Increased expression level of Hsp70 in the inner ears of mice by exposure to low frequency noise

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Abstract

Previous studies showed that people in urban areas are possibly exposed to 60-110 dB of low frequency noise (LFN) defined as noise of ≤100 Hz in their daily life. Previous studies also showed increased health risks by exposure to high levels (130-140 dB) of LFN in animals. However, little is known about the health effects of exposure to an ordinary level of LFN. We biochemically and immunohistochemically assessed the effects of exposure to inaudible LFN for mice (12 hours/day of 100 Hz LFN at 95 dB for 5 days), at a level to which people are possibly exposed in daily life, on a murine inner ear by targeting 9 stress-reactive molecules. There was more than a 5-fold increased transcript level of *heat shock protein 70 (Hsp70)* in the whole inner ear exposed to LFN. However, the transcript levels of the other 8 stress-reactive molecules including *Hsp27* and *Hsp90* were comparable in LFN-exposed and unexposed murine inner ears. Only the transcript level of *Cebpβ* among the previously reported 4 transcriptional activators for *Hsp70* expression was more than 3-fold increased by LFN exposure. *Hsp70* transcript expression levels in the inner ears 3 days after LFN exposure were comparable to those in unexposed inner ears. The protein level of Hsp70, but not the levels of Hsp27 and Hsp90, was also increased in the vestibule by LFN exposure. However, hearing levels as well as expression levels of Hsp70 protein in the cochleae were comparable in LFN-exposed mice and unexposed mice. Our results demonstrated that the inner ear might be one of the organs that is negatively affected by stress from inaudible LFN exposure. Moreover, LFN exposure might increase Hsp70 expression level via *Cebpβ* in the inner ear. Thus, Hsp70 and Cebpβ levels could be candidates of biomarkers for response to LFN exposure.

Key words:
Low frequency noise; Inner ear; Heat shock protein 70; Cebpβ; Vestibule

***Abbreviations*:**
LFN: low frequency noise; HSP: heat shock protein

1. Introduction

People in urban areas are generally exposed to 60-110 dB of low frequency noise (LFN) defined as noise of ≤100 Hz in their daily life including the workplace (Berglund et al., 1996). LFN consisting of audible and inaudible components generally derived from the daily environment including noise from air conditioning, road traffic and wind turbines has been reported to cause health disturbances including dizziness in humans (Baliatsas et al., 2016; Schmidt and Klokker 2014). However, previous studies showed a limited effect of LFN on hearing level in humans (Mohr et al. 1965; Slarve and Johnson 1975). In addition to exposure to LFN including audible and inaudible components, sole exposure to inaudible LFN has been shown to cause the development of nystagmus and body sway but not hearing loss in humans (Evans and Tempest 1972; Johnson 1973, 1980; Takigawa et al., 1988). These findings in humans suggest that LFN affects the vestibule rather than the cochlea. However, the health risk of LFN exposure in humans remains controversial (Baliatsas et al., 2016). Occupational and environmental standards have not been established in most countries.

Basic study using animals is useful for assessing the health risk of LFN exposure because of ethical limitations in human studies. High levels (130-140 dB) of inaudible LFN exposure for rats for 2-72 hours induced apoptosis in cardiac myocytes and hippocampus cells (Pei et al., 2011, Zhang et al., 2016). Those studies have shown increased health risks from exposure to high levels of inaudible LFN. However, further studies on the biological effects of exposure to inaudible LFN for animals at a level to which people are possibly exposed in daily life for a short period are also needed for health risk assessment.

Biomarkers are strong tools for assessing health risks as well as stages of diseases. Although biochemical studies on LFN exposure are essential for the development of biomarkers and preventive therapies, such studies have been limited compared to physiological and morphological studies. There is still no candidate biomarker for LFN-mediated health risk. Previous studies showed that Tnf, Il1β, Il6, Gstα1, Nqo1, Hmox1 and heat shock proteins (HSPs) including Hsp27, Hsp70 and Hsp90 are molecules regulated by environmental stresses such as allergens, ultraviolet light and heat (Abed et al., 2015; Bolton, 2005; Lanneau et al., 2010). However, no stress-reactive molecule that responds to LFN exposure has been reported.

Our previous physiological and histological studies in mice showed that exposure to inaudible 100 Hz LFN at 70 dB for one month resulted in the development of impaired balance with damage to the inner ear (Tamura et al., 2012; Ohgami et al., 2017). In this study, we biochemically investigated the effect of ordinary levels of exposure to inaudible LFN for mice for a short period (12 hours/day of 100 Hz LFN at 95 dB for 5 days) on expression levels of various stress-reactive molecules in the inner ears of wild-type mice.

2. Materials and methods

2.1. Mice preparation

Wild-type ICR mice were originally obtained from Charles River Laboratories, Inc. Three-week-old mice were obtained for experiments by self-breeding (total of 44 mice). Males and females were used for quantitative RT-PCR analysis, and only males were used for histological and auditory brain-stem response (ABR) analyses. All experiments were authorized by the Animal Care and Use Committee in Nagoya University (approval number: 28251, 29031) and the Institutional Recombinant DNA Experiment Committee in Nagoya

University (approval number: 16–73, 17–65) and followed the Japanese Government Regulations for Animal Experiments.

2.2. LFN exposure

Figure 1 shows the system of exposure to inaudible LFN for mice (100 Hz at 95 dB) (Fig. 1A-C) and the sampling schedule from mice (Fig. 1D). Control mice were kept in a mice cage with background noise of 100 Hz at 60 dB (Fig. 1B). Expression levels of *Hsp70* in murine inner ears were comparable in the mice sited in the LFN exposure spot (five times for 12 hours) without LFN exposure (n=3) and the control (n=3) littermate mice in the rack, indicating a limited effect of replacement from the mouse rack to the exposure room on *Hsp70* expression level. To investigate the health effect of inaudible LFN, we generated 100 Hz LFN (Fig. 1B) with a setting of 0.1-ms rise-fall, 600-ms interval and 10-ms flat by a sound stimulator (DPS-725, Dia medical Systems Co., LTD, Japan) and outputted the noise from a speaker (powered subwoofer). The level of LFN was monitored by a noise level meter (Type 6224 with an FFT analyzer, ACO Co., LTD, Japan) in every experiment.

2.3. Quantitative RT-PCR analysis

Quantitative RT-PCR analysis was performed using whole murine inner ears that were dissected after LFN exposure and stored at -80°C. A single inner ear was homogenized with a multi-beads shocker (Yasui Kikai) in 500 μl of Isogen II (Nippon Gene, 311-07361), and RNAs were purified according to the manufacturer's protocol. From the RNAs, cDNAs were synthesized with Primescript RT Master Mix (Takara, PR36A) followed by quantitative PCR with SYBR green (Roche, 04913850001) on a Thermal Cycler Dice Real Time system (Takara, TP800). Primers are presented in Supplemental Table S1. The Ct value was obtained from the average of triplicated PCR reactions. Gene expression was normalized with *Hprt* expression, and then the values in tissues isolated from the animals exposed to LFN were normalized with the average values in tissues from non-exposed control animals (Yajima et al., 2015).

2.4. Histological analysis

Inner ears were dissected from mice after intra-cardiac perfusion with 4% paraformaldehyde in phosphate buffered saline (PBS). The inner ears were further fixed with 4% paraformaldehyde in PBS overnight at 4°C, followed by decalcification with 10% EDTA for 2 days at 4°C. Then the inner ears were embedded in paraplast and sectioned at 4 μm. Antigen retrieval was performed on re-hydrated sections with 10 mM citrate buffer (pH 6.0) or with 10 mM Tris-1 mM EDTA buffer (pH 9.0, DAKO, #S2367, for anti-Myosin7a antibody) at 94°C for 10 minutes in immunohistochemical analysis. Rabbit anti-HSP70 polyclonal antibody (Santa Cruz, sc-33575), rabbit anti-Hsp27 (Hspb1) polyclonal antibody (Enzo, ADI-SPA-801), rabbit anti-HSP90 polyclonal antibody (Cell Signaling, #4875), rabbit anti-Myosin7a polyclonal antibody (Proteus, #25-6790) and Alexa Fluor 488- or Alexa Fluor 594-labeled donkey anti-rabbit IgG (Invitrogen) were used. Anti-HSP70 and anti-Myosin7a antibodies were diluted in Can Get Signal (TOYOBO) and the others were diluted in PBS with 0.1% Tween20. After immunoreactions, the sections were counterstained with 4', 6-diamidino-2-phenylindole (DAPI). We used inner ears from *HSP70*-transgenic mice, in which the human *HSP70* gene was introduced under control of the β-actin promoter (Plumier et al., 1995), as a positive control of our immunohistochemical analysis using the anti-HSP70 polyclonal antibody (Supplemental Fig. S1).

2.5. ABR analysis

Before and after exposure to LFN, we performed ABR measurements (AD Instruments Pty. Ltd.) as described previously (Ohgami et al., 2010; Ohgami et al., 2016). We used wild-type ICR mice at 3 weeks of age. Tone burst stimuli were measured 5 dB-stepwise from 0 dB SPL to 90 dB SPL. The threshold was judged by the appearance of the lowest level of I-IV waves of ABR.

2.6. Statistical analysis

Statistical analyses were performed by JMP Pro (version 11.0.0; SAS Institute Inc., Cary, NC, USA) as described previously (Ohgami et al., 2016). Since the ABR thresholds of all frequencies were discontinuous valuables, we performed the Mann-Whitney *U* test to evaluate statistical differences of hearing levels between the two groups. We performed the unpaired *t*-test by Excel to evaluate statistical differences of transcript expression levels between the two groups. In this study, values of $p < 0.05$ were considered statistically significant.

3. Results

3.1. Effects of LFN exposure on transcript expression levels of stress-reactive molecules in the whole inner ear

After five repeated exposures to 100 Hz LFN at 95 dB for 12 hours with a distance of 40 cm between the speaker and mice cage (Fig. 1), transcript expression levels of 9 stress-reactive molecules (*Tnf, Il1β, Il6, Gstα1, Nqo1, Hmox1, Hsp27, Hsp70* and *Hsp90*) in the inner ear were examined by quantitative RT-PCR (Fig. 2). The transcript expression levels of *Hsp70*, but not those of 8 stress-reactive molecules including *Hsp27* and *Hsp90*, in the inner ears from mice just after the fifth LFN exposure were significantly higher than those in the inner ears from unexposed control mice (Fig. 2A). *Hsp70* transcript expression levels in the inner ears 3 days after the LFN exposure were comparable to those in unexposed inner ears (Fig. 2B).

3.2. Effects of LFN exposure on transcript expression levels of regulatory molecules for Hsp70 in the whole inner ear

We next examined expression levels of four transcriptional regulators for *Hsp70* (*Hsf1, Stat1, Nfκb* and *Cebpβ*) (Guzhova et al., 1997; Stephanou et al., 1999; Stephanou and Latchman, 2005). Transcript expression levels of *Cebpβ,* but not those of the other 3 regulatory molecules, in inner ears just after the fifth LFN exposure were significantly higher than those in unexposed control inner ears (Fig. 3).

3.3. Effect of LFN exposure on protein expression levels of Hsp70 in the vestibule and cochlea

Protein expression levels of Hsp27, Hsp70 and Hsp90 in the vestibule and cochlea after exposure to LFN for 5 days were examined by immunohistochemical analysis (Figs. 4 and 5). Protein expression levels of Hsp70 in the utricles just after the fifth LFN exposure were higher than those in the utricles from unexposed control mice (Fig. 4B). We confirmed the presence of hair cells by expression of Myosin7a, a hair cell marker (Fig. 4D). Expression of Hsp70 was detected in both the cytoplasm and nucleus (Fig. 4B; Kose et al., 2012; Song et al., 2016; Welch and Feramisco, 1984). Protein expression levels of Hsp27 and Hsp90 were comparable in the utricles from LFN-exposed mice and unexposed mice (Fig. 4A, C). On the other hand, protein expression levels of Hsp70 in addition to Hsp27 and Hep90 were

comparable in cochleae from LFN-exposed mice and unexposed mice (Fig. 5). Expression patterns of Hsp70 were comparable in apical, middle and basal turns of the cochleae.

3.4. Effect of LFN exposure on hearing level in mice

Hearing levels of mice just after the fifth LFN exposure (12 hours/day of 100 Hz LFN at 95 dB for 5 days) and control mice were finally examined by ABR (Fig. 6). There were no differences in hearing levels at 4 k, 12 k, 20 k and 32 k Hz frequency sounds between LFN-exposed mice and unexposed mice.

4. Discussion

HSPs are molecular chaperones expressed in all cells (Lanneau et al., 2010). Expression of HSPs is induced by stresses such as fever, UV irradiation, and cytotoxic drugs (Lanneau et al., 2010). In this study, we showed that there was a more than 5-fold increased expression level of *Hsp70* in the whole inner ear after exposure for 5 days to LFN at a level to which we are possibly exposed in our daily life, while the expression levels of 8 other stress-reactive molecules including *Hsp27* and *Hsp90* were comparable in LFN-exposed mice and unexposed mice. The increased *Hsp70* levels after exposure to LFN for 5 days returned to unexposed levels 3 days later. We also confirmed that there was an approximately 2.5-fold significant increase of *Hsp70* level by LFN exposure for just 1 day (Supplemental Fig. S2A). The increased *Hsp70* levels after exposure to LFN for 1 day again returned to unexposed levels 3 days later (Supplemental Fig. S2B). These results suggest that *Hsp70* could be a candidate of biomarkers that sensitively rises and falls in response to LFN-mediated stress.

There was a temporary increase of Hsp70 expression after exposure to LFN for a short period in this study. Further study to examine the expression level of Hsp70 after exposure to LFN for a long period is needed in the future because there are many sources of chronic noise below the frequencies of human sensitivity that have been associated with health problems.

Among 4 kinds of transcriptional activators for *Hsp70* expression (Guzhova et al., 1997; Stephanou et al., 1999; Stephanou and Latchman, 2005), there was more than 3-fold increase of *Cebpβ* level by LFN exposure for 5 days. There was an approximately 3-fold significant increase of *Cebpβ* by LFN exposure for just 1 day (Supplemental Fig. S2C). These results suggest not only that *Cebpβ* is associated with the LFN-mediated increase of *Hsp70* but also that *Cebpβ* could also be a candidate of biomarkers for LFN exposure. While only *Cebpβ* transcript increased after LFN exposure, post-transcriptional modifications of transcription factors also regulate the expression of *Hsp70* (for example Hsf1; Gomez-Pastor et al., 2018). Therefore, we cannot exclude the other three factors that did not show a transcriptional increase as transcriptional regulators of *Hsp70* after LFN exposure.

A previous mouse study showed that an increased level of Hsp70 expression in the cochlea, an organ for hearing, by audible noise exposure results in hearing loss (Gong et al., 2012; Gratton et al., 2011; Roy et al., 2013). However, there has been no study showing an increased expression level of Hsp70 in the vestibule, an organ for balance, by exposure to any kind of noise. We immunohistochemically showed that there was an increased level of Hsp70 expression particularly in the endolymphatic side of utricle hair and supporting cells in the vestibule by LFN exposure, while levels of Hsp70 expression in the cochleae and hearing levels were comparable in LFN-exposed mice and unexposed mice. Our results showing that Hsp70 was expressed in the vestibule but not in the cochleae partially correlate with physiological results showing that inaudible LFN exposure resulted in the development of impaired balance but not hearing loss in our previous studies (Ohgami et al., 2017; Tamura et al., 2012) and the present study. While these studies suggest LFN induced stress in the vestibule, the reason for the increased level of Hsp70 expression in that region remains unknown. Since a previous study showed increased Hsp70 secretion from supporting cells around hair cells in the utricle by heat exposure (May et al., 2013), LFN exposure may

promote extracellular secretion of Hsp70. Further study is needed to clarify the mechanism and biological significance of increased Hsp70 expression level after exposure to LFN at a level we are possibly exposed to in our daily life for a short period.

In conclusion, we demonstrated for the first time that inaudible LFN exposure increases the expression level of Hsp70, which might be activated via *Cebpβ*, in the vestibule of the inner ear, suggesting that the vestibule is one of target organs for LFN. These stress-reactive molecules could be candidates of biomarkers for LFN exposure.

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References

Abed, D.A., Goldstein, M., Albanyan, H., Jin, H., Hu, L., 2015. Discovery of direct inhibitors of Keap1-Nrf2 protein-protein interaction as potential therapeutic and preventive agents. Acta Pharm. Sin. B. 5, 285-299. doi: 10.1016/j.apsb.2015.05.008.

Baliatsas, C., van Kamp, I., van Poll, R., Yzermans, J., 2016. Health effects from low-frequency noise and infrasound in the general population: Is it time to listen? A systematic review of observational studies. Sci. Total Environ. 557-558, 163-169. doi: 10.1016/j.scitotenv.2016.03.065.

Berglund, B., Hassmén, P., Job, R.F., 1996. Sources and effects of low-frequency noise. J. Acoust. Soc. Am. 99, 2985-3002.

Bolton, A.E., 2005. Biologic effects and basic science of a novel immune-modulation therapy. Am. J. Cardiol. 95, 24C-29C.

Evans, M.J., Tempest, W., 1972. Some effects of infrasonic noise in transportation. J. Sound Vib. 22, 19-24.

Johnson, D.L., 1973. Various aspects of infrasound. In: Pimonow, L., eds., Proceedings of Colloquium of Infrasound. Centre National de la Recherche Scientifique, Paris, pp. 337-356.

Johnson, D.L., 1980. The effects of high level infrasound. In: Moller, H.; Rubak, P., eds., Proceedings of the conference on low frequency noise and hearing. Aalborg University Center, Aalborg, pp. 47-60.

Kose, S., Furuta, M., Imamoto, N., 2012. Hikeshi, a nuclear import carrier for Hsp70s, protects cells from heat shock-induced nuclear damage. Cell 149, 578-589. doi: 10.1016/j.cell.2012.02.058.

Gomez-Pastor, R., Burchfiel, E.T., Thiele, D.J., 2018. Regulation of heat shock transcription factors and their roles in physiology and disease. Nat. Rev. Mol. Cell Biol. 19, 4-19. doi: 10.1038/nrm.2017.73.

Gong, T.W., Fairfield, D.A., Fullarton, L., Dolan, D.F., Altschuler, R.A., Kohrman, D.C., Lomax, M.I., 2012. Induction of heat shock proteins by hyperthermia and noise overstimulation in hsf1 -/- mice. J. Assoc. Res. Otolaryngol. 13, 29-37. doi: 10.1007/s10162-011-0289-9.

Gratton, M.A., Eleftheriadou, A., Garcia, J., Verduzco, E., Martin, G.K., Lonsbury-Martin, B.L., Vázquez, A.E., 2011. Noise-induced changes in gene expression in the cochleae of mice differing in their susceptibility to noise damage. Hear. Res. 277, 211-226. doi: 10.1016/j.heares.2010.12.014.

Guzhova, I.V., Darieva, Z.A., Melo, A.R., Margulis, B.A., 1997. Major stress protein Hsp70 interacts with NF-kB regulatory complex in human T-lymphoma cells. Cell Stress Chaperones 2, 132-139.

Lanneau, D., Wettstein, G., Bonniaud, P., Garrido, C., 2010. Heat shock proteins: cell protection through protein triage. ScientificWorldJournal 10, 1543-1552. doi: 10.1100/tsw.2010.152.

May, L.A., Kramarenko, I.I., Brandon, C.S., Voelkel-Johnson, C., Roy, S., Truong, K., Lanneau, S.P., Monzack, E.L., Lee, F.S., Cunningham, L.L., 2013. Inner ear supporting cells protect hair cells by secreting HSP70. J. Clin. Invest. 123, 3577-3587. doi: 10.1172/JCI68480.

Mohr, G.C., Cole, J.N., Guild, E., Gierke, von, H.E., 1965. Effects of low frequency and infrasonic noises on man. Aerospace Med. 36, 817-827.

Ohgami, N., Ida-Eto, M., Shimotake, T., Sakashita, N., Sone, M., Nakashima, T., Tabuchi, K., Hoshino, T., Shimada, A., Tsuzuki, T., Yamamoto, M., Sobue, G., Jijiwa, M., Asai, N., Hara, A., Takahashi, M., Kato, M., 2010. c-Ret-mediated hearing loss in mice with Hirschsprung disease. Proc. Natl. Acad. Sci. U S A. 107, 13051-13056. doi: 10.1073/pnas.1004520107.

Ohgami, N., Oshino, R., Ninomiya, H., Li, X., Kato, M., Yajima, I., Kato, M., 2017. Risk Assessment of Neonatal Exposure to Low Frequency Noise Based on Balance in Mice. Front. Behav. Neurosci. 11, 30. doi: 10.3389/fnbeh.2017.00030.

Ohgami, N., Yajima, I., Iida, M., Li, X., Oshino, R., Kumasaka, M.Y., Kato, M., 2016. Manganese-mediated acceleration of age-related hearing loss in mice. Sci. Rep. 6:36306. doi: 10.1038/srep36306.

Pei, Z.H., Chen, B.Y., Tie, R., Zhang, H.F., Zhao, G., Qu, P., Zhu, X.X., Zhu, M.Z., Yu, J., 2011. Infrasound exposure induces apoptosis of rat cardiac myocytes by regulating the expression of apoptosis-related proteins. Cardiovasc. Toxicol. 11, 341-346. doi: 10.1007/s12012-011-9126-y.

Plumier, J.C., Ross, B.M., Currie, R.W., Angelidis, C.E., Kazlaris, H., Kollias, G., Pagoulatos, G.N., 1995. Transgenic mice expressing the human heat shock protein 70 have improved post-ischemic myocardial recovery. J. Clin. Invest. 95, 1854-1860.

Roy, S., Ryals, M.M., Van den Bruele, A.B., Fitzgerald, T.S., Cunningham, L.L., 2013. Sound preconditioning therapy inhibits ototoxic hearing loss in mice. J. Clin. Invest. 123, 4945-4949.

Schmidt, J.H., Klokker, M., 2014. Health effects related to wind turbine noise exposure: a systematic review. PLoS One. 9, e114183. doi: 10.1371/journal.pone.0114183.

Slarve R.N., Johnson D.L., 1975. Human whole - body exposure to infrasound. Aviation, Space, and Environmental Medicine 46, 428-431.

Stephanou, A., Isenberg, D.A., Nakajima, K., Latchman, D.S., 1999. Signal transducer and activator of transcription-1 and heat shock factor-1 interact and activate the transcription of the Hsp-70 and Hsp-90beta gene promoters. J. Biol. Chem. 274, 1723-1728.

Stephanou, A., Latchman, D.S., 2005. Opposing actions of STAT-1 and STAT-3. Growth Factors. 23, 177-182.

Song, H., Kim, W., Kim, S.H., Kim, K.T., 2016. VRK3-mediated nuclear localization of HSP70 prevents glutamate excitotoxicity-induced apoptosis and Aβ accumulation via enhancement of ERK phosphatase VHR activity. Sci. Rep. 12, 38452. doi: 10.1038/srep35245.

Takigawa, H., Hayashi, F., Sugiura, S., Sakamoto, H., 1988. Effects of infrasound on human body sway. Journal of Low Frequency Noise and Vibration 7, 66-73.

Tamura, H., Ohgami, N., Yajima, I., Iida, M., Ohgami, K., Fujii, N., Itabe, H., Kusudo, T., Yamashita, H., Kato, M., 2012. Chronic exposure to low frequency noise at moderate levels causes impaired balance in mice. PLoS One 7, e39807. doi: 10.1371/journal.pone.0039807.

Yajima, I., Kumasaka, M.Y., Ohnuma, S., Ohgami, N., Naito, H., Shekhar, H.U., Omata, Y., Kato, M., 2015. Arsenite-mediated promotion of anchorage-independent growth of HaCaT cells through placental growth factor. J. Invest. Dermatol. 135, 1147-1156. doi: 10.1038/jid.2014.514.

Welch, W.J., Feramisco, J.R., 1984. Nuclear and nucleolar localization of the 72,000-dalton heat shock protein in heat-shocked mammalian cells. J. Biol. Chem. 259 4501–4513.

Zhang, M.Y., Chen, C., Xie, X.J., Xu, S.L., Guo, G.Z., Wang, J., 2016. Damage to Hippocampus of Rats after Being Exposed to Infrasound. Biomed. Environ. Sci. 29, 435-442. doi: 10.3967/bes2016.056.

Figure captions

Fig. 1. Experimental method and design for LFN exposure. A photograph of the method for LFN exposure (**A**), sound spectrum with (**C**) or without (**B**) LFN exposure and a schema (**D**) for sampling just after the fifth LFN exposure (12 hours/day of 100 Hz LFN at 95 dB for 5 days) are presented.

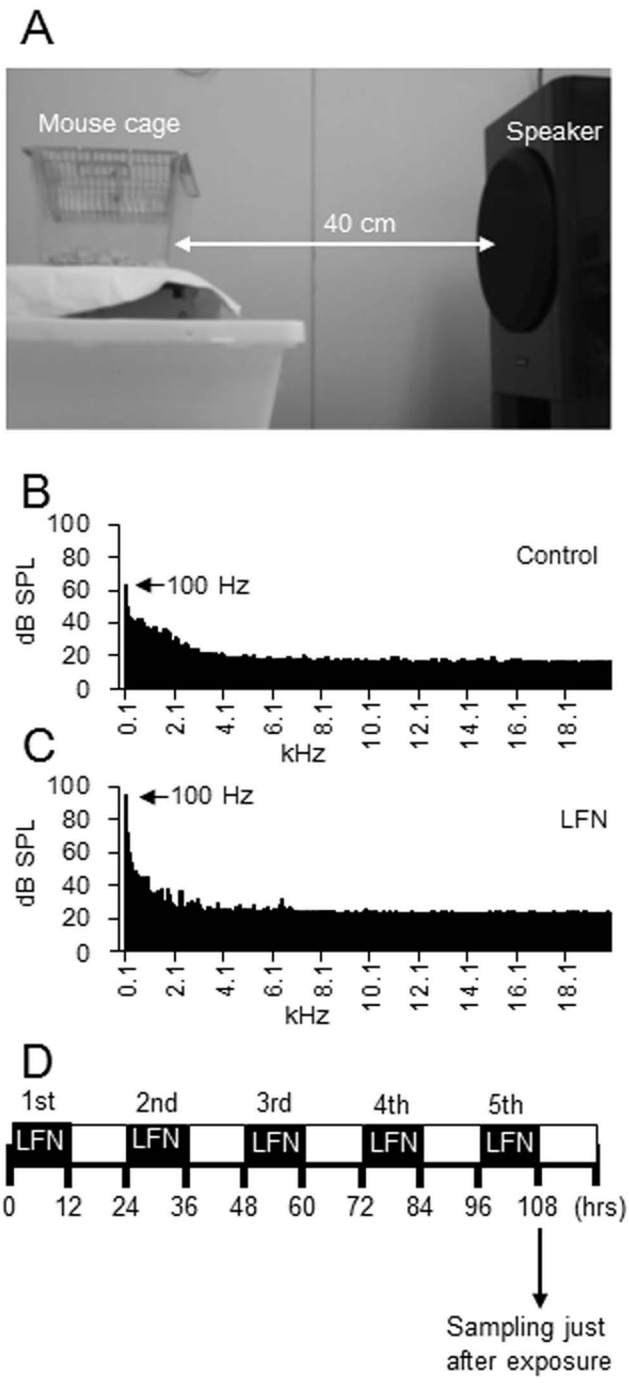
Fig. 2. Transcript expression levels of stress-reactive molecules in the inner ear. Transcript expression levels [mean ± standard deviation (SD)] of *Tnf, Il1β, Il6, Gstα1, Nqo1, Hmox1, Hsp27, Hsp70* and *Hsp90* in whole inner ears from mice just after the fifth LFN exposure (n=6, L, closed bars) and in whole inner ears from unexposed control mice (n=6, C, open bars) are presented (**A**). Transcript expression levels of *Hsp70* in whole inner ears from mice 3 days after the fifth LFN exposure (n=6, L, closed bars) and in whole inner ears from unexposed control mice (n=6, C, open bars) are presented (**B**). *: significantly different (p<0.05) by Student's t-test.

Fig. 3. Expression levels of 4 transcriptional activators for *Hsp70* expression. Expression levels (mean ± SD) of *Hsf1, Stat1, Nfκb* and *Cebpβ* transcripts in whole inner ears from mice just after the fifth LFN exposure (n=6, L, closed bars) and in whole inner ears from unexposed control mice (n=6, C, open bars) are presented. **: significantly different (p<0.01) by Student's t-test.

Fig. 4. Protein expression levels of Hsp70 in the vestibule. Protein expression levels of Hsp27 (**A**, red), Hsp70 (**B**, green), Hsp90 (**C**, red) and Myosin7a (D, Red) in utricles (vestibule) from mice just after the fifth LFN exposure (right, n=3) and in utricles from unexposed control mice (left, n=3) are presented. Successive sections from the same sample were immune-labeled with different antibodies. Blue: DAPI staining of nuclei. Brackets: vestibular hair cells. Yellow arrow: Hsp70 expression in the endolymphatic side of hair and supporting cells in the utricle. Scale bars: 20 μm.

Fig. 5. Protein expression levels of Hsp70 in the organ of Corti. Protein expression levels of Hsp27 (A, red), Hsp70 (B, green) and Hsp90 (C, red) in the organ of Corti (cochlea) from mice just after LFN exposure (right, n=3) and in the organ of Corti from unexposed control mice (left, n=3) are presented. Blue: DAPI staining of nuclei. Scale bar: 20 μm.

Fig. 6. Hearing levels in LFN-exposed mice and unexposed mice. Hearing levels of mice before (**A**) and just after (**B**) the fifth LFN exposure (n=4, black dots and lines) and of unexposed control mice (n=4, gray dots and lines) are presented. Minimum sound pressure levels (dB SPL, mean ± SD) that evoked an ABR signal in mice at 4k, 12k, 20k and 32k Hz sounds are shown.



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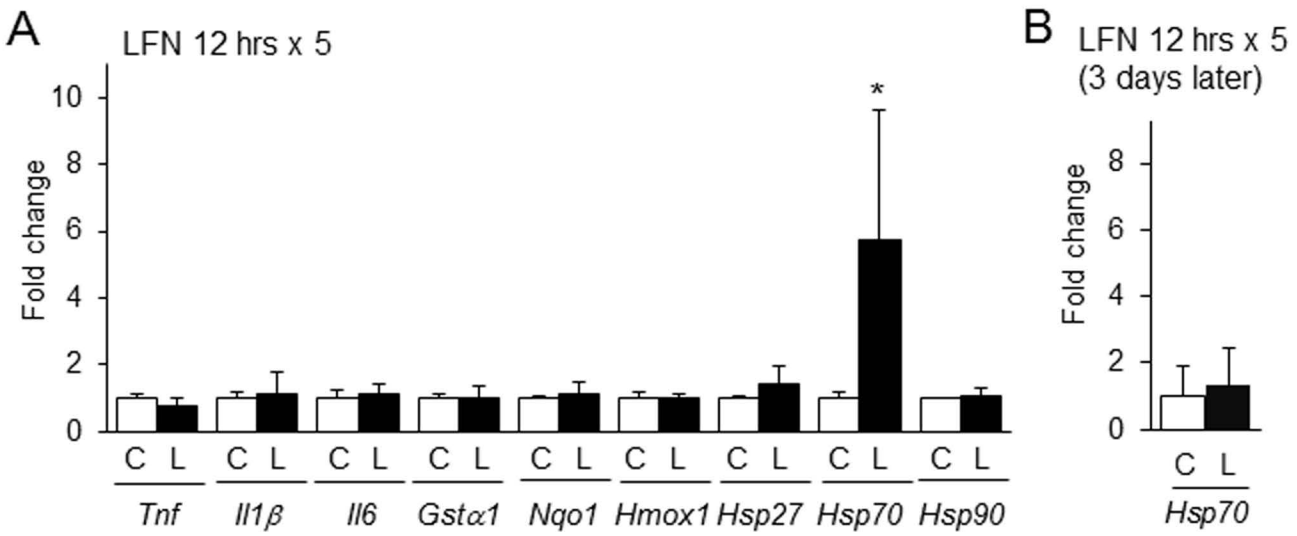
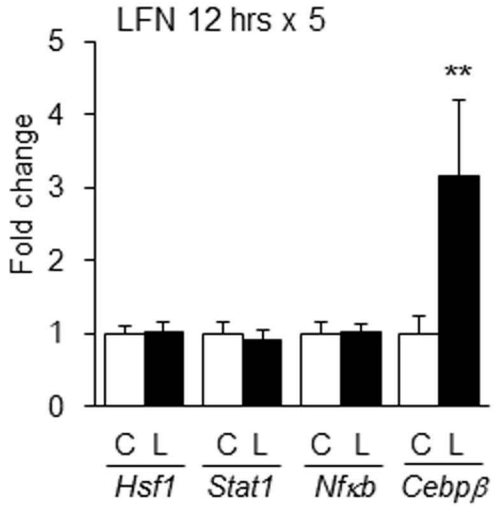
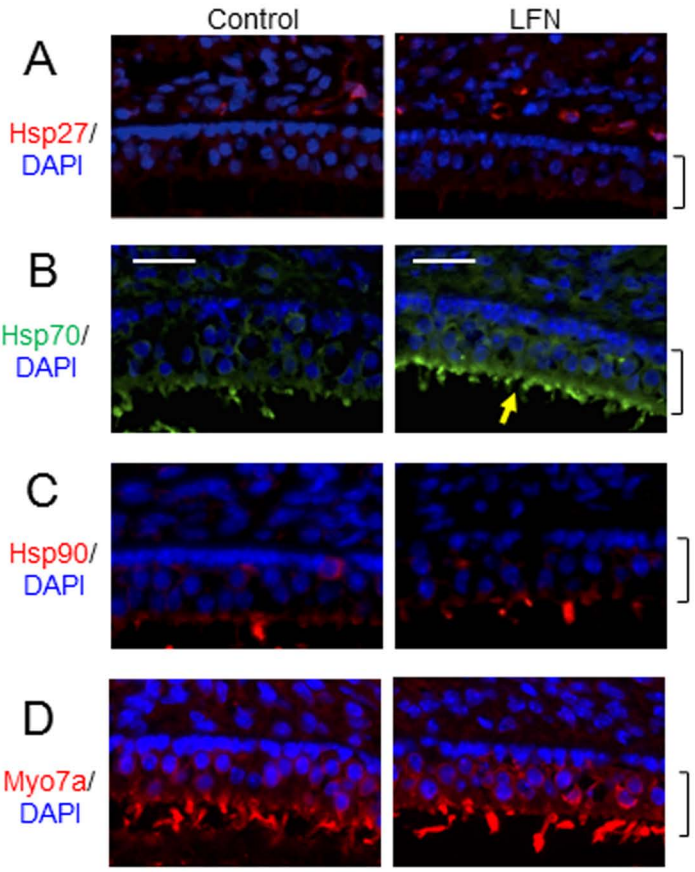


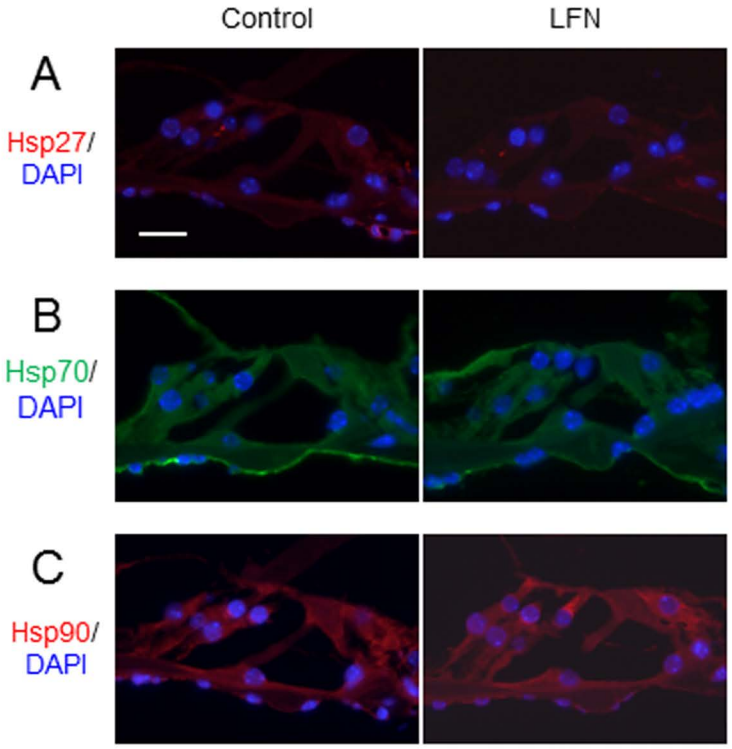
Ninomiya et al. Figure 2



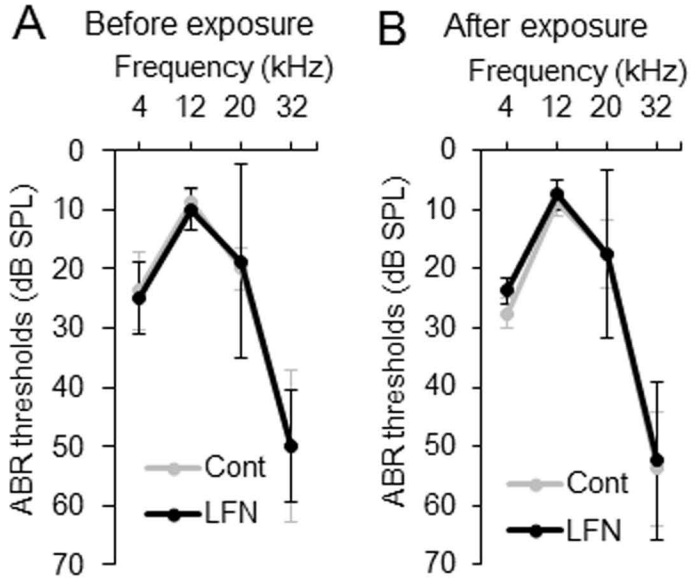
Ninomiya et al. Figure 3



Ninomiya et al. Figure 4 (color)



Ninomiya et al. Figure 5 (color)

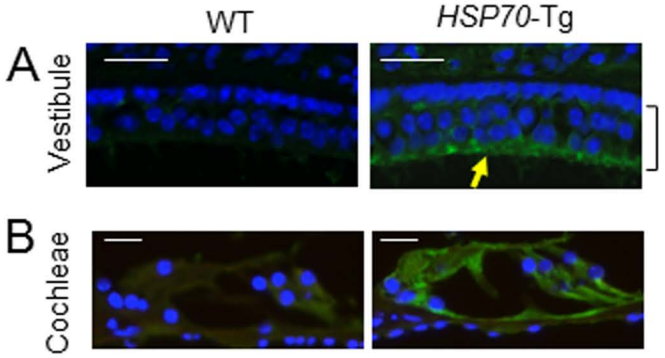


Ninomiya et al. Figure 6

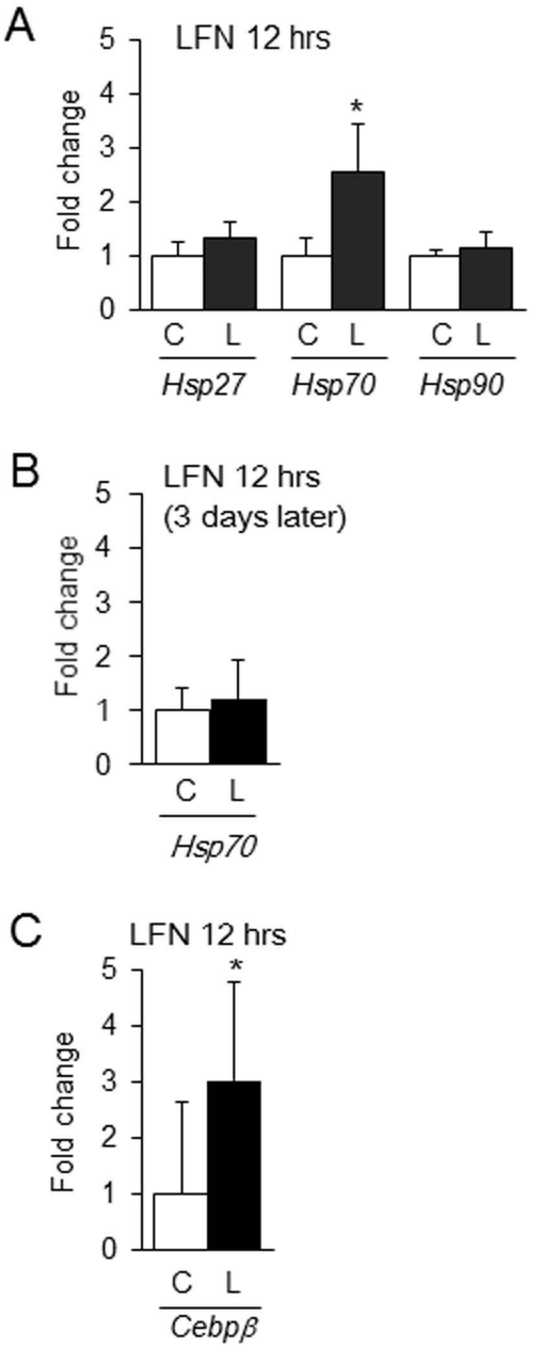
Supplementary figure captions

Supplementary Fig. S1. Protein expression levels of Hsp70 in the vestibule and the organ of Corti from *HSP70*-transgenic mice. Protein expression levels of Hsp70 (green) in the utricles (vestibule) (A) and in the organ of Corti (cochleae) (B) from *HSP70*-transgenic mice (*HSP70*-Tg) (Plumier et al., 1995) and from control wild-type mice (WT) are presented. Blue: DAPI staining of nuclei. Black bracket: vestibular hair cells. Yellow arrow: Hsp70 expression in the endolymphatic side of hair and supporting cells in the utricle. Scale bars: 20 μm.

Supplementary Fig. S2. Quantitative RT-PCR analysis after LFN exposure for 1 day (12 hours). Expression levels (mean ± SD) of *HSP* transcripts in whole inner ears from mice just after single LFN exposure (100 Hz LFN at 95 dB for 12 hours) (n=6, L, closed bars) and in whole inner ears from unexposed control mice (n=6, C, open bars) are presented (**A**). Expression levels of *Hsp70* transcript in whole inner ears from mice 3 days after single LFN exposure (n=5, L, closed bars) and in whole inner ears from unexposed control mice (n=5, C, open bars) are presented (**B**). Expression levels (mean ± SD) of *Cebpβ* transcript in whole inner ears from mice just after single LFN exposure (n=8, L, closed bars) and in whole inner ears from unexposed control mice (n=8, C, open bars) are presented (**C**). *: significantly different ($p<0.05$) by Student's t-test.



Ninomiya et al. Figure S1



Ninomiya et al. Figure S2

Table S1: Sequences of primers used in this study

Names of genes	Forward primer (5'-3')	Reverse primer (5'-3')
Hprt	TTATCAGACTGAAGAGCTACTGTAATGATC	TTACCAGTGTCAATTATATCTTCAACAATC
Tnf	TATGGCCCAGACCCTCACA	GGAGTAGACAAGGTACAACCCATC
Il1 β	TCCAGGATGAGGACATGAGCAC	GAACGTCACACACCAGCAGGTTA
Il6	CAACGATGATGCACTTGCAG	CTCCAGGTAGCTATGGTACTCCAGA
Gst α1	CGCCACCAAATATGACCTCT	CCTGTTGCCCACAAGGTAGT
Nqo1	TTCTCTGGCCGATTCAGAGT	GGCTGCTTGGAGCAAAATG
Hmox1	AAGCCGAGAATGCTGAGTTC	GCCGTGTAGATATGGTACAAGGA
Hsp27 (*Hspb1*)	AAGGAAGGCGTGGTGGAGAT	TTCGTCCTGCCTTTCTTCGT
Hsp70 (*Hspa1a, Hspa1b*)	ATGGACAAGGCGCAGATCC	CTCCGACTTGTCCCCCAT
Hsp90 (*Hsp90aa1*)	AATTGCCCAGTTAATGTCCTTGA	CGTCCGATGAATTGGAGATGAG
Hsf1	AACGTCCCGGCCTTCCTAA	AGATGAGCGCGTCTGTGTC
Stat1	TCACAGTGGTTCGAGCTTCAG	GCAAACGAGACATCATAGGCA
Nf κb2	CTGGTGGACACATACAGGAAGAC	ATAGGCACTGTCTTCTTTCACCTC
Cebp β	GGGTTGTTGATGTTTTTGGTTT	GAAACGGAAAAGGTTCTCAAAA