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Prophylactic Effect if Growth Hormone on Zebrafish Auditory Hair Cell Damage

Mackenzie C. Perkins
Western Kentucky University, Mackenzie.Perkins969@topper.wku.edu

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PROPHYLACTIC EFFECTS OF GROWTH HORMONE ON ZEBRAFISH
AUDITORY HAIR CELL DAMAGE

A Capstone Experience/Thesis Project

Presented in Partial Fulfillment of the Requirements for

the Degree Bachelor of Science with

Honors College Graduate Distinction at Western Kentucky University

By

Mackenzie C. Perkins

*****

Western Kentucky University

2013

CE/T Committee:

Dr. Michael Smith, Advisor

Dr. Nancy Rice

Dr. Leslie Baylis

Approved by

_____
Advisor
Department of Biology

ABSTRACT

Growth hormone (GH) has been shown to play a role in and improve hair cell regeneration when injected intraperitoneally in zebrafish post-sound exposure. The purpose of this study was to examine whether exogenous GH has a prophylactic effect on auditory hair cell damage when injected prior to acoustic trauma. Groups of zebrafish were injected with either GH or buffer. Immediately following the injection, auditory hair cell damage was induced through exposure to acoustic overstimulation. Hearing tests were then performed on the fish by measuring auditory evoked potentials. Then, the fish ears were dissected either immediately post-trauma or at one, two, and three days following acoustic exposure. The dissected zebrafish saccules were stained with fluorescein-conjugated phalloidin and visualized under flourescence microscopy. Hearing loss and hair cell damage was reduced following trauma in GH-treated fish in comparison to buffer-treated fish. The results show that exogenous growth hormone has a prophylactic effect on acoustically-induced zebrafish auditory hair cell damage.

Keywords: growth hormone, hair cell, regeneration, zebrafish, saccule, inner ear

Dedicated to my family and friends for their encouragement

and support throughout my life.

"A woman who fears the Lord, she shall be praised.

Give her of the fruit of her hands,

And let her own works praise her in the gates."

Proverbs 31:30-31

ACKNOWLEDGEMENTS

I would like to thank those who have been such a support during my undergraduate education. First, I would like to thank my parents, sisters, and Jason. With their love, understanding and support, I am able to make my aspirations a reality. I would like to thank Dr. Michael Smith for allowing me to join his research group, challenging me intellectually and guiding me through my undergraduate education. I deeply appreciate his patience that he has exhibited throughout my Honor's Capstone thesis process. I would like to thank Dr. Huifang Sun for her patience in training and education within my project and her motivating words during the duration of my project.

VITA

March 25. 1991 ....Born - Evansville, Indiana

2009..Hopkinsville High School, Hopkinsville, Kentucky

2009..Acceptance into HHMI Genome Discovery Program
                                            at Western Kentucky University

2010..Acceptance into Dr. Smith's Lab

FIELDS OF STUDY

Major Field: Biology

Minor Field: Psychology

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CHAPTER 1

INTRODUCTION

Nearly 10 million Americans suffer from measurable hearing impairment and

related speech disorders, while 1 million Americans are functionally deaf (Mitchell,

2006). Preventative measures and therapeutics are needed to delay the onset of deafness.

The sensory cells of the inner ear that transduce acoustical into neural signals are called

hair cells. Loss of these hair cells results in hearing loss. Understanding the cellular

pathways of auditory hair cell death and, potentially, regeneration, is a step towards

successful development of therapeutics for humans.

Mechanosensory hair cells in the vertebrate inner ear receive sound and vibrations

from the environment. Stereocilia located on the apical surface of the hair cell are

deflected back and forth when vibrations travel through the fluids of the inner ear. Such

deflections open ion channels on the surface of the hair cell creating a neural signal that is

sent to the auditory nerve and then the brain. Hair cells are located in the inner ear and

the lateral line system of vertebrates which function in hearing and balance (Fettiplace &

Ricci, 2006), thus when hair cells are damaged or lost, it can lead to deficits in both

hearing and balance. Prolonged noise exposure, ototoxic drugs, and age can cause

damage to auditory hair cells (Corwin & Cotanche, 1988). Auditory hair cell

regeneration of lost hair cells does not occur in mammals, thus deafness is permanent. In

contrast, regeneration of auditory hair cells does occur in fishes, birds, reptiles and amphibians (Corwin & Cotache, 1988).

The fish auditory system is comprised of three pairs of endorgans called the utricle, lagena and saccule. The saccule is the most distinguished as the sound detector relative to the other two sound organs, the utricle and lagena (Popper and Fay, 1993). Each inner ear endorgan contains sensory epithelia with hair cells, the apical portion of which are composed of stereociliary hair bundles, which contact an overlying calcareous otolith. Saccular hair cells have been shown to regenerate following acoustic trauma in goldfish and zebrafish (Smith et al. 2006; Sun et al. 2011), and this regeneration has also led to functional hearing recovery in goldfish (Smith et al. 2006).

Zebrafish (*Danio rerio*) has been used as a model organism for hair cell regeneration because the structure and function of their inner ears are similar to other vertebrates (Popper & Fay, 1999). Specifically zebrafish have similar inner ear sensory surfaces found in goldfish (Platt, 1993). Prolonged noise exposure leads to hair cell death and damage to the hearing organs of zebrafish (Schuck & Smith, 2009). Acoustical trauma leads to an increase in apoptotic cells and hair cells missing stereocilia in the saccule, and a decrease in hair cell bundle density (Smith et al., 2006). This damage in the saccule is followed by increased cell proliferation and hair cell regeneration. At fourteen days post-sound exposure, hair cell bundle counts reached pre-noise exposure levels in zebrafish, showing that they are a good model for hair cell regeneration (Schuck & Smith, 2009).

Through microarray analysis of zebrafish saccules following acoustic trauma, previous findings have shown a 64-fold increase in growth hormone (GH) transcripts

peaking at 2 days post-sound exposure (Schuck et al., 2011). Intraperitoneal injection of GH has been shown to improve hair cell regeneration in zebrafish post-sound exposure (Sun et al., 2011). The fact that GH can assist in the proliferation and recovery of hearing in zebrafish may have potential use in therapeutic treatment for auditory hearing loss in humans.

GH is a secretory protein produced in the anterior pituitary gland as a member of the superfamily polypeptide regulators (Tanner, 1972). Primarily, GH is thought to promote growth in children and adolescents, yet GH also functions to regulate growth, differentiation, development and metabolism of most tissues (Bidlingmaier and Strasburger, 2010). GH is involved in the proliferation and differentiation of cells occurring in the neural, immune, reproductive, alimentary, and respiratory tissues and within the muscular, skeletal and cardiovascular systems (Harvey, 2010). The possibility of GH preventing auditory hair cell damage (prophylactic effect) has not yet been assessed. The purpose of this study was to examine whether exogenous GH has a prophylactic effect on auditory hair damage and hearing loss in zebrafish.

CHAPTER 2

METHODS

Animals

Adult breeder zebrafish (*Danio rerio*) was obtained from commercial suppliers and maintained in a 170-L aquarium tank with constant temperature (25°C) and on a light/dark schedule of 12-hour rotation. All work was done under the supervision and approval of the Institutional Animal Care and Use Committee of Western Kentucky University.

Experimental design

The testing of the prophylactic effect using GH was through injection, sound exposure, dissection, microscopy analysis and statistical analysis. Sixty-four zebrafish were divided into two groups. One group was injected intraperitoneally with buffer. The other group was injected intraperitoneally by carp growth hormone (GenWay at San Diego, CA) at a concentration of 20 μg/gram fish body mass. Immediately after injection, the fish was exposed to 150 Hz tone at a source level of 179 dB re 1 μPa root mean squared (RMS) measured at 1 cm directly above an underwater speaker (University Sound UW-30) for 40 hours.

There are four different time points of the sound exposure, the first one beginning at the termination of the sound exposure. The next three are the three consecutive days post-sound exposure. Data was collected at all four post-sound exposure days.

Auditory evoked potentials

Hair cell recovery leads to hearing recovery, which is measured by determining the fish's hearing threshold across the frequencies which the fish can detect (Smith et al., 2006). Through the use of the auditory evoked potential (AEP) technique, the neural response to auditory stimuli is recorded to measure hearing thresholds to produce audiograms for fish and other organisms (Corwin et al., 1983). AEP recording allows one to examine the extent of function of the ear based on the hair cell bundle density. It is expected that hreshold shifts will increase with the decrease of normal hair cell bundles on the saccule of goldfish (Smith et al., 2006).

Each fish was anaesthetized with MS-222 (tricaine methanosulfonate), restrained in a mesh sling and suspended under water in a 19-L plastic vessel. Each fish's head was 6 cm below the surface of the water and 22 cm above the speaker. Three stainless steel subdermal electrodes (27 ga, Rochester Electro-Medical Inc., Tampa, FL) recorded the auditory evoked potentials. The reference electrode was inserted subdermally 2 mm deep into the medial dorsal surface of the head between the anterior portion of the eyes. The recording electrode was inserted subdermally 2 mm into the dorsal midline surface of the fish approximately halfway between the anterior insertion of the dorsal fin and posterior edge of the operculae, directly over the brainstem. A ground electrode was inserted in the musculature on the tail of the fish.

Sound stimuli were presented and AEP waveforms collected using SigGen and BioSig software running on a TDT physiology apparatus (Tucker-Davis Technologies Inc., Alachua, FL). Sounds were computer generated via TDT software and passed through a P1000 power amplifier (Hafler, Tempe, AZ) connected to a University Sound UW-30 underwater speaker (Electro-Voice, Burnsville, MN). Tone bursts were 15, 10, and 5 ms in total duration for 0.1 and 0.25, 4 and 6, and 0.8–4 kHz tones, respectively. Each tone pip had a 2 ms rise and fall time and were gated through a Hanning window similar to the conditions of other AEP studies (Smith et al., 2004; Smith et al., 2004; Smith et al., 2006). Responses to each tone burst at each SPL were collected using the BioSig software package, with 400 responses averaged for each presentation. Auditory thresholds were determined at 6 frequencies for each fish (0.1, 0.25, 0.4, 0.8, 1.5, and 3 kHz). The SPLs of each presented frequency were confirmed using a calibrated underwater hydrophone (calibration sensitivity of –195 dB re 1 V/mPa; 63 dB, 0.02–10 kHz, omnidirectional, GRAS Type 10CT, Denmark), placed in the same location where fish were held during AEP recording. Auditory thresholds were determined by visual inspection of AEP as in previous studies (Smith et al., 2004; Smith et al., 2004).

Inner ear morphology

Following AEP, subjects were euthanized by overdose with MS-222. The heads were removed and fixed with 4% paraformaldehyde overnight at 4°C. The heads were washed in 0.1 M phosphate buffer and dissected using microscopic dissection techniques. The dissected zebrafish left and right saccules were trimmed and incubated with fluorescein-conjugated phalloidin (Alexa Fluor 488 phalloidin, Molecular

Probes/Invitrogen, Carlsbad, CA, USA). Saccules were mounted with a cover slip with

Prolong Gold antifade reagent with 4',6-diamidino-2-phenylindole (DAPI) to stain nuclei.

Auditory hair cells were visualized under a fluorescence microscope. Images were

taken of the saccules using a 10x objective and 100x objective using a Zeiss Axioplan 2

(Germany) epiflourescent microscope and a Zeiss MRm digital CCD camera. To count

the auditory hair cell numbers, five distinct locations along the rostral-causal axis were

chosen (5%, 25%, 75%, and 95% starting from the rostral end; Smith et al., 2006) (Figure

1). Each distinct location measures 1600 mm^2. The auditory hair cells were characterized

into different morphotypes (Figure 2). The number of normal hair cells, new hair cell

bundles, and damaged hair cells were counted for each location. Normal hair cells were

characterized to have standard length and height of stereocilia. Damaged hair cells were

characterized through the fractured and few stereocilia. The newly formed hair cells were

similar to the normal hair cells yet were stunted in height (Schuck & Smith, 2009).

Statistical analysis

Using analysis of variance (ANOVA), the differences between buffer- and GH-

injected fish for all the time points were quantified. Separate ANOVAs were completed

for hair cell bundle counts for each hair cell morphotype in each distinct location along

the rostral-caudal axis and for each frequency of the AEP. The ANOVAs were completed

using Systat (SYSTAT 13; Systat Software Inc., Chicago, IL).
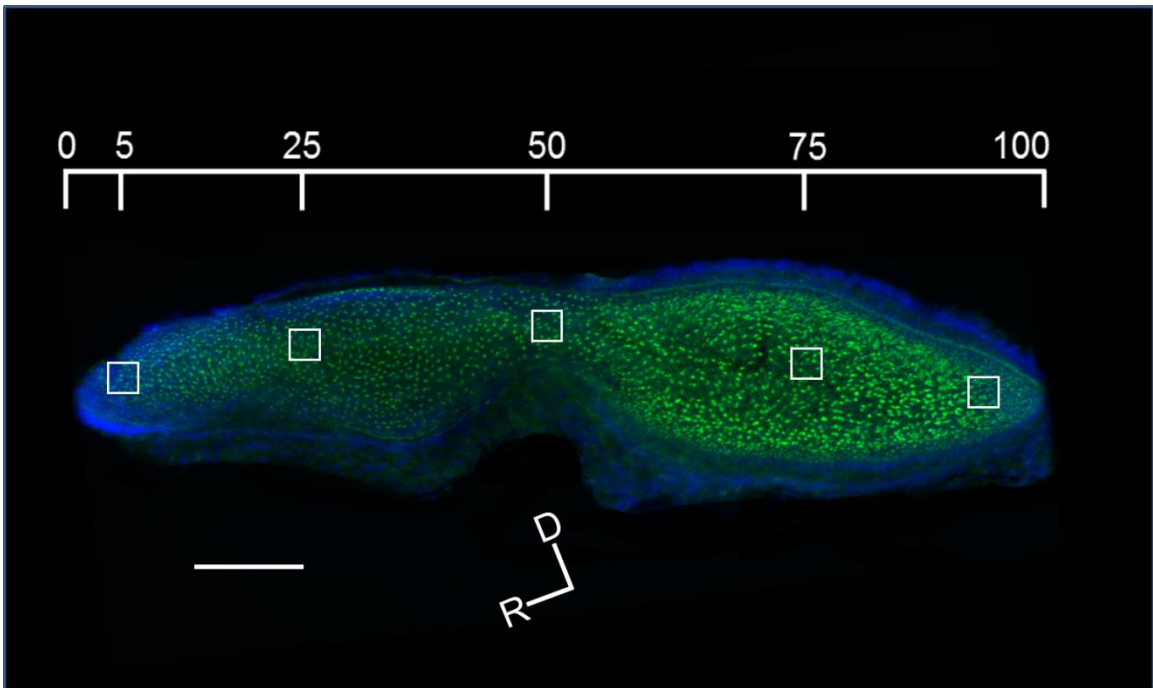
Figure 1. Phalloidin (green)- and DAPI (blue)-labeled baseline zebrafish saccule. The numbers of hair cell bundles were counted in 40 µm X 40 µm boxes at five predetermined areas along the rostral-caudal axis: 5, 25, 50, 75 and 90% of the distance from the rostral end of the saccule (white boxes). D = dorsal, R = rostral. Scale bar = 100 µm.
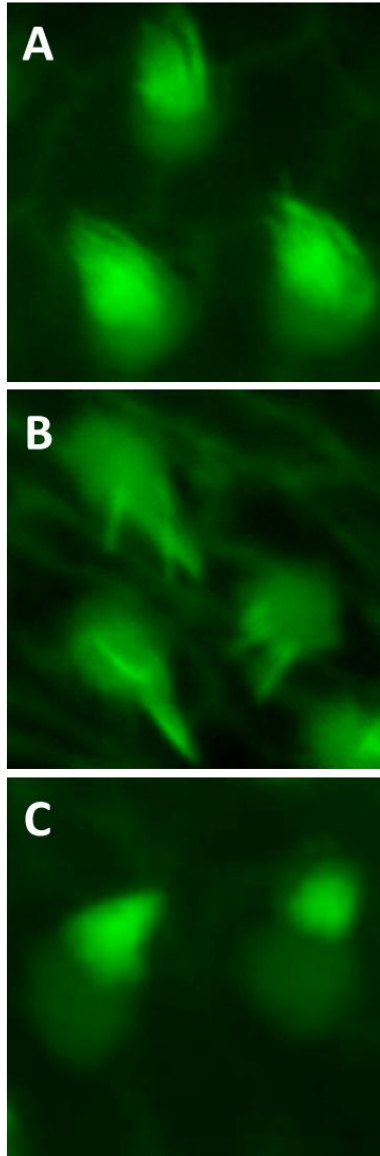
Figure 2. High power photomicrographs (100X) of phalloidin-labeled hair cells located on the saccule of the zebrafish. A) Normal hair cell bundles with intact, long stereocilia. B) Damaged hair cell bundles with broken, disordered, or sparse stereocilia. C) Newly-formed hair cell bundles with compact and well-ordered short hair cell bundles.

CHAPTER 3

RESULTS

GH prophylaxis reduces hair cell bundle damage

The mean density of hair cell bundles in the whole zebrafish saccule under normal

conditions is approximately 32-37 hair cells/1600 μm^2. This hair cell density has a

rostral-caudal shift across the saccule though, with the lowest mean ($\pm$ S.E.) density at the

25% location (26.4$\pm$ 4.6), and the highest at the 90% location (51.3 $\pm$ 3.3) in baseline

animals.

Immediately after sound exposure, the hair cell bundles were significantly

reduced, with hair cell bundle densities of 13-17 hair cells/1600 μm^2. When the total hair

cell bundle density was compared between the GH-injected and buffer-injected fish, there

was fewer total hair cell bundles at PSED1, PSED2, and PSED3 found in the buffer

group (P<0.05) (Figure 3A). Similarly, there was significantly fewer normal hair cell

bundles in buffer- compared to GH-treated fish at PSED1 and PSED2 (P<0.05) (Figure

3B). The numbers of hair cell bundles began to recover toward control levels in both

groups by PSED2 (Figure 5). At PSED 2, the hair cell bundles in GH group recovered to

baseline levels yet the buffer group still had not recovered to the normal levels by PSED
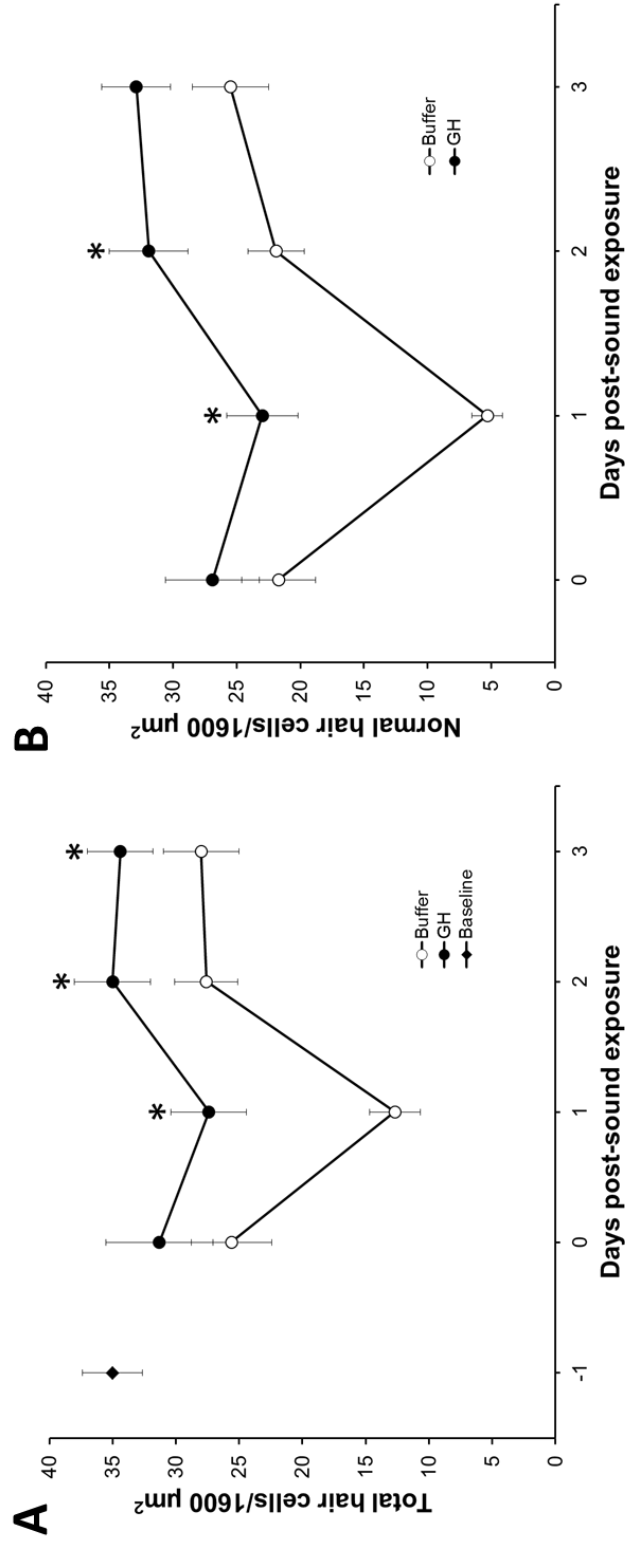
3 (Figure 3A).

After sound exposure, the buffer-injected fish had greater numbers of damaged

hair cell bundles compared to the GH-injected fish at PSED 1 and PSED 2 (P<0.05)

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(Figure 4A). Minimal damage was found at PSED3. Furthermore, the number of new hair cell bundles were greater in buffer-injected fishes compared to the GH group at PSED 3 ($P<0.05$) (Figure 4B). The quantity of newly formed hair cell bundles peaked at PSED 2 in both groups.

Effects of GH on auditory thresholds

Auditory evoked potentials (AEPs) were recorded from GH-injected and buffer-injected zebrafish (Figure 5A, B, C, and D). Significant threshold differences occurred between the GH and buffer group at PSED2 at the frequencies of 100, 250, 400 and 3000 Hz ($P<0.05$). Similarly, at PSED3 hearing thresholds were greater for buffer-injected compared to GH-injected fish at the frequency 400 Hz ($P<0.05$).

There was a significant inverse linear regression relationship between hair cell density and temporary threshold shift in the zebrafish. This relationship was significant for both buffer- and GH-injected fish (Figure 7). In general, the GH group exhibited higher hair cell bundle densities and lower temporary threshold shifts, and the opposite was true of the buffer group. This relationship suggests that the GH group was more sensitive to sound (i.e., had lower hearing thresholds) because they had greater densities of hair cells.

Figure 3. Mean (± S.E.) total (A) and normal (B) phalloidin-labeled hair cell bundles of buffer- (white) and GH-injected (black) saccules across all rostral-caudal regions at respective post-sound exposure days (* $p < 0.05$, n= 9).
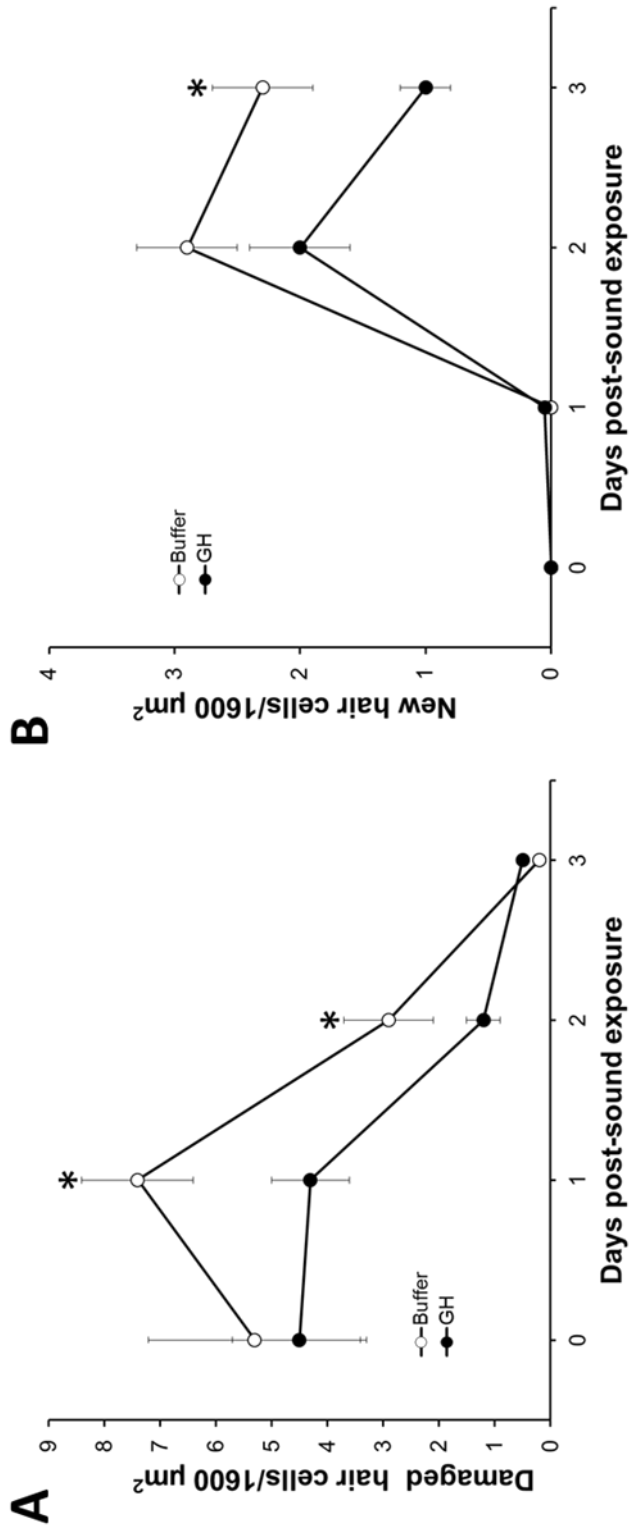
Figure 4. Mean (± S.E.) damaged (A) and newly formed (B) phalloidin-labeled hair cells of buffer- (white) and GH-injected (black) saccules across all rostral-caudal regions at respective post-sound exposure days (* p < 0.05, n= 9).
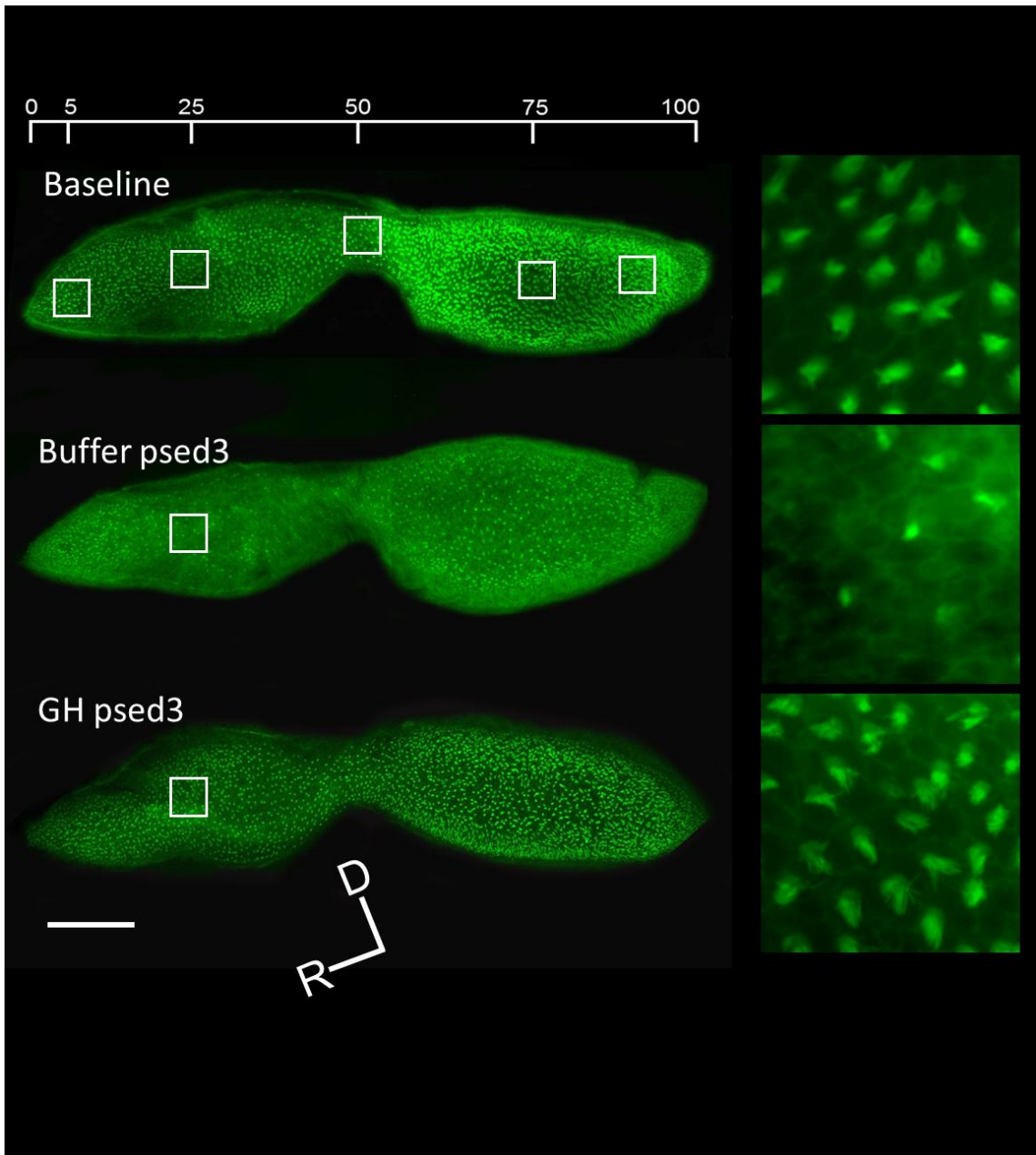
Figure 5. Phalloidin-labeled hair cell bundles of baseline, buffer- and GH-treated zebrafish saccules at post-sound exposure day 3 (psed3). R= rostral, D = dorsal. Scale bar = 100 μm. High power photomicrographs (100X) of phalloidin-labeled hair cells at 25% along the rostral-caudal axis are to the right of the whole saccules. A) Baseline fish (no noise exposure). B) Buffer-injected fish following sound exposure at psed3. C) GH-injected fish following sound exposure at psed3.
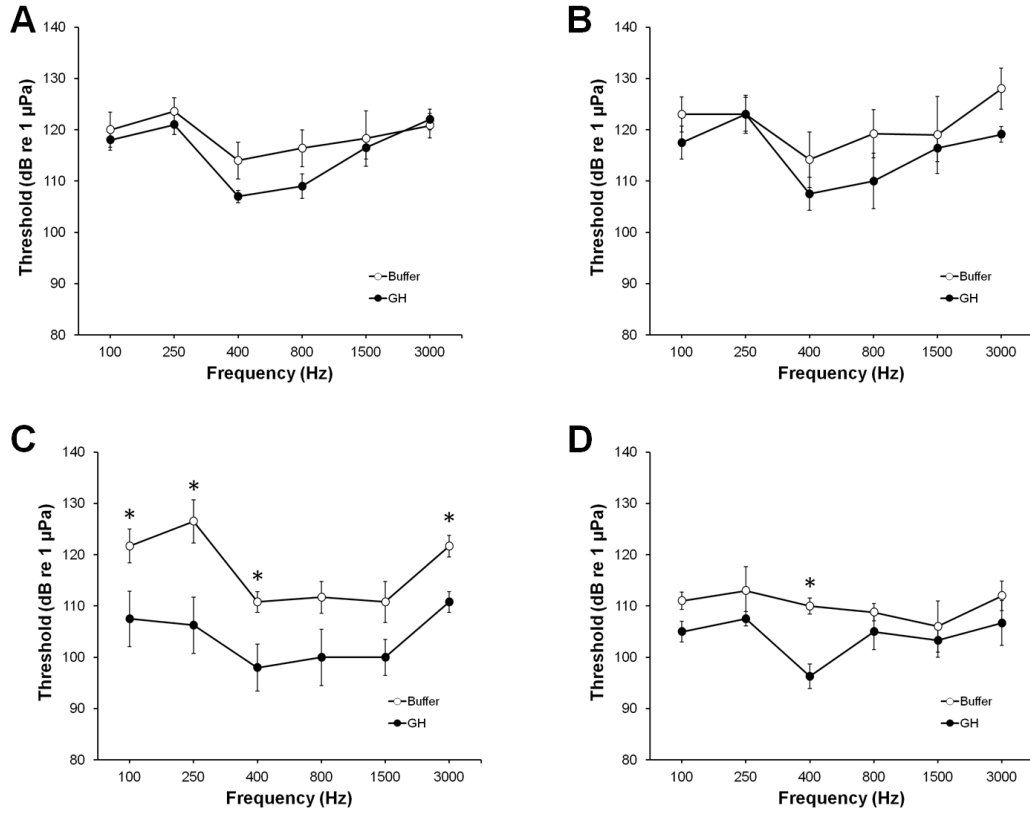
Figure 6. Mean (± S.E.) auditory thresholds following sound exposure at the following time points: A) post-sound exposure day 0 (psed0), B) psed1, C) psed2, and D) psed3. Audiograms were used to compare auditory function in GH-treated (black) and buffer-treated (white) fish (*p<0.05, n = 9-11).
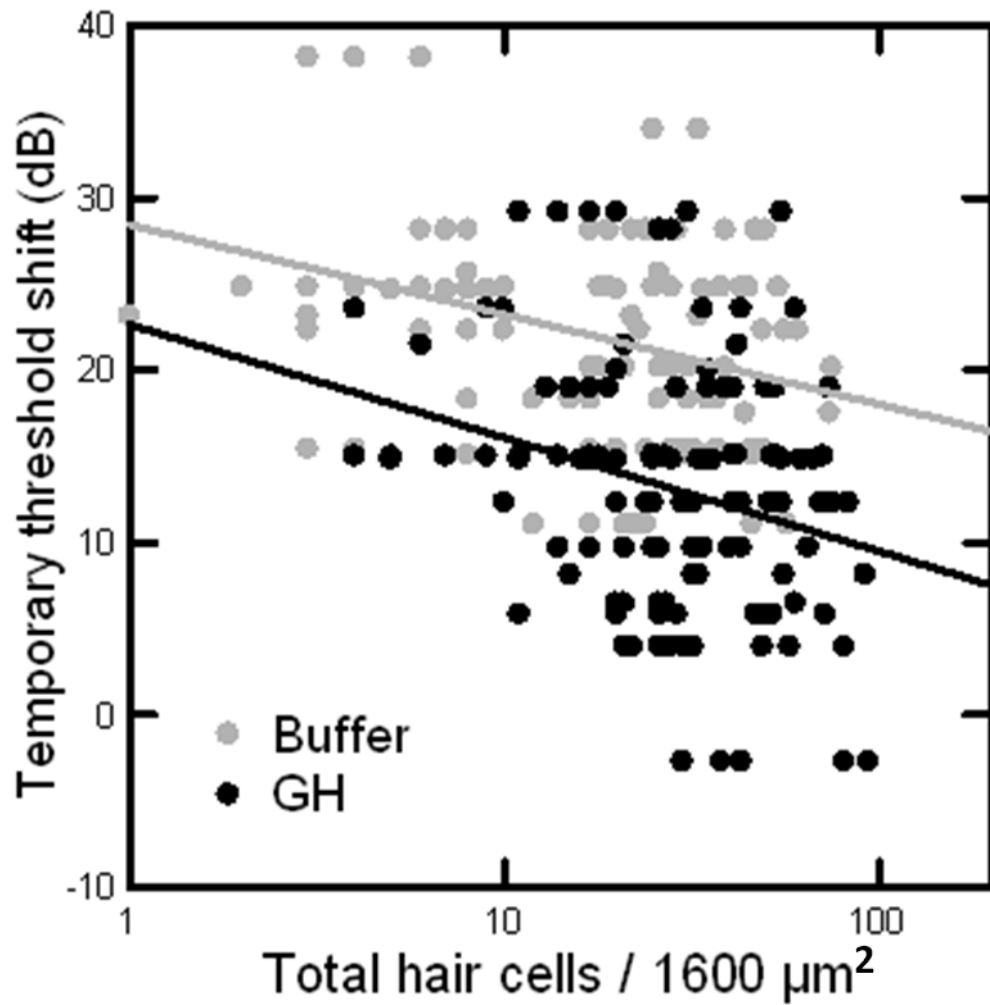
Figure 7. Regression relationship for temporary threshold shifts and total hair cell bundle counts within 40 x 40 µm counting locations for GH (black) and buffer (gray) groups. The linear regression equation for the buffer group is TTS= -0.108 (Total hair cells) + 24.6; $p<0.05$) and for the GH group is TTS=0.092 (Total hair cells) + 16.28; $p<0.05$).

CHAPTER 4

DISCUSSION

GH reduces hair cell bundle damage

The control hair cell densities found in this study were similar to those found in other studies examining the zebrafish saccule (Schuck and Smith, 2009; Sun et al., 2011; Wang, 2012). Through this study, we found that the injection of GH in zebrafish prior to sound exposure led to faster recovery of hair cell bundle density compared to the buffer-injected group. The GH group had less damaged hair cell bundles and more intact hair cells compared to the buffer group. These results suggest that GH injection prior to sound exposure may have prevented some hair cell loss, thus there was less formation of new hair cells compared to buffer-injected controls. An explanation for these results could be that GH inhibits the hair cells from being ejected from the epithelia following hair cell damage from sound exposure. Avoiding this ejection, hair cells would be able to repair the damage caused by sound exposure through other regenerative mechanisms. Self-repair has been seen in the rat utricle's hair cell stereocilia following damage (Zheng et al., 1999). Following sound exposure, the hair cell density in the GH-treated group had already recovered to baseline levels by PSED 2. The buffer-treated group hair cell densities at PSED3 had still not reached baseline levels (Figure 3). Newly-formed hair bundles increased in PSED 2 for both groups with the buffer group having a higher number of newly-formed hair cell bundles. Numbers of newly-formed hair cells in both

the GH and buffer groups peaked at PSED 2, while in a previous study in which zebrafish were injected with GH after sound exposure, the GH group peaked at PSED2, but the buffer group peaked at PSED3 or later (Wang, 2012). Similarly, at PSED 3, there were significantly greater numbers of newly-formed hair cell bundles in the buffer group compared to the GH group which is opposite of what was seen in previous studies of GH injections following sound exposure (Figure 4B) (Wang, 2012). It is counterintuitive that there would be less newly formed hair cells in GH-injected fishes, since newly-formed zebrafish saccular hair cells actually increased at PSED2 in a previous study in which GH was injected after sound exposure instead of before (Sun et al., 2011). One potential explanation for this is that GH injection prevents hair cell loss, thus fewer newly formed hair cells would be needed to replace those that were lost.

Comparing this time course of saccular hair cell density shifts with those of other experiments examining hair cell regeneration in fish, GH appears to decrease the recovery time. For example, in goldfish, saccular hair cell bundle density returned to control levels within eight days following acoustic trauma (Smith et al., 2006). In zebrafish, the hair cell bundle regeneration following acoustic trauma was seven days (Schuck and Smith, 2009). The current data also exhibited regeneration and recovery trends of hair cell bundles that were similar to those seen in a previous study of GH injection following sound exposure in zebrafish (Wang, 2012). In the previous study, zebrafish saccular total hair cell density decreased at PSED1 (Wang, 2012), similar to what I found in the buffer group (Figure 4A). While the GH group decreased in the total hair cells present, this density was significantly higher than the buffer group (Figure 4B). This supports the idea that GH may prevent hair cell loss through not allowing the hair

cells to undergo apoptosis and be ejected from the epithelia. In summary, GH may produce higher hair cell bundle densities through preventing normal post-traumatic hair cell bundle loss (Figure 4), but this is only one possibility.

Two possible explanations for the effect of GH on hair cell regeneration are decreased cell death and increased cell proliferation and subsequent regeneration. As mentioned previously, there were significantly greater numbers of newly-formed hair cell bundles in the buffer group compared to the GH group at PSED 3 (Figure 4B). Furthermore, the GH group had a decrease in the total hair cells present compared to baseline controls, yet the hair cell bundle density is significantly higher than the buffer group (Figure 3A). One explanation for this is that GH is preventing the apoptosis of normal hair cell bundles. GH injection post-sound exposure has been found to decrease apoptosis within the saccular epithelia (Sun et al., 2011; Wang, 2012). GH has been found to reduce apoptosis in multiple tissue types. Human recombinant growth hormone (rhGH) has the ability to lessen the effects of cell damage and apoptosis caused by morphine in the hippocampal neural cells within in the brain of mice embryo (Svensoon et al., 2008). GH has also been found to reduce apoptosis in neutrophils of post-surgical patients (Decker et al., 2005). I suggest that the mechanism potentially used by hair cells is through the prevention of apoptosis.

Even though the suggested mechanism is apoptosis, cell proliferation is still active in regenerating auditory epithelia. GH injected prior to sound exposure speeds up the recovery process through increased cell proliferation (Sun et al., 2011; Wang, 2012). In order for new hair cells to develop, surrounding supporting cells must proliferate via mitosis and then differentiate into a hair cell. Proliferation plays a dominant role in

regeneration of hair cells in the lateral line of chick basilar papilla (Mackenzie and Raible, 2012). Cell proliferation has also been found to increase in the zebrafish saccule after GH injection (Sun et al., 2011; Wang, 2012). This is not altogether surprising since GH has been known to increase cell proliferation in other tissues such as mammary tissues (Kaulsay et al., 2001). The specific cellular pathways that are activated during the process of hair cell proliferation and apoptosis are unknown. Through further examination, we could potentially determine the explanation of the prophylactic effect of growth hormone on zebrafish auditory hair cell damage.

Effects of GH on auditory thresholds

I found that GH-injection resulted in lower hearing thresholds than the buffer group. Although mean thresholds were consistently higher in buffer-injected fish compared to GH-injected fish, these differences were greater at lower frequencies than higher frequencies. This is because a low frequency sound exposure stimulus was used (150 Hz), and thus areas of the ear that are more sensitive to low frequencies would be expected to be more damaged. Previous research has established that the fish saccule has a tonotopic organization- that is; certain regions of hair cells are sensitive to low frequencies and other regions to higher frequencies. Intense lower frequency tones can damage the caudal end of the goldfish saccule and higher frequency tones damage the rostral end of the saccule, suggesting there is a rostral-caudal shift in frequency sensitivity along the saccule (Smith et al., 2011).

While some studies have examined sound-induced hair cell loss in fishes, and other studies have examined hearing loss in fishes, very few studies have studied both simultaneously in order to understand the relationship between hair cell loss and hearing

loss. The lower hearing thresholds of GH-injected fish could be related to the higher hair cell density found in these fish. Normal hearing thresholds in fish vary across frequencies. After noise exposure, these thresholds are elevated. The difference (in decibels of sound pressure level) between control and post-exposure thresholds are referred to as threshold shifts, or temporary threshold shifts (TTS) in fishes since this shifts have not been found to be permanent (Smith et al., 2004). TTS measurements allow for establishing a relationship between hair cell and hearing loss.

Previous studies that have examined the hearing loss of goldfish exposed to sound exposures of similar intensities found threshold shifts similar to that of my study-approximately 10-20 decibels (Smith et al., 2004; Smith et al., 2006; Smith et al., 2011). Noise exposure does not produce long-term hearing loss in fishes, since thresholds have been shown to recover almost to control levels within one to two weeks (Smith et al., 2004; Smith et al., 2006). A significant linear relationship was found between hair cell loss and hearing loss in sound-exposed goldfish, with threshold shifts increasing with greater hair cell loss (Smith et al., 2011). Similarly, my results show that with an increase in the number of total hair cells, the TTS decreases (i.e., the zebrafish's sensitivity to sound is increased with the increase of hair cell bundles on the auditory epithelia). In summary, the results show that there is a significant relationship between morphological and physiological effects of inner ear damage due to noise exposure in zebrafish.

Further research

In summary, the results suggest that GH can mitigate zebrafish auditory hair cell damage and its associated hearing loss. Thus, GH not only appears to play an important role in the regeneration process of the zebrafish hair cells, but also has a prophylactic

effect against acoustically-induced hair cell loss. GH exhibits significant and similar effect when injected either prior to sound exposure or post-sound exposure. The prophylaxis of GH needs to be further explored through examining the mechanisms of hair cell proliferation and apoptosis to determine how GH prevents damage. Furthermore, to identify whether GH is necessary for zebrafish hair cell regeneration, a GH antagonist will be used in an attempt to block the positive effects of GH in future experiments. The genes involved within this GH pathway leading to hair cell regeneration have yet to be identified and would be crucial to further determine the mechanism of regeneration. Next Generation Sequencing experiments have been used to analyze the mRNA of the zebrafish inner ear tissues during the process of auditory hair cell regeneration. The goal is to examine the gene expression patterns in the zebrafish inner ear to determine which genes are vital for effective regeneration of hair cells following damage due to sound exposure.

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