kit POD; Roche, Mannheim, Germany) to visualize cell death mechanisms.[31] After removing the paraffin (2× Rotihistol [Carl Roth, Karlsruhe, Germany] for 10 min) and rehydrating in a descending ethanol series (90% and 70% each for 5 min) and distilled water (5 min), a pretreatment with 5% proteinase K (20.5 μg/mL in 10 mM trishydroxymethylaminomethane, pH 7.5, Roche; 100 μL per slice for 10 min) was performed.[32] DNA strand breaks were provoked via deoxyribonuclease I recombinant (100 U/mL; Roche) used as a positive control as proposed by the manufacturer (Roche). A 3% H_2O_2 solution (dissolved in methanol) was applied for 5 minutes to block endogenous nucleases and avoid false-positive results. Each slice was incubated for 60 minutes with 50 μL TUNEL reaction mixture [diluted 1:2 with phosphate buffered saline (PBS)] at 37°C in a humidified chamber. To obtain a light microscopic analysis, each slice was incubated for 30 minutes with 50-μL converter POD, with diaminobenzidine as substrate (50 μL per slice for 10 min). After washing in PBS and distilled water, dehydration in an ascending alcohol series (70% ethanol, 90% ethanol, and 100% isopropanol, each for 1 min) was performed. Slices were stored in Rotihistol until mounting with Roti Histokitt (Carl Roth).

TUNEL cell counting and statistical analysis

The stained slices were microscopically magnified (250×, Axiovert 25C; Carl Zeiss, Göttingen, Germany) and colored photomicrographs were taken using a digital camera (Canon Eos 1000D, Tochigi, Japan). Pictures were standardized ("autocontrast" function, Adobe Photoshop CS3 Extended, Version 10.0, 2007, USA) and the number of TUNEL-positive cells[33] was counted in particular grids within VCN (0.22 mm × 0.17 mm), the fusiform layer of DCN (0.16 mm × 0.1 mm), and the ICC (0.45 mm × 0.33 mm) [Figure 2]. The brain areas were defined in accordance with the mouse brain atlas of Paxinos and Franklin.[30] The grid was manually placed in the center of the structure after randomization of the experimental groups. Equivalent regions of interest were investigated in earlier studies for determination of noise-induced neuronal cell loss as well as noise-induced apoptosis after a single noise trauma in VCN, DCN, and ICC.[18,19] To avoid double counting the same cell in different layers, every second slice was added to the histological analyses.[24] The cell counting was performed manually. To reduce technical and methodical errors to a minimum, the slides were counted after randomization and the counter was "blind" to the structure and the group.

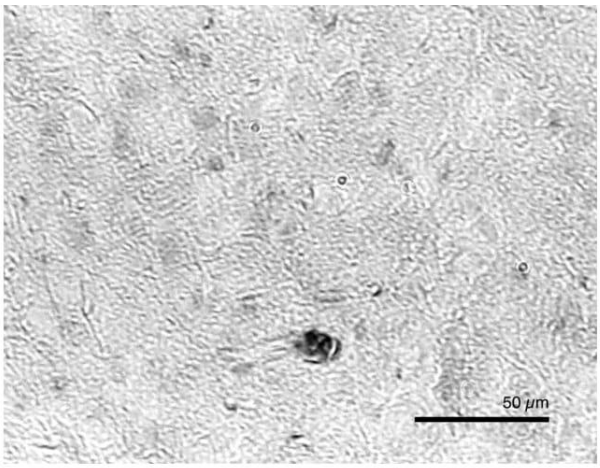

Figure 2: TUNEL-positive cells in a VCN grid. A VCN grid (0.22 mm × 0.17 mm) captured at 250× magnification. A single TUNEL-positive cell is seen in the grid (7-day group-single). TUNEL = terminal deoxynucleotidyl transferase deoxyuridine triphosphate nick end labeling; VCN = ventral and dorsal cochlear nucleus.

The statistical procedure was chosen accordingly to earlier investigations about TUNEL in CN and ICC after a single noise exposure.[19] The mean of TUNEL-positive cells (sum of TUNEL-positive cells divided by the number of grids) and standard error (SE) were calculated for all groups and structures. Data from the double noise exposure groups was tested for significant differences against the corresponding single noise exposure groups. Data from the 14-day groups was tested for significant differences against the corresponding 7-day groups.

The data distribution was statistically tested by Kolmogoroff–Smirnoff test. Normally distributed data was tested for significant differences by using the *t* test, otherwise the Mann–Whitney *U* test (*u*-test) was applied. The significance levels were set to $P < 0.05$ for all tests. The Tukey honestly significant difference (HSD) *post hoc* test was used to counteract the problem of multiple statistical comparisons and minimize the false discovery rate.[34] SPSS software (SPSS Statistics, Version 20; IBM, Chicago, IL, USA) was used for all statistical calculations. Results with statistical significance at the level of $P < 0.05$ were marked with an asterisk. Results without statistical significance were marked with "n.s." (not significant).

Results

Ventral cochlear nucleus

In VCN, 54 TUNEL-positive cells were counted in 34 slices analyzed in 7-day group-single (45 TUNEL-positive cells in 40 slices in 7-day group-double) and 93 TUNEL-positive cells were found in 29 slices in 14-day group-single (102

TUNEL-positive cells in 28 slices in 14-day group-double). No statistically significant differences in the mean of TUNEL-positive cells per grid were found within the 7-day groups (VCN: 7-day single = 1.59 ± 0.30 SE; 7-day double = 1.13 ± 0.23; $P_{\text{VCN-7-day}} = 0.156$ [*u*-test]) nor within the 14-day groups (VCN: 14-day single = 3.21 ± 0.61 SE; 14-day double = 3.64 ± 0.48; $P_{\text{14-day}} = 0.579$ [*t*-test]). The mean of TUNEL-positive cells was statistically significant elevated in the 14-day group compared to 7-day group ($P_{\text{single}} = 0.021$ [*u*-test] and $P_{\text{double}} = 0.000$ [*u*-test], respectively) [Figure 3].

Dorsal cochlear nucleus

In DCN, 20 slices (15 TUNEL-positive cells in total) of 7-day group-*single* (21 slices [14 TUNEL-positive cells] of 7-day group-*double*) and 34 slices (80 TUNEL-positive cells) of 14-day group-*single* (32 slices [75 TUNEL-positive cells] of 14-day group-*double*) were included in the statistical analysis. No statistically significant differences in the mean of TUNEL-positive cells per grid were found within the 7-day group (DCN: 7-day single = 0.75 ± 0.28 SE; 7-day double = 0.67 ± 0.20; $P_{\text{DCN-7-day}} = 0.883$ [*u*-test]) nor within the 14-day group (DCN: 14-day single = 2.35 ± 0.35 SE; 14-day double = 2.34 ± 0.35; $P_{\text{DCN-14-day}} = 0.953$ [*u*-test]). The mean of TUNEL-positive cells was statistically significant elevated in the 14-day group compared to the 7-day group ($P_{\text{single}} = 0.001$ [*u*-test] and $P_{\text{double}} = 0.000$ [*u*-test], respectively) [Figure 4].

Central nucleus of the inferior colliculus

In ICC, 29 slices were analyzed in 7-day group-*single* (63 TUNEL-positive cells) (30 slices in 7-day group-*double* [57 TUNEL-positive cells]) and 38 slices in 14-day group-*single* (197 TUNEL-positive cells) (28 slices in 14-day group-*double* [146 TUNEL-positive cells]). No statistically significant differences in TUNEL-positive cells per grid were found within the 7-day group (ICC: 7-day single = 2.17 ± 0.42 SE; 7-day double = 1.90 ± 0.27; $P_{\text{ICC-7-day}} = 0.883$ [*u*-test]) nor within the 14-day group (ICC: 14-day single = 5.18 ± 0.66 SE; 14-day double = 5.21 ± 0.88; $P_{\text{ICC-14-day}} = 0.978$ [*t*-test]). The increase in TUNEL in the 14-day

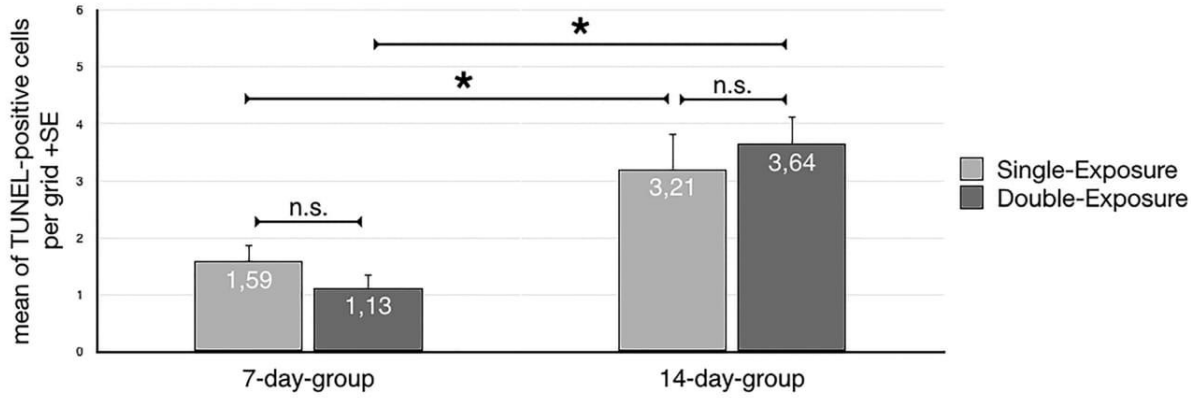

Figure 3: Ventral cochlear nucleus. The mean of TUNEL-positive cells and standard error in the ventral cochlear nucleus 1 (7-day groups) or 2 weeks (14-day groups) after the initial noise exposure in the single (light gray) and double exposure groups (dark gray). No differences in the incidence of TUNEL-positive cells were found between single and double-trauma group neither in the 7-day nor 14-day group (n.s.). TUNEL was increased in the 14-day groups compared to 7-day groups (asterisk). n.s. = not significant; TUNEL = terminal deoxynucleotidyl transferase deoxyuridine triphosphate nick end labeling.

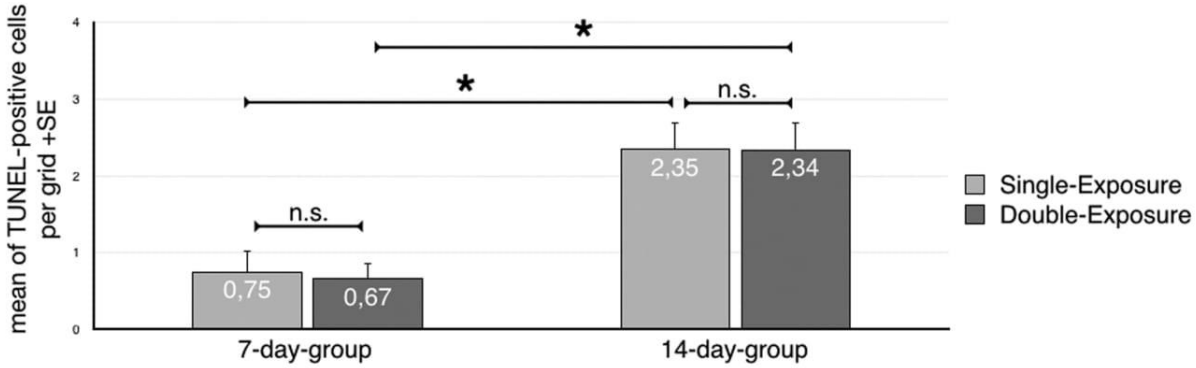

Figure 4: Dorsal cochlear nucleus. The mean of TUNEL-positive cells and standard error in the dorsal cochlear nucleus 1 (7-day groups) or 2 weeks (14-day groups) after the initial noise exposure in the single (light gray) and double exposure groups (dark gray). Compared to 7-day groups, TUNEL was increased in the 14-day groups (asterisk). No differences in the incidence of TUNEL-positive cells were found between single and double-trauma group neither in the 7-day nor 14-day group (n.s.). n.s. = not significant; TUNEL = terminal deoxynucleotidyl transferase deoxyuridine triphosphate nick end labeling.

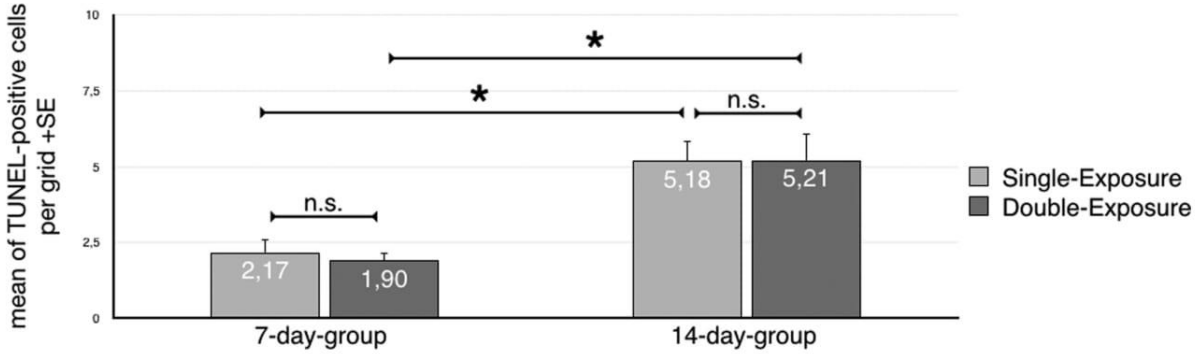

Figure 5: Inferior colliculus. Mean of TUNEL-positive cells and standard error in the central nucleus of the inferior colliculus 1 (7-day groups) or 2 weeks (14-day groups) after the initial noise exposure in the single (light gray) and double exposure groups (dark gray). Compared to 7-day groups, TUNEL was increased in the 14-day groups (asterisk). No differences in the incidence of TUNEL-positive cells were found between single and double-trauma group neither in the 7-day nor 14-day group (n.s.). n.s. = not significant; TUNEL = terminal deoxynucleotidyl transferase deoxyuridine triphosphate nick end labeling.

group was statistically significant compared to the 7-day group within the single exposure group ($P_{single} = 0.000$ [*t*-test]) and double exposure group ($P_{double} = 0.005$ [*u*-test]) [Figure 5].

Discussion

The present study investigated the incidence of cell death mechanisms in the VCN, DCN, and ICC immediately and 1 week after a second noise exposure at traumatizing level. Compared to a corresponding single noise exposure, the influence of the second noise trauma on cell death mechanisms was very limited in the investigated basal structures of the central auditory pathway. Surprisingly, an increase in TUNEL-positive cells was seen in the 14-day groups independent of the number of sound exposures in all investigated areas. This might be a long-term effect upon the first noise exposure rather than a consequence of the second noise trauma.

Influence of a second noise trauma on the incidence of apoptosis in VCN, DCN, and ICC

Our group showed a direct impact of a single noise exposure on apoptosis in the VCN, DCN, and ICC and described the time course of cell death over 1 week post-exposure.[19] In the present study, a second noise trauma was performed 1 week after the first trauma [Figure 1], when the peak of cell death after the first noise exposure had already been exceeded in VCN, DCN, and ICC.[19] It was shown that the incidence of apoptosis immediately increased after the first noise exposure in VCN and ICC.[19] The present second noise exposure could not alter cell death mechanisms in VCN, DCN, and ICC in the same intensity. No increase in TUNEL was seen immediately after the second noise exposure (7-day group-*double*) compared to the corresponding single group (7-day group-*single)* [Figures 3–5]. It was shown that the incidence of apoptosis on day 7 after a single noise exposure was either decreased to pretraumatic level (VCN) or slightly upregulated (ICC and DCN) compared to unexposed controls.[19] Therefore, the small number of TUNEL-positive cells in the present 7-day groups is less surprising, especially in the small analyzed area of the DCN. In the present investigation, on day 7 after repeated noise exposure (14-day group-*double*) [Figure 1], the incidence of TUNEL was increased compared to 7-day group-*double*. This increase seems to be mainly triggered by factors other than the second noise exposure as it was also seen in the corresponding single groups [Figures 3–5].

With regard to the present results, it should be considered for different reasons that a second traumatizing noise exposure might not be processed in the basal structures of the central auditory pathway in the same manner as the first noise trauma. First of all, the previous studies have shown that the present noise paradigm leads to a permanent threshold shift in NMRI mice with a loss of hair cells in the cochlear frequency range of noise stimulation that extends over time.[3,23,35-38] Due to the identical noise trauma characteristic of the first and second noise exposure, it seems plausible that the frequency range of the second noise application has its largest impact on the tonotopic region in the organ of corti that shows destroyed hair cells due to the first noise exposure. Concerning hair cell function as mechanoreceptor and afferent neuron of the VIII cranial nerve, the second noise trauma might not be transduced to the central auditory pathway in the same intensity. Second, a massive reduction in cell densities had been shown 1 week after a single noise exposure in an earlier study (neuronal loss of 39% in DCN, 30% in VCN, and 31% in ICC, respectively), exactly the moment in time when the second noise exposure was performed in the present study.[18] Besides deafferentation due to a noise-damaged cochlea, neuronal loss is hypothesized as a direct consequence of noise.[18] Third, noise mainly affects acoustic-sensitive neurons but the CN integrates also the nonauditory inputs.[39] As a consequence after noise damage, the CN might change in responsiveness to nonauditory stimuli.[40] The compensatory stronger nonauditory input to the CN and the ICC could lead to a weaker sensitivity to a second auditory stimulation. The interaction of several of these effects might protect the central auditory pathway from further damage and contribute to

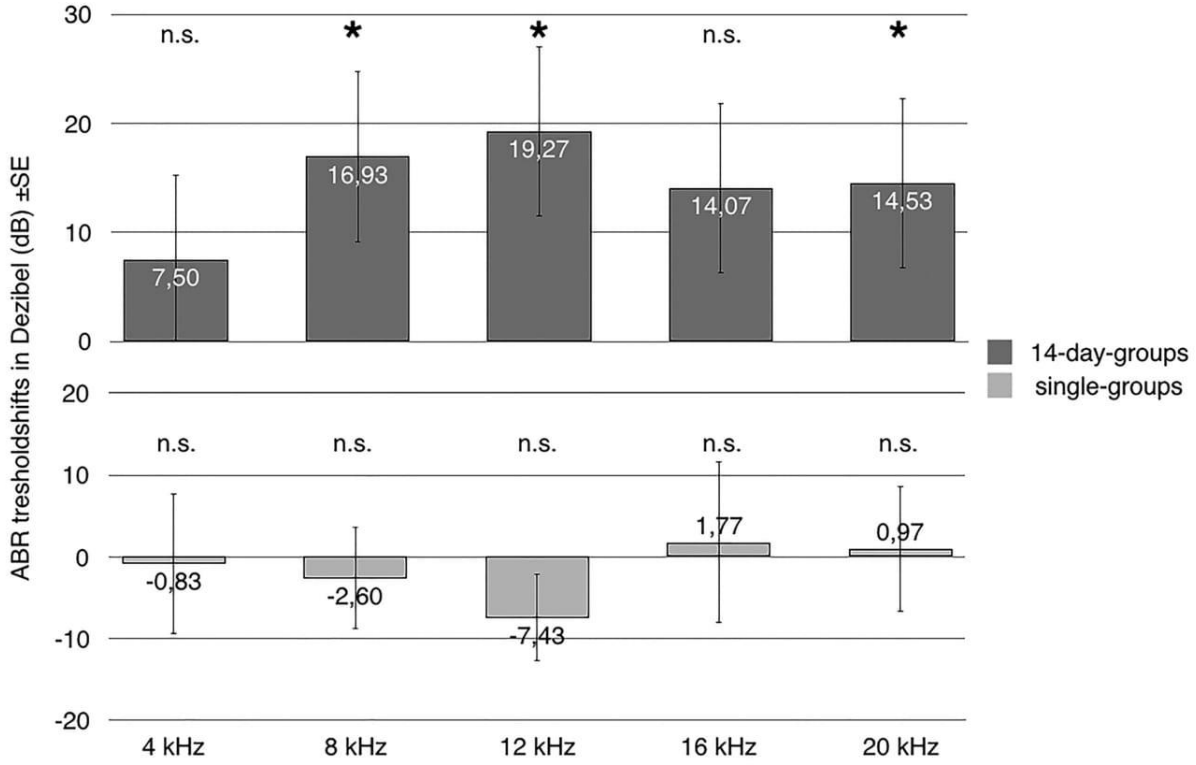

Figure 6: ABR measurements. ABR threshold shifts at different frequencies of the present 14-day groups (dark gray) and the present single groups (light gray). A threshold shift was seen in the 14-day group-double compared to the corresponding single group in all measured frequencies (dark gray) and statistically significant at 8 kHz ($P = 0.045$), 12 kHz ($P = 0.013$), and 20 kHz ($P = 0.04$). No big changes in ABR thresholds were seen in the 14-day group-single compared to the 7-day group-single (light gray). Either the recovery at 4 kHz ($P = 0.923$), 8 kHz ($P = 0.665$), and 12 kHz ($P = 0.235$) or the worsening at 16 kHz ($P = 0.855$) and 20 kHz ($P = 0.904$) had statistical significance. Data from earlier investigations of our group.[23] ABR = auditory brainstem recording.

explain the present findings in TUNEL in the double group compared to the single group. Auditory brainstem recordings from earlier investigations performed in the present 14-day groups are shown in Figure 6. The additional shift in hearing thresholds was dramatically smaller after a second noise exposure (compared to the first one) and supports the hypothesis of a weaker responsiveness to a second noise exposure.[23] Furthermore, calcium was statistically significantly elevated upon a second noise trauma in VCN, DCN, and ICC.[23] Its role as a mediator in the generation of necrosis and apoptosis was recently described but calcium is also dose dependent highly involved in neuroprotective mechanisms and might have a protective function in the present study.[23,41-46] Calcium might also modulate neuronal nitric oxide synthase and calcium-binding protein calretinin, which increased after single and multiple noise trauma in the CN.[22] Nitric oxide is discussed to play a role in cytotoxicity, cell death, and hearing disorders but can be cell protective depending on its dose.[22,47] The changes on a molecular level might have a protective role during the second noise exposure in the present study. It might hold true that homeostatic mechanisms induced by the first trauma help to protect the CN and ICC from further damage, at least during a specific timeslot. These long-term effects might contribute to the present increase in TUNEL in the 14-day group independent of the number of exposures.

Long-term effects of the initial noise exposure on apoptosis in VCN, DCN, and ICC

It was earlier hypothesized that noise-induced apoptosis and cell loss seem to terminate 1 week after a single noise trauma in the VCN, whereas it was still observed in DCN and ICC.[18,19] However, the incidence of TUNEL was increased in the present study in VCN, DCN, and ICC in the 14-day group, apparently independently of the second noise exposure (no differences between the single and double groups). There are different reasons for the present increase in TUNEL in the VCN, DCN, and ICC 2 weeks post-exposure. Neuronal degeneration in the CN varies with the age of the animals as well as trauma etiology.[9,48] Therefore, the time course of cell death after cochlea removal might not totally match with the noise-induced deafferentation. Nevertheless, cochlea removal or destruction led to increased TUNEL and a continuing or reflating process of cell death beyond 1-week post-exposure in the basal structures of the auditory system.[48,49] Consequently, the present increase in TUNEL in the 14-day group might rather represent long-term effects of the first noise trauma than a consequence of noise itself. This might also explain the findings of earlier auditory brainstem recording measurements that did not show big differences in hearing thresholds in the present single groups [Figure 6].[23] These long-term effects might include deafferentation and neuroplasticity due to a noise-damaged

organ of corti with cochlea damage and changed mechanotransduction after hair call loss.[35,37,50] Deafferentation, reorganization, and neuroplasticity take place around 2 weeks post-lesion and go along with apoptosis.[51,52] This is provided by lacking plasticity markers within 1 week post-exposure in the VCN but their upregulation between 15 and 30 days post-exposure.[51] Statistically significant degeneration was found 1-week post-exposure and progressed for weeks post-exposure in the CN.[8,9,53,54] The sustained increase of choline acetyltransferase activity in the CN granular region after trauma could be consistent with the formation of new cholinergic synapses or upregulation of existing cholinergic synapses upon granule cells and could have got caught by the present 14-day group.[55-57] Furthermore, excitatory neurotoxicity in the mammalian central nervous system is largely mediated by excessive release of glutamate that might also contribute to a raising cell death in VCN, DCN, and ICC post-exposure.[58,57] Axonal sprouting like reactive axonal growth was described to occur from weeks up to months after trauma in the DCN and contributes to the process of reorganization.[54,59] It was shown that the present noise paradigm led to an increase of spontaneous activity in CN and ICC that reflects a more slowly developing phenomenon and occurs secondarily after induction.[38,60-63] The neuronal hyperactivity as compensatory mechanism for lost excitatory network inputs might push apoptosis as seen in the present 14-day group.[38,64] However, the ICC might profit from inhibitory interneurons that project from the CN to higher structures and possibly have protecting function.[19] This theory is provided by earlier investigations about TUNEL and neuronal activity that was much lower in the ICC than in the CN.[19,64] Apoptosis in the ICC may be derived, prolonged, and stimulated from its external inputs. The most powerful of these inputs would be the (hyperactive) inputs from the DCN, as these project directly to the contralateral ICC.[64-68]

Conclusion

The present investigation showed an increase in TUNEL 2 weeks after the initial noise trauma independent of the number of noise exposures. It seems that the first noise trauma has a long-lasting effect on apoptotic mechanisms in the CN and ICC. These results might help to understand the underlying mechanisms of different psychoacoustic phenomena in NIHL such as tinnitus, hyperacusis, or reduced speech perception.

Financial support and sponsorship

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Conflicts of interest

There are no conflicts of interest.

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12. Curriculum Vitae

Mein Lebenslauf wird aus datenschutzrechtlichen Gründen in der elektronischen Version meiner Arbeit nicht veröffentlicht.

13. Publication list

Publication 1:

Strübing I, Gröschel M, Schwitzer S, Ernst A, Fröhlich F, Jiang D, Boyle P, Basta D. Neuroprotective effect of near-infrared light in an animal model of CI surgery. *Audiol Neurotol.* 2020: Vol. 26: 95–101.

Publication 2:

Basta D, Gröschel M, **Strübing I**, Boyle P, Fröhlich F, Ernst A, Seidl R. Near-infrared-light pre-treatment attenuates noise-induced hearing loss in mice. *PeerJ.* 2020: Vol. 8: e9384.

Publication 3:

Fröhlich F, Gröschel M, **Strübing I**, Ernst A, Basta D. Apoptosis in the cochlear nucleus and inferior colliculus upon repeated noise exposure. *Noise Health.* 2018: Vol. 20: 223–231.

Publication 4:

Fröhlich F, Basta D, **Strübing I**, Ernst A, Gröschel M. Time course of cell death due to acoustic overstimulation in the mouse medial geniculate body and primary auditory cortex. *Noise Health.* 2017. Vol. 19: 133–139.

Publication 5:

Fröhlich F, Ernst A, **Strübing I**, Basta D, Gröschel M. Apoptotic mechanisms after repeated noise trauma in the mouse medial geniculate body and primary auditory cortex. *Exp Brain Res.* 2017: Vol. 235: 3673–3682.

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