Research Article

Study of the Mechanisms by Which Aminoglycoside Damage Is Prevented in Chick Embryonic Hair Cells

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ABSTRACT

A major side effect of aminoglycoside antibiotics is mammalian hair cell death. It is thus intriguing that embryonic chick hair cells treated with aminoglycosides at embryonic day (E) 12 are insensitive to ototoxicity. To exclude some unknown factors in vivo that might be involved in preventing aminoglycoside damage to embryonic hair cells, we first cultured chick embryonic basilar papilla (BP) with an aminoglycoside antibiotic in vitro. The results indicated that the hair cells were almost intact at E12 and E14 and were only moderately damaged in most parts of the BP at E16 and E18. Generally, hair cells residing in the approximate and abneural regions were more susceptible to streptomycin damage. After incubation with gentamicin-conjugated Texas Red (GTTR), which is typically used to trace the entry route of aminoglycosides, GTTR fluorescence was not remarkable in hair cells at E12, was weak at E14, but was relatively strong in the proximal part of BP at E18. This result indicates that the amounts of GTTR that entered the hair cells are related to the degrees of aminoglycoside damage. The study further showed that the fluorescence intensity of GTTR decreased to a low level at E14 to E18 after disruption of mechanotransduction machinery, suggesting that the aminoglycoside entry into hair cells was

mainly through mechanotransduction channels. In addition, most of the entered GTTR was not found to be colocalized with mitochondria even at E18. This finding provides another reason to explain why embryonic chick hair cells are insensitive to aminoglycoside damage.

Keywords: antibiotic toxicity, mechanotransduction channels, transient receptor potential ion channel, chicken cochlea, mitochondria Huanju Bai and Xi Wang contributed equally to this work.

INTRODUCTION

Aminoglycosides, polycationic water-soluble compounds, are highly effective in gram-negative infections (Guthrie 2008). Nevertheless, a large number of patients treated with aminoglycosides have suffered from irreversible hearing loss (Fee 1980; Rizzi and Hirose 2007). Aminoglycosides can interact with ribosomal RNAs in mitochondria, as they do in microbial ribosomes (Hobbie et al. 2008; Qian and Guan 2009). After the binding of aminoglycosides, the electron transport chain in mitochondrial ribosomes is disrupted and some free radicals are released (Sugahara et al. 2006; Warchol 2010). These radicals subsequently activate the c-Jun N-terminal kinase (JNK) and cause the release of cytochrome-c from the mitochondria, leading to the activation of caspases and nucleases and hair cell apoptosis. Thus, the mitochondrion is an important target organelle for aminoglycoside injury (Sugahara et al. 2006; Warchol 2010).

Aminoglycosides enter the cell by permeation through mechanotransduction (MET) channels that

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possess a larger pore size of approximately 1.25 nm in diameter compared with the size of aminoglycosides (Farris et al. 2004; Marcotti et al. 2005; Luk et al. 2010). In addition to the MET channel, some members of the transient receptor potential (TRP) family show nonselective permeability to cations and small organic molecules, including gentamicin (Meyers et al. 2003; Corey et al. 2004; Myrdal and Steyger 2005), and they were also considered to be transduction channel candidates (Cuajungco et al. 2007; Alharazneh et al. 2011; Stepanyan et al. 2011). However, mice lacking some groups of TRP channels have normal transduction currents and hearing, arguing against the participation of TRP channels in hair cell transduction, including Trpa1 (Kwan et al. 2006; Wu et al. 2016).

Our recent study indicated that the auditory epithelia in the chick BP are insensitive to an aminoglycoside antibiotic that was injected into the yolk sac at E12 (Jiang et al. 2016). Given that aminoglycosides exhibit serious ototoxicity in the cochlea of posthatch chicks (Stone et al. 1996; Shang et al. 2010) and in mammals, including newborn human babies (Village 1999; Selimoglu 2007; Guthrie 2008; Kent et al. 2014), the mechanism by which embryonic hair cells in the chick BP are insensitive to the aminoglycoside needs to be studied, as it might provide suggestions on how to prevent hair cells from suffering from aminoglycoside injury and may impart knowledge on how aminoglycoside antibiotics enter the hair cells without harming them.

Although it is unclear how chick embryonic hair cells are protected from aminoglycoside damage, it is relatively clear how the transducer apparatus is formed and how hearing ability occurs during the development of chick or mouse inner ears (Si et al. 2003; Jones et al. 2006; Housley et al. 2006). In the developing chick BP, the transducer apparatus, including Myosin VIIa and plasma membrane Ca^{2+} -ATPase, is assembled jointly at embryonic day (E) 11, and a robust transduction current can be recorded at E12 (Si et al. 2003). Incipient responses to airborne sounds emerge in cochlear ganglion neurons at approximately E14; however, responses with frequency selectivity occur from E16 to E18 (Jones et al. 2006). Thus, the timing of the transducer apparatus packaging and the occurrence of hearing ability in chick hair cells do not correspond accurately to the time when the hair cells are insensitive to aminoglycoside damage (Jiang et al. 2016), and the mechanisms by which the chick embryonic hair cells are protected from aminoglycoside damage remain elusive.

To address the above issue, the present study first confirmed our previous results by culturing embryonic BP with an aminoglycoside antibiotic *in vitro* to exclude some potential factors in the chick embryo that might be involved in preventing aminoglycoside damage to

embryonic hair cells. We found that after incubation with GTTR, fluorescence was weak in the hair cells in the chick BPs at E12 and E14, but relatively strong in the proximal part of BP at E16 or E18. After MET transduction was disrupted, we studied the changes in the entry of GTTR into the hair cells from E14 to E18. Finally, we studied whether GTTR that entered into the hair cells was colocalized with the mitochondria.

MATERIALS AND METHODS

Animal Care

Fertilized eggs were obtained from the Chinese Academy of Agricultural Sciences. The eggs were hatched in a humidified incubator at 37.5 °C until E12, E14, E16, and E18. The chicken eggs were staged as previously described (Hamburger and Hamilton 1992). All the experiments performed in this study were conducted in accordance with the Beijing Laboratory Animal Welfare and Ethics Review guidelines, and all the procedures were approved by the Animal Management Committee of the College of Life Sciences, Beijing Normal University.

Culture of the Basilar Papilla

Embryonic chickens (E12 to E18) were dissected out, and they were then euthanized by rapid decapitation using a pair of dissecting scissors. The heads were placed in a glass bottom Petri dish on an ice bag. Cochlear ducts were dissected under aseptic conditions as previously described (Honda et al. 2014; Stepanyan et al. 2006). The BPs were then placed in ice-cold sterile hanks buffered saline solution (HBSS; Sigma-Aldrich). Each BP was cultured in 24-well culture plates coated with a thin layer of collagen type I rat tail (Corning, 354236), and prefilled with 300 μl of DMEM (Gibco, 11995-065) medium per well, supplemented with 1 % fetal bovine serum (FBS, Gibco, 16000-044), 1 % L-glutamine (Gibco, 25030081), and penicillin G (1500 U/mg, Amresco, E480). The BPs were incubated at 37 °C in 95 % air/5 % CO_2 for 3 days. The medium was replaced every day. In experimental group, streptomycin dissolved in sterile saline (78 μM , Sigma-Aldrich) was added to the medium for 2 days (the same volume of sterile saline was added for the control). Such treatment can damage all auditory hair cells in the chick BP in a proximal-to-distal sequence within 3 days after streptomycin addition. Streptomycin was used as it is often used in previous reports to injure hair cells of chick BP *in vitro* (Stone et al. 1996; Shang et al. 2010). After 1 day recovery (without streptomycin in culture medium), the BPs were fixed with buffered 4 % paraformaldehyde for 4 h at 4 °C. After they were washed with phosphate-buffered saline (PBS), the tegmentum vasculosum was dissected off. The remaining

tissue of BP was used for further study. For each of the studied groups, five to ten BPs were performed.

GTTR Uptake into Hair Cells

Gentamicin (Sigma-Aldrich) was conjugated with Texas Red succinimidyl esters (Molecular Probes, OR) and was purified as described in the literature (Tao and Segil 2015). To prepare the stock solution, GTTR was dissolved in 0.01 M PBS (0.5 mg/mL). GTTR was chosen because it has been used in corresponding studies on cultured mouse cochlea (Alharazneh et al. 2011; Stepanyan et al. 2011). GTTR allows for live imaging of drug uptake into hair cells (Wang and Steyger 2009), and it is not absorbed by supporting cells (Alharazneh et al. 2011).

Freshly obtained BPs (E12–E18), as described above, were transferred into a GTTR working solution (15 μ M, dissolved in 0.01 M PBS). With reference to a previous report (Alharazneh et al. 2011) and our preliminary experiment, the GTTR working solution was chosen from among two other concentrations, i.e., 7.5 μ M and 30 μ M. The other two concentrations demonstrated a rapid (shorter than 10 min at 30 μ M) or slow (beyond 1 h at 7.5 μ M) uptake of GTTR into the hair cells. To avoid entry of GTTR into hair cells via MET channels, BPs were treated with 1, 2-bis (o-aminophenoxy) ethane-N, N, N', N'-tetra-acetic acid (BAPTA, Thermo Fisher, B6769), which was added into the culture media (1 to 10 mM, dissolved in DMSO and diluted with Ca^{2+} -free HBSS (catalog no. 14175, Invitrogen, containing 0.5 mM of MgCl_2). BAPTA has been effectively used in previous reports to disrupt MET transduction, as it breaks tip links of hair cells in mammalian and nonmammalian species, including the chick (Assad et al. 1991; Zhao et al. 1996; Beurg et al. 2006; Stepanyan and Frolenkov 2009; Stepanyan et al. 2011). After the BPs were preincubated in the BAPTA solution for 10 min and washed 2–3 times in HBSS, they were incubated with the above GTTR working solution. The dosages of BAPTA were determined based on a previous report on the chick (Zhao et al. 1996) and our preliminary experiments (for the dose-response curves, five drug concentrations were chosen). Special care was taken to use the identical protocol for all of the samples to be compared. For each group, at least five to ten BPs were used in parallel.

After incubation, the BPs were rinsed with standard HBSS (containing Ca^{2+}) to remove the nonloaded GTTR. The BPs were immersed in 4 % paraformaldehyde for 4 h at 4 °C. After fixation, the BPs were washed several times in PBS, placed onto microscope slides coated with poly-L-lysine, and cover-slipped with antifade solution (Applygen). The amount of GTTR that entered the hair cells was assessed by comparing the fluorescence intensity.

Isolated Hair Cell Culture and Incubation with Mitochondrion Dye

Isolated hair cells were prepared as previously described in the literature (Tao and Segil 2015). Briefly, the freshly dissected BPs were digested with 0.25 % trypsin (Invitrogen) in DMEM at 37 °C for 5 min and incubated with 10 % FBS (Life Technologies) in DMEM to stop the enzymatic digestion. To make a single-cell suspension, the BPs were triturated with a P200 pipette for approximately 5 min. The suspension was centrifuged for 5 min at 2000 r/min. The supernatant was discarded, the cells were resuspended in the fresh culture medium, and the suspension was cultured on the coverslips coated with poly-L-lysine at $1 \times 10^9/\text{L}$ in 6-well culture plates filled with the culture medium (1 mL per well). The cell cultures were incubated at 37 °C with 5 % CO_2 for 24 h. The culture medium was replaced with 15 μ M GTTR working solution and was incubated for 1 h. After three washes with PBS, the cultured cells were incubated with the mitochondrion dye MitoTracker® Deep Red FM (500 nM, dissolved in DMSO) for 30 min at 37 °C with 5 % CO_2 . The concentration of mitochondrion dye and incubation time with the hair cells were determined in our preliminary experiments. After staining, the cells were washed three times with PBS and were fixed in freshly prepared 4 % paraformaldehyde for 30 min at room temperature. After the cultured cells were washed with PBS, they were cover-slipped with antifade solution (Applygen).

Immunohistochemistry

Before all of the immunoreactions, the tissues or cells were inhibited with blocking solution (5 % normal donkey or bovine serum in 0.05 % Triton X-100 in PBS, pH 7.4) for 30 min at room temperature. The following primary and secondary antibodies were used: rabbit anti-myosin VI (1:500, Proteus BioSciences, 25-6791), and Alexa 647-conjugated donkey anti-rabbit IgG (1:200, Jackson ImmunoResearch, cat. 711-606-152). In addition, to detect hair cell stereociliary bundles, direct immunofluorescence was performed with fluoresce-conjugated phalloidin, a label for filamentous actin (5 units/mL, Invitrogen, cat. A12379). The specificity of used antibodies in the chick cochlea was reported previously (Jiang et al. 2016), or confirmed from our staining patterns which were similar to related reports (Heller et al. 2002; Cotanche and Kaiser 2010).

Microscopic Imaging and Quantitative Analysis

The labeled whole-mount BPs and isolated cells were examined and imaged with an inverted Zeiss Axio

Observer Z1 fluorescence microscope or an inverted Zeiss LSM700 laser-scanning confocal microscope under a $\times 20$ objective or $\times 100$ oil objective. Using the LSM software (Carl Zeiss, Germany), the pinhole was adjusted to obtain optical sections of 2 μm thickness. For each hair cell, all of the stacks with 2 μm thickness were taken from the apical to basal planes along the Z-axis to ensure a complete single layer of hair cells to be analyzed, and they were combined into an image. The labeled cells in the combined images were compared among the studied groups. ImageJ and Adobe Photoshop were used to analyze and manage the images.

For age-dependent susceptibility to aminoglycoside ototoxicity, we evaluated labeling with myosin VI (staining for hair cell bodies), phalloidin (staining for stereocilia bundles of hair cells), and DAPI (staining for cell nuclei) along the whole BP. Given that the hair cell body remains despite stereocilia bundle loss after gentamicin treatment (Gale et al. 2002), the disappearance of both myosin VI-labeled cell body and phalloidin-labeled stereocilia bundle of hair cells was used for quantifying the hair cell loss. The whole-mount BP was divided into three parts (proximal, middle, and distal). The examined measurements (such as cell damage or cell labeling) were quantified in three square ($350 \times 350 \mu\text{m}$) regions along the proximal-distal axis (1/4, 2/4, and 3/4 from the proximal end) and regarded as the representative values for the proximal, middle, and distal parts, respectively (Fig. 1A, B). The values were compared among the studied groups. To examine possible gradients in the widthwise direction (from neural to abneural), the regions examined were further divided into neural and abneural halves (Fig. 1A, B).

For GTTR loading, the subdivision of BP and the sampling methods were similar to those described above, and the amount of GTTR taken up by the hair cells was assessed via the intensity of GTTR fluorescence (excited at 532 nm), the pixels in the ROIs (ImageJ, NIH). After the background intensities were subtracted, the fluorescence intensities for at least 50 hair cells in each area examined were averaged. The averaged values were compared among the studied groups. We chose only hair cells that were labeled with myosin VI or phalloidin and that had normal epithelial-like morphology for data analysis.

All data are presented as the mean $\pm$ standard deviation (SD). Before statistical analysis, the distributions of dependent variables were tested for normality (Tukey's hoc tests). One-way ANOVA and Student's *t* test were used to compare the differences of examined measures in the studied groups. Differences were considered either nonsignificant ($p > 0.05$) or significant at various levels ($*p < 0.05$ and $**p < 0.01$).

RESULTS

Survival of Hair Cells in the Cultured Embryonic Chicken BP After Treatment with Streptomycin

After incubation with streptomycin for 2 days, the hair cells labeled with myosin VI were almost intact in the three parts of the BP (proximal, middle, and distal) at E12 and E14 and in the distal part at E16 (Fig. 2 for E14, not shown for E12 and E16). Hair cells were almost all injured and lost in the proximal part of the BP at E18 after treatment with streptomycin (Fig. 3F1), but they were only moderately damaged, and quite a number of hair cells (myosin VI+) still remained in the middle and distal parts of the BP (Fig. 3B1, D1). In addition, phalloidin staining for stereociliary bundles indicated that the orientation or distribution of the stereociliary bundles of hair cells in the BP at E12 or E14 and in the distal part of the BP at E16 did not appear to change between the streptomycin-treated and control groups (Fig. 2A2–F2). However, the stereociliary bundles were almost all lost in the other streptomycin-treated groups (Fig. 3A2–F2). In contrast, DAPI staining did not show notable differences in staining intensity or patterns, and there was a lack of marked apoptotic bodies among all of the groups studied (Figs. 2A3–F3 and 3A3–F3).

Because DAPI was used to label the nuclei of all BP cells, a distinction between neural and abneural regions was possible according to differences in internuclear spacing and the distribution of nuclei. Thus, the division of the neural and abneural sides was mainly based on the DAPI staining (Figs. 2 and 3). Given that the stacked or combined images were obtained from the apical to basal planes (with 2 μm thickness for each plane) of the hair cells along the Z-axis, the stained nuclei (DAPI+) of the hair cells (myosin VI+) should be included among the nuclei collected by the laser-scanning confocal microscope. Thus, the remained hair cells (myosin VI+) might not be apoptotic (Figs. 2 and 3).

To quantify the streptomycin damage, hair cells in each part of the BP were counted and compared among the streptomycin-treated groups and the control groups (for each group, $n = 4$). The sizes and locations of the sampled areas in the BP for quantity analysis are shown in Fig. 1. All of the hair cells were counted in each of the sampled areas (which covered both the neural and abneural parts of the BP). The densities of the hair cells (the numbers of hair cells relative to the sizes of the regions examined) (cell number per $10,000 \mu\text{m}^2$) in the neural and abneural sides in the control and streptomycin-treated BPs at E16 and E18 are shown in Table 1. We compared the percentage changes in the densities of the hair cells between the

FIG. 1. Low magnification of chick basilar papillae (BP) labeled for myosin VI at embryonic day (E) 18. **A** and **B**: The BPs were incubated in culture media supplemented with 1 % fetal bovine serum for 3 days without streptomycin (**A** No Strep, 3DIV) or were incubated for 2 days with streptomycin addition and recovery for 1 day without streptomycin (**B** 2d + Strep/1 day recovery). Proximal is toward the left and abneural is toward the bottom. For quantitative analysis, the whole BP was divided into three parts (proximal, middle, and distal). In the widthwise direction, each part of the BP was further divided into neural and abneural halves (along the dashed white lines). The

studied measures (such as the cell number for damage) were quantified in three boxed regions ($350 \times 350 \mu\text{m}$) along the proximal-distal axis (1/4, 2/4, and 3/4 from the proximal end). Scale bar = $200 \mu\text{m}$ for **A** and **B**. After a 3-day culture without streptomycin, some hair cells in the proximal part were lost, but they remained intact in the middle and distal parts. In contrast, after treatment with streptomycin for 2 days, besides hair cell loss in the proximal part, some cells in the middle and distal parts were lost. Nevertheless, a large number of hair cells remained in the middle and distal parts, especially in the neural side

abneural and neural regions after treatment with streptomycin, and the results indicated that they decreased more significantly in the abneural region than those in the neural region (except the distal part at E16) (Table 1). These data suggested that more hair cells were damaged and lost in the abneural region than those in the neural region after treatment with streptomycin.

We also examined and compared the densities of the total hair cells (myosin VI+) in the sampled areas (shown in Fig. 1, hair cells in the abneural and neural sides were all counted, but they were not distinguished between the two sides) between the streptomycin treated and control groups in the three parts of the BP from E12 to E18. The results indicated no significant differences between the streptomycin treated and control groups at E12 and E14 (Fig. 2G, H; for E12: proximal, $t(6) = 0.001$, $p = 0.999$; middle, $t(6) = 0.244$, $p = 0.819$; distal, $t(6) = 0.352$, $p = 0.742$); for E14: proximal, $t(6) = 0.221$, $p = 0.837$; middle, $t(6) = 0.234$, $p = 0.852$; distal, $t(6) = 0.253$, $p = 0.872$). However, the densities of the total hair cells indicated significant differences at E16 and E18 except the group in the distal part at E16 (Fig. 3G, H; for E16: proximal, $t(6) = 26.408$, $p = 0.000$; middle, $t(6) = 8.253$, $p = 0.001$; distal, $t(6) = 1.341$, $p = 0.251$; for E18: proximal, $t(6) = 7.140$, $p = 0.002$; middle, $t(6) = 9.80$, $p = 0.001$; distal, $t(6) = 10.948$, $p = 0.007$).

GTTR Uptake and GTTR Localization in Embryonic Chick Hair Cells

After incubation with GTTR for 30 min, we examined and compared the distribution of GTTR fluorescence in the hair cells between the streptomycin treated and control groups from E12 to E18. No hair cells labeled with GTTR fluorescence were clearly distinguished from the background at E12 (Fig. 4A2–C2'). Hair cells labeled with GTTR fluorescence could be clearly identified at E14, but the GTTR fluorescence was not strong. The intensity of GTTR fluorescence increased at E16, and some granules of aggregated GTTR (punctate fluorescence) were observed in the hair cells. At E18, the intensity of the GTTR fluorescence was strongest, and the granules of aggregated GTTR were richly distributed throughout the whole cell (Fig. 5A2–C2'). In addition, phalloidin-stained stereociliary bundles were seen at the top of the hair cells from E12 to E18 (Figs. 4A4–C4' and 5A4–C4'), and the stereociliary bundles were regularly located in the hair cells, even in the proximal part of the BP at E18. These data indicated that the hair cells did not suffer significant injury from incubation with gentamicin-conjugated Texas Red.

The sampled areas for quantitative analysis in the BP are shown in Fig. 1. At least 50 cells were randomly chosen from the abneural and neural sides in each

FIG. 2. Chick basilar papillae (BP) at embryonic day (E) 14 after a 2-day culture with streptomycin (Str) and a 1-day recovery. The BPs, divided into distal (A1–B4), middle (C1–D4), and proximal (E4–F4), were labeled for myosin VI (A1–F1), phalloidin (A2–F2), and DAPI (A3–F3), and the merged images are shown in A4–F4. Streptomycin-treated BPs showed no differences compared with the control. Scale

bar=100 μm for A1–F4. The positions of the presented images in the BP are approximately indicated in the boxed areas in Fig. 1. The orientation for neural (N) or distal (D) is shown in F4 for A1–F4. G and H, comparison of the densities of hair cells in the proximal, middle, and distal parts between the Str-treated and control (treated with sterile saline) groups at E12 and E14. Error bars=SD

FIG. 3. Chick basilar papillae (BP) at embryonic day (E) 18 after a 2-day culture with streptomycin (Str) and a 1-day recovery. The BPs, divided into distal (A1–B4), middle (C1–D4), and proximal (E4–F4), were labeled for myosin VI (A1–F1), phalloidin (A2–F2), and DAPI (A3–F3), and the merged images are shown in A4–F4. Compared to the control BPs, phalloidin stained stereocilia bundle of hair cells were lost in the three parts of the streptomycin treated BPs, and hair cells labeled for myosin VI were almost all lost in the proximal part of BP, but a large number

of hair cells remained in the middle and distal parts. Scale bar=100 μm for A1–F4. The positions of the presented images in the BP are approximately indicated in the boxed areas in Fig. 1. The orientation for neural (N) or distal (D) is shown in A4 to F4. G and H, comparison of the densities of hair cells in the proximal, middle, and distal parts between the Str-treated and control (treated with sterile saline) groups at E16 and E18. Error bars=SD. Significant differences in the hair cell density are indicated by “**” ($p < 0.05$) or “***” ($p < 0.01$)

TABLE 1

Comparison of the percentage changes in the densities of the hair cells (number per $10,000 \mu\text{m}^2$) between the abneural and the neural regions after treatment with streptomycin*

Ages	Groups	Proximal		Middle		Distal	
		Neural	Abneural	Neural	Abneural	Neural	Abneural
E16	Control	46.52 $\pm$ 5.05	38.57 $\pm$ 7.64	116 $\pm$ 6.55	71.44 $\pm$ 12.23	132.33 $\pm$ 2.51	80.35 $\pm$ 0.48
	Streptomycin	47.65 $\pm$ 3.85	17.23 $\pm$ 2.08	70.83 $\pm$ 3.33	28.32 $\pm$ 3.73	115.98 $\pm$ 8.82	67.66 $\pm$ 7.80
	Percentage change	2.4 $\pm$ 0.66	-54.33 $\pm$ 7.73	-38.76 $\pm$ 5.18	-56.54 $\pm$ 2.53	-12.35 $\pm$ 7.06	-15.79 $\pm$ 9.21
	Student's <i>t</i> test	<i>t</i> (6) = 7.139, <i>P</i> = 0.002**		<i>t</i> (6) = 5.331, <i>P</i> = 0.006**		<i>t</i> (6) = 0.522, <i>P</i> = 0.629	
E18	Control	61.53 $\pm$ 2.66	47 $\pm$ 2.00	70.01 $\pm$ 2.09	55 $\pm$ 2.65	118.02 $\pm$ 9.05	84.76 $\pm$ 5.80
	Streptomycin	6.56 $\pm$ 0.67	2.73 $\pm$ 0.45	32.84 $\pm$ 2.73	11.63 $\pm$ 1.54	57.70 $\pm$ 5.73	26.66 $\pm$ 3.39
	Percentage change	-89.32 $\pm$ 1.20	-94.19 $\pm$ 0.89	-52.98 $\pm$ 5.24	-78.90 $\pm$ 1.88	-50.75 $\pm$ 7.58	-68.55 $\pm$ 3.14
	Student's <i>t</i> test	<i>t</i> (6) = 5.649, <i>P</i> = 0.005**		<i>t</i> (6) = 8.054, <i>P</i> = 0.001**		<i>t</i> (6) = 3.755, <i>P</i> = 0.02*	

*The chick basilar papillae at embryonic day (E) 16 and 18 were cultured with 78 μM streptomycin or the sterile saline (control). The basilar papilla was divided into proximal, middle and distal parts. **Percentage change = [(Streptomycin-Control)/(Control)]*100. For each group, *n* = 4

part of the BP, respectively (for each group, *n* = 4–6 BPs), and the intensities of GTTR fluorescence (after the background intensities were subtracted) in the hair cells were compared between the neural and abneural sides of BP. They did not show any significant differences from E12 to E16, except those in the distal part at E16 (Fig. 4F *t* (4) = -3.665, *p* = 0.042). However, they were significantly higher in the abneural side than in the neural side in the middle (*t* (6) = -3.804, *p* = 0.041), and distal (*t* (6) = -3.677, *p* = 0.032) parts at E18 (Fig. 5D). After the intensities of GTTR in the abneural and neural sides were combined (hair cells in the examined regions were all counted together, and they were not distinguished between the abneural and neural sides), we analyzed the changes of the combined values with age. As shown in Fig. 5E, the fluorescence intensities of GTTR entered the hair cells in each part of the BP increased significantly from E12 to E18 (proximal: *F* (3, 20) = 129.4, *p* < 0.0001; middle: *F* (3, 20) = 1104.7, *p* < 0.0001; distal: *F* (3, 20) = 52.54, *p* < 0.0001), and the fluorescence intensities of GTTR in the proximal part of BP were higher than those in the distal part at E16 or in the middle and distal parts at E18 (*p* < 0.01).

Given that the mitochondrion is an important target organelle for aminoglycoside ototoxicity (Sugahara et al. 2006; Warchol 2010), we determined whether the GTTR that entered the hair cells (myosin VI+) was colocalized with the mitochondria. As shown in Fig. 6, the mitochondria were specifically labeled with a dye, MitoTracker® Deep Red FM (500 nM, stained for 30 min). At E14, diffuse GTTR fluorescence (no granules of aggregated GTTR) was observed in hair cells with weak GTTR fluorescence over their nuclei (Fig. 6A1–A6). GTTR fluorescence was obviously stronger at E16 and E18 than at earlier ages, and some granules of aggregated GTTR were observed in the hair cells (Fig. 6B1–C6), as described above (Fig. 5A2–C2').

Generally, the stained mitochondria (in green) were not overlapped with the GTTR taken up (in red) (Fig. 6). We examined the percentages of the overlapped areas (in yellow) that were occupied by both mitochondria and the aggregated GTTR to the whole areas occupied by mitochondria within a hair cell, and they were 0 at E14, 1.25 ± 0.75 % at E16, and 2.51 ± 0.93 % at E18 (the number of analyzed cells was ~ 30 for each group).

The Entry Routes of GTTR into Embryonic Hair Cells

To determine the entry routes of GTTR into embryonic hair cells, BAPTA was used to disrupt MET transduction. As the above results indicated that embryonic hair cells were most susceptible to aminoglycoside damage in the

FIG. 4. Uptake of gentamicin-conjugated Texas Red (GTTR) in hair cells in the chick basilar papilla (BP) at embryonic day (E) 12. The BPs were incubated with GTTR (15 μ M) for 30 min at 37 $^{\circ}$ C. The BPs, divided into distal (A1–A5'), middle (B1–B5'), and proximal (C1–C5'), were labeled for myosin VI (A1–C1'), GTTR (A2–C2'), phalloidin (A3–C3'), and DAPI (A4–C4'), and the merged images are shown in A5–C5'. A1'–C5' are the amplified areas boxed in A1–C5. Scale bar in C5 = 100 μ m for A1–C5 and in

C5' = 20 μ m for A1'–C5'. The positions of the presented images in the whole BP are approximately indicated in the boxed areas in Fig. 1. No hair cells labeled for GTTR fluorescence are clearly distinguished from the cell background. D–F, comparison of the fluorescence intensities of GTTR in hair cells in the abneural and neural parts of BP from E12 to E16. Error bars = SD. Significant differences in the intensity of GTTR fluorescence are indicated by *** ($p < 0.05$) or **** ($p < 0.01$).

abneural region of the proximate part of BP, the analysis of the entry routes of GTTR into embryonic hair cells was focused on the abneural region of the proximate part in all of the studied groups. After preincubation of

the hair cells with BAPTA (1.5 mM) for 10 min, and then incubation with GTTR (15 μ M) for 30 min as described above, the fluorescence intensity of GTTR entered into the hair cells decreased obviously in the groups from

FIG. 5. Uptake of gentamicin-conjugated Texas Red (GTTR) in hair cells in the chick basilar papilla (BP) at embryonic day (E) 18. The BPs were incubated with GTTR (15 μ M) for 30 min at 37 $^{\circ}$ C. The BPs, divided into distal (A1–A5'), middle (B1–B5'), and proximal (C1–C5'), were labeled for myosin VI (A1–C1'), GTTR (A2–C2'), phalloidin (A3–C3'), and DAPI (A4–C4'), and the merged images are shown in A5–C5'. A1'–C5' are the amplified areas boxed in A1–C5. Scale bar in C5 = 100 μ m for A1–C5 and in C5' =

20 μ m for A1'–C5'. The positions of the presented images in the whole BP are approximately indicated in the boxed areas in Fig. 1. D, comparison of the fluorescence intensities of GTTR in hair cells in the abneural and neural parts of BP at E18 (D). E, comparison of the fluorescence intensities in hair cells from E12 to E18, when hair cells in the abneural and neural sides were combined and not distinguished. Error bars=SD. Significant differences are indicated by “***” ($p < 0.01$)

E14 to E18, compared to the controls (Fig. 7). Although the fluorescence intensity of GTTR entered the hair cells in the controls varied from E14 to E18, the

decreased intensity of GTTR after preincubation with BAPTA kept a similar low level (approximately 1.5×10^{-2} arb. units).

FIG. 6. The distribution of gentamicin-conjugated Texas Red (GTTR) entered hair cells in relation to that of mitochondria at embryonic days (E) 14 to E18. The isolated cells from 0.25 % trypsin-digested chick basilar papillae were incubated at 37 °C with GTTR for 1 h and were incubated with a mitochondrion dye (MitoTracker® Deep Red FM, 500 nM) for 30 min. The images were acquired using an inverted Zeiss LSM700 laser-scanning confocal microscope under a $\times 100$ oil objective. A1–C1, cell nuclei staining for DAPI. A2–C2,

hair cell staining for myosin VI. A3–C3, mitochondria staining for MitoTracker® Deep Red FM. A4–C4, fluorescence of GTTR that entered the hair cells. A5–C5, merged images for the mitochondrion dye and GTTR fluorescence. A6–C6, merged images from four color channels. The boxed areas in A3–C6 are further amplified in the insert at the top right of the same image. Scale bar in C6 = 5 μ m for A1–C6. Most of the entered GTTR was not observed to be overlapping with the stained mitochondria

DISCUSSION

The present results first indicate that the cultured embryonic hair cells are insensitive to streptomycin damage at E12 or E14 in the whole BP and at E16 in the distal part of the BP. Although some hair cells were injured after treatment with streptomycin for 2 days, a large number of hair cells remained alive in the other studied groups except in the proximal part of BP at E18. These results were in stark contrast with reports that showed that hair cells were completely lost along the whole BP in posthatch chicks 2 days after treatment with streptomycin or gentamicin (Stone et al. 1996; Shang et al. 2010). Our present data are largely consistent with the *in vivo* results, which indicated that hair cells were not damaged following a single injection of gentamicin (400 mg/kg) into the yolk sac at E12 (Jiang et al. 2016). Our results clearly indicated that hair cells at E18 were more susceptible to aminoglycoside damage in the abneural side (Fig. 3B1, D1) or in the proximal region (Fig. 3F1, H). Correspondingly, GTTR entry into the hair cells was not notable in hair cells at E12, weak at E14, and relatively strong at E16 or E18. In addition, the fluorescence intensities of GTTR entered the hair

cells in the abneural side or in the proximal BP at E18 were significantly higher than those in the neural side (except proximal BP) or in the middle/distal BP (Fig. 5). It is not clear why there were no significant differences in the fluorescence intensity of GTTR between the abneural and neural sides in the proximal BP at E18, but it might be because a relatively large amount of GTTR had entered the hair cells from both the abneural and neural sides, and thus any significant differences in the fluorescence intensity of GTTR were not easily detected.

The avian auditory papilla contains two classes of sensory cells, tall hair cells in the neural side and short hair cells in the abneural side. Among these, short hair cells correspond to mammalian outer hair cells, since both are essentially located on the same area of the basilar membrane, and both have a similar pattern of innervation (Tilney and Saunders 1983; Waguespack et al. 2007; Tan et al. 2013). It has been shown that avian short (or mammalian outer) hair cells and basal hair cells are more susceptible to noise, free-radical, or gentamicin damage (Ryals and Rubel 1982, 1985; Corwin and Cotanche 1988; Sha et al. 2001; Alharazneh et al. 2011). These reports are consistent with our results indicating that hair cells

◀ **FIG. 7.** The entry routes of gentamicin-conjugated Texas Red (GTTR) into hair cells in the abneural region of the proximal part of the basilar papilla (BP) at embryonic days (E) 14 to 18. The BPs were treated with BAPTA (1.5 mM) for 10 min and were then incubated with GTTR (15 μ M) for 30 min at 37 °C. They were labeled for myosin VI (A1–F1), GTTR (A2–F2), phalloidin (A3–F3), and DAPI (A4–F4); the merged images are shown in A5–F5. Scale bar= 25 μ m for A1–F5. GTTR fluorescence was obviously reduced after treatment with BAPTA in contrast with the treatment with DMSO (for control). G–I, the fluorescence intensities were compared among the control group and the groups treated with BAPTA. Error bars=SD. Significant differences in the intensity of GTTR fluorescence are indicated by “*” ($p < 0.05$) or “***” ($p < 0.01$)

in the abneural side or in the proximal BP are more susceptible to aminoglycoside damage at E18. It has been proposed that high open probability, MET channel conductance, and low levels of antioxidants in hair cells explain these differential susceptibilities (Sha et al. 2001; Ricci et al. 2003).

In addition, our results have indicated that most GTTR that entered into the hair cells are not colocalized with the mitochondria at any of the studied ages (from E14 to E18). This result might suggest greatly decreased aminoglycoside damage to hair cells (Cunningham et al. 2002). However, the corresponded studies in mammalian hair cells are lacked in the literature; therefore, it needs to be further determined whether the decreased colocalization of mitochondria with aminoglycosides can reduce aminoglycoside damage in embryonic chick hair cells.

After addition of BAPTA into the cultured medium for chick BPs, the fluorescence intensity of GTTR entered hair cells decreased to a similar low level (approximately 1.5×10^{-2} arb. units) from E14 to E18. These data support the view that METs, not Trpa1, are the main transduction channels into hair cells (Kwan et al. 2006; Wu et al. 2016).

It is believed that the perception of sound initiates from the mechanical deflection of the mechanosensitive stereocilia bundle of hair cells and causes ion channels at the bundle tips to open rapidly (Hudspeth 1982; Si et al. 2003). As mentioned above, the transducer apparatus including myosin VIIa has been assembled jointly at E11, and hair cell stereociliary bundles with an orderly hexagonal array have been observed at E12 (Si et al. 2003). In addition, using the whole-cell patch-clamp technique, inward and outward currents were observed in developing hair cells at E12 (Jones et al. 2006). Nevertheless, our results on large entry of GTTR into hair cells did not correlate with the time of assembly of the transducer apparatus nor with the occurrence of the MET transduction current.

Although stereociliary bundles in hair cells were found at E12 by phalloidin labeling, there is a

systematic pruning of microvilli on the apical aspects of the basolateral membrane and a decreasing distance between stereocilia per bundle size from E12 onwards (Si et al. 2003). Responses with sound frequency selectivity only occur from E16 to E18 (Jones et al. 2006), and such frequency selection is related to the fine temporal and precise spatial assembly of the transducer apparatus. Our study demonstrated that the prevention of aminoglycoside injury to chick embryonic hair cells was most likely caused by the limited uptake of aminoglycosides before E18. The age of E18 is therefore a critical time point, for example, a time when the fine temporal and precise spatial assembly of the transducer apparatus is completed or almost completed. Before this time point, hair cells are insensitive to aminoglycoside damage due to the limited amount of aminoglycoside entry into hair cells.

Although the molecular components of the mechanotransduction apparatus are highly similar between mammals and birds (Kurima et al. 2002; Vreugde et al. 2002; LeMasurier and Gillespie 2005; Gillespie and Müller 2009; Pepermans and Petit 2015), the mature mechanotransduction apparatus and mature MET currents in mice occur between P6 and P14 (Bryant et al. 2003; Housley et al. 2006; Waguespack et al. 2007). However, it should be noted that some studies have indicated a profound ototoxic effect on hair cell function after treatment with aminoglycosides during the last 15 days of gestation in guinea pigs (Dumas and Charachon 1982; Wang and Liou 1994) or 1 day after birth in cats and rodents (Shepherd and Martin 1995; Henley and Rybak 1995). In addition, hearing loss has been reported at an early age in children following aminoglycoside treatment during pregnancy (Ganguin and Rempt 1970; Prazić and Salaj 1975; Donald et al. 1991). These data indicate that hair cell death caused by aminoglycoside ototoxicity occurs before the mechanotransduction apparatus matures in mice (Lim and Anniko 1985; Housley et al. 2006). In contrast, death occurs only after the mechanotransduction apparatus is fully assembled or well matured (around E18) in the chick, as discussed above. These differences may reflect the differences in molecular constitution and physiological properties of the mechanotransduction apparatus between mammals and birds.

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COMPLIANCE WITH ETHICAL STANDARDS

Conflict of Interest The authors declare that they have no competing interests.

Abbreviations: BP – Basilar papilla; GTTR – Gentamicin-conjugated Texas Red; MET – Mechano-electrical transduction; TRPA1 – Transient receptor potential (TRP) ion channel 1; BAPTA-2-bis (o-aminophenoxy) ethane-N, N, N', N'-tetra-acetic acid; FM1-43 – (N- (3-triethylammoniumpropyl)- 4- (4-(dibutylamino) styryl) pyridiniumdibromide

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