

筑波大学

博士（医学）学位論文

# **Ceramide/sphingomyelin cycle involvement in gentamicin-induced cochlear hair cell death**

(ゲンタマイシン内耳有毛細胞障害における  
セラミド/スフィンゴミエリン・サイクルの関与)

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筑波大学大学院博士課程人間総合科学研究科

**LE NGUYEN UYEN CHI**

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# ABSTRACT

## Purpose

Ceramide, a sphingolipid metabolite, regulates diverse cellular processes including apoptosis, cell senescence, the cell cycle, and cellular differentiation. Exogenously administered ceramide reportedly increased cochlear hair cell death due to gentamicin-induced ototoxicity. Two major ceramide generation pathways are known: the sphingomyelin pathway and the de novo generation pathway. In the sphingomyelin pathway, sphingomyelin is hydrolyzed to ceramide by 3 types of sphingomyelinases (acid, neutral, and alkaline). Of these, magnesium-dependent neutral sphingomyelinase (nSMase) has been identified as a prime candidate for ceramide synthesis. Sphingomyelin synthase (SMS) can also potentially regulate ceramide levels. SMS reconverts ceramide produced by nSMase to sphingomyelin. In the de novo ceramide biosynthesis pathway, serine palmitoyltransferase (SPT) is the first and rate-limiting enzyme. Ceramide generation is derived from the de novo biosynthesis pathway by coordinated activation of SPT and ceramide synthase, an endpoint catalyst of ceramide production. This study was designed to investigate the possible involvement of nSMase, SMS, or SPT in hair cell death due to gentamicin.

## **Material and Method**

The basal turns of the organ of Corti of Sprague–Dawley rats were dissected on postnatal days 3 to 5. Cochlear cultures were exposed to media containing 35  $\mu$ M gentamicin for 48 hours to assess the effects of GW4869 (an nSMase inhibitor), 2-hydroxyoleic acid (an SMS activator), and myriocin (an SPT inhibitor). At the end of the culture, hair cells were fixed by paraformaldehyde and stained with phalloidin to count hair cells. The hair cells were double-immunostained by anti-nSMase2 antibody and phalloidin to detect the presence of nSMase2 in hair cells. nSMase activity of the basal turn of the organ of Corti cells during gentamicin exposure was measured using an nSMase assay kit.

## **Result**

Immunostaining of nSMase2 in the organ of Corti revealed the presence of nSMase2 in the inner and outer hair cells. The level of nSMase activity of the hair cell extracts from the organ of Corti explants during gentamicin exposure was significantly increased at 20 minutes and 48 hours after the initiation of gentamicin exposure. The effects of GW4869, 2-hydroxyoleic acid and myriocin on gentamicin-induced hair cell damage were examined. Hair cell loss was significantly decreased in the presence of GW4869 or 2-hydroxyoleic acid. Myriocin had no significant effect against gentamicin-induced hair cell loss.

## **Discussion**

Ceramide has attracted great attention because of its possible roles in cochlear hair cell apoptosis. In a ceramide/sphingomyelin cycle, nSMase hydrolyzes sphingomyelin to ceramide and SMS synthesizes sphingomyelin from ceramide. Thus, ceramide levels may be balanced by the activities of these 2 enzymes. We have here documented, for the first time, the immunolocalization and enzymatic activity of nSMase in the cochlea. Gentamicin exposure increased the enzymatic activity of nSMase. A specific inhibitor of nSMase or activation of SMS decreased gentamicin-induced hair cell death. Our results suggested that the ceramide/sphingomyelin cycle exists in the organ of Corti and that the balance in this cycle is important for determination of hair cell fate in gentamicin ototoxicity. An SPT inhibitor did not prevent hair cell death due to gentamicin-induced ototoxicity. This result indicated that the de novo generation of ceramide might not be involved in gentamicin-induced ototoxicity.

## **Conclusion**

The present findings strongly suggest that the ceramide/sphingomyelin cycle plays an important role in the generation mechanism of gentamicin-induced ototoxicity.

# INTRODUCTION

Sphingolipids, the essential components of cellular membranes, are involved in diverse cellular functions. Sphingolipids comprise a wide variety of molecules containing a long-chain sphingoid base, sphingosine. Ceramide is composed of sphingosine in N-linkage to a variety of acyl groups. Ceramide induces apoptosis, differentiation, senescence, autophagy, and migration as a lipid mediator. Thus, ceramide occupies the central position of the sphingolipid network (Hannun and Obeid 2008). Roles for ceramide as a cell death-inducing factor have also been suggested in a number of pathological conditions including cancer, neurodegeneration, diabetes, microbial pathogenesis, obesity, and inflammation (Wu et al. 2007; Zeidan and Hannun 2007). Moreover, ceramide involvement has been suggested in cochlear hair cell loss induced by gentamicin through apoptosis (Nishimura et al. 2010).

Ceramide can be formed through sphingomyelinase-dependent catabolism of sphingomyelin, as well as via the de novo synthesis pathway by serine palmitoyltransferase (SPT). Inversely, ceramide can be converted to sphingomyelin by sphingomyelin synthase (Pewzner-Jung et al. 2006). Because ceramide shows toxicity against the cochlea, we supposed that generation of ceramide through the above-mentioned synthesis pathways may affect hair cell damage.

Sphingomyelin, a key component of membrane microdomain, is abundant in mammalian nervous system. In mammalian cells, three types of sphingomyelinases (acid, neutral, and alkaline) exist.

Of these, magnesium-dependent neutral sphingomyelinase (nSMase) has been identified as a prime candidate for ceramide synthesis. Although 3 nSMases (nSMase1, nSMase2, and nSMase3) exist (Hofmann et al. 2000; Krut et al. 2006; Tomiuk et al. 1998), nSMase2 is responsible for most of the nSMase activity in many organs, and thus, is the principal generator of ceramide (Aubin et al. 2005; Stoffel et al. 2005). Overexpression of nSMase2 in hepatocytes reportedly resulted in a 40% decrease in SM levels and a concomitant 60% increase in ceramide levels (Karakashian et al. 2004; Marchesini et al. 2003). Although ceramide augments the apoptotic death of outer hair cells (Nishimura et al. 2010), whether nSMase is involved in ceramide generation in gentamicin-induced ototoxicity has not been revealed.

Sphingomyelin synthase (SMS) can also potentially regulate ceramide levels. SMS reconverts ceramide produced by nSMase to sphingomyelin. Namely, catalysis by nSMase and SMS forms a ceramide/sphingomyelin cycle. However, the role of the ceramide/sphingomyelin cycle has not yet been reported.

In the de novo ceramide biosynthesis pathway, SPT is the first and rate-limiting enzyme. Ceramide generation is derived from the de novo biosynthesis pathway by coordinated activation of SPT and ceramide synthase, an endpoint catalyst of ceramide production.

Considering a possible role of ceramide in gentamicin-induced ototoxicity, we hypothesized that hair cell loss could be prevented by modifying the ceramide biosynthesis pathways. In this study, we

examined the functions of nSMase, SMS, and SPT in gentamicin-induced ototoxicity.

# **MATERIALS AND METHODS**

## **Organ of Corti collection**

The basal turns of the organ of Corti were dissected from Sprague-Dawley rats on postnatal days 3 to 5 and cultured according to the methods of Van de Water and Ruben (1974) and Sobkowicz et al. (1993).

All animal procedures were carried out according to the guidelines of the Laboratory Animal Research Center of the University of Tsukuba.

## **Culture techniques**

Cochlear explants were maintained in Dulbecco's modified Eagle's medium with 10% fetal bovine serum (FBS), 25 mM HEPES, and 30 U/mL penicillin and cultured in an incubator at 37°C with 5% CO<sub>2</sub> and 95% humidity. Cochlear cultures were maintained in the above-described medium overnight (8 - 12 hours) and then exposed to a medium containing 35 µM gentamicin for 48 hours to assess the effects of sphingolipid metabolites (Nakamagoe et al. 2010; Nishimura et al. 2010; Tabuchi et al. 2007).

## Pharmacological treatments

GW4869 (an nSMase inhibitor), 2-hydroxyoleic acid (2OHOA, an SMS activator), and myriocin (an SPT inhibitor) were purchased from Sigma Japan (Tokyo, Japan). The tested concentrations of GW4869, 2OHOA, and myriocin were 1 to 50  $\mu$ M, 10nM to 1 mM and 1 pM to 50  $\mu$ M, respectively. The tested concentrations were determined based on the previous experiments on the neuronal cells, red blood cells, vascular smooth muscle cells, fibroblasts, glioma cells, lung adenocarcinoma cells and liver cells (Babenko and Kharchenko 2012; Cubi et al. 2013; Gbotosho et al. 2013; Liao et al. 2013; Martin et al. 2013; Qin et al. 2012). The highest concentration of myriocin was determined at 50  $\mu$ M because of its solubility. GW4869 was routinely stored at  $-80^{\circ}\text{C}$  as a 1.5 mM stock suspension in dimethyl sulfoxide (DMSO; Sigma, St Louis, MO, USA). Immediately before use, the suspension was solubilized by addition of 5% methane sulfonic acid (MSA; Sigma). The suspension was mixed and warmed at  $37^{\circ}\text{C}$  until clear (Luberto et al. 2002). 2OHOA was dissolved in DMSO at a stock concentration of 25 mg/mL and stored at  $-20^{\circ}\text{C}$ . Myriocin was dissolved in methanol at a stock concentration of 2 mg/mL and stored at  $-20^{\circ}\text{C}$ .

## **Cytochemistry**

After 48-hour culture, the explants were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) for 20 minutes and then permeabilized with 5% TritonX-100 (Sigma) in PBS with 10% FBS for 10 minutes (Nishimura et al. 2010; Tabuchi et al. 2007; Tabuchi et al. 2010). The specimens were stained with phalloidin with a conjugated Alexa Fluor phalloidin probe (1:100; Molecular Probes, Carlsbad, CA, USA) at room temperature for 1 hour. Phalloidin is a specific marker for cellular F-actin and labels the stereociliary arrays and cuticular plates of hair cells (Nakamagoe et al. 2010; Tabuchi et al. 2007).

Hair cells were characterized as missing if no stereocilia and/or no cuticular plate were observed by phalloidin staining. Quantitative results were obtained by evaluating 30 outer hair cells associated with 10 inner hair cells in a given microscopic field (Nakamagoe et al. 2010; Tabuchi et al. 2007). The average of 3 separate counts was used to represent each culture.

## **Immunostaining of hair cells by anti-nSMase2 antibody**

Rabbit polyclonal anti-nSMase2 (H-195) (sc-67305) was purchased from Santa Cruz Biotechnology (Dallas, TX, USA), and donkey anti-rabbit FITC (711-095-152), from Sigma.

The hair cells were removed from the culture media, rinsed twice in PBS, and fixed in 4%

paraformaldehyde for 20 minutes, and then double-immunostained. After removal of the fixative, the cells were incubated in 0.1% Triton X-100 and 10% FBS at room temperature for 15 minutes.

The primary anti-nSMase2 antibody was diluted 1:100 in PBS and incubated with cells overnight at 4°C. After 6 rinses in PBS for 5 minutes each time at room temperature, cells were incubated with FITC-conjugated secondary antibody (diluted 1:100 in PBS) for 45 minutes in a dark chamber and then rinsed extensively in PBS at room temperature. The specimens were stained with phalloidin with a conjugated Alexa Fluor phalloidin probe (1:100) at room temperature for 1 hour. One drop of SlowFade Gold Antifade reagent (Invitrogen, Carlsbad, CA, USA) was added, and the slide was sealed with nail polish. The immunofluorescence reaction was followed and documented with an Axiophot microscope (Carl Zeiss, Oberkochen, Germany).

### **nSMase assay**

nSMase activity of the basal turn of the organ of Corti cells during gentamicin exposure was measured using an nSMase assay kit (K-1800; Echelon, Salt Lake City, UT, USA) as described in the manufacturer's protocol. Briefly, organ of Corti cells were harvested and washed with PBS. After the cells had been homogenized, 60 µg total protein was used to examine the nSMase activity. One hundred-microliter samples were mixed with 100 µL reaction buffer and 5 mM dithiothreitol (Thermo

Scientific, Rockford, IL, USA) to block any acid sphingomyelinase activity. The samples were incubated at 37°C for 4 hours. The enzymatic activity was calculated from the graph's slope of the absorbance of light at 595 nm against the concentration of sphingomyelinase and standardized by milligrams of protein (mU/mg, representing the specific enzymatic activity).

### **Data analyses and statistics**

All data were expressed as the means  $\pm$  SEMs. Statistical analysis was performed using unpaired *t*-tests or 1-way analysis of variance (ANOVA) followed by Bonferroni post-hoc tests, as required (StatView 5.0). Probability (*p*) values below 0.05 were considered significant.

# RESULTS

## Expression of nSMase2 in cochlear hair cells

Immunostaining of nSMase2 in organ of Corti cells revealed the presence of nSMase2 in the inner and outer hair cells (Fig. 1).

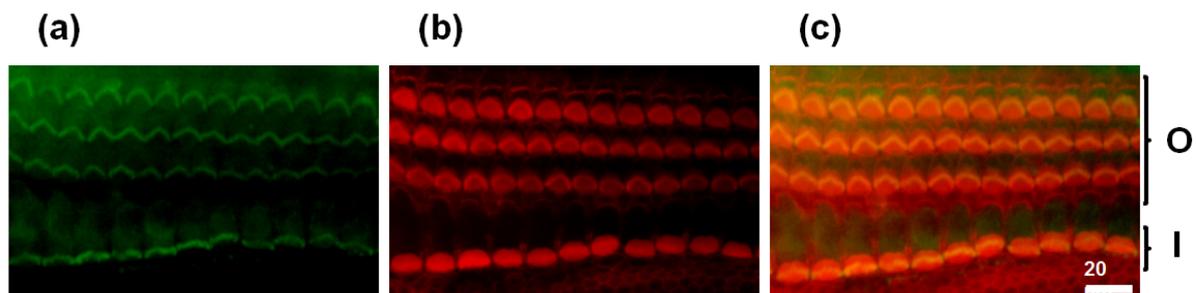


Fig. 1 Localization of nSMase2 in cochlear explants (a). nSMase 2 stained with anti-nSMase 2 antibody (indicated in green) (b). Hair cells stained with phalloidin (indicated in red) (c). Merged images of nSMase2 and hair cells. The outer and inner hair cells stained with nSMase2. O: outer hair cell. I: inner hair cell

## **GM activated nSMase in organ of Corti cells**

Changes in nSMase activity during gentamicin exposure were examined using the organ of Corti explants.

Twenty minutes after the initiation of gentamicin exposure, enzymatic activity of the hair cell extracts significantly increased ( $n = 6$  samples of protein,  $p < 0.001$ ), to nearly 3-fold those of the non-gentamicin controls. Thereafter, nSMase activity gradually declined, but at 48 hours, the level of nSMase activity was significantly ( $n = 6$ ,  $p < 0.001$ ) higher (344%) than that of the non-gentamicin controls (1-way ANOVA and Bonferroni post-hoc test; Fig. 2).

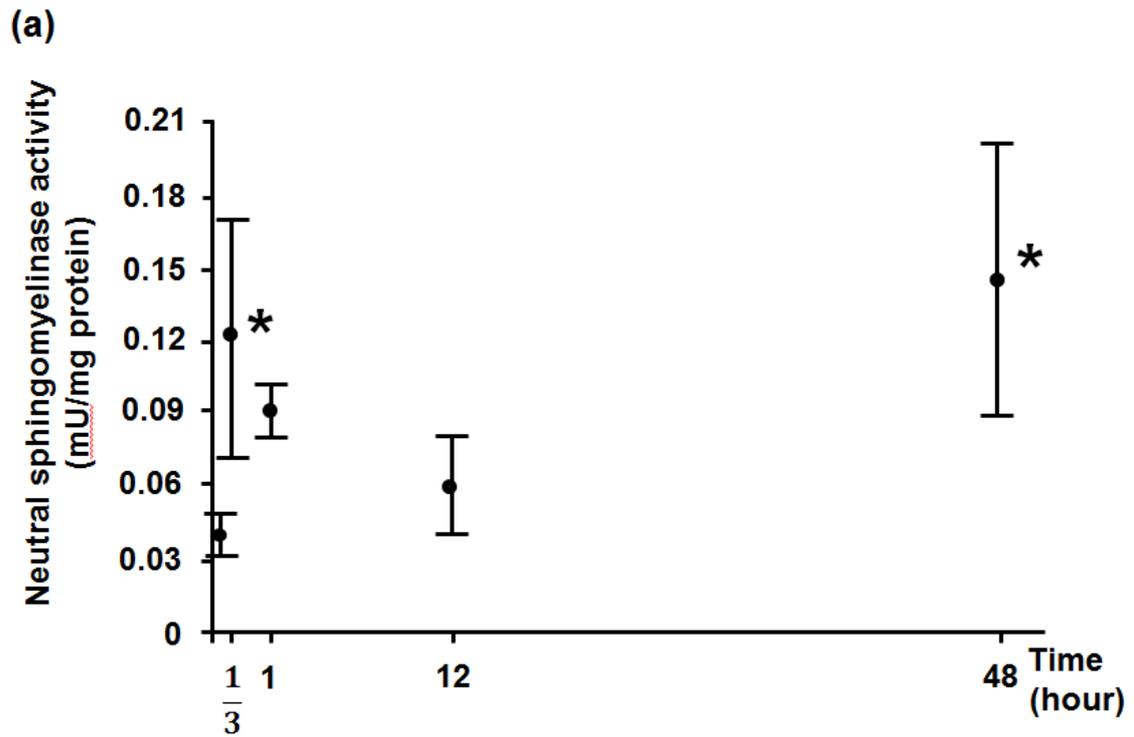


Fig. 2 Characterization of nSMase enzymatic activity (a). Enzymatic assays of cochlear hair cell extracts significantly increased at 20 minutes (292%) and at 48 hours (344%) after exposure to 35  $\mu$ M gentamicin (1-way ANOVA and Bonferroni post-hoc test: \* $p < 0.05$  versus the non-gentamicin control group at 0 hour). The enzymatic activity expressed as mU/mg protein

## Control pharmacological study

Before the gentamicin experiments, the effects on hair cells of 1  $\mu\text{M}$  to 50  $\mu\text{M}$  GW4869, 10 nM to 100  $\mu\text{M}$  2OHOA, and 1 pM to 50  $\mu\text{M}$  myriocin, and of 0.01% MSA, 0.25% DMSO, and 1% methanol as solvents were examined. In the control explants maintained in the media containing these agents for 48 hours without exposure to gentamicin, almost all hair cells, in both the inner and the outer rows, were present. Namely, hair cell loss was not statistically significant when explants were cultured for 48 hours in the media containing each agent without gentamicin (data not shown). Furthermore, we also confirmed that the hair cell loss did not differ significantly between the explants exposed to media containing 35  $\mu\text{M}$  gentamicin with 0.01% MSA, 0.25% DMSO, or 1% methanol for 48 hours and those exposed to media containing 35  $\mu\text{M}$  gentamicin alone (data not shown).

## **Inhibition of nSMase protected hair cells against gentamicin-induced ototoxicity**

The effects of GW4869, an nSMase inhibitor, on gentamicin-induced hair cell damage were examined.

GW4869 significantly decreased outer hair cell loss induced by gentamicin at 35  $\mu\text{M}$  ( $n = 12$  explants,  $p = 0.03$ ), 40  $\mu\text{M}$  ( $n = 15$ ,  $p < 0.001$ ), 45  $\mu\text{M}$  ( $n = 13$ ,  $p < 0.001$ ) and 50  $\mu\text{M}$  ( $n = 16$ ,  $p < 0.001$ ) when compared with the control group exposed to gentamicin alone (1-way ANOVA and Bonferroni post-hoc test; Fig. 3).

**Fig. 3** Effects of GW4869 on gentamicin-induced hair cell loss (a). Quantitative analysis of outer hair cell loss. GW4869 protected the cochlea at a concentration of 35 to 50  $\mu\text{M}$  (1-way ANOVA and Bonferroni post-hoc test:  $*p < 0.05$  versus the gentamicin control group)

(b-k). Representative photographs of each group (b). Solvent alone (c). Gentamicin control (35  $\mu\text{M}$  gentamicin alone) (d). 35  $\mu\text{M}$  gentamicin plus 1  $\mu\text{M}$  GW4869 (e). 35  $\mu\text{M}$  gentamicin plus 10  $\mu\text{M}$  GW4869 (f). 35  $\mu\text{M}$  gentamicin plus 20  $\mu\text{M}$  GW4869 (g). 35  $\mu\text{M}$  gentamicin plus 30  $\mu\text{M}$  GW4869 (h). 35  $\mu\text{M}$  gentamicin plus 35  $\mu\text{M}$  GW4869 (i). 35  $\mu\text{M}$  gentamicin plus 40  $\mu\text{M}$  GW4869 (j). 35  $\mu\text{M}$  gentamicin plus 45  $\mu\text{M}$  GW4869 (k). 35  $\mu\text{M}$  gentamicin plus 50  $\mu\text{M}$  GW4869

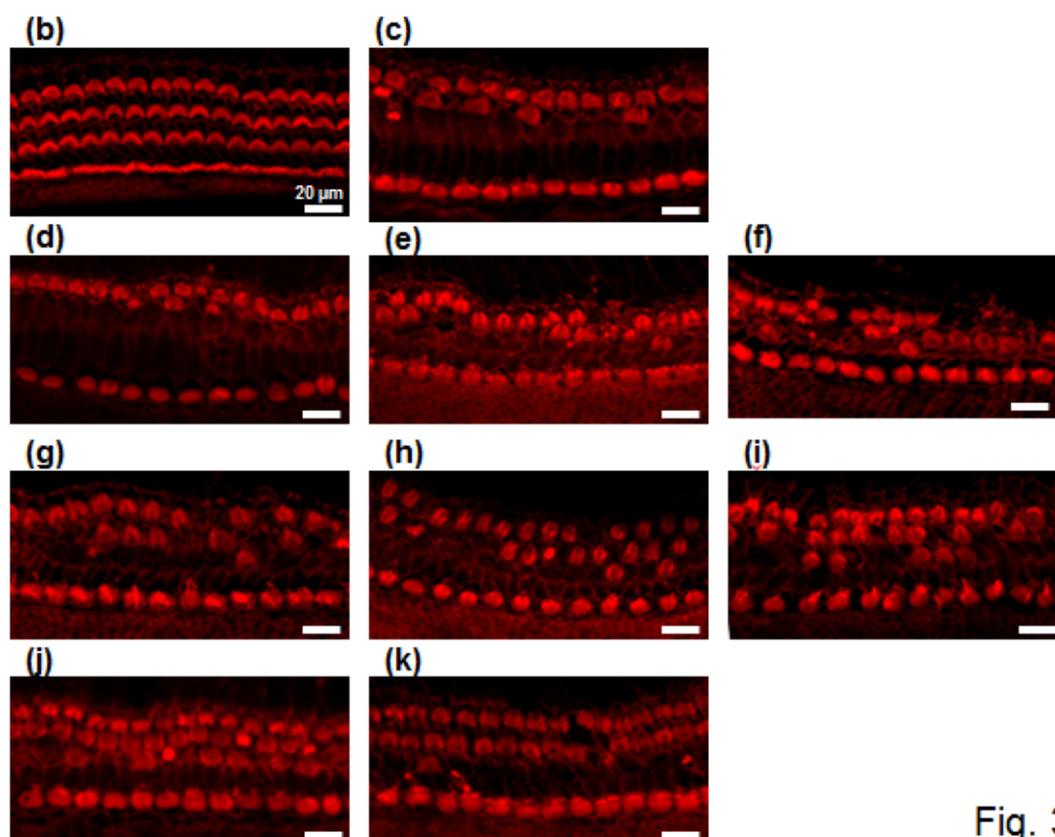
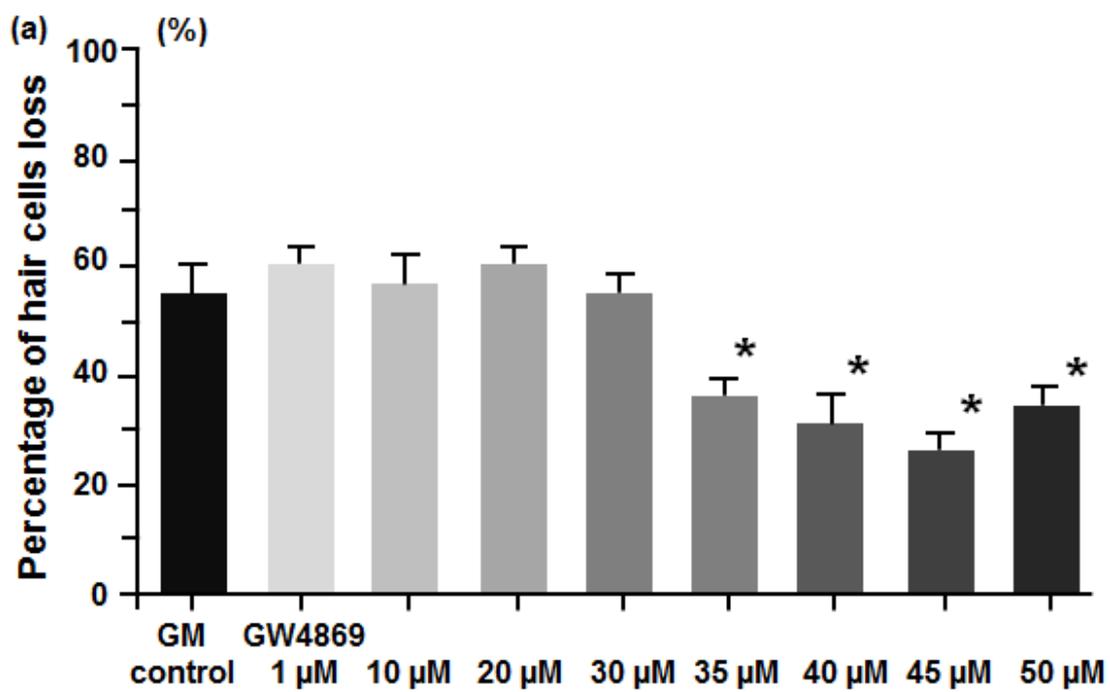


Fig. 3

## Activation of SMS protected hair cells against gentamicin-induced ototoxicity

The effects of 2OHOA, an SMS activator, on gentamicin-induced hair cell damage were examined.

2OHOA significantly decreased outer hair cell loss induced by gentamicin at 10  $\mu\text{M}$  ( $n = 11$  explants,  $p < 0.001$ ) and 100  $\mu\text{M}$  ( $n = 15$ ,  $p < 0.001$ ) when compared with the control group exposed to GM alone (1-way ANOVA and Bonferroni post-hoc test; Fig. 4).

**Fig. 4** Effects of 2OHOA on gentamicin-induced outer hair cell loss (a). Quantitative analysis of outer hair cell loss. 2OHOA protected the cochlea at a concentration of 10 nM to 100  $\mu\text{M}$  (1-way ANOVA and Bonferroni post-hoc test:  $*p < 0.05$  versus the gentamicin control group) (b-h). Representative photographs of each group (b). Solvent alone (c). Gentamicin control (35  $\mu\text{M}$  gentamicin alone) (d). 35  $\mu\text{M}$  gentamicin plus 10 nM 2OHOA (e). 35  $\mu\text{M}$  gentamicin plus 100 nM 2OHOA (f). 35  $\mu\text{M}$  gentamicin plus 1  $\mu\text{M}$  2OHOA (g). 35  $\mu\text{M}$  gentamicin plus 10  $\mu\text{M}$  2OHOA (h). 35  $\mu\text{M}$  gentamicin plus 100  $\mu\text{M}$  2OHOA

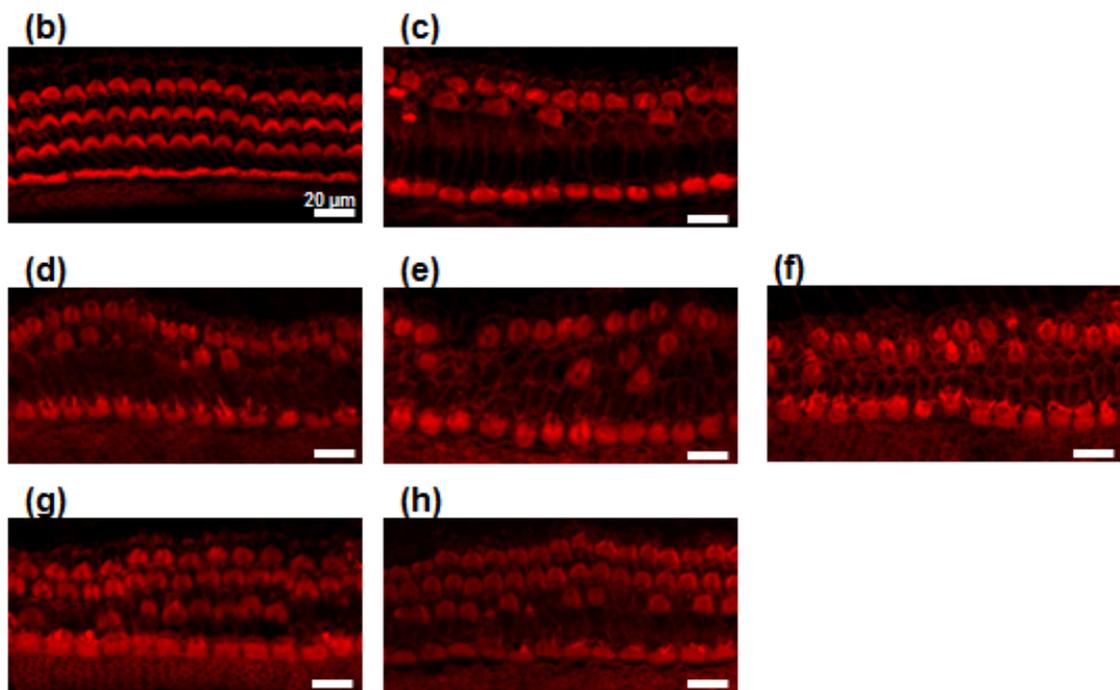
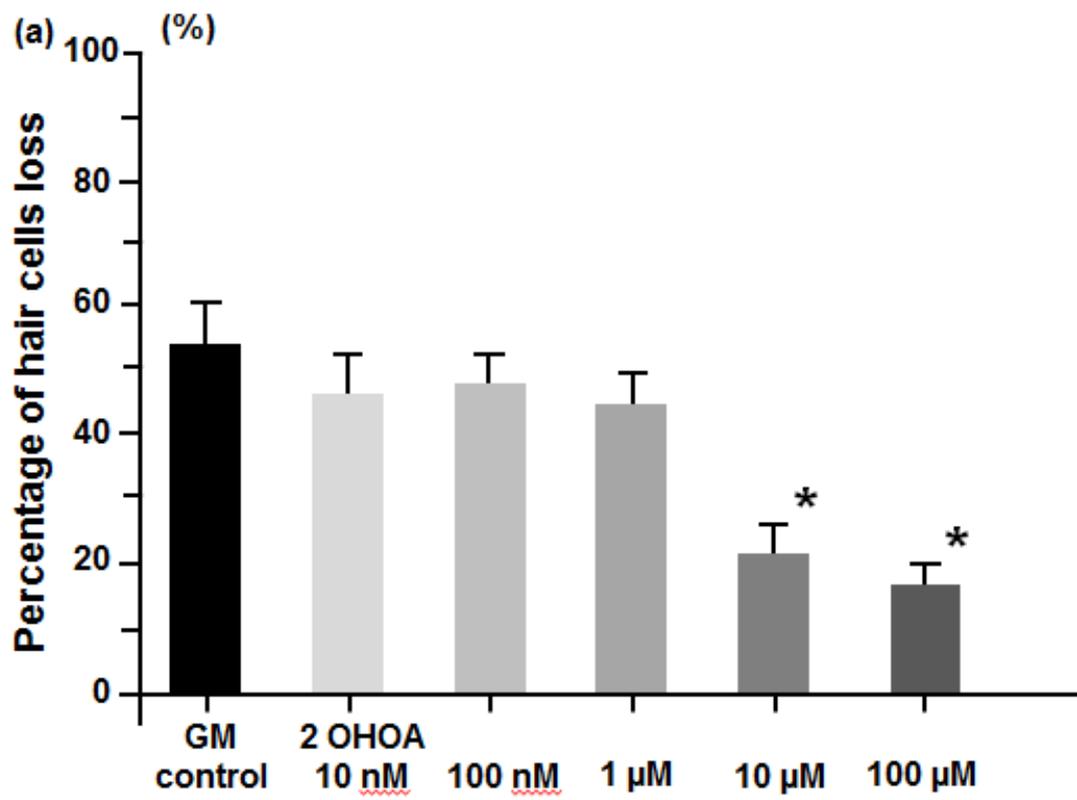


Fig. 4

## **Inhibition of SPT had no effect on gentamicin-induced hair cell loss**

The effects of myriocin, an SPT inhibitor, on gentamicin-induced hair cell damage were examined.

Myriocin had no significant effect on the degree of hair cell survival at 1 pM to 50  $\mu$ M when compared

with gentamicin alone (1-way ANOVA; Fig. 5)

**Fig. 5** Effects of myriocin on gentamicin-induced outer hair cell loss (a). Quantitative analysis of outer hair cell loss. Myriocin had no effect on gentamicin-induced outer hair cell loss at a concentration of 1 pM to 50  $\mu$ M

(b-l). Representative photographs of each group (b). Solvent alone (c). Gentamicin control (35  $\mu$ M gentamicin alone) (d). 35  $\mu$ M gentamicin plus 1 pM myriocin (e). 35  $\mu$ M gentamicin plus 10 pM myriocin (f). 35  $\mu$ M gentamicin plus 100 pM myriocin (g). 35  $\mu$ M gentamicin plus 1 nM myriocin (h). 35  $\mu$ M gentamicin plus 10 nM myriocin (i). 35  $\mu$ M gentamicin plus 100 nM myriocin (j). 35  $\mu$ M gentamicin plus 1  $\mu$ M myriocin (k). 35  $\mu$ M gentamicin plus 10  $\mu$ M myriocin (l). 35  $\mu$ M gentamicin plus 50  $\mu$ M myriocin

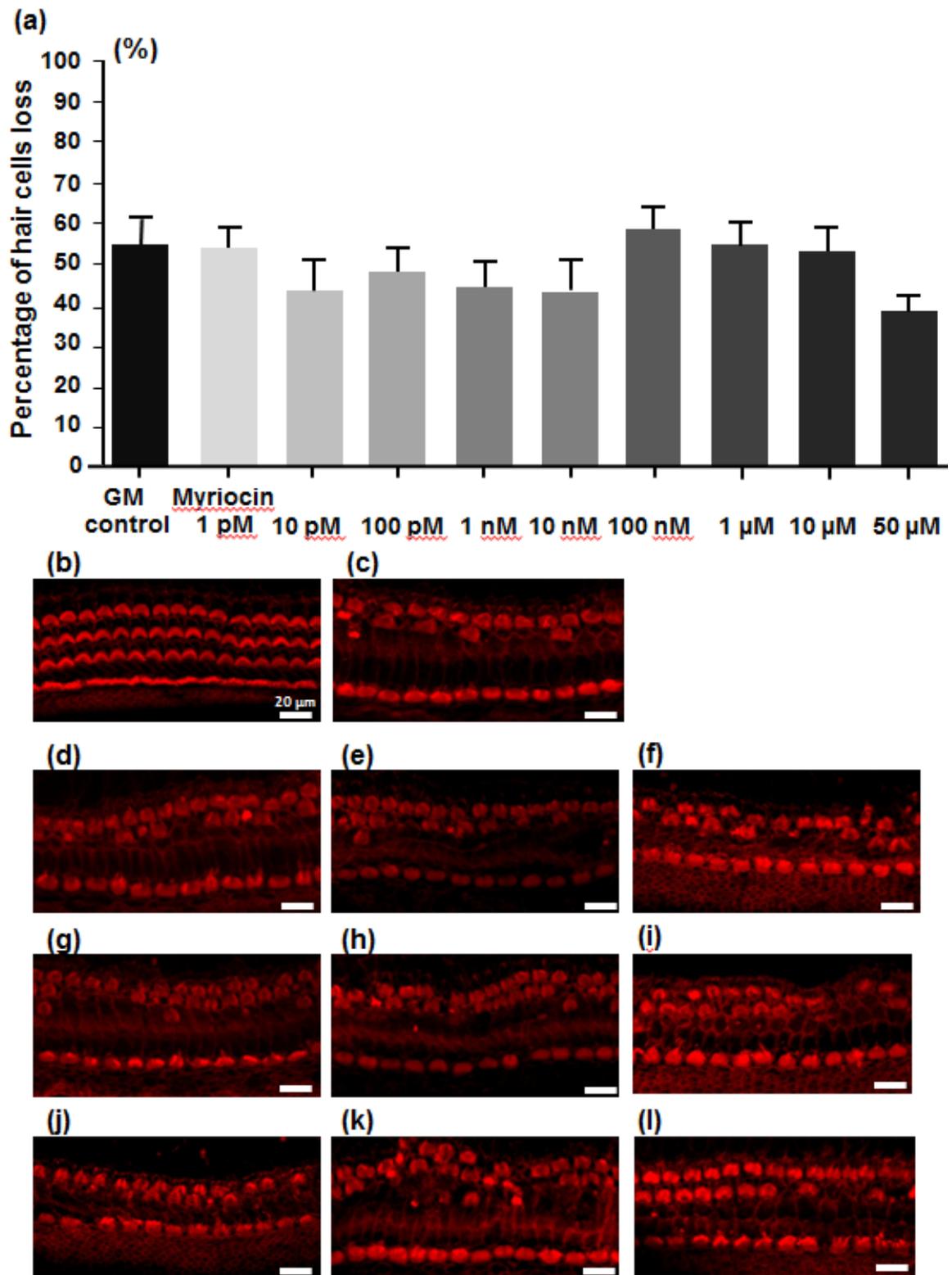


Fig. 5

## DISCUSSION

Gentamicin is well known as an ototoxic agent. Gentamicin causes both inner and outer hair cell death. The toxicity depends on its concentration. At low concentration, the outer hair cells are the only site of the damage. The loss of outer hair cells is often more evident in the first row than the second or third row (Nakamagoe et al. 2011). At high concentration, gentamicin induces both outer and inner hair cell loss. In our study, we conducted the gentamicine experiments at 35  $\mu$ M for 48 hours, which induced about 50% outer hair cell loss while inner hair cells remained intact.

Ceramide has attracted great attention because of its possible roles in cochlear hair cell apoptosis (Nishimura et al. 2010). Better understanding of the ceramide formation system might offer relevant insights into cochlear hair cell death.

Most of nSMase2 is localized at the plasma membrane, the primary site of action of this enzyme in rat hepatocytes (Karakashian et al. 2004). Our immunofluorescence study detected existence of nSMase2 in hair cells. Importantly, nSMase2 is responsible for the main function of nSMase (Aubin et al. 2005; Karakashian et al. 2004; Marchesini et al. 2003; Stoffel et al. 2005). Additionally, gentamicin exposure increased the enzymatic activity of nSMase. GW4869, a specific inhibitor of nSMase, decreased gentamicin-induced hair cell death. On the basis of these findings, ceramide generated by nSMase through the hydrolysis of sphingomyelin may be involved in gentamicin-induced ototoxicity.

In addition, activation of SMS by 2OHOA protected hair cells against gentamicin. Some researchers speculated on the existence of a ceramide/sphingomyelin cycle. In this cycle, nSMase hydrolyzes sphingomyelin to ceramide and SMS synthesizes sphingomyelin from ceramide. Thus, ceramide levels are balanced by the activities of these 2 enzymes. Our results suggested that such a ceramide/sphingomyelin cycle exists in the organ of Corti and that the balance in this cycle is important for determination of hair cell fate in gentamicin ototoxicity.

We have here documented, for the first time, the immunolocalization and enzymatic activity of nSMase in the cochlea. On the basis of the kinetics, the increase in enzymatic nSMase activity was divided into 2 phases. In the early phase, the total cellular nSMase activity increased by 292% at 20 minutes of gentamicin exposure. Possibly, the nSMase activity rapidly increased as an initial response against gentamicin exposure. Thereafter, the nSMase activity gradually declined. In the late phase, at 48 hours after gentamicin exposure, the level of nSMase activity was 344% higher than that in the non-gentamicin-exposed controls.

nSMase is reportedly activated by diverse stress stimuli including reactive oxygen species,  $\alpha$ -TNF, IL-1 $\beta$ , H<sub>2</sub>O<sub>2</sub> and promotes an increase in cellular ceramide levels over a period of minutes to hours (Clarke et al. 2007; Gu et al. 2013; Hannun et al. 2001; Mathias et al. 1998; Wiegmann et al, 1994). Martinez-Salgado (2004) showed that generation of reactive oxygen species occurs in two phases in gentamicin ototoxicity. We supposed that activation of nSMase was observed in two phases because

gentamicin induced cellular stress, such as formation of reaction oxygen species, in two phases. However, because of lack of more frequent measurements in nSMase enzymatic activity experiment, we could not elucidate the precise mechanism of action underlying these biphasic elevations of neutral sphingomyelinase activity.

In contrast to the results obtained with GW4869 and 2OHOA, myriocin, an SPT inhibitor, known as an inhibitor of the first enzyme in the de novo biosynthesis pathway, did not prevent hair cell death due to gentamicin-induced ototoxicity. This result indicated that the de novo generation of ceramide might not be involved in gentamicin-induced ototoxicity. However, several researchers reported that generation of ceramide via de novo biosynthesis occurred much more slowly than via the sphingomyelin hydrolase pathway (Bose et al. 1995; De Luca et al. 2005; Mullen et al. 2011). Because our study examined results over a time course of 48 hours, they may indicate that the de novo biosynthesis pathway is activated after a longer period.

The ceramide/sphingomyelin cycle is important in determination of hair cell fate based on our results. Ceramide, generated this cycle, induced hair cell death. However, one limitation of this study is that the levels of ceramide cannot be measured in the organ of Corti due to the tiny explants. If the levels of ceramide were measured, the data would have confirmed our hypothesis.

In conclusion, the present findings strongly suggest the involvement of a ceramide/sphingomyelin cycle in gentamicin-induced cochlear ototoxicity. Gentamicin-induced

ototoxicity may be ameliorated by modifying ceramide synthesis through this cycle.

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**Conflict of interest** The authors declare that they have no conflict of interest.

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