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Letter to the Editor

Reevaluating the use of aminoglycoside antibiotics in behavioral studies of the lateral line

Van Trump et al. (2010) recently demonstrated that the aminoglycoside antibiotic gentamicin, previously thought to be selectively toxic to hair cells of the canal neuromasts (CN) of the fish lateral line system, is additionally toxic to hair cells of the superficial neuromasts (SN). The authors used the fluorescent vital dyes DASPEI (2-(4-(dimethylamino)styryl)-*N*-ethylpyridinium iodide) and FM1-43 *n*-(3-triethylammoniumpropyl)-4-(4-(dibutylamino) styryl) pyridinium dibromide to visualize by fluorescence microscopy the survival of hair cells in the CN and SN of zebrafish (*Danio rerio*) and Mexican blind cave fish (*Astyanax fasciatus*) treated in a solution of 0.001% gentamicin sulfate for 24 h. Extensive hair cell death was observed in populations of CN and SN of gentamicin-treated animals of both species compared to control animals. On the basis of this result, the authors concluded, (p. 49), "(1) That hair cells in the SNs of the lateral line can no longer be regarded as functionally resistant to gentamicin toxicity, (2) that this drug should therefore no longer be used as a pharmacological tool for selective blocking of CN, but not SN hair cells, and (3) that the conclusions of some previous studies need to be re-evaluated." Using an alternative approach to themselves reevaluate a 15-year-old assumption in the literature drawn from SEM imaging (e.g., Song et al., 1995), Van Trump et al. (2010) convincingly demonstrated that gentamicin in fact damages a high proportion of both SN and CN, similar in effect to another commonly used aminoglycoside, streptomycin (e.g., Kaus, 1987; Blaxter and Fuiman, 1989; Montgomery et al., 1997, 2003). Toward further caution in the interpretation of lateral line behavioral studies that have used aminoglycoside antibiotics to chemically ablate SN and/or CN without rigorous verification of treatment efficacy, we are inspired to share our own recent observations on *limitations* in the efficacy of both gentamicin and streptomycin in damaging the lateral line systems of three fish species.

The images presented here (Figs. 1–3) are drawn primarily from two projects. Images comprising Fig. 1 were obtained over the course of a series of experiments designed to examine the role of the lateral line system in swimming behaviors of goldfish (*Carassius auratus*) and juvenile steelhead trout (*Oncorhynchus mykiss*). During these experiments, prior to behavioral testing, fish were held for 24 h in 10 L buckets of aerated water containing one of several concentrations of streptomycin sulfate, expected on the basis of previously published reports to chemically ablate both CN and SN (Kaus, 1987; Montgomery et al., 1997, 2003). Following treatment, fish were tested in a behavioral assay, labeled with DASPEI, anesthetized, and examined under a fluorescent dissecting microscope to assess the extent of streptomycin-induced hair cell death. Labeling patterns in streptomycin-treated fish were compared to labeling patterns in untreated fish. Fig. 1A–C display goldfish treated with 0% (untreated), 0.01% and 0.05% streptomycin, respectively; Fig. 1D–F display juvenile steelhead treated at the same concentrations. The 0.05% streptomycin treatment (cf. Montgomery et al., 1997) appeared to ablate both the CN and SN of the goldfish (Fig. 1C), but left a number of partially intact neuromasts (primarily CN) in the steelhead (Fig. 1F). The lower 0.01% concentration left a number of partially or wholly intact CN and SN in animals of both species, as indicated by persistent DASPEI labeling in these animals (Fig. 1B and E). While the mechanism of DASPEI uptake is unknown, possible entry routes include the transduction channel and apical endocytosis, both of which are transduction-dependent processes such that DASPEI will only be taken up by active hair cells (Van Trump et al., 2010; Raible, personal communication). Although DASPEI intensity is an indirect method for hair cell assessment, studies in zebrafish have demonstrated that DASPEI intensity is proportional to direct counts of labeled hair cells (Harris et al., 2003; Coffin et al., 2009). The observed changes in DASPEI labeling at increasing streptomycin concentrations (Fig. 1) are thus consistent with increased but incomplete hair cell loss in *O. mykiss* at 0.05% and in both *O. mykiss* and *C. auratus* at 0.01%.

Images comprising Figs. 2 and 3 were obtained over the course of a project designed to evaluate the role of the lateral line system in sound source localization by a marine species, the plainfin midshipman (*Porichthys notatus*). While an influential theoretical paper published several decades ago posited that the lateral line must be critical for sound source localization by fish (Van Bergeijk, 1964), more recent theories (e.g., the "phase model") have posited that auditory mechanisms of the inner ear may be sufficient for source localization (see Sand and Bleckmann, 2008; Rogers and Zeddies, 2008). Thus, in order to test the hypothesis that fish without a functioning lateral line system would retain the ability to localize a biologically relevant signal emanating from a distant underwater speaker, test animals were treated with gentamicin or streptomycin in an attempt to chemically ablate both CN and SN. Fish were treated in seawater (salinity approximately 23–28 parts per thousand) containing 0.001% gentamicin

Abbreviations: CN, canal neuromast; DASPEI, 2-(4-(dimethylamino) styryl)-*N*-ethylpyridinium iodide; FM1-43, *n*-(3-triethylammoniumpropyl)-4-(4-(dibutylamino)styryl) pyridinium dibromide; SN, superficial neuromast.

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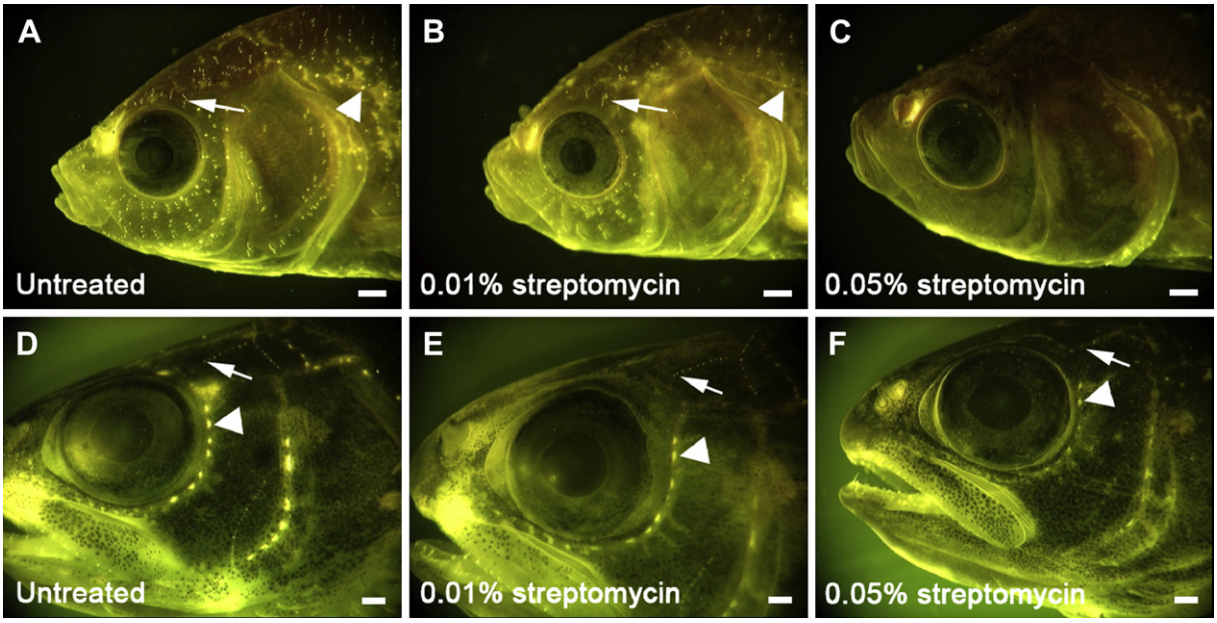

Fig. 1. DASPEI-labeled SN (arrows) and CN (arrowheads) on the heads of goldfish (*Carassius auratus*) (A–C) and steelhead (*Oncorhynchus mykiss*) (D–E). Fish were treated in 10 L buckets of aerated water for 24 h with streptomycin sulfate at concentrations of (A,D) 0% (untreated) (B,E) 0.01%, or (C,F) 0.05%. Higher streptomycin concentrations resulted in increased hair cell death, evidenced by a reduction in the number of labeled neuromasts, although a number of partially intact neuromasts remained in the steelhead at the highest concentration tested (F). All scale bars = 1.0 mm.

sulfate for 24 h (after Van Trump et al., 2010) or 0.05% streptomycin sulfate for 3 h (after Montgomery et al., 1997). As in the goldfish and steelhead experiments, treated and untreated midshipman were then labeled with DASPEI and examined under a fluorescent dissecting microscope to assess the extent of aminoglycoside-induced hair cell death. Fig. 2 displays DASPEI-labeled images of the anterior dorsal trunk lateral line from gentamicin- (Fig. 2A) and streptomycin-treated (Fig. 2B) animals. Extensive neuromast survival was evident in both cases. Although this result was consistent with reduced uptake of aminoglycosides in seawater noted in past investigations (Blaxter and Fuiman, 1989; see below), it was also consistent with the limited efficacy of streptomycin noted above in *O. mykiss* at the same 0.05% concentration (Fig. 1C) and in both *O. mykiss* and *C. auratus* at a lower 0.01% concentration (Fig. 1B and E). We thus subsequently probed the effect of higher aminoglycoside concentrations, treating additional fish in seawater containing doubled concentrations of gentamicin (0.002%) for 24 h or streptomycin (0.1%) for 3 h. As before, extensive neuromast survival was evident (Fig. 2C and D), with 20 or fewer missing or damaged neuromasts (out of several hundred neuromasts total) in each fish examined, regardless of aminoglycoside type or concentration (mean = 11.6, s.d. = 5.5, $n = 8$ fish). Since comorbid nonsensory effects have been associated with exposure to high concentrations of aminoglycosides (Kaus, 1987; see Janssen, 2000; Coffin, personal observation), we elected to abandon aminoglycosides altogether as a means of lateral line ablation, eventually selecting a physical method of ablation – direct application of a liquid nitrogen-chilled probe to SN and surgically exposed CN – to enable the completion of our sound source localization experiments.

Liquid nitrogen ablation of SN has been discussed previously (e.g., Montgomery et al., 2003); extension of the technique to CN in midshipman was straightforward. Briefly, a liquid nitrogen-cooled copper probe (diameter ~1 mm) was applied for ~2 s to each of the well-defined stitches of SN on the head, trunk, and caudal fin of anesthetized midshipman (see Greene, 1899). Cranial lateral line canals were then opened with

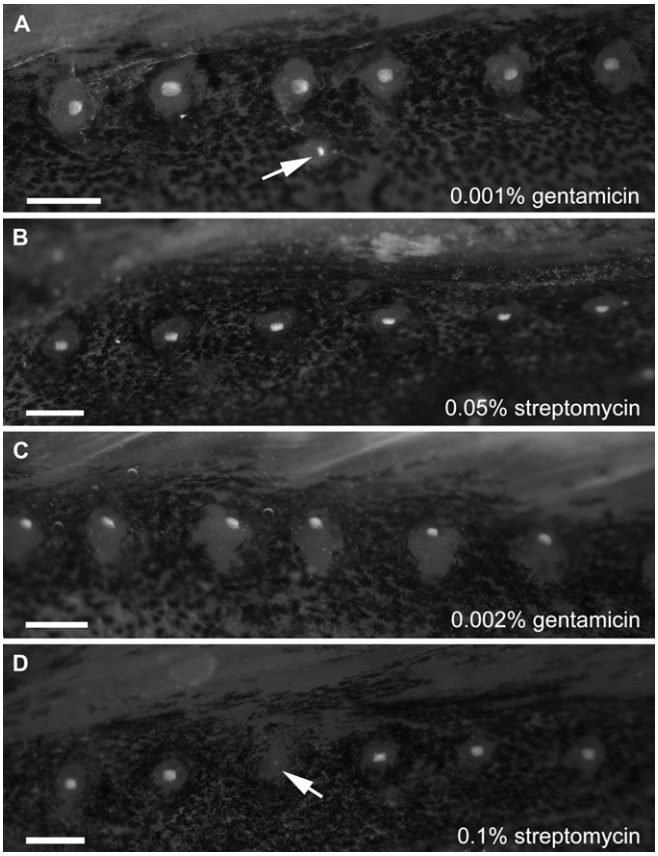

Fig. 2. DASPEI-labeled neuromasts from the dorsal lateral line of plainfin midshipman (*Porichthyus notatus*), just ventral to the dorsal fin. Fish were treated with (A) 0.001% gentamicin sulfate for 24 h, (B) 0.05% streptomycin sulfate for 3 h, (C) 0.002% gentamicin sulfate for 24 h, or (D) 0.1% streptomycin sulfate for 3 h. Neuromasts were largely intact in all animals examined, although some damage was noted (indicated by arrows in A and D). Images were taken with a Leica MZFLIII stereomicroscope and a Leica DFC350FX CCD-cooled camera. All scale bars = 0.5 mm.

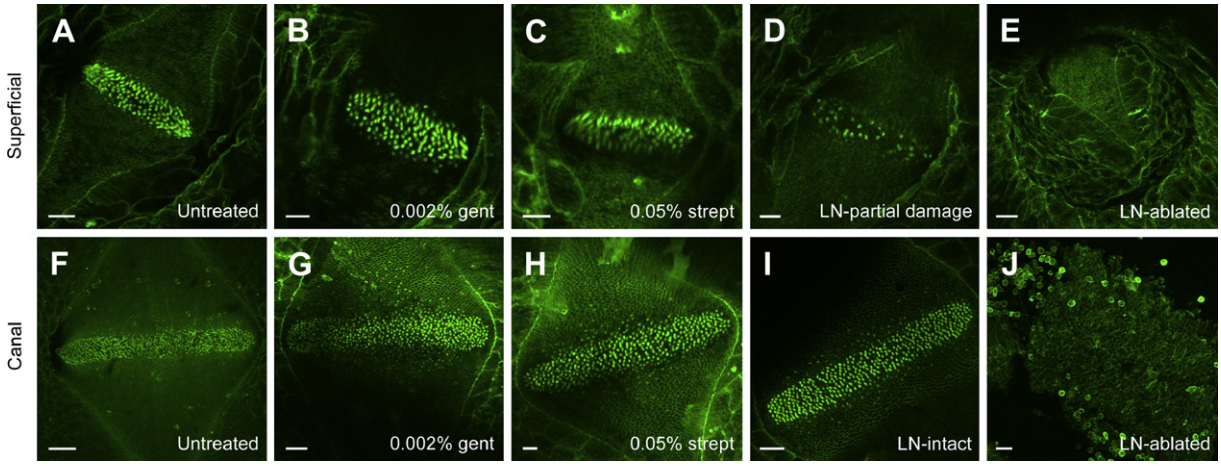

Fig. 3. Confocal brightest-point projections of midshipman SN (A–E) and CN (F–J) labeled with Alexa 488 phalloidin, which labels the actin-rich hair bundles at the apical end of each hair cell. (A) Intact SN from an untreated fish, (B) SN treated with 0.002% gentamicin are largely intact, as are (C) SN treated with 0.05% streptomycin. (D) SN partially or (E) completely damaged by liquid nitrogen (LN). (F) Intact CN from an untreated fish, (G) intact CN treated with 0.002% gentamicin, (H) intact CN treated with 0.05% streptomycin, (I) intact CN from a fish treated with LN ablation, as an example of a neuromast skipped during the procedure, (J) LN-ablated CN. Scale bars in A, E, and $G = 20$ μm, bars in B, C, D, H, and $J = 10$ μm, bars in F and I = 30 μm.

microdissection scissors and exposed CN were treated with a liquid nitrogen-cooled surgical steel probe (diameter ~0.5 mm). After CN treatment the canals were closed with superglue and the fish was resuscitated by flushing water over the gills until normal righting behavior resumed. For comparison of the pharmacological and physical methods, Fig. 3 displays images obtained by confocal microscopy of phalloidin-labeled SN (Fig. 3A–E) and CN (Fig. 3F–J) hair bundles. Little to no hair bundle damage was observed in any SN or CN exposed to either streptomycin or gentamicin, as demonstrated by SN or CN treated with 0.002% gentamicin (Fig. 3B and G, respectively) or 0.05% streptomycin (Fig. 3C and H, respectively). Physical ablation using liquid nitrogen, in contrast, successfully damaged both SN (Fig. 3E) and CN (Fig. 3J), although neuromasts were occasionally incompletely damaged or spared if the cooled probe was not properly applied (Fig. 3D and I).

While the ototoxicity of aminoglycoside antibiotics in the vertebrate inner ear (Li et al., 1995; Kil et al., 1997; Forge and Li, 2000) and in the neuromasts of the lateral line system of some fish species (e.g., zebrafish: Harris et al., 2003; Ton and Parng, 2005; Coffin et al., 2009; Owens et al., 2009; Van Trump et al., 2010) is well established, rigorous validation of aminoglycoside-induced damage to lateral line hair cells in many model species is lacking. Using a fluorescent vital dye to assess neuromast survival in streptomycin- or gentamicin-treated fish of two freshwater species (juvenile *O. mykiss, C. auratus*) and a marine species (*P. notatus*), we have provided evidence that many lateral line hair cells may survive aminoglycoside exposure. While streptomycin treatment at the higher 0.05% concentration (but not the lower 0.01% concentration) appeared to ablate a majority of hair cells in juvenile steelhead and especially goldfish, the hair cells of the midshipman lateral line appeared extraordinarily resistant to aminoglycoside toxicity. Although previous investigators have noted that hair cell uptake of aminoglycosides may be reduced in seawater by high levels of Ca^{2+} (Blaxter and Fuiman, 1989; see also Coffin et al., 2009), streptomycin has been used in behavioral studies of lateral line function in at least two other marine species (*Clupea harengus*, Blaxter and Fuiman, 1989; *Pagothenia borchgrevinki*, Montgomery et al., 1997). While changes in behavior following streptomycin treatment reported in those studies were attributed to a loss of lateral line function, Janssen (2000) has suggested that behavioral changes observed following pharmacological treatment may be partially attributable to comorbid nonsensory effects; our data indicate that this possibility is worth further consideration.

Although caution is certainly warranted in generalizing our results and those of Van Trump et al. (2010) to other species and treatment paradigms, Van Trump et al.'s (2010) investigation offered a valuable caveat on the importance of rigorous verification of treatment efficacy, which we would underscore: While pharmacological manipulation of the lateral line seems straightforward methodologically , comparative biologists studying lateral line-mediated behavior must understand precisely how – and to what extent – treatment has altered sensory anatomy before any functional inferences should be drawn. In species with thousands of distributed neuromasts (e.g., *C. auratus*) and/or extensive canal systems (e.g., *O. mykiss*), pharmacological treatment with aminoglycosides or other ototoxic agents (e.g., cobalt chloride; but see Janssen, 2000) may prove the only feasible treatment option. In species with localized and accessible SN and CN (e.g., *P. notatus*), however, physical ablation, which avoids the potential comorbid nonsensory effects of aminoglycosides and can provide for a more thorough ablation of hair cells, may prove a more effective alternative.

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