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Injury-Induced Neuronal Turnover with Zinc Sulfate Affects Cilitated Olfactory Sensory Neurons more than Microvillous Olfactory Sensory Neurons in the Adult Zebrafish

James Hentig
Western Michigan University, jhentig@nd.edu

Injury-Induced Neuronal Turnover with Zinc Sulfate Causes Differential Effects in Olfactory Sensory Neuronal Subtypes in the Adult Zebrafish

James T. Hentig and Christine A. Byrd-Jacobs
Department of Biological Sciences
Western Michigan University
1903 W Michigan Ave
Kalamazoo, MI 49008-5410 USA

James.t.hentig@wmich.edu
Christine.byrd@wmich.edu

Abstract

Zinc sulfate is a known olfactory toxicant, although its effects on the olfactory epithelium of zebrafish have not been examined. Previous observations utilizing Triton X-100 found selective retention of some olfactory sensory neurons and loss of others. Based on those findings, we hypothesized that the retention of the ability to sense amino acids, detected by microvillous olfactory sensory neurons, and loss of the ability to perceive bile salts, detected by ciliated olfactory sensory neurons, is a universal response to chemical ablation. Fish were treated with zinc sulfate and survived for 2, 5, 10 and 14 days post treatment and processed for histological, immunohistochemical, ultrastructural, and behavioral analysis.

Severe morphological changes to the olfactory organ were observed 2 days following exposure, accompanied by a significant decrease in anti-calretinin staining in the olfactory epithelium. Lamellae of the olfactory organ appeared fused, and there was obvious inflammation of the epithelium, with large vacuoles within the cells. The structure of the olfactory organ returned to near-control morphology with a lamellar arrangement by 5 days after chemical exposure; however, the amount of anti-calretinin labeling in the olfactory epithelium did not return to control levels until 10 days after exposure.

Scanning electron microscopy revealed the sensory region was absent of ciliated structures, but microvilli could be seen on the apical surface at 2 days after zinc sulfate exposure. Sensory ciliated structures began to return 5 days after exposure, though the population was scarce and the structures appeared thinner. Treated organs returned to near control structure by 10 days following exposure.

An assay quantifying the fish response to an amino acid mixture or a bile salt mixture was utilized to examine olfactory-mediated behavior. Behavior before and after odor exposure was recorded and number of turns was compared. Unlesioned controls responded by significantly increasing their turning behavior in response to both amino acids and bile salt mixtures. At 2 days following exposure, the fish appeared to detect amino acids but their turning behavior was not significantly different from pre-odor behavior. The ability to perceive amino acids returned 10 days after exposure, while the ability to perceive bile salts remained lost. When given 14 days of recovery, the ability to perceive bile salts returned. Thus, chemical ablation of the olfactory epithelium with zinc sulfate results in degeneration of the olfactory organ and removal of most olfactory sensory neurons within 2 days. The ability to perceive amino acids was regained prior to the ability to detect bile salts. Thus, zinc sulfate appears to have a greater affect on ciliated olfactory sensory neurons than on microvillous olfactory sensory neurons.

1. Introduction

The zebrafish, *Danio rerio,* is a long-standing model widely used in developmental and neuroplasticity studies. In the adult zebrafish nervous system, the rate of recovery from damage is well documented. The olfactory system, however, is unlike most other systems as it is an open sensory system exposed to the environment. The easy access allows exposure of the olfactory organs to a wide variety of olfactotoxicants, coupled with the system's natural neuronal turnover and ability to restore function, makes for an excellent model to examine neuroplasticity and toxicology.

In zebrafish, the olfactory system consists of the olfactory organs, termed rosettes, and the olfactory nerves that project to the olfactory bulbs in the brain. The rosette consists of olfactory epithelial tissue, a cell-dense pseudostratified columnar epithelium with a variety of cell types, and a non-sensory epithelium. The olfactory epithelium is comprised of ciliated and non-ciliated basal cells, goblet cells, microvillous supporting cells, and subsets of olfactory sensory neurons (OSNs) (Byrd and Brunjes, 1995, Hansen and Zeiske, 1998). The olfactory sensory epithelium of zebrafish and other teleosts possess 3 types of OSNs: ciliated OSNs, microvillous OSNs, and crypt neurons (Hansen and Zielinski, 2005). These OSN subtypes display distinct physiological and morphological differences. Ciliated OSNs, which respond to bile salts and are responsible for social-mediated behavior, are recognized by their distinctive placement deep within the epithelium and possession of a long, thin dendrite with 3-7 protruding cilia (Hansen and Zeiske, 1998). Microvillous OSNs respond to amino acids, are responsible for feeding behavior, and are categorized by a thick dendrite and cell body found in the middle of the epithelium, with 10-30 microvilli per knob (Lipschitz and Michel, 2002, Hamdani et. al. 2001). Crypt OSNs are oblong in shape, with no dendrite, and are found near the apical surface

of the OE (Hansen and Zeiske, 1998). The OSN subtypes are dispersed throughout the sensory epithelium, though they innervate specific regions of the olfactory bulb and mediate the described behaviors.

The effects of a wide variety of toxins on the olfactory epithelium have been well studied (Cancalon et. al 1982, Ghosh and Mandel 2014, Holbrook et. al 2014). In particular, zinc sulfate has been studied extensively in several species. The effects of zinc sulfate on the olfactory epithelium are well known and have been shown in rodents and humans to cause anosmia and degradation of the olfactory epithelium (McBride et. al 2003, Slotnick et. al 2000). A series of studies have been done on the effects of zinc sulfate, as well as a variety of other heavy metals, in catfish (Cancalon et. al 1982). The results showed that while a degree of degradation of the olfactory epithelium was observed among all heavy metals, zinc sulfate was found to cause the most severe damage.

Previous work in zebrafish has shown that after a single treatment of Triton X-100, there is rapid degeneration, a disruption in morphology, and regeneration when given time to recover (Iqbal and Byrd-Jacobs, 2010). Olfactory-mediated behavior was examined following chronic treatment of Triton X-100. Fish retained the ability to sense amino acids, but lost the ability to respond to bile salts (Paskin and Byrd-Jacobs, 2012). This work was expanded to examine the effects of a single Triton X-100 treatment on glomerular plasticity and olfactory-mediated behavior (White et al., 2015). Similar to the chronic study, the response to amino acids was never lost, but the response to bile salts was lost at 4 days post-lesioning and regained 10 days after treatment.

We hypothesized that retention of the ability to sense amino acids and loss of the ability to perceive bile salts is a universal response to chemical ablation. This study aimed to

demonstrate this phenomenon utilizing zinc sulfate as the chosen xenobiotic through analysis of histology, immunocytochemistry, scanning electron microscopy, and behavioral analyses. We expected to see a rapid injury-induced neuronal degeneration and regeneration in the lesioned olfactory organs. Based on our previous results with detergent applications, we expected to see the retention of the ability to perceive amino acids with the loss of bile salts perception.

2. Materials and Methods

2.1. Chemical Lesioning with Zinc Sulfate:

Fish were anesthetized in 0.03% MS222 (methane sulfonate salt, Sigma) until unresponsive to a tail pinch. Fish received intranasal administration of 2µl of 1M zinc sulfate (Sigma) in dH2O to the right olfactory organ. A thin strip of Vaseline was applied between the nostrils to limit leakage to the left side, which was untreated to serve as an internal control. Fish were placed on ice to allow for a 3-minute exposure to the zinc sulfate before being returned to a recovery tank and examined after 2, 5, 10, or 14 days.

2.2. Tissue Processing:

Unlesioned control and chemically lesioned fish were euthanized by overexposure to 0.03% MS222. They were then placed in 4% paraformaldehyde for 24 hours before being measured, sexed, and dissected. Whole heads were dissected and decalcified with RDO (Electron Microscopy Sciences), dehydrated through ascending ethanol washes, and embedded in paraffin. Semi-serial, 10µm sections were mounted on positively charged slides.

2.3. Histology and Immunohistochemistry

Some sections were stained following typical hematoxylin and eosin protocols and coverslipped with DPX (Sigma-Aldrich) to observe morphology. Other sections were labeled with cell-specific markers using immunohistochemistry following typical protocols. Briefly, sections were rehydrated and treated with 3% H2O2 in dH2O to remove endogenous peroxidases. Non-specific binding was blocked with 2% BSA and 0.4% Triton X-100 in phosphate-buffered saline (PBS) for 1 hour at room temperature. Slides were incubated in a humid chamber at 4°C for 24 hours in anti-calretinin (Santa Cruz; 1:1000 made in blocking solution). Following PBS rinses, sections were incubated at room temperature for 1 hour in biotinylated anti-goat IgG (Vector; 1:100 made in blocking solution) Following PBS rinses, they were treated with ABC solution (Vector) for 1.5 hrs and exposed to DAB Solution (Vector) until sufficient staining was observed. Sections were dehydrated and coverslipped with DPX.

2.4. Optical Density Measurements:

Using SPOT Software 5.0, images were taken of anti-calretinin labeled sections at 20x and converted to 8-bit gray scale. Using ImageJ software, the amount of antibody labeling was estimated by calculating the optical density of staining. To do this, the gray area intensity of the sensory area of three lamellae per section from three alternating semi-serial sections of each fish was measured and averaged. The background gray area intensity was also measured, and the optical density for that fish was calculated using the formula: OD=log(background intensity/average intensity of measured regions). The percent difference between sides was calculated and comparisons between groups were made using ANOVA with Tukey post hoc

analysis. A significance level of 0.05 was used. A sample size of 3 fish was used at each time point.

2.5. Scanning Electron Microscopy:

Control and chemically lesioned fish were euthanized with an overdose of 0.03% MS222 at 2, 5, and 10 day time points. Whole fish were fixed in 3% glutaraldehyde in PBS at 4°C for 48 hours. Fish were decapitated, and heads were rinsed in PBS before undergoing a secondary fixation of 1% osmium tetroxide in PBS at room temperature for 1 hour. Heads were then dehydrated through ascending ethanol rinses and Hexamethyldisilazane before being left to air-dry for 24 hours. Olfactory organs were then dissected, mounted, sputtered with gold, and imaged with a Hitachi S-4500 scanning electron microscope.

2.6. Behavior Assay:

2.6.1. Treatment groups

Treatment groups for this assay included control fish and fish 2 days, 10 days, and 14 days following zinc sulfate treatment. Controls were not treated, while treated groups were exposed to 1M zinc sulfate as described in section 2.1; however, the olfactory organs of fish in those treatment groups were treated bilaterally with 1M zinc sulfate to examine the effects on olfactory acuity.

2.6.2. Testing:

Olfactory responses were observed using a behavioral assay as previously described (Paskin and Byrd-Jacobs, 2012) Fish were placed individually into testing tanks on the appropriate day following exposure to zinc sulfate. They were allowed 1.5 hours to acclimate to the tank between

all trials. A digital video recorder was set above the testing site to capture behavioral data. Fish were exposed to an amino acids mixture (alanine, cysteine, histidine, methionine, and valine; 10mM each, all from Sigma) or bile salts mixture (taurocholic acid, taurodeoxycholic acid, taurochenodexoycholate, lithocholic acid, glycocholic acid, and glycochenodeoxycholate; 10mM each, all from Sigma). Odorants were delivered through a tube on one side of the testing apparatus while water was simultaneously delivered through another tube on the opposing side. For each trial, whether an odorant or water was delivered was determined with a random number generator. Behavior was assessed 30 seconds prior to odorant delivery, and 30 seconds following delivery. For some control fish, a trial of water was delivered simultaneously on both sides of the test apparatus as a mechanical control.

2.6.3. Quantification:

Behavior trials were recorded, and swimming behavior was analyzed by observing the number of turns fish made pre-odorant delivery (pre-trial) and post-odorant delivery (trial). For each treatment group, data was averaged, and comparisons of pre-trial and trial were made using a two-way ANOVA and Tukey's post hoc. A significance level of 0.05 was used.

3. Results

3.1. Time course of degeneration and regeneration of the zebrafish olfactory epithelium following exposure to zinc sulfate

Hematoxylin and eosin staining and anti-calretinin labeling were utilized to examine structural changes and presence of OSNs, respectively, in lesioned olfactory organs. Anti-calretinin is a marker that is used to label mature OSNs, although it is debated whether both ciliated and microvillous OSNs express the antigen. Unlesioned control organs were symmetrical with

lamellae projecting out from the central raphe (Fig. 1A). Control olfactory organs had heavy anti-calretinin positive labeling of densely packed mature OSNs (Fig. 1A'). At 2 days following treatment, there was severe morphological disruption of the olfactory organ (Fig. 1B) and an observed decrease in anti-calretinin labeled OSNs (Fig. 1B'). The organ had lost most anatomical resemblance and there was obvious inflammation of the epithelium. Lamellae of lesioned organs appeared fused, and large vacuoles could be seen within the cells. Anti-calretinin labeling was minimal and found in isolated pockets. Following 5 days after treatment, the epithelium appeared thin (Fig. 1C), and inflammation appeared to have decreased allowing for a closer resemblance to control morphology. Anti-calretinin labeling began to return 5 days after exposure (Fig. 1C'), although it still appeared diminished. When permitted 10 days recovery following zinc sulfate exposure, the anatomical structure appeared fully intact, and the epithelium appeared to have regained a control appearance (Fig. 1D). Anti-calretinin labeling of 10 day organs showed densely packed OSNs (Fig. 1D') and resembled that of control.

We measured the amount of antibody labeling as a means to infer the amount of OSNs in the olfactory organ. Densitometry was used to quantify the levels of anti-calretinin through the degeneration and regeneration time course described above. Unlesioned controls had 10.76±10.91 SEM percent difference between the right and the left olfactory organ (Fig. 2) showing that there was some variation in amount of label between right and left olfactory organs under

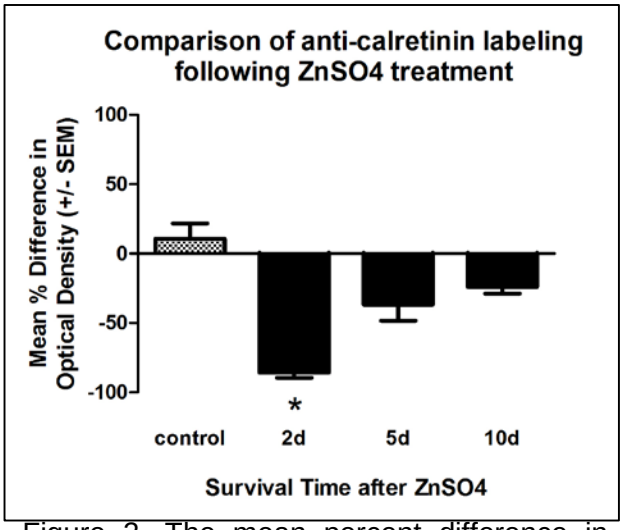

Figure 2. The mean percent difference in calretinin immunoreactivity between treated and internal-control sides was compared between survival groups using optical density. There was a significant decrease in anti-calretinin labeling at 2d after $ZnSO_4$ treatment. By 5d after chemical ablation, the amount of anti-calretinin labeling was not different from controls. * = $P<0.05$.

control conditions. In the lesioned side at 2 days following chemical ablation with zinc sulfate, anti-calretinin positive labeling had significantly decreased to -85.6%±3.73% (Fig. 2). Following 5 days after exposure, anti-calretinin labeling appeared diminished (-36.66%± 11.78%; Fig. 2), though it began approaching control levels. The presence of anti-calretinin labeling resembled control levels more closely after 10 days after treatment (-24.16%±4.57%; Fig. 2), although the optical density of anti-calretinin positive labeling did not return completely to that of unlesioned controls.

3.2. Scanning Electron Microscopy

Lamellae of unlesioned control organs were observed with scanning electron microscopy from an *en face* view. Clear separations of sensory and non-sensory regions were witnessed as an evident ridge formed by distinctly different ciliated structures (Fig. 3A). The non-sensory region possessed cilia that were considerably longer in comparison to sensory cilia. The sensory regions of unlesioned organs displayed densely packed mats of ciliated OSNs covering the epithelium (Fig. 3B). Two days following zinc sulfate exposure, ciliated structures in the sensory regions were no longer present but microvilli were seen on the apical surface. Observations following 2 days after exposure (Fig. 3C) to zinc sulfate was of an organ containing only microvillous olfactory sensory neurons with a complete absence of sensory ciliated structures. When given 5 days following exposure (Fig. 3D), cilia were now observed, although they appeared thinner, shorter, and in considerably less quantity compared to control. However, when the organ was permitted 10 days after exposure to recover (Fig. 3E), the sensory regions were again densely covered in ciliated structures. It is relevant to note that following exposure, non-sensory cilia appeared to be undamaged (Fig. 3C). An alternative morphology was infrequently observed. The

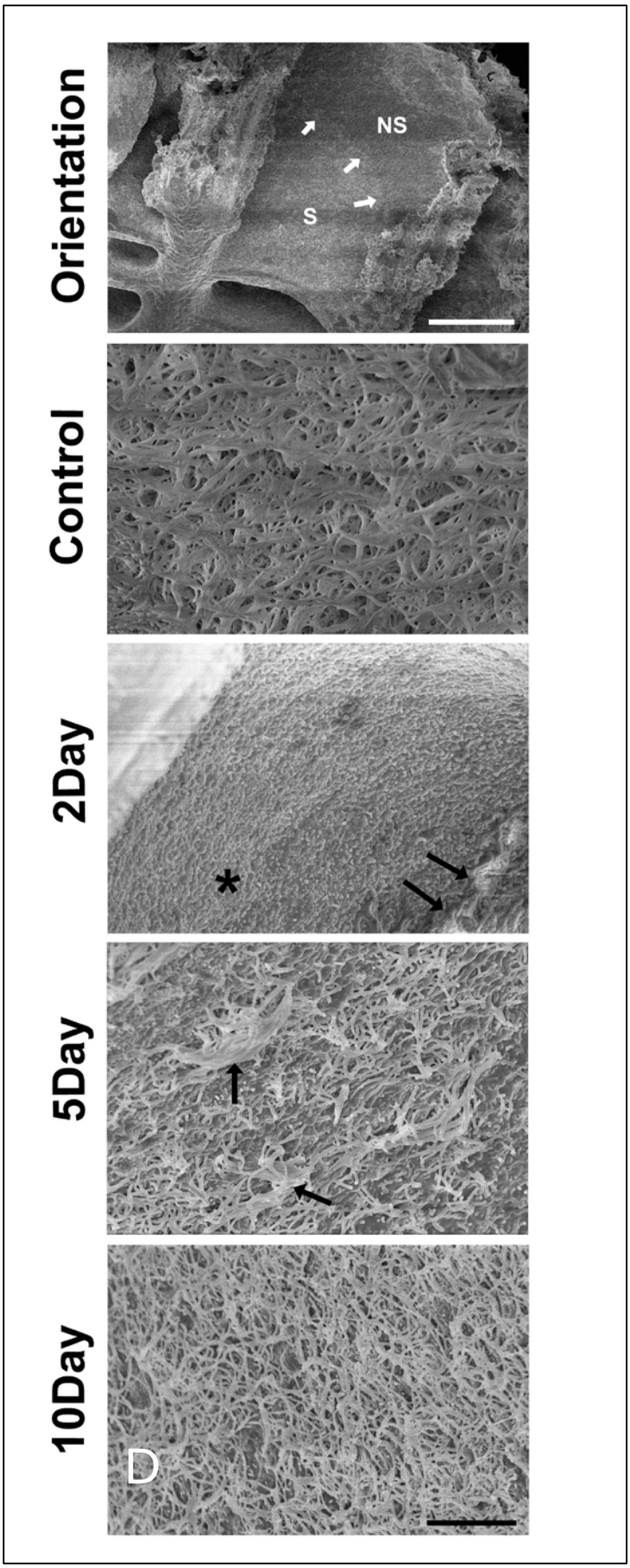

Figure 3. A) The sensory (S) and non-sensory (NS) regions of a lamella, with a defined separation (arrows), are shown in a control fish. B) The surface of control olfactory epithelia was densely packed with cilia and microvilli from OSNs. C) At 2D after zinc sulfate exposure the olfactory epithelial surface appeared to contain only microvilli, with sensory cilia lacking (*). Non-sensory cilia remained (arrows). D) On the surface of the olfactory epithelium of 5D fish, intermittent cilia (arrows) were present across the mat of microvilli. E) The sensory region appeared to be densely packed with cilia and microvilli, similar to control tissue, by 10D. Scale bars= 7μm

olfactory organ appeared barren with irregularly shaped concentric circles with raised ridges formed across the entire organ. Similar structures were described as microridges of the supporting cells following mercuric chloride olfactory toxicity in *Labeo rohita* (Ghosh, 2014).

3.3. Behavioral examination

Water administered to control fish through both tubes in the testing apparatus as a mechanical control showed there was no significant response in the absence of odor (data not shown, P=28.56; N=6). Unlesioned control fish increased their

number of turns following delivery of the amino acids mixture or bile salts mixture (Fig. 4A, $P<0.05$; N=5). An anosmic control with complete occlusion of the nostrils was performed by White et al.(2015) demonstrating that olfaction, not gustation, was tested in this experimental paradigm. At 2 days after exposure, there appeared to be a subset at 2d in response to an amino acid mixture; responders (A, 2D) and non-responders (A, 2D**) though no significant response was seen to either amino acids or bile salts (Fig. 4AB, $P>0.05$; N=6). Given 10 days following exposure, there was a significant response to amino acids (Fig. 4A, $P<0.05$), but no significant response was observed following exposure to bile salts (Fig. 4B, $P>0.05$; N=6). Only after 14 days following exposure were significant responses observed following exposure to both amino acids and bile salts (Fig. 4AB, $P<0.05$; N=6). Thus, the perception of both amino acids and bile salts were lost at 2 days after treatment, the ability to sense amino acids had returned at 10 days, but it was not until 14 days following exposure that the detection of bile salts recovered.

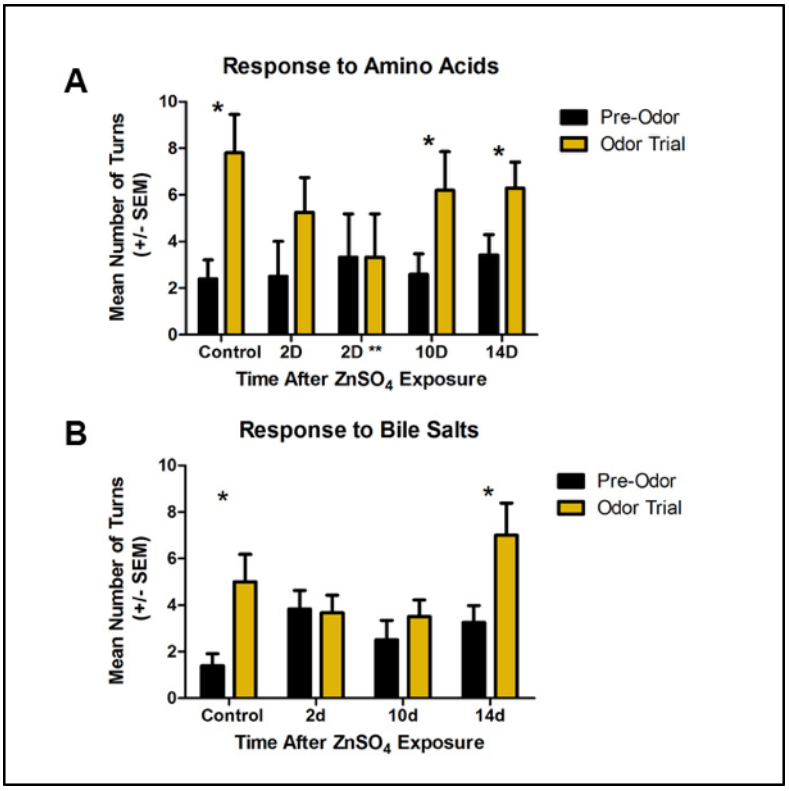

Figure 4. Behavioral responses were compared before (pre-odor) and after delivery (odor trial) of an amino acid mixture (A) or a bile salt mixture (B). Control fish made significantly more turns after exposure to either mixture. At 2d following zinc sulfate treatment, fish did not show a statistically significant response to either mixture. There appeared to be a subset at 2d in response to an amino acid mixture; responders (A, 2D) and non-responders (A, 2D**) though neither group was significant. However, at 10d after chemical ablation fish made more turns following amino acid delivery, while the perception of bile salts had not yet returned. Given 14 days following exposure the ability to perceive bile salts regained. * = $P<0.05$.

4. Discussion

Our results demonstrate that a single exposure to zinc sulfate has a rapid effect on the sensory epithelium of the olfactory organ of the zebrafish causing severe morphological changes and disruption of olfactory-mediated behavioral responses. When allowed a short recovery time following exposure, inflammation of the olfactory organ decreases, regains anatomical resemblance, responds with neuronal turnover, and regains olfactory-mediated behavior. This rapid degeneration and regeneration is similar to that shown previously following both single and chronic applications of Triton X-100, although there were differences in epithelial response. Triton X-100 resulted in a thinning of the epithelium, while zinc sulfate initially resulted in an inflammation of the epithelium prior to the thinning of the tissue (Iqbal and Byrd-Jacobs, 2010, Paskin and Byrd-Jacobs 2012, White et. al, 2015).

There are progressive morphological changes seen in the olfactory epithelium of the zebrafish after exposure to zinc sulfate. There is a dramatic inflammation response at 2 days following exposure to zinc sulfate. Our observations coincide with the inflammation observed after exposure to other heavy metals as noted by Ghosh with mercuric chloride (2014). After 5 days following zinc sulfate, inflammation had decreased and the anatomical structure began to resemble control. While the initial response was of inflammation, at day 5 the epithelium was thinning and similar to observations in Triton X-100 applications (Iqbal and Byrd-Jacobs 2010). The olfactory organ had morphological restoration following 10 days after zinc sulfate. The organ had regained a control appearance in shape, size, and presence of ultrastructure. The recovery of the zebrafish olfactory system is remarkably faster than other models such as rodents.

The effect of zinc sulfate on cellular ultrastructure led to the loss of ciliated structures with the retention of microvilli. Scanning electron microscopy revealed there is a rapid loss of sensory cilia as early as 2 days following zinc sulfate exposure, while non-sensory cilia and microvilli remain intact. The loss of sensory cilia and the preservation of microvilli and non-sensory cilia have been observed elsewhere leading to the possibility of a specific response (Ghosh and Mandel, 2014). Microvillous OSNs were observed beginning at 2 days following exposure and throughout the time course. Sensory ciliated structures began to recover as early as 5 days following exposure, though they appeared shorter, thinner, and sparsely distributed. It was not until 10 days following exposure, that sensory ciliated structures were observed in densities that appeared similar to control levels.

The behavior assay suggested a loss of perception of both bile salts and amino acids immediately following zinc sulfate exposure. When given 10 days following a single treatment of zinc sulfate, the ability to detect amino acids had returned, while the ability to detect bile salts was still absent. Only when allotted 14 days following exposure was the ability to perceive bile salts regained. While we did not see a statistically significant response, we observed more turns in our 2 day treatment group to amino acids than to bile salts. This response combined with the ultrastructure analysis leads us to believe microvillous OSNs functionality might be near the end stages of recovery by 2 days following exposure. This conclusion is strengthened by the behavioral response conducted by White et. al. (2015) with a significant response to amino acids 4 days following exposure to Triton X-100. Nevertheless, the recovery of ultrastructure followed by function of microvillous OSNs preceded the respective recovery of ciliated OSNs. This response has been shown in earlier studies through both chronic and single applications of detergent. The ability to detect amino acids, which are detected by microvillous OSNs, was

retained, while the ability to sense bile salts, distinguished by ciliated OSNs, was not (Iqbal and Byrd-Jacobs 2010, Paskin and Byrd-Jacobs 2012, White et. al. 2015). Notably, we observed more turns in our pre-trial 2 day treatment group to amino acids than to bile salts. Anxiety and alterations in sensory input have been demonstrated in mice (Lepicard et. al. 2000). We postulate this might be a behavioral response to limitations in the ability of the fish to fully perceive their surroundings.

Several studies have demonstrated that retention of olfactory-mediated food-finding behavior is often primary over olfactory-mediated social or reproductive preservation. Utilizing methyl bromide-induced olfactory lesions, rats exhibited responses to odorants indicating feeding behavior at 3 days following exposure (Youngentob et. al. 1997). This repeated pattern suggests an evolutionary process to preserve one's immediate needs over olfactory-mediated social and predatory cues. There are postulations as to why ciliated OSNs seem to be more vulnerable. A plausible explanation is that ciliated and microvillous neurons have a separate origin during development (Saxena et. al. 2013).

We demonstrate that the olfactory epithelium can rapidly repair after zinc sulfate insult. Exposure to zinc sulfate results in an observable temporary loss of ciliated OSNs with the preservation of microvillous OSNs. The complete degeneration and regeneration of the olfactory organ and function happens within 14 days. The effects of zinc sulfate are similar to the effects of detergent, although subtle differences exist. In sum, it appears that ciliated OSNs of zebrafish are more affected by toxic insult that microvillous OSNs.

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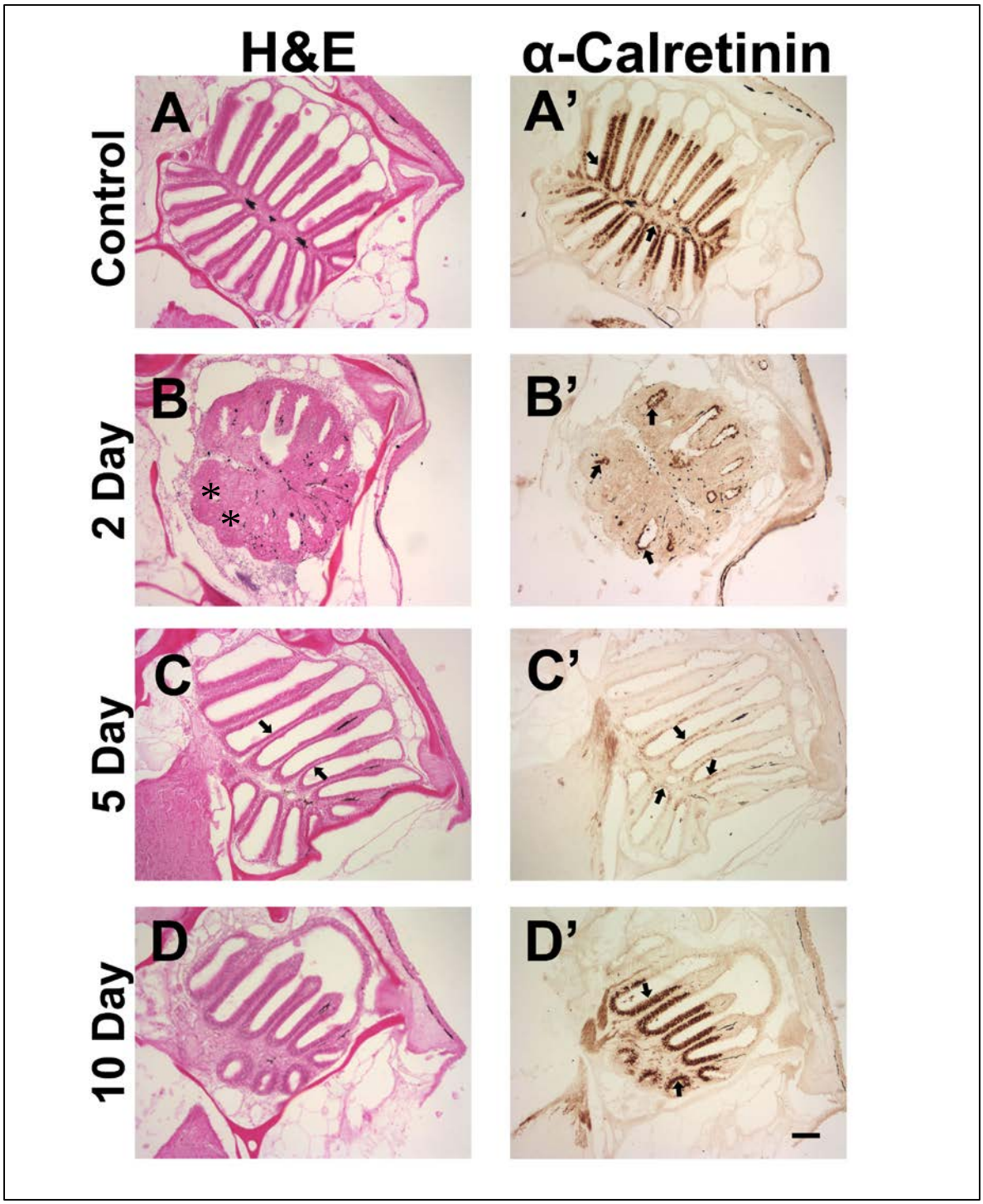

Figure 1. Unlesioned control olfactory organs display a semi-symmetrical shape with radiating lamellae (A) and dense anti-calretinin labeling along the sensory portion of the olfactory epithelium (A', arrows). Olfactory organs 2 days following zinc sulfate exposure were inflamed with fusing of lamellea (B, asterisk) and anti-calretinin labeling (B', arrows) was diminished and confined to the apical surface of the epithelium. After 5 days, the olfactory epithelium (C, arrows) was noticeably thinner than control tissue, and anti-calretinin labeling showed a return of olfactory sensory neurons (C', arrows), although it appeared to be less than control levels. The morphology of the olfactory organ 10 days after zinc sulfate resembled that of control (D), and anti-calretinin labeling appeared to resemble control levels in amount and intensity (D', arrows). Scale bars= 100μm