a small increase in plasma vasopressin (Fig. 5). Additionally, AQP2 was not resolved but stored within the cytoplasmic endosome in the endolymphatic sac after AVP stimulation. This finding indicates that AQP2 can be translocated again to the luminal side by medical treatments, which may lead new ideas to cure patients with Meniere's disease.

Finally, we would like to speculate about the possible causes for attacks associated with inner ear pathology in Meniere's disease. It has been reported that Meniere's disease is usually triggered by immune, infectious, traumatic or other insults to the inner ear, in association with a small misplaced malfunctioning endolymphatic sac (21,22). Among these insults, immune-mediated responses in the inner ear endo-organs, such as the endolymphatic sac, stria vascularis and spiral ligament, are considered to be the main bases for the fluid homeostatic disorder in Meniere's disease (23,24). Certain virus infections, such as varicella-zoster, Epstein–Barr and adenovirus infections, of the endolymphatic sac in early childhood represent other bases for the dysfunction of endolymph absorption (25,26). Taken together with the present data, it is suggested that autoimmune responses and/or virus infections could cause damage to the V2R regulatory genes in the endolymphatic sac, resulting in V2R-cAMP-PKA-AQP2 activation and endosomal trapping of AQP2. In the V2R-cAMP-PKA-AQP2 activated inner ear, endolymphatic hydrops could gradually be generated and Reissner's membranes could become ruptured (27) after even a small elevation in plasma vasopressin as a result of stress, thereby resulting in attacks of Meniere's disease. The laterality of Meniere's disease could not be decided by the level of plasma vasopressin but by the laterality of inner ear molecular pathology mentioned above. The data of V2R up-regulation in Meniere's disease in the previous study (4) and AQP2 up-regulation in Meniere's disease in the present study were obtained from human *in vivo*. However, all the evidence for AQP2 translocation in the endolymphatic sac in the present study was demonstrated in humans *in vitro*. Therefore, there are limitations in the present study and further genetic studies in mice and clinical observations in patients with Meniere's disease are required to confirm the significant relationship among the inner ear molecular pathology, endolymphatic hydrops and Meniere's attacks.

In conclusion, in the pathogenesis of inner ear hydrops resulting in vertigo attacks accompanied by hearing loss and tinnitus in Meniere's disease, plasma vasopressin elevation as a result of stress and subsequent V2R-cAMP-PKA-AQP2 activation and endosomal trapping of AQP2 in the endolymphatic sac might be essential as the basis of this disease.

Meniere's disease, associated with vertigo, fluctuating hearing loss and tinnitus as a result of inner ear pathology, has been proposed to occur especially in people living a stressed lifestyle (28). Indeed, this disease is provoked by a poor adaptation to physical and/or psychogenic stress in daily life (29). We consider that the results obtaned in the present study will encourage continued investigations that help to ascertain ideal treatments for the inner ear in Meniere's disease, ranging from psychotherapy for leading a stressless life to gene therapy for stress hormone receptor-related molecules via an endolymphatic sac approach.

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References

1 Yamakawa K. Uber die pathologische Veranderung bei einem Meniere-Kranken. *J Otolaryngol Jpn* 1938; **44**: 2310–2312.
2 Hallpike CS, Cairns H. Observations on the pathology of Meniere's syndrome. *J Laryngol Otol* 1938; **53**: 625–655.
3 Ishiyama G, Lopez IA, Ishiyama A. Aquaporins and Meniere's disease – review. *Curr Opin Otolaryngol Head Neck Surg* 2006; **14**: 332–336.
4 Kitahara T, Doi K, Maekawa C, Kizawa K, Horii A, Kubo T, Kiyama H. Meniere's attacks occur in the inner ear with excessive vasopressin type-2 receptors. *J Neuroendocrinol* 2008; **20**: 1295–1300.
5 Kitahara T, Maekawa C, Kizawa K, Horii A, Doi K. Plasma vasopressin and V2 receptor in the endolymphatic sac in patients with delayed endolymphatic hydrops. *Otol. Neurotol.* 2009; **30**: 812–819.
6 Committee on Hearing and Equilibrium. Committee on hearing and equilibrium guidelines for diagnosis and evaluation of therapy in Meniere's disease. *Otolaryngol Head Neck Surg* 1995; **113**: 181–185.
7 Claes J, Van de Heyning PH. A review of medical treatment for Meniere's disease. *Acta Otolaryngol Suppl* 2000; **544**: 34–39.
8 Kitahara T, Takeda N, Mishiro Y, Saika T, Fukushima M, Okumura S, Kubo T. Effects of exposing the opened endolymphatic sac to large doses of steroids to treat intractable Meniere's disease. *Ann Otol Rhinol Laryngol* 2001; **110**: 109–112.
9 Kitahara T, Kondoh K, Morihana T, Okumura S, Mishiro Y, Kubo T. Surgical management for special cases of intractable Meniere's disease: unilateral cases with intact canals and bilateral cases. *Ann Otol Rhinol Laryngol* 2004; **113**: 399–403.
10 Kitahara T, Kubo T, Okumura S, Kitahara M. Effects of endolymphatic sac drainage with steroids for intractable Meniere's disease: a long-term follow-up and randomized controlled study. *Laryngoscope* 2008; **118**: 854–861.
11 Schmittgen TD, Zakrajsek BA, Mills AG, Gorn V, Singer MJ, Reed MW. Quantitative reverse transcription polymerase chain reaction to study mRNA decay: comparison of endpoint and real-time methods. *Anal Biochem* 2000; **285**: 194–204.
12 Kumagami H, Loewenheim H, Beitz E, Wild K, Schwartz H, Yamashita K, Schultz J, Paysan J, Zenner HP, Ruppersberg JP. The effect of anti-diuretic hormone on the endolymphatic sac of the inner ear. *Pflugers Arch* 1998; **436**: 970–975.
13 Tanaka M, Watanabe Y, Yoshimoto K. Regulation of relaxin 3 gene expression via cAMP-PKA in a neuroblastoma cell line. *J Neurosci Res* 2009; **87**: 820–829.
14 Takeda T, Kakigi A, Saito H. Antidiuretic hormone (ADH) and endolymphatic hydrops. *Acta Otolaryngol Suppl* 1995; **519**: 219–222.

15 Aoki M, Asai M, Nishihori T, Mizuta K, Ito Y, Ando K. The relevance of an elevation in the plasma vasopressin levels to the pathogenesis of Meniere's attack. *J Neuroendocrinol* 2007; **19**: 901–906.
16 Takeda T, Takeda S, Kitano H, Okada T, Kakigi A. Endolymphatic hydrops induced by chronic administration of vasopressin. *Hear Res* 2000; **140**: 1–6.
17 Lim JS, Lange ME, Megerian CA. Serum antidiuretic hormone levels in patients with unilateral Meniere's disease. *Laryngoscope* 2003; **113**: 1321–1326.
18 Yazawa Y, Kitahara M. Bilateral endolymphatic hydrops in Menière's disease: review of temporal bone autopsies – review. *Ann Otol Rhinol Laryngol* 1990; **99**: 524–528.
19 Kitano H, Suzuki M, Kitanishi T, Yazawa Y, Kitajima K, Isono T, Takeda T, Kimura H, Tooyama I. Regulation of inner ear fluid in the rat by vasopressin. *Neuroreport* 1999; **10**: 1205–1207.
20 Taguchi D, Takeda T, Kakigi A, Takumida M, Nishioka R, Kitano H. Expressions of aquaporin-2, vasopressin type 2 receptor, transient receptor potential channel vanilloid (TRPV)1, and TRPV4 in the human endolymphatic sac. *Laryngoscope* 2007; **117**: 695–698.
21 Shambaugh GE Jr, Clemis JD, Arenberg IK. Endolymphatic duct and sac in Meniere's disease. I. Surgical and histopathologic observations. *Arch Otolaryngol* 1969; **89**: 816–825.
22 Gutierrez F, Moreno PM, Sainz M. Relationship between immune complex and total hemolytic complement in endolymphatic hydrops. *Laryngoscope* 1994; **104**: 1495–1498.
23 Hughes GB, Kinney SE, Barna BP, Calabrese LH. Autoimmune reactivity in Meniere's disease: a preliminary report. *Laryngoscope* 1983; **93**: 410–417.
24 Yamanobe S, Harris JP. Inner ear-specific autoantibodies. *Laryngoscope* 1993; **103**: 319–325.
25 Yazawa Y, Suzuki M, Hanamitsu M, Kimura H, Tooyama I. Detection of viral DNA in the endolymphatic sac in Ménière's disease by in situ hybridization. *ORL J Otorhinolaryngol Relat Spec* 2003; **65**: 162–168.
26 Selmani Z, Marttila T, Pyykkö I. Incidence of virus infection as a cause of Meniere's disease or endolymphatic hydrops assessed by electrocochleography. *Eur Arch Otorhinolaryngol* 2005; **262**: 331–334.
27 Schuknecht HF. Pathophysiology of endolymphatic hydrops. *Arch Otorhinolaryngol* 1976; **212**: 253–262.
28 Watanabe I. Ménière's disease with special emphasis on epidemiology, diagnosis and prognosis – review. *ORL J Otorhinolaryngol Relat Spec* 1980; **42**: 20–45.
29 Takahashi M, Ishida K, Iida M. Analysis of lifestyle and behavioral characteristics in Meniere's disease patients and a control population. *Acta Otolaryngol* 2001; **121**: 254–256.

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BEHAVIORAL ASSESSMENT AND IDENTIFICATION OF A MOLECULAR MARKER IN A SALICYLATE-INDUCED TINNITUS IN RATS

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Abstract—Tinnitus is a non-observable phantom sensation. As such, it is a difficult condition to investigate and, to date, no effective treatment has been developed. To approach this phantom sensation, we aimed to develop a rat behavioral model of tinnitus using salicylate, an active component of aspirin known to induce tinnitus. We also aimed to establish a molecular marker of tinnitus by assessing the expression of transient receptor potential cation channel superfamily V-1 (TRPV1) in the rat auditory pathway during salicylate-induced tinnitus. Animals were trained to perform "an active avoidance task": animals were conditioned by electrical footshock to move to the other side of the conditioning box when hearing a sound. Animals received a single injection of saline or salicylate (400 mg/kg i.p.) and false positive responses were measured 2 h after injection as the number of movements during a silent period. The number of responses in salicylate-treated animals was highest when the conditioned stimulus was 60 dB sound pressure level (SPL) and 16 kHz. This indicates that animals could feel tinnitus 2 h after salicylate injection, equivalent to that induced by 60 dB SPL and 16 kHz. By means of real-time PCR and western blot analysis, TRPV1 expression was significantly upregulated in spiral ganglion cells 2 h after salicylate injection and this upregulation together with the increase in the number of false positive responses was significantly suppressed by capsazepine (10 mg/kg i.p.), a specific antagonist of TRPV1. This suggests that salicylate could induce tinnitus through activation of TRPV1 in the rat auditory pathway.

Key words: animal model, TRPV1, spiral ganglion, dorsal cochlea nucleus.

Many people have experienced a sensation of ringing in their ears when no external sound is present. Typically, this sensation of tinnitus is associated with a reversible cause and subsides over a period of time ranging from a few seconds to a few days. However, in 5–15% of the general population, the tinnitus is unremitting (Heller, 2003). Chronic tinnitus is more prevalent among seniors (12% after the age of 60) than in young adults (5% in the age group 20–30), but can occur at any age. In 1–3% of the general population, tinnitus is perceived as loud enough to affect the quality of life (Eggermont and Roberts, 2004). However, no effective therapeutic strategy for such intractable tinnitus has been established.

There are at least two obvious reasons why it is still so hard for clinicians to treat intractable tinnitus. One reason is that, since Jastreboff and Sasaki (1994) proposed an animal behavioral model of tinnitus based on an active avoidance task of drinking water with electrical footshock, better feasibility of the model has been discussed. The first purpose of the present study was, therefore, to develop a rat behavioral model to enable the objective evaluation of tinnitus. Salicylate, an active component of aspirin, is well known to induce an acute and transient type of tinnitus (Cazals, 2000; Eggermont and Roberts, 2004). According to the recent good work of climbing a pole with electrical footshock by Guitton et al. (2003), our model using salicylate was developed with modifications of a much easier active avoidance task. These previous and present tinnitus models will be addressed again in the first paragraph in Discussion.

The other reason is that the molecular mechanism of tinnitus generation in the auditory pathway has not been clarified yet. The second purpose of the present study was, therefore, to identify molecular markers to understand the molecular mechanism of salicylate-induced tinnitus in the rat auditory pathway. Salicylate inhibits cyclo-oxygenase activity (Christie et al., 1998) and cyclo-oxygenase inhibition leads to the *in vitro* activation of a nociceptive receptor transient receptor potential cation channel superfamily V-1 (TRPV1) (Fosslien, 1998; Hwang et al., 2000; Caterina et al., 1997; Benham et al., 2003). TRPV1 is located in the mouse inner ear ganglia and is upregulated *in vivo* after noxious challenges of kanamycin (Kitahara et al., 2005a). Furthermore, cochlear background activity is increased by inner ear perfusion of capsaicin, a TRPV1 agonist and is suppressed by capsazepine, a TRPV1 specific antagonist (Zhou et al., 2006). We examined changes in mRNA and protein levels of TRPV1 in the salicylate-treated rat auditory pathway. Although salicylate-induced tinnitus is acute and transient, we believe that by elucidating the molecular mechanism of salicylate-induced tinnitus we can provide insight into chronic intractable tinnitus.

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Abbreviations: ABR, auditory brainstem response; BRCx, brain cortex; B2m, beta-2 microglobulin; CT, cycle threshold; DCN, dorsal cochlear nucleus; DMSO, dimethyl sulfoxide; RT, room temperature; SG, spiral ganglion; SPL, sound pressure level; TRPV1, transient receptor potential cation channel superfamily V-1.

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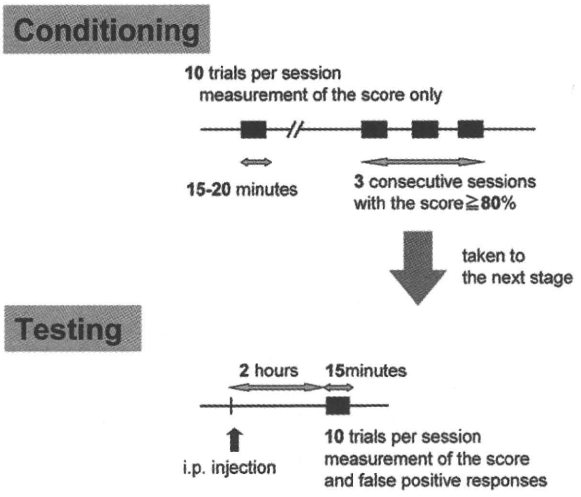

Fig. 1. Schematic representation of the behavioral protocol. In "conditioning," animals were conditioned to move in response to a sound stimulation. The conditioning procedure requires up to seven sessions lasting 15–20 min. When conditioned (criterion, three consecutive sessions with an active avoidance score ≥80%), animals were taken to the next stage, "testing" (day 0). The "testing" behavioral protocol consisted of a daily measurement (on four consecutive days, days 1–4) of the correct responses to sound (active avoidance score) and moves during inter-trial periods (false positive responses) in a 15 min session. Saline or salicylate was injected daily 2 h before the testing session.

EXPERIMENTAL PROCEDURES

Experimental procedures involving animals were performed according to animal ethical guidelines and were approved by Osaka University, School of Medicine (certificate number: 0755). All efforts were made to minimize the suffering to animals and to limit the number of animals used. A total of 99 male Wistar rats (Japan SLC, Hamamatsu, Shizuoka, Japan), weighting between 150 and 200 g, were used. Animals were individually housed in a temperature-controlled room on a constant 12 h light/dark cycle. All behavioral tests were conducted during the animals' activity period (dark phase) at approximately the same time each day. Food and tap water were available throughout the experiments.

Behavioral assessment

Animals were trained to perform "an active avoidance task," according to the protocol of Guitton et al. (2003) (Fig. 1). Both "conditioning" and "testing" were performed in a conditioning box that had an electrified floor, which was divided in two by a low wall, 3 cm high. The conditioning box was in a soundproof room. A 5 s pure tone sound was used as the "conditioned stimulus" and a 3.7 mA electrical footshock was given for a maximum of 30 s as the "unconditioned stimulus." The interval between conditioned and unconditioned stimuli was 1 s. The footshock was stopped when animals correctly escaped from the unconditioned shock to the opposite side of the cage. The inter-trial interval or silent period was at least 1 min. The level of performance over 10 trials, or "active avoidance score," was assessed by the ratio of how many times the rat moved correctly in response to the conditioned sound. In the "conditioning" stage, animals were considered to be conditioned when the active avoidance score reached at least 80% in three consecutive sessions. When conditioned, animals were taken to the next "testing" stage (day 0).

Testing was performed once daily for 4 days, at the same time each day (day 1–4). Animals received a single daily injection of saline or sodium salicylate (400 mg/kg i.p.) (Sigma, St. Louis, MO, USA) for 3 days (day 1–3), according to the previous reports of Jastreboff and Sasaki (1986); Rüttiger et al. (2003) and Im et al. (2007). Injections were performed 2 h before behavioral measurements. On the fourth day, they received no treatment. The behavioral protocol consisted of a daily measurement of the active avoidance score and of false positive responses. The "false positive responses" represent the number of movements to the opposite side of the cage during the inter-trial interval, when there was no sound. Trials were randomized and electric footshocks were presented only if animals didn't move in response to sounds. Whatever the results of the active avoidance score and false positive responses were, each session included 10 trials and lasted 15 min. Both the active avoidance score and false positive responses were measured in the same session.

The most appropriate conditioned stimulus was determined by the following pilot experiments. Animals were divided into four groups (n=6 in each group) according to conditioned stimuli of 4, 10, 16 or 40 kHz (60 dB sound pressure level (SPL)), and false positive responses were measured on the third day after salicylate injection. Animals were also divided into three groups (n=6 in each group) according to conditioned stimuli of 20, 60 or 80 dB SPL (16 kHz) and false positive responses were measured as above.

Auditory brainstem response (ABR) recording

The ABR was measured with a Neuropack-4 (Nihon Koden Co., Shinjuku, Tokyo, Japan). The active platinum electrode was inserted at the vertex, and reference electrodes at both pinnae of the ears. Binaural, open fielded stimuli of click were generated through a TDH-49 headphone attached to the animal's ears. The rat ABR consisted of a series of III to V vertex-positive peaks within the first 6 ms from the onset of the stimulus, and they were called I to V. The III wave was detected at the lowest stimulus intensity, so the threshold was defined as the lowest stimulus intensity to elicit a reliable III wave.

Hematoxylin and eosin staining

Serial inner ear sections from saline controls and salicylate-treated rats were stained with hematoxylin and eosin to determine if salicylate treatment caused any overt damage to the inner ear. Morphological structures in the organ of Corti and the spiral ganglion (SG) were microscopically observed.

Analysis of mRNA levels

Animals were divided into five groups: a saline i.p. injection control group, a 2 h post-salicylate i.p. injection group, a 12 h post-salicylate injection group, a 24 h post-salicylate injection group, and a 72 h post-salicylate injection group (n=6 in each group).

The procedures of tissue preparation for real-time PCR have already been described in our previous papers (Kitahara et al., 2005a,b). Animals were deeply anesthetized with pentobarbital and the SG, dorsal cochlear nucleus (DCN) and brain cortex (BRCx) were immediately dissected under a stereomicroscope in chilled buffered saline and then frozen in dry ice powder. The DCN region is thought to be one of the main structures involved in tinnitus (Eggermont and Roberts, 2004) and it was carefully dissected according to the coordinates of the Paxinos and Watson brain atlas; rostral coordinate, bregma: −10.52 mm, and caudal coordinate, bregma: −11.60 mm (Paxinos and Watson, 1986). The BRCx region does not include the auditory cortex. Total RNA was extracted using an RNeasy Mini Kit (Qiagen, Venlo, Netherlands) according to the manufacturer's instructions.

PCR was performed using oligonucleotide primers for TRPV1 (Takara Bio Inc., Otsu, Shiga, Japan) and beta-2 microglobulin (B2m) (Takara), as shown in Table 1, and products were quantified by SYBR Green PCR reagents (Applied Biosystems, Foster City, CA, USA). B2m was assayed as a control housekeeping gene. The PCR mixture included 10 μl of 2× SYBR Premix Taq,

Table 1. Gene-specific primers for PCR of rat transient receptor potential cation channel superfamily V type 1 (TRPV1) and β-2 microglobulin (B2m)

TRPV1 (accession no. NM_031982)
Forward primer 5′-ACTCCTGACGGCAAGGATGAC-3′
Reverse primer 5′-ACCCACATTGGTGTTCCAGGTAG-3′
Estimated size 81 bp
B2m (accession no. NM_012512)
Forward primer 5′-CCTGGCTCACACACTGAATTCACAC-3′
Reverse primer 5′-AACCGGATCTGGAGTTAAACTGGTC-3′
Estimated size 163 bp

0.8 μl of each gene-specific primer (5 μM), 6.8 μl of dH2O, 0.4 μl of 50× ROX Reference Dye and 2 μl of cDNA (250 ng) in a final volume of 20 μl. The conditions used were 95 °C for 10 s, 40 cycles at 95 °C for 5 s and 60 °C for 30 s, 95 °C for 15 s, 60 °C for 1 min and 95 °C for 15 s. The amplification plots from fluorescent emission data collected during PCR were constructed using the ABI7900 model software (Applied Biosystems).

The number of PCR cycles was recorded until the fluorescence intensity exceeded the pre-determined threshold. The quantification of the initial amounts of template molecules relied on this number of PCR cycles, which is termed the cycle threshold (CT). The dCT represents the CT of the target gene normalized to the rat endogenous B2m ($dCT=CT_{target}-CT_{B2m}$). Relative quantification of the mRNA levels of target genes (=fold range) was calculated using the 2^{-ddCT} method, where $ddCT=(CT_{target}-CT_{B2m})A-(CT_{target}-CT_{B2m})B$ (Schmittgen et al., 2000). For example, changes in gene expression of TRPV1 among groups CONT, SA2H, SA12H, SA24H and SA72H ($GROUP_x$) within each region were quantified as the fold range: 2^{-ddCT} ($ddCT=(CT_{TRPV1}-CT_{B2m})_{GROUPx}-(CT_{TRPV1}-CT_{B2m})_{CONT}$).

Protein examination

TRPV1 protein levels were assayed using standard western blotting techniques (Kitahara et al., 2005a,b). Briefly, tissues of each region (SG, DCN, BRCx) were obtained from a saline i.p. injection control group, a 2 h post-salicylate i.p. injection group and a 24 h post-salicylate injection group ($n=8$ in each group) through the same procedure for real-time PCR in the present study. Then, the tissues were added to lysis buffer (1% NP-40, 150 mM NaCl, 1 mM EDTA, 10 mM PBS (pH 7.4), 0.25 mM DTT, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 10 μg/ml leupeptin) and homogenized gently on ice using a Polytron tissue homogenizer (Brinkmann Instruments, Westbury, NY, USA). The samples were boiled for 1 min, transferred to clean Eppendorf tubes on ice and centrifuged at 10,000 g for 30 min. Supernatant was transferred to clean tubes, and protein concentration was determined using a protein assay kit (Pierce, Rockford, IL, USA). The protein extracts (20 μg for each lane) were subjected to 12% SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose filters (Amersham Biosciences, Piscataway, NJ, USA) at 500 mA for 1 h. The filters were pre-blocked in 0.1 M PBS containing 0.2% Tween 20 and 5% non-fat dried milk for 3 h at room temperature (RT), probed overnight with TRPV1 primary antibody (anti-rabbit polyclonal; diluted 1:1000; Alpha Diagnostic International, San Antonio, TX, USA) and then washed with Blotto solution (50 mM Tris (pH 7.4), 0.9% NaCl, 0.5% Tween 20) for 1 h. Filters were then incubated with HRP-conjugated secondary antibody (Dako, Carpenteria, CA, USA) for 1 h and washed with Blotto solution for 1 h. Protein bands were visualized using Supersignal Ultra chemiluminescence substrate (Pierce), assessed on ECL film (Amersham Biosciences) and quantified by laser densitometry (Quantity One Software, Bio-Rad Lab, Hercules, CA, USA). The density of staining with anti-β actin monoclonal antibody (Oncogene Research Products, Boston, MA, USA: diluted 1:500) was used to normalize the TRPV1 densitometric determination of each sample.

Tissue preparation procedures for immunohistochemistry have already been described in our previous papers (Kitahara et al., 2005a,b). Temporal bones were obtained from two adult male rats from each of a saline i.p. injection control group and a 2 h post-salicylate i.p. injection group. The animals were euthanized with sodium pentobarbital (100 mg/kg i.p.) and perfused transcardially with 0.1 M PBS, followed by paraformaldehyde-lysine-periodate fixative. Temporal bones were post-fixed in 4% paraformaldehyde for 24 h at RT, decalcified in 10% formic acid, neutralized overnight in 5% sodium sulfate, infiltrated with OCT compound and sectioned on a cryostat at 5 μm in a plane that produces mid-modiolar sections. The sections were thaw mounted on slides. The 5 μm cryostat sections were incubated sequentially in the following solutions at RT: 0.1% TritonX-100 and 2% bovine serum albumin (BSA) in 0.1 M PBS for 2 h; antisera against TRPV1 (anti-rabbit polyclonal; diluted 1:1000; Alpha Diagnostic International) in the above solution for 48 h; 0.1 M PBS for 15 min; biotinylated goat anti-rabbit IgG (diluted 1:250; Vector Laboratories, Burlingame, CA, USA) in 2% BSA in 0.1 M PBS for 24 h; 0.1 M PBS for 15 min; Vectastain ABC reagent (Vector Laboratories) for 1 h; 0.1 M PBS for 15 min; 5 mg/ml diaminobenzidine tetrahydrochloride (DAB)/0.01% H_2O_2 in 0.05 M Tris buffer for 5 min. Sections were then examined under a light microscope. For negative controls, primary antibodies were either pre-absorbed with each control peptide (1:50) or the primary antibody was omitted.

Antagonist treatment

To elucidate the direct involvement of TRPV1 in the mechanism of tinnitus generation, a specific antagonist of TRPV1, capsazepine (Sigma) and its vehicle, 50% dimetyl sulfoxide (DMSO) (Sigma) were used. Group I was a saline+DMSO group, group II was a salicylate+DMSO group, group III was a salicylate+capsazepine group and group IV was a saline+capsazepine group. DMSO or capsazepine was administrated 0.5 h after the first injection of saline or salicylate. Effects of capsazepine within the dose range of 0–10 mg/kg (De Schepper et al., 2008) were evaluated behaviorally by counting false positive responses (cf. 2.1 in Experimental Procedures) and morphologically by real-time PCR (cf. 2.3 in Experimental Procedures).

Statistical analysis

In the present paper, the statistical significance of changes among groups was analyzed using one-way ANOVA with Bonferroni *t*-test except for capsazepine treatment. Two-way ANOVA with Bonferroni *t*-test was used to test the hypotheses that salicylate effect is blocked by capsazepine. All the data were presented as mean±SE and *P*-values under 0.05 were considered significant (SPSS Inc., Chicago, IL, USA).

RESULTS

Behavioral assessment

In pilot experiments, animals were divided into four groups ($n=6$ in each group) according to conditioned stimuli of 4, 10, 16 or 40 kHz (60 dB SPL), and false positive responses were measured on the third day after salicylate injection. Each number of false positive responses was 0.6±0.4 (4 kHz), 2.8±0.8 (10 kHz), 4.2±0.9 (16 kHz), or 0.6±0.4 (40 kHz) and increased significantly when the conditioned stimulus was 16 kHz (* $P=0.004$) (Fig. 2A). Then, animals were also divided into three groups ($n=6$ in each group) according to conditioned stimuli of 20, 60 or 80 dB SPL (16

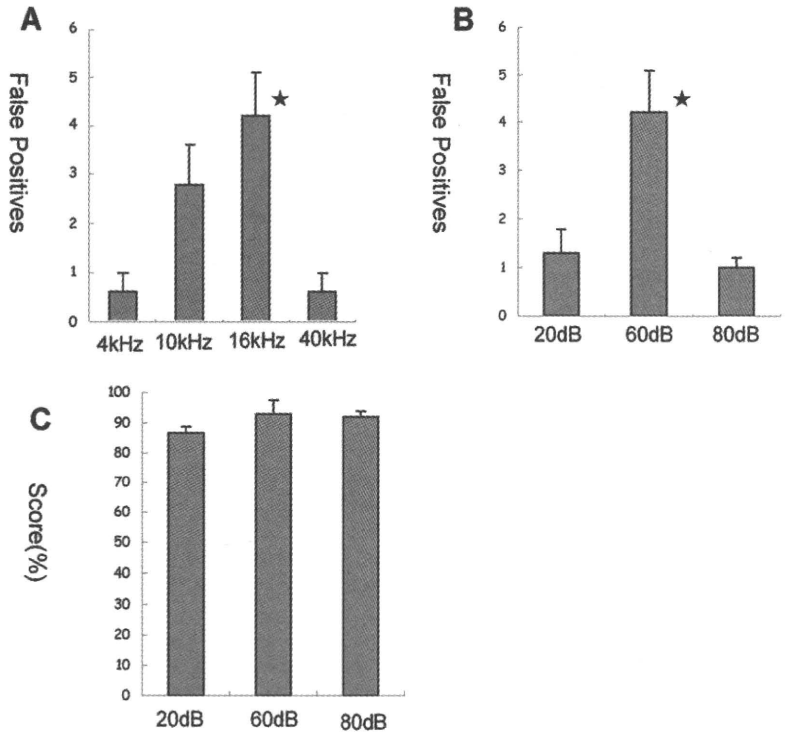

Fig. 2. The most appropriate kHz and dB sound pressure level (SPL) for the conditioning stimulus in the rat salicylate-induced tinnitus model. (A) Animals were divided into four groups (n=6 in each group) according to conditioning stimuli of 4, 10, 16 or 40 kHz (60 dB SPL). The number of false positives were measured on the third day after salicylate injection and increased significantly when the conditioning stimulus was 16 kHz (* P<0.005). (B) Animals were divided into three groups (n=6 in each group) according to conditioning stimuli of 20, 60 or 80 dB SPL (16 kHz). The number of false positives between these groups on the third day after salicylate injection increased significantly when the conditioning stimulus was at 60 dB SPL (* P<0.05). (C) The percentage of correct responses (score %) to sound of 20, 60 or 80 dB SPL (16 kHz) (active avoidance score). There were no significant differences between all three groups.

kHz) and false positive responses were measured as above. Each number of false positive responses was 1.3±0.5 (20 dB SPL), 4.2±0.9 (60 dB SPL), or 1.0±0.2 (80 dB SPL) and increased significantly when the conditioned stimulus was 60 dB SPL (* P=0.013) (Fig. 2B). The active avoidance score showed no significant change among all these groups, though (Fig. 2C). From these experiments, a 16 kHz and 60 dB SPL pure tone sound was adopted as the most appropriate conditioned stimulus for the present Wistar rat study.

Animals in the control group showed no significant change either in the active avoidance score (Fig. 3A) or in the false positive responses (Fig. 3B) from day 0 to day 4. The active avoidance scores of control and experimental

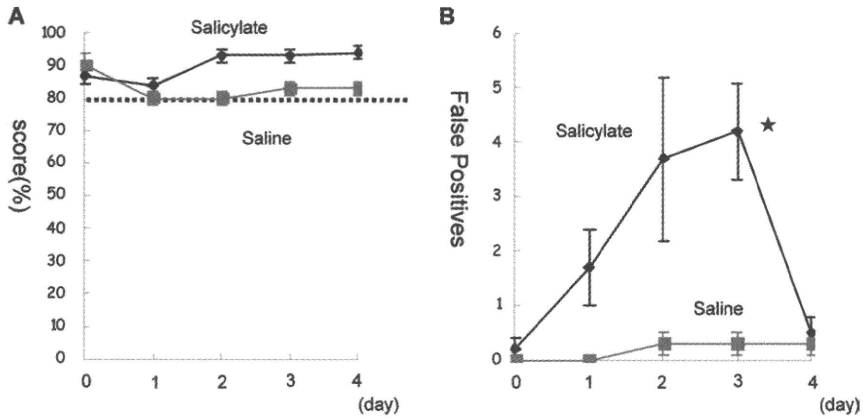

Fig. 3. The active avoidance score (A) and false positive responses (B) in salicylate-treated rats. (A) The percentage of correct responses to sound (active avoidance score %) measured before (day 0), during (day 1–3), and after injections of saline or salicylate (400 mg/kg i.p.) (day 4). The active avoidance score % remained stable (≥80%) in both groups during the experimental period. (B) The number of abnormal responses during silent periods (false positives). Injections of salicylate significantly increased the number of false positives on the third day (day 3) (* P<0.005). A complete recovery was seen when the treatment was stopped on the fourth day (day 4).

animals remained stable (≥80%) during the whole period of saline and salicylate administration. This suggests that salicylate application did not cause any obvious functional damage to cochlear endo-organs. False positive responses increased gradually after salicylate treatment with a maximum at day 3 (day 1: 0.2±0.2, day 2: 1.7±0.7, day 3: 4.2±0.9, day 4: 0.5±0.3, * P=0.002) and returned to the control value at day 4. This suggests that salicylate treatment caused a phantom sound sensation or "tinnitus" in rats, with a maximum at day 3 that endured for one day at most.

ABR assessment

We examined if the salicylate induced the inner ear dysfunction by means of ABR. We used the III wave in the measurement of ABR threshold, because this was most detectable at lower-intensity stimuli reference. No remarkable differences were observed between mean ABR threshold of control and salicylate-treated rats (data not shown). This suggests that salicylate treatment did not cause any obvious functional damage to cochlear endo-organs.

Morphological assessment

Neither hair cells nor SG cells showed any remarkable changes between control and salicylate-treated rats (data not shown). This suggests that salicylate treatment did not cause any obvious morphological damage to cochlear endo-organs.

Expression of TRPV1 in the auditory pathway and BRCx

TRPV1 mRNA levels in the SG were significantly upregulated 2 h (2.39±0.18 fold: * P=0.0005), returned to control levels 12 h (0.81±0.02 fold: P=0.105), significantly suppressed 24 h (0.35±0.03 fold: ** P=0.0002) and returned to control levels 72 h post-treatment (0.91±0.05 fold: P=0.130) (Fig. 4A). TRPV1 mRNA levels in the DCN were significantly suppressed 2 h (0.53±0.03 fold: * P=0.0001) and 12 h (0.64±0.03 fold: * P=0.0001) post-treatment, respectively and returned to control levels 24 h post-treatment (1.01±0.05 fold: P=0.810) (Fig. 4B). TRPV1 mRNA levels in the BRCx did not show any significant change after salicylate treatment (Fig. 4C).

TRPV1 protein levels in the inner ear

Fig. 5 illustrates western blot results for TRPV1 and the relative protein levels (TRPV1/actin in CONT=1) in the rat SG, DCN and BRCx. Similar to the results for mRNA levels, TRPV1 protein levels in the SG were significantly increased 2 h after salicylate treatment (2.94±0.72: * P=0.022) and returned to control levels 24 h after salicylate treatment (0.89±0.65: P=0.068) (Fig. 5A). In spite of a slight reduction 2 h after salicylate treatment (0.61±0.48: * P=0.044), TRPV1 protein levels in the DCN were significantly increased 24 h after salicylate treatment (1.56±0.60: ** P=0.038) (Fig. 5C). TRPV1 protein levels in the BRCx did not show any significant change after salicylate treatment (Fig. 5D).

TRPV1 immunoreactivity in the salicylate-treated group was clearly enhanced in almost all SG cells compared with that in the saline-control group (Fig. 5B). The dense immunoreactivity extended beyond the perinuclear region and into the somata of ganglion cells. All TRPV1 immunoreactivity was eliminated by pre-absorption with an excess of blocking peptide (data not shown).

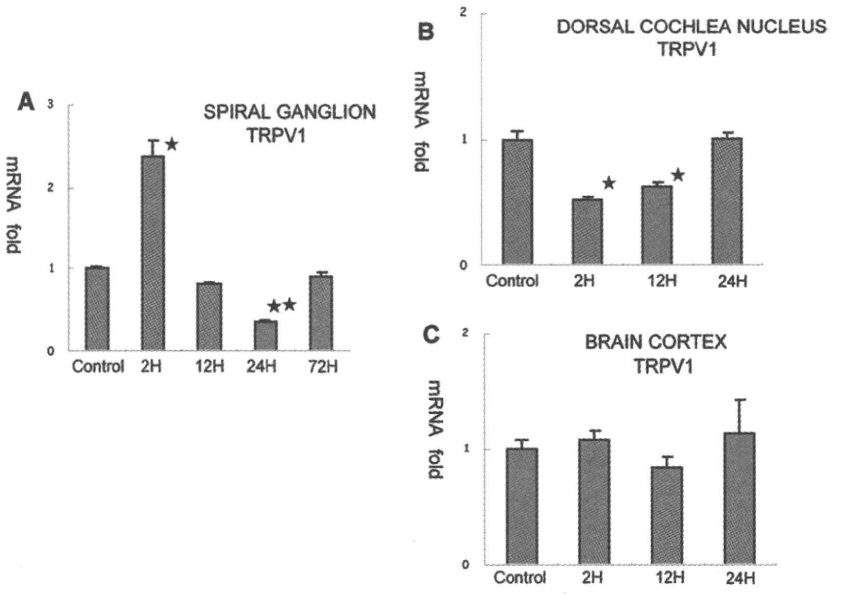

Fig. 4. Salicylate-induced changes in transient receptor potential cation channel superfamily V-1 (TRPV1) mRNA levels in the rat spiral ganglion (SG) (A), dorsal cochlear nucleus (DCN) (B) and brain cortex (BRCx) (C). (A) TRPV1 mRNA levels in SG were significantly upregulated 2 h (* P<0.001), returned to control levels 12 h, significantly suppressed 24 h (** P<0.0005) and returned to control levels 72 h post-treatment. (B) TRPV1 mRNA levels in the DCN were significantly suppressed at the 2 and 12 h post-treatment (* P<0.0005) and had returned to control levels 24 h post-treatment. (C) TRPV1 mRNA levels in the BRCx did not show any significant change after salicylate treatment.

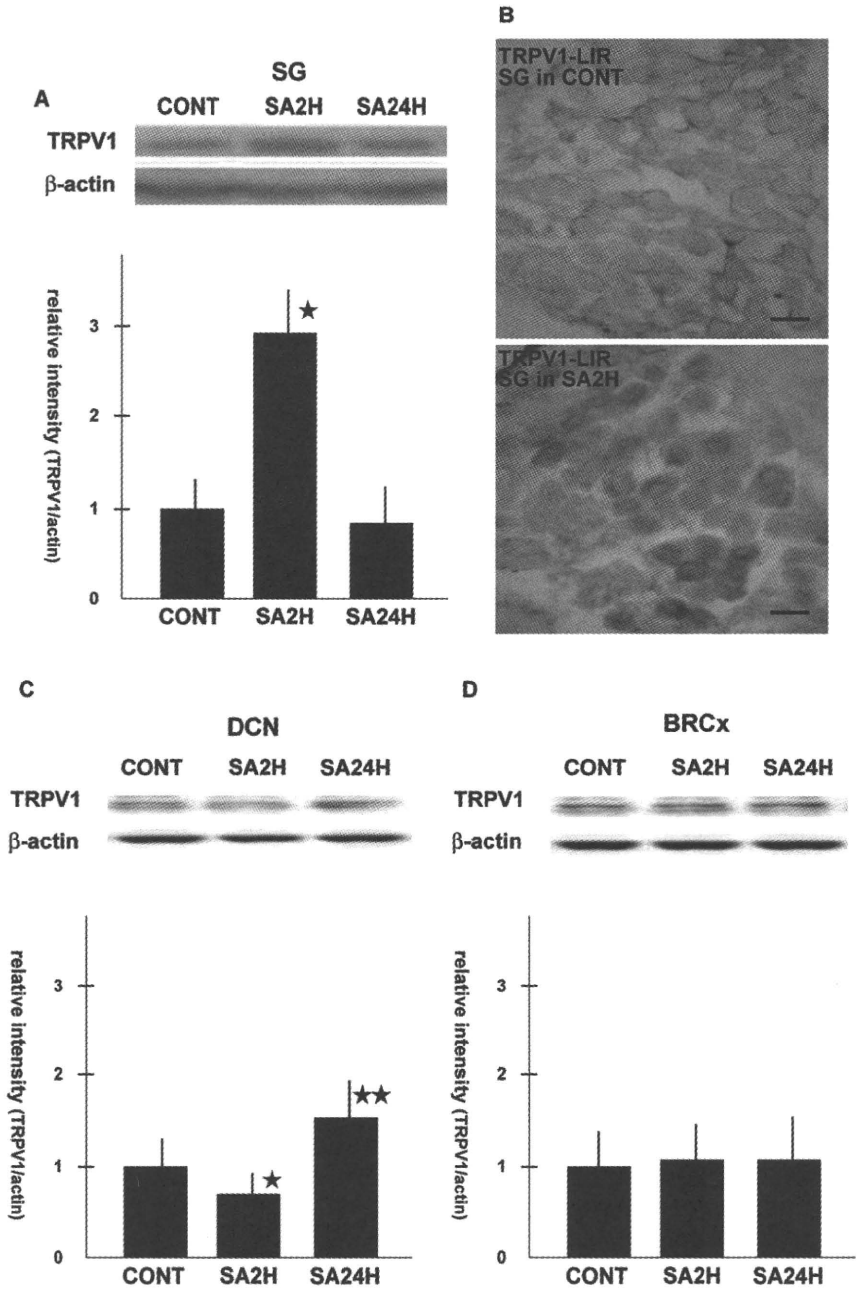

Fig. 5. Western blot analysis (A, C, D) and immunohistochemistry (B) of salicylate-induced changes in TRPV1 protein levels in the rat SG, DCN and BRCx. (A) TRPV1 protein levels in SG were significantly increased 2 h after salicylate treatment (SA2H) (* $P<0.05$) and had returned to control levels (CONT) 24 h after salicylate treatment (SA24H). (B) TRPV1-like immunoreactivity (LIR) in the salicylate-treated group (SA2H) was clearly enhanced in almost all SG cells compared with that in the saline-control group (CONT). (C) In spite of a slight reduction 2 h after salicylate treatment (SA2H) (* $P<0.05$), TRPV1 protein levels in the DCN were significantly increased 24 h after salicylate treatment (SA24H) (** $P<0.05$). (D) TRPV1 protein levels in the BRCx did not show any significant change after salicylate treatment.

Effect of TRPV1 antagonist

Behavioral assessment showed that false positive responses were increased 2 h post-treatment in group II (salicylate+DMSO) (3.7±1.2 responses) compared with control group I and that this increase was significantly suppressed in group III (salicylate+capsazepine) (0.8±0.5 responses, * $P=0.015$) (Fig. 6A). Examination of TRPV1 mRNA levels in the SG showed that mRNA was upregulated 2 h post-treatment in group II (salicylate+DMSO) (3.38±1.13 fold) compared with the control group I and that this upregulation was significantly suppressed in group III (salicylate+capsazepine) (0.85±0.07 fold, * $P=0.020$) (Fig. 6B). Capsazepine suppressed the salicylate-induced molecular changes in the SG in a dose dependent manner (Fig. 6C).

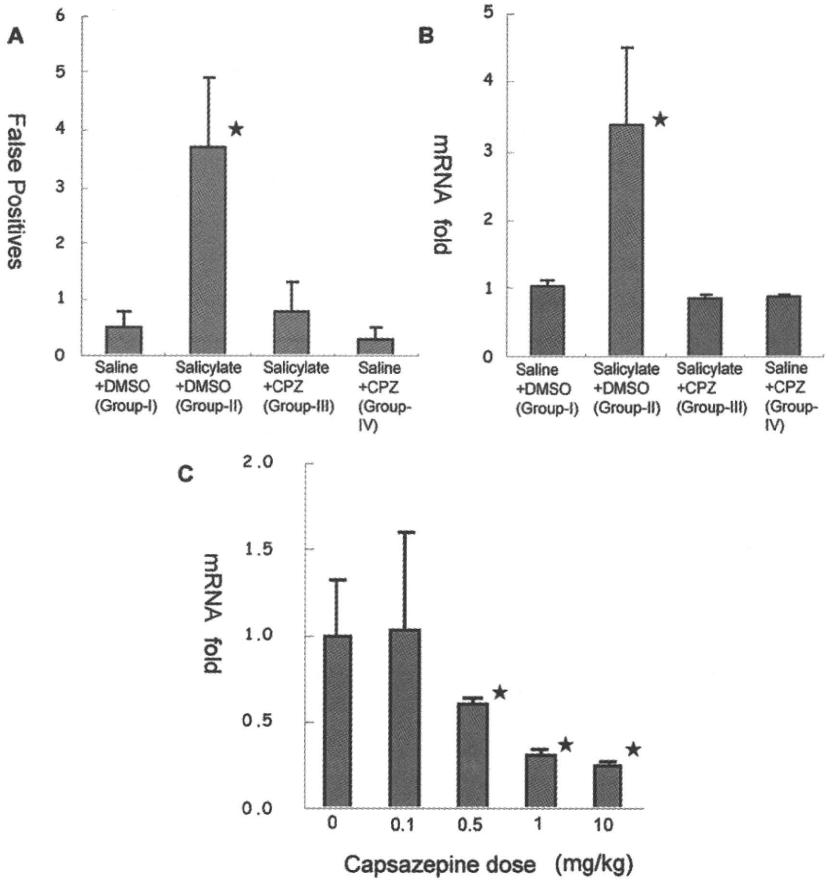

Fig. 6. Capsazepine (CPZ) blocked both the salicylate-induced increase of false positives and upregulation of TRPV1 mRNA in the rat SG. Dimethyl sulfoxide (DMSO) or CPZ (10 mg/kg i.p.) was administrated 0.5 h after the first injection of saline or salicylate. (A) False positives were increased 2 h post-treatment in group II (salicylate+DMSO) and this increase was significantly suppressed in group III (salicylate+CPZ) (* $P<0.05$). (B) TRPV1 mRNA was upregulated in the SG 2 h post-treatment in group II (salicylate+DMSO) and this upregulation was significantly suppressed in group III (salicylate+CPZ) (* $P<0.05$). (C) CPZ (0–10 mg/kg) suppressed the salicylate-induced upregulation of TRPV1 mRNA in the SG in a dose dependent manner (* $P<0.05$).

DISCUSSION

One of the reasons why it is still so hard for clinicians to cure intractable tinnitus is that appropriate animal models of tinnitus have not yet been established. To overcome this deficiency, we present here an animal behavioral model of tinnitus. Validating a behavioral procedure in animals to assess the presence of tinnitus is an unusual and difficult task to which many research groups have devoted many years. Among these groups, Jastreboff and Sasaki (1994) proposed an animal model based on an active avoidance paradigm; animals were conditioned by electrical footshock to drink water when hearing a sound. Recently, Guitton et al. (2003) demonstrated an animal model involving escape to a climbing pole to avoid electrical footshock when hearing a sound. In the present study, we further developed Guitton's model with modifications of a much easier active avoidance task of escaping to the next room instead. Adult or even aged animals were able to quickly step over the wall to move to the other side of the box. The conditioning box should also be set in a soundproof room, because it was important for animals to clearly hear both the conditioned sound and salicylate-induced phantom sound.

Previous studies in tinnitus kHz have revealed that salicylate induces an acute, relatively high frequency and transient type of tinnitus. It ranges from 10 to 16 kHz (Bauer et al., 1999; Guitton et al., 2003; Zheng et al., 2006; Yang et al., 2007). In the present study, false positive responses increased significantly when the conditioned stimulus was 16 kHz compared with 4, 10 and 40 kHz. Previous studies in tinnitus dB SPL have also shown that salicylate caused tinnitus of around 60 dB SPL (Bauer et al., 1999; Guitton et al., 2003; Rüttiger et al., 2003; Zheng et al., 2006; Yang et al., 2007). In the present study, false positive responses also increased significantly when the conditioned stimulus was 60 dB SPL compared with 20 dB SPL and 80 dB SPL. Judging from these pilot experiments, together with previous papers, 16 kHz and 60 dB SPL pure tone sound was the most similar to the sound of 400 mg/kg salicylate-induced tinnitus in the present Wistar rat study.

Another reason why it is still so hard for clinicians to cure intractable tinnitus is that the molecular mechanism of tinnitus generation in the auditory pathway has not yet been clarified. We therefore aimed to establish such a molecular marker. The history of TRP channels in hearing and balance is characterized at great length by the hunt for the elusive transduction channel of sensory hair cells. Such pursuit has not resulted in unequivocal identification of the transduction channel, but nevertheless revealed a number of candidates, such as TRPV4, TRPN1, TRPA1, and TRPML3. Based on mutations in the corresponding mouse genes, TRPV4 (Tabuchi et al., 2005; Cuajungco et al., 2007) and TRPML3 (van Aken et al., 2008) are possible candidates for human hearing, and potentially also balance disorders. In the present study, we focused especially on TRPV1, a member of the non-specific cation ion channel receptor family, which responds to various kinds of noxious pain, such as capsaicin, inflammation, heat, low pH and hypo-osmolarity (Caterina et al., 1997; Benham et al., 2003), because it is expressed in the mouse inner ear ganglia and is upregulated by noxious challenges of kanamycin (Kitahara et al., 2005a). Interestingly, tinnitus is the sensation of a sound in the ear without an external source, similar to phantom pain (Bartels et al., 2007).

In the present study, TRPV1 mRNA levels in the SG were significantly upregulated 2 h post-treatment, significantly downregulated 12–24 h post-treatment and had returned to control levels by 72 h post-treatment. The reasons of discrepancy in these molecular results of mRNA and protein level could be explained by the time lag between mRNA and protein synthesis and/or different sensitivity in mRNA and protein experiments. According to the animal behavioral model of tinnitus, salicylate-induced tinnitus was maximal 2 h post-injection and had disappeared by 24 h post-injection. Furthermore, capsazepine, a TRPV1 antagonist, demonstrated a significant suppression of false positive response increase and of TRPV1 mRNA upregulation in the SG. Taken together, these data suggest that tinnitus in salicylate-treated animals could be caused through the activation of the nociceptive receptor, TRPV1 in the SG. Therefore, we hypothesize that tinnitus is a type of phantom pain sensation in the inner ear (Bartels et al., 2007). The mechanism of TRPV1 regulation in the SG has not been clarified yet. However, TRPV1 is auto-regulated via neurotrophic factors in damaged dorsal root ganglia (Acheson et al., 1995; Anand et al., 2006; Szallasi et al., 2006). In the present study, the blockade of salicylate-induced TRPV1 upregulation in the SG by capsazepine suggests that TRPV1 was also auto-regulated via neurotrophic factors in the salicylate-treated SG (Hansen et al., 2001; Zha et al., 2001; Shepherd et al., 2005; Kitahara et al., 2006). Known TRPV1 antagonists (capsazepine, BCTC and thio-BCTC) were also able to block the response of TRPM8 (Behrendt et al., 2004), which shares many functional and pharmacological properties with TRPV1 (Weil et al., 2005). Although TRPM8 has never been reported to be located in the inner ear, the possible role of TRPM8 in hearing and/or tinnitus should be discussed after further studies of TRPM8 in the inner ear.

The following mechanisms of salicylate-induced TRPV1 activation and tinnitus generation are suggested: TRPV1 and 5-lipoxygenase are co-expressed by SG cells in the inner ear (Balaban et al., 2003). Salicylate, an active component of aspirin, inhibits cyclo-oxygenase activity (Christie et al., 1998) and this cyclo-oxygenase inhibition leads to an excess of intracellular arachidonic acid, which is metabolized by 5-lipoxygenase pathways (Fosslien, 1998). These findings suggest that the resultant increase in arachidonic acid products, such as hydroperoxyeicosatetraenoic acid and hydroxyeicosatetraenoic acid, has the potential to depolarize SG cells by activation of TRPV1 (Hwang et al., 2000). This may either lower their threshold for spike generation or increase their sensitivity to suprathreshold activation and mimic the discharge pattern during low level natural stimulation. Actually, a couple of physiological studies of TRPV1 in the cochlea were reported. Zheng et al. revealed that activation of TRPV1 increases the threshold of the cochlear action potential, but decreases both cochlear microphonic and electrically-evoked otoacoustic emissions (Zheng et al., 2003). Zhou et al. demonstrated that perfusion with capsaicin alone produced a dose-dependent increase of the 900 Hz peak ratio (power normalized re the overall spectrum) of the ensemble background activity (Zhou et al., 2006). The capsaicin effect was attenuated during concurrent perfusion with capsazepine. These findings are consistent with the hypothesis that TRPV1 activation increases background activity of SG cells and support a role of TRPV1 in gating spontaneous and evoked auditory nerve excitability.

In contrast, the peak TRPV1 protein levels in the DCN were delayed relative to the peak levels in SG cells. This delay might indicate the mechanism for the alteration from peripheral tinnitus to central tinnitus and/or the mechanism of chronic tinnitus. There is an interesting case of a patient with intractable chronic tinnitus, who underwent temporal bone removal surgery, however, this treatment failed to cure the tinnitus (House, 1964). It is extremely difficult to identify the site of chronic tinnitus, because it may alternate between the periphery and the CNS, like phantom pain sensation. Further studies addressing the mechanisms of central tinnitus and/or chronic tinnitus are needed.

According to the morphological data, salicylate treatment caused no obvious morphological damage to cochlear hair cells or SG cells. Furthermore, from the behavioral study, the active avoidance score remained stable during the whole period of salicylate injections. Together with the ABR data, this suggests that salicylate application caused no obvious functional damage to the auditory system. However, Guitton et al. (2005) reported that administering salicylate led to transient hearing loss by means of compound action potential (CAP) threshold shifts, directional preponderance of otoacoustic emission (DPOAE) recordings and score measurements. This hearing loss was evaluated around 40 dB SPL at 16 kHz (Cazals, 2000; Guitton et al., 2005) and might not be comparable to the results in the present study, which used 60 dB SPL and 16 kHz sound stimuli.

CONCLUSION

In conclusion, we developed a rat behavioral model of salicylate-induced tinnitus and identified a molecular marker of salicylate-induced tinnitus in the rat auditory pathway. These findings could make "phantom tinnitus" clearly observable and easily accessible. We believe that these findings are important for understanding the mechanism of tinnitus generation and for elucidating de novo treatments for intractable tinnitus.

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REFERENCES

Acheson A, Conover JC, Fandl JP, DeChiara TM, Russell M, Thadani A, Squinto SP, Yancopoulos GD, Lindsay RM (1995) A BDNF autocrine loop in adult sensory neurons prevents cell death. Nature 374:450–453.

Anand U, Otto RW, Casula MA, Day NC, Davis JB, Bountra C, Birch R, Anand P (2006) The effect of neurotrophic factors on morphology, TRPV1 expression and capsaicin responses of cultured human DRG sensory neurons. Neurosci Lett 399:51–56.

Balaban CD, Zhou J, Li HS (2003) Type 1 vanilloid receptor expression by mammalian inner ear ganglion cells. Hear Res 175:165–170.

Bartels H, Staal MJ, Albers FWJ (2007) Tinnitus and neural plasticity of the brain. Otol Neurotol 28:178–184.

Bauer CA, Brozoski TJ, Rojas R, Boley J, Wyder M (1999) Behavioral model of chronic tinnitus in rats. Otolaryngol Head Neck Surg 121:457–462.

Behrendt HJ, Germann T, Gillen C, Hatt H, Jostock R (2004) Characterization of the mouse cold-menthol receptor TRPM8 and vanilloid receptor type-1 VR1 using a fluorometric imaging plate reader (FLIPR) assay. Br J Pharmacol 141:737–745.

Benham CD, Gunthorpe MJ, Davis JB (2003) TRPV channels as temperature sensors. Cell Calcium 33:479–487.

Caterina MJ, Schumacher MA, Tominaga M, Rosen TA, Levine JD (1997) The capsaicin receptor: a heat-activated ion channel in the pain pathway. Nature 389:816–824.

Cazals Y (2000) Auditory sensori-neural alterations induced by salicylate. Prog Neurobiol 62:583–631.

Christie MJ, Vaughan CW, Ingram SL (1998) Opioids, NSAIDs and 5-lipoxygenase inhibitors act synergistically in brain via arachidonic acid metabolism. Inflamm Res 48:1–4.

Cuajungco MP, Grimm C, Heller S (2007) TRP channels as candidates for hearing and balance abnormalities in vertebrates. Biochim Biophys Acta 1772(8):1022–1027.

De Schepper HU, De Man JG, Ruyssers NE, Deiteren A, Van Nassauw L, Timmermans JP, Martinet W, Herman AG, Pelckmans PA, De Winter BY (2008) TRPV1 receptor signaling mediates afferent nerve sensitization during colitis-induced motility disorders in rats. Am J Physiol Gastrointest Liver Physiol 294:245–253.

Eggermont JJ, Roberts LE (2004) The neuroscience of tinnitus. Trends Neurosci 27:676–682.

Fosslien E (1998) Adverse effects of nonsteroidal anti-inflammatory drugs on the gastrointestinal system. Ann Clin Lab Sci 28:67–81.

Guitton MJ, Caston J, Ruel J, Johnson RM, Pujol R, Puel JL (2003) Salicylate induces tinnitus through activation of cochlear NMDA receptors. J Neurosci 23:3944–3952.

Guitton MJ, Pujol R, Puel JL (2005) m-Chlorophenylpiperazine exacerbates perception of salicylate-induced tinnitus in rats. Eur J Neurosci 22:2675–2678.

Hansen MR, Zha XM, Bok J, Green SH (2001) Multiple distinct signal pathways, including an autocrine neurotrophic mechanism, contribute to the survival-promoting effect of depolarization on spiral ganglion neurons in vitro. J Neurosci 21:2256–2267.

Heller AJ (2003) Classification and epidermology of tinnitus. Otolaryngol Clin North Am 36:239–248.

House WF (1964) Transtemporal bone microsurgical removal of acoustic neurinomas: report of cases. Arch Otolaryngol 80:617–667.

Hwang SW, Cho H, Kwak J, Lee SY, Kang CJ (2000) Direct activation of capsaicin receptors by products of lipoxygenases: endogenous capsaicin-like substances. Proc Natl Acad Sci U S A 97:6155–6160.

Im GJ, Jung HH, Chae SW, Cho WS, Kim SJ (2007) Differential gene expression profiles in salicylate ototoxicity of the mouse. Acta Otolaryngol 127:459–469.

Jastreboff PJ, Sasaki CT (1986) Salicylate-induced changes in spontaneous activity of single units in the inferior colliculus of the guinea pig. J Acoust Soc Am 80:1384–1391.

Jastreboff PJ, Sasaki CT (1994) An animal model of tinnitus: a decade of development. Am J Otol 15:19–27.

Kitahara T, Li HS, Balaban CD (2005a) Changes in transient receptor potential cation channel superfamily V (TRPV) mRNA expression in the mouse inner ear ganglia after kanamycin challenge. Hear Res 201:132–144.

Kitahara T, Li HS, Balaban CD (2005b) Regulation of mitochondrial uncoupling proteins in mouse inner ear ganglion cells in response to systemic kanamycin challenge. Neuroscience 135:639–653.

Kitahara T, Kizawa K, Horii A, Kubo T, Balaban CD (2006) Changes in TRPV1 expression in the rat auditory pathway after salicylate challenge. In: Abstracts of the 36th Annual Meeting Society for Neuroscience. Program#/Poster# 45.12/E38.

Paxinos G, Watson C (1986) The rat brain in stereotaxic coordinates. New York: Academic Press.

Rüttiger L, Ciuffani J, Zenner HP, Knipper M (2003) A behavioral paradigm to judge acute sodium salicylate-induced sound experience in rats: a new approach for an animal model on tinnitus. Hear Res 180:39–50.

Schmittgen TD, Zakrajsek BA, Mills AG, Gorn V, Singer MJ, Reed MW (2000) Quantitative reverse transcription-polymerase chain reaction to study mRNA decay: comparison of endpoint and real-time methods. Anal Biochem 285:194–204.

Shepherd RK, Coco A, Epp SB, Crook JM (2005) Chronic depolarization enhances the trophic effects of brain-derived neurotrophic factor in rescuing auditory neurons following a sensorineural hearing loss. J Comp Neurol 486:145–158.

Szallasi A, Cruz F, Geppetti P (2006) TRPV1: a therapeutic target for novel analgesic drugs? Trends Mol Med 12:545–554.

Tabuchi K, Suzuki M, Mizuno A, Hara A (2005) Hearing impairment in TRPV4 knockout mice. Neurosci Lett 382:304–308.

van Aken AFJ, Atiba-Davis M, Marcotti W, Goodyear RJ, Bryant JE, Richardson GP, Noben-Trauth K, Kros CJ (2008) TRPML3 mutations cause impaired mechano-electrical transduction and depolarization by an inward-rectifier cation current in auditory hair cells of varitint-waddler mice. J Physiol 586:5403–5418.

Weil A, Moore SE, Waite NJ, Randall A, Gunthorpe MJ (2005) Conservation of functional and pharmacological properties in the distantly related temperature sensors TRPV1 and TRPM8. Mol Pharmacol 68:518–527.

Yang G, Lobarians E, Zhang L, Turner J, Stolzberg D, Salvi R, Sun W (2007) Salicylate induced tinnitus: behavioral measures and neural activity in auditory cortex of awake rats. Hear Res 226:244–253.

Zha XM, Bisop JF, Hansen MR, Victoria L, Abbas PJ, Mouradian MM, Green SH (2001) BDNF synthesis in spiral ganglion neurons is constitutive and CREB-dependent. Hear Res 156:53–68.

Zheng J, Dai C, Steyger PS, Kim Y, Vass Z, Ren T, Nuttall AL (2003) Vanilloid receptors in hearing: altered cochlear sensitivity by vanilloids and expression of TRPV1 in the organ of corti. J Neurophysiol 90(1):444–455.

Zheng Y, Lee HS, Smith PF, Darlington CL (2006) Neuronal nitric oxide synthase expression in the cochlear nucleus in a salicylate model of tinnitus. Brain Res 1123:201–206.

Zhou J, Balaban CD, Durrant JD (2006) Effect of intracochlear perfusion of vanilloids on cochlear neural activity in the guinea pig. Hear Res 218:43–49.

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ORIGINAL ARTICLE

Effect of water-soluble coenzyme Q10 on noise-induced hearing loss in guinea pigs

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Abstract

Conclusion. The results of this study indicate that coenzyme Q10 reduces cochlear oxidative stress induced by acoustic overstimulation. *Objective.* We investigated the effects of coenzyme Q10 on noise-induced hearing loss in guinea pigs. *Materials and methods.* Animals received water-soluble coenzyme Q10 intraperitoneally 2 h before noise exposure. Seven days after noise exposure (130 dB sound pressure level for 3 h), the auditory brainstem response (ABR) threshold shift and cochlear hair cell damage were assessed. *Results.* We observed that the ABR threshold shift was significantly less in the coenzyme Q10 group than in the vehicle control group. In addition, the percentage of missing outer hair cells was lower in the coenzyme Q10 group than in the control group. Moreover, 2 days after administration of coenzyme Q10, increased antioxidative activity in the cochlea, as measured by analysis of hydroxy radical scavenging activity by electron spin resonance was observed.

Keywords: *Coenzyme Q10, reactive oxygen species, cochlear protection, noise trauma, electron spin resonance, guinea pig*

Introduction

Many recent studies have reported that reactive oxygen species (ROS) play a major role in noise-induced deafness. Noise exposure increases the levels of ROS in the cochlear tissue [1] and results in cochlear damage. Noise-induced hearing loss can be reduced by treatment with antioxidants [2]. In our previous study, we reported that perilymphatic application of the antioxidative reagent edaravone reduced noise-induced hearing loss in guinea pigs [3]. However, antioxidative drugs are difficult to use in treatment of chronic acoustic trauma because of side effects. Coenzyme Q10 (2,3 dimethoxy-5-methyl-6-decaprenyl 4-benzoquinone) is a vitamin-like antioxidant that reacts with oxygen radicals and lipoperoxides to prevent damage to biomolecules in various tissues and cell compartments [4]. However, coenzyme Q10 is extremely lipid-soluble, and absorption by the body is not easy. Recently, water-soluble coenzyme Q10 was developed to improve coenzyme Q10 absorption [5].

Coenzyme Q10 is used in the treatment of a variety of disorders related to suboptimal cellular energy metabolism and oxidative injury such as Parkinson's disease and congestive heart failure [6]. Based on the antioxidant properties of coenzyme Q10, we hypothesized that it can protect the cochlea from acoustic trauma. The aim of the present study was to evaluate the effects of water-soluble coenzyme Q10 on noise-induced hearing loss in guinea pigs.

Materials and methods

Animals

Thirty male Hartley guinea pigs (350–400 g; Chiyoda Development Inc., Tokyo, Japan) with normal Preyer's reflexes and normal tympanic membranes were used in this study. Auditory brainstem response (ABR) thresholds of all animals were evaluated before the experiments, and animals with abnormal ABR thresholds were excluded. The animals were assigned to one of two groups. In the

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coenzyme Q10 group ($n=10$), water-soluble coenzyme Q10 (20 mg/kg) was administered intraperitoneally (i.p.) 2 h before noise exposure. In the vehicle control group ($n=10$), the same dosage of vehicle was administered i.p. Seven days after noise exposure, ABR threshold was assessed again. We also assessed cochlear hair cell damage. Four animals were used for immunohistochemical analysis of 4-hydroxy-2-nonenal (4-HNE), and six animals were used for hydroxy radical extinction assay.

This study was reviewed by the Committee for the Ethics of Animal Experiments of Yamaguchi University School of Medicine and was carried out according to the Guidelines for Medicine and the Law (No. 105) and Notification No. 6 of the Japanese government.

Drug

Water-soluble coenzyme Q10 and its solvent were a gift of Eisai Co. Ltd (Tokyo, Japan).

Noise exposure

Guinea pigs under pentobarbital anesthesia (33 mg/kg, i.p.) were exposed to intense band noise centered at 4 kHz for 3 h (130 dB sound pressure level, SPL). The noise we used was designed to cause permanent threshold shifts and to damage cochlear hair cells at the basal end of the second turn. Each animal was immobilized, and a speaker was centered over the animal's head at a distance of 15 cm. Sound intensity was monitored with a sound-level meter (NA-60; Rion, Tokyo, Japan) positioned near the external auditory canal.

Auditory brainstem response

The ABR threshold was examined under xylazine (16 mg/kg, i.p.) and ketamine (16 mg/kg, i.p.) anesthesia. Responses were recorded between subcutaneous stainless steel electrodes located at the vertex (positive) and antinion (negative); the lower back served as the ground. The sound stimuli consisted of 2, 4, and 8 kHz tone bursts (rise–fall time 2 ms, duration 8 ms). Stimuli were presented through a 10 cm tube that connected an earphone to the external auditory canal. The stimulus intensity was evaluated with an NA-60 sound-level meter adjacent to the tip of the tube. Responses to 500 stimuli were recorded with a signal processor (Synax 1100; NEC Corp., Tokyo, Japan). The ABR threshold was defined as the lowest stimulus intensity to produce a reliable waveform of three to five peaks.

Histopathology

Seven days after sound exposure, animals were anesthetized and killed with an overdose of pentobarbital. The temporal bones of all animals were removed. Each cochlea was opened at the apex and oval window and fixative (4% paraformaldehyde in 0.01 M phosphate-buffered saline, PBS) was perfused into the cochleae. The organ of Corti of each inner ear was removed, permeabilized with 0.3% Triton X-100 for 10 min, and subsequently incubated with fluorescein isothiocyanate-conjugated phalloidin (1:50 dilution; Sigma-Aldrich Corp., St Louis, MO, USA) at room temperature for 1 h. After a rinse in PBS, specimens were mounted in Vectashield (Vector Laboratories, Inc., Burlingame, CA, USA). Surface structures were observed under fluorescence microscopy (Nikon Corp., Tokyo, Japan). Missing outer hair cells in the cochlear second turn between 45% and 70% distance from the apex were quantified by two investigators and presented as a percentage of defects of the outer hair cells.

Immunohistochemistry for 4-HNE

We examined 4-HNE production in animals in the coenzyme Q10 group ($n=2$) and the control group ($n=2$). In the coenzyme Q10 group, water-soluble coenzyme Q10 (20 mg/kg) was administered i.p. 2 h before noise exposure. In the vehicle control group, the same dosage of vehicle was administered i.p. Animals were anesthetized and killed with an overdose of pentobarbital 48 h after noise exposure. The temporal bones of all animals were removed, transferred to 4% paraformaldehyde, and kept in fixative overnight. The tissue was decalcified in 5% ethylene diamine tetraacetic acid (EDTA, pH 7.2) for approximately 14 days at 4°C. The lateral walls of the cochleae were removed under a microscope. Specimens were rinsed in PBS, incubated in methanol for 20 min at −20°C, and then hydrated in PBS containing 0.1% Triton X-100 for 10 min. Specimens were soaked in blocking solution (1% bovine serum albumin, 0.4% normal goat serum, 0.4% normal horse serum, 0.4% Triton X-100 in PBS) for 2 h at 4°C and then incubated in a 1:100 dilution of anti-4-HNE mouse monoclonal antibody (Oxis International, Inc., Portland, OR, USA) for 12 h at 4°C. The specimens were rinsed in PBS and incubated at 4°C in a 1:100 dilution of Alexa Fluor568-conjugated goat anti-mouse IgG (Molecular Probes, Eugene, OR, USA) for 8 h. They were then rinsed in PBS and embedded in a semi-water-soluble resin (Immuno-Bed; Polysciences, Inc., Warnington, PA, USA) and cut into 2 μm thick sections. Sections were counterstained with 4;6-diamino-2-phenylindole (DAPI; Vector Laboratories, Inc.). Immunolabeling

was visualized under bright-field illumination with a fluorescence microscope equipped with a 20 × objective (Nikon).

Hydroxy radical extinction assay

We examined hydroxy radical scavenging activity after the administration of coenzyme Q10. At 24 h after administration of water-soluble coenzyme Q10, animals were anesthetized and killed with an overdose of pentobarbital. The cochleae were removed from the temporal bone and immediately frozen in liquid nitrogen. Each specimen was added to 0.5 ml PBS, crushed by sonication on ice, and centrifuged at 3000 rpm. The supernatant fluid was extracted and immediately frozen in liquid nitrogen.

Scavenging activity against HO was measured by electron spin resonance (ESR) as described by Noda et al. [7]. Conditions for ESR spectrometry (JES-FR80; JEOL, Tokyo, Japan) were as follows: magnetic field, 335.0±5 mT; power, 4.0 mW; modulation frequency, 100 kHz; frequency, 9.415 GHz; modulation amplitude, 0.079 mT; time scan, 2 min; and time constant, 0.3 s. For the assay, all solutions were dissolved in distilled water, and 50 µl of 10 mg/ml sample, 50 µl of 92 mM 5,5-dimethyl-1-pyrroline-N-oxide (DMPO), 50 µl of 0.6% H_2O_2, and 50 µl of 16.9 × 10.3 mg/ml of $FeCl_2$ were mixed and put into a flat cell. At 60 s after adding hydrogen peroxide at room temperature (23°C), ESR spectra of DMPO-OH spin adduct were recorded.

Statistical analysis

Differences in pre- and post-exposure ABR thresholds, percentages of missing outer cells, and hydroxy radical extinction assay results between the coenzyme Q10 group and the vehicle control group were analyzed by t test with StatView version 4.5 J for Macintosh (Abacus Concepts Inc., Berkeley, CA, USA). $p<0.05$ was accepted as statistically significant.

Results

ABR thresholds

ABR thresholds before and after sound exposure are shown in Figure 1. There was no difference between the coenzyme Q10 group and the vehicle control group before sound exposure. ABR thresholds of all animals increased after sound exposure. However, the threshold shift was smaller in the coenzyme Q10 group than in the vehicle control group at all frequencies (2, 4, and 8 kHz).

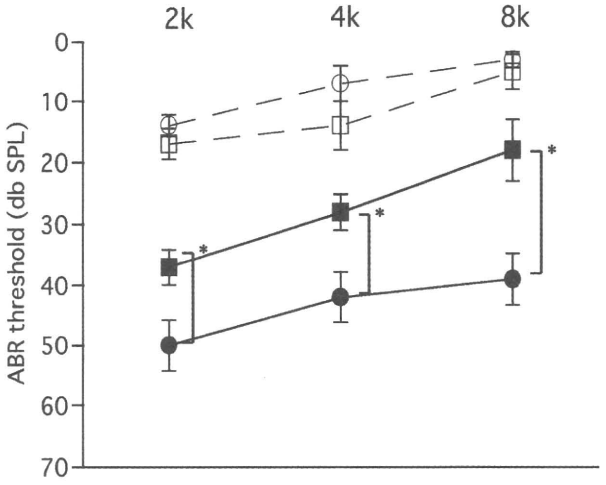

Figure 1. Auditory brainstem response (ABR) thresholds of the coenzyme Q10 group ($n=10$, squares) and the control group ($n=10$, circles) before and after noise exposure. Broken lines indicate ABR thresholds before sound exposure and solid lines indicate thresholds at 1 week after sound exposure. Error bar: ±1 SEM. *$p<0.05$.

Number of outer hair cells

The surface structure of the organ of Corti (second cochlear turn) is shown in Figure 2. Three rows of outer hair cells and a single row of inner ear hair cells

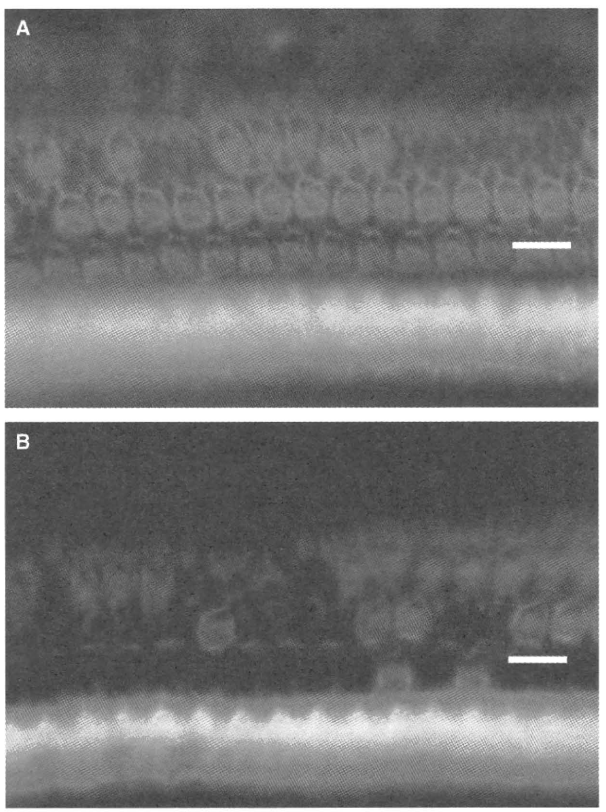

Figure 2. Surface structure of the organ of Corti 1 week after sound exposure. Fluorescence microscopy shows that the defects of the outer hair cells appear less severe in the coenzyme Q10 group (A) than in the vehicle control group (B). Bars = 20 µm.

were visualized by phalloidin fluorescence. The inner hair cells of all ears were relatively well preserved after sound exposure (date not shown). On the other hand, all animals showed a decreased number of outer cells in the second cochlear turn. However, the decrease was less in the coenzyme Q10 group than in the vehicle control group. Most hair cell damage was found between 45% and 70% distance from the apex. Therefore, the extent of hair cell damage induced by noise was analyzed only for that region. The number of missing outer hair cells was presented as a percentage (Figure 3). The percentage of missing outer hair cells in the coenzyme Q10 group was significantly lower than that in the vehicle control group.

Immunohistochemistry for 4-HNE

All sections for 4-HNE assessment were taken from the main lesion of the second turn (approximately 9–12 mm from the apex). Slight staining was observed in the stria vascularis in untreated control animals (Figure 4A). At 48 h after noise exposure, the vehicle control group showed increased 4-HNE immunoreactivity (Figure 4B). However, the coenzyme Q10 group showed little immunoreactivity (Figure 4C). These results suggest that administration of water-soluble coenzyme Q10 can limit the generation of peroxidation products in response to free radicals.

Hydroxy radical extinction assay

Cochlear radical scavenging activities of the coenzyme Q10 group and the vehicle control group are shown in Figure 5. Scavenging activity in the coenzyme Q10 group was significantly greater than that in the vehicle control group, suggesting that the coenzyme Q10 enhances the cochlear capacity to scavenge the hydroxy radical.

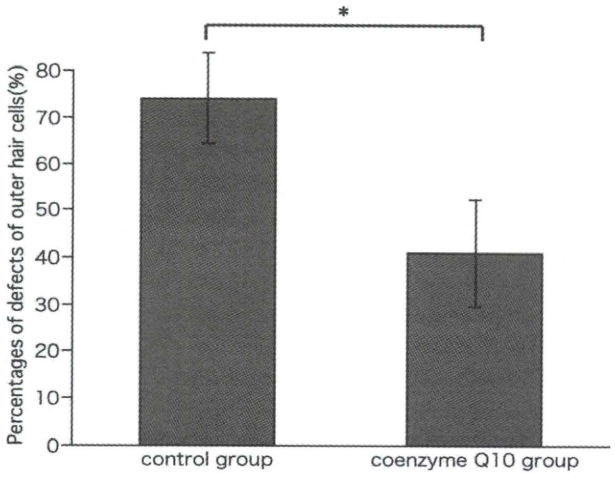

Figure 3. Percentages of missing outer hair cells between 9.3 mm and 14.3 mm from the apex 1 week after sound exposure. In the coenzyme Q10 group, the percentage is lower than that in the vehicle control group. Error bar: ±1 SEM. $*p < 0.05$.

Discussion

In the present study, ABR thresholds in both groups increased significantly, and all animals showed a decrease of outer hair cells at the second cochlear turn 7 days after sound exposure. Outer and inner hair cells in the apex, third, and basal turn were almost preserved, because the noise in this study was designed to damage cochlear hair cells at the basal end of the second turn [2]. Takemoto et al. reported a temporary threshold shift in guinea pigs exposed to intense sound at 130 dB SPL [3]. We used the same system to generate a sound at 130 dB SPL for 3 h to cause a permanent threshold shift.

A dose of 30–1200 mg/day of coenzyme Q10 can be taken with minimal side effects and low potential for drug interaction [6]. Kasparová et al. used coenzyme Q10 at 20 mg/kg/day in a model of Huntington's disease [8]. Therefore, we set a dose of 20 mg/kg that is the maximum level to use clinically. Coenzyme Q10 is a safe drug, so we assumed that this drug has a role as a preventive medical agent. Thus, we administered it only before the noise exposure.

Noise exposure increases ROS in the cochlea [1]. ROS induce histological and functional damage in the cochlea [2]. Free radicals damage cellular molecules such as lipids, proteins, and DNA. The mechanisms of cochlear damage are related to lipid oxidation, protein oxidation, and DNA damage [2,9–11]. Coenzyme Q10 inhibits lipid peroxidation by either scavenging free radicals directly or by reducing α-tocopheroxyl radical to α-tocopherol [12,13]. Coenzyme Q10 protects membrane proteins against oxidation [14]. Coenzyme Q10 also inhibits DNA oxidation in rat liver mitochondria [15] and inhibits DNA strand breaks in human lymphocytes [16]. In the cochlea, coenzyme Q10 presumably prevents lipid oxidation, protein oxidation, and DNA damage.

To elucidate the mechanism of coenzyme Q10 antioxidative activity in the cochlea, we performed two assays. The first evaluated the production of 4-HNE in the cochlea after noise exposure. The second evaluated hydroxy radical scavenging activity after the administration of coenzyme Q10. We evaluated immunostaining for 4-HNE, a phospholipid peroxidation product generated by the reaction of free radicals with the plasma membrane. In the present study, staining was mainly observed in the stria vascularis. Tanaka et al. also showed the formation of 4-HNE in the organ of Corti after noise exposure and 4-HNE staining was clearly observed in the stria vascularis [17]. Our results indicate that

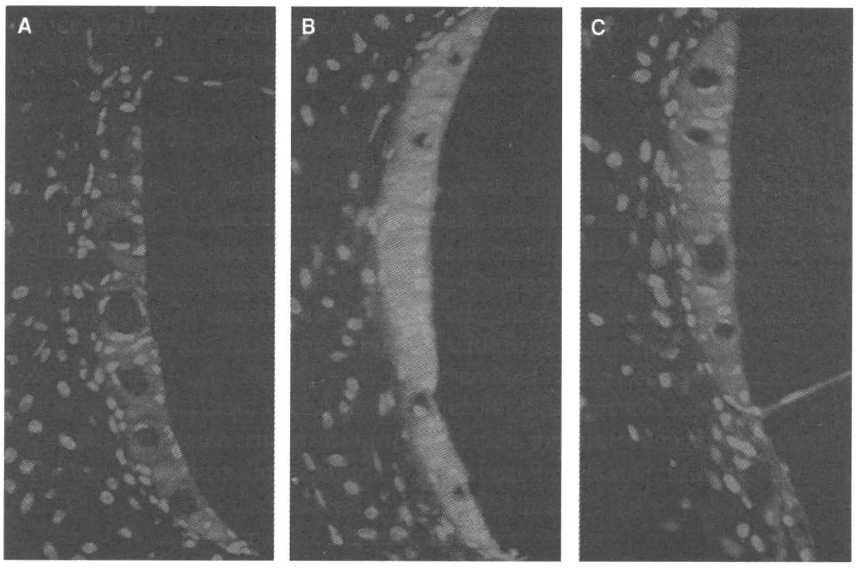

Figure 4. Immunoreactivity for 4-HNE in the stria vascularis after noise exposure. Nuclei were visualized with DAPI counterstaining. Sections were taken from the main lesion of the second turn of the cochlea. (A) Untreated control; (B) 48 h after exposure with vehicle; (C) 48 h after exposure with coenzyme Q10.

administration of coenzyme Q10 can limit the generation of phospholipid peroxidation products in response to free radicals.

Radical scavenging activity can be measured by a decrease of DMPO spin adducts by ESR. ESR has been used to assess radical scavenging activities of antioxidants such as vitamin E, vitamin C, tissues (e.g. muscle), and some foods [18,19]. We found that coenzyme Q10 exerts radical scavenging activity in the cochlea. Thus, coenzyme Q10 increases radical scavenging activity in cochlea and protects the cochlea by limiting the generation of phospholipid peroxidation products.

Sergi et al. reported that coenzyme Q10 significantly decreases noise-induced compound cochlear action potential threshold shifts and attenuates noise-induced outer hair cell loss [20]. The study was the first report that elucidated the effect of coenzyme Q10 in acoustic trauma. However, these authors did not assess the action mechanism for the protective effects, sufficiently. The present study is the first to show the mechanism of coenzyme Q10 protection against noise-induced hearing loss.

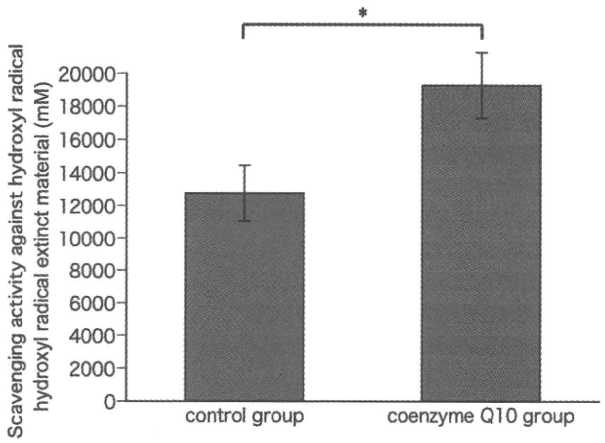

Figure 5. Hydroxyl radical scavenging activity in the cochlea in the coenzyme Q10 group and the vehicle control group. Hydroxyl radical scavenging activity is greater in the coenzyme Q10 group than in the vehicle control group. Error bar: ± 1 SEM. $^*p < 0.05$.

We used a water-soluble type of coenzyme Q10. Coenzyme Q10 is lipid-soluble, and absorption by the body is not easy. Recently, water-soluble coenzyme Q10 was developed to improve coenzyme Q10 absorption. Water-soluble coenzyme Q10 showed higher uptake when administered in the fasting state or with food compared with lipid-soluble coenzyme Q10 in a rat and human study [5].

In the inner ear, there are many factors that cause permanent disability such as inflammatory disease and acoustic injury. Coenzyme Q10 constitutes a useful treatment for the inner ear.

Conclusion

In conclusion, this study has shown that administration of coenzyme Q10 may prevent noise-induced hearing loss by protecting the cochlea from oxidative stress.

References

[1] Yamane H, Nakai Y, Takayama M, Iguchi H, Nakagawa T, Kojima A. Appearance of free radicals in the guinea pig inner ear after noise-induced acoustic trauma. Eur Arch Otorhinolaryngol 1995;252:504–8.

[2] Yamasoba T, Schacht J, Shoji F, Miller JM. Attenuation of cochlear damage from noise trauma by an iron chelator, a

free radical scavenger and glial cell line-derived neurotrophic factor in vivo. Brain Res 1999;815:317–25.
[3] Takemoto T, Sugahara K, Okuda T, Shimogori H, Yamashita H. The clinical free radical scavenger, edaravone, protects cochlear hair cells from acoustic trauma. Eur J Pharmacol 2004;487:113–6.
[4] Turunen M, Olsson J, Dallner G. Metabolism and function of coenzyme Q. Biochim Biophys Acta 2004;1660:171–99.
[5] Nukui K, Yamagishi T, Miyawaki H, Kettawan A, Okamoto T, Sato K. Comparison of uptake between PureSorb-Q40 and regular hydrophobic coenzyme Q10 in rats and humans after single oral intake. J Nutr Sci Vitaminol 2007;53:187–90.
[6] Bonakdar RA, Guarneri E. Coenzyme Q10. Am Fam Physician 2005;72:1065–70.
[7] Noda Y, Anzai K, Mori A, Kohno M, Shinmei M, Packer L. Hydroxyl and superoxide anion radical scavenging activities of natural source antioxidants using the computerized JES-FR30 ESR spectrometer system. Biochem Mol Biol Int 1997;42:35–44.
[8] Kasparová S, Sumbalová Z, Bystrický P, Kucharská J, Liptaj T, Mlynárik V, et al. Effect of coenzyme Q10 and vitamin E on brain energy metabolism in the animal model of Huntington's disease. Neurochem Int 2005;48:93–9.
[9] Kopke R, Allen KA, Henderson D, Hoffer M, Frenz D, Van de Water T. A radical demise. Toxins and trauma share common pathways in hair cell death. Ann N Y Acad Sci 1999;884:171–91.
[10] Pirvola U, Xing-Qun L, Virkkala J, Saarma M, Murakata C, Camoratto AM, et al. Rescue of hearing, auditory hair cells, and neurons by CEP-1347/KT7515, an inhibitor of c-Jun N-terminal kinase activation. J Neurosci 2000;20:43–50.
[11] Ylikoski J, Xing-Qun L, Virkkala J, Pirvola U. Blockade of c-Jun N-terminal kinase pathway attenuates gentamicin-induced cochlear and vestibular hair cell death. Hear Res 2002;163:71–81.
[12] Forsmark P, Aberg F, Norling B, Nordenbrand K, Dallner G, Ernster L. Inhibition of lipid peroxidation by ubiquinol in submitochondrial particles in the absence of vitamin E. FEBS Lett 1991;285:39–43.
[13] Lass A, Sohal RS. Electron transport-linked ubiquinone-dependent recycling of alpha-tocopherol inhibits autooxidation of mitochondrial membranes. Arch Biochem Biophys 1998;352:229–36.
[14] Forsmark-Andree P, Dallner G, Ernster L. Endogenous ubiquinol prevents protein modification accompanying lipid peroxidation in beef heart submitochondrial particles. Free Radic Biol Med 1995;19:749–57.
[15] Ernster L, Dallner G. Biochemical, physiological and medical aspects of ubiquinone function. Biochim Biophys Acta 1995;1271:195–204.
[16] Tomasetti M, Littarru GP, Stocker R, Alleva R. Coenzyme Q10 enrichment decreases oxidative DNA damage in human lymphocytes. Free Radic Biol Med 1999;27:1027–32.
[17] Tanaka K, Takemoto T, Sugahara K, Okuda T, Mikuriya T, Takeno K, et al. Post-exposure administration of edaravone attenuates noise-induced hearing loss. Eur J Pharmacol 2005;522:116–21.
[18] Tanabe K, Masuda K, Hirayama A, Nagase S, Kono I, Kuno S. Effect of spontaneous exercise on antioxidant capacity in rat muscles determined by electron spin resonance. Acta Physiol (Oxf) 2006;186:119–25.
[19] Kaneyuki T, Noda Y, Traber MG, Mori A, Packer L. Superoxide anion and hydroxyl radical scavenging activities of vegetable extracts measured using electron spin resonance. Biochem Mol Biol Int 1999;47:979–89.
[20] Sergi B, Fetoni AR, Paludetti G, Ferraresi A, Navarra P, Mordente A, et al. Protective properties of idebenone in noise-induced hearing loss in the guinea pig. Neuroreport 2006;17:857–61.

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Research Report

Attenuation of progressive hearing loss in a model of age-related hearing loss by a heat shock protein inducer, geranylgeranylacetone

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ABSTRACT

Mechanisms of age-related hearing loss (ARHL) have not been elucidated as aging processes are extremely complex. Although oxidative stress and apoptotic cell death are involved in progression of ARHL, number of trial to treat ARHL is limited. Heat shock response is characterized by induction of heat shock proteins (HSPs) in response to stresses such as heat shock, which diminishes during aging. HSPs act as molecular chaperones, and some HSPs also inhibit apoptotic pathways. Here, we examined age-related expression of HSPs in the cochlea of ARHL model DBA/2J mice and control CBA/N mice. Western blot assay revealed that CBA/N mice showed constant expression of Hsp70 and Hsp110 with age, but not in DBA/2J mice. The result suggests that pharmacological upregulation of HSPs might attenuate ARHL. We administered DBA/2J mice with food containing geranylgeranylacetone (GGA) that induces HSPs in the cochlea, and found that its administration suppresses ARHL examined by ABR test and histological examination though protection is specific for the apical part of the cochlea. These results demonstrate that dietary supplementation of GGA could be an effective therapeutic strategy for treatment of ARHL.

1. Introduction

Age-related hearing loss (ARHL) known as "presbycusis" is a major pathogeny of acquired progressive hearing loss. It is reported that about one third of people aged older than 65 years may be affected by ARHL (Yamasoba et al., 2007). Although the mechanism of ARHL has not been fully understood since aging processes are extremely complex, oxidative stress (Seidman et al., 2002) and increase of apoptosis (Someya et al., 2006) seem to contribute to age-related cochlea pathology. There are several reports in which ARHL was treated by administration of antioxidants (Seidman, 2000) and caloric restriction (Someya et al., 2006), but the total number of trials to treat ARHL is limited.

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Abbreviations: ABR, auditory brainstem response; ARHL, age-related hearing loss; Cdh23, cadherin 23; GGA, geranylgeranylacetone; HSF1, heat shock transcriptional factor 1; HSPs, heat shock proteins; IHC, inner hair cell; JNK, Junuas N-terminal kinase; OHC, outer hair cell; PBS, phosphate-buffered saline; SGCs, spiral ganglion cells; SV, stria vascularis

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Various inbred mouse strains have been investigated to elucidate mechanisms of ARHL. Among 80 inbred mouse strains, 18 strains exhibit early onset hearing loss before 3 months of age, and 16 strains exhibit hearing loss at older ages (Zheng et al., 1999). Interestingly, at least 10 inbred strains including DBA/2J have the ahl allele that contributes to ARHL (Johnson et al., 1997, 2000). These mice are homozygous for the defective ahl allele of the gene encoding cadherin 23 (Cdh23), which is hair cell-specific cadherin and is suggested to regulate the activity of mechanically-gated ion channels in hair cells (Zheng and Johnson, 2001; Siemens, et al., 2004). In strains harboring this allele, cell death of hair cell is observed at different time points during aging. Cdh^{ahl} allele is, however, not the only pathogenesis of the ARHL model (Johnson and Zheng, 2002; Nemoto et al., 2004). DBA/2J was reported to have other loci contributing to early onset ARHL (Willott and Erway, 1998; Ohlemiller, 2006). Although mechanisms are unclear, DBA/2J is a useful model for age-related progressive hair cell death and hearing loss.

Several studies demonstrated that heat shock response, which heat shock transcription factor 1 (HSF1) is activated and induces a set of heat shock proteins (HSPs), diminishes during aging (Fawcett et al., 1994; Morley and Morimoto, 2004) and chaperone activity is reduced in age-associated disorders

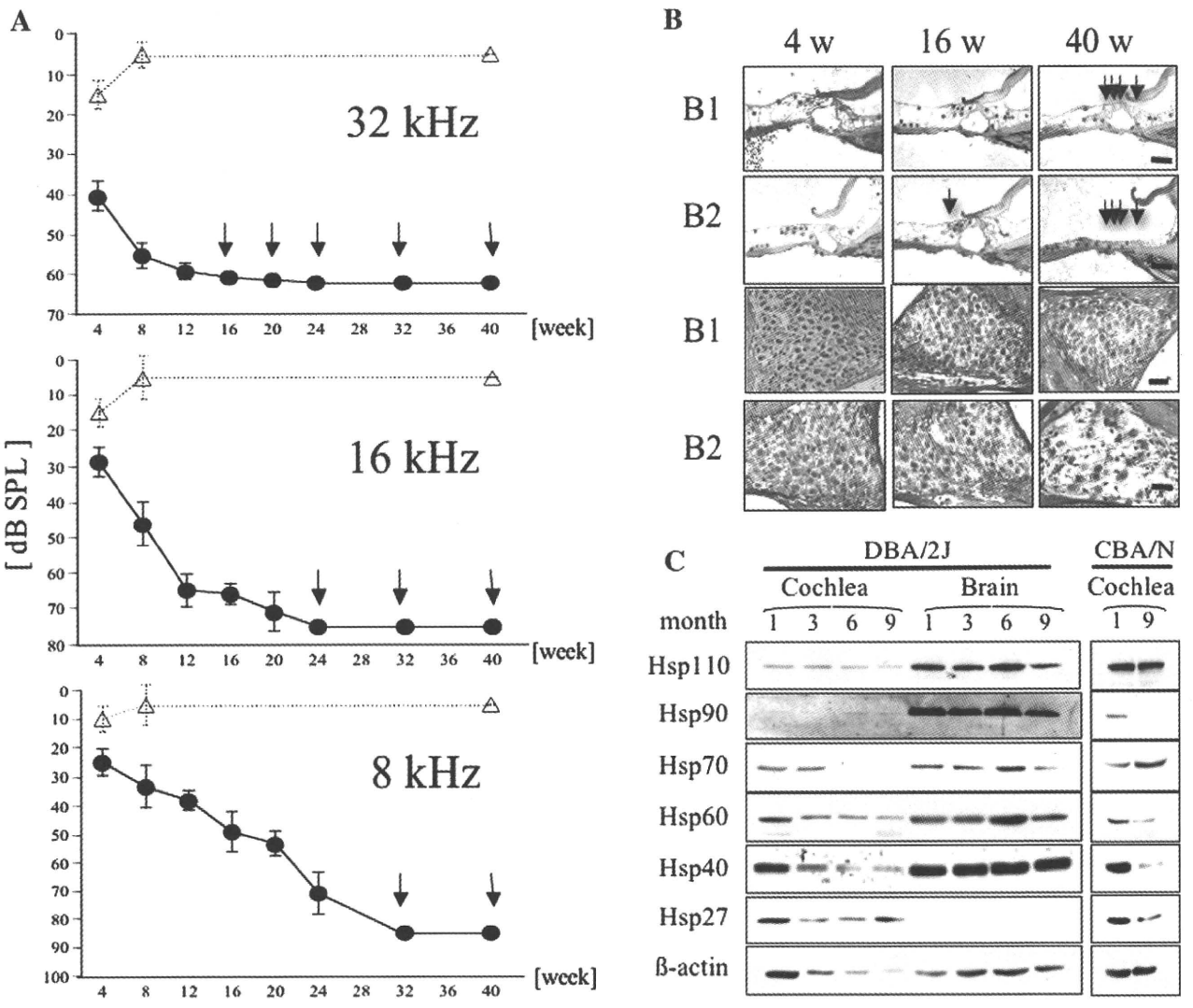

Fig. 1 – Comparison of ABR thresholds and HSPs expression between two strains during aging. (A) Change of ABR thresholds during aging. The ABR test was analyzed from 4 to 40 weeks weeks of age in CBA/N (open triangles) and DBA/2J (closed circle) at three frequencies (32, 16 and 8 kHz). CBA/N were tested at 4, 8 and 40 weeks ($n=3$, respectively) and DBA/2J were tested every 4 weeks except for 28 and 36 weeks ($n=7$, respectively). Arrows show insensitive to stimuli. Error bar: ±1 SEM. (B) The progressive defects of hair cells and SGCs in DBA/2J H&E stain of the cochlea in DBA/2J at 4, 16 and 40 weeks ($n=4$, respectively). Corti's organs and spiral ganglion were stained with H&E and displayed in gray scale. B1: lower apex. B2: lower base. Arrows show the defect of hair cells. Bars = 20 μm. (C) Western blot analysis of HSPs. Pairs of cochlea and brain of two strains at 1, 3, 6 and 9 months of age were used for extracts ($n=4$, respectively). Equal amounts of proteins were loaded onto each lane (80 μg).