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Topographical and drug specific sensitivity of hair cells of the zebrafish larvae to aminoglycoside-induced toxicity

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ABSTRACT

The hair cells of the lateral line system of fishes are morphologically and physiologically similar to the hair cells of the mammalian inner ear, also sharing its molecular characteristics. For this reason, it has been used as a powerful animal model to analyze *in vivo* ototoxicity. In this work, we examined the dose-dependent effects of two potent ototoxic aminoglycosides, neomycin and gentamicin, on the hair cells of two selected neuromasts (L1 and T1, the first of the trunk and the terminal located in the fin, respectively) of the lateral line in the ET4 transgenic zebrafish line. The hair cells of this strain selectively and constitutively display fluorescence. The fish were treated for 24 h at different doses (1, 2.5, 5, 10 and 100 μM levels) of both aminoglycosides. Immediately after treatment the morphology and the number of cells in L1 and T were analyzed under a fluorescence microscope. The results show that neomycin and gentamicin have different effects on the hair cell death at the same concentration, showing also different toxicity in L1 and T1 neuromasts. The toxicity observed in the hair cells of T1 neuromast was less than in L1 especially for the gentamicin treatment. These results demonstrate different sensitivity of hair cells of the lateral line to ototoxic drugs according to topographical localization and suggest the *in vivo* assay of the L1 neuromast of zebrafish larva and low doses of neomycin as an ideal model to study ototoxicity induced by aminoglycosides.

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1. Introduction

The damage of hair cells in the inner ear, due to environmental or genetic factors, results in hearing loss or even deafness and balance disorders. Among the external factors, environmental toxins, exposure to several chemical compounds or drugs, and high sound levels should be considered (Namdaran et al., 2012). After injury, adult mammals, including Homo sapiens, do not replace or regenerate injured hair cells (Oesterle and Stone, 2008; Warchol, 2010). Conversely, aquatic vertebrates replace lost hair cells in response to environmental injuries. So, fish models, especially zebrafish, are currently used as a model to study both the normality of the inner ear and the pathophysiology of hearing defects (Whitfield, 2002).

The zebrafish is a powerful animal model for quantitatively and qualitatively studies on hair cells *in vivo* (Ou et al., 2010, 2012). This small teleost, like all aquatic vertebrates, has hair cells on the body surface forming a sensory system called the lateral line system (LLS) (Coombs et al., 1989; Ghysen and Dambly-Chaudière, 2007). The functional units of the lateral line are the neuromasts which contain several sensory hair cells surrounded by supporting cells and mantle cells (Fig. 1). The lateral line hair cells are developmentally, morphologically, and physiologically similar to the hair cells of the mammalian inner ear and share many of their characteristics and molecular constituents; interestingly, inactivation of genes affecting human hereditary deafness also cause loss of hair cell function in zebrafish (Coombs et al., 1989; Coffin et al., 2004; Nicolson, 2005).

It is well known that aminoglycosides, such as gentamicin and neomycin, cause irreversible ototoxicity, gentamicin being primarily vestibulotoxic and neomycin cochleotoxic (Selimoglu, 2007). Similarly, both aminoglycosides also cause death of zebrafish neuromast hair cells with a dose-dependent difference in damage (Froehlicher et al., 2009; Owens et al., 2009), and *via* multiple processes that form an interactive network of cell death signaling cascades involving mitochondrial p53 of Bax and Bcl2 (Coffin et al., 2013a,b).

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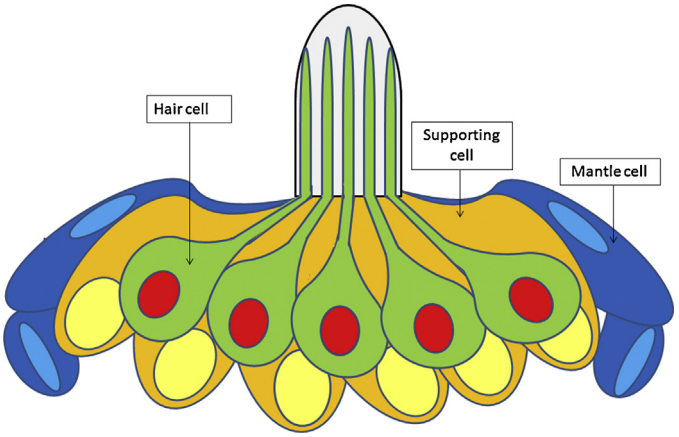

Fig. 1. Schematic representation of the structure of a neuromast. Neuromast consists of a cluster of pear-shaped sensory cells (hair cells) surrounded by long, slender supporting cells and laterally by the mantle cell. The kinocilia and stereocilia of the hair cells project into a jellylike substance (cupola) that bends in response to water displacement.

In zebrafish, the LLS develops in a stereotyped way resulting in a regular and constant position of the neuromasts along the head and body of the animal (Raible and Kruse, 2000; Ghysen and Dambly-Chaudière, 2004). Moreover, in the zebrafish larva, the number of superficial neuromasts is relatively few, easy to observe and readily accessible to manipulation; thus, most studies on hair cells toxicity have been carried out on zebrafish larva (Buck et al., 2012; Mackenzie and Raible, 2012; Ou et al., 2010, 2012; Vlasits et al., 2012). The stereotyped arrangement of neuromasts makes LLS particularly interesting in studying hair cells because the expected location of each neuromast is known, thus allowing for detection of missing neuromasts or the alterations in hair cells within them that can be quickly identified.

In the present research we have sought to determine the minimal dose of the aminoglycosides gentamicin and neomycin able to induce maximal toxicity and whether or not these aminoglycosides have identical effects in neuromasts, or whether these effects depend on their position within LLS. Neuromasts from the trunk were selected for this study: the anteriorly positioned L1 and T at the tail. The main goal of the study was to provide an experimental model of ototoxicity using the hair cells of the LLS as a model, sampling one unique neuromast and one unique toxic.

2. Materials and methods

The transgenic strain ET4 of zebrafish (Parinov et al., 2004) was used in this study. The animals were kindly provided by Prof. A.J. Hudspeth (Laboratory of Sensory Neuroscience, Rockefeller University, NY, USA) and bred in the fish facility of the CISS (Centro di Ittiopatologia Sperimentale per la Sicilia) of the University of Messina under standard conditions (for details see Germanà et al., 2010). Naturally spawned eggs were collected according to Kimmel et al. (1995), cleaned, and maintained in system water at 28.5 °C at a density of 50 per 85-mm Petri dish.

Six hundred ET4 zebrafish larvae of 6 days post fertilization (dpf) were divided into groups of 40 animals each, and exposed to different doses of gentamicin and neomycin for 24 h. Gentamicin sulphate (Fluka, G0200000) and neomycin sulphate (Sigma, N6386) were added to 6 ml of embryo medium [1 mM $MgSO_4$, 120 mM KH_2PO_4, 74 mM Na_2HPO_4 1 mM $CaCl_2$, 500 mM KCl, 15 mM NaCl, and 500 mM $NaHCO_3$ in dH_2O] in each well tissue culture plate to make final concentrations of 0, 1, 2.5, 5, 10, 100 μM, and incubated for 24 h in room incubator. After treatment, the larvae were rinsed three times quickly in fresh medium to remove the antibiotics and placed in fresh medium.

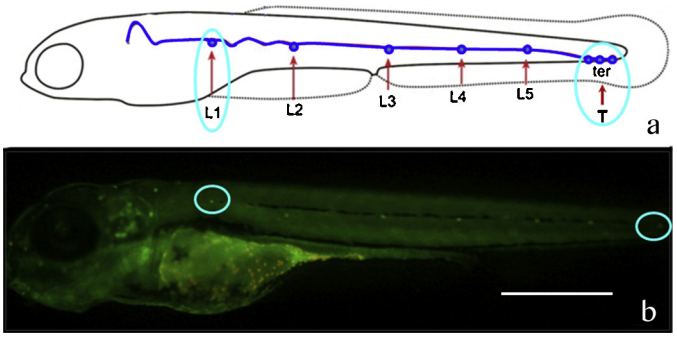

Fig. 2. (a) Scheme of the posterior lateral line in zebrafish larva at 6 days post fertilization (dpf) showing the first neuromast of the trunk (denominated L1) and the terminal one (denominated T). (b) ET4 zebrafish larva at 6 dpf showing encircled L1 and T neuromasts (scale bar 1 cm).

Visualization and counting of hair cells was achieved within 1 h of the endpoint time of drug incubation. In each larva, the number of fluorescent hair cells was counted under fluorescence microscopy Spinning Disk (Leica DMI 6000B) within two specific neuromasts: L1 (first of the posterior lateral line) and T (terminal) (Fig. 2). The total number of surviving hair cells was compared among control and experimental groups, among different doses of the same compound, and among identical doses of the two compounds tested.

Each experiment was performed at least three times independently, and all values are presented as mean ± standard deviation of triplicates. Quantifications of the green fluorescent protein positive hair cells positive are represented as percentage of the standard deviation. For each aminoglycoside and neuromast, averages for control and treated larvae were compared within each group. The same statistical analysis was used to compare among control groups with L1 neomycin and gentamicin treatment and groups of controls with T neomycin and gentamicin. To compare each condition, a one-way ANOVA was used to analyze for statistical significance. Values of $P \leq 0.05$ were considered to be significant.

3. Results

In the lateral line system of 6 dpf ET4 zebrafish larvae, the neuromasts L1 and T (Fig. 2) were analyzed to investigate the effects after 24 h of exposition to different doses, from 1 to 100 μM, of two ototoxic aminoglycosides (gentamicin and neomycin). The number of surviving hair cells in these two selected neuromasts was assessed by counting the fluorescent hair cells.

Typically, in the green fluorescent protein positive cells of the untreated control, neuromasts were arranged as a rosette formed by approximately 12 hair cells. The number of surviving hair cells decreased in both L1 and T neuromast cells and also decreased in a dose-dependent manner for both gentamicin and neomycin (Fig. 3). After treatment with neomycin, and in comparison with the control, the percentages of surviving hair cells in L1 decreased progressively and significantly ($P < 0.05$) to values of 65%, 50%, 47%, 24%, and 16% with concentrations of 1 μM, 2.5 μM, 5 μM, 10 μM and 100 μM, respectively (Fig. 4a). In the T neuromast, the percentage of survival cells was higher than in L1, the values being 65%, 59%, 51%, 36%, and 27% with concentrations of 1 μM, 2.5 μM, 5 μM, 10 μM and 100 μM, respectively. Dose-dependent hair cell loss was statistically significant ($P < 0.05$) only at a concentration of 2.5 μM (Fig. 4b).

Regarding gentamicin treatment, the toxic effects were less potent than those observed for neomycin in both neuromasts under consideration. In L1, in comparison with the control, the percentage

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