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Pathology of the inner ear after acoustic injury

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Abstract We examined the impact of acoustic injury on the cochlea by assessing auditory brain-stem response (ABR) threshold shifts, outer hair cells, and the number of synaptic ribbons before and after noise exposure. The ABR threshold was elevated immediately after noise exposure, but recovered over time; 14 days after noise exposure, the ABR threshold was approximately 15 dB higher than before exposure. There were few damaged outer hair cells. The number of synapses directly under inner hair cells markedly decreased immediately after noise exposure, but then exhibited early signs of recovery. Twenty-four hours after noise exposure, the number of synapses was higher than before exposure. Subsequently, synapse number decreased. Seven days after noise exposure, synapes number was lower than before exposure. In this acoustic injury model, although the synapses were damaged, as described in the previous reports on the temporary threshold shift (TTS), the regeneration of synapses was promoted soon thereafter. There appears to be a point during the repair process when the number of synapses temporarily increases. This model is considered to be a partially reversible synaptic damage model. This study revealed an association between the intensity of noise exposure and the severity of functional and histological damage.

Key words: synaptic ribbons, noise exposure, temporary threshold shift

Introduction

In Europe and the United States, hearing loss due to acoustic injury is regarded as extremely critical and has become a major social issue. Acoustic injury causes mechanical and metabolic disturbances in the cochlea[1]. After acoustic injury, the hearing threshold is elevated temporarily (temporary threshold shift [TTS]) or permanently (permanent threshold shift [PTS]), depending on the intensity and duration of injury[2,3]. Elucidation of the pathology of hearing loss due to acoustic injury is necessary for the development of appropriate treatment protocols. In this study, we developed an acoustic injury model and used it to examine temporal and histological changes in hearing ability following damage.

Methods

Hartley albino guinea pigs with normal Preyer's reflex and tympanic membranes (350-400 g; Chiyoda Kaihatsu Co., Ltd., Tokyo, Japan) were used. Within 1-2 days before the experiments, auditory brain-stem response (ABR) tests were performed to confirm that the animals exhibited no right/left differences in hearing ability. All experiments were performed based on a protocol approved by the Yamaguchi University Science Research Center Institute of Laboratory Animals, and complied with the Animal Welfare Act and the *Guidelines for the Care and Use of Laboratory Animals*.

Assessment of hearing ability

Before noise exposure, and 3 hours, 12 hours, 24 hours, 3 days, 7 days and 14 days after exposure, ABR tests were conducted to assess hearing ability. The tests were performed under general anesthesia with medetomidine (intraperitoneally at 1 mg/kg), xylazine (intraperitoneally at 2 mg/kg), and pentobarbital sodium (intraperitoneally at 24 mg/kg). In ABR tests, platinum needle electrodes were inserted under the skin of the animals. Measurements were taken with the cathode at the glabellar midline, the anode at the vertex, and the ground electrode under the skin of the trunk, and responses were recorded. The stimulating tone was 8-kHz tone bursts for 0.8 seconds with a 0.2-second interval. Acoustic stimulation was applied a total of 500 times. A signal processor was used for recording. In order to apply acoustic stimulation, a 10-cm tube was connected to an earphone and inserted into the external auditory canal. ABR test results were recorded using a Synax 1100 signal processor (NEC Co., Tokyo, Japan). The ABR threshold was defined as the lowest measurable level of stimulation among 3-5 waveforms.

Infliction of acoustic injury

While guinea pigs were anesthetized as described above and fixed in an anechoic chamber, a speaker was placed at a location 10 cm above the middle of the head. Near both external auditory canals of the animals, sound pressure was measured in the flat mode with a sound-level meter (NA-60, Rion, Tokyo, Japan) in order to homogenize the conditions. Animals were exposed to high-intensity noise (110 dB sound pressure level [SPL] octave-band noise centered at 4 kHz) for 3 hours.

Histological examination of synaptic ribbons

Before noise exposure, and 3 hours, 12 hours, 24 hours, 3 days, and 7 days after exposure, animals were transcardially perfused with 200 mL of physiological saline after deep anesthesia as described above, and transcardial perfusion fixation was immediately performed with 4% paraformaldehyde. Next, animals were decapitated to extract the temporal bones. The cochlear apex and the oval window of the extracted temporal bones were opened, and 4% paraformaldehyde was injected into the cochlea, followed by post-fixation for 12 hours at room temperature. The portion between 45% and 70% of the distance from the cochlear apex to the basal turn (cochlear second turn) was taken out, irrigated with phosphate-buffered saline (PBS), blocked at 4℃ for 2 hours (1% bovine serum albumin, 0.4% caprine serum, and 0.4% equine serum), and hydrated with PBS with 0.3% Triton X-100 for 10 minutes. For staining of synaptic ribbons, C-Terminal Binding Protein-2 (CtBP2; BD Biosciences, Franklin Lakes, NJ, USA) (4℃, 24 hours, 1:200) was used as the primary antibody, and Alexa Fluor 594 goat anti-mouse immunoglobulin G (IgG) (Molecular Probe, Eugene, OR, USA) (4℃, 24 hours, 1:200) was used as the secondary antibody. In order to confirm whether synaptic ribbons stained with CtBP2 were contiguous with the cochlear nerve terminals, anti-neurofilament heavy polypeptide antibody (MILLIPORE, Billerica, MA, USA) (4℃, 12 hours, 1:200) and anti-chicken IgY-NL-493 (R&D SYSTEM, Minneapolis, MN, USA) (4℃, 12 hours, 1:200) were used as the primary and secondary antibodies, respectively, for staining of cochlear nerves. Next, nuclear staining of the nerves was performed with 4,6-diamidino-2-phenylindole (DAPI; Vector Laboratories), and specimens were observed under a fluorescence microscope (KETENCE, Biozero, Osaka, Japan). By observing synaptic ribbons directly under the inner hair cells in the portion between 45% and 70% of the distance from the cochlear apex to the basal turn (cochlear second turn), the number of synaptic ribbons per 10 inner hair cells was determined in the portion with the lowest density of synaptic ribbons. Synaptic ribbons were observed in the cochlear second turn because injury caused by exposure to octave-band noise centered at 4 kHz is most severe in turn that region[4].

Histological examination of outer hair cells

Before noise exposure, and fourteen days after noise exposure, the temporal bones were extracted under deep anesthesia as described above. A pore was formed in the apical turn of the cochlea of the extracted temporal bones, and the perilymph space was

perfused with 4% paraformaldehyde from the oval window, followed by postfixation for 12 hours at room temperature. The organ of Corti was extracted and infiltrated with 0.3% Triton X-100 for 10 minutes. Next, the specimens were stained with 1 μg/mL fluorescein isothiocyanate-conjugated phalloidin (Sigma, St. Louis, MO, USA) in PBS for 1 hour. After irrigation with PBS, nuclei were stained with DAPI, and the specimens were observed under a fluorescence microscope. By observing the outer hair cells in the cochlear second turn, the portions with the lowest density of outer hair cells were assessed.

Statistical analysis

All data are expressed as mean and standard deviation. Statistical analysis was performed by Fisher's protected least significant difference test using the StatView software package for Macintosh, version 4.5J (Abacus Concepts, Berkeley, CA, USA). A P value < 0.05 was considered to indicate significance.

Results

ABR thresholds before and after noise exposure

Although the ABR threshold was elevated from approximately 5 dB before noise exposure to approximately 45 dB immediately after exposure, recovery of the threshold was subsequently observed over time. The ABR threshold 14 days after noise exposure was approximately 15 dB higher than the threshold before exposure (Figure 1).

Loss of outer hair cells after noise exposure

No loss of outer hair cells was observed 14 days after noise exposure (Figure 2).

Changes in the number of synaptic ribbons after noise exposure

A decrease in synaptic ribbons was observed 3 hours after noise exposure. However, the number of ribbons gradually increased afterwards, and was significantly higher 24 hours after noise exposure than before exposure. Subsequently, the number of synaptic ribbons gradually decreased, and was slight-

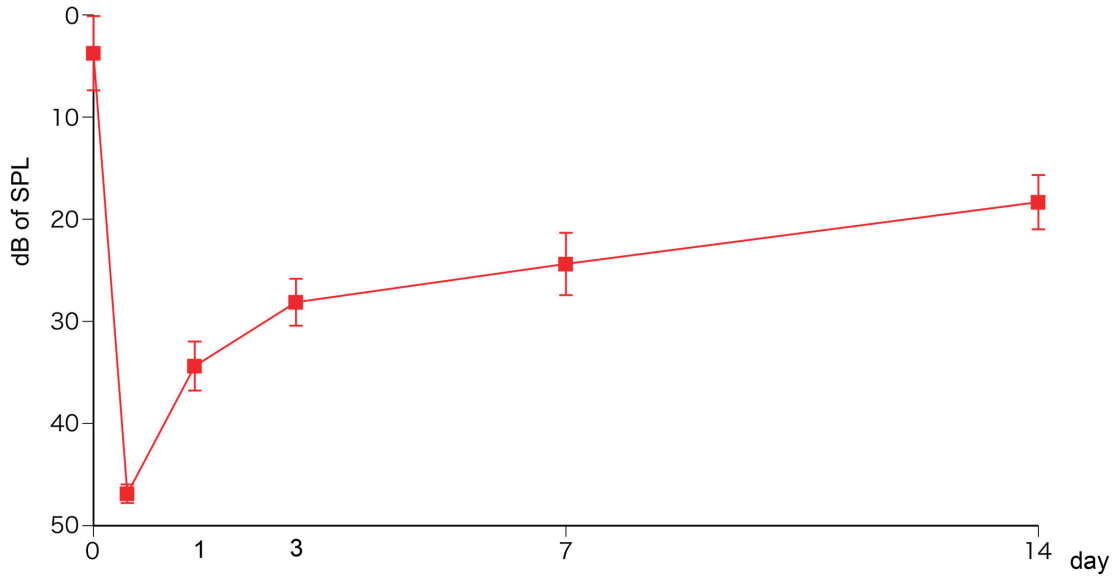

Figure 1: ABR thresholds

ABR thresholds measured at 8 kHz before and after noise exposure are shown (n = 8). The vertical axis represents thresholds measured in decibels (dB) of SPL, and the horizontal axis represents the number of days before and after noise exposure. The thresholds are approximately 5 dB before noise exposure, and approximately 45 dB immediately after noise exposure. Despite subsequent gradual recovery, thresholds do not reach the levels before noise exposure. Data represent the means ± SEM.

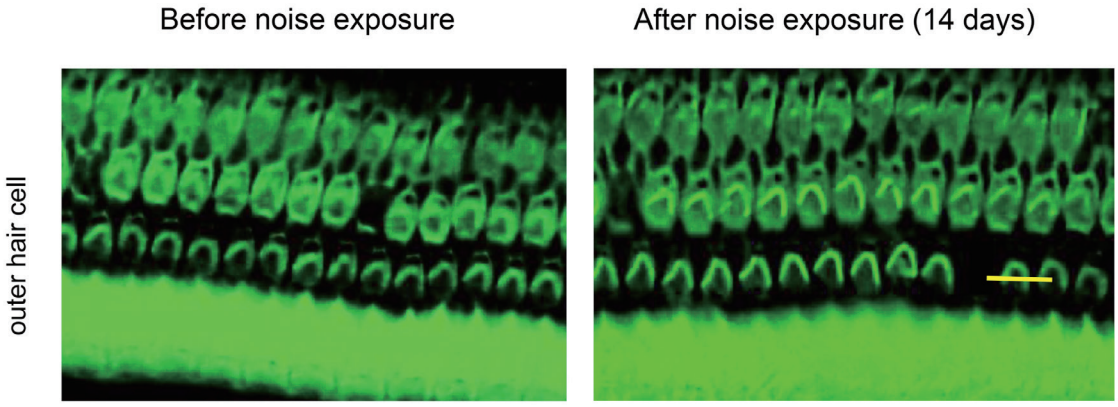

Figure 2: Outer hair cells in the organ of Corti in the cochlear second turn after noise exposure

This image shows outer hair cells in a specimen of cochlear second turn in the organ of Corti from a cochlea obtained before noise exposure and 14 days after noise exposure. No loss of outer hair cells is observed. Scale bar = 20 μm

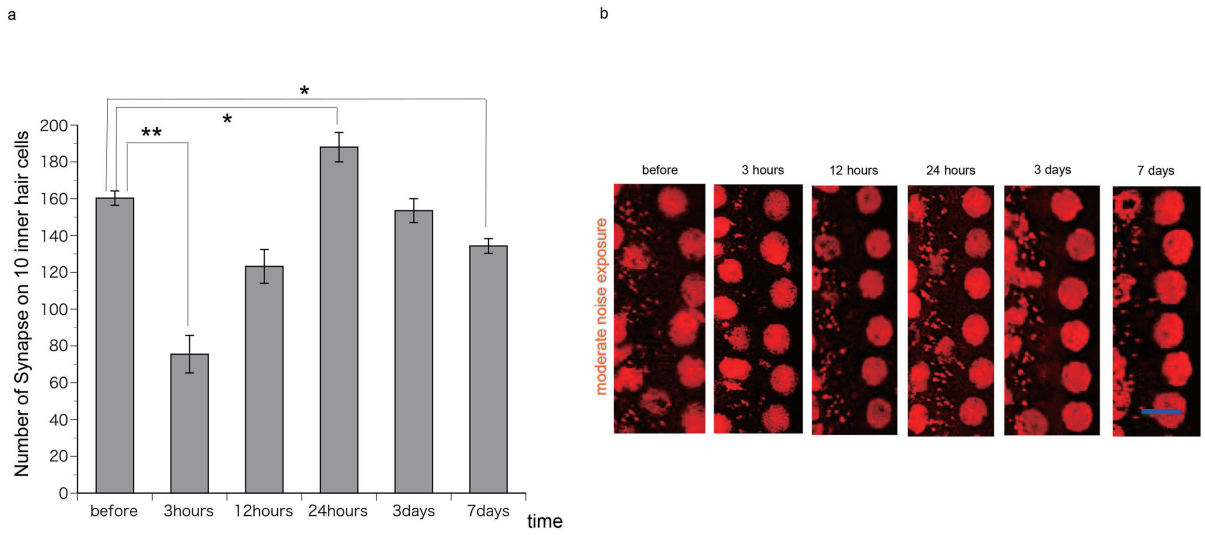

Figure 3: Changes in the number of synaptic ribbons after noise exposure

(a) This figure shows the number of synaptic ribbons per 10 inner hair cells in the cochlear second turn in the organ of Corti of cochleas before exposure and 3 hours, 12 hours, 24 hours, 3 days, and 7 days after noise exposure (n = 6). The vertical axis represents the number of synaptic ribbons per 10 inner hair cells, and the horizontal axis represents time after noise exposure. Error bars indicate ± SEM.

(b) The number of synaptic ribbons in the cochlear second turn in the organ of Corti of the cochleas compared in Figure 3(a) (n = 6). Although a decrease in synaptic ribbons was observed 3 hours after noise exposure, the number gradually increased afterwards. The number of synaptic ribbons was higher 24 hours after noise exposure than before exposure, and subsequently gradually decreased. The number of synapses was slightly lower 7 days after noise exposure than before exposure. Scale bar =10 μm

ly lower 7 days after noise exposure than before exposure (Figure 3).

Discussion

The synaptic ribbon is a synapse located between the hair cell and the auditory nerve fiber[4], and CtBP2 is a component protein of the synaptic ribbon[5,6]. Anti-CtBP2 antibody has been used to assess synaptic ribbons directly under the inner hair cells by researchers in multiple fields[7]. In the past, assessment of synapses directly under hair cells was primarily performed using electron microscopy[8], and it was difficult to determine the number of synapses per hair cell. However, the use of anti-CtBP2 antibody has made it relatively easy to assess synapses directly under hair cells. In our experiments, we also used this method to assess synaptic ribbons directly under inner hair cells.

Mechanism of acoustic injury

In this study, we assessed thresholds by performing ABR tests at 8 kHz on the basis of previous report that exposure to octave-band noise centered at 4 kHz affects ABR thresholds at frequencies higher than 4 kHz[9], and our previous observation that ABR thresholds at 8 kHz are strongly affected by exposure to octave-band noise centered at 4 kHz[10,11].

Unlike TTS, in our acoustic-damage model the ABR threshold gradually recovered but was not fully restored to the level before damage. However, no damage to outer hair cells was observed in association with surface preparation. Previous studies have reported that the rates of loss of outer hair cells and ABR thresholds are not coherent[12,13], possibly because even though outer hair cells appear normal, microstructures such as mechano-electrical transduction channels and synapses have actually been damaged, resulting in loss of cellular function[13]. In this study, a recovery in the number of synapses was observed immediately after noise exposure, and the number of synapses 24 hours after noise exposure was higher than the number before exposure. Subsequently, the number of synapses decreased and was slightly lower 7 days after noise exposure than before exposure. According to recent studies, the formation of synapses occurs in a short period of time, ranging from 30 minutes to a few hours[14]. Moreover, the number of synapses decreases 24 hours after noise exposure during TTS[15]. In our study, the number of synapses 24 hours after noise exposure was higher than before exposure; this result contradicts those of previous reports. The number of peripheral vestibular synapses increases under weightless conditions in the vestibular area[16]. Thus, the number of synapses in affected regions temporarily increases, to an extent that depends on the types and severity of stress. We hypothesized that the same mechanism might apply to the cochlea. In other words, although synapses are damaged in this acoustic damage model, as shown in previous reports on TTS, formation of synapses is immediately promoted, and the number of synapses temporarily increases during the repair process 24 hours after noise exposure. The finding that the number of synapses 7 days after noise exposure was slightly lower than before exposure was consistent with the incomplete recovery of the ABR threshold measured at 8 kHz 7 days after noise exposure. Thus, this model can be considered to be a partially reversible synaptic damage model. Although synapses are temporarily regenerated in excess after the number of synapses is decreased by damage, non-functional synapses may ultimately undergo apoptosis and be eliminated. This report is the first report that the number of synapses changes with passage of time after damage.

For treatment of this type of acoustic trauma, administration of drugs that promote recovery of synapses may be effective. Future studies are required to determine how to efficiently deliver those drugs to the inner ear.

Conflict of Interest

The authors state no conflict of interest.

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