Stress is irrelevant to the onset and exacerbation of sensorineural hearing loss: Evaluation using various types of stress models in mice

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Abstract
Chronic stress has been argued to produce sensorineural hearing loss in human beings. To elucidate the influence of stress on hearing loss, we tested changes in hearing ability in various types of stress-model mice, such as water-immersion restraint stress (WIRS, 3 h/day, for 4 weeks), social defeat stress (SDS, 10 min/day, for 2 weeks), and social isolation stress (SIS, for 4 weeks). No marked changes in hearing ability and the number of cochlear hair cells lost were observed in mice exposed to these different types of stress. In addition, these stresses did not exacerbate temporary hearing impairment induced by noise (8 kHz octave band noise, 90 dB sound pressure level, 1 h). Taken together, our data suggest that chronic stress did not cause hearing loss in mice. These findings reveal that chronic stress is irrelevant to the onset and exacerbation of sensorineural hearing loss at least in mice.

Introduction
Human beings are simultaneously exposed to social stress in their daily lives. Repeated exposure to social and psychological stress has been shown to disturb health, and is considered to cause lifestyle-related diseases, such as hyperinsulinemia, hyperglycemia, cardiovascular diseases, and obesity [1,2]. Additionally, the involvement of stress in the development of psychiatric disorders, such as depression, has been widely investigated in animals [3,4]. Moreover, several studies have proposed that exposure to chronic social stress produces sudden sensorineural hearing loss (SNHL) in human beings [5,6].

Sudden SNHL is an acute dysfunction of the inner ear that is characterized by sudden hearing loss and potential progression to complete deafness. Elderly people are primarily affected, but it can occur at any age. Sudden SNHL is usually classified as idiopathic, because the causative factor is not identified in most cases [7–10]. Impaired hearing ability greatly detracts from the quality of life of individuals, and may trigger psychiatric disorders, such as cognitive dysfunction [11,12]. Moreover, since most SNHL induced by transiently altered hearing function is irreversible, SNHL is becoming a global social problem.

The present study aimed to test the hypothesis that chronic stress increases the risk of developing hearing loss. In addition, we set out to verify whether noise-exposure hearing loss is exacerbated by chronic stress.

Materials and methods
Animals: All experiments used here met the Guidelines of the Japanese Society for Pharmacology and were approved by the Committee for Ethical Use of Experimental Animals at Setsunan University. Adult male Std-ddY mice (SHIMIZU Laboratories Supplies Co., Ltd, Kyoto, Japan), weighing 22–26 g, were used for preparation of stress models.
in phosphate-buffered saline for whole-mount cochlear processing. To visualize hair cells, we incubated the specimens with a solution containing 0.3% Triton X-100 and Alexa-Fluor 568-conjugated phalloidin (1:100 dilution; Invitrogen, Carlsbad, CA, USA) for 30 min at room temperature. The stained specimens were observed under a confocal fluorescence microscope using the FV1000D system (Olympus, Tokyo, Japan) for counting the numbers of missing hair cells in the cochleae. The ratio of missing-to-total hair cells was expressed as a percentage.

**Data analysis:** The results are presented as the means ± S.E.M from several separate experiments. Differences were assessed by using analysis of variance. Data were analyzed by means of one-way ANOVA, and a least significant difference post-hoc test was used to evaluate the statistical differences between groups.

**Results**

Changes in body weight after stress treatment: To confirm whether animals were exposed to sufficient stress under the current experiments, we measured the body weight of animals during the stress treatments. In mice treated with WIRS or SDS, the body weight decreased markedly during the treatment. However, SIS-exposed mice demonstrated a significant increase in body weight during the treatment (Figure 2).

**Auditory brainstem response (ABR) recording:** To determine the hearing ability of the animals, we measured their ABR under anesthesia induced by chloral hydrate (500 mg/kg, i.p.), as previously reported [13]. The threshold of ABR was determined at the frequencies of 4, 12, and 20 kHz, by means of a 5-dB SPL minimum-size step-down from the maximum amplitude. The hearing threshold was defined as the lowest stimulus intensity that produced wave I in the ABR test.

**Noise-induced hearing impairment:** To establish noise-induced hearing impairment, the sound was generated and amplified as previously described [13]. Each animal was placed in a cage. The mice were then exposed to an octave-band noise, centered at 8 kHz, at a 90-dB sound pressure level, for 1 h, within a sound chamber. As a control, naïve animals were placed in the same cage, but without the noise.

**Quantitative assessment of hair cell loss:** After the final ABR test, animals were decapitated and cochleae were immersion-fixed with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) for 2 h at room temperature after rapid removal of the temporal bone and opening of the round and oval windows. The cochleae were then decalcified in 4% EDTA solution at room temperature for at least 2-7 days. Under a microscope, the specimens including organ of Corti were dissected subjected to social defeat for 7 consecutive days. Control mice were kept alone in transparent plastic cages for 7 consecutive days. For SIS, mice were housed individually for 4 weeks.

**Figure 1. Experimental schedules.** All animals underwent 1 week of habituation before stress treatment. (a) Water-immersion restraint stress (WIRS): animals treated with WIRS for 4 weeks were subjected to measurement of auditory brainstem response (ABR) and body weight. (b) Social defeat stress (SDS): animals were exposed to SDS for 1 week and then subjected to measurement of ABR and body weight. (c) Social isolation stress (SIS): animals were treated with SIS for 4 weeks and then subjected to measuring of ABR and body weight.

**Figure 2. Effect of stress treatment on body weight.** Graphs denote the average of body weight. Values are the means ± S.E.M. from 4 independent experiments performed under the same experimental conditions. **P < 0.01**, significantly different from the value obtained for animals not exposed to stress treatment.
Effect of stress treatment on hearing ability: To assess if physical or social stress increases the risk of developing hearing loss, hearing ability was determined in mice treated with WIRS, SDS, and SIS (Figure 3). No significant impairment in hearing ability occurred at 4, 12, and 20 kHz in any of the stress groups (WIRS, SDS, and SIS).

Effect of stress treatment on noise-induced temporary hearing impairment: We next assessed the effect of stress treatment on noise-induced temporary hearing impairment (Figure 4). The ABR threshold was assessed at the frequencies of 4, 12, and 20 kHz on immediately after (day 0) or 7 days after noise exposure. Exposure to noise at a 90-dB sound pressure level for 1 h produced a temporary threshold shift in ABR immediately after noise exposure. Already on day 1 after noise exposure, ABR returned to the level of animals without exposure to noise. WIRS, SDS, or SIS did not exacerbate the noise-induced hearing impairment, at least on the days tested.

Effect of stress treatment on cochlear outer hair cells damaged after noise exposure: To examine whether stress treatment exacerbates hair cell damage induced by noise exposure, we stained and counted the damaged outer hair cells in the cochleas of noise-exposed animals (Figure 5). In animals exposed to noise alone, 2-4% of outer hair cells in the cochleas disappeared. The damage to the outer hair cells was not significantly exacerbated by WIRS, SDS, or SIS.

**Figure 3.** Effect of stress treatment on hearing ability. Animals were treated with stress and then the auditory brainstem response (ABR) threshold was measured. Graphs denote the average of threshold shift at each frequency. Values are the means ± S.E.M. from 4 independent experiments performed under the same experimental conditions.
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**Discussion**

In this study, we evaluated whether physical and social stresses are involved in the onset or exacerbation of SNHL. Using various types of stress models in mice, our current study demonstrated that exposure to severe stress was not accompanied by impairment of hearing ability. In addition, evidence for the irrelevance of severe stress to the exacerbation of noise-induced hearing loss came from current findings that severe stress treatment produced no significant effect on the temporary threshold shift of ABR and the hair cell loss in mice exposed to noise at a 90-dB sound pressure level. Therefore, the present study, using stress model mice, could not demonstrate data supporting the previous hypothesis that chronic stress increases the risk of developing hearing loss.

We first performed experiments to confirm whether animals were exposed to sufficient stress during our experimental procedures. Changes in the body weight of animals have been reported to be a convenient index for stress exposure [14,15]. A marked decrease in the body weight occurs in WIRS- and SDS-exposed animals [16]; in contrast, SIS promotes food intake and body weight gain [15,17]. These previous reports strongly support that in the present study, animals exposed to WIRS, SDS, or SIS were useful as stress models.

In these stress model animals, hearing impairment and cochlear
头发细胞损伤没有被观察到，至少在试验期间。存在一些可能的原因：实验期可能太短而不能影响听觉功能，即使压力诱导的生理反应可能作为压力模型；此外，单次压力可能没有产生必要的和足以影响听觉的条件。在人类和实验中，压力暴露在现代社会中是常见的。因此，听力损失可能由压力暴露导致，包括各种类型的联合压力。实验期可能过短而不能影响听觉功能，尽管压力引起的物理损伤。

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