Gentamicin is ototoxic to all hair cells in the fish lateral line system

William J. Van Trump, Sheryl Coombs, Kyle Duncan, Matthew J. McHenry

Department of Ecology & Evolutionary Biology, 321 Steinhaus Hall, University of California, Irvine, CA 92697, USA
Department of Biological Sciences, J. P. Scott Center for Neuroscience, Mind & Behavior, 434 Life Science Center, Bowling Green State University, Bowling Green, OH 43403, USA

**Abstract**

Hair cells of the lateral line system in fish may differ in their susceptibility to damage by aminoglycoside antibiotics. Gentamicin has been reported to damage hair cells within canal neuromasts, but not those within superficial neuromasts. This finding, based on SEM imaging, indicates a distinction in the physiology of hair cells between the two classes of neuromast. Studies concerned with the individual roles of canal and superficial neuromasts in behavior have taken advantage of this effect in an attempt to selectively disable canal neuromasts without affecting superficial neuromast function. Here we present an experimental test of the hypothesis that canal neuromasts are more vulnerable to gentamicin than superficial neuromasts. We measured the effect of gentamicin exposure on hair cells using vital stains (DASPEI and FM1-43) in the neuromasts of Mexican blind cave fish (*Astyanax fasciatus*) and zebrafish (*Danio rerio*). Contrary to the findings of prior studies that used SEM, gentamicin significantly reduced dye uptake by hair cells of both canal and superficial neuromasts in both species. Therefore, lateral line hair cells of both neuromast types are vulnerable to gentamicin ototoxicity. These findings argue for a re-evaluation of the results of studies that have used gentamicin to differentiate the roles of the two classes of neuromast in fish behavior.

**Keywords:** Aminoglycoside antibiotics, DASPEI, FM1-43

**1. Introduction**

Aminoglycoside antibiotics are ototoxic to hair cells within the inner ear and lateral line system of vertebrates (*Wersäll*, 1960; *Kroese and van den Bercken*, 1982; *Forge and Schacht*, 2000; *Ernst et al.*, 1994). Because these antibiotics provide a cost-effective treatment for bacterial infections, biomedical researchers have investigated whether hair cells may be protected from this ototoxicity (*Forge and Schacht*, 2000). Comparative biologists have utilized aminoglycosides to experimentally ablate the lateral line system to examine its role in behavior (e.g. *Montgomery et al.*, 1997; *Coombs et al.*, 2001). It is therefore of interest to both biomedical and comparative research that some hair cells in the fish lateral line system have been reported to be resistant to damage by the aminoglycoside gentamicin (*Song et al.*, 1995).

Hair cells are found in two classes of lateral line receptor organs, canal neuromasts (CNs) and superficial neuromasts (SNs) (Fig. 1). In both classes, a gelatinous cupula covers the apical surface of the hair cells. The cupula transmits the motion of the surrounding fluid to the underlying hair cells to allow fish to sense water flow (*Dijkgraaf*, 1963). CNs are recessed within channels within the dermal layer and range in diameter from 100 to 500 μm with tens to hundreds of hair cells. SNs reside on the surface of the skin and are smaller (~50 μm in diameter) with around a dozen hair cells (*Münz*, 1989). The two classes are innervated separately and have distinct developmental origins (*Webb and Shirey*, 2003; *Ghysen and Damby-Chaudière*, 2004; *López-Schier and Hudspeth*, 2005; *Nuñez et al.*, 2009). In addition, the response properties of the two receptors differ, which creates two submodalities within the lateral line system. SNs generally are sensitive to low-frequency (<30 Hz) deflections that are representative of water velocity and CNs respond best to high-frequency deflections that are dependent on pressure gradients (*Coombs and van Netten*, 2006). Although these differences can largely be understood as a result of the mechanical filtering properties of the overlying cupula and canal, it remains possible that differences in the physiology of hair cells contribute to their distinct properties.

*Song et al.* (1995) found that the hair cells within CNs are damaged by exposure to gentamicin while those within SNs were not. Although the hair cells of both classes are similar in ultrastructure, scanning electron microscopy (SEM) revealed that exposure to gentamicin results in damage to the ciliary bundles of only the hair cells within canal neuromasts. This difference in susceptibility has
been used to distinguish hair cell types in fish (Yan et al., 1991) and mammals (Lindeman, 1969). Thus, Song’s result (later replicated by Montgomery et al., 1997; Baker and Montgomery, 1999a; and Coombs et al., 2001 in different species) provided the basis for the hypothesis that the hair cells within SNs are a physiologically distinct type from those within CNs. The goal of the present study was to use vital-staining techniques (Raible and Kruse, 2000; Meyers et al., 2003) to examine the effect of gentamicin on hair cell viability in CNs and SNs.

A number of styryl pyridinium dyes, like DASPEI (2-((4-(dimethylamino)styryl)-N-ethylylpyridinium iodide) and FM1-43 ((n-(3-triethylammoniumpropyl)-4-(4-(dibutylamino)styryl) pyridinium dibromide), allow for the visualization of hair cells in vivo. FM1-43 enters hair cells by rapid entry through the mechanotransduction channels at the stereociliary tips of hair cells (reviewed in Meyers et al., 2003). When the mechanotransduction channels are blocked or otherwise compromised, this dye fails to permeate the cell; as such, dye uptake indicates functioning hair cells. Similarities in conformation and labeling behavior make it likely that DASPEI is also rapidly taken up through the mechanotransduction channels. FM1-43 has an additional advantage in that it will fluoresce much longer than DASPEI (e.g. over many days, Meyers et al., 2003). Thus, FM1-43 can be applied shortly before drug treatment and then visualized afterwards to determine if the hair cells survive the treatment.

In this study, the effects of gentamicin on the lateral line system were explored in zebrafish (Danio rerio) and the Mexican blind cave fish (Astyanax fasciatus). The zebrafish has emerged as a major model for studying the effects of aminoglycoside antibiotics on hair cells (Harris et al., 2003; Murakami et al., 2003; Santos et al., 2006; Owens et al., 2007; Chiu et al., 2008). The adults have little pigmentation in the head, which allows canal neuromasts to be visualized. The Mexican blind cave fish (also known as Astyanax mexicanus) completely lacks pigmentation, has thousands of large neuromasts, and is a common subject for studying the role of the lateral line system in hair cells (e.g. von Campenhausen et al., 1981; Weissert and von Campenhause, 1981; Tyke 1990; Baker and Montgomery, 1999a; Sharma et al., 2009). Additionally, cave fish have been used in studies of hair cell damage and recovery (Repass and Watson, 2001; Berg and Watson, 2002). Therefore, both of these species are ideal animal models for investigation of the effects of gentamicin on hair cells within the lateral line.

2. Materials and methods

2.1. Animals

Experiments were conducted independently at Bowling Green State University with Mexican blind cave fish and at the University of California at Irvine with zebrafish. Fish at both locations were maintained with standard techniques for these species. Mexican blind cave fish (standard length: 4–6.7 cm) were obtained from commercial aquarium suppliers and maintained in freshwater at 20–25 °C in darkness. Zebrafish (AB line, standard length: 2.7–3.5 cm) were raised in a colony in the lab of MJM, where they were maintained at 27–29 °C on a 14/10 h light/dark cycle. The Institutional Animal Care and Use Committees of both universities approved all protocols for experiments and fish care.

2.2. Gentamicin treatment

The effect of gentamicin on the number of neuromasts was assessed by a comparison between treated and control groups. Fish within both groups were handled to the same degree and were placed in a well-aerated aquarium for 24 h. During this period, the control group was exposed to standard tank water, whereas the water for the treatment group included a 0.001% concentration of gentamicin sulfate (Fisher Scientific, Pittsburgh, PA). This treatment was a lower concentration and a shorter exposure than in previous studies (Song et al., 1995 used 0.002% for 4 days) because we learned from a pilot study that a 0.002% gentamicin concentration prevented dye labeling in all neuromasts in both species within 24 h of exposure. Our 0.001% treatment assured that some neuromasts would still label, thus permitting a qualitative comparison between CNs and SNs and maximizing the potential for observing differential effects.

2.3. Vital dye labeling

Two vital stains were used to determine the presence of viable hair cells within neuromasts. Both stains were applied by exposing animals to a bath solution of dye under darkened conditions. Treatment and control fish of both species were stained with 0.008% DASPEI (Invitrogen Molecular Probes, Eugene, OR) solution for 10 min (Mexican blind cave fish) or 1 h (zebrafish) immediately following the 24 h gentamicin or sham exposure. In order to
determine if measured differences in DASPEI staining after gentamicin exposure resulted from damage to the integrity of the hair cell or a mere blockage of transduction channels, we stained additional groups of control and treated zebrafish to 3 μM FM1-43 (Invitrogen Molecular Probes, Eugene, OR) 1 h prior to the gentamicin or sham treatment. This stain allowed us to measure the number of pretreatment cells that survived gentamicin exposure.

2.4. Imaging

All imaging was performed on adult fish anesthetized with 200 mg l⁻¹ tricaine methanesulfonate (i.e. MS-222 or Finquel; Argent Labs, Redmond, WA). Neuramets were observed under epifluorescent illumination. Observations on Mexican blind cave fish were conducted on a stereomicroscope (Olympus SXZ12, New Hyde Park, NY) with a GFP filter set (excitation 450–490 nm and barrier 515 nm) to visualize DASPEI. Photographs of these fish were captured with a CCD camera (Olympus Q Color-3 with Image Pro Software, V. 6). A similar microscope (Zeiss Discovery V.20 with GFP filter set and an AxioCam HRC camera, Carl Zeiss, Thornwood, NY) was used to visualize DASPEI in zebrafish. FM1–43 was made visible on this microscope using the same filter set. All observations were made within 1.5 h of the completion of the 24 h treatment.

2.5. Counting neuromasts

Three methods for counting neuromasts were used due to differences in microscope optics, the degree of pigmentation in the two species, and the specificity of the two stains. Individual hair cells were most visible by DASPEI staining in zebrafish, which allowed us to differentiate brightly stained, presumably active hair cells, from weakly stained inactive hair cells and surrounding support cells (as in Harris et al., 2003). A zebrafish neuromast stained with DASPEI was scored as “viable” if it possessed more than two brightly stained hair cells, “damaged” if it included one or two brightly stained hair cells, or “non-viable” if no brightly stained cells could be identified. Individual hair cells were not visible in DASPEI-stained cavefish and we consequently counted every neuromast with visible labeling without differentiating the intensity of labeling. Therefore, neuromast counts for the blind cave fish included both weakly and strongly labeled neuromasts with varying numbers of viable hair cells. We similarly counted all identifiable neuromasts within a specified region in FM1–43 stained zebrafish because it was difficult to obtain reliable hair cell counts at 24 h following labeling. This was due to diffusion of the dye resulting in the labeling of nearby cells around the neuromasts. However, neuromasts were clearly identifiable under high magnification, as observed previously (Santos et al., 2006).

Neuramets from all over the body were included in our counts. For DASPEI-stained Mexican blind cave fish, CNs were sampled from the supraorbital, infraorbital, post otic, preopercular, mandibular canals on the head and from the main trunk canal on the body (see Fig. 2). Superficial neuromasts were sampled from the ventral surface of the lower jaw (mandibular tip location) and along the rostral-most portion of the trunk canal from the fifth trunk canal scale through the tenth. SNs from scales dorsal and ventral to each of these trunk scales were also sampled. Because zebrafish possess far fewer neuromasts compared to blind cavefish, we were able to score all visible CNs over the entire body and all SNs on the trunk for both DASPEI and FM1–43 stained fish. On the head, samples of SNs from DASPEI-stained fish were limited to a randomly selected group of 50 because they were more numerous and distributed with greater variability in this region. In FM1–43 stained fish, samples of SNs were restricted to the ventral section of the preopercu-
These results were obtained despite using doses and exposure durations much lower than those previously reported to cause destruction of ciliary bundles in CN, but not SN hair cells (Song et al., 1995). Moreover, this finding is supported by the work of two independent laboratories, using three quantification procedures, with two vital stains, and in two species. These findings have implications for our understanding of aminoglycoside ototoxicity and the behavioral roles of the two submodalities of the lateral line system.

4.1. Ototoxic effects of gentamicin in lateral line hair cells

In contrast to our findings, studies that have visualized neuromasts with SEM reported morphological disruption of hair cells in CNs, but not SNs in three different species: oscar Astronotus ocellatus (Song et al., 1995; Montgomery et al., 1997), Mexican blind cave fish, A. fasciatus (Baker and Montgomery, 1999a) and mottled sculpin, Cottus bairdi, (Coombs et al., 2001). This pattern even persisted for several days following gentamicin.

**Fig. 2.** DASPEI staining of the lateral line system in a Mexican blind cave fish from the control (A–E) and treatment (F–J) groups. These photographs were taken from lateral (A, F), and dorsal (B, G) perspectives of the head, a ventral view of the mandible (C, H), a lateral view of the trunk (D, I), and a single trunk canal neuromast (E, J) showing the pattern of weak surround labeling in a treated neuromast (J).
exposure, until CN hair cells eventually regenerated, approximately 10–20 days after the initial gentamicin treatment (Coombs et al., 2001). Therefore, the prior evidence showing a differential effect of gentamicin is compelling. Understanding the discrepancy between vital dye and SEM results requires a consideration of what each method indicates. Gentamicin is capable of disabling a neuromast either by blocking the mechanotransduction channels (Kroese and van den Bercken, 1982; Forge et al., 1989; Moczydlowski et al., 1985; Ernst et al., 1994), or triggering a process that leads to the degeneration of apical cilia, and hair cell apoptosis (Williams et al., 1987; Forge and Schacht 2000). Either mechanism disrupts hair cell function and therefore prohibits DASPEI staining, but only cilia degeneration or apoptosis would generate the observed SEM findings. Therefore, the discrepancy between the present DASPEI staining results and previous findings could be explained if gentamicin blocks the channels of both CN and SN hair cells, but causes cilia degeneration in CNs only. Under such conditions, neither group of hair cells would be labeled by DASPEI and only CN hair cells would appear damaged in SEM. However, our results from FM1-43 staining oppose this interpretation. Neuromasts pre-labeled with FM1-43 were significantly less numerous in the treatment group compared to the control in both CNs and SNs (Figs. 4, 5C, and Table 3). This suggested that in addition to possibly

Fig. 3. The location of weakly labeled superficial neuromasts is visible in an individual zebrafish that was treated with gentamicin and subsequently stained with DASPEI. The stain fluoresces strongly in one or two active hair cells and weakly in surrounding cells within the neuromast. (A) 14 neuromasts are arranged in a vertical series, called a stitch (dashed box). Only one neuromast is revealed to have more than a single active hair cell (arrowhead). (B) A detailed view of two weakly labeled neuromasts illustrates staining of a single (above) and no (below) active hair cells.

Fig. 4. FM1-43 staining of the lateral line system in the head of a zebrafish from the control (A) and treatment (B) groups. These images were produced about 24 h after staining to allow the time necessary for the gentamicin treatment. Note the high contrast of neuromasts in the control fish (A). Staining can be observed in ~18 superficial neuromasts along the operculum (dashed ellipse). In contrast, treated fish (B) retain fewer neuromasts with labeled hair cells.
blocking the mechanotransduction channels, gentamicin exposure leads to loss of hair cells in both types of neuromasts. Although gentamicin is ototoxic to all hair cells in the lateral line system (Fig. 5), developmental differences between CNs and SNs could create different patterns of damage from gentamicin exposure. CNs appear in embryonic development and, unlike SNs, do not increase in numbers as the fish matures (Webb and Shirey, 2003; López-Schier and Hudspeth, 2005; Nuñez et al., 2009). It is therefore likely that an adult fish will possess some SNs that contain a greater proportion of functionally immature and regenerating hair cells than in its CNs. Immature and recently regenerated hair cells are resistant to aminoglycoside antibiotics (Dai et al., 2006; Hashino and Salvi, 1996; Murakami et al., 2003) because they lack a complete compliment of functioning mechanotransduction channels. These channels provide the major means for entry by the antibiotics to damage the cell (Kroese et al., 1989; Marcotti et al., 2005). If gentamicin exposure ceased prior to the maturation of these cells, then SN could appear resistant to gentamicin by virtue of possessing a greater number of immature and regenerating hair cells.

![Graphs of hair cell counts](image-url)

Fig. 5. Comparison of numbers of neuromasts visible with vital staining. For each fish, we included counts of superficial and canal neuromasts as the sum from all regions of the body. The variation among individuals is represented by the boxplots that denote the median (central line), 25th and 75th percentiles (outer margins of box) and the range (whiskers). Counts of canal (A) and superficial (B) neuromasts were visualized with DASPEI in Mexican blind cavefish from both treatment (n = 5) and control (n = 6) groups (Table 1). DASPEI staining in zebrafish revealed a similar difference between treatment (n = 8) and control (n = 8) groups in both canal (C) and superficial (D) neuromasts. These counts included the sum of viable and damaged neuromasts (Table 2). A similar difference was measured between treatment (n = 5) and control (n = 5) fish stained with FM1-43 in both canal (E) and superficial (F) neuromasts (Table 3). In all cases, a two-factor ANOVA found significant differences between the treatment and control groups.

### Table 1

Number of neuromasts stained with DASPEI in Mexican blind cavefish.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Individual</th>
<th>Cranial</th>
<th>Trunk</th>
<th>Mandible</th>
<th>Trunk</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CN</td>
<td>SN</td>
<td>CN</td>
<td>SN</td>
<td>CN</td>
</tr>
<tr>
<td>1</td>
<td>48</td>
<td>32</td>
<td>17</td>
<td>89</td>
<td>1</td>
</tr>
<tr>
<td>2</td>
<td>29</td>
<td>32</td>
<td>14</td>
<td>88</td>
<td>2</td>
</tr>
<tr>
<td>3</td>
<td>6</td>
<td>10</td>
<td>0</td>
<td>22</td>
<td>3</td>
</tr>
<tr>
<td>4</td>
<td>12</td>
<td>26</td>
<td>11</td>
<td>43</td>
<td>4</td>
</tr>
<tr>
<td>5</td>
<td>34</td>
<td>21</td>
<td>18</td>
<td>47</td>
<td>5</td>
</tr>
<tr>
<td>Mean</td>
<td>26</td>
<td>24</td>
<td>12</td>
<td>58</td>
<td>1</td>
</tr>
<tr>
<td>1SD</td>
<td>23</td>
<td>9</td>
<td>7</td>
<td>30</td>
<td>1</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Control</th>
<th>Individual</th>
<th>Cranial</th>
<th>Trunk</th>
<th>Mandible</th>
<th>Trunk</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CN</td>
<td>SN</td>
<td>CN</td>
<td>SN</td>
<td>CN</td>
</tr>
<tr>
<td>1</td>
<td>50</td>
<td>24</td>
<td>30</td>
<td>69</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>46</td>
<td>52</td>
<td>24</td>
<td>107</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>50</td>
<td>48</td>
<td>42</td>
<td>165</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>45</td>
<td>50</td>
<td>29</td>
<td>119</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>52</td>
<td>62</td>
<td>41</td>
<td>124</td>
<td></td>
</tr>
<tr>
<td>Mean</td>
<td>49</td>
<td>45</td>
<td>33</td>
<td>106</td>
<td></td>
</tr>
<tr>
<td>1SD</td>
<td>14</td>
<td>14</td>
<td>7</td>
<td>41</td>
<td></td>
</tr>
</tbody>
</table>

The treatment group was exposed to a gentamicin solution (see Section 2 for details).
4.2. Gentamicin and the role of lateral line submodalities in fish behavior

Gentamicin has been used as a tool for selectively blocking CNs to test the role of this submodality in fish behavior. Our finding that gentamicin is ototoxic to both CNs and SNs is cause for the results of these studies to be re-evaluated. Because gentamicin disables both submodalities, we predict that gentamicin-treated fish should behave similarly to fish treated with other non-selective blockers of lateral line function, such as streptomycin sulfate (Kaus, 1987) or cobalt chloride (Karlsen and Sand, 1987). This prediction is consistent with the results of some previous studies, whereas others require re-interpretation. Here we survey the findings of a few studies that have investigated the relative contributions of CN and SN submodalities by gentamicin treatment.

4.2.1. Object entrainment

Montgomery et al. (2003) found that trout (Oncorhynchus mykiss) entrain their swimming behind a cylinder in flow to a similar degree when treated with either gentamicin or streptomycin. This behavior occurred less often in treated fish than in the control group. The authors concluded that both classes of neuromast contributed to entrained swimming under the assumptions that (1) both submodalities were blocked with streptomycin but (2) only the CN submodality was blocked with gentamicin. Our results suggest that the second assumption is incorrect and that, while the lateral line system contributes to this behavior (as confirmed by Liao, 2006), the relative role of each submodality remains inconclusive.

4.2.2. Rheotaxis

The ability of fish to orient to flow, rheotaxis, has been ascribed exclusively to sensory input from the SN submodality. Ablation of the entire lateral line system by treatment with either cobalt chloride or streptomycin sulfate caused three species of fish (including the Mexican blind cave fish) to be less capable of orienting their body towards flow (Montgomery et al., 1997; Baker and Montgomery, 1999a,b). Despite our prediction that gentamicin should have a similar influence, the number of individuals exhibiting rheotaxis was significantly greater for the gentamicin group than the cobalt chloride or streptomycin treated groups. Furthermore, there were no significant differences between control and gentamicin-treated fish at any flow velocity. Thus, it is difficult to reconcile our current findings and predictions with the gentamicin results of these studies. Nevertheless, the conclusion that rheotaxis is mediated by SNs at low current velocities may still be correct. This is because the physical ablation of SNs revealed that fewer individuals exhibited rheotaxis than in control groups.

4.2.3. Prey capture

Previous studies have suggested distinct roles for the two lateral line submodalities during prey capture. Coombs et al. (2001) found that prey-orienting responses to a vibrating sphere were significantly reduced in gentamicin-treated mottled sculpin (C. baikdi)
References


