Sub-lethal concentrations of waterborne copper are toxic to lateral line neuromasts in zebrafish (*Danio rerio*)

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Abstract

In teleosts, the lateral line system is composed of neuromasts containing hair cells that are analogous to those present in the inner ear of all vertebrates. In the zebrafish embryo and early larva, this system is composed of the anterior lateral line (ALL), which covers the head, and the posterior lateral line (PLL), present in the trunk and tail. The mechanosensory hair cells found in neuromasts can be labeled in vivo using fluorescent dyes such as 4-di-2-Asp (DiAsp) or FM1-43. We have studied the effects of water-borne copper exposure on the function of the lateral line system in zebrafish larvae. Our results show that transient incubation of post-hatching larvae for 2 h with non-lethal concentrations of copper (1–50 μM $CuSO_4$) induces cellular damage localized to neuromasts, apoptosis, and loss of hair cell markers. This effect is specific to copper, as other metals did not show these effects. Since hair cells in fish can regenerate, we followed the reappearance of viable hair cells in neuromasts after copper removal. In the PLL, we determined that there is a threshold concentration of copper above which regeneration does not occur, whereas, at lower concentrations, the length of time it takes for viable hair cells to reappear is dependent on the amount of copper used during the treatment. The ALL behaves differently though, as regeneration can occur even after treatments with concentrations of copper an order of magnitude higher than the one that irreversibly affects the PLL. Regeneration of hair cells is dependent on cell division within the neuromasts as damage that precludes proliferation prevents reappearance of this cell type.

Keywords: Zebrafish; Lateral line; Mechanosensory cells; Copper toxicity; Hair cell regeneration

1. Introduction

In zebrafish, the lateral line system develops during the early larval stages and consists of a regular array of neuromasts present on the body surface that are in direct contact with the environment (Ghysen and Dambly-Chaudiere, 2004). Within the neuromast, there are mechanosensory cells, called hair cells, surrounded by supporting cells and perhaps other, unidentified cell types. These hair cells are similar to those present in the vertebrate ear and, due to being exposed to the environment, they become readily labeled in live fish with styryl dyes such as 4-Di-2-Asp (DiAsp) and FM1-43 (Collazo et al., 1994; Nishikawa and Sasaki, 1996). The primary mechanism of dye entry remains unresolved however, occurring either by endocytosis (Seiler and Nicolson, 1999; Griesinger et al., 2002) or via the mechanotransduction channels present on the apical surface of hair cells (Gale et al., 2001; Meyers et al., 2003; Corey et al., 2004). Hair cells in the inner ear of mammals are very sensitive to compounds including aminoglycoside antibiotics and anticancer platinum-based drugs (Nakashima et al., 2000; Schweitzer, 1993). Zebrafish lateral line hair cells have also been shown to be susceptible to these drugs (Williams and Holder, 2000; Murakami

Abbreviations: hpf, hours post-fertilization; dpf, days post-fertilization; ALL, anterior lateral line; PLL, posterior lateral line; PCNA, proliferating cell nuclear antigen; GFP, green fluorescent protein; LC50, lethal concentration 50; PI, propidium iodide

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et al., 2003; Harris et al., 2003; Ton and Parng, 2005), further demonstrating functional parallels with hair cells of the mammalian inner ear. In contrast to mammals though, hair cells in anamniotes are able to regenerate after damage, suggesting either the presence of a population of progenitors or a higher degree of plasticity of the supporting cells, which could divide and/or transdifferentiate into hair cells (Balak et al., 1990).

Few studies have addressed the potential toxicity of metal ions in hair cells. Some metals such as cadmium and mercury can block or suppress ion channel activity (Griesinger et al., 2002; Liang et al., 2003). Zebrafish embryos treated with acute levels of cadmium had edema, curved body, and lesions in the exposed epithelia (Blechinger et al., 2002). In that work, a transgenic fish line that contains GFP under the control of a heat shock 70 gene promoter provided information on the cellular stress response induced by the presence of cadmium. Cadmium in the medium elicited expression of the reporter in liver, gills, the pronephric duct and, interestingly, the lateral line (Blechinger et al., 2002). Another report has shown that copper that was transiently present in the water during embryogenesis of fathead minnows adversely affected chemosensation (Carreau and Pyle, 2005). These studies become relevant when considering the amounts of copper present in the aquatic environment. For example, metal-contaminated freshwater bodies can present between 0.5 and 1.5 μM dissolved copper, and this metal has the potential to bioaccumulate (Villavicencio et al., 2005; Vinot and Pihan, 2005; Reash, 2004). A common source of copper in the environment is runoff from agriculture, where compounds such as copper sulfate are used as pesticides (Oliveira-Filho et al., 2004).

Copper is a micronutrient essential for life, yet it can become toxic to many cell types at elevated concentrations. Varying the copper load in the diet can have important consequences on fish physiology (Clearwater et al., 2002), but other experiments have shown that copper can accumulate in fish if it is present in excess amounts in the water (Grosell et al., 2003; Handy, 2003; P.H. and M.A., unpublished results). Our goal is to determine whether there is any consequence to the physiology of post-hatching larvae when exposed to sub-lethal amounts of copper dissolved in water for short periods of time. Our results show that transient exposures of fish to low micromolar concentrations of copper in the growth medium produce no significant effect on gross morphology or survival. However, the lateral line system is extremely sensitive to these treatments.

In this work, we identify the doses and times of copper exposure that cause reversible and irreversible damage to the zebrafish lateral line hair cells. In larvae exposed for 2 h to concentrations greater than 1 μM of $CuSO_4$, we found loss of hair cell markers and loss of labeling with styryl dyes. The capacity of hair cells to regain functionality after the treatment is dose-dependent. In the posterior lateral line, exposure to 50 μM or higher $CuSO_4$ permanently eliminates functional hair cells though the anterior lateral line can regenerate even after treatments with 400 μM $CuSO_4$. This suggests the existence of a copper-sensitive mechanism for replacing damaged or dead hair cells in zebrafish posterior lateral line neuromasts.

2. Materials and methods

2.1. Maintenance of zebrafish and exposure to metals

A breeding colony of the Tübingen wild-type strain of zebrafish (*Danio rerio*) was maintained at 28.5 °C on a 14-h light/10-h dark cycle (Westerfield, 1994). All embryos were collected by natural spawning, staged according to Kimmel et al. (1995) and were raised at 28 °C in E3 medium (5 mM NaCl, 0.17 mM KCl, 0.33 mM $CaCl_2$, 0.33 mM $MgSO_4$, and 0.1% Methylene Blue) in petri dishes (Haffter et al., 1996). We express the larval ages in hours post-fertilization (hpf) or days post-fertilization (dpf). We chose to perform all treatments at 76 hpf since at this stage, the primary lateral line is completely developed and functional (Raible and Kruse, 2000). Copper was added as $CuSO_4$ (Merck, Darmstadt, Germany) to E3 medium, larvae were exposed for the required amount of time and subsequently rinsed a minimum of three times in fresh medium. For testing other metals we used the following salts: $CuSO_4$, $ZnSO_4$, $FeCl_3$, $AgNO_3$, $SnCl_2$, $MnCl_2$, $CoCl_2$, and $CdCl_2$ (Merck Chemicals, Darmstadt, Germany). All protocols involving animals have been reviewed by the Animal Welfare and Ethics Committees of the University of Chile.

2.2. Detection of functional neuromasts

Neuromast hair cells were labeled by incubating the zebrafish larvae in 5 mM 4-(4-diethylaminostyryl)-*N*-methylpyridinium iodide (4-di-2-Asp, DiAsp, Sigma D-3418), or in 2 μM [*N*-(3-triethylammeniumpropyl)-4-(4-[dibutylamino]pyridinium)dibromide] (FM1-43, Molecular Probes T-35356), in E3 medium. Labeled larvae were rinsed several times with E3 medium and anaesthetized with MS222 (3-aminobenzoic acid ethyl ester, methanesolfonate salt, Sigma) for observation under epifluorescence. This technique allowed us to quantify the number of viable hair cells before and after treatment. DiAsp-positive hair cells were counted in two specific neuromasts in each larva: one from the ALL (O) and one from the PLL (P1) (see neuromast nomenclature in Harris et al., 2003).

2.3. Proliferation assays and detection of cell death

Larvae were processed for immunohistochemistry (see below) using a rabbit anti-PCNA (proliferating cell nuclear antigen) antibody (Santa Cruz SC-56, dilution 1/500). Secondary antibody was a goat rhodamine (TRITC)-conjugated anti-rabbit IgG (Jackson Immunoresearch), at a 1/200 dilution.

For detection of dying cells, larvae were stained with the vital dye Acridine Orange (acridinium chloride hemi- (zinc chloride), Sigma A1121), (Abrams et al., 1993). Embryos were incubated for 5 min in 2 μg/ml Acridine Orange, washed in distilled water several times and anaesthetized with MS-222 for observation. For quantification of the effects, Acridine Orange-labeled cells were counted in the O and P1 neuromasts in at least 10 larvae for each condition. For statistical analysis we used one-way ANOVA or the Kruskal-Wallis one-way ANOVA on Ranks test (SigmaStat 3.1 software).

2.4. Regeneration assay

For each experiment, 15 larvae were incubated in Petri dishes with 1, 10, 50 μM $CuSO_4$ or in E3 medium for 2 h. The samples were washed three times with E3 and stained with DiAsp immediately and after 24, 48, 96 and 120 h to check for hair cell viability with DiAsp. The number of anterior lateral line (ALL), posterior lateral line (PLL), secondary posterior lateral line (2PLL), and terminal neuromasts positive for DiAsp staining were counted on one side of each larva. From these values, the mean

and standard deviation were calculated for the different conditions. We counted the number of labeled neuromasts per larva; in these cases, a single labeled hair cell is sufficient to score the neuromast as positive. One side of each larva was counted and a minimum of 15 larvae was scored for each experiment.

2.5. Whole mount immunohistochemistry

Larvae were staged and fixed overnight in 4% paraformaldehyde in phosphate-buffered saline (PBS). Embryos were rinsed three times for 5 min in PBS and then incubated in methanol at −20 °C for 1 h, rinsed two times for 5 min in PBS and washed one time for 1 h in distilled water. The embryos were then incubated in acetone for 7 min, washed one time in distilled water, two times in PBS plus 0.1% Tween 20 (polyoxyethylene-sorbitan monolaurate; Sigma, USA) for 5 min, and transferred to blocking solution (20% lamb serum, 1% dimethylsulfoxide, 0.1% Tween 20 in PBS) for 1 h. Embryos were then incubated overnight at 4 °C with the mouse monoclonal anti-acetylated tubulin antibody (Sigma, USA) used at a 1:1000 dilution. They were then washed four times for 20 min in PBS-Tween 20, incubated for 30 min in blocking solution and incubated overnight at 4 °C in goat anti-mouse IgG, horseradish peroxidase (HRP) linked whole antibody (Molecular Probes G21040), or Alexa Fluor 488-conjugated chicken anti-mouse IgG (Molecular Probes A21200), both diluted 1:200 in blocking solution. They were then rinsed four times for 20 min in PBS plus Tween 20. For the HRP conjugated secondary antibody, the embryos were transferred to peroxidase staining solution (0.03% diaminobenzidine dissolved in PBS) for 30 min and then to peroxidase staining solution with 0.015% H_20_2. After the peroxidase reaction was detected, the embryos were rinsed three times in PBS and photographed.

Live larvae were viewed in a Leica MZ12 dissecting scope or in a Zeiss microscope and photographed using a Magnafire digital camera (Optronix). For fluorescence microscopy, embryos were incubated for 10 min in 10 μg/ml Propidium Iodide in PBS. For visualization of fluorescent antibody stains shown in Fig. 4 we used a confocal microscope (LSM META510, Carl Zeiss) and a Plan-Apochromat 63X/1.4 oil lens. Z stacks were obtained every 6 μm and between 50 and 65 sections were merged for composing the final image. Digital images were handled with the Photoshop 7.0 for Macintosh.

3. Results

3.1. Larval neuromasts are damaged by external copper

In the course of our studies on copper toxicity during zebrafish development, we determined that zebrafish larvae (2–5 days post-fertilization, dpf) do not show any adverse effects when exposed to medium containing $CuSO_4$ at concentrations of 1–50 μM for up to 2 h. The LC50 value we have obtained for 2-h exposures at 72 hpf is reached at 280.16 μM $CuSO_4$ (P.H., Viviana Gallardo, and M.A., unpublished results). Though we failed to detect any morphological abnormalities after this transient exposure to copper, more careful examination of fish incubated in 50 μM $CuSO_4$ showed the presence of moderate tissue damage in the form of a regularly spaced pattern of protrusions arising from the sides of the body, structures never seen in control larvae (Fig. 1A and B). This effect of copper was dose-dependent since higher concentrations of $CuSO_4$ (e.g., 100 μM, Fig. 1C) produced more visible damage. The extruded material appeared to be cellular debris coinciding in position to where the lateral line neuromasts are located in larvae of this age (compare to Fig. 1D).

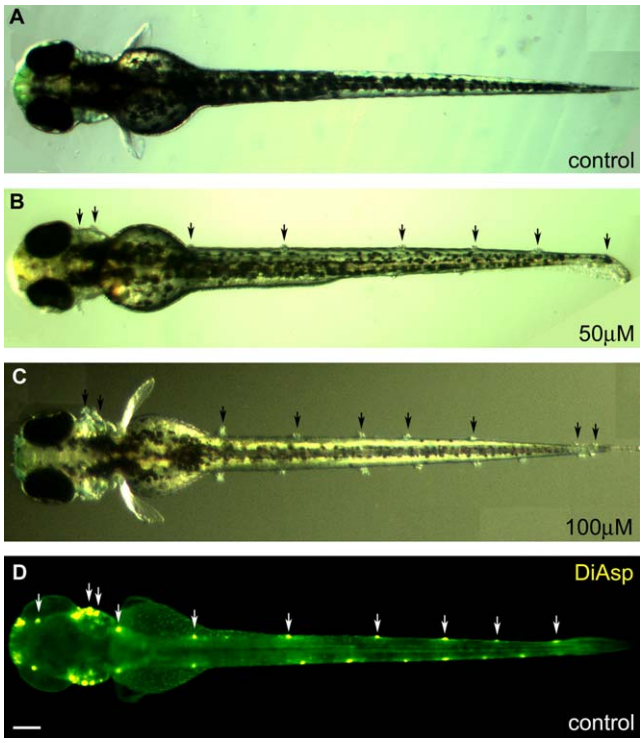

Fig. 1. Copper in the water disrupts neuromasts. Dorsal views of 78 hpf larvae. A, Control without added copper. B, Larva incubated for 2 h in 50 μM $CuSO_4$. C, Larva incubated for 2 h in 100 μM $CuSO_4$. The arrows show damaged neuromasts. D, Distribution of neuromasts in an untreated larva after staining with the vital dye 4-Di-2-Asp (DiAsp). Arrows show intact neuromasts labeled with DiAsp. Bar in D = 35 μm.

To more precisely define the effects of copper on neuromasts, we used the vital dyes DiAsp and FM1-43 to test for hair cell viability after the different treatments with copper, with both dyes producing identical results. Neuromasts in control larvae are readily labeled with DiAsp (Fig. 2A), a method that allows us to detect individual live hair cells. Exposing larvae to $CuSO_4$ dissolved in the water at concentrations beginning at 1 μM for 2 h is sufficient to completely prevent neuromasts from being labeled with DiAsp (Fig. 2B). 50 μM-treated larvae show occasional, non-specific labeling with DiAsp, that is coincident with the structural tissue damage that appears after this treatment (Fig. 2C). When larvae were exposed for 2 h in 0.5 μM $CuSO_4$ there was no observable effect on DiAsp or FM1-43 labeling (data not shown). To determine whether neuromast cells die due to the treatment, we incubated larvae in Acridine Orange, which identifies dying cells in live fish (Williams and Holder, 2000). Untreated larvae show a few labeled cells in some neuromasts (Fig. 2D), due to the constant turnover of cells that occurs in these organs (Williams and Holder, 2000). While no appreciable difference was observed in larvae treated with 1 μM $CuSO_4$ (Fig. 2E), staining appeared to increase substantially when they were treated with 50 μM $CuSO_4$ (Fig. 2F).

These results led us to more closely examine the effects of copper on the cells that compose the neuromasts by

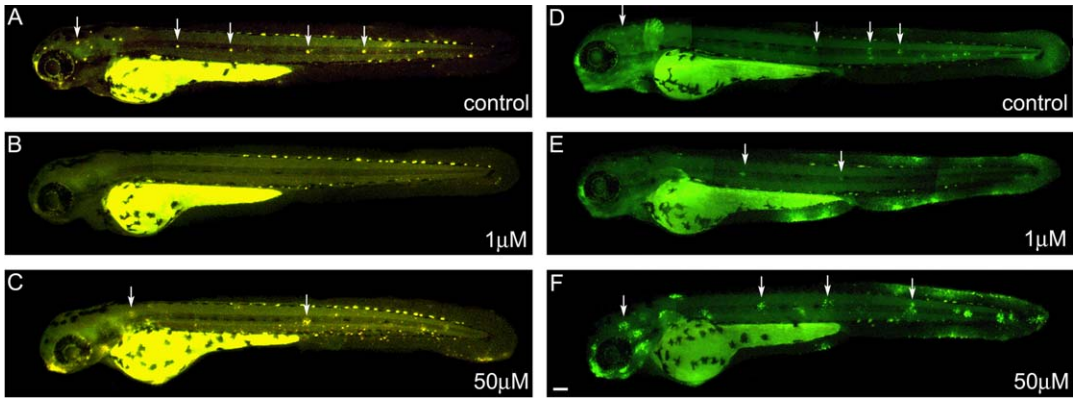

Fig. 2. Loss of viability and cell death after copper treatments. Lateral views of 78 hpf larvae after 2 h of copper exposure. A, B, C, DiAsp labeled larvae. A, Control without copper. Arrows show viable neuromasts. B, Incubation with 1 μM $CuSO_4$ produces loss of label in hair cells. C, At 50 μM no labeled neuromasts are observed, but non-specific occasional label suggests structural damage (arrows). D, E, F, Acridine Orange (AO) labeled larvae. D, Control untreated larva. A few neuromasts show staining. D, Larvae treated with 1 μM $CuSO_4$. No differences with control are observed. F, 50 μM $CuSO_4$ treated larva. Arrows show strong label in dead cells. Bar in F = 35 μm.

observing these organs under the microscope after treating fish with 1–50 μM $CuSO_4$ for 2 h, and then comparing them with untreated fish (Fig. 3A–I). The neuromasts were observed unstained or labeled with DiAsp or Acridine Orange (Fig. 3A–I). In control larvae, neuromasts show a rosette pattern in which cells are arranged in concentric fashion (Fig. 3A), the central cells incorporate DiAsp (Fig. 3B), and, in most cases, no label is seen with Acridine Orange (Fig. 3C). Neuromasts in fish treated with 1 μM copper do not present observable morphological differences, or an increase in apoptosis compared with controls (Fig. 3D and F), even though they fail to incorporate DiAsp (Fig. 3E). After exposure to 50 μM $CuSO_4$, the neuromasts are disorganized as the remaining cells appear swollen and misshapen (Fig. 3G), there is no specific DiAsp labeling (Fig. 3H), and nuclei are strongly labeled with Acridine Orange (Fig. 3I). To quantify the effect of copper on cell death in the lateral line cells, we first counted the number of DiAsp-labeled hair cells (±SD) present in two specific neuromasts, O in the ALL and P1 in the PLL. The O neuromast contains, on average, 7.0 (±2.21) and the P1 neuromast 6.6 (±1.73) hair cells at 76 hpf, the time-

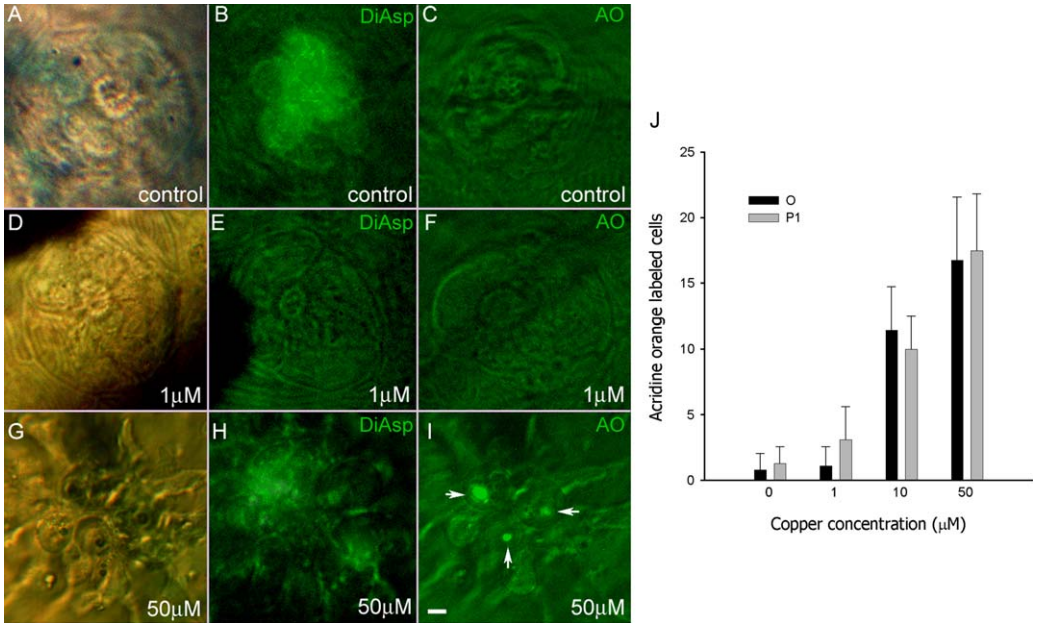

Fig. 3. DIC microscopy of 78 hpf larval neuromasts treated with copper. A, D, G, non-fluorescence DIC image. B, E, H, DiAsp staining. C, F, I, Acridine Orange staining. A, B, C, control without copper. Typical neuromast structure (A), positive DiAsp (B) and negative Acridine Orange staining (C). D, E, F, 1 μM copper. Normal neuromast structure (D) and negative label for DiAsp (E) and Acridine Orange (F). G, H, I, 50 μM copper. Neuromasts show extensive cell damage (G), which is confirmed by the non-specific DiAsp label, associated with cell debris (H) and the specific staining of the apoptotic cell nuclei with Acridine Orange (arrows in I). Bar in I = 3 μm. J, Quantification of Acridine Orange labeled cells in the O and P1 neuromasts after copper treatments. See text for details.

point we performed the incubations with copper. Thus, there is no significant ($p > 0.05$) difference in the number of hair cells between these neuromasts. After the copper treatments, we counted the number of Acridine Orange-labeled cells in both the O and the P1 neuromasts. For each copper concentration the O and P1 neuromasts showed a similar trend. In this experiment, we found a significant increase in Acridine Orange labeling as the concentration of copper is increased (Fig. 3J). Thus, we conclude that hair cell viability is progressively lost when larvae are exposed to concentrations of $CuSO_4$ higher than 1 μM and that cell death is induced significantly as the concentration of $CuSO_4$ increases.

Though we observed that in 1 μM or higher copper-treated larvae, the neuromast cells do not take up DiAsp or FM1-43, we wished to determine whether the hair cells are lost after exposure. We performed immunostains using the anti-acetylated tubulin antibody, which labels only the centrally located hair cells (Fig. 4). Larvae were treated with copper and prepared for antibody labeling after 2 h of exposure; we performed immunostains using a peroxidase-coupled or a fluorescent secondary antibody. In the latter case we also counterstained with Propidium Iodide (PI) to label all of the nuclei, and we imaged the cells using a confocal microscope. Control neuromasts show the typical structure of a central hair cell bundle crowned by a kinocilium (Fig. 4A and D). The cells are arranged with a typical rosette pattern, as seen by the disposition of PI-labeled nuclei (Fig. 4D). Neuromast cells from larvae treated with 1 μM $CuSO_4$ are not labeled with acetylated tubulin, though there is some remaining stain in the vicinity of where the hair cells should be found (Fig. 4B and E). There is no effect on the underlying lateral line nerve, which is also labeled with anti-acetylated tubulin, or in the rosette arrangement of the neuromast cells (Fig. 4E). Neuromasts treated with 50 μM $CuSO_4$ show a complete absence of acetylated tubulin stain in the position the hair cells should occupy and the rosette pattern of nuclei appears disrupted. The underlying nerve does not appear to be affected (Fig. 4C and F).

3.2. Sensitivity of neuromasts to copper compared with other metals

In our studies, we found that the effects of $CuSO_4$ and another copper salt, $CuCl_2$, produced identical outcomes (data not shown). To determine whether the observed effects were specific to copper, we tested the viability of hair cells (by DiAsp incorporation) and the induction of cell death (by Acridine Orange staining) after treatment with various other metals for 2 h starting at 76 hpf (Table 1). Zinc ($ZnSO_4$) produced effects similar to those induced

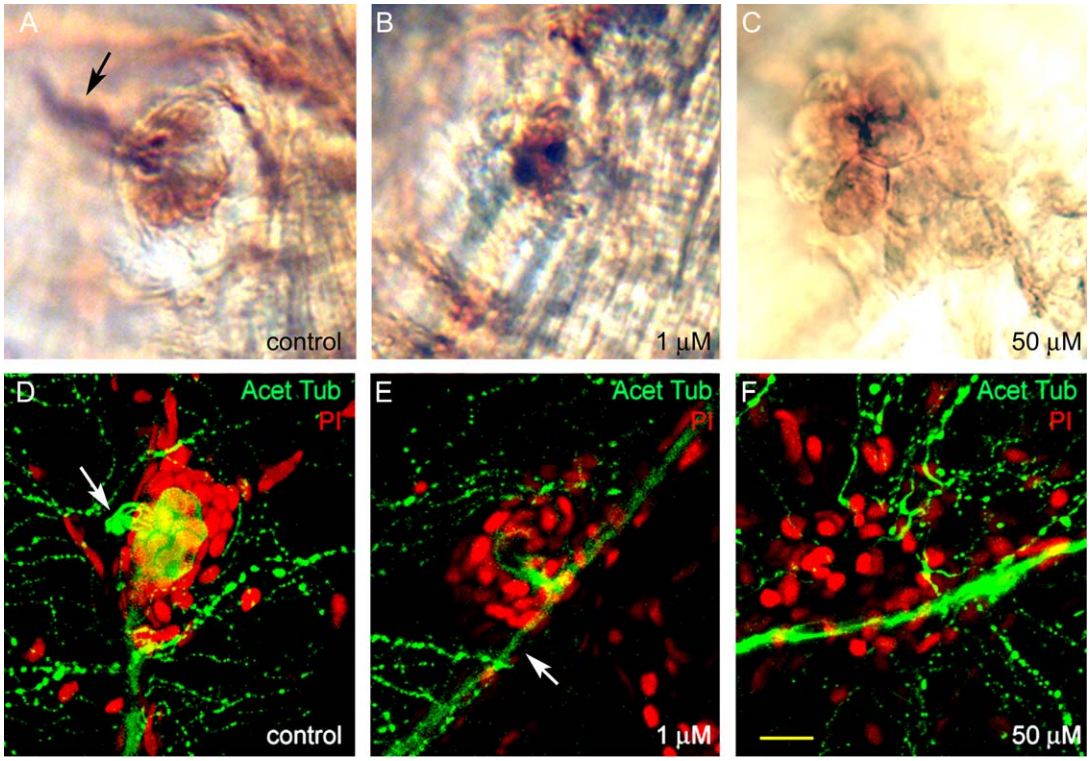

Fig. 4. Anti-acetylated tubulin immunohistochemistry in larvae exposed between 76 and 78 hpf to copper. A–C, Anti-acetylated tubulin (Acet Tub) antibody detected with horseradish peroxidase (HRP) linked secondary antibody. D–F, Same as above but a fluorescein-linked secondary antibody was used and images were obtained by confocal microscopy. Nuclei were stained with Propidium Iodide (PI). A and D, Control without added copper; arrows indicate kinocilium. B and E, 1 μM copper; arrow in E indicates the lateral line nerve, which is not affected by copper. Note the absence of hair cells. C and F, 50 μM copper. Note the disorganization in the neuromast, evidenced by dispersed nuclei and the loss of the rosette pattern. Bar is 10 μm for D, E and F and 12.5 μm for A, B, and C.

Table 1
The effect of different metals on functionality and survival of hair cells

Metal	Concentration (μM)	DiAsp label	Acridine Orange label
Cu^{++}	1	–	–
	50	–	+
	250	–	+
Zn^{++}	1	+	–
	50	–	–
	250	–	+
Fe^{+++}	1	+	–
	50	–	–
	250	–	–
Ag^{+}	1	–	–
	50	–	–
	250	–	–
Mn^{++}	1	+	ND
	50	+	ND
	250	ND	ND
Co^{++}	1	+	ND
	50	+	ND
	250	ND	ND
Cd^{++}	1	+	ND
	50	+	ND
	250	ND	ND
Sn^{++}	1	+	ND
	50	+	ND
	250	ND	ND

Larvae were treated beginning at 76 hpf for 2 h with the indicated metal and larvae were assayed for incorporation of the vital dyes DiAsp and Acridine Orange. In the case of the DiAsp assay, minus and plus signs indicate absence or presence of label. For Acridine Orange stains, minus signs indicate no effect compared to control and plus signs indicate increased staining. Salts used were $CuSO_4$, $ZnSO_4$, $FeCl_3$, $AgNO_3$, $SnCl_2$, $MnCl_2$, $CoCl_2$, and $CdCl_2$. ND, not determined.

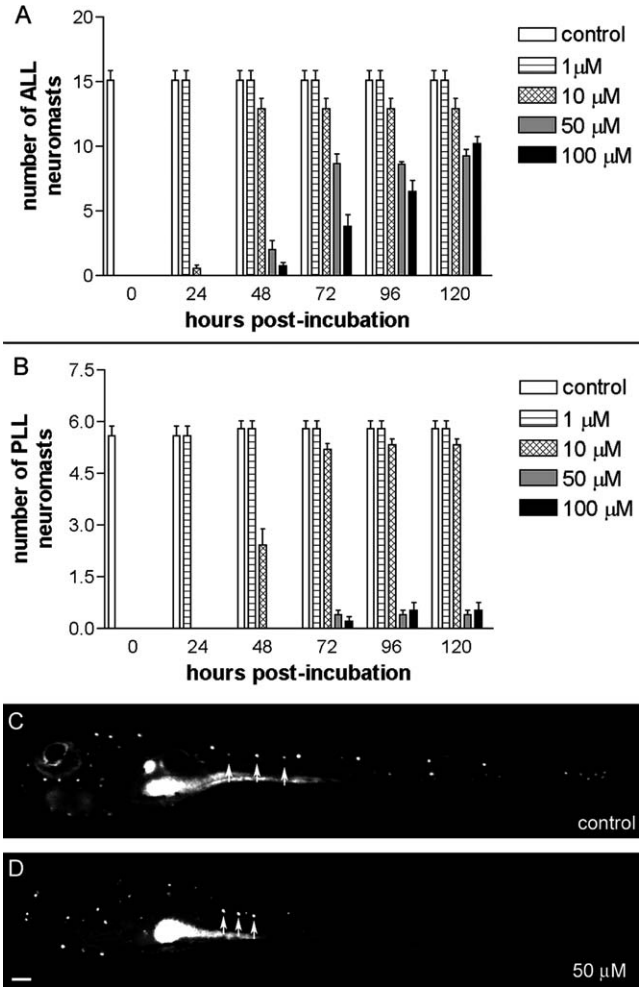

Fig. 5. Larvae of 76 hpf were exposed to different copper concentrations for 2 h. Neuromast viability was monitored daily by DiAsp labeling from 1 to 5 days post-incubation. We counted both anterior (ALL, A) and posterior (PLL, B) lateral line DiAsp-positive neuromasts (see Section 2). C, Control larva stained with DiAsp at 8 dpf. Arrows indicate neuromasts of the secondary lateral line. D, Larva treated with 50 μM of copper for 2 h at 76 hpf and stained with DiAsp five days after treatment. Note the presence of recovered ALL but not PLL primary lateral line neuromasts. Arrows indicate secondary lateral line neuromasts that appear in the treated larva. White bar in D is 35 μm.

by copper but the concentration required to inhibit DiAsp incorporation was 50 μM or higher, while Acridine Orange stain was only induced above 250 μM. Iron ($FeCl_3$) also prevented DiAsp staining at 50 μM but did not induce cell death even at 250 μM; however, this concentration was lethal to the larvae. Silver ($AgNO_3$) caused loss of DiAsp incorporation at 1 μM and higher but we never observed increased Acridine Orange label in these treatments. Incubation with up to 50 μM of Mn, Co, Cd, and Sn did not cause loss of function in hair cells and therefore, higher concentrations were not tested.

3.3. Regeneration of neuromasts after copper treatment is concentration dependent

To determine whether the effect of copper on neuromast structure is permanent or reversible, we assayed for recovery of DiAsp staining after the metal is removed from the incubation medium. Larvae were treated for 2 h with varying concentrations of $CuSO_4$ and, after rinsing at least three times in copper-free medium, DiAsp-labeled neuromasts were counted until 5 days post-incubation (Fig. 5). A neuromast was considered to be regenerated when we detected DiAsp staining in at least one cell in the neuromast. In our analysis, we detected differences between the recovery of ALL (head) and PLL (trunk and tail) neuromasts. ALL neuromasts always recovered (Fig. 5A), even at 400 μM, the highest concentration tested (data not shown). We found that after exposure to 1 or 10 μM $CuSO_4$, PLL neuromasts are able to recover after the treatment, the time of recovery being proportional to the concentration of copper used (Fig. 5B). However, for the most part, PLL neuromasts were permanently lost at concentrations of 50 μM or higher, with the exception of the terminal neuromasts, those residing at the tip of the tail. At 5 days post-incubation, the secondary lateral line develops in all larvae regardless of the concentration of copper used (Fig. 5D and not shown). This indicates that the general health of the fish and the capacity for generation of

neuromasts arising from alternative sources is not lost in treated larvae.

Since we observe the reappearance of functional PLL hair cells 1–2 days after treating larvae for 2 h with 1 or 10 μM $CuSO_4$ (Fig. 5B), we wished to know if they represented newly born cells in the neuromasts. We examined cell proliferation in the neuromasts by using the mitotic marker Proliferating Cell Nuclear Antigen (PCNA) in control neuromasts and after treatment with copper (Fig. 6). We found, that proliferating cells can be detected in ALL, PLL and 2PLL neuromasts in control larvae (Fig. 6A) and in 1 and 10 μM $CuSO_4$-treated larvae (not shown), while 50 μM-treated fish showed label only in the ALL, and in the 2PLL (Fig. 6B). PCNA-labeled cells can be normally found in the periphery of the neuromast (Fig. 6A, inset). To know whether the number of proliferating cells changed after treatment with copper, we counted the number of PCNA-labeled cells in the O and P1 neuromasts 15 h after copper exposure. This allowed us to assay proliferation at a timepoint midway between the treatment and the recovery of viable hair cells (Fig. 5). When fish are incubated in 1 μM $CuSO_4$, significantly more proliferating cells are found comparing both the O and P1 neuromasts to controls (Fig. 6C, $p < 0.001$). As higher concentrations of $CuSO_4$ were used, the O and P1 neuromasts behave differently. While the ALL neuromast (O) shows proliferating cells at 10 and 50 μM, the PLL neuromast (P1) showed PCNA labeled cells at 10 μM but they were almost never detected at 50 μM $CuSO_4$. These results, combined with the Acridine Orange staining experiments and the hair cell regeneration assays, suggest that low concentrations of copper induce cell death followed by proliferation and recovery of viable hair cells in PLL neuromasts. Moreover, higher copper concentrations (e.g., 50 μM $CuSO_4$) irreversibly affect the capacity of cells in the neuromast to regenerate hair cells. In the ALL, $CuSO_4$ induces cell death to a similar extent, but proliferation and recovery of hair cells can still occur at concentrations of copper that abolish this process in the PLL.

4. Discussion

We have shown that the presence of waterborne copper can cause profound effects on the function, survival, and regeneration capacity of mechanosensory cells of the zebrafish lateral line. None of the concentrations used in this study are acutely lethal, though we did not try to raise the treated fish to search for potential long-term effects of these transient copper treatments.

The neuromasts are mechanosensory units of the lateral line that contain at least two cell types: sensory hair cells, which are able to transduce mechanical stimuli, and supporting cells (Ghysen and Dambly-Chaudiere, 2004). In larvae, hair cells are directly exposed to the surrounding medium and they present active endocytosis of compounds present in the water. They can be easily visualized in vivo because they can take up fluorescent molecules such as DiAsp or FM1-43 (Collazo et al., 1994; Seiler and Nicolson, 1999). Several authors have proposed that the mechanotransduction capacity of lateral line hair cells in zebrafish neuromasts is only achieved 3–4 days after fertilization as it is at this time when they become innervated (Raible and Kruse, 2000) and when larval behaviors are consistent with a functional lateral line (Nicolson et al., 1998).

There is strong evidence that lateral line hair cells are equivalent to hair cells present in the ear (Pichon and Ghysen, 2004). In anamniotes, ear and lateral line hair cells are able to regenerate after damage and it is believed that a population of precursors remains undifferentiated throughout life and allows for constant turnover of these sensory mechanoreceptors (Balak et al., 1990). Previous studies

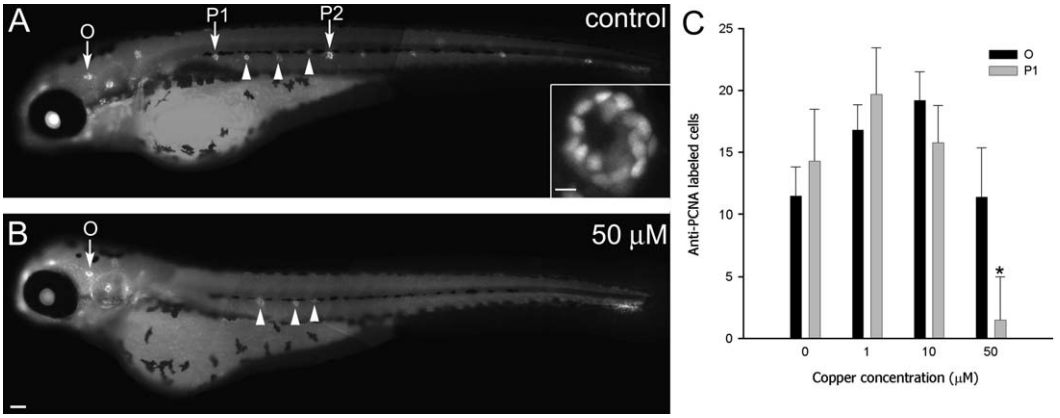

Fig. 6. Immunohistochemistry against PCNA in 93 hpf larvae, analyzed 15 h after copper exposure. A, Control larva shows PCNA label in all neuromasts: PLL neuromasts (e.g., P1 and P2), ALL neuromasts (e.g., O neuromast) and 2PLL neuromasts (arrowheads). Inset in A is a close up image of a PCNA-stained neuromast showing the peripherally stained nuclei. B, Larva treated with 50 μM $CuSO_4$. Note presence of labeled cells in the ALL (e.g., O neuromast) and 2PLL neuromasts (arrowheads), but absence of staining in the PLL. Bar indicates 35 μm in B and 6 μm in the A inset. C, Quantification of anti-PCNA labeled cells in the O and P1 neuromasts in larvae exposed to different copper concentrations. The number of P1 labeled cells at 50 μM was significantly different compared to numbers for all other concentrations (asterisk, $p < 0.001$).

have shown that aminoglycosides, which induce hair cell death in the inner ear of all vertebrates, can also damage and ablate zebrafish neuromast hair cells in a dose-dependent manner (Murakami et al., 2003), and that hair cells reappear 12–24 h post-treatment (Harris et al., 2003). Work in amphibians has shown that regeneration of hair cells occurs by differentiation of supporting cells that lie within the neuromast (Balak et al., 1990).

In our experiments, incubating zebrafish larvae for only 2 h in copper eliminates hair cell viability. The concentration required for this effect is 1 μM $CuSO_4$, more than two orders of magnitude less than the LC50 for zebrafish larvae of the same age. This treatment abrogates labeling with the vital dyes DiAsp and FM1-43 and produces loss of hair cell-specific antigens in the neuromasts (Fig. 4). Our interpretation of this analysis is that hair cells undergo cell death shortly after exposure with 1 μM copper. Despite this effect, there is a subsequent increase in proliferation and viable hair cells can be seen 24 h after the copper is removed. Furthermore, our tests with larvae exposed to increasing concentrations of copper show a clear dose-dependent effect: larvae treated with 10 μM $CuSO_4$ show increased cell death and a longer period for recovery of hair cells compared to 1 μM copper. Therefore, the higher concentration is affecting a larger population of cells than the lower one, and it is possible that affected cells include those that proliferate and have the capacity to generate new hair cells.

When larvae are treated with 50 μM $CuSO_4$, the damage is not only visible in the live fish as bursting neuromast cells, but we also see a strong increase in the number of apoptotic cells, near-complete absence of proliferation in PLL neuromasts after copper is removed, and failure of PLL hair cells to reappear. This result would seem to indicate that high doses of the metal are able to destroy both the hair cells and most of the cells that have the capacity to generate new hair cells in these neuromasts. The precursors presumably inhabit the second tier of cells in the neuromast, under or beside the hair cells (Balak et al., 1990; Williams and Holder, 2000), and thus could be affected directly by the high concentrations of the metal. It is also possible that supporting cells undergo programmed cell death if hair cells are rapidly destroyed. Our current research is aimed at discriminating whether regenerated hair cells are descendants of supporting cells that are able to transdifferentiate or whether they are an as-of-yet unidentified population of stem-like cells.

The ALL showed an important difference with the PLL in these experiments. While neuromasts in both areas of the lateral line showed comparable numbers of apoptotic cells for each condition, proliferation levels followed different profiles in the PLL vs. the ALL as copper concentrations were varied (Fig. 6). In the PLL, numbers of proliferating cells increased in fish treated with 1 μM $CuSO_4$, and then diminished to almost undetectable levels at 50 μM $CuSO_4$. In contrast, in the ALL of fish treated with 50 μM copper we detected abundant numbers of proliferating cells. This difference correlates well with our observations on regeneration of hair cells in the ALL vs. the PLL at 50 μM $CuSO_4$ (Fig. 5). In the P1 neuromast, where we rarely observed PCNA labeled cells after treatment with 50 μM $CuSO_4$, we did not detect regeneration of hair cells. In contrast, the presence of PCNA positive cells in the O neuromast correlates with the presence of viable hair cells after the treatment. While we do not know the reason for the difference in regenerative capacity between the ALL and PLL, it is interesting to note that ALL neuromasts become embedded in pits or canals in the head (Webb and Shirey, 2003), possibly offering more protection from external toxicants. Furthermore, it is important to point out that in all of our experiments, including those involving very high doses of copper (100–400 μM), we observed the appearance of the secondary lateral line after treatment (Fig. 6B). Thus, the secondary lateral line primordium, which is not yet functional at the times we performed the treatments (Sapède et al., 2001), may also be protected from the effects of external copper.

Copper is well known to be a severely cytotoxic metal, generating oxidative stress and cellular damage if it accumulates beyond the chelating capacity of the cell (Gaetke and Chow, 2003). Our results suggest that copper is gaining access to hair cells in sufficient amounts to produce cell death in a matter of minutes or hours. Toxicity through oxidative stress has been observed for aminoglycosides, which activates an apoptotic cell death signaling pathway in hair cells due to the induction of free radicals and reactive oxygen species (Forge and Li, 2000; Forge and Schacht, 2000). Moreover, the anticancer drug cisplatin has also been shown to be extremely ototoxic in mammals due to entry into hair cells and induction of oxidative stress (Clerici et al., 1996), in part through sequestration of antioxidant molecules in the cell (Ravi et al., 1995). Cisplatin is also toxic to zebrafish lateral line hair cells (Ton and Parng, 2005). If copper accumulates within hair cells, the effects will be predictably similar to those described.

How copper enters these cells presents an interesting problem. Previous studies have attempted to resolve this issue for compounds that are also specifically toxic to hair cells such as aminoglycosides, but the mechanism of uptake of these drugs still remains unclear. Both ingression through apical endocytosis (Richardson et al., 1997) and via the mechanotransduction channels (Gale et al., 2001) have been proposed. This issue is further obscured because of the strong link between endocytosis and mechanotransduction in hair cells: mutations in the Myosin VII gene affect both processes and block entry of aminoglycosides into hair cells (Enrnest et al., 2000). In zebrafish larvae, immature hair cells show increased resistance to these compounds (Murakami et al., 2003) suggesting a correlation between mechanoreceptor maturity and drug ingression. Mechanotransduction channels are permeable to mono- and divalent cations and can be blocked by neomycin, a positively charged antibiotic (Ohmori, 1985). Though the channel may be non-selective for cations, copper is partic-

ularly toxic to hair cells as, with the exception of silver, many other metals do not show the same effects in our assays when used at similar concentrations. Interestingly, cisplatin may enter cells through a high-affinity copper transporter (Ishida et al., 2002). In zebrafish, the high affinity copper transporter Ctr1, is widely expressed during the early life stages of zebrafish (Mackenzie et al., 2004), and thus, Ctr1 could be mediating, at least partially, the accumulation of copper in neuromast hair cells. Moreover, this transporter is capable of transporting silver as well (Lee et al., 2002), a property that correlates with the toxicity that both metals produce in hair cells (see Table 1).

A recent report showed that transient exposure to copper during embryonic development in fathead minnows can alter the capacity of the fish to respond to chemosensory cues (Carreau and Pyle, 2005). Chemosensation is acquired through specialized cells present in the olfactory epithelium and on the body surface, directly exposed to the environment as are hair cells. It is interesting to note that these cells are also labeled readily with styryl dyes, suggesting that the mechanisms of toxicosis in these two cell types could be shared.

Our results incorporate a new level of complexity in the analysis of the consequences of moderate amounts of copper in the aqueous environment. Transient exposure to low levels of copper in the water may not be affecting the immediate survival of fish, but sensory organs, such as the lateral line, may show impairment or damage under these conditions. We aim to determine whether even lower doses, perhaps with longer exposures, can have similar effects in larval or adult fish. Moreover, it will be important to consider these studies when analyzing human patients presenting imbalances in copper metabolism due to disease or diet.

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