(Soti and Csermely, 2002). When proteins are damaged due to various stresses, HSPs undergo cytoprotective function by acting as molecular chaperones that stabilize denatured proteins and facilitate their refolding and degradation (Lindquist, 1986; Morimoto, 1998). HSF1 and HSPs exist in the inner ear (Neely et al., 1991; Gower and Thompson, 1997, Fairfield et al., 2002) and play a protective role against cochlear damage induced by sound exposure, oto-toxic drugs and heat stress (Yoshida et al., 1999; Oh et al., 2000; Sugahara et al., 2003; Fairfield et al., 2005). There, however, is no report which indicates the correlation ARHL with HSPs.

Geranylgeranylacetone (GGA), an acyclic polyisoprenoid, has been used clinically as an oral anti-ulcer drug since 1984. GGA is easy to cross the blood–brain barrier because GGA is the lipid-soluble reagent (announced by interview form Eisai Co., Ltd.). GGA enhances activation of HSF1 and induces HSPs and shows protective effects in many organs without any histotoxic effect (Hirakawa et al., 1996; Kikuchi et al., 2002; Hirota et al., 2000; Fujiki et al., 2003; Ishii et al., 2003; Mikuriya et al., 2005; Otaka et al., 2007). Here, we demonstrate that HSPs expression is altered during aging and administration of GGA suppresses ARHL and hair cell loss in DBA/2J.

2. Results

2.1. *Comparison of hearing ability and HSPs expression in the cochlea of aged DBA/2J and CBA/N mice*

We examined ABR, and found that CBA/N mice did not show any hearing loss at all frequencies until 40 weeks whereas DBA/2J mice showed severe hearing loss even at 4 weeks (Fig. 1A). DBA/2J mice became completely insensitive to 32 kHz at 16 weeks, to 16 kHz at 24 weeks, and to 8 kHz at 32 weeks. Coronal sections of the cochlea in DBA/2J mice showed apparent progressive loss of cell in Corti's organ and spiral ganglion cells (SGCs) (Fig. 1B) as previously reported (Ohlemiller and Gagnon, 2004). We examined expression of HSPs in the cochlea because attenuation of expression of HSPs is known to be related to accumulation of damaged proteins, and to increased cell death during aging (Verbeke et al., 2001). In the cochlea of DBA/2J mice, all expressions of HSPs except for Hsp27 seemed to be reduced during aging (Fig. 1C). Expression of Hsp90, Hsp60, Hsp40, and Hsp27 also decreased in the cochlea of 9 month old CBA/N mice.

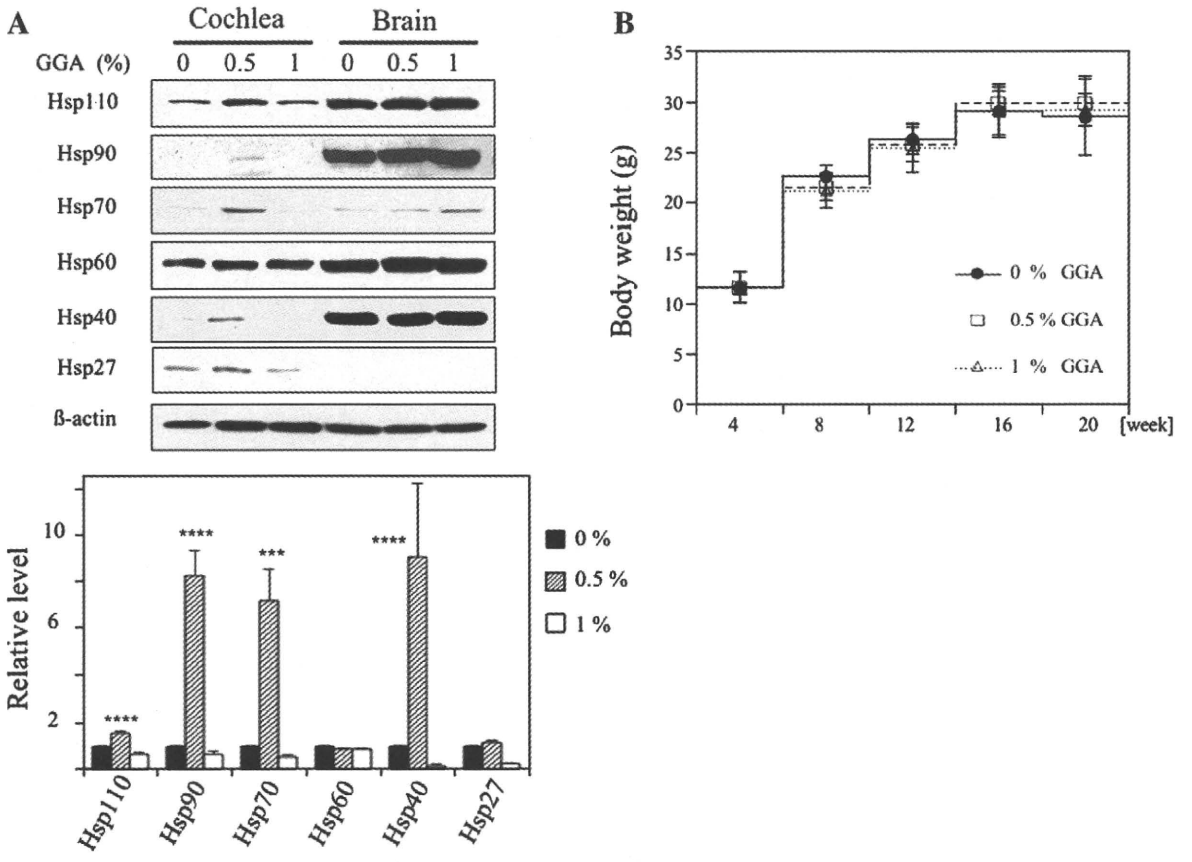

Fig. 2 – Effect of GGA on expression of HSPs. (A) Western blot analysis of HSPs in DBA/2J treated with GGA food at three concentrations (0, 0.5 and 1%) for 8 weeks. Pairs of cochlea and brain were obtained at 12 weeks of ages ($n=4$, respectively). The bands were quantified and the values were normalized to that of β-actin in the same sample. The values shown in the graphs were adjusted so that the expression in 0% would be 1.0. ***$P<0.001$, ****$P<0.0001$, Error bar: ±1 SEM. (B) Body weight of animals treated with GGA at concentrations of 0% (closed circles), 0.5% (open boxes) and 1% (open triangles) ($n=6$, respectively). Error bars: ± SD.

Interestingly, Hsp70 expression increased and Hsp110 expression was constant in the cochlea of 9 month-old CBA/N mice.

2.2. *Effect of GGA on expression of HSPs in the cochlea*

We found that Hsp110, Hsp90, Hsp70 and Hsp40 was highly expressed when mice were administered with 0.5% GGA food (Fig. 2A) whereas we did not find any induction by 1% GGA. GGA food did not show any effect on body weight (Fig. 2B). We next examined localization of major molecular chaperone Hsp70 which is induced in old CBA/N and plays critical role in age-related neurodegenerative diseases (Soti and Csermely, 2002) by using immunohistochemical analysis. We found that Hsp70 was highly expressed in both the outer hair cells (OHCs) and inner hair cells (IHCs) after GGA administration (Figs. 3, A–F). In control SGCs, Hsp70 was expressed and localized in both the cytoplasm and cytoplasmic foci (Figs. 3G, I and K), and was induced after GGA administration (Figs. 3, M, O and Q). Hsp70 was also expressed in the stria vascularis (SV) (Figs. 3, H, J, L, N, P and R).

2.3. *Effect of GGA on the ABR thresholds and the survival of IHC and OHC*

We examined auditory brain stem response (ABR) of DBA/2J mice administered with 0%, 0.5% or 1.0% GGA food. We found that administration of 0.5% GGA food inhibits hearing loss until 8 weeks at 32 kHz, at 12 weeks at 16 kHz, and until 20 weeks at 8 kHz (Fig. 4). Interestingly, administration of 1.0% GGA food, which expresses less HSPs, has significant inhibitory effect at 32 kHz in 8 weeks and 8 kHz in 8 and 12 weeks.

We then performed histological examination of the cochlea of 20 week-old DBA/2J mice, and found that administration of 0.5% or 1% GGA food suppressed cell loss of OHC and IHC in both upper and lower apex that they correspond to≈1–15 kHz on the frequency map (Viberg and Canlon, 2004) (Figs. 5A, 5B and Table1). Consistent with the results from ABR, hair cell loss in the upper apex was much inhibited in mice administered with 0.5% GGA food more than that in mice administered with 1.0% GGA food. However inhibition of hair cell loss was not observed in the upper and lower base that they correspond to >15 kHz.

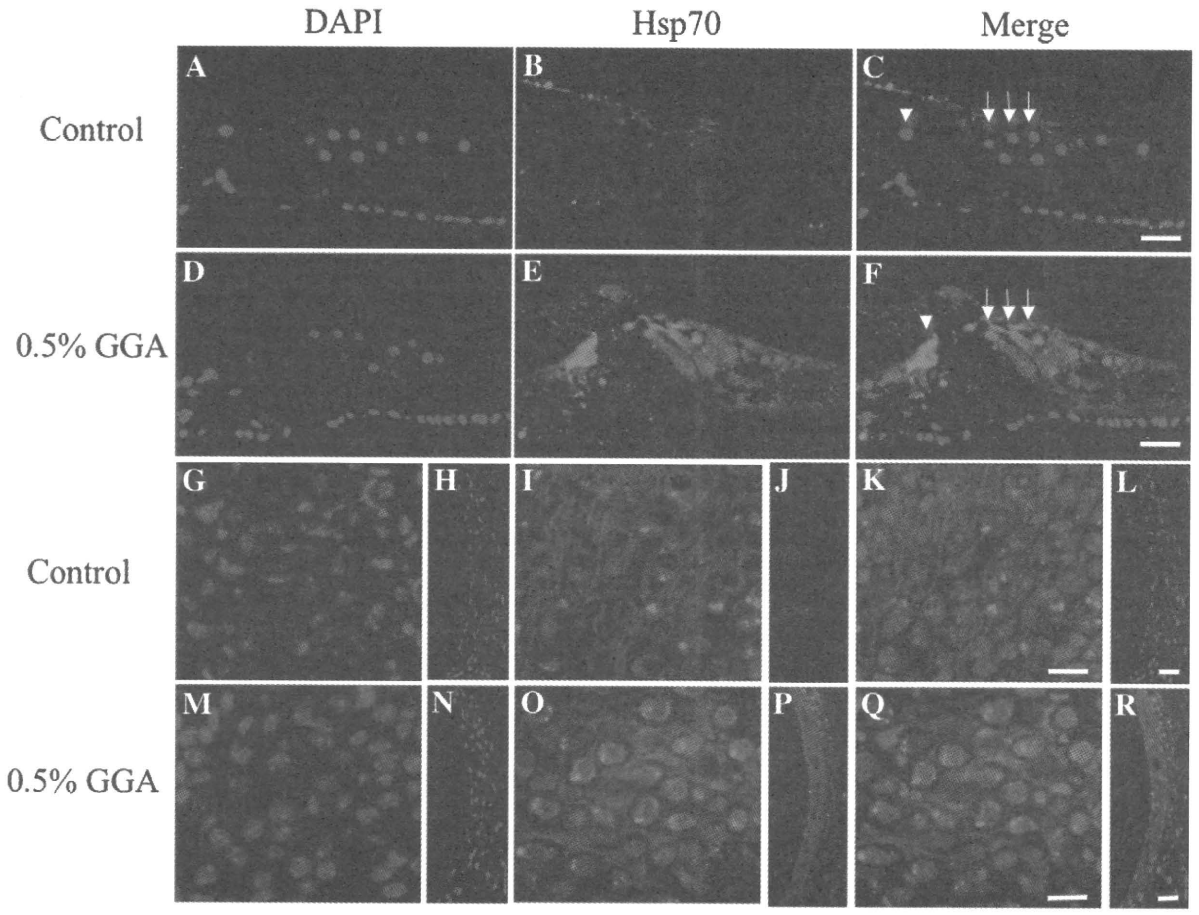

Fig. 3 – Localization of Hsp70 upregulated by GGA. Immunostaining with antibody against Hsp70 (red channel) and counter-staining with DAPI (blue channel) in the coronal section at 8 weeks of ages in the cochlea of DBA/2J mice given 0% or 0.5% GGA for 4 weeks ($n=4$, respectively). These figures observed in the turn at lower apex. A–F show the region of Corti's organ, G, I, K, M, O and Q show SGCs and H, J, L, N, P and R show stria vascularis. Arrows show OHCs. Arrowheads show IHCs. Bars=20 μm.

3. Discussion

Expression of Hsp70 and Hsp110 in the whole cochlea of 9 month-old CBA/N mice did not decrease whereas it decreased in that of 9 month-old DBA/2J mice. Thus, high expression of Hsp70 and Hsp110 in the cochlea might be important for maintain hearing ability. Hsp70 is a major molecular chaperone that facilitates protein folding and prevents misfolding (Parsell and Lindquist, 1993), and plays critical roles in age-related neurodegenerative diseases such as Alzheimer disease (Soti and Csermely, 2002). Decreased expression of Hsp70 in the cochlea might lead to accumulation of misfolded protein during aging. Hsp110 also prevents protein misfolding of proteins as it interacts with Hsp70 (Easton et al., 2000). Therefore, we thought that pharmacological upregulation of Hsp70 and Hsp110 might attenuate age-related vulnerability in DBA/2J mice. β-actin was also decreased with aging though equal amount of protein was loaded to all lanes (Fig. 1C), which was considered to be due to the change in the proportion of component cells of cochlea with aging (Fig. 1B). We should have used alternative control antibody.

We previously showed that oral administration of GGA induces major HSPs in the cochlea of guinea pig (Mikuriya et al., 2005). Here we showed that administration of GGA food upregulates HSPs in the cochlea of mice, and that it suppresses hearing loss and hair cell death in one of ARHL models. It is known that GGA enhances activation of HSF1 (Hirakawa et al., 1996; Otaka et al., 2007). HSF1 exists as an inactive form in the rodent cochlea in non-stress condition and is activated by heat stress (Fairfield et al., 2002). Therefore we considered that GGA could induce HSPs in the cochlea. Probably, increased levels of HSPs may inhibit age-related accumulation of misfolded proteins. It is also possible that HSPs might prevent apoptotic cell death of hair cells since Hsp90, Hsp70 and Hsp27 inhibit apoptotic cell death by binding directly to proapoptotic molecules such as januas N-terminal kinase (JNK), protease activating factor-1 (Apaf-1), and cytochrome c (Mosser et al., 1997; Beere et al., 2000; Saleh et al., 2000; Bruey et al., 2000).

Interestingly, we showed that administration of 0.5% GGA food upregulated HSPs whereas that of 1.0% GGA food did less HSPs or tend to decline them (Fig. 2A). However administration of 1.0% GGA food suppresses hearing loss with the significant difference at 32 kHz in 8 weeks and 8 kHz in 8 and 12 weeks and attenuates hair cell losses in apex turn (Figs. 4,5). These results indicate that effects of GGA are mediated through HSP-dependent and HSP-independent pathways. After 12 weeks of ages, HSP-dependent protection is major factor because HSPs were upregulated only by 0.5%

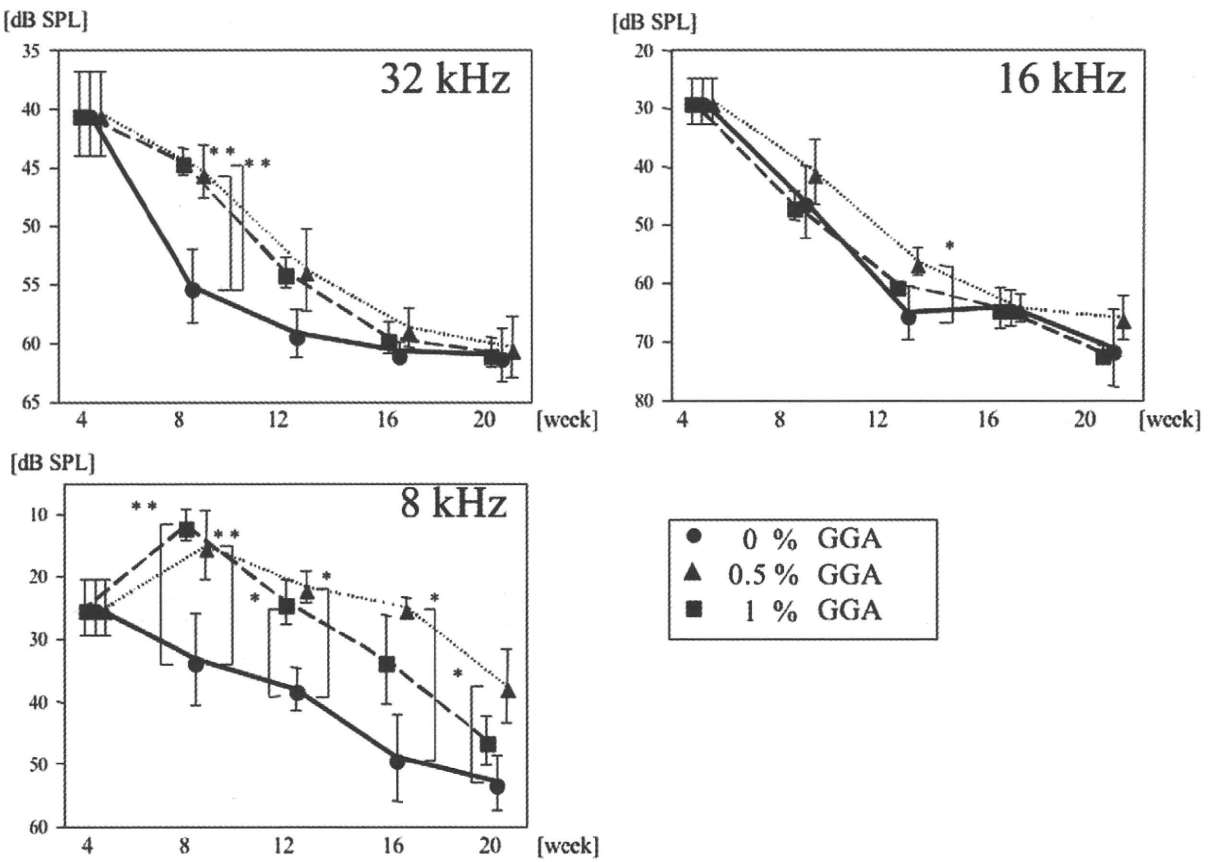

Fig. 4 – Protective effect of GGA against ARHL in DBA/2J mice. ABR test was analyzed from 4 to 20 weeks of age at three frequencies (32, 16, and 8 kHz). Mice were given GGA foods from 4 to 20 weeks of ages. 0% GGA (closed circles), 0.5% GGA (closed triangles) 1% GGA (closed boxes) ($n=6$, respectively). *$P<0.05$, **$P<0.01$, Error bar: ±1SEM.

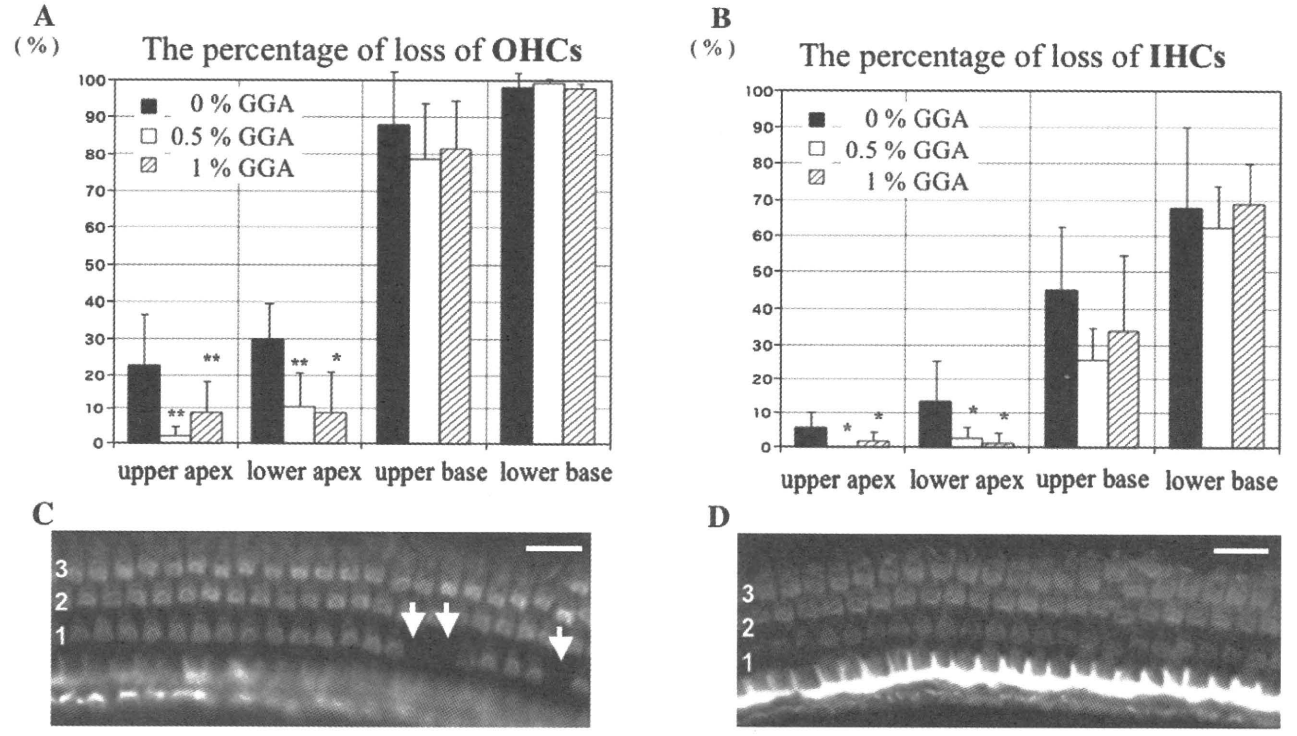

Fig. 5 – Protective effect of GGA on cochlear hair cells. After the final ABR test at 20 weeks of age, we calculated the percentages of the missing hair cells ($n=6$, respectively). The percentages are shown divided into 4 parts; upper and lower apex, upper and lower base. The loss of OHCs and the loss of IHCs are shown (Figs. 5A and B). Asterisks show significant difference against 0% GGA as a control. *$P<0.05$, **$P<0.01$, Error bar: ±1SEM. The pictures of surface preparation are shown in Figs. 5C and B. There are three rows of OHCs (numbers 1–3) and arrows show the deficits of OHCs. (C) The structure which obtained from mice treated by 0% GGA (D) The structure which obtained from mice treated by 0.5% GGA Bars=20 μm.

GGA though we evaluated them only at 12 weeks of ages (Fig. 2A). Before 12 weeks of ages, both HSP-dependent and HSP-independent pathways may work together. It is reported that GGA has a cytoprotective role by elevating prostaglandin E2 (Kurihara et al., 1996), inducing neuronal nitoric oxide synthase (nNOS) (Rokutan et al., 2000), inducing thioredoxin (Hirota et al., 2000) and by affecting mitochondrial function (Aron et al., 2001). One of theories of aging is that oxidative stress generated inside mitochondria damage key mitochondrial components (Balaban et al., 2005). Such damage accumulates with time, leads to mitochondrial dysfunction. Therefore the effect of GGA on thioredoxin and mitochondria may be important as HSP-independent pathways. Although caloric restriction that postulated to reduced oxidative stress suppress apoptosis in cochlear cell and prevent ARHL (Someya et al., 2006), we excluded a possibility of weight loss in mice administered with GGA food (Fig. 2B).

There are other discrepancies in ABR thresholds and hair cell count. We consider that hair cells at 20 weeks of ages given 1% GGA may not have cellular function whereas cell death is prolonged by the effect via HSP-independent pathways example for thioredoxin and mitochondria as described above. If we observed hair cells at older age, we might show that progression of hair cells death which has no function become

Table 1 – Average value for the loss of OHCs and IHCs

	OHC	0% GGA	0.5% GGA	1% GGA	0% GGA SEM.	0.5% GGA SEM.	1% GGA SEM.
A	Upper apex	22.87	2.324	8.89	±13.63	±2.062	±8.89
	Lower apex	30.06	10.51	8.755	±9.319	±9.902	±11.92
	Upper base	87.92	78.8	81.52	±14.35	±14.89	±12.94
	Lower base	98.33	99.52	97.91	±3.83	±0.65	±1.22
	IHC						
B	Upper apex	5.662	0	1.925	±4.237	±0	±2.226
	Lower apex	13.47	2.63	1.25	±11.82	±2.962	±2.5
	Upper base	45.03	26	34.1	±17.19	±8.768	±20.24
	Lower base	67.86	62.39	69.03	±22.24	±11.24	±10.64

clearer in 1% GGA group. We could not success to find protective effect at higher frequencies during this study. DBA/2J mice show severe early onset hearing loss and hair cell loss at all frequency and these events initial at higher frequency as same as common ARHL. Because of the severe damage at higher frequency during the present study, we considered that protection by GGA at 32 and 16 kHz during study is partial whereas it is continuous at 8 kHz. In addition, results about ABR and hair cell count may indicate complex action by GGA since the mechanism of cochlear cell death in DBA/2J has not been elucidated yet. Late-onset or moderate ARHL model may show the effect of GGA more clearly.

1% GGA food induced Hsp70 expression in the brain whereas it did not induce major HSPs in the cochlea. On higher dose condition, similar result is reported previously, but the reason is unclear yet. They have also reported that administration of higher dose of GGA (>1000 mg/kg, which corresponding to >1% concentration of GGA food) showed hepatic toxicity in mice (Katsuno et al., 2005). But our results suggest that an optimal dose may be low enough to attenuate ARHL. As GGA has several beneficial effects as described above, administration of GGA would be a new therapeutic approach for age-related progressive cochlear diseases.

4. Experimental procedures

4.1. *Animals*

Fifty-seven male DBA/2J mice (aged from 4 to 40 weeks) and seventeen male CBA/N mice (aged from 4 to 40 weeks) were used in this study. DBA/2J mice were the offspring of parents purchased from Jackson Lab (Jackson Lab, Bar Harbor, ME). Mice were housed in cages in a room maintained at 24 °C on a 12-h light/dark cycle without noise. Both DBA/2J mice and CBA/N mice which were used in Fig. 1 were given foods as pellet chow whereas mice used in Figs 2–5 were done as powdered rodent chow. All experimental protocols were reviewed by the Committee for Ethics on Animal Experiments of Yamaguchi University Graduated School of Medicine. All experiments were carried out in accordance with these guidelines and the Japanese Federal Law No. 105 and Notification No 6.

4.2. *GGA food*

GGA granules (as Selbex®; Eisai, Tokyo, Japan) were mixed with powdered rodent chow at concentrations of 0.5 and 1%, which corresponding to 400–600 mg/kg/day and 900–1000 mg/kg/day during the study, respectively (Katsuno et al., 2005). GGA food was administered to animals from 4 weeks of age until the end of the study.

4.3. *Western blot analysis*

The method of western blotting using whole cochlea was previously described (Mikuriya et al., 2005). In brief, mice were immediately decapitated under deep anesthesia by inhalation of diethyl ether. Cochleae were immediately dissected and frozen in liquid nitrogen (both cochleae from one mouse). The tissue specimens were homogenized in NP-40 lysis buffer (150 mM NaCl, 1% Nonidet-P 40, 50 mM Tris–HCl, pH 8.0, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, 1 μg/ml pepstatin). Equal amounts of protein were subjected to SDS-PAGE, and then were transferred onto nitrocellulose membranes. The membranes were blotted with antiserum against mouse Hsp110 (α-Hsp110b), Hsp60 (αmHsp60-1), human Hsp40 (hHsp40a) (Fujimoto et al., 2004), human HSP90 (α-Hsp90d) (Katsuki et al., 2004), mouse Hsp-27c (Takaki et al., 2007), Hsp70 (W27, Santa Cruz Biotechnology) and β-actin (AC-15, Sigma, St. Louis, MO, US). Horseradish peroxidase-conjugated goat antibody against rabbit and mouse IgG was used as a second antibody. The signals were visualized using an enhanced chemiluminescence system (Amersham Pharmacia, NJ, USA). To compare expression of HSPs between two strains during aging, a pair of cochleae and brain were obtained at 1, 3, 6 and 9 months of age (n=4, respectively). To investigate the effect of GGA on expression of HSPs, a pair of cochlea and brain of DBA/2J given GGA food at each concentration were obtained at 12 weeks (n=4, respectively). Brain tissue was used as a control because it includes many neurons and dietary GGA can induce HSPs in brain (Katsuno et al., 2005). Western blot assay were done three times with same samples and quantified by Image J version 1.38× software (National institute of health, USA). Then the values were normalized to that of β-actin in the same sample.

4.4. *Auditory function analysis*

We used auditory brain stem response (ABR) for hearing assessment. The ABR thresholds of all animals were assessed under pentobarbital anesthesia (25 mg/kg, intraperitoneal). The 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 32, 16 and 8 kHz tone bursts (rise–fall time 2 ms, duration 4 ms). The stimuli were presented through a 5-cm tube connecting an earphone to the external auditory canal. The stimulus intensity was evaluated with a sound-level meter (NA-60; Rion) adjacent to the tip of the tube. The responses to the 500 stimuli were recorded using PowerLab systems (AD Instruments, NSW, Australia). The ABR thresholds were defined as the lowest stimulus intensity that produced a reliable peak 3 or 5 in the ABR waveforms. To compare the change of hearing during aging, CBA/N mice were tested at 4, 8 and 40 weeks (n=3, respectively) and DBA/2J mice were tested every 4 weeks from 4 to 40 weeks except for 28 and 36 weeks (n=7). To investigate the effect of GGA on ARHL, DBA/2J mice given GGA food at each concentrations were assessed from 4 to 20 weeks (n=6, respectively).

4.5. *Histopathology and immunohistochemistry*

Mice were anesthetized and killed under deep anesthesia with inhalation of diethyl ether and perfused transcardially with fixative (4% paraformaldehyde, PFA, in 0.1 M phosphate buffer, pH 7.3). The temporal bones of all animals were removed and each cochlea was opened at the apex, base, and oval window. After the fixative was gently perfused from base to apex with a micropipette, cochleae were soaked with fixative for 6 h. After rinsing in phosphate-buffered saline (PBS), the specimens

were decalcified in 10% EDTA (pH 7.2) for 3 days. For histopathology, specimens were embedded in a semi-water-soluble resin (Immuno-Bed; Polysciences, Washington, DC, USA) and blocks were cut into 3 μm thick sections, following staining with hematoxylin and eosin. To examine histopathology in DBA/2J, mice were killed at 4, 16, 40 weeks of age (n=4, respectively). For immunochemistry, non-specific binging sites were blocked with blocking solution (1% bovine serum albumin, 0.4% normal goat serum, 0.4% normal horse serum, 0.4% Triton X-100 in PBS) for 12 h at 4 °C and incubated in a 1:100 dilution of antibody against HSP70 (Kawazoe et al., 1999) for 12 h at 4 °C. After rinsing in blocking solution and incubation in a 1:100 dilution of Alexa Fluor® 594-conjugated goat anti-rabbit IgG (Molecular Probes) for 12 h at 4 °C, the sections were then washed in blocking solution and embedded in a semi-water-soluble resin and cut into 3-μm thick sections. Sections were counterstained with 4′, 6-diamino-2-phenylindole (DAPI) (Vectashield; Vector Laboratories, Burlingame, CA, USA). All slides in this study were visualized using Axioplan 2 microscope and an AxioCam HR CCD with Axiovision 3.1 software (Carl Zeiss, Canada). To examine the localization of induced HSP70, the differences between 0% and 0.5% GGA was observed at the turn at lower apex where there are a fewer loss of cells at 8 weeks (n=4, respectively) because DBA/2J shows premature cell loss in hair cell and spiral ganglion and differences of the number of component cells may directly influence the immunofluorescent intensity.

4.6. *Hair cell count*

The method of hair cell count with mouse cochlea was previously reported (Sugahara et al., 2003). Briefly, after the final ABR test, cochleae of each group were dissected under deep anesthesia with inhalation of diethyl ether (n=6, respectively). After being fixed in PFA and rinsed with PBS, cochleae were permeabilized with 0.3% Triton X-100 for 10 min and were subsequently incubated with fluorescein isothiocyanate conjugated phalloidin (1:50 dilution; Sigma, St. Louis, MO, USA) at room temperature for 1 h. After rinsing in PBS, cochleae were dissected into 4 half-turn pieces (Viberg and Canlon, 2004). The modioluses were removed as much as possible and put onto slides with several sections well separated, and mounted with Vectashield®. After digital images were taken with a microscope, the lengths of basal membrane were measured three times, and hair cells including all three rows of OHC and IHC were counted for each 0.25 mm from the apex. These data were converted into a cochlea frequency map and plotted to a graph. Finally, the percentages of the missing hair cells were shown as upper apex, lower apex, upper base, lower base, which corresponded to successive 20% (≈0–1 mm), 30% (≈1–3 mm), 30% (≈3–4.5 mm), and 20% (≈4.5–6 mm) from the apex (Viberg and Canlon, 2004; Lang et al., 2006).

4.7. *Statistical analysis*

Data regarding ABR thresholds, the percentage of missing hair cells, and relative level was expressed as means±SEM. Data about the change of weight was expressed as mean±SD. The differences among these parameters were analyzed using a StatView version 4.5 J software package for Macintosh (Abacus Concepts, Berkley, CA, USA). The data in Figs. 2, 4, 5 was assayed using Dunnett's post hoc test for multiple comparisons. A value of $P<0.05$ was accepted as statistically significant.

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Unilateral intra-perilymphatic infusion of substance P enhances ipsilateral vestibulo-ocular reflex gains in the sinusoidal rotation test

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ABSTRACT

Previous studies have reported localization of substance P (SP) within the inner ear and that SP exists abundantly within vestibular endorgans. While SP's functional role in the inner ear remains unclear, SP can act as a neuromodulator in the CNS and directly influences neuronal excitability. We hypothesized that SP might influence neuronal excitability within the vestibular periphery. The present study used the sinusoidal rotation test to investigate the influence of SP after its local application in the guinea pig unilateral inner ear. A tiny hole was made adjacent to the round window in the right ears of Hartley white guinea pigs that had normal tympanic membranes and Preyer reflexes. An osmotic pump infused SP (10^{-4} M, 10^{-3} M, and 10^{-2} M), neurokinin-1 (NK-1) receptor antagonist (10^{-3} M) alone, or SP (10^{-3} M) + NK-1 receptor antagonist (10^{-3} M) through this hole, with rotation tests performed before, and 12 h and 24 h after the treatment. Results were used to calculate the vestibulo-ocular reflex (VOR) gains. After administration of 10^{-3} M and 10^{-2} M SP, significant increases in the VOR gains were noted at 12 h after treatment, with these gains disappearing by 24 h after treatment. This increase was not observed when there was simultaneous NK-1 receptor antagonist administration. There were also no changes in the VOR gains noted after administration of 10^{-4} M SP or the NK-1 receptor antagonist alone. These results indicate the possibility that SP may act on vestibular endorgans as an excitatory factor via the NK-1 receptors.

Patients with peripheral vestibular disorder sometimes suffer from prolonged unsteadiness when they shake their heads, walk in the dark, or ride on a bicycle. During the aging process, proprioceptive and visual information decreases and vestibular compensation does not work well, which can lead to increases in unsteadiness in the elderly. By 2050, every third person in Japan will be classified as elderly. Therefore, it is essential to find a way to aid in the functional recovery of the vestibular periphery.

Recently, we investigated substance P (SP), an undecapeptide belonging to a class of neuropeptides called tachykinins [8]. Starting in the 1980s, and continuing through the 1990s, many studies reported information on the existence of abundant amounts of SP in the vestibular endorgans [3,5,13,15,21,22,24,28,31,32]. However, the functional role of SP in the vestibular periphery is still unknown. SP often acts as a neuromodulator in the CNS [19,26], can directly influence the neuronal excitability [1,25], and has also been shown to have an effect on synaptic plasticity [17]. Furthermore, SP has neurotrophic and neuroprotective actions [10,29]. We hypothesized that SP might play an important role in the vestibular periphery, and therefore, might be useful in the functional recovery of the vestibular periphery. The aim of the present study was to investigate the influence of locally applied SP in the unilateral inner ear of guinea pigs.

This study used 26 Hartley white guinea pigs with normal tympanic membranes and normal Preyer reflexes. The study protocol was reviewed by the Yamaguchi University School of Medicine's Committee for Ethics in Animal Experiments and was carried out in accordance with the Guidelines for Animal Experiments of the Yamaguchi University School of Medicine and Law No. 105 and Notification No. 6 of the Japanese government.

The five experimental groups included: 10^{-4} M SP administered group ($n=4$), 10^{-3} M SP administered group ($n=4$), 10^{-2} M SP administered group ($n=6$), 10^{-3} M neurokinin-1 (NK-1) receptor antagonist ([D-Pro^2, D-Trp^{7,9}]-SP) administered group ($n=4$), and the 10^{-3} M SP + 10^{-3} M NK-1 receptor antagonist administered group ($n=8$).

Xylazine (16 mg/kg, i.p.)–ketamine (16 mg/kg, i.p.) anesthesia was induced, followed by injection of 1.5 ml lidocaine HCl into the right postauricular region for the purpose of local anesthesia. Body temperature was maintained at 37 °C. The mastoid bulla was opened by a postauricular incision to allow for visualization of the round window with a surgical microscope. A hole

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was made adjacent to the round window with a perforating burr (Proxxon, 0.5 mm diameter; Kiso Power Tool, Osaka, Japan). A polyethylene catheter (0.2 mm inner diameter, 0.5 mm outer diameter; Natsume Co., Ltd., Tokyo, Japan) filled with artificial perilymph (113.5 mM NaCl, 5.4 mM KCl, 2.0 mM $CaCl_2$, 1.0 mM $MgCl_2$, 10.0 mM glucose, and 10.0 mM *N*-2-hydroxyethylpiperazine *N'*-2-ethanesulfonic acid) was connected to an osmotic pump (Model 2002, Alza Co., Palo Alto, CA) that was filled with drug and then inserted into the hole. The length of the polyethylene catheter was adjusted so that it would deliver the artificial perilymph that was contained in the catheter over a 12-h period. At the end of the 12-h artificial perilymph infusion, the pump then infused the drug. During the surgical procedure, after the pump was placed under the skin on the animal's back, the wound was washed with saline, followed by the introduction of a small amount of piperacillin sodium (PIPC) in order to prevent infection. After closure, PIPC (40 mg/kg) was injected intramuscularly, and oxytetracycline HCl ointment was applied to the wound. During the operation, the animal's body temperature was maintained at 37 °C, and for 24 h following the operation, each animal was kept warm through the use of an electric blanket.

During the experimental procedure, we observed static symptoms, such as spontaneous nystagmus and head deviation. To measure the vestibulo-ocular reflexes (VOR), sinusoidal rotation tests were performed before treatment, and 12 h and 24 h after treatment. The VOR gains were calculated using our previously reported analysis system [27]. For the purpose of immobilizing the guinea pig, a cage designed to hold the animal still during experiments was mounted on top of a turntable apparatus (Daiichi Medical, Tokyo, Japan). The animal's head was fixed firmly with both auricles held between sponge-covered plates that held both of the acoustic meati horizontally so that the midpoint of a straight line joining the lateral semicircular canals was located on the rotation axis of the turntable. We set up an infrared CCD camera (Nagashima Medical, Tokyo, Japan) so that it was perpendicular to the sagittal plane of the guinea pig's head and in a plane that was parallel to the rotational plane of the turntable apparatus. By opening an aperture on the left side of the head cage, we were able to videotape (mini DV format, Canon, Tokyo, Japan) the eye movements of guinea pigs in the dark using the infrared CCD camera. We stored the video images on a computer (Power Mac G4, Apple Computer, CA, USA). Each image was converted to an image file using QuickTime 4.0 (Apple Computer). For the automatic analysis of guinea pig eye movement, we created a macro for use with the National Institutes of Health (NIH) Image analysis software (http://rsb.info.nih.gov/nih-image/). Our macro is available at http://www.cc.yamaguchi-u.ac.jp/~ent/gankyu3d/ikeda.html. After capturing the eye movement on the computer with this macro, we removed any unnecessary portions from the images, and set thresholds to ensure that there would be clear outlines of the pupil. The *X–Y* center of the pupil was analyzed, with the horizontal and vertical components of eye movements then calculated. We calculated the slow-phase velocities, found the maximum slow-phase velocity, and then calculated the horizontal VOR gain by dividing the maximum slow-phase velocity by the peak angular velocity. Rotation test conditions were 0.1 Hz, with a peak angular velocity of 60 deg/s. To evaluate vestibular function, we defined the "gain ratio" as follows: VOR gain on the treated side/VOR gain on the untreated side. Differences in gain ratios between the before treatment, 12 h and 24 h after treatment in each group were evaluated by Friedman's test with the significance set at $P<0.05$. All data are shown as mean ± S.E.

After performing physiological evaluations in the 10^{-3} M SP administered group, each animal was deeply anesthetized with pentobarbital and then immediately decapitated. The temporal bone was dissected, followed by excision of the ampulla of the lateral semicircular canal and utricular macula, with the specimens then soaked in 4% paraformaldehyde in 0.1 M phosphate buffer for 1 h. The samples were decalcified in 10% ethylenediamine-tetraacetic acid for 1 h, and then rinsed in 0.01 M phosphate-buffered saline. Specimens were embedded in Immuno-Bed®, with 2-μm-thick sections then cut, stained with hematoxylin and eosin, and visualized by light microscopy.

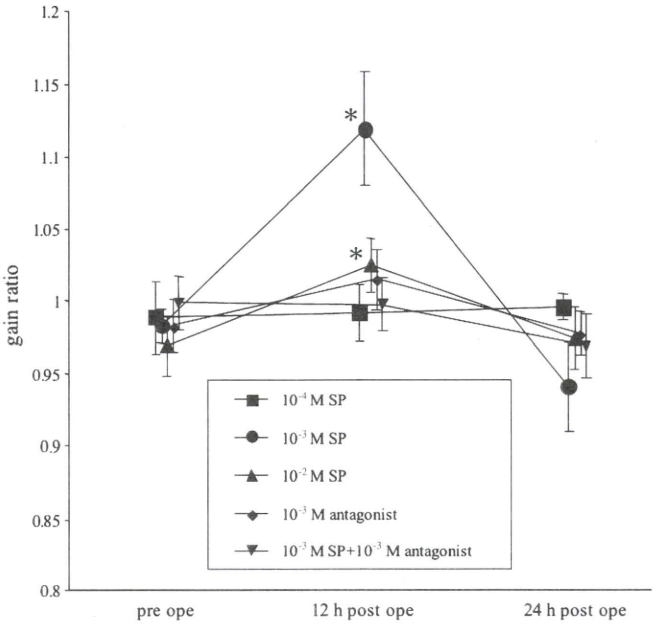

Fig. 1. Gain ratio changes in each group. No significant statistical changes were observed between the gain ratios for any of the examined times in the 10^{-4} M substance P infusion group, in the 10^{-3} M neurokinin-1 receptor antagonist infusion group, and in the 10^{-3} M substance P + 10^{-3} M neurokinin-1 receptor antagonist infusion group. Significant statistical differences were observed between the gain ratios for each of the examined times in the 10^{-2} M and 10^{-3} M substance P infusion groups. SP: substance P. *$P<0.05$. Error bar indicates mean ± S.E.

Neither spontaneous nystagmus nor head deviation was observed in any of the animals during the experimental period. In the 10^{-4} M SP group, no statistical changes were observed in the gain ratio for any of the examined times. In the 10^{-3} M SP and the 10^{-2} M SP groups 12 h after treatment, the gain ratios were statistically higher than those seen in either the pretreatment or the 24 h after treatment groups. No statistical changes for the gain ratios were observed for either the NK-1 receptor antagonist alone or the 10^{-3} M SP + 10^{-3} M NK-1 receptor antagonist groups at any of the examined time points (Fig. 1).

In 10^{-3} M SP infused animals, there were also no obvious histological changes noted during the light microscopic examinations for either the utricular macula or the crista ampullaris of the lateral semicircular canal (data not shown).

In order to evaluate the action of SP without any general anesthesia influence, the current study was designed so that the SP infusion began 12 h after the treatment. While an intraperilymphatic infusion of SP caused an excitatory action within the vestibular periphery, we observed no static symptoms such as spontaneous nystagmus or head deviation. Though, we could observe this excitatory action as directionally preponderance using sinusoidal rotation test. In previous electrophysiological studies, it has been shown that SP acts as an excitatory neurotransmitter or as a facilitatory neuromodulator on the vestibular ganglion cells [6]. Our in vivo data are not inconsistent with the previous in vitro data. In the present study, we used 10^{-2} M, 10^{-3} M, and 10^{-4} M

SP. Concentrations were chosen based on previous results obtained from cell culture and organ culture studies in the rat spiral ganglion neurons [9,12]. The excitatory effect of SP was most remarkable in the 10^{-3} M concentration group. But this effect did not show linear change. We speculated about the possibility that higher concentration of SP (10^{-2} M) might act on some receptors non-specifically and we could not detect pure SP effect using sinusoidal rotation test. Interestingly, this SP-induced effect was not observed at 24 h after treatment. The reason why this effect was only observed just after beginning the SP infusion may be due to compensation by the vestibular system. In lower vertebrates, it is well known that vestibular compensation is very quickly established. In the guinea pig, spontaneous nystagmus disappeared within 48 h after unilateral labyrinthectomy [4,23]. In the present study, the imbalance caused by the SP infusion might have been very small, and thus, was very quickly compensated.

The excitatory effect of SP was suppressed by simultaneous administration of the NK-1 receptor antagonist, which suggests that SP may influence the neuronal excitability in the vestibular periphery via the NK-1 receptor. The NK-1 receptor antagonist alone exhibited no influence within the vestibular periphery. One possibility is the limitation of vestibular testing we used. Such rotational testing is not a test for detecting direct effect of drugs applied to the inner ear. Therefore, the effect of NK-1 receptor antagonist may not be detected by sinusoidal rotation test. Another possibility is that, under normal conditions, the NK-1 receptor may not have a role in the main excitatory transmission within the vestibular periphery. SP may act as a neuromodulator via the NK-1 receptor, as the NK-1 receptor is widely distributed within the central and peripheral nervous system [11,16]. Many previous studies have documented the localization of SP in the vestibular endorgans. In the guinea pig, SP-like immunoreactivity has been observed around hair cells in the macula utriculi and within the nerve chalice on the slope of the crista ampullaris [28]. Although Ylikoski et al. and Scarfone et al. have reported that SP exists in the nerve chalices of type I vestibular hair cells and in the distal end of the nerve chalices, respectively, there have been no reports on the existence of NK-1 receptors within the vestibular periphery [21,31]. Thus, our data support the existence of NK-1 receptors within the vestibular endorgans pharmacologically.

Calyx and dimorphic units in the central and intermediate zones of the crista ampullaris have been said to be mainly sensitive to head rotation [7]. While, SP has been demonstrated predominantly in the peripheral zone of the crista ampullaris [28,31]. Dimorphic units supply both type I and type II hair cells [7], and the neural connection of type I and type II hair cells has been demonstrated under confocal laser scanning microscopy [30]. We speculate that SP modulation mainly occurring in the peripheral zone may influence afferent signaling from the central and intermediate zones induced by head rotation via neural connections.

SP is capable of causing excitatory actions within the vestibular periphery. Previously, it has been shown that SP possesses neurotrophic actions such as the facilitation of neurite extension in cultured neuroblastoma cells [14]. In addition, it has been demonstrated that SP exhibits a neuroprotective action against the neurotoxic effect of beta-amyloid protein on hippocampal cells, or against quinolinic acid-induced excitotoxic death in the striatal neurons [2,20]. However, SP showed no obvious influence within the vestibular morphology when samples were examined under a light microscope in our study. This suggests that there is a possibility that SP could be used as a treatment by directly applying it into the inner ear of patients suffering from peripheral vestibular dysfunction. To confirm the use of this possible treatment, electron microscopic examinations will need to be undertaken in the future. Glutamate, an excitatory neurotransmitter in the vestibular periphery, sometimes causes excitotoxicity in hair cells when excessive amounts of glutamate are applied. Morphologically, postsynaptic dendrites swelling occur [18]. SP also shows an excitatory action, synaptic changes should be examined by electron microscope to confirm the safety of SP.

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