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Cell proliferation follows acoustically-induced hair cell bundle loss in the zebrafish saccule

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ABSTRACT

Fishes are capable of regenerating sensory hair cells in the inner ear after acoustic trauma. However, a time course of auditory hair cell regeneration has not been established for zebrafish. Adult zebrafish (*Danio rerio*) were exposed to a 100 Hz pure tone at 179 dB re 1 μPa RMS for 36 h and then allowed to recover for 0–14 days before morphological analysis. Hair cell bundle loss and recovery were determined using phalloidin to visualize hair bundles. Cell proliferation was quantified through bromodeoxyuridine (BrdU) labeling. Immediately following sound exposure, zebrafish saccules exhibited significant hair bundle damage (e.g., splayed, broken, and missing stereocilia) and loss (i.e., missing bundles and lesions in the epithelia) in the caudal region. Hair bundle counts increased over the course of the experiment, reaching pre-treatment levels at 14 days post-sound exposure (dpse). Low levels of proliferation were observed in untreated controls, indicating that some cells of the zebrafish saccule are mitotically active in the absence of a damaging event. In sound-exposed fish, cell proliferation peaked two dpse in the caudal region, and to a lesser extent in the rostral region. This proliferation was followed by an increase in numbers of cuticular plates with rudimentary stereocilia and immature-like hair bundles at 7 and 14 dpse, suggesting that at least some of the saccular cell proliferation resulted in newly formed hair cells. This study establishes a time course of hair cell bundle regeneration in the zebrafish inner ear and demonstrates that cell proliferation is associated with the regenerative process.

1. Introduction

Mammalian cochlear hair cells are damaged or lost due to loud or prolonged noise-exposure (Lim, 1976; Lindeman and Bredberg, 1972; Stockwell et al., 1969), ototoxic drugs (Lim, 1976; Theopold, 1977), and age (Coleman, 1976; Keithley and Feldman, 1982). Limited hair cell regeneration has been reported in mammalian utricular sensory epithelium via mitosis *in vitro* (Warchol et al., 1993; Zheng et al., 1997) and via non-mitotic hair cell replacement *in vivo* (Rubel et al., 1995). Hair cell replacement has also been reported in cultured embryonic mouse cochleae and neonatal supporting cell cultures (Kelley et al., 1995; White et al., 2006); however, hair cell regeneration does not appear to take place in the sensory epithelium of the intact postnatal cochlea (Roberson and Rubel, 1994) in mammals, leading to permanent deafness.

In contrast, non-mammalian vertebrates such as fish and birds spontaneously regenerate hair cells in both the vestibular and auditory portions of the inner ear (Cotanche, 1987b; Corwin and Cotanche, 1988; Ryals and Rubel, 1988; Lombarte et al., 1993; Weisleder and Rubel, 1993; Smith et al., 2006). Following noise- or drug-induced trauma in the avian ear, supporting cells appear capable of giving rise to new hair cells through mitosis (Corwin and Cotanche, 1988; Hashino and Salvi, 1993; Raphael, 1992; Ryals and Rubel, 1988; Stone and Cotanche, 1994) or direct transdifferentiation (Adler and Raphael, 1996; Baird et al., 1996; Baird et al., 2000; Roberson et al., 1996, 2004; Taylor and Forge, 2005). Post-embryonic hair cell production has been observed in the lateral line neuromast organs of amphibians and fish (Stone, 1937; Wright, 1947; Tester and Kendall, 1969; Corwin, 1986; Corwin et al., 1989). Hair cell regeneration has been reported in the saccule and lateral line of urodele amphibians (Jones and Corwin, 1996; Taylor and Forge, 2005), and in the crista ampullaris of lizards (Avallone et al., 2003).

Similarly, hair cell regeneration occurs in fishes. This has been demonstrated in the goldfish saccule (Smith et al., 2006), the oscar utricle and lagena (Lombarte et al., 1993), and the zebrafish lateral

Abbreviations: ANOVA, analysis of variance; BrdU, bromodeoxyuridine; cm, centimeters; d, days; DAPI, 4′,6-diamidino-2-phenylindole; dB, decibels; DT, direct transdifferentiation; dpse, days post-sound exposure; FITC, fluorescein isothiocyanate; h, hour; HCL, hydrochloric acid; Hz, hertz; L, liter; μm, micron; M, molar; mm, millimeter; min, minute; μPa, microPascal; MS-222, tricaine methanesulfonate; n, number (i.e., sample size); N, normal; PBS, phosphate buffered saline; RMS, root mean square

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line (Harris et al., 2003). Although hair cell regeneration has been reported in the zebrafish lateral line exposed to copper sulfate or aminoglycosides (Song et al., 1995; Harris et al., 2003; Hernandez et al., 2007), the regenerative abilities of the zebrafish inner ear hair cells have not been characterized.

The zebrafish (*Danio rerio*) is an emerging model organism for vertebrate inner ear development and deafness (Whitfield, 2002), and genetic diseases associated with hearing loss such as Usher 1B syndrome (Ernest et al., 2000), Long QT syndrome (Arnaout et al., 2007) and Branchio-oto-renal syndrome (Kozlowski et al., 2005). The zebrafish is a useful model organism because the basic structure and function of the fish inner ear is similar to that of other vertebrates (Popper and Fay, 1999) and mammals share homologous genes with zebrafish that are known to affect inner ear structure and/or function. For instance, the zebrafish Mariner mutant possesses a missense mutation in the gene encoding Myosin VIIA and presents functional and morphological hair cell defects that are similar to those found in mice defective in Myosin VIIA (Ernest et al., 2000). *Foxi1*(aka *Fkh10*), a gene expressed in otic precursor cells, is necessary for normal inner ear development in both mice (Hulander et al., 1998, 2003) and zebrafish (Solomon et al., 2003). *Atoh1* (atonal homolog 1), a gene also known as *Math1*, is a key regulator of differentiation of precursor cells that become hair cells in mice (Bermingham et al., 1999; Zheng and Gao, 2000). The presence of zebrafish atoh1 homologs are prominent during development and are necessary for hair cell fate selection in the lateral line and inner ears of zebrafish (Itoh and Chitnis, 2001; Millimaki et al., 2007). Since zebrafish share inner ear developmental and differentiation genes with mammals, examination of gene expression in the zebrafish during naturally occurring hair cell regeneration may uncover targets for genetic manipulation in mammals to provide new ways to treat deafness.

The purpose of this study was to establish the time course and extent of hair cell regeneration in the zebrafish saccule in response to acoustic trauma, in preparation for subsequent work exploring gene expression patterns during this process. In addition, we wanted to determine whether hair cell bundle recovery is associated with mitosis in the adult zebrafish saccule, as mitosis is associated with hair cell regeneration following acoustic trauma in the avian basilar papilla (Corwin and Cotanche, 1988; Hashino and Salvi, 1993; Raphael, 1992; Ryals and Rubel, 1988; Stone and Cotanche, 1994). We chose to examine the saccule specifically since, of the three otolithic organs (utricle, saccule, and lagena), the saccule has been most fully characterized as a sound detector in fishes (reviewed in Popper and Fay, 1973, 1999).

2. Materials and methods

2.1. Experimental animals

Wild Type adult breeder zebrafish were obtained from a commercial supplier (Segrest Farms, Gibsonton, FL) and maintained in 170-L flow-through aquaria under conditions of constant temperature (25 °C) and a 12-h light/12-h dark schedule. Fish total lengths ranged from 36 to 44 mm. All work was done under the supervision of the Institutional Animal Care and Use Committee of Western Kentucky University.

2.2. Acoustic exposure

Zebrafish were exposed to a 100 Hz tone at a source level of 179 dB re 1 μPa root mean squared (RMS) at 1 cm directly above the center of the speaker. A preliminary study had shown that this stimulus was capable of producing significant hair bundle damage in the saccule. The sound was generated by a B&K Precision function generator (4017A) connected to a 5.3 amp/200 watt Audiosource monoblock amplifier and University Sound UW-30 underwater speaker placed in a 19-L sound exposure chamber. A total of 56 fish were sound-exposed for 36 h at 24.5–25 °C. Three sound exposure experiments were performed. One for phalloidin labeling and two for BrdU labeling. Fish in the bromodeoxyuridine (BrdU) labeling experiment were divided into two groups. Following sound exposure, fish in the first group were allowed to recover for 0, 2, and 4 days before dissection, and fish in the second group were allowed to recover 1, 3, and 10 days before dissection. Two groups of sound-exposed fish were needed because the process used to label BrdU incorporation was too time-consuming to allow consecutive daily dissections.

2.3. Phalloidin labeling

Hair bundles were quantified through visualization of stereocilia stained with Alexa Fluor 488-conjugated phalloidin at 0, 2, 7, and 14 days post-sound exposure (dpse), plus controls ($n = 5$ per group). Phalloidin binds with actin, a primary component of hair stereociliary bundles and cuticular plates, permitting visual identification of hair cells. Fish were euthanized with an overdone of tricaine methanesulfonate (MS-222, Argent, Redmond, WA), a fish anesthetic, and the heads were removed and fixed with 4% paraformaldehyde overnight. After rinsing 4× 10 min in 0.1 M phosphate buffered saline solution (PBS), the inner ears were dissected out of the head, the saccules isolated from the ears, and excess tissue was trimmed away to allow the saccules to lie flat. Saccules were then placed in concavity wells and incubated in 1:100 fluorescein phalloidin (Invitrogen) in PBS at room temperature in a dark box for 30 min. Following incubation, saccules were mounted on glass slides using Prolong Gold Antifade reagent with DAPI (Invitrogen), which allows visualization of nuclei. Slides were viewed under a Zeiss Axioplan 2 epifluorescent microscope with FITC and DAPI filters. Images were captured using an AxioCam MRm camera under 5, 10, 20, and 100× objectives. Images were analyzed with Zeiss Axiovision 4.4 software. Saccular length was determined for each sample, and 2500 μm^2 sampling squares were placed along the center length of the saccule at 5, 25, 50, and 75% of the length from the rostral tip of the saccule (Fig. 1). The hair cell bundle densities at the most caudal tip of saccules (90–100% of length measured from the rostral tip) were too high to accurately quantify; therefore, we chose not to attempt quantification of hair bundle density in this area of the saccule. Phalloidin-labeled hair bundles fluoresced green under the FITC filter, allowing their number to be counted in each square. Due to extensive DAPI staining of nuclei through multiple layers of cells, we abandoned attempts to quantify numbers of nuclei. However, we did note qualitative de-

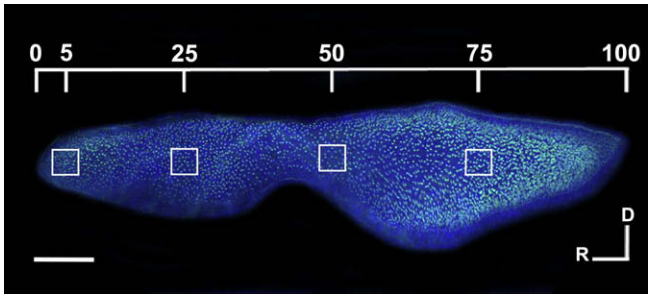

Fig. 1. Hair cell bundle count locations on the zebrafish saccule. Hair bundle counts were sampled at four predetermined locations: 5%, 25%, 50%, and 75% of the total saccular length, as measured from the rostral tip. A 2500 μm^2 box was placed at each sampling area and labeled hair cell bundles were counted within each box to determine hair cell density. D = dorsal, R = rostral; scale bar = 100 μm.