

2013

# Progenitor activity within the zebrafish lateral line following cisplatin toxicity

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**PROGENITOR ACTIVITY WITHIN THE ZEBRAFISH LATERAL LINE  
FOLLOWING CISPLATIN TOXICITY**

**by**

**Laura G. Street**

**A Capstone Project  
submitted in partial fulfillment of the  
requirements for the degree of:**

**Doctor of Audiology**

**Washington University School of Medicine  
Program of Audiology and Communication Sciences**

**May 16, 2014**

**Approved by:**

**Mark Warchol, Ph.D., Capstone Project Advisor  
Kevin Ohlemiller, Ph.D., Second Reader**

*Abstract: Supporting cell proliferation patterns were assessed in the three most posterior neuromasts of zebrafish larvae following treatment with 1000  $\mu$ M cisplatin. Proliferation was upregulated 48 to 72 hours post-exposure, but survival of internal supporting cell progeny was low three days after treatment. This finding may account for why hair cell regeneration is delayed or absent after cisplatin administration.*

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## ACKNOWLEDGMENTS

First and foremost, I would like to thank my mentor, Dr. Mark Warchol, for giving me the privilege of working and studying in his lab this past semester. As an audiologist-in-training, I felt it was necessary to educate myself on the progress that has been made in the area of hair cell regeneration. Hearing impaired patients are always looking for a “miracle cure,” and I think audiologists should be prepared to step in and explain the possibilities that unlocking hair cell regeneration may or may not hold for their patients. That and I simply wanted to know if I’d still have a job in ten years. Thank you, Dr. Warchol, for not only teaching me about this important and topical line of research but also exhibiting the characteristics that I look for in a mentor. You are not only extremely intelligent but undeniably kind, genuine, patient, and funny.

I would also like to thank Dr. Kevin Ohlemiller for serving as my second reader, teaching me how to work with patients of the rodent kind, and giving me a foundation in basic science and genetic research. The classes you and Dr. Warchol taught have enabled me to think more critically about the hearing research I read.

Finally, I would like to thank the other members of the Sensory Regeneration Lab—Dr. Matthew Barton, Dr. Tejbeer Kaur, Angie Schrader, Judy Speck, and Rose Veile. All of them assisted me at one time or another with a question that I had, and they were always quick to check and make sure I hadn’t blown myself up (along with their experiments) whenever a loud sound went off in the lab. Additional thanks are owed to Angie Schrader who gave me a crash course in immunohistochemistry procedures, slide preparation, and confocal microscopy.

## DEDICATIONS

This Capstone project is dedicated to Brian Aldana, whose unselfishness and ongoing good will have enabled me to pursue countless opportunities that would have been unavailable to me had I been on this life’s journey alone. You continue to be the most beautiful person I know, and your kindness and intelligence consistently inspire me to become more involved in the world in which I live.

I would also like to dedicate this project to my parents who were my first teachers. Whenever someone compliments me, I know my actions have been a reflection of you.

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## INTRODUCTION

The potential for hair cell regeneration therapies is currently gaining public interest and awareness. In a recent study conducted by Ali, Williams, Jackson, and Pau (2012), a questionnaire was distributed to members of the British Association of Otorhinolaryngologists, and 11% of responders reported that patients had specifically asked them about hair cell regeneration and stem cell therapies. Audiologists should know that the patients most likely to benefit from the advancement of hair cell regeneration research are those that have damage limited to the level of the hair cell. Deterioration of cochlear cells and structures other than hair cells, as seen in animal models experiencing noise-induced (Wang, Hirose, and Liberman, 2002) and age-related (Ohlemiller, Dahl, and Gagnon, 2010) hearing loss, would not be targeted for repair with hair cell regeneration therapies. In the present study, an additional limitation of the hair cell replacement mechanism is uncovered using five day post-fertilization zebrafish treated with cisplatin.

## LITERATURE REVIEW

### I. Cisplatin Toxicity

Cis-platinum (II) diamine dichloride, commonly referred to as *cisplatin*, is a chemotherapeutic agent that was first introduced in the 1970s to treat ovarian, testicular, cervical, bladder, lung, and head and neck tumors (reviewed in Gratton and Smyth, 2004; Yorgason, Fayad, and Kalinec, 2006; Rybak, 2007; Rybak, Mukherjea, Jajoo, and Ramkumar, 2009). The mechanism by which cisplatin appears to treat soft tissue cancers is through the creation of intrastrand cross-links in the deoxyribonucleic acid (DNA) of mitotically-active cells. When the nuclear DNA of

dividing cells is adducted in this fashion, DNA replication is inhibited and apoptosis or programmed cell death is initiated if these cross-links are not repaired (e.g., by a nucleotide excision repair system). Similar to other platinum-based chemotherapy agents, cisplatin has several undesirable side effects including ototoxicity, nephrotoxicity, neurotoxicity, emesis, and nausea. Animal models have shown that cisplatin also damages inner ear hair cells, supporting cells, spiral ganglion cells, the spiral ligament, and the stria vascularis, specifically. Despite these known side effects, physicians continue to prescribe cisplatin to cancer patients because of its unparalleled effectiveness against soft tissue cancers and because neoplasms have a low likelihood of developing resistance to cisplatin compared to other chemotherapy drugs.

## **II. Hair Cell Function, Death, and Regeneration**

Hair cells are mechanosensory receptor cells found within the hearing, vestibular, and lateral line organs of vertebrate species. When the stereocilia along the apical surfaces of hair cells are displaced, neural processes surrounding the hair cells generate electrical impulses that are transmitted to the brain (reviewed in Hudspeth, 1997). These neural inputs are used to inform the organism that sound waves, head movements, changes in body orientation, or surrounding water currents have been detected (reviewed in Matsui & Ryals, 2005 and Salvi, 2008).

Hair cell loss contributing to hearing and/or balance disturbances can result from extreme environmental stress. Damaging amounts of noise, aminoglycosides, and cisplatin have all been shown to increase the level of reactive oxygen species (ROS) present inside hair cells and can initiate apoptosis when an unidentified threshold of ROS has been reached (Ou, Raible, and Rubel, 2007; reviewed in Gratton and Smyth, 2004; Yorgason et al., 2006; Rybak, 2007; Stone and Contanche, 2007; Rybak et al., 2009). Since auditory hair cells are rarely produced after

embryogenesis in mammalian species (reviewed in Oesterle and Stone, 2008 and Warchol, 2011), the result of cochlear hair cell death is permanent sensory hearing loss.

Unlike their mammalian counterparts, birds, reptiles, amphibians, and fish all maintain the capacity to regenerate cochlear hair cells beyond the late embryonic period. Regeneration of hair cells following exposure to damaging levels of noise and ototoxic drugs has also been noted in all four animal classes (reviewed in Oesterle and Stone, 2008; Warchol, 2011). Why hair cell regeneration persists in nonmammals but not mammals is unknown, but one theory proposes that mammals made an evolutionary tradeoff at some point in their history – the ability to regenerate cochlear hair cells for the complex hearing organ needed to achieve high frequency sensitivity (Warchol, 2011). Regardless of whether or not this theory is true, one of the main challenges to treating hearing loss in humans will undoubtedly be the need to regenerate cochlear hair cells without interfering with the intricate structure of the mammalian organ of Corti.

Within the sensory epithelia of the auditory, vestibular, and lateral line systems, both sensory hair cells and nonsensory supporting cells are present. Sensory hair cell somata contact the luminal surface of the epithelium, and supporting cell somata are interspaced between them. When hair cell regeneration is not taking place, nonsensory supporting cells are in a state of quiescence. Their somata contact the luminal and basal surfaces of the epithelium, and their nuclei are located more towards the basal side. Compared to sensory hair cells in avian and fish species, nonsensory supporting cells are morphologically simple, exhibiting a noncomplex cytoskeleton and few organelles. As hair cell death occurs, it is these seemingly undifferentiated supporting cells that replenish the lost population of hair cells through either direct transdifferentiation or mitotic proliferation (reviewed in Stone and Cotanche, 2007; Oesterle and Stone, 2008; and Brignull, Raible, and Stone, 2009).

During direct transdifferentiation, dying hair cells are replaced by adjacent supporting cells that have undergone phenotypic conversion and transformed into new hair cells. This mechanism, at least in theory, is capable of replacing lost hair cells so long as the endogenous population of supporting cells is not depleted. Surprisingly, what appears to limit the utility of this mechanism has less to do with the number of supporting cells remaining and more to do with the number of essential functions assigned to supporting cells. For instance, not only do supporting cells serve as precursor cells during direct transdifferentiation but they also contribute to the endocochlear potential through potassium (re)cycling and provide structural support to hair cells, spiral ganglion terminals, and the sensory epithelium as a whole. If direct transdifferentiation was the only method by which nonmammalian species were able to regenerate lost hair cells, then the sensory epithelium would effectively collapse and deteriorate whenever it experienced a massive hair cell lesion. For this reason, direct transdifferentiation appears to have evolved as an "early response" or a "quick fix" for replacing a few lost hair cells.

When extensive hair cell damage occurs, supporting cells leave growth-arrest, reenter the cell cycle, and undergo mitosis. The two cells resulting from the division may then differentiate into a symmetrical pair of two new hair cells, a symmetrical pair of two new supporting cells, or an asymmetrical pair comprised of one new hair cell and one new supporting cell. Current thinking suggests that these three outcomes are likely balanced and highly regulated so as to ensure that the correct number of hair cells and supporting cells are regenerated without jeopardizing the important role supporting cells play in maintaining inner ear homeostasis. At present, it is unclear whether three different populations of supporting cells exist (i.e, a population devoted to direct transdifferentiation, a population devoted to mitotic proliferation, and a population devoted to standard supporting cell functions) or if all supporting cells possess the potential to

perform all three functions based on local cell signaling. Since supporting cells serve as the replacement stock for dying hair cells (Williams and Holder, 2000) regardless of the hair cell replacement mechanism used, supporting cells can be viewed as a type of progenitor cell if not a true stem cell.

### **III. The Benefits of Using a Zebrafish Model**

Neuromasts are mechanosensory organs found in fish and amphibian species. They contain a cluster of centrally-located hair cells surrounded by intervening supporting cells. When neuromasts are organized into species-specific patterns on the bodies of fish, the result is the lateral line system (Ledent, 2002; Dambly-Chaudière et al., 2003; Ghysen and Dambly-Chaudière, 2004; Chiu, Cunningham, Raible, Rubel, and Ou, 2008). The lateral line system is used to sense water disturbances in a fish's immediate environment, and it plays an important role when fish are swimming in schools, socially interacting with one another, and localizing predators and prey (Dambly-Chaudière et al., 2003; Ghysen and Dambly-Chaudière, 2004).

The embryonic posterior lateral line of zebrafish (*Danio rerio*) is comprised of between seven and eight neuromasts that extend from the head of the fish to the tip of the tail (Ledent, 2002; Dambly-Chaudière et al., 2003; Ghysen and Dambly-Chaudière, 2004). As described in Ledent (2002), development of the embryonic posterior lateral line is complete by three days post-fertilization (dpf); however, the lateral line continues to develop as a fish matures. Starting between three and four dpf, new neuromasts appear interspersed between the previously developed neuromasts and posterior lateral line neuromasts begin to migrate ventrally. The three most posterior neuromasts, in particular, rearrange themselves with the penultimate neuromast moving to a more ventral position than the surrounding two. This movement seemingly appears in anti-

cupation of the developing bend in a fish's tail. Ledent stresses that postembryonic growth of the zebrafish lateral line is highly dependent upon a fish' size rather than its age. When a fish reaches approximately 10-12 millimeters in length, its posterior lateral line neuromasts begin to contribute to the formation of stitches where new neuromasts appear to bud off and travel away from existing neuromasts in a dorsoventral fashion.

Despite the level of postembryonic growth seen in the zebrafish lateral line, it is a practical model for studying hair cell death and regeneration for several reasons (Chiu et al., 2008). First, lateral line hair cells are structurally and functionally comparable to those found within the mammalian inner ear despite obvious differences in cellular organization (i.e., neuromasts vs. the organ of Corti). Second, like inner ear hair cells, lateral line hair cells are vulnerable to ototoxic drugs such as aminoglycosides and cisplatin. Unlike cochlear hair cells, however, neuromast hair cells do not require invasive techniques to be accessed. They are superficially located on the body surfaces of fish. An additional benefit to working with zebrafish larvae is that they are optically transparent and therefore amenable to immunofluorescent dyes taken up by hair cells (Quigley and Parichy, 2002; Dambly-Chaudière et al., 2003). Finally, zebrafish larvae are an ideal laboratory specimen because they exhibit rapid development and high fecundity when adulthood is reached (Quigley and Parichy, 2002; Chiu et al., 2008). By five days old, zebrafish larvae have developed an average of  $10 \pm 2.7$  hair cells per neuromast (Genualdi, 2012), and this number doubles by ten days of age (Williams and Holder, 2000).

#### **IV. Previous Research on Hair Cell Regeneration within the Zebrafish Lateral Line**

Several lines of research suggest that hair cell replacement within the zebrafish lateral line is a highly regulated process sustained by a population of proliferating supporting cells that

differentiate into hair cells. López-Schier and Hudspeth (2006) were the first to characterize hair cell regeneration patterns in ET4 transgenic zebrafish following treatment with neomycin. According to the authors, transgenic zebrafish of the ET4 line are identical to wild-type zebrafish in all ways except for the expression of a green fluorescent protein in lateral line hair cells and in progenitor cells shortly before they differentiate into hair cells. Using live imaging techniques, López-Schier and Hudspeth monitored hair cell regeneration in the neuromasts of fourteen ET4 transgenic zebrafish and found that, prior to their final mitosis, progenitor cells appeared in positions perpendicular to the neuromasts' axes of hair cell polarization. Progenitor cells were thus seen in positions anterior to and posterior to neuromast hair cells when hair cells were regenerating along a dorsoventral axis or vice versa. Given this finding, the authors concluded that hair cell orientation in zebrafish larvae is partially based upon the positioning of progenitor cells which are, in turn, situated in the neuromast depending upon the axis of hair cell planar polarity. Production of the Vangl2 protein was then found to govern kinocilia and stereocilia arrangement atop regenerating hair cells.

Although López-Schier and Hudspeth did not directly investigate direct transdifferentiation versus mitotic proliferation methods of hair cell replacement in the zebrafish lateral line, they did hypothesize that direct transdifferentiation would be unlikely to dominate over mitotic proliferation for two reasons. First, the time course for hair cell replacement following treatment with neomycin sulfate did not seem accelerated or extensive enough to suggest that supporting cells were transdifferentiating. Second, a single neuromast contains roughly 30 supporting cells. If direct transdifferentiation had been occurring to a substantial degree, this small population of cells would have been depleted or significantly reduced in the two day's time it took to replace the ablated hair cells.

Additional experiments by Mackenzie and Raible (2012) have also suggested that mitotic regeneration is the primary mechanism by which lateral line hair cell replacement is performed in zebrafish. Five dpf zebrafish larvae were treated with aminoglycosides or copper (II) sulphate at concentration levels known to result in near complete recovery of lateral line hair cells. After treatment with these ototoxic compounds, larvae were incubated for two days in flubendazole, a drug that has limited toxicity to hair cells but has been shown to inhibit microtubule assemblage during chromosome segregation. After larval incubation in flubendazole, Mackenzie and Raible noted a significant reduction in hair cell regeneration and a significant increase in phosphorylated histone H3 (PH3) expression in progenitor cells. Since PH3 is expressed in mitotic cells transitioning from the gap 2 phase to metaphase (a stage during mitosis), the authors concluded that flubendazole appears to hinder hair cell regeneration by arresting cellular division—not by blocking direct transdifferentiation. If flubendazole had prevented precursor cells from transdifferentiating, the authors believe they would have seen an increase in precursor cell numbers and not progenitor cell numbers. For this reason, the authors conclude that mitotic proliferation is the dominant mechanism responsible for lateral line hair cell replacement after ototoxic injury.

Presently, two distinct supporting cell populations have been proposed to contribute to the regeneration of lateral line hair cells in zebrafish – an internal supporting cell population located in the center of zebrafish neuromasts and two to three layers of peripheral (or mantle) supporting cells lying along the periphery of each neuromast. During an experiment conducted by Ma, Rubel, and Raible (2008), five dpf zebrafish larvae were treated with neomycin and then incubated in the Notch inhibitor DAPT. Following neomycin treatment and incubation in DAPT, internal supporting cell proliferation was found to significantly increase and peripheral supporting cell proliferation was found to decrease inside lateral line neuromasts. Excess

regeneration of hair cells was then noted shortly thereafter. Based on these results, Ma et al. suggested that internal supporting cells undergo mitotic division and differentiation in order to replace lost hair cells and peripheral supporting cells only divide when the structural integrity of a neuromast is compromised or additional growth is required.

Studies have shown that hair cell replacement within the zebrafish lateral line occurs as part of a fish's natural development and in response to damaging insults such as tail amputation and ototoxic exposure (Williams and Holder, 2000; see Stone and Cotanche, 2007, for a review). Following treatment with aminoglycosides and limited amounts of copper (II) sulphate, for instance, near complete restoration of lateral line hair cells has been observed within 24-72 hours of recovery (Harris et al., 2003; Ma et al., 2008; Mackenzie and Raible, 2012). Only a handful of studies, however, have investigated the effects of cisplatin on zebrafish lateral line hair cells.

## **V. Justification for the Present Study**

Understanding the mechanisms underlying the initiation and maintenance of hair cell regeneration in animal models is important as the knowledge gained may contribute to the development of human hair cell replacement procedures via gene therapies, stem cell therapies, or possibly drug therapies that induce endogenous supporting cells to transdifferentiate into hair cells (Mizutari et al., 2013; reviewed in Brigande and Heller, 2009; Groves, 2010; Warchol, 2011). While many additional factors need to be considered before one can readily assess the clinical utility of hair cell regeneration in humans, Matsui and Ryals (2005) and Ryals (2009) hypothesize that the renewal of a few fully functional and innervated hair cells within the cochlea would likely result in improved hearing aid benefit even if the complete restoration of normal hearing is not possible. In addition, patients suffering from the effects of vestibulotoxic

drugs (e.g., gentamicin) may also be better able to compensate if hair cell regeneration in the vestibular organs can be induced.

Given that cisplatin is toxic to hair cells, proliferating cells, and supporting cells, it seems plausible that cisplatin would severely limit or completely shutdown the regenerative process in non-mammalian species. Interestingly, Slattery and Warchol (2010) came to that conclusion in a study examining the capacity of chick hair cells to regenerate in the presence of cisplatin. In their study, cochlear and utricular organotypic cultures taken from 7-14 day post-hatch chicks were incubated in 0.2 to 20.0 micromolar ( $\mu\text{M}$ ) cisplatin for 24 hours and permitted to recover for up to seven days. After cisplatin administration, the authors noted a dose-dependent increase in the amount of hair cell death and a dose-dependent decrease in the amount of cell proliferation observed. In addition, cisplatin-treated cultures did not show an increase in hair cell numbers compared to controls even after being incubated in the Notch inhibitor DAPT. Given these findings, the authors concluded that cisplatin appears to have a toxic effect on both hair cells and supporting cells in the avian inner ear for at least seven days following cisplatin injury.

To determine whether or not comparable effects were observed in the zebrafish lateral line, Genualdi (2012) treated five dpf zebrafish larvae with 1000  $\mu\text{M}$  cisplatin for four hours and then allowed them to recover for up to 10 days. After the cisplatin exposure, neuromast hair cell counts were found to decrease to approximately 10-20% of those seen in undamaged controls and they did not fully recover. Surviving and regenerated hair cell numbers only reached 60% of those seen in the undamaged controls 10 days-post injury. When incubated in 50  $\mu\text{M}$  DAPT, however, a twofold increase in the number of regenerated hair cells was noted as early as three days post-injury. Nevertheless, the resulting hair cell numbers were still so low ( $0.08 \pm 0.20$  hair

cells per neuromast) that the addition of DAPT following cisplatin treatment did not appear to enhance the regenerative response in any meaningful way.

Mackenzie and Raible (2012) obtained similar findings to Genualdi (2012) using a much lower dose of cisplatin (50  $\mu$ M). Following treatment with cisplatin for 24 hours, 5 dpf zebrafish larvae exhibited incomplete hair cell replacement at 96 hours post-injury with the experimental group displaying approximately 25% fewer hair cells than the control group. In addition, the regenerative response following cisplatin was noted to be delayed compared to that following treatment with various aminoglycosides.

Collectively, these findings suggest that either a.) zebrafish internal and/or peripheral supporting cells are less susceptible to cisplatin than are chick inner ear progenitors (Genualdi, 2012) or b.) lateral line supporting cells have some sort of robust DNA repair mechanism that is not found in the chick inner ear (Mackenzie and Raible, 2012). In discussing the persistence of hair cell regeneration in the zebrafish lateral line, however, it is important to keep in mind that hair cell replacement is both delayed and less robust following treatment with cisplatin than following treatment with aminoglycosides or copper sulphate. At present, only a few studies have investigated supporting cell proliferation patterns within the zebrafish lateral line after toxic damage. The studies that have focused primarily on the response initiated by aminoglycosides. No studies have assessed the resulting proliferation patterns resulting from cisplatin exposure. For this reason, the goal of the present study was to characterize the time course and patterning of nonsensory supporting cell proliferation within the zebrafish lateral line post-cisplatin treatment.

## RESEARCH QUESTIONS AND HYPOTHESES

Since antineoplastic agents are known to terminate mitotically-active cells through the initiation of apoptosis, the following Capstone Project sought to characterize the effects of cisplatin on proliferating supporting cells within the zebrafish lateral line. Specifically, we examined whether cisplatin was toxic to dividing inner and peripheral supporting cells as well as lateral line hair cells and, if so, what consequences this toxicity appeared to have on their proliferation patterns compared to untreated controls. The null and alternative hypotheses for this project were as follows:

- $H_0$ : Average supporting cell counts obtained for control versus experimental neuromasts would not be significantly different for any recovery period following treatment with cisplatin.
- $H_1$ : Average supporting cell counts obtained for control versus experimental neuromasts would be significantly different for one or more recovery periods following treatment with cisplatin.

## MATERIALS AND METHODS

### I. Zebrafish Husbandry

Zebrafish embryos were obtained from AB wild type pairings of adult fish housed in the fish facility at Washington University in St. Louis. The embryos were then maintained in a tissue culture incubator at 28.5°C in embryo medium (EM: 1 mM  $MgSO_4$ , 0.15 mM  $KH_2PO_4$ , 0.04 mM  $Na_2HPO_4$ , 1 mM  $CaCl_2$ , 0.5 mM  $KCl$ , 15 mM  $NaCl$ , and 0.7 mM  $NaHCO_3$ , pH 7.2). At 2-3 days

post-fertilization (dpf), embryogenesis had ended and the specimens were considered larvae (Ledent, 2002; Dambly-Chaudière et al., 2003). According to the guidelines set forth by the Institutional Animal Research Committee at Washington University in St. Louis, zebrafish larvae are not viewed as vertebrate animals and do not require approval for experimentation. Zebrafish larvae were then housed in 85 millimeter (mm) Petri dishes and fed live rotifers once per day. Prior to initiation of the experiments, the Petri dishes were tapped lightly to ensure that the fish had a normal startle response.

## **II. Cisplatin Treatment**

Five dpf zebrafish larvae (N = 85) were incubated in a 1000  $\mu$ M solution of cisplatin (Sigma Aldrich) for four hours at 28.5°C. The chosen treatment protocol was selected as previous work by Ou et al. (2007) and Genualdi (2012) indicated that it was appropriate for killing the majority of lateral line hair cells. Age-matched controls (N = 51) were then incubated in cisplatin-free EM under the same conditions.

## **III. Cell Proliferation Assay**

In order to characterize supporting cell proliferation at different points in time following cisplatin treatment (or lack thereof), zebrafish larvae were rinsed in fresh EM three times over thirty minutes and then incubated in a 5 mM solution of bromodeoxyuridine (BrdU) in EM. BrdU is a nucleotide analog that is incorporated into the cellular DNA of a specimen's cells during the S-phase prior to mitosis. As such, it can be used to label cells preparing to undergo mitotic division. For the present experiment, two labeling paradigms were employed (Figure 1).

### **A. BrdU Pulse-Fix Protocol**

A BrdU pulse-fix protocol was used to identify all progenitor cells undergoing division during the final 24 hours prior to fixation. For the pulse-fix paradigm, both control and experimental fish were permitted to recover for 24, 48, 72, or 96 hours in fresh EM following cisplatin or mock treatment. During the final 24-hour survival period, fish were then incubated in 5 mM BrdU in EM prior to fixation.

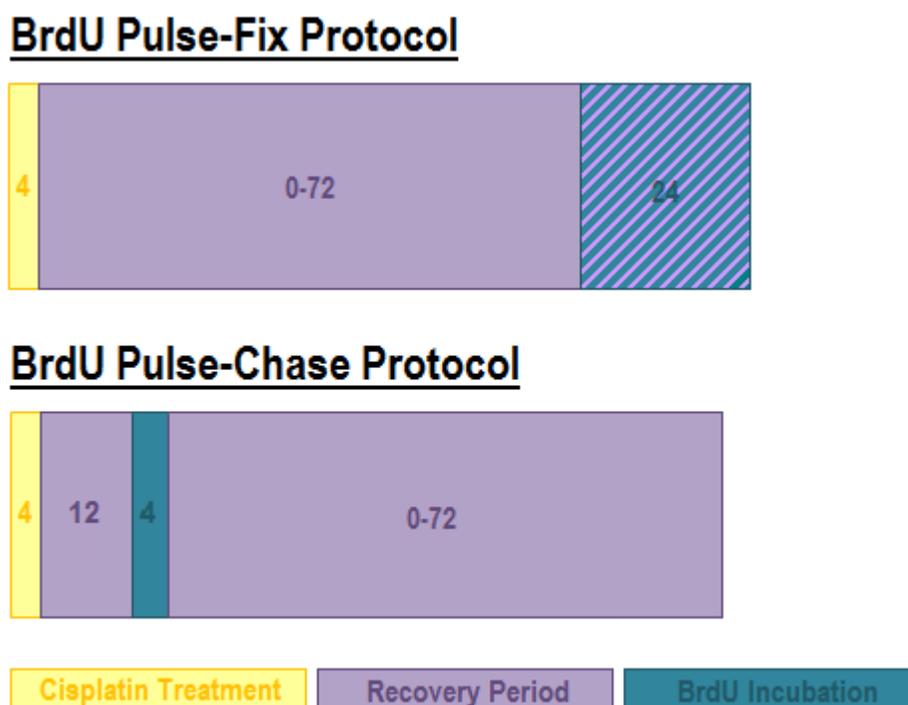
Approximately twenty fish were randomly assigned to each of the eight conditions in the BrdU pulse-fix paradigm (cisplatin treatment x four survival periods + mock treatment x four survival periods = eight conditions); however, some specimens were lost during cisplatin treatment or immunoprocessing. No fewer than eight and no more than nineteen fish contributed to the data set for each condition.

### **B. BrdU Pulse-Chase Protocol**

A BrdU pulse-chase protocol was used to identify all progenitor daughter cells that managed to survive the cisplatin treatment for up to three days. Since BrdU is incorporated into the cellular DNA of mitotically-active cells, all progeny arising from initial progenitor divisions contain the BrdU analog and can be tracked. For the pulse-chase paradigm, experimental fish were treated with cisplatin for four hours; permitted to recover for 12 hours; incubated in 5 mM BrdU for four hours; and then permitted to recover for an additional 0, 24, 48, or 72 hours in fresh EM. This protocol was selected because proliferation levels were found to be at their highest approximately 15 hours post-neomycin injury (Ma et al., 2008).

Approximately 20 fish were randomly assigned to each of the four conditions in the BrdU pulse-chase paradigm (cisplatin treatment x four survival periods = four conditions). Ultimately,

no fewer than 10 and no more than 11 fish contributed to the data set for each condition. Additional fish were processed for each of the four conditions but could not be included in the final data sets due to time constraints.



**Figure 1. Bar graphs illustrating the main differences between the two experimental protocols. A.** For the BrdU Pulse-Fix protocol, animals were treated with cisplatin (CPPD) for four hours, permitted to recover between 24-96 hours, and incubated in BrdU during their final 24 hour survival period. **B.** For the BrdU pulse-chase protocol, animals were treated with CPPD for four hours, allowed to recover for 12 hours, incubated in BrdU for four hours, and then allowed to recover for an additional 0-72 hours.

#### IV. Fixation and Immunohistochemistry Procedures

All fish, regardless of the BrdU-labeling protocol employed, were sacrificed with an overdose of 0.02% Tricaine and fixed in 4% paraformaldehyde in phosphate buffered saline (PBS) overnight. Following fixation, zebrafish larvae were rinsed for five minutes five times in

PBS. The specimens were then incubated in 2 normal (N) hydrochloric acid (H-Cl) for 45 minutes to make their fixed tissues more permeable to antibody application. After incubation in 2 N H-Cl, the specimens were rinsed for five minutes three times in PBS and then transferred into a blocking solution containing 5% normal horse serum and 95% PBS with 1% Triton X-100 detergent and 1% dimethyl sulfoxide (DMSO) to further increase tissue permeability for two hours. As is the case with all studies employing immunohistochemistry techniques, specimens were blocked prior to application of the primary antibodies in order to diminish nonspecific binding of the primary antibodies and to improve the resulting signal-to-noise ratio. When specimens are blocked in this fashion, the likelihood that the primary antibody will bind to the wrong antigen epitopes or protein locations is reduced (Jantzie, Tanay, and Todd, 2007).

After each specimen was blocked, the fish were incubated in PBS containing mouse anti-BrdU primary antibodies (1:50) overnight at room temperature along with 1% Triton X-100 and 5% normal horse serum. Anti-BrdU primary antibodies bind to BrdU antigens incorporated into a specimen's DNA. Primary antibodies then serve as new epitopes for secondary antibodies conjugated with fluorescent molecules. One benefit to using an indirect immunohistochemistry procedure such as this is that multiple fluorescent secondary antibodies can bind to a single primary antibody and, thus, amplify the fluorescent signal observed (Jantzie et al., 2007; Carter and Shieh, 2010a). Additional Triton X-100 and normal horse serum were then used for the same reasons discussed previously.

Following incubation in the primary antibody, the fish were rinsed for five minutes five times in PBS and incubated for 2 hours at room temperature in PBS containing Alexa 488-conjugated goat anti-mouse (1:500) secondary antibodies and 4',6-diamidino-2-phenylindole (DAPI 1:500). Anti-mouse Alexa 488 antibodies bind to mouse anti-BrdU antibodies and cause all mito-

tically-active nuclei to fluoresce green. The counter stain DAPI binds to the A-T regions in cellular DNA and stains all cell nuclei blue. By staining all cell nuclei along the surface of the fish blue, the investigator was better able to identify cellular “landmarks” and, thus, more readily recognize where cell proliferation was taking place within the lateral line. Upon completion of the immunofluorescent antibody staining protocol, the zebrafish larvae were mounted on standard microscope slides and coverslipped. Care was taken to ensure that the tail surfaces of the specimens were lying flat and facing upwards. Positioning the fish in this manner was important as it provided a clear shot of the lateral line neuromasts during confocal imaging.

## **V. Imaging**

To assess the degree of supporting cell proliferation following cisplatin treatment, a Carl Zeiss LSM 700 Confocal Microscope with a 20x objective lens was used to image the three most posterior neuromasts of each specimen. Confocal microscopy was selected for this purpose as it is the gold standard for imaging thick sections and small intact organisms such as zebrafish (Carter and Shieh, 2010b). Images were then captured using Zeiss proprietary software (ZEN) with the averaging parameter set to two and the acquisition speed set to seven. The gain of the microscope was set to the maximum value permissible before pixel saturation occurred (i.e., between 550-800 gain). All images were subsequently analyzed using Volocity.

As described in Carter and Shieh (2010b), a confocal microscope works by passing a laser through a fluorescently stained specimen at a specified depth. The laser illuminates the selected plane of interest, and emitted light from the specimen is received by a detector positioned confocal to the laser. A confocal microscope essentially scans very thin sections of a specimen and produces a stack of images referred to as a *Z-stack*. *Z-stacks* are then used to

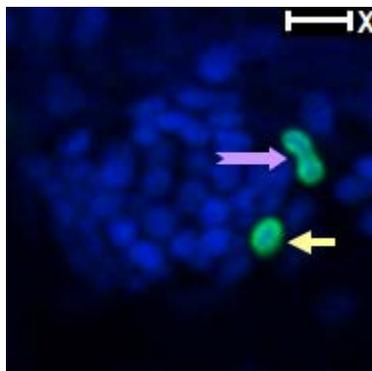
construct 2D or 3D images of the specimen on a computer. In the present experiment, the first image in a Z-stack was set to a depth where the specimen's neuromasts first became visible under the microscope. The last image in the Z-stack was then set to a depth inside the specimen where the neuromasts were no longer visible. The last image in the Z-stack captured an outline of the specimen's tail but did not represent the reverse side of the fish. Care was taken to ensure that the reverse side of the fish was not imaged as any proliferation occurring on that side would have contaminated the resulting 2D image.

## **VI. Data Quantification**

Counts of mitotically-active cells were performed for the three most posterior neuromasts of each fish using Volocity and the XYZ image mode. Volocity's XYZ image mode was selected over the Extended Focus image mode as rotating through each image of the Z-stack made it easier to disambiguate cases where a lot of cellular proliferation was taking place. Both single and dividing BrdU+ cell counts were made (Figure 2) as well as BrdU+ internal and peripheral supporting cells counts. Internal supporting cell counts were performed with relative ease as they were fairly straight forward to identify within the body of a neuromast. Peripheral supporting cell counts, however, proved to be more difficult as skin lesions about the neuromast appeared to arise from the cisplatin exposure. To help maintain consistency across neuromasts when counting BrdU+ internal and peripheral supporting cells, the following criteria were developed:

1. BrdU+ cells were counted as internal supporting cells when they a.) exhibited small round or cylindrical nuclei and b.) were located centrally within the neuromast.
2. BrdU+ cells were counted as peripheral supporting cells when they a.) exhibited larger cylindrical nuclei and b.) were situated in either the outermost layer of neuromast cells or

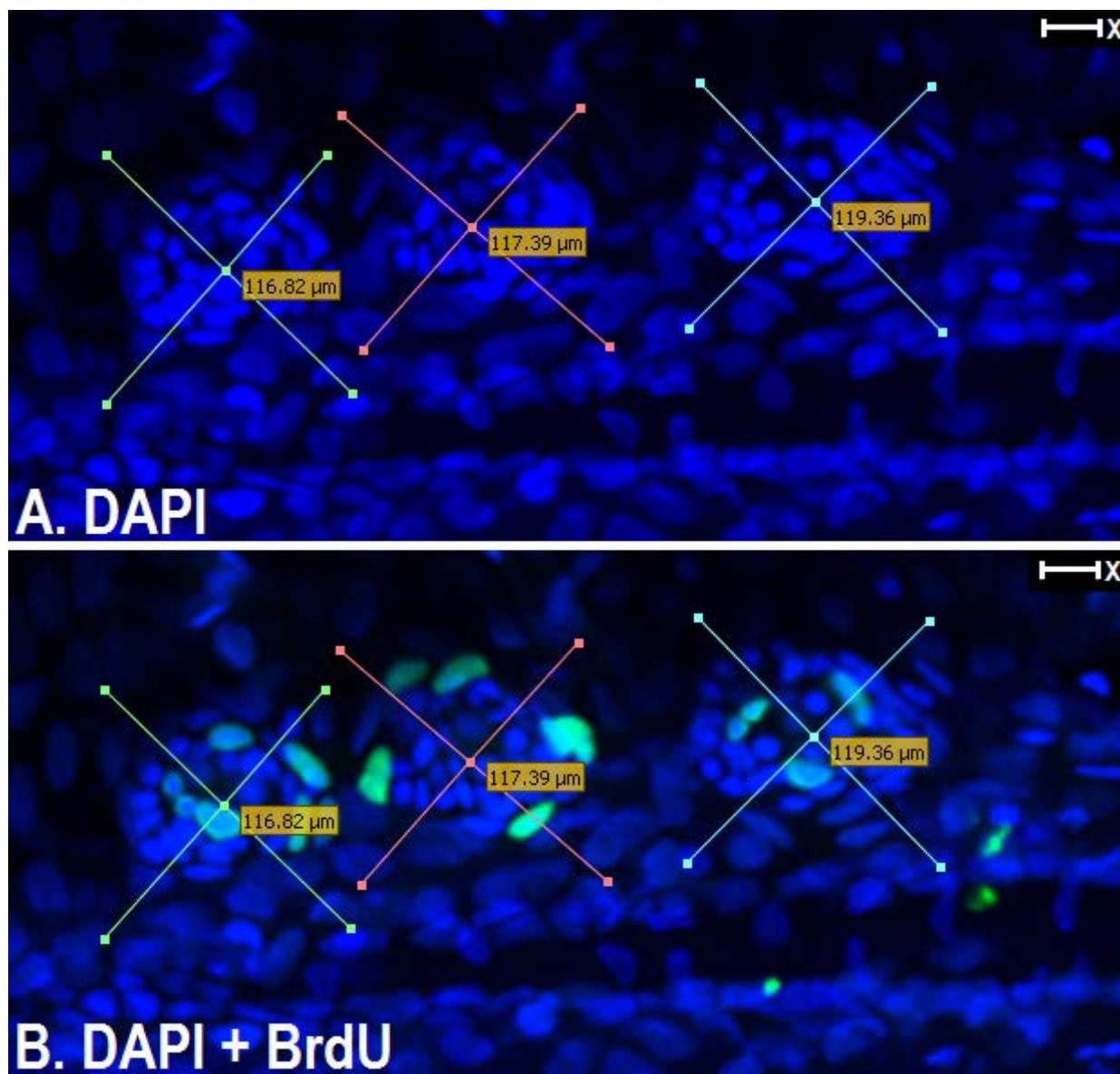
the one to two cell layers immediately beyond the periphery of the neuromast. Large nebulous BrdU+ cells were never included in the peripheral cell counts unless they appeared by other BrdU+ peripheral cells in a clear semicircular pattern about the neuromast.



**Figure 2. Cell division inside a zebrafish neuromast.** Cells entering S-phase are labeled with BrdU, and surrounding cell nuclei are counterstained with DAPI. The long arrow with a pointed tail identifies a cell undergoing division at the time of fixation. The short arrow points to a single cell that has entered S-phase. Scale bar = 10 micrometers ( $\mu\text{m}$ ).

Following peripheral and internal supporting cell counts, each neuromast was divided into four quadrants (anterior, ventral, posterior, and dorsal) to assess directional proliferation patterns. Given that existing neuromasts contribute to the formation of dorsolateral stitches (Ledent, 2002), the present study assessed proliferation patterns along the anteroposterior and dorsoventral axes. The four neuromast quadrants were created in Volocity using the line tool and the Alexa channel turned off. The Alexa channel was turned off so as to reduce bias when drawing the quadrant lines. The number of proliferating cells present in each quadrant was then assessed with the Alexa channel turned back on. When a BrdU+ cell fell into more than one quadrant, it was assigned to the quadrant that housed the majority of its nucleus. This procedure

is illustrated in Figure 3 below. While not initially intended for this purpose, the quadrant supporting cell counts provided a nice check for consistency as they were performed separately from the type supporting cell counts. If the investigator was consistent in making the BrdU+ supporting cell counts, both the quadrant and the type supporting cell counts should have been the same. In cases where the two counts were not equivalent, the investigator returned to the particular neuromast in question and performed the two counts again. Rarely did the two counts differ by more than one or two cells.



**Figure 3. Creating quadrants for directional proliferation counts.** In panels A and B, cells entering S-phase are labeled with BrdU and surrounding cell nuclei are counterstained with DAPI. **A.** Intersecting lines are drawn over the three most posterior neuromasts of each specimen using Volocity's line tool and the BrdU channel turned off. **B.** Both channels (DAPI + BrdU) are then turned on to make cell counts for the individual quadrants. The numbers in the figures represent the total length of each pair of intersecting lines. Scale bars = 10  $\mu\text{m}$ .

## **VII. Data Analysis and Statistical Procedures**

Descriptive and inferential statistical analyses were conducted using Microsoft Excel. All resulting data are presented as means  $\pm$  one standard deviation. Where appropriate, two-tailed t-tests and one way analyses of variance (ANOVAs) were performed to assess statistical differences amongst the control and experimental data after each assigned recovery period. Multiple comparisons tests, such as Tukey's test, could not be performed due to the statistical limitations of Excel. Differences were considered statistically significant when  $p < 0.05$ . Upon completion of data analysis, Excel was also used to generate representative graphs for the data. In all graphs, errors bars represent standard deviations.

## **RESULTS**

### **I. Results for the BrdU Pulse-Fix Experiment**

The relative amount of cell proliferation occurring in the three most posterior neuromasts of 5 dpf zebrafish larvae following treatment with 1000  $\mu\text{M}$  cisplatin or cisplatin-free EM for four hours was assessed at 24, 48, 72, and 96 hours post-treatment (Figure 4). All cells undergoing DNA replication during the final 24 hours of survival were labeled by incubating the fish in 5 mM BrdU. Immediately following incubation in BrdU, the fish were anesthetized and fixed. Figure 5 displays the mean number of peripheral and internal supporting cells that had incorporated BrdU at the four assessment points. Descriptive data for all figures presented in this paper are provided in the supplemental tables located in the appendix.

### **A. Moderate Levels of Proliferation Occur in Untreated Control Neuromasts**

As was observed in Williams and Holder (2000) and Ma et al. (2008), control neuromasts appeared to exhibit ongoing proliferation during the four survival periods. The reason for this constant turnover of cells is unknown but it has likely developed out of necessity. Unlike auditory and vestibular hair cells, lateral line hair cells are directly exposed to damaging agents being situated on the exterior surface of a fish's body.

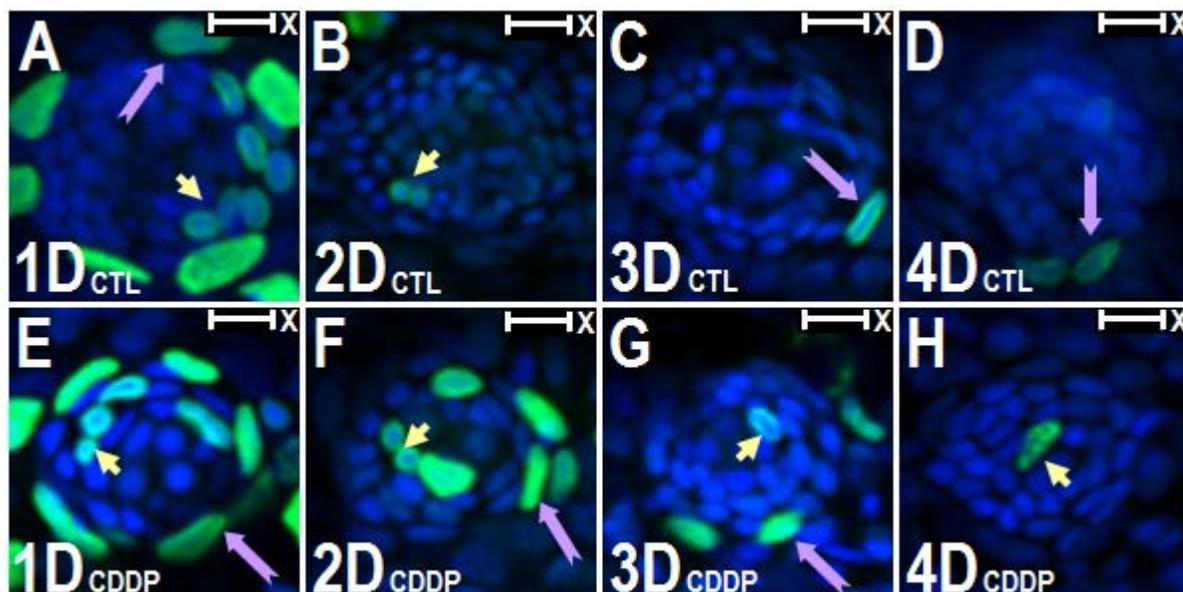
### **B. Proliferation is Transiently Upregulated in Cisplatin-Treated Neuromasts**

Overall proliferation levels declined for both the control and experimental groups during the first 24-96 hours after cisplatin administration. Cisplatin-treated neuromasts, however, displayed significantly more BrdU+ peripheral and internal supporting cells than did control neuromasts at the 48 and 72 hour assessment points ( $p < 0.05$  or  $0.005$  for peripheral and internal supporting cell counts,  $p < 0.0005$  for total supporting cell counts). This suggests that, compared to control animals, the number of cells entering S-phase is temporarily upregulated during the 48-72 period following cisplatin treatment. When experimental animals were assessed another 24 hours later, proliferation levels had returned to control levels.

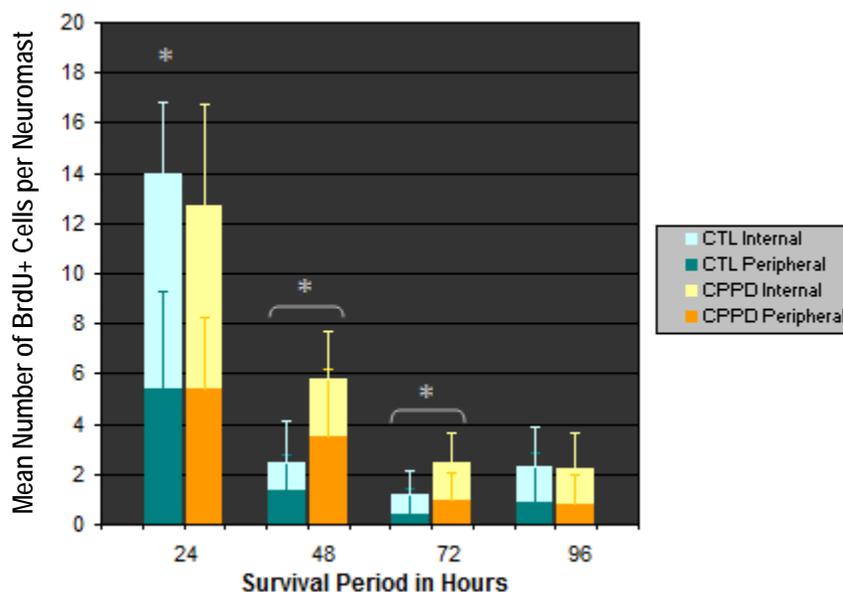
### **C. Both Supporting Cell Subpopulations Contribute Equally to the Proliferative Response**

BrdU+ internal supporting cell counts did not differ significantly from BrdU+ peripheral supporting cell counts at any time point in any group save for one exception. Control neuromasts displayed significantly more BrdU+ internal cells than peripheral cells at the 24 hour assessment point ( $p < 0.05$ ). These findings are in stark contrast to those reported by Ma et al. (2008). Fifteen days after neomycin treatment, when the proliferative response had peaked, BrdU+

internal supporting cell counts outnumbered BrdU+ peripheral supporting cell counts by a factor of approximately 2:1. Interestingly, Williams and Holder (2000) and Harris et al. (2003) noted the reverse trend following neomycin treatment although they did not provide exact counts for the two populations of cells.



**Figure 4. Supporting cell proliferation in the three most posterior neuromasts of zebrafish after treatment and one to four days (1D-4D) of recovery.** In panels A-H, cells entering S-phase are labeled with BrdU and surrounding cell nuclei are counterstained with DAPI. **A-D.** Images depict representative neuromasts from control (CTL) animals following mock treatment, a recovery period, and a 24 hour BrdU pulse immediately prior to fixation. The number of additional BrdU+ cells appearing in neuromasts after the first day of recovery declines rapidly. **E-H.** Images depict representative neuromasts from cisplatin-treated (CDDP) animals following a recovery period and a 24 hour BrdU pulse immediately prior to fixation. The number of additional BrdU+ cells appearing in neuromasts after the first day of recovery declines more gradually in the cisplatin-treated fish compared to controls. Long arrows with pointed tails indicate sample peripheral supporting cells, and short arrows indicate sample internal supporting cells. Scale bars = 10 micrometers ( $\mu\text{m}$ ).

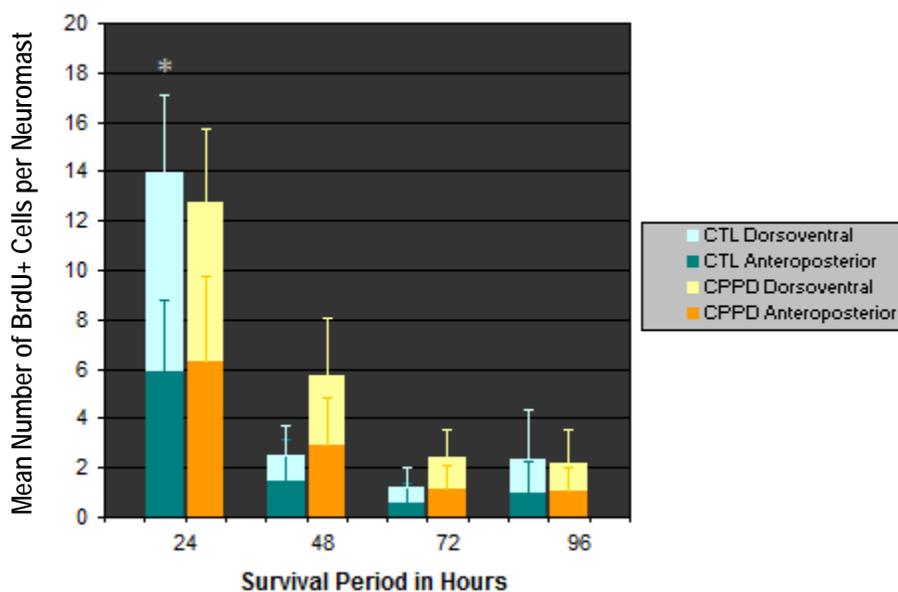


**Figure 5.** Time course graph showing the mean number of internal and peripheral BrdU+ cells at each of the 24-96 hour assessment points for the control (CRL) and cisplatin-treated (CPPD) animals in the BrdU pulse-fix experiment. Compared to controls, a transient but significant increase in the mean number of added BrdU+ internal and peripheral supporting cells is seen between 48 and 72 hours after cisplatin treatment in the experimental group. Mean BrdU+ internal supporting cell counts do not significantly differ from mean BrdU+ peripheral supporting cell counts except for in the controls at the 24 hour assessment point. Error bars represent positive standard deviations (N = 8-19 fish per condition, 3 neuromasts per fish). Asterisks with brackets denote significant differences ( $p < 0.05$  or  $0.005$ ) between the control and experimental groups. Solitary asterisks indicate a significant difference ( $p < 0.05$ ) between the mean internal and peripheral supporting cell counts.

#### **D. Proliferation is Homogenously Distributed within Zebrafish Neuromasts**

Previous research has indicated that developmental growth and supporting cell proliferation could occur along either a dorsoventral or anteroposterior axis (Ledent, 2002; López-Schier and Hudspeth, 2006). For this reason, individual neuromasts were divided into four quadrants so that proliferation patterns could be assessed along the two axes. As displayed in Figure 6, BrdU+

cell counts for the dorsoventral axis were not significantly higher than BrdU+ cell counts for the anteroposterior axis except for in the 24 hour control group ( $p < 0.05$ ).



**Figure 6.** Time course graph showing the mean number of dorsoventral and anteroposterior BrdU+ cells per neuromast at each of the 24-96 hour assessment points for the control (CRL) and cisplatin-treated (CPPD) animals in the BrdU pulse-fix experiment. The mean number of additional cells entering S-phase along the dorsoventral axis is not significantly higher than the mean number of additional cells entering S-phase along the anteroposterior axis except for in the controls at the 24 hour assessment point. Error bars represent positive standard deviations ( $N = 8-19$  fish per condition, 3 neuromasts per fish). The solitary asterisk denotes a significant difference ( $p < 0.05$ ) between the mean dorsoventral and anteroposterior cell counts.

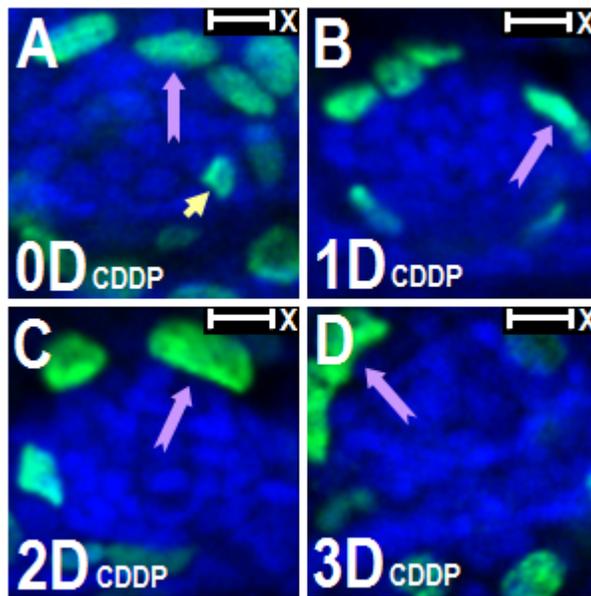
## II. Results for the BrdU Pulse-Chase Experiment

Five dpf zebrafish larvae were treated with  $1000 \mu\text{M}$  cisplatin for four hours and then incubated in  $5 \text{ mM}$  BrdU for the same period of time 12 hours post-treatment. Prior to fixation, fish were permitted to recover for 0, 24, 48, or 72 hours in fresh EM (Figure 7). All BrdU+ progenitor cells and their progeny were then counted in the three most posterior neuromasts of

each zebrafish. Figure 8 displays the mean number of peripheral and internal supporting cells that exhibited BrdU at the four assessment points.

### **A. Fewer BrdU+ Internal Supporting Cells Survive the Cisplatin Exposure than BrdU+ Peripheral Supporting Cells**

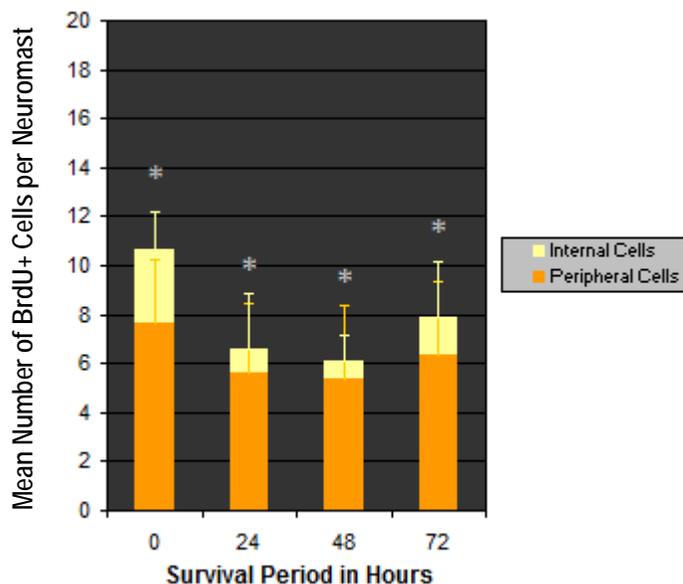
During a BrdU pulse-chase experiment, BrdU+ cell counts are expected to increase gradually over time as BrdU carrying progenitors divide. In the present experiment, the reverse trend was noted with both the BrdU+ internal and BrdU+ peripheral supporting cell counts declining between 0 and 48 hours after cisplatin. Interestingly, the mean BrdU+ counts for the peripheral supporting cells were significantly higher ( $p < 0.5 \times 10^{-8}$ ) than those obtained for the internal supporting cells at every time point. This is the opposite of what was observed for the cisplatin-treated animals in the BrdU pulse-fix experiments.



**Figure 7. Supporting cell proliferation in the three most posterior neuromasts of zebrafish after cisplatin treatment, a four-hour BrdU pulse (12 hours after cisplatin), and zero to three days (0D-3D) of recovery.**

Cells entering S-phase are labeled with BrdU and surrounding cell nuclei are counterstained with DAPI.

**A-D.** Images depict representative neuromasts from cisplatin-treated (CDDP) animals in the BrdU pulse-chase experiment. The number of BrdU+ cells gradually declines with longer survival periods, and BrdU+ internal supporting cells are noticeably absent for survival periods longer than a day. Long arrows with pointed tails indicate sample peripheral supporting cells, and the single short arrow indicates a sample internal supporting cell. Scale bars = 10 micrometers ( $\mu\text{m}$ ).

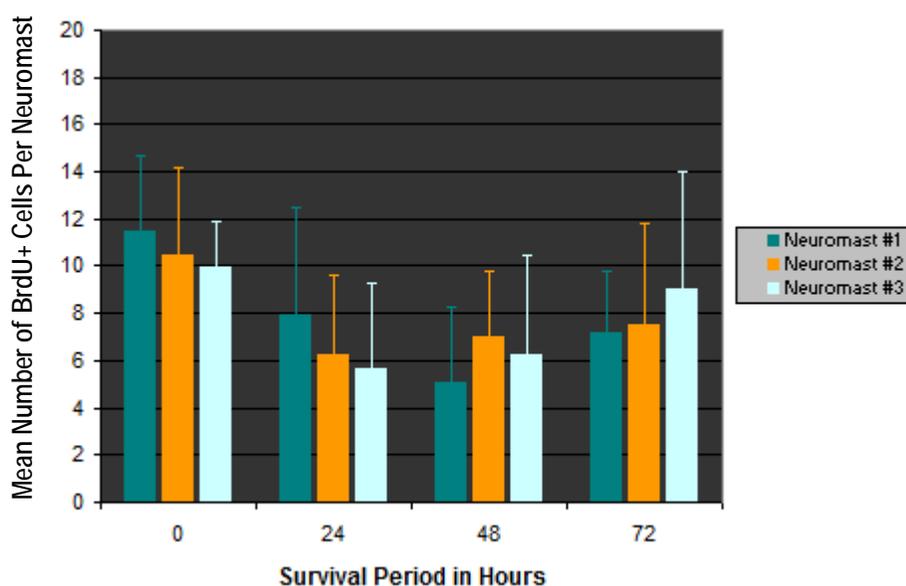


**Figure 8.** Time course graph showing the mean number of internal and peripheral BrdU+ cells per neuromast at each of the 0-24 hour assessment points for the cisplatin-treated animals in the BrdU pulse-chase experiment. Mean BrdU+ peripheral supporting cell counts are significantly higher than mean BrdU+ internal supporting cell counts at all of the assessment points. Error bars represent positive standard deviations (N = 10-11 fish per condition, 3 neuromasts per fish). Solitary asterisks indicate a significant difference ( $p < 0.5 \times 10^{-8}$ ) between the mean internal and peripheral supporting cell counts.

### III. Results for Individual Neuromasts

Previous research has shown that the number of hair cells regenerated following ototoxic injury is not uniform across lateral line neuromasts and that the number of regenerated hair cells is highly dependent upon the original size of the damaged neuromasts (Ma et al. 2008; Genualdi, 2012). According to Ledent (2002), lateral line neuromasts in zebrafish larvae can vary in size depending upon whether they were developed early as part of the embryonic lateral line or later as part of the maturing lateral line. In the present experiment, differing levels of supporting cell proliferation were noted but not found to be significant amongst the three most posterior neuromasts in the control and experimental groups (ANOVAs,  $p > 0.05$ ). Data from the BrdU pulse-

chase experiment are provided for illustrative purposes (Figure 9). Since proliferating supporting cells have been implicated as hair cell and supporting cell progenitors (Williams and Holder, 2000; Harris et al., 2003; Ma et al., 2008; reviewed in Stone and Contanche, 2007; Oesterle and Stone, 2008; Warchol, 2011; and Brignull et al., 2009), it is possible that these subtle differences reflect natural size variations in lateral line neuromasts as well.



**Figure 9.** Time course graph showing the mean number of BrdU+ cells counted in each of the three most posterior neuromasts in the BrdU pulse-chase experiment. One way ANOVAs were performed and did not reveal significant differences amongst the three most posterior neuromasts at any of the assessment points. Error bars represent positive standard deviations (N = 10-11 fish per condition, 3 neuromasts per fish).

## DISCUSSION

### I. Initial Proliferation Levels in Controls May Be Due to Ongoing Development

Compared to control data published in other studies, the level of proliferation noted in our control animals is mostly consistent with previous findings. Harris et al. (2003) and Ma et al. (2008) assessed cell proliferation in the neuromasts of 4-5 dpf zebrafish larvae fixed after a 24 hour survival period, and they rarely found more than two BrdU-labeled cells per neuromast. This number is substantially lower than the average number of BrdU+ cells we observed per neuromast at this time point ( $13.95 \pm 4.64$  cells per neuromast) but comparable with those observed at the 48 to 96 hour time points. It should be noted, however, that both Harris et al. (2003) and Ma et al. (2008) used a higher concentration of BrdU (10 mM) and a shorter pulse (one hour).

Another explanation that may account for this apparent discrepancy in control data is that 5-6 dpf larvae are still undergoing developmental growth during this period. In experiments conducted by Ma et al. (2008), five dpf controls exhibited as much as a 20% increase in FM1-43X-labeled, myosin VI-labeled, and HCS-1-labeled hair cells during the first 48 hours following mock treatment. Moderate levels of *atoh1* and *notch3* transcripts as well as low levels of *deltaA* transcripts were also detected up to this point in time in controls. The authors attributed these findings to possible hair cell addition resulting from ongoing lateral line development.

### II. Increased Proliferation in Cisplatin-Treated Animals is Delayed and Prolonged

The initiation of increased supporting cell proliferation after treatment with 1000  $\mu\text{M}$  cisplatin was delayed compared to that following treatment with 400-500  $\mu\text{M}$  neomycin. Between

12 and 21 hours post-neomycin exposure, a significant increase in BrdU+ supporting cells was observed in experimental neuromasts. This increase was then downregulated to near control levels by 24 hours post-injury (Harris et al., 2003; Ma et al., 2008). In the present study, cisplatin-induced proliferation was not seen until 24 hours after the reported starting point for neomycin. In addition, the time course for cisplatin-induced proliferation was approximately 24 hours longer than that observed for neomycin treatment.

### **III. Proliferation Patterns May Be Due to Extensive Cell Death in Cisplatin-Treated Neuro-masts**

The lack of localized proliferation observed in the BrdU pulse-fix and BrdU pulse-chase experiments (BrdU pulse-chase data were not shown) suggests that hair cell loss occurs with concomitant supporting cell loss. The reason why Ma et al. (2008) recorded higher BrdU+ counts for the internal supporting cell population is likely due to the fact that neomycin specifically targets hair cells and not supporting cells. If cisplatin targets both hair cells and dividing supporting cells, then equivalent proliferation levels would be expected between the internal and peripheral supporting cell populations at some point following treatment. Internal supporting cells would need to divide to replace lost hair cells, and peripheral supporting cells would need to divide to replace lost inner supporting cells.

In the BrdU pulse-fix experiment, the 24 hour control animals were the only ones to exhibit significantly more BrdU+ internal and BrdU+ dorsoventral cells. Why this was the case is not clear. Perhaps this difference reflects a period of active neuromast growth at 5-6 dpf that is not seen over the next 72 hours.

#### **IV. Many BrdU+ Internal Supporting Cells Do Not Survive Cisplatin Exposure**

The purpose of the BrdU pulse-chase experiment was to keep a running tabulation of approximately how many BrdU+ cells were produced after cisplatin treatment in the three most posterior neuromasts. Every time a BrdU+ progenitor cell divides, the BrdU analog persists in its daughter cells, and so BrdU+ cell counts are expected to become increasingly larger with progressively longer periods of survival—particularly if the progeny of those progenitor cells are induced to divide. Interestingly, BrdU+ cell counts did not continuously increase with each added recovery period of 24 hours in the present experiment. In fact, the reverse trend was observed between 0 and 48 hours after BrdU incubation. Since fewer BrdU+ cells were counted than expected with the pulse-chase protocol, this suggests that many of the daughter cells resulting from early mitotic divisions do not survive the cisplatin treatment even with extended recovery periods. This is the opposite of what Ma et al. (2008) observed when treating lateral line neuromasts with neomycin and indicates that cisplatin is toxic to both hair cells and supporting cells while neomycin is not.

Another interesting finding was the change in BrdU+ counts obtained for the internal versus peripheral supporting cell populations seen in Figures 5 and 8. In Figure 5, there were no significant differences between the two cell populations for BrdU incorporation in the experimental animals. In Figure 8, however, the mean BrdU+ counts for the peripheral cells were significantly higher than the mean BrdU+ counts for the internal cells at all of the survival periods. These findings suggest that internal supporting cell progenitors are far more susceptible to the effects of cisplatin than are peripheral supporting cells. Why this may be the case is unclear, but it is possible that peripheral supporting cells are less affected because they only begin dividing

after some unknown threshold of internal supporting cell death. A later time point of division may, therefore, permit peripheral cell nuclei to have less direct exposure to cisplatin.

## **V. Regenerative Processes within the Zebrafish Lateral Line May Be Impaired Due to Limited Progenitor Survival**

As was discussed previously, cisplatin appears to impair the hair cell regenerative processes of both the chick inner ear and the zebrafish lateral line, although the former seems to be far less capable of overcoming the insult than the latter. In the chick inner ear, hair cell regeneration was not seen in cisplatin-treated organotypic cultures for as many as seven days post-exposure (Slattery and Warchol, 2010). Hair cell regeneration in the zebrafish lateral line, however, was identified but found not to be complete by 10 days post-cisplatin treatment (Genualdi, 2012; Mackenzie and Raible, 2012). While the proliferative responses of both species are different, it is possible that both are impaired by the same underlying mechanism – cisplatin’s toxicity to hair cells, supporting cells, and proliferating cells in general.

Results from the present study indicate that a transient increase in supporting cell proliferation is seen between 48 and 72 hours post-treatment with 1000  $\mu\text{M}$  cisplatin, but many of these putative progenitors do not appear to survive long enough to contribute to the regenerative process in a meaningful way. This seems particularly true for BrdU<sup>+</sup> internal supporting cells which have been hypothesized to divide and give rise to new neuromast hair cells (Ma et al., 2008). The reason why progenitor progeny fail to survive after treatment with cisplatin is obscure but presumably there are lingering toxic effects from the cisplatin exposure that affect the internal supporting cell population similar to the hair cell population (Ou et al., 2007). At 96 hours post-treatment, the number of supporting cells entering S-phase in the three most posterior

neuromasts of the zebrafish lateral line returns to control levels and hair cell replacement is still incomplete. Based on these observations, the delayed regenerative response observed in zebrafish larvae may be related to limited internal supporting cell survival. Over time, when lingering toxic effects from cisplatin have waned, the less susceptible peripheral supporting cells may gradually restore the hair cell regenerative mechanism by dividing, migrating, and differentiating into new internal supporting cells inside neuromasts (Harris et al., 2003).

## **FUTURE DIRECTIONS**

### **I. Identifying the Mechanism of Internal Supporting Cell Death**

Findings from the present study have suggested that internal supporting cells are more susceptible to injury by cisplatin than are peripheral supporting cells in the zebrafish lateral line. The method by which cisplatin effectively removes inner supporting cell progeny from zebrafish neuromasts is not known, but the creation of intrastrand cross-links is a likely culprit given the data collected. Since internal supporting cells undergo mitosis at an earlier time point after hair cell death than do peripheral cells, the development of DNA adducts and the inhibition of DNA synthesis would explain why they are comparatively more susceptible. Future research endeavors should explore the manner in which inner supporting cells are targeted by cisplatin (i.e., DNA cross-link formation versus upregulation of ROS) and how long this mechanism remains in effect and delays the regeneration of lateral line hair cells. Recommended analytic tools include adduct-specific monoclonal antibodies (used in mice in Liedert, Pluim, Schellens, and Thomale, 2006 and Dzagnidze et al., 2007) and TUNEL staining (used in zebrafish in López-Schier and

Hudspeth, 2006) as they would permit one to assess the rate of DNA platination and supporting cell apoptosis, respectively.

## **II. Comparing Results with Those Obtained in Mature Animals**

Zebrafish larvae are currently a popular choice for performing hair cell regeneration studies for many of the reasons discussed previously. Larvae, however, are still in the process of maturing. If the goal is to make hair cell regeneration therapy a viable treatment option for both children and adults, then studies should work to identify animal models that are easy to work with and that maintain a regenerative response in their adult form. Until recently, this was difficult in the mature zebrafish due to its opaque constitution. The *casper* line developed by White et al. (2008), however, has offered a unique opportunity. In the *casper* line, black melanophores and reflective iridophores are absent from the epidermal, dermal, and hypodermal layers of the fish. These mutations give the adult zebrafish a transparent appearance that makes it ideal for studies utilizing immunofluorescence. In addition, the *casper* line does not exhibit premature mortality compared to wild-type controls, and it maintains all of the benefits associated with wild-type larvae. Prior to comparing the regenerative response of adults versus larvae, however, future research should address whether or not the *casper* line mutations have any effect on hair cell death and regenerative processes in young larvae.

## CONCLUSION

Hair cell regeneration research in animal models is important as the knowledge gained may contribute to the development of human hair cell replacement therapies as well as highlight some of the limitations associated with these potential interventions. In the present study, five dpf zebrafish larvae were treated with 1000  $\mu\text{M}$  cisplatin for four hours and then a.) allowed to recover for 24-96 hours before receiving a pulse of BrdU or b.) pulsed with BrdU 12 hours after cisplatin treatment and then permitted to recover for 0-72 hours. Results indicate that cisplatin induces both hair cell and supporting cell death as an increased and homogenous spread of proliferation was noted between 24-48 hours after cisplatin in the three most posterior neuro-masts of the experimental group. While supporting cell proliferation was observed to be elevated during this time, long-term survival of internal supporting cell progeny was not. Considering that supporting cells are the progenitors of replacement hair cells, these findings may explain why hair cell regeneration is either absent or delayed post-cisplatin treatment. These results have identified a clear deficit in the hair cell regenerative mechanism.

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## APPENDIX

## Supplemental Tables Containing Descriptive Data for the BrdU Pulse-Fix and Pulse-Chase Experiments

| Table 1. Mean Control Data from the BrdU Pulse-Fix Experiment |             |               |      |       |             |      |       |          |          |          |
|---|-------------|---------------|------|-------|-------------|------|-------|----------|----------|----------|
| Survival Period   | Neuromast N | Peripheral SC |      |       | Internal SC |      |       | Ant/Post | Dor/Vent | Total SC |
|   |             | Singlet       | Pair | Total | Singlet     | Pair | Total |          |          |          |
| 1 Day   | 21          | 5.14          | 0.14 | 5.43  | 7.19        | 0.67 | 8.52  | 5.90     | 8.05     | 13.95    |
| 2 Day   | 44          | 1.25          | 0.05 | 1.34  | 1.05        | 0.05 | 1.14  | 1.48     | 1.00     | 2.48     |
| 3 Day   | 47          | 0.40          | 0.00 | 0.40  | 0.51        | 0.13 | 0.77  | 0.57     | 0.60     | 1.17     |
| 4 Day   | 22          | 0.82          | 0.05 | 0.91  | 0.95        | 0.23 | 1.41  | 1.00     | 1.32     | 2.32     |

| Table 2. Standard Deviations for Mean Control Data from the BrdU Pulse-Fix Experiment |             |               |      |       |             |      |       |          |          |          |
|---|-------------|---------------|------|-------|-------------|------|-------|----------|----------|----------|
| Survival Period   | Neuromast N | Peripheral SC |      |       | Internal SC |      |       | Ant/Post | Dor/Vent | Total SC |
|   |             | Singlet       | Pair | Total | Singlet     | Pair | Total |          |          |          |
| 1 Day   | 21          | 3.29          | 0.48 | 3.85  | 2.80        | 0.80 | 2.91  | 2.91     | 3.11     | 4.64     |
| 2 Day   | 44          | 1.33          | 0.21 | 1.41  | 1.66        | 0.21 | 1.68  | 1.70     | 1.22     | 2.30     |
| 3 Day   | 47          | 1.01          | 0.00 | 1.01  | 0.91        | 0.34 | 1.00  | 0.83     | 0.83     | 1.22     |
| 4 Day   | 22          | 1.59          | 0.21 | 1.93  | 1.59        | 0.43 | 1.53  | 1.23     | 2.01     | 2.83     |

| Table 3. Mean Experimental Data from the BrdU Pulse-Fix Experiment |             |               |      |       |             |      |       |          |          |          |
|--|-------------|---------------|------|-------|-------------|------|-------|----------|----------|----------|
| Survival Period  | Neuromast N | Peripheral SC |      |       | Internal SC |      |       | Ant/Post | Dor/Vent | Total SC |
|  |             | Singlet       | Pair | Total | Singlet     | Pair | Total |          |          |          |
| 1 Day  | 21          | 5.24          | 0.10 | 5.43  | 6.62        | 0.33 | 7.29  | 6.29     | 6.43     | 12.71    |
| 2 Day  | 25          | 3.36          | 0.08 | 3.52  | 1.92        | 0.16 | 2.24  | 2.92     | 2.84     | 5.76     |
| 3 Day  | 33          | 0.97          | 0.00 | 0.97  | 1.39        | 0.03 | 1.45  | 1.09     | 1.33     | 2.42     |
| 4 Day  | 30          | 0.77          | 0.00 | 0.77  | 1.23        | 0.10 | 1.43  | 1.03     | 1.17     | 2.20     |

| Table 4. Standard Deviations for Mean Experimental Data from the BrdU Pulse-Fix Experiment |             |               |      |       |             |      |       |          |          |          |
|--|-------------|---------------|------|-------|-------------|------|-------|----------|----------|----------|
| Survival Period  | Neuromast N | Peripheral SC |      |       | Internal SC |      |       | Ant/Post | Dor/Vent | Total SC |
|  |             | Singlet       | Pair | Total | Singlet     | Pair | Total |          |          |          |
| 1 Day  | 21          | 2.79          | 0.30 | 2.80  | 3.79        | 0.58 | 4.05  | 3.51     | 3.01     | 5.65     |
| 2 Day  | 25          | 2.46          | 0.40 | 2.63  | 1.68        | 0.37 | 1.94  | 1.96     | 2.32     | 3.55     |
| 3 Day  | 33          | 1.13          | 0.00 | 1.13  | 1.06        | 0.17 | 1.20  | 1.01     | 1.14     | 1.56     |
| 4 Day  | 30          | 1.22          | 0.00 | 1.22  | 1.22        | 0.40 | 1.48  | 1.00     | 1.34     | 1.86     |

**Table 5. Mean Experimental Data from the BrdU Pulse-Chase Experiment**

| Survival Period | Neuromast N | Peripheral SC |      |       | Internal SC |      |       | Ant/Post | Dor/Vent | Total SC |
|-----------------|-------------|---------------|------|-------|-------------|------|-------|----------|----------|----------|
|                 |             | Singlet       | Pair | Total | Singlet     | Pair | Total |          |          |          |
| 1 Day           | 33          | 7.55          | 0.06 | 7.67  | 2.42        | 0.27 | 2.97  | 5.30     | 5.33     | 10.64    |
| 2 Day           | 33          | 5.58          | 0.03 | 5.64  | 0.91        | 0.03 | 0.97  | 3.42     | 3.18     | 6.61     |
| 3 Day           | 33          | 5.36          | 0.00 | 5.36  | 0.76        | 0.00 | 0.76  | 3.39     | 2.73     | 6.12     |
| 4 Day           | 30          | 6.17          | 0.10 | 6.37  | 1.53        | 0.00 | 1.53  | 4.27     | 3.63     | 7.90     |

**Table 6. Standard Deviations for Mean Experimental Data from the BrdU Pulse-Chase Experiment**

| Survival Period | Neuromast N | Peripheral SC |      |       | Internal SC |      |       | Ant/Post | Dor/Vent | Total SC |
|-----------------|-------------|---------------|------|-------|-------------|------|-------|----------|----------|----------|
|                 |             | Singlet       | Pair | Total | Singlet     | Pair | Total |          |          |          |
| 1 Day           | 33          | 2.54          | 0.24 | 2.61  | 1.41        | 0.45 | 1.53  | 2.20     | 2.12     | 3.03     |
| 2 Day           | 33          | 2.89          | 0.17 | 2.86  | 2.26        | 0.17 | 2.26  | 2.19     | 2.11     | 3.89     |
| 3 Day           | 33          | 3.03          | 0.00 | 3.03  | 1.00        | 0.00 | 1.00  | 2.11     | 1.99     | 3.43     |
| 4 Day           | 30          | 2.95          | 0.55 | 3.00  | 2.29        | 0.00 | 2.29  | 2.59     | 2.17     | 4.05     |