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The Effects of Growth Hormone in the Inner Ear of Zebrafish (*Danio rerio*) during Hair Cell Regeneration

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THE EFFECTS OF GROWTH HORMONE IN THE INNER EAR OF ZEBRAFISH (*DANIO RERIO)* DURING HAIR CELL REGENERATION

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THE EFFECT OF GROWTH HORMONE IN THE INNER EAR OF ZEBRAFISH (*DANIO RERIO)* DURING HAIR CELL REGENERATION

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Directed by: Michael E. Smith, Claire A. Rinehart, and Nancy A. Rice

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Although deafness is a universal problem, effective treatments have remained elusive. In order to develop potential treatments, an overall understanding of the cellular process of auditory hair cell regeneration, which occurs in fish but not mammals, must be established. A previous microarray analysis and qRT-PCR validation of noise-exposed zebrafish showed that growth hormone (GH) was significantly upregulated during the process of auditory hair cell regeneration. Thus, GH may play an important role during hair cell regeneration. However, cellular effects of exogenous GH in the zebrafish auditory hair cell regeneration have not been examined after noise exposure. To understand the effect of GH in hair cell regeneration, adult zebrafish were exposed to a 150 Hz pure tone at a source level of 179 dB re 1 μPa RMS for 36 hours. Afterward the fish were immediately injected intraperitoneally with carp recombinant GH (20 μg/gram of body mass) or buffer (0.1 M, pH 7.4 phosphate buffer) and then placed in a recovery tank. The effect of GH on apoptosis in fish inner ear end organs were examined using TUNEL-labeling. Cell proliferation was measured by BrdU incorporation assay. Hair cell regeneration was determined by phalloidin-labeling to allow visualization of hair cell stereociliary bundles. After GH injection, the numbers of TUNEL-labeled cells showed a

significant decrease in all three inner ear end organs (saccule, lagena, utricle), suggesting GH may suppress hair cell death induced by acoustic trauma. Higher levels of cell proliferation were also observed in the ears of GH-injected fish, indicating that GH is capable of activating cell mitosis in the zebrafish auditory system. Following sound exposure, the GH-injected group exhibited greater numbers of saccular hair cell bundles compared to the buffer-injected group. These results indicate that GH promotes hair cell regeneration following acoustic damage. Future studies are needed to examine the potential therapeutic benefits of GH in the mammalian ear.

CHAPTER 1

Background:

The loss of auditory hair cells is a major cause of hearing impairment that affects approximately 278 million people in the world and at least 37 million people in the United States (Pleis and Lethbridge-Cejku, 2006). Although it is a universal problem, effective therapeutics for hearing loss have still remained indefinite. Most sensorineural hearing loss results from the loss the sensory cells of the ear. How to restore these lost sensory cells and make them return to normal function has become an important issue for the development of potential treatments for deafness.

The sensory cells found in the organ of Corti in the cochlea are called hair cells. These hair cells can be damaged and or destroyed by noise exposure (Hamernik et al., 1984), ototoxic drugs (Theopold, 1977; Wanamakers et al., 1998; Monge et al., 2006), infection, autoimmune disorders, and aging (Keithley and Feldman, 1982). In mammals, such hair cell loss cannot be replaced spontaneously in the organ of Corti, and this can cause permanent hearing loss (Richardson et al. 1995). Auditory hair cells that are exposed to very loud sounds or noises of lower intensity for longer periods can cause irreversible threshold shifts and noise-induced hearing loss (NIHL; Saunders et al, 1991). NIHL is usually studied using animal models because noise exposure studies in humans are unethical and since the genetics of animal models is tractable and can be manipulated. For instance, intense sounds or ototoxic drugs have been shown to cause hearing threshold shifts in birds (Corwin and Cotanche, 1988; Saunders et al., 1991; Saunders et al., 1992; Park et al., 2002) and mammals (Prosen et al, 1990). Therefore, optimal animal models can be very effective tools for studying NIHL.

While hair cell loss produces permanent hearing loss in mammals, these lost sensory hair cells can regenerate in other vertebrates (e.g., birds, amphibians, and fishes). For example, hair cell regeneration is not only found in lateral line neuromast organs of amphibians and the inner ear of reptiles and amphibians (Stone, 1937; Corwin et al., 1989; Avallone et al., 2003), but also in the avian cochlea (Weisleder and Rubel, 1992; Cotanche, 1999).

Hair cell regeneration can also occur in the inner ear and lateral line of fishes (Lombarte et al., 1993; Harris et al., 2003; Smith et al., 2006; Schuck and Smith, 2009). A fish's ear is composed of three semicircular canals and three pairs of otolithic end organs (the utricle, lagena, and saccule) which contain sensory hair cells found in patches referred to as maculae (Fay and Popper, 2000). Auditory hair cells in the fish inner ear can be damaged via acoustic overexposure and cause NIHL (Smith et al., 2004a b; Smith et al., 2006). Scholik & Yan (2001) found that hearing recovered after acoustic trauma and this recovery was dependent upon the noise exposure duration and frequency. This recovery was likely the result of regeneration of lost hair cells.

Hair cell regeneration in the teleost inner ear following noise exposure was first studied in the goldfish (*Carassius auratus*; Smith et al., 2006). After 2 days of noise exposure, significant saccular hair cell and hearing loss occurred, but significant recovery of both hair cells and hearing capabilities occurred within 8 days. Similarly, hair cell regeneration occurs in zebrafish. Schuck and Smith (2009) found that numbers of hair cell bundles recovered back to control levels in the zebrafish saccule at seven days post-noise exposure. This recovery in hair cell number was preceded by an increase in

auditory epithethial cell proliferation suggesting that newly regenerated hair cells are produced at least partially via mitosis in zebrafish.

The inner ear epithelia in birds and fishes are composed of three layers: the basal lamina, supporting cells, and hair cells. Supporting cells lay on top of the basal lamina and surround hair cells which occur on the apical surface of the epithelia where their stereocilia protrude (Oesterle and Stone, 2008). When hair cells undergo trauma followed by apoptosis, they are ejected out of the epithelium. Following this ejection, newly formed hair cells are regenerated but the source of these new hair cells is not always clear. Bhave et al. (1995) concluded there are two methods that cause this regeneration: the new hair cells can be transdifferentiated from the supporting cell precursors, or quiescent supporting cells divide and then differentiate into new supporting cells and hair cells. Thus, there are multiple potential pathways for the regeneration of newly formed hair cells, including supporting cell proliferation, direct transdifferentiaton, and also direct hair cell repair (reviewed in Walshe and Walsh, 2003).

Supporting cell proliferation (mitosis) refers to those newly grown hair cells that resulted from cell division of supporting cells, followed by differentiation into hair cells. The fact that hair cells regenerate via this mechanism is based on evidence of hair cells which are labeled with a mitotic marker like [H^3] methyl-thymidine or bromodeoxyuridine (BrdU) and colocalization of supporting cells undergoing mitosis and newly formed hair cells (Warchol et al., 1993).

Direct transdifferentiation occurs when supporting cells differentiate into a hair cell without undergoing mitosis. While it has been shown that mitosis is definitely involved in hair cell regeneration in the avian ear, direct transdifferentiation of hair cells

from supporting cells has also been reported (Corwin and Oberholtzer, 1997; Staecker & Van De Water, 1988; Shiang et al., 2010).

Direct hair cell repair means that damaged hair cells are able to self-repair (intracellular repair) without mitosis and without transdifferentiating from other cell types. Hair cell damage may include loss or damage of the stereociliary bundle without loss of the cell body. Zheng et al. (1999) found evidence of direct hair cell repair in rat utricle explants. A substantial number of utricular hair cells survived gentamicin exposure even though some lost their stereocilia. These partially damaged hair cells can survive for a prolonged time and regrow stereocilia.

The zebrafish (*Danio rerio*) is a useful vertebrate model for the study of inner ear, organ development, hearing disease, and genetic defects (Whitfield, 2002; Kimmel 1989; Zon, 1999). It is an important animal model for hearing loss studies because many genetic mutations related to the inner ear development have been identified (Malicki et al., 1996), and the readily accessible transparent embryos allow for rapid screening for different mutants (Sprague et al., 2001). In addition, the zebrafish inner ear has sensory hair cells and auditory mechanisms that are similar to those of other vertebrates (Fay and Popper, 2000), and also share homologous genes with mammals. All the known zebrafish homologs of mammalian genes can be found on the Zebrafish Information Network (http://zfin.org).

Mammals and fish share several homologous genes that can promote hair cell regeneration. Atoh1, Rb1, and p27kip1 are three examples. Atoh1 (atonal homolog 1a), also called Math1, is a key gene that regulates the differentiation of mice hair cells (Zheng and Gao, 2000). After the deletion of tumor suppressor Rb1 (retinoblastoma 1) in

mice, hair cells proliferated and maintained a highly differentiated state (Sage et al., 2005). Disruption of the cyclin-dependent kinase inhibitor p27kip1 (cdkn1b) caused the proliferation of cochlear hair cells in postnatal mice (Löwenheim et al., 1999). Fish hair cells regenerate naturally on their own following trauma. Understanding the process of, and genes regulated during hair cell regeneration in fishes may help us develop therapeutics to promote hair cell regeneration in mammals.

A previous microarray analysis of noise-exposed zebrafish in our lab showed that a number of genes were significantly up- or down-regulated in the zebrafish inner ear at two and four days post-sound exposure. Growth hormone (GH) was the greatest up-regulated gene and subsequent quantitative Real-Time PCR (qRT-PCR) validated this result (Schuck, 2007).

The purpose of this thesis is to study the cellular effects of exogenous growth hormone (GH) in zebrafish auditory hair cell regeneration after noise exposure and establish possible gene networks and biochemical signaling pathways that may be related to hair cell regeneration. Chapter 2 describes the experiments that measure the effect of GH injection on apopotosis, cell proliferation, and hair cell regeneration in the end organs of the zebrafish ear after sound exposure. Chapter 3 discusses the results found in Chapter 2, and Chapter 4 is a summary of the overall findings in this thesis and possible directions for further studies.

CHAPTER 2

Introduction:

Growth hormone (GH), a common secretory protein, is produced from the anterior pituitary gland (Okada and Kopchick, 2001) and has been found to have effects on cellular proliferation, differentiation, and metabolism (Isaksson and Jansson, 1982; Davidson, 1987; Casanueva, 1992). GH is also a member of cytokine superfamily of polypeptide regulators (Bravo and Heath 2000). For instance, van den Eijnden and Strous (2007) found autocrine GH is related to the function of development, cell proliferation and differentiation in tumor cells.

GH can also promote the regeneration of a number of different tissues. GH has been reported to stimulate muscle (Ullman et al., 1989) and bone regeneration (Cacciafesta et al., 2001). In liver regeneration, GH also played an important role (Pennisi et al., 2004). More importantly, GH has displayed therapeutic effects on nerve regeneration in rat (Kanje et al., 1988; Jung et al., 1998), and of wound healing acceleration in human burn patients (Herndon et al., 1990; Gilpin et al., 1994). GH has been used to increase the rate of fish growth (Johnsson et al., 1999; Martin-Smith, K.M. et al., 2004; Rønsholdt and McLean., 2004), but the effect of GH on the regeneration of any specific fish tissue type has not yet been examined.

Growth factors and mitogens are known to regulate hair cell regeneration as well. For example, insulin-like growth factor-I (IGF-I) and insulin causes cell proliferation of the sensory epithelium in the mature avian ear (Oesterle et al., 1997). In the ear of a cichlid fish (*Astronotus ocellatus*), GH was found to increase supporting cell proliferation (Presson and Kim, 2001). Similarly, a previous study in our lab showed that GH was

greatly up-regulated in the zebrafish ear following noise exposure and that the timing of this upregulation coincided with a peak in cell proliferation in the zebrafish ear during the process of hair cell regeneration (Schuck, 2007). A follow-up study showed a significant increase in cell proliferation in the zebrafish ear following intraperitoneal injection of growth hormone (Michael Smith, unpublished data). These previous studies suggest that growth hormone may play a critical role during auditory hair cell regeneration in zebrafish.

The purpose of this study is to examine the effects of growth hormone in the zebrafish ear following sound-exposure. Specifically, I examined the effect of carp GH-injection on cell proliferation, apoptosis, and hair cell bundle density in the sound-exposed zebrafish ear in order to quantify any potential therapeutic effects.

Methods:

Experimental animals

Ninety adult zebrafish were obtained from commercial suppliers (Fishy Business, Bowling Green, KY) and maintained in 170-L flow-through aquarium under constant temperature (25 °C) and a 12 hours light/12 hours dark cycle. All experiments were done under the approval of the Institutional Animal Care and Use Committee of Western Kentucky University.

Experimental design

In all experiments, groups of six fish were exposed to sound (see acoustic exposure description below) and then immediately injected intraperitoneally with either

carp recombinant growth hormone (GH; 20 μg/gram of body mass) or buffer (0.1 M, pH 7.4 phosphate buffer) and then placed in a recovery tank. Apoptosis and cell proliferation were measured at 1 day following acoustic trauma (post-sound exposure day 1=psed1). Hair cell densities were measured at post-sound exposure day 2 (psed2). These time points were chosen since our previous studies showed a peak in cell proliferation in the zebrafish saccule at 2 days post-trauma (Schuck and Smith, 2009) and near-complete hearing recovery occurs between 7 and 14 days post-trauma (Smith et al., 2004a; Smith et al., 2006).

Growth hormone injection

First, carp recombinant growth hormone powder (ProSpec, Israel) was dissolved in Nanopure water at a final concentration of 0.5 μg/μl. This GH solution was injected into the fish immediately after sound exposure. First, a light dose (approximately 10 μg in 1 ml water) of tricaine methanesulfonate (MS-222, Argent, Redmond, WA) was used to sedate the fish and then each fish was injected intraperitoneally with carp GH at 20 μg per gram of body mass. Fish were allowed to recover from injection in a small container containing fish tank water. After fish recovered to normal movement, they were put back into their normal aquaria until needed for specific experimental time points.

Acoustic exposure

Zebrafish were exposed to a 150 Hz pure tone at a source level of 179 dB re 1 μPa root mean squared (RMS). The noise was generated by an underwater speaker (University Sound UW-30) and function generator (4017A, B&K Precision) attached

with 5.3 amp/200 watt Audiosource monoblock amplifier. All fish were exposed for 36 hours at 25ºC and placed in a 19-L sound exposure aquarium. From previous studies, this protocol has resulted in significant hair cell loss in the zebrafish saccule (Smith and Schuck, 2009).

Determination of cell death

In order to detect death of hair cells (apoptosis), after sound exposure, treated fish were euthanized with an overdose of MS-222 (n=6 per group), and the heads were removed. Both inner ears were dissected out and immediately fixed in 4% paraformaldehyde. After washing with 0.1 M pH 7.4 phosphate buffer (PB), the intact saccules, lagenaes, and utricles were then trimmed out from the ears and placed on adhesive poly L-lysine coated slides. Apotosis was detected using the standard protocol of the ApopTag Fluorescein In Situ Apoptosis Detection Kit (S7710, Millipose). Tissues were post-fixed in pre-chilled ethanol:acetic acid (2:1) for 6 minutes at -20℃ and then washed 3 X 5 minutes with 0.1 M PB. Then equilibration buffer was applied directly on the slide and incubated at room temperature for at least 10 seconds. The buffer was rinsed and TdT (terminal deoxyribonucleotidyl transfer) enzyme was added on the slide and incubated in a humidified incubator at 37℃ for 1 hour. Stop/wash buffer was applied, agitated for 15 seconds, and then incubated at room temperature for 10 minutes. After rinsing 3 X 5 minutes with 0.1 M PB, anti-digocigenin conjugate (fluorescein) was added to the slides and incubated in a humidified container in dark at room temperature for 1 hour. The slides were washed with 0.1 M PB 3 X 5 minutes again and mounted with the Prolong Gold Antifade reagent with DAPI (P36931, Invitrogen) and then a cover-slip

was placed on top. Labeled cells were counted in sections manually under a Zeiss compound microscope at 10X and 100X magnification.

Determination of cell proliferation

Following sound exposure and injection with either GH or buffer, fish groups (n=6 per group) were allowed to recover for 8 hours in the tank and were used for BrdU incorporation assay with the standard protocol of Amersham Cell Proliferation Kit (RPN20LR, GE Healthcare). Bromodeoxyuridine (BrdU) is a synthetic thymidine analog and can be incorporated into cellular DNA during S-phase, thus allowing detection of cell proliferation. Fish were injected intraperitoneally with a BrdU labeling reagent and then placed in a separate tank. After 16 hrs, fish were euthanized with an overdose of MS-222, their heads were removed, and their inner ears were immediately dissected out and fixed in 4% paraformaldehyde for 1 hour. After washing with 0.1 M PB, the intact saccules, lagenaes, and utricles were trimmed of excess tissue and placed on adhesive poly L-lysine coated slides and then incubated for 1 hour at room temperature in mouse monoclonal anti-BrdU antibody. After washing in 0.1 M PB 3 X 5 minutes again, tissues were incubated with a 1:200 dilution of Alexa Fluor 488-conjugated goat anti-mouse IgG (A11001, Invitrogen) for 1 hour at room temperature in a dark box. Finally, the tissues were washed 3 X 5 minutes with 0.1 M PB and these end organs were then mounted with Prolong Gold Antifade reagent with DAPI (P36931,Invitrogen) and then cover-slipped. Low (10X objective) and high (100X objective) power images of the saccule, lagenae and utricle were viewed under FITC and DAPI filters of a Zeiss Axioplan 2 epifluorescent microscope and merged pictures were photographed by the AxioCam MRm camera. The

qualitative evaluation of cell proliferation was determined by counting Alexa Fluor 488-labeled cells in the whole saccule, laganea, and utricle.

Quantification of hair cells bundles:

Two days after sound exposure and the injection, groups of fish (n=6 per group) were euthanized with an overdose of tricaine methanesulfonate. The heads removed and fixed in 4% paraformaldehyde at 4°C overnight. Then they were washed 3 X 10 minutes with 0.1 PB, and the inner ear maculaes (saccules, lagenaes, and utricles) were dissected out of the head and excess tissue trimmed. All tissue was incubated in concavity wells with a 1:100 dilution of fluorescein phalloidin (F432, Invitrogen) in phosphate buffer (PB) at room temperature in a dark box for 1 hour. After incubation, end organs were placed on glass slides mounted with Prolong Gold Antifade DAPI mount reagent (P36931, Invitrogen) and then a cover-slip was placed on top. Low power images (10X objective) of the saccule, lagenae and utricle were viewed under FITC and DAPI filters of an Zeiss Axioplan 2 epifluorescent microscope and photographed with an AxioCam MRm camera. Hair cell bundle counts were obtained from 5 preselected locations along the rostral-caudal axis of the saccular epithelium. Each location was 30 X 30 μm boxes which were placed over digital images in Photoshop using scaling parameters of the microscope. Under the FITC filter, the phalloidin-labeled hair cell stereocilia fluoresced green which allowed easy quantification of their numbers. DAPI staining (blue color) allowed the visualizing of cell nuclei. Previous work showed a direct correlation between hair cell bundle loss and hair cell nuclei loss following noise exposure (Smith et al., 2006), thus for this project only hair cell bundles were quantified. Hair cell morphotypes were

quantified as 1) normal hair cell bundles with intact, long stereocillia, 2) damaged hair cell bundles which had broken, disordered, or sparse stereocillia, 3) presumed newly formed hair cell bundles which were compact, well-ordered, and much shorter than whole normal hair cell bundles (less than half of the length of normal hair cell bundles), and 4) bundleless hair cells lacking stereociliary bundles and exposing the cuticular plates.

Data analysis

Analysis of variance (ANOVA) was used to test for differences between buffer- and GH-injected fish for all of the endpoints quantified. Separate ANOVAs were done for each location along the rostral-caudal axis for hair cell bundle counts, for each hair cell morphotype in each location, and for each end organ (saccule, lagena, utricle) for BrdU- and TUNEL-labeled cells.

Results:

Effect of GH on apoptosis in the zebrafish ear

To test whether growth hormone (GH) is able to suppress cellular death after sound-induced trauma, high power photomicrographs (100X) served as an internal confirmation of TUNEL-labeling in the sensory epithelium of the inner ears of GH or buffer-treated zebrafish and showed that TUNEL-labeling was localized to cell nuclei (Fig. 1). The numbers of TUNEL-labeled cells of GH-injected fish decreased significantly in saccules, lagenae, and utricles of zebrafish at post sound exposure day 1 (psed1; Figs. 2 and 3; $p < 0.01$, $n = 12$). Thus, GH may prevent apoptosis in zebrafish ears exposed to acoustic trauma or at least change the rate at which apoptosis occurs.

Effect of growth hormone on auditory cell proliferation

To test whether growth hormone (GH) is able to promote cellular proliferation after sound-induced damage, a BrdU incorporation assay was used to examine cellular proliferation in the sensory epithelium of zebrafish inner ears of GH- and buffer-treated groups. High power images of each GH- or buffer-injected group were used to verify the correct BrdU staining protocol at psed 1 (Fig. 4). Baseline proliferation was examined in normal zebrafish without sound exposure and injection. Low numbers of BrdU-labeled cells were detected in the baseline group, indicating that a little proliferation occurs in normal zebrafish inner ears in order to maintain homeostasis (Fig. 5; saccule, n=12).

After sound exposure, the numbers of BrdU-labeled hair cells of the GH-injected group were significantly greater in all three zebrafish ear end organs (saccule, lagena and utricle) compared to buffer-injected controls (Fig. 6; $p < 0.001$, $n = 12$). This was especially true in the saccule which had more than a 10-fold increase in BrdU-labeled cells in the GH-treated group compared to buffer-injected controls (Figs. 5 and 6; $p < 0.001$, $n = 12$).

Effect of growth hormone on hair cell bundle regeneration

Hair cell number counts were obtained from 5 preselected locations of the saccular epithelium (Fig. 7). Following sound exposure, phalloidin staining of saccules showed there was a significant loss of hair cell bundles at areas of 25%, 50% and 75% along the rostral-caudal axis (as described in the methods) of buffer-injected fish ($p < 0.01$, $n = 12$; Fig. 8). Density of hair cell bundles increased in the GH-injection group at

post-sound exposure day 2 (psed 2) compared to controls (Fig. 9). Specifically, significant increases occurred at 25, 50 and 75% of the total distance from the rostral tip of the saccules ($p < 0.01$, $n = 12$). No significant differences were observed in the extreme rostral and caudal tip respectively (ie., at the 5% and 90% of the total distance from rostral tip). Phalloidin staining revealed the variation in the morphology of different hair cells found in the experiment (Fig. 10). Control fish exhibited whole normal hair cell bundles with intact, long stereocilia. Sound-exposed fish had some damaged hair cell bundles which had broken, disordered, or sparse stereocilia. Presumed newly formed hair cell bundles were compact, well-ordered, and much shorter than whole normal hair cell bundles (less than half of the length of normal hair cell bundles). Bundleless hair cells lacked stereociliary bundles so that cuticular plates were exposed. Numbers of normal hair bundles and newly formed hair bundles increased significantly in the GH-treated group at the regions of 25, 50, and 75% of the distance from the rostral tip of saccules at psed 2 (Figs.11, 12, and 13; $p < 0.01$, $p < 0.001$, and $p < 0.001$, respectively, $n=12$). This result indicates GH may prevent hair cell bundle loss and stimulate the proliferation of new hair cells following noise exposure. No significant differences were found in numbers of damaged hair bundles and bundleless hair cells.

Table 1. Experimental design to examine the effects of GH injection on apoptosis (TUNEL), cell proliferation (BrdU), and hair cell bundle density (phalloidin) in the zebrafish ear following sound exposure. GH=growth hormone; psed=post-sound exposure day; SE=sound exposure. This design was used for each end organ (saccule, utricle, lagena) for TUNEL, and BrdU, but only the saccule was examined with phalloidin staining.

Treatment (day)	**Experiment**		
	TUNEL	**BrdU**	**phalloidin**
Baseline (no SE)	6	6	6
Buffer (psed1)	6	6	6
GH (psed1)	6	6	6
Buffer (psed2)	6	6	6
GH (psed2)	6	6	6
Total fish = 90	30	30	30

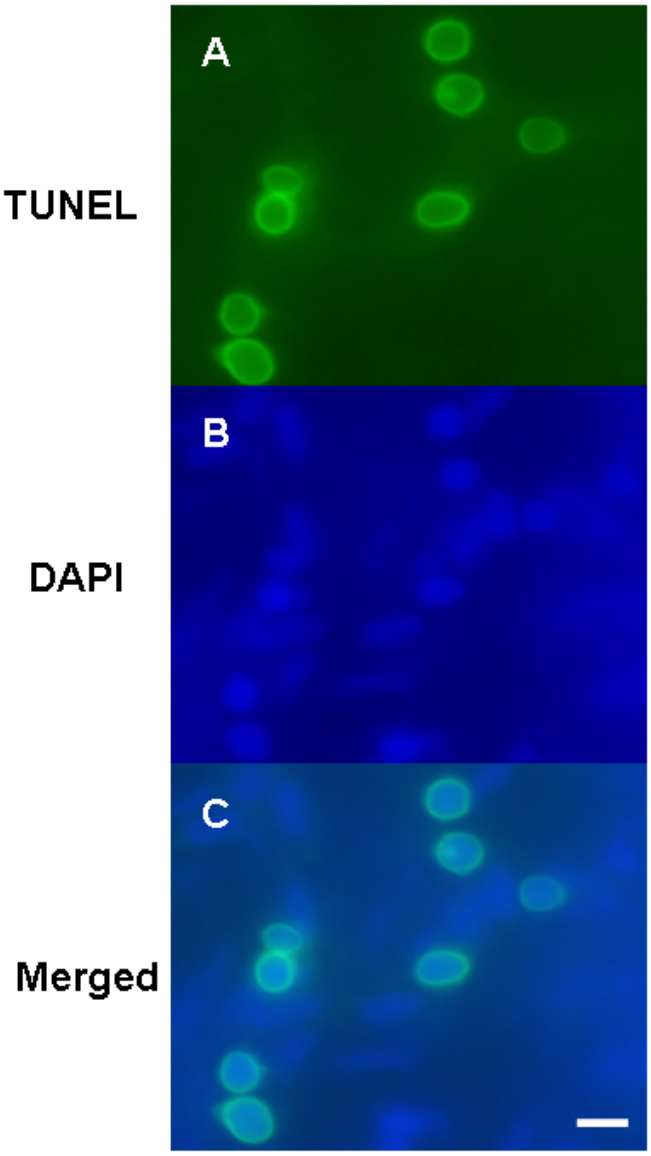

Figure 1.

High power photomicrographs (100X) of TUNEL-labeled cells in the saccule at post-sound exposure day 1 (psed1). A) TUNEL-labeled cells, B) DAPI-stained cell nuclei, C) TUNEL and DAPI merged cells. Scale bar = 5 μm.

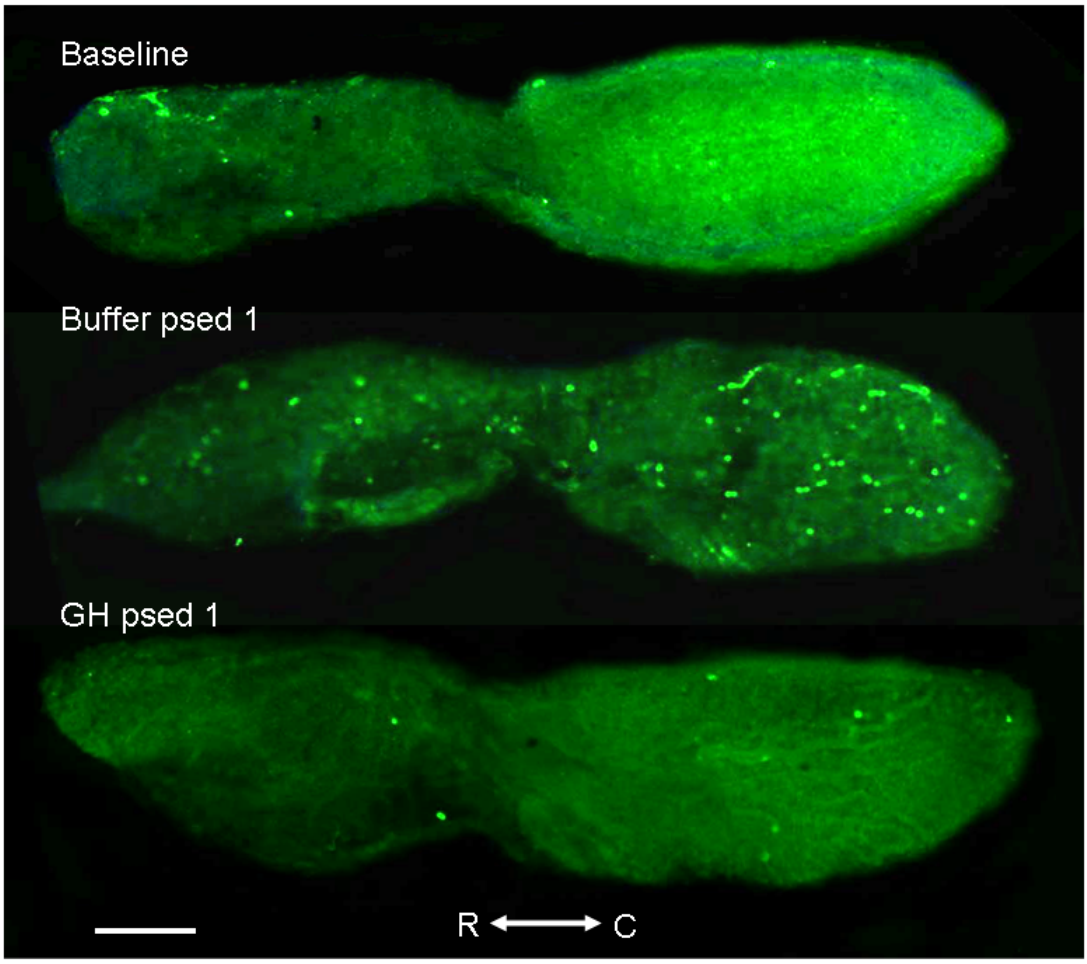

Figure 2.

TUNEL-labeled cells of the saccules of baseline, buffer- and GH-injected zebrafish at post-sound exposure day 1 (psed1). R= rostral, C = caudal. Scale bar = 100 µm.

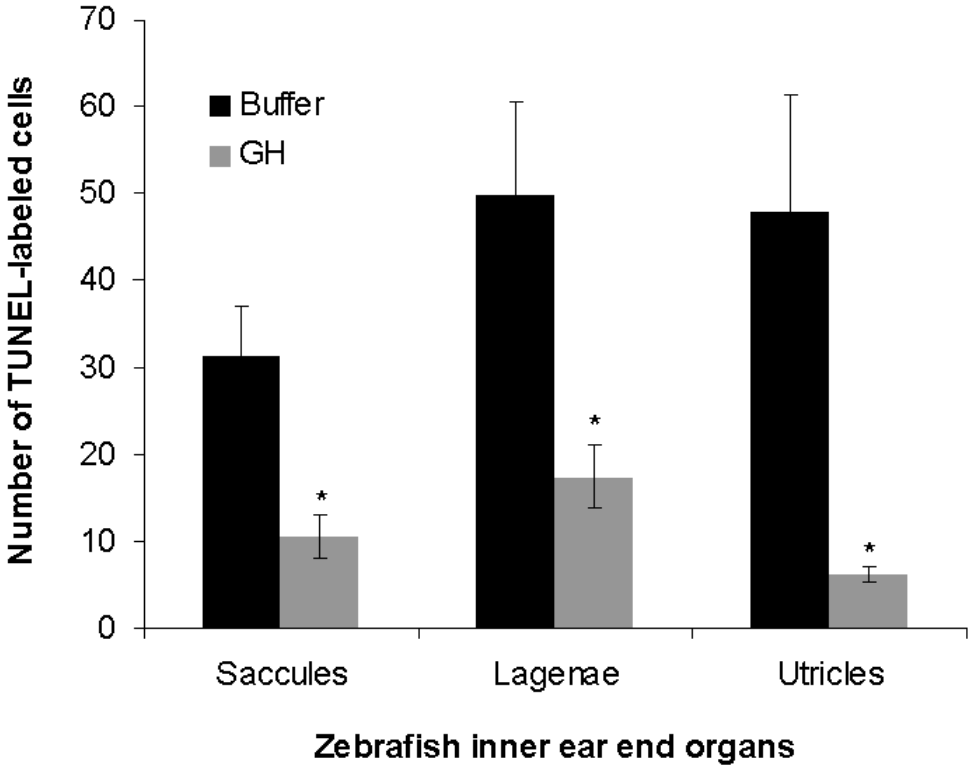

Figure 3.

Mean (± S.E.) TUNEL-labeled cells in the saccules, lagenae and utricles of buffer- (black) and GH-injected (gray) zebrafish following 2 days of sound exposure (dpse2). (* $p < 0.001$, $n = 12$).

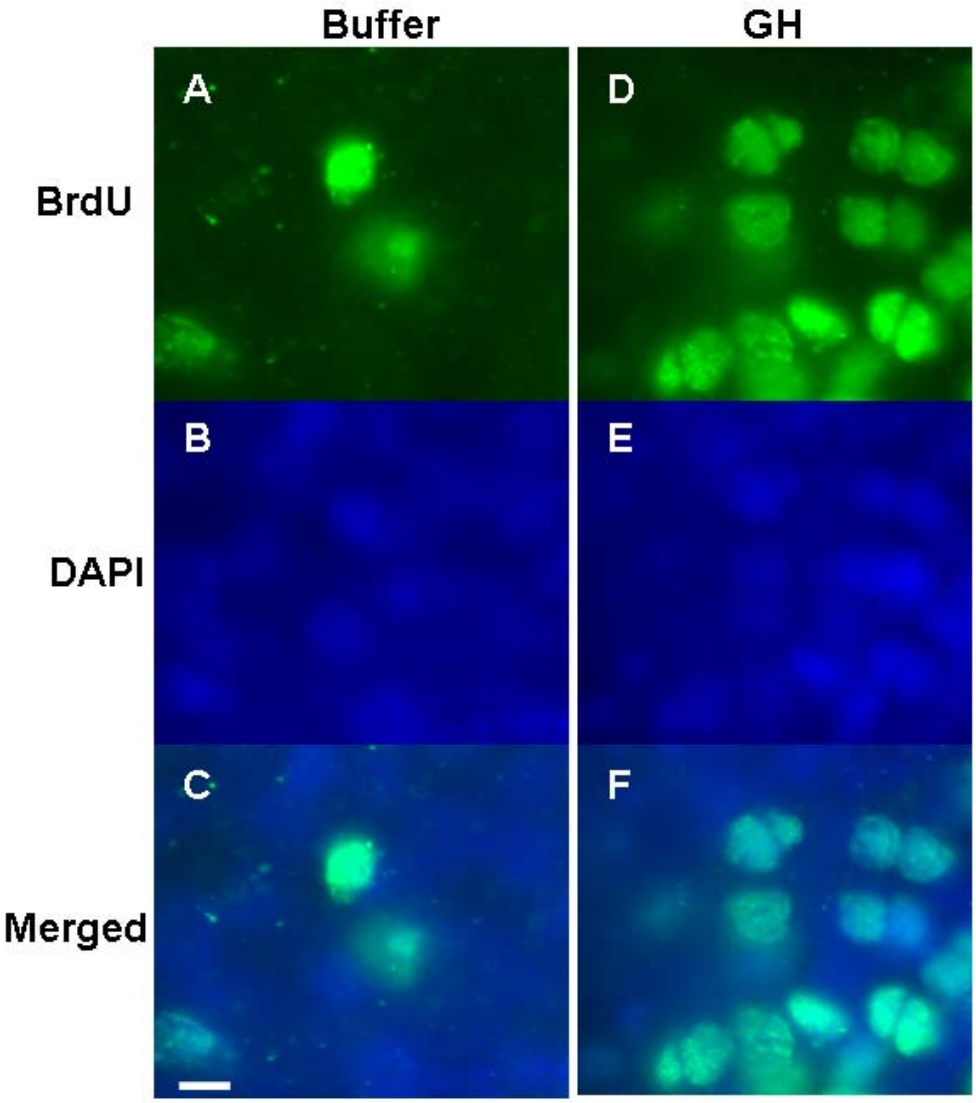

Figure 4.

High power photomicrographs (100X) of BrdU-labeled cells in the zebrafish saccule. Buffer- and GH-injected examples of BrdU-labeled (panels A and D), DAPI (panels B and E), and merged (panels C and F). Scale bar = 5 μm.

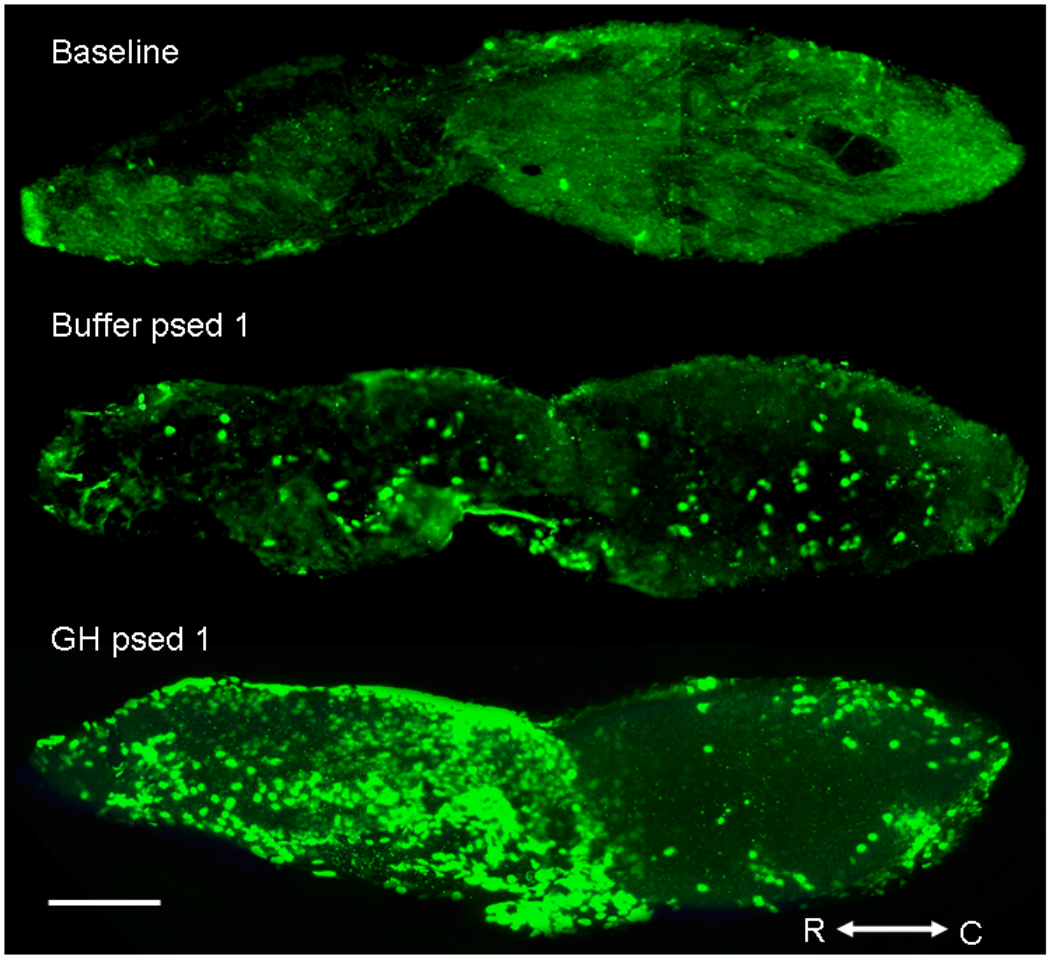

Figure 5.

BrdU-labeled hair cells of baseline, buffer- and GH-treated zebrafish saccules at post sound exposure day 1 (psed1). R= rostral, C = caudal. Scale bar = 100 μm.

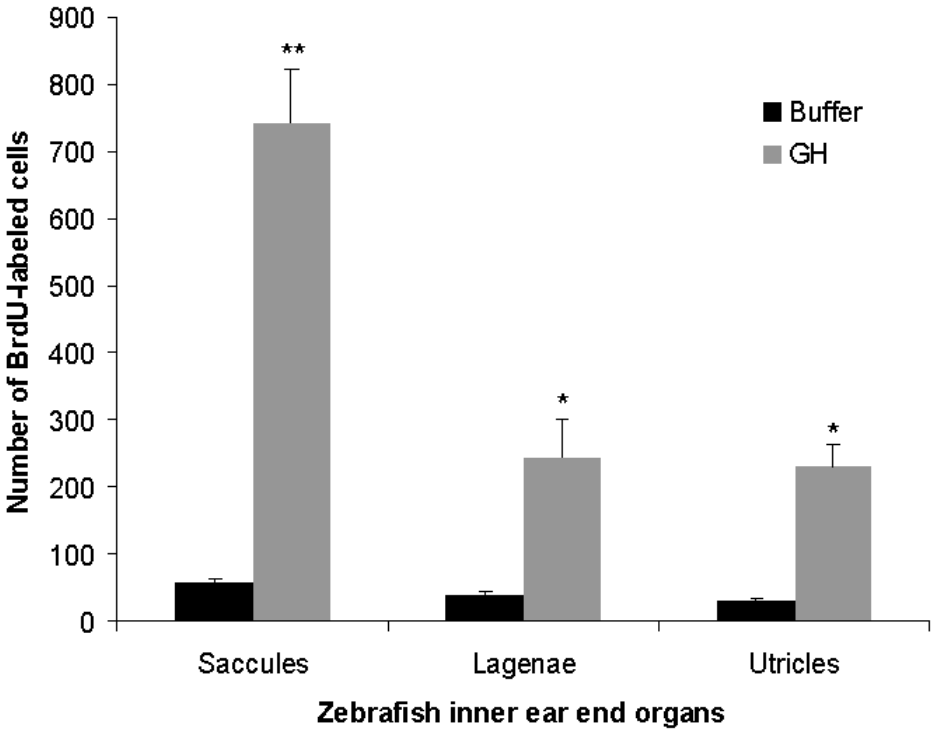

Figure 6.

Mean (± S.E.) BrdU-labeled cells in saccules, lagenas and utricles of buffer- (black) and GH-injected (gray) zebrafish at post-sound exposure day 1 (psed1). (* $p < 0.01$, ** $p < 0.001$, n= 12).

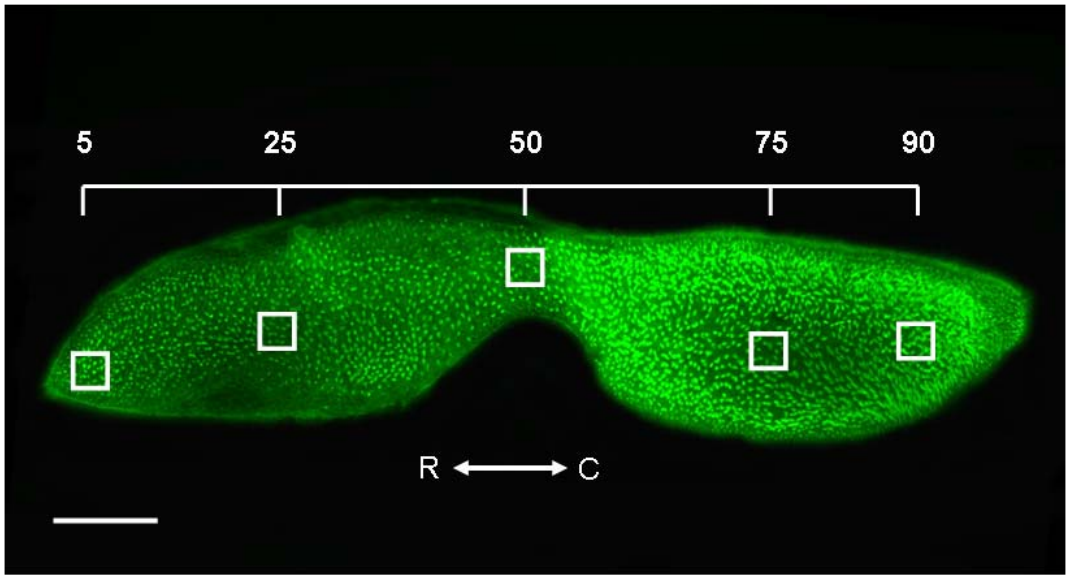

Figure 7.

Phalloidin-labeled hair cell bundle counting areas overlaid of a baseline zebrafish saccule. The numbers of hair cell bundles were counted in 30 μm X 30 μm boxes at five predetermined areas along the rostral-caudal axis: 5, 25, 50, 75 and 90% of the distance from the rostral end of the saccule. D = dorsal, R = rostral. Scale bar = 100 μm.

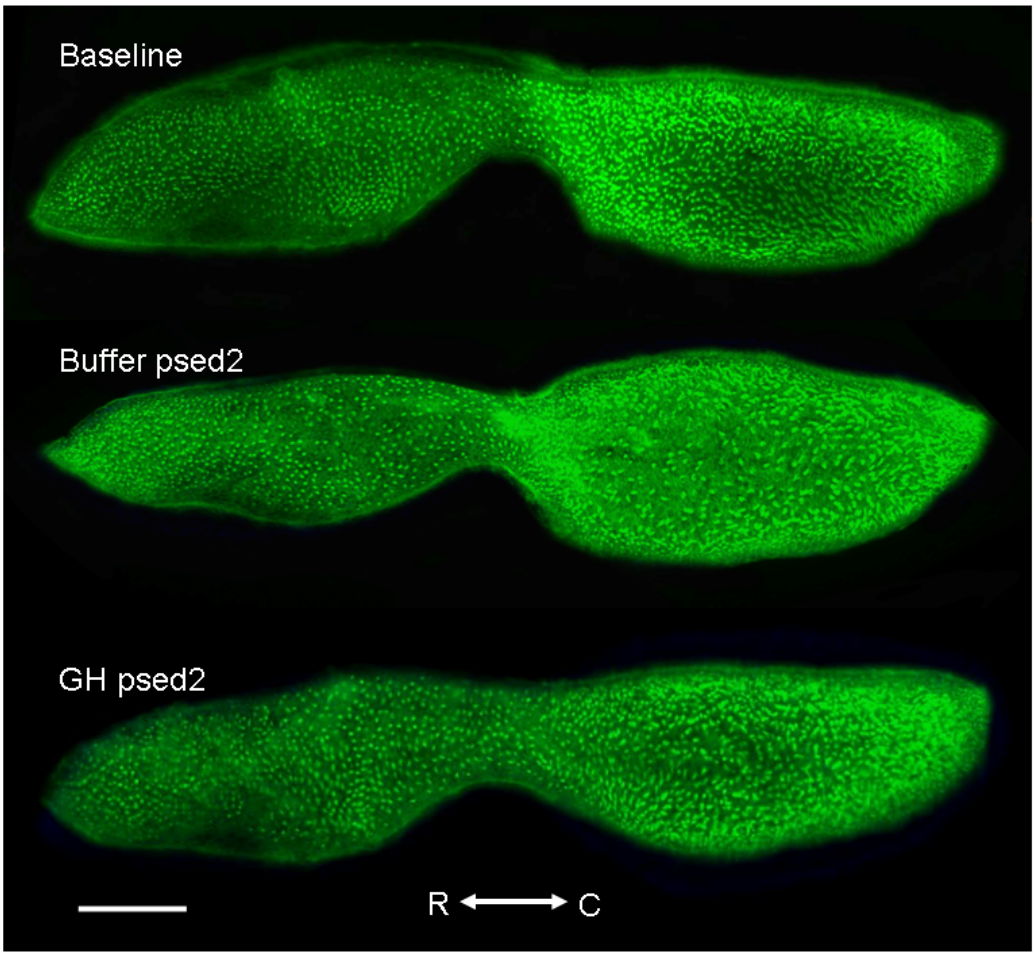

Figure 8.

Phalloidin-labeled hair cell bundles of baseline, buffer- and GH-treated zebrafish saccules at post-sound exposure day 2 (psed2). R= rostral, C = caudal. Scale bar = 100 μm.

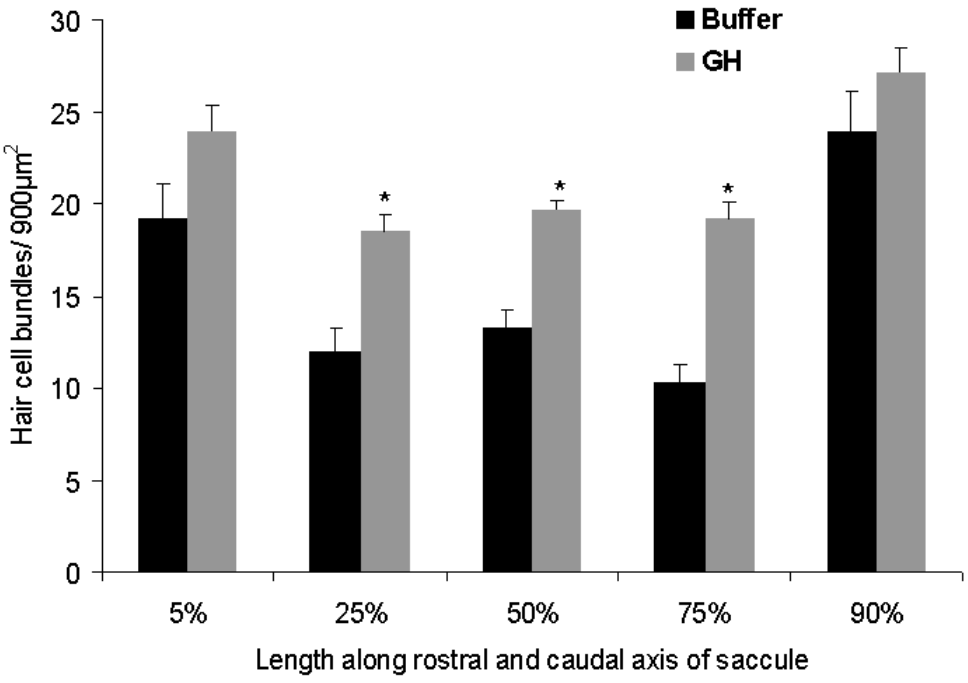

Figure 9.

Mean (± S.E.) phalloidin-labeled hair cell bundles of buffer- (black) and GH-injected (gray) saccules at the 5, 25, 50, 75 and 90% rostral-caudal regions at post-sound exposure day 2 (psed2) (* $p < 0.001$, n= 12).

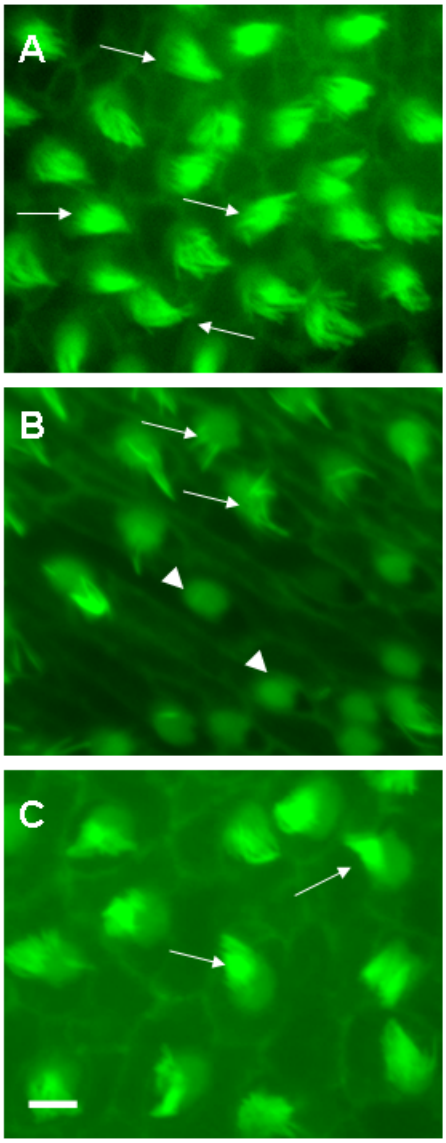

Figure 10.

High power photomicrographs (100X) of phalloidin-labeled hair cells at 25% along the rostral-caudal axis. A) Baseline fish (no noise exposure). Normal hair cell bundles with intact, long stereocilia (arrows). B) Buffer-injected fish following sound exposure (psed2). Damaged hair cell bundles with broken, disordered, or sparse stereocilia (arrows), or bundleless hair cells missing all stereocilia (arrowhead) are evident. C) GH-injected fish at psed2. Presumed newly formed hair cell bundles with compact and well-ordered short hair cell bundles (arrows). Scale bar = 5μm.

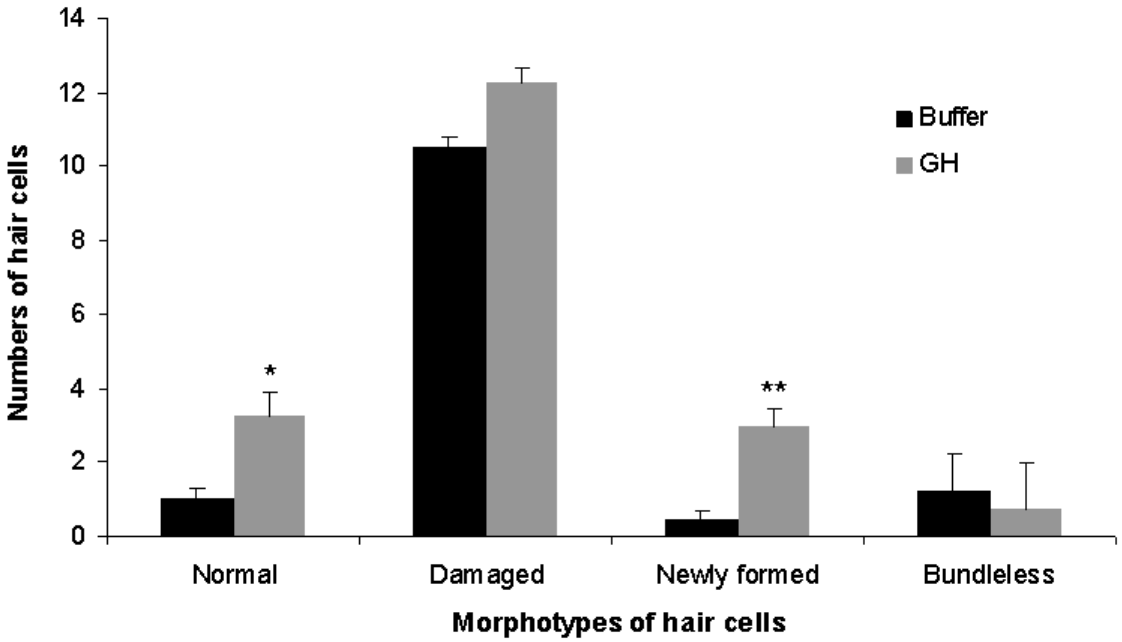

Figure 11.

Mean (± S.E.) normal, damaged, newly formed, and bundleless phalloidin-labeled hair cells of buffer- (black) and GH-injected (gray) saccules at the 25% rostral-caudal region at post-sound exposure day 2 (psed2) (* $p < 0.01$, ** $p < 0.001$, n= 12).

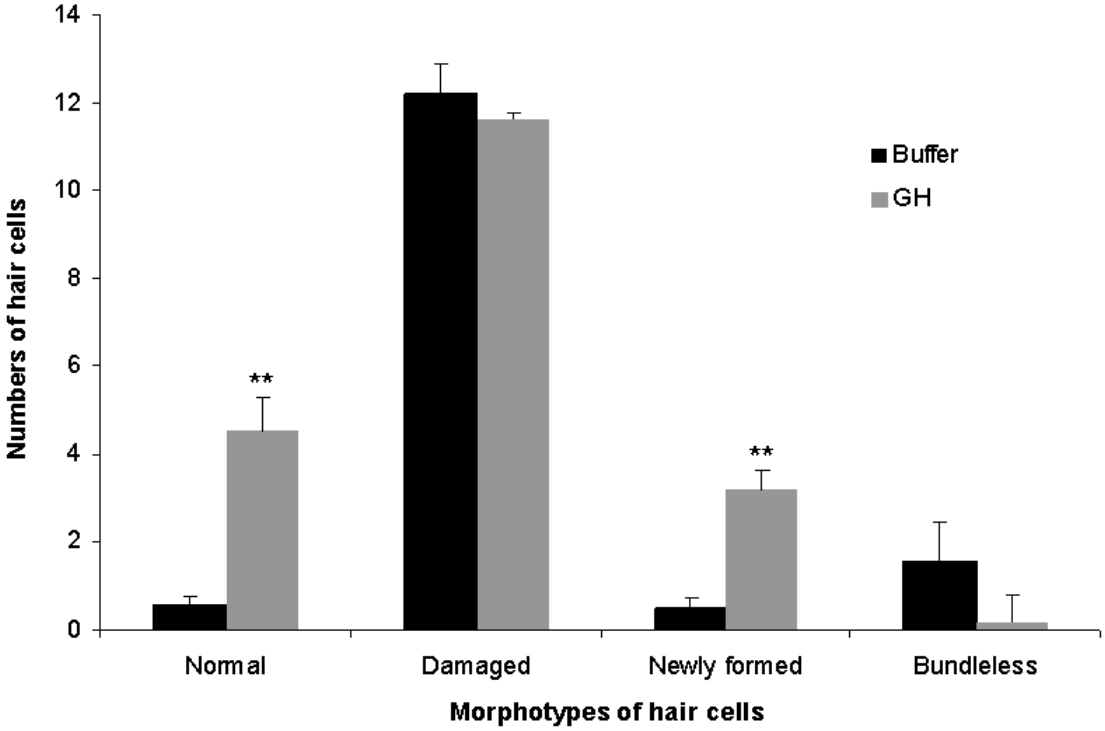

Figure 12.

Mean (± S.E.) normal, damaged, newly formed, and bundleless phalloidin-labeled hair cells of buffer- (black) and GH-injected (gray) saccules at the 50% rostral-caudal region at post-sound exposure day 2 (psed2) (** $p < 0.001$, n= 12).

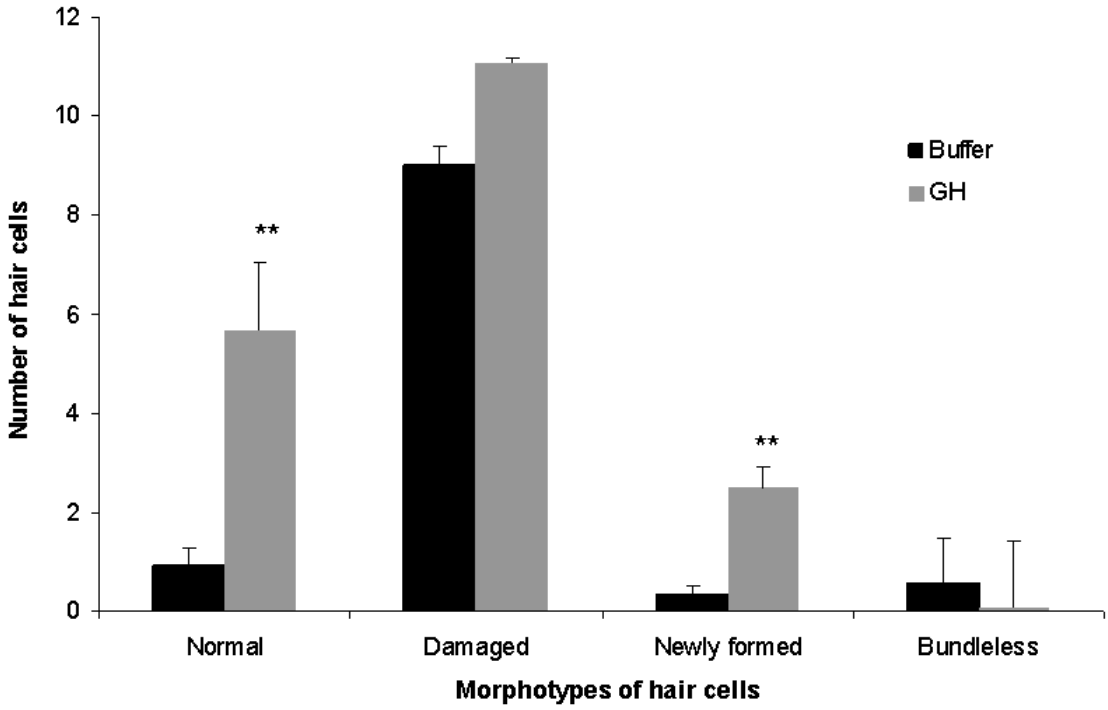

Figure 13.

Mean (± S.E.) normal, damaged, newly formed, and bundleless phalloidin-labeled hair cells of buffer- (black) and GH-injected (gray) saccules at the 75% rostral-caudal region at post-sound exposure day 2 (psed2) (** $p < 0.001$, n= 12).

CHAPTER 3

Discussion:

Effect of growth hormone on apoptosis in the zebrafish ear

In earlier studies, ototoxic drugs (such aminoglycoside antibiotics) induced apoptosis of the vestibular or cochlear hair cells of guinea pigs (Nakagawa et al., 1997; Lang and Liu, 1997; Nakagawa et al., 1998). Noise exposure also induces apoptosis in the goldfish (*Carrasius auratus*) ear (Smith et al., 2006). Concurrent with this apoptosis is hair cell and hearing loss. In this study, the results of the TUNEL assay suggest that growth hormone is able to suppress such apoptotic cell death in all three inner ear end organs in zebrafish at one day after sound exposure.

While the effects of GH on cell proliferation has been well documented for many tissues, studies on the effects of GH on apoptosis is rare and our discovery is novel for the teleost ear. Svensson et al. (2008) found human recombinant growth hormone (rhGH) was able to counteract the effects of morphine-induced cell damage and apoptosis in mouse fetus hippocampal neurons, which indicated that GH may help to suppress apoptosis in mouse brain. GH has also been reported to decrease apoptosis in neutrophils of post-surgical patients (Decker et al. 2005). GH or other molecules in cellular GH pathways may be a useful prophylactic for preventing hearing loss. While systemic administration of GH could cause problems like acromegaly, direct injection into the mammalian cochlea, which is an enclosed, fluid-filled organ, may be a useful means of preventing hair cell loss in individuals who are undergoing or recently experienced trauma to the ear (such as taking aminoglycoside antibiotics or exposure to explosive blasts).

Effect of growth hormone on auditory cell proliferation

In previous work, an increase in cellular proliferation was observed after prolonged sound exposure in the zebrafish saccule, with a peak at psed2 (post sound exposure day 2; Schuck et al., 2009). Simultaneously, growth hormone mRNA was found to be greatly elevated, also with a peak at psed2 (Schuck, 2007). The current study showed that GH injection increases cell proliferation in all end organs of the zebrafish ear following noise exposure. The saccules of GH-treated zebrafish showed cell proliferation increased over 10-fold compared to those of buffer-treated fish 24 hours post-sound exposure. The fact that cell proliferation was greatest in the saccule compared to the utricle and lagenae may suggest that the sound exposure had the greatest effect on the saccule, although phalloidin-labeling of hair cells in zebrafish utricles and lagenae following sound exposure is still needed. This result supports our hypothesis that the saccule is probably the most important of the zebrafish inner ear end organs for hearing. The results also suggest that supporting cell proliferation is an evoked cellular response to the loss of adjacent hair cells in the inner ear, in an attempt to repair the damage (reviewed in Cotanche and Lee, 1994).

The striking difference between the buffer group and the GH group showed that GH has potent effects on promoting cellular proliferation in the zebrafish inner ear. However, the underlying mechanism of GH-mediated proliferation in the inner ear is yet to be elucidated. One of the growth factors that GH is known to stimulate is insulin-like growth factor-I (IGF-I). Oesterle et al. (1997) found that IGF-I and insulin can promote cell proliferation in the mature avian inner ear. Growth hormone has also been reported to increase proliferation of supporting cells in the inner ear of the cichlid fish *Astronotus*

ocellatus (Presson and Kim, 2001). They found that numbers of BrdU-labeled cells in the saccules of GH-treated fish were 1.5 and 2 times greater than controls, compared to the over 10-fold increase found in the current study. One potential reason for this difference is that we used twice the dosage of GH (20 μg instead of 10 μg GH/gram of body mass). Another potential source of variation in the effect of GH is the source. Carp GH was used in this study. Salmon GH was used in the Presson and Kim (2001) study for *Astronotus ocellatus*. Since carp and zebrafish are very closely related fishes, it is possible that carp GH is more effective than salmon GH at promoting cell proliferation in zebrafish. In addition, while I exposed the experimental zebrafish to acoustic trauma, which has been shown to increase endogenous GH (Schuck, 2007), Presson and Kim (2001) did not.

Effect of growth hormone on auditory hair cell density

The hair cell bundle densities reported here for zebrafish are comparable to those of other studies (Higgs et al., 2001, Schuck and Smith, 2009). Hair cell bundle density is greatest close to the rostral and caudal tips and decreases at the center portion of the saccule (Fig. 9). This result is consistent with the previous observations in our lab for zebrafish and goldfish.

This study shows that growth hormone (GH) is capable of stimulating zebrafish hair cell bundle density within two days following sound exposure. In previous studies, hair cell bundles regenerated to control levels in the goldfish saccule after eight days following acoustic trauma (Smith et al., 2006), and after 7 days following acoustic trauma in the zebrafish saccule (Schuck and Smith, 2009). This study showed that GH is able to

accelerate hair cell regeneration, with less damaged hair cell bundles and more newly growth hair cell bundles in zebrafish inner ears post-sound exposure, compared to the buffer group (Figs. 11, 12, 13). However, it has yet to be determined whether and how fast GH can help hair cell bundle density return to normal levels post-sound exposure. At least at the time point of psed2, there were damaged hair bundles observed in the GH group, indicating that longer time is likely necessary for the GH group to fully regenerate new hair cells or repair the damaged stereocilia following sound exposure.

Two days post-sound exposure, the rostral and caudal tip regions (5% and 90% along the axis from rostral tip) of the saccule showed slight hair cell loss, with little damaged hair cells and cuticular plates (Fig. 9). While significant numbers of hair cells were lost in the central region of saccule (25, 50 and 75% of the total distance from rostral tip), suggesting that this region is more susceptible to trauma induced by low frequency sounds (Fig. 9). There were many damaged or lost hair cells in all three of these central regions of the saccules of buffer-injected fish. This result is similar to previous studies in which a loud 100 Hz tone exposure for 2 days significantly decreased hair cell bundle density in the central rostral and cadual regions in goldfish (Smith et al., 2006) and zebrafish (Schuck et al., 2009). In the current study, two days post-sound exposure, GH-injected fish showed a significant increase in hair cells in this same central area compared to the buffer group. This area was also associated with a notable increase in normal hair cell bundles and newly formed hair cell bundles (Figs. 11, 12, 13). There are two potential explanations for this-either GH increased the rate of hair cell regeneration or prevented some hair cell loss. Since GH-injection resulted in both

increase cell proliferation and reduced apoptosis, both of these explanations are possibilities.

CHAPTER 4

Summary and future studies:

Growth hormone reduces apoptosis, increases cell proliferation, and increases hair cell density in the zebrafish ear during the process of hair cell regeneration. This is consistent with the reports of the regenerative effects of GH on other tissues, but this is the first time that the effects of GH on the auditory system have been thoroughly examined.

So far, we have only examined the changes in the inner ear sensory epithelia of zebrafish after sound exposure. However, we do not know if the newly grown hair cells in the zebrafish ear are functional. Hearing capabilities (hearing thresholds) can be determined by recording auditory evoked potentials (AEP). AEP is a common non-invasive method that is used to measure neural responses to auditory stimuli immediately after sound exposure in some vertebrates and fish (Corwin et al., 1982; Kenyon et al., 1998; Higgs et al., 2001; Mann et al., 2001; Smith et al., 2004a, b; Wysocki and Ladich, 2005). In the future studies, AEP would be a good method to see if GH not only promotes hair cell regeneration, but also functional hearing recovery following acoustic trauma.

Longer term BrdU studies are also needed to see if BrdU is incorporated into hair cells as well as surrounding supporting cells. This method would prove that those hair cells resulted from a mitotic event and not just direct transdifferentiation of supporting cells to hair cells. For example, parvalbumin or calretinin (both of them are hair cell markers) and BrdU double-staining of hair cells might show that newly grown hair cells are directly divided from supporting cells through cell proliferation/mitosis

(Zheng and Gao, 1997). The current study provided only one time point per experiment (dpse1 for TUNEL, dpse1 for BrdU, and dpse2 for hair cell density). Longer time course studies of the effects of GH-treatment on the zebrafish inner ear are needed to examine if GH can promote complete recovery of hair cells and hearing.

REFERENCES

Avallone, B., Porritiello, M., Esposito, D., Mutone, R., Balsamo, G., Marmo, F., 2003. Evidence for hair cell regeneration in the crista ampullaris of the lizard *Podarcis sicula*. Hear. Res. 178:79-88.

Bhave S.A., Stone J.S., Rubel E.W., Coltrera, M.D., 1995. Cell cycle progression in gentamicin damaged avian cochleas. J. Neurosci. 15:4618–4628.

Brave, J. and Heath, J.K., 2000. Receptor recognition by gp130 cytokines. EMBO. J. 19: 2399-2411.

Cacciafesta, V. and Dalstra, M., 2001. Growth hormone treatment promotes guided bone regeneration in rat calvarial defects. Eur. J. Orthod. 23: 733-740.

Casanueva, F.F., 1992. Physiology of growth hormone secretion and action. Endocrinol. Metab. Clin. North. Am. 21:483-517.

Corwin, J.T., Bullock, T.H. and Schweitzer, J., 1982. The auditory brainstem response in five vertebrate classes. Electroencephalogr. Clin. Neurophysiol. 54:629-641.

Corwin, J.T. and Cotanche, D.A., 1988. Regeneration of sensory hair cells after acoustic trauma. Science 240:1772-1774.

Corwin, J.T., Balak, K.J., and Borden, P.C., 1989. Cellular events underlying the regenerative replacement of lateral line sensory epithelia in amphibians. In: Coombs, S., Gorner, P., Munz, P.H. (Eds.), The Mechanosensory Lateral Line: Neurobiology and Evolution. Springer, New York, pp 161–183.

Corwin, J.T. and Oberholtzer, J.C., 1997. Fish n' chicks: Model recipes for hair cell regeneration? Neuron. 19:951-954.

Cotanche, D.A. and Lee, K.H., 1994. Regeneration of hair cells in the vestibulocochlear system of birds and mammals. Neurobiology 4:509-514.

Cotanche, D.A., 1999. Structural recovery from sound and amino-glycoside damage in the avian cochlea. Audio. Neurootol. 4:271-285.

Davidson, M.B., 1987. Effect of growth hormone on carbohydrate and lipid metabolism. Endocr. Rev. 8:115-131.

Decker, D., Springer, W., Tolba, R., Lauschke, H., Hirner, A. and von Ruecker, A., 2005. Perioperative treatment with human growth hormone down-regulates apoptosis and increases superoxide production in PMN from patients undergoing infrarenal abdominal aortic aneurysm repair. Growth Horm. IGF Res.15:193-199.

Fay, R.R. and Popper, A.N., 2000. Evolution of hearing in vertebrates: The inner ears and processing. Hear. Res., 149:1-10.

Gilpin, D.A., Bch, M.B., Barrow, R.E., Rutan, R.L., Broemeling, L., and Herndon, D.N., 1994. Recombinant human growth hormone accelerates wound healing in children with large cutaneous burns. Ann. Surg. 220:19-24.

Hamernik, R.P., Turrentine, G., Roberto, M., Salvi, R., and Henderson, D., 1984. Anatomical correlates of impulse noise-induced mechanical damage in the cochlea. Hear. Res. 13:229-247.

Harris, J.A., Cheng, A.G., Cunningham, L.L, MacDonald, G., Raible, D.W., and Rubel, E.W., 2003. Neomycin-induced hair cell death and rapid regeneration in the lateral line of zebrafish (*Danio rerio*). J. Assoc. Res. Otolaryngol. 4: 219-234.

Herndon, D.N., Barrow, R.E., Kunkel, K.R., Broemeling, L. and Rutan, R.L.,1990. Effects of recombinant human growth hormone on donor-site healing in severely burned children. Ann. Surg., 212:424-429.

Higgs, D.M., Souza, M.J., Wilkins, H.R., Presson, J.C. and Popper, A.N., 2001. Age- and size-related changes in the inner ear and hearing ability of the adult zebrafish (*Danio rerio*). J. Assoc. Res. Otolaryngol. 3:174-184.

Johnsson, J.I., Petersson, E., Jönsson, E., Järvi, T., Björnsson, B. TH., 1999. Growth hormone-induced effects on mortality, energy status and growth: a field study on brown trout (*Salmo trutta*). Functional Ecology. 13:514-522.

Jung, G.W., Spencer, E.M., Lue, T.F., 1998. Growth hormone enhances regeneration of nitric oxide synthase-containing penile nerves after cavernous nerve neurotomy in rats. J. Urol. 160:1899-1904.

Kanje, M., Skottner, A., Lundborg, G., 1988. Effects of growth hormone treatment on the regeneration of rat sciatic nerve. Brain Res. 475:254-258.

Keithley, E.M. and Feldman, M.L., 1982. Hair cell counts in an age-graded series of rat cochleas. Hear. Res. 8:249–262.

Kenyon, T.N., Ladich, F., and Yan, H.Y., 1998. A comparative study of hearing ability in fishes: the auditory brainstem response approach. J. Comp. Physiol. A. 182:07-318.

Isaksson, O.G. and Jansson, J.O., 1982. Growth hormone stimulates longitudinal bone growth directly. Science 216:1237-1239.

Kimmel, C.B., 1989. Genetics and early development of zebrafish. Trends. Genet. 5:283-288.

Lang, H. and Liu, C., 1997. Apoptosis and hair cell degeneration in the vestibular sensory epithelia of the guinea pig following a gentamicin insult. Hear. Res. 111:177-184.

Lombarte, A., Yan, H.Y., Popper, A.N., and Chang, J.S., 1993.Damage and regeneration of hair cell ciliary bundles in a fish ear following treatment with gentamicin. Hear. Res. 64:166-174.

Löwenheim, H., Furness, D.N., Kil, J., Zinn, C., Gultig, K., Fero, M.L., Frost, D., Gummer, A.W., Roberts, J.M., Rubel, E.W., Hackney, C.M., and Zenner, H.P., 1999. Gene disruption of p27(Kip1) allows cell proliferation in the postnatal and adult organ of Corti. Proc. Natl. Acad. Sci. USA 96:4084–4088.

Malicki, J., Schier, A.F., Solnica-Krezel, L., Stemple, D.L., Neuhaus, S.C.F., Stainier, D.Y.R., Abdelilah, S., Rangini, Z., Zwartkuis, F., and Driever, W., 1996. Mutations affecting the development of the zebrafish ear. Development 123:275-283.

Mann, D.A., Higgs, D.M., Tavolga, W.N., Souza, M.J. and Papper, A.N., 2001. Ultrasound detection by clupeiform fishes. J. Acoust. Soc. Am. 109:3048-3054.

Martin-Smith, K.M., Armstrong, J.D., Johnsson, J.I., Björnsson, B. TH., 2004. Growth hormone increases growth and dominance of wild juvenile Atlantic salmon without affecting space use. J. Fish Biol. 65:156-172.

Monge, A., Nagy I., Bonabi, S., Schmid, S., Gassmann, M., and Bodmer, D., 2006. The effect of erythropoietin on gentamicin-induced auditory hair cell loss. Laryngoscope 116:312-316.

Nakagawa, T., Yamane, H., Shibata, S., and Nakai, Y., 1997. Gentamicin ototoxicity induced apoptosis of the vestibular hair cells of guinea pigs. Eur. Arch. Otorhinolaryngol. 254:9-14.

Nakagawa, T., Yamane, H., Takayama, M., Sunami, K., and Nakai, Y., 1998. Apoptosis of guinea pig cochlear hair cells following chronic aminoglycoside treatment. Eur. Arch. Otorhinolaryngol. 255:127-131.

Oesterle, E.C., Tsue, T.T., Rubel, E.W., 1997. Induction of cell proliferation in avian inner ear sensory epithelia by insulin-like growth factor-I and insulin. J. Comp. Neurol. 380:262-274.

Oesterle, E.C. and Stone, J.S., 2008. Hair cell regeneration: Mechanisms guiding cellular proliferation and differentiation. In: Salvi, R.J., Popper, A.N., Fay, R.R. (Eds.), Hair cell regeneration, repair, and protection. Springer, New York, pp. 141-197.

Ohashi, S., Kaji H., Abe, H., and Chihara, K., 1995. Effect of fasting and growth hormone (GH) administration on GH receptor (GHR) messenger ribonucleic acid (mRNA) and GH-binding protein (GHBP) mRNA levels in male rats. Life Sci. 57:1655-66.

Okada, S. and Kopchick, J.J., 2001. Biological effects of growth hormone and its antagonist. Trends. Mol. Med. 7:126-132.

Park, D.L., Girod, D.A., and Durham, D., 2002. Avian brainstem neurogenesis is stimulated during cochlear hair cell regeneration. Brain Res. 949:1-10.

Pennisi, P.A., Kopchick, J.J., Thorgeirsson, S., LeRoith, D., and Yakar, S., 2004. Role of growth hormone (GH) in liver regeneration. Endocrinology 10:4748-4755.

Pleis J.R. and Lethbridge-Cejku M., 2007. Summary health statistics for U.S. adults: National Health Interview Survey, 2006. National Center for Health Statistics. Vital Health Stat 10 (235):1-153.

Presson, J., and Kim, A., 2001. Growth hormone increases supporting cell proliferation in a cichlid fish. Assoc. Res. Otolaryngol. Abs.:21476.

Prosen, C.A., Moody, D.B., Stebbins, W.C., Smith, D.W., Sommers, M.S., Brown, J.N., Altschuler, R.A. and Hawkins, J.E., Jr., 1990. Apical hair cells and hearing. Hear. Res. 44:179-194.

Richardson, W.J., Greene, C.R., Malme, Jr., C.I., and Thomson, D.H., 1995. Marine Mammals and Noise. Academic Press, New York.

Rønsholdt, B. and McLean, E., 2004. Effect of growth hormone and salbutamol on growth performance, fillet proximate composition and pigmentation of rainbow trout (*Oncorhynchus mykiss*). Aquaculture. 229:225-238.

Ryals, B.M. and Rubel, E.W., 1988. Hair cell regeneration after acoustic trauma in adult Coturnix quail. Science 240:1774–1776.

Sage, C., Huang, M., Karimi, K., Gutierrez, G., Vollrath, M.A., Zhang, D.S., Garcia-Anoveros, J., Hinds, P.W., Corwin, J.T., Corey, D.P., and Chen, Z.Y., 2005. Proliferation of functional hair cells in vivo in the absence of the retinoblastoma protein. Science 307:1114-1118.

Saunders, J.C., Adler, H. J., and Pugliano, F.A., 1992. The structural and functional aspects of hair cell regeneration in the chick as a result of exposure to intense sound. Exp. Neurol. 115:13-17.

Saunders, J. C., Cohen, Y.E., Szymko, Y.M., 1991. The structural and functional consequences of acoustic injury in the cochlea and peripheral auditory system: A five year update. J. Acoust. Soc. Am. 90:147-155.

Scholik, A.R. and Yan, H.Y., 2001. The effects of underwater noise on auditory sensitivity of a cyprinid fish. Hear. Res. 152:17-24.

Schuck, J.B., 2007. Auditory hair cell regeneration and gene expression in noise-exposed zebrafish (*Danio rerio*). Masters thesis, Western Kentucky University. 116 pp.

Schuck, J.B. and Smith, M.E., 2009. Cell proliferation follows acoustically-induced hair cell bundle loss in the zebrafish saccule. Hear. Res. 253:67-76.

Shang, J., Cafaro, J., Nethmer, R., and Stone, J., 2010. Supporting cell division is not required for regeneration of auditory hair cells after ototoxic injury in vitro. J. Assoc. Res. Otolaryngol. 11:203-222.

Smith, M.E., Kane, A.S., and Popper, A.N., 2004a. Noise-induced stress response and hearing loss in goldfish (*Carrasius auratus*). J. Exp. Biol. 207:427-435.

Smith, M.E., Kane, A.S., and Popper, A.N., 2004b. Acoustical stress and hearing sensitivity in fishes: does the linear threshold shift hypothesis hold water? J. Exp. Biol. 207:3591-3602.

Smith, M. E., Coffin A.B, Miller, D.L., and Popper A.N., 2006. Anatomical and functional recovery of the goldfish (*Carassius auratus*) ear following noise exposure. J. Exp. Biol. 209:4193-4202.

Sprague, J., Doerry, E., Douglas, S., and Westerfield, M., 2001. The Zebrafish Information Network (ZFIN): a resource for genetic, genomic and developmental research. Nucleic Acids Res. 29:87-90.

Staecker, H. and Van De Water, T.R., 1998. Factors controlling hair cell regeneration/repair in the inner ear. Curr. Opin. Neurobiol. 8:480-487.

Stone, L.S., 1937. Further experimental studies of the development of lateral-line sense organs in the amphibians observed in living preparations. J. Comp. Neurol. 68:83–115.

Svensson, A., Bucht, N., Hallberg, M., Nyberg, F., 2008. Reversal of opiate-induced apoptosis by human recombinant growth hormone in murine foetus primary hippocampal neuronal cell cultures. Proc. Natl. Acad. Sci. 105:7304-7308.

Theopold, H.M., 1977. Comparative surface studies of ototoxic effects of various aminoglycoside antibiotics on the organ of Corti in the guinea pig: a scanning electron microscopic study. Acta Otolaryngol. 84:57–64.

Ullman, M., Alameddine, H., Skottner, A., and Oldfors, A., 1989. Effects of growth hormone on skeletal muscle. II. Studies on regeneration and denervation in adult rats. Acta. Physiol. Scand. 135:537-543.

van den Eijnden, M. J. and Strous, G. J., 2007. Autocrine growth hormone: effects on growth hormone receptor trafficking and signaling. Mol. Endocrinol. 21:2832-2846.

Wachol, M.E., Lambert, P.T., Goldstein, B. J., Forge, A., and Corwin, J.T., 1993. Regenerative proliferation in inner ear sensory epithelia taken from adult guinea pigs and humans. Science 259:1619-1622.

Walshe, P., Walsh, M., and Walsh, R.M., 2003. Hair cell regeneration in the inner ear: a review. Clin. Otolaryngol. 28:5-13.

Wanamaker, H.H., Gruenwald, L., Damm, K.J., Ogata ,Y., and Slepecky, N., 1998. Dose-related vestibular and cochlear effects of transtympanic gentamicin. Am. J. Otol. 19:170-179.

Weisleder, P., and Rubel, E.W., 1992. Hair cell regeneration in the avian vestibular epithelium. Exp. Neurol.115:2-6.

Whitfield, T.T., 2002. Zebrafish as a model for hearing and deafness. J. Neurobiol. 53:157-171.

Wysocki, L.E. and Ladich, F., 2005. Effects of noise exposure on click detection and the temporal resolution ability of the goldfish auditory system. Hear. Res. 201: 27-36.

Zheng, J.S., Gilbert, K., and Gao, WQ., 1999. Immunocytochemical and morphological evidence for intracellular self-repair as an important contributor to mammalian hair cell recovery. J. Neurosci. 19:2161-2170.

Zheng, J.L. and Gao, W.Q., 2000. Overexpression of Math1 induces robust production of extra hair cells in postnatal rat inner ears. Nat. Neurosci. 3:580-586.

Zon, L.I., 1999. Zebrafish: A new model for human disease. Genome Res. 9:99-100.