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Astrogliosis in the Adult Zebrafish Olfactory Bulb during Repetitive Peripheral Damage

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ASTROGLIOSIS IN THE ADULT ZEBRAFISH OLFACTORY BULB DURING REPETITIVE PERIPHERAL DAMAGE

by

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Traumatic brain injuries (TBIs), particularly if repetitive, cause massive disruptions of brain homeostasis, significant loss of neurons, and may result in death. Astrocytes are a type of cell that maintain brain homeostasis and neuronal health. Astrogliosis is a continuum of morphological and functional changes of astrocytes in response to damage. This can be neuroprotective or neurotoxic depending on the severity and type of injury. Global astrocyte scarring is typical of neurotoxic astrogliosis and is the source of secondary injury after TBIs. The adult zebrafish olfactory system is known for its capacity to recover from trauma, however, astrocytes in this system remain unexplored. Furthermore, it is unknown if astrogliosis will occur after repetitive damage to the periphery. This study examines this, and my hypothesis is that astrogliosis will occur. Indeed, it was shown that this does occur, but this process attenuated as the damage continued, and no scarring was evident. This is in contrast to what would be expected of mammalian astrocytes, which would have remained in their astrogliosis morphology and cause scarring. Since astrocytes are crucial mediators for recovery from trauma, further study of these cells in zebrafish may lead to novel medical treatments.

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CHAPTER I

INTRODUCTION

Glia and Astrogliosis

The nervous system consists two general cell types: neurons and glia. Communicating neurons converge to form neuronal circuits with very large amounts of neurons working in concert with each other to dictate the overall function of the organism. Neuronal circuits are incredibly complex and any deviation of the structure of a circuit can lead to physiological changes including sensory hallucinations, cognitive deficits, and death. It is crucial that neurons and neuronal circuits are maintained and supported correctly to ensure proper organismal function, and the cell type that is largely responsible for this is glia.

Glia are specialized to perform various roles. Astrocytes, the most common type of glia, are generally tasked with maintaining brain homeostasis and neuronal health. Astrocytes have been shown to influence synapse formation, function, and structure (Pérez-Alvarez & Araque, 2013; Verkhratsky & Nedergaard, 2014) and have been demonstrated to be crucial to neuronal health (Sofroniew & Vinters, 2010; Khakh & Sofroniew, 2015; Macvicar & Newman, 2015). Importantly, astrocytes sense subtle changes to their environment and adjust it accordingly. In the presence of noxious

stimuli, like brain trauma and damage to neurons, astrocytes undergo a characteristic change in appearance and function termed “astrogliosis”.

Astrogliosis is described as a continuum of morphological and molecular changes in astrocytes when responding to a noxious environment (reviewed in Burda & Sofroniew, 2014; Pekny, Wilhelmsson, & Pekna, 2014; Burda, Bernstein, & Sofroniew, 2016). The extent of astrogliosis depends on the method and severity of injury. At one end of the astrogliosis spectrum, there are subtle changes in astrocyte branching and size, they secrete molecules that encourage synapse reconstruction, and neuronal metabolic support is emphasized. At the other end of the spectrum there is massive proliferation of astrocytes, scar formation, and permanent molecular changes to the local environment causing secondary damage to the tissue.

One important constituent of astrogliosis is the glial scar that is present in moderate to severe astrogliosis (Sofroniew, 2009; Anderson et al., 2013; Burda, Bernstein, & Sofroniew, 2016). The scar is formed by newly proliferated astrocytes and can be identified using antibodies against glial fibrillary protein, a typical marker of astrocytes. If a high amount of GFAP persists in the tissue, it is deemed a glial scar. Currently, glial scars are heavily studied due to its interesting properties. The scar acts as a physical barrier to neuroplasticity, likely has a role in neurotoxicity, yet may also be crucial for axon regrowth (Anderson et al., 2016). Many questions remain that pertain to the glial scar as it is both beneficial and detrimental to overall nervous system health. One question that is particularly interesting is: are there differences in astrogliosis and glial scar formation between species?

Zebrafish as a Model Organism

Humans have evolved to have a low degree of neuroplasticity when compared to lower vertebrates, which hinders the ability to recover from trauma. For example, human spinal cord injuries (SCIs) are commonly considered irreversible (Chen & Levi, 2017; Eckert & Martin, 2017). Zebrafish, however, are able to fully recover from a SCI without medical intervention (Becker et al, 1997; Ghosh & Hui, 2018; Noorimotlagh et al, 2017). This remarkable neuroplasticity is seen in other areas of the zebrafish brain including the telencephalon and olfactory bulb (März et al, 2011; Ganz & Brand, 2016; Trimpe & Byrd-Jacobs, 2016).

Since astrocytes support neurons and influence synapse formation, they are likely crucial mediators of neuroplasticity. Astrocytes are also morphologically different in lower vertebrates when compared to mammals (Kálmán, 1998; Kálmán, 2002; Grupp, Wolburg, & Mack, 2010). Therefore, since astrocytes are major mediators of neuroplasticity, and differences in the astrocyte morphology and neuroplasticity ability exist between mammals and lower vertebrates, zebrafish astrocytes likely possess functional differences in the presence of trauma. Indeed, previous studies of astroglia (a broad categorization under which astro*cytes* reside), in zebrafish have shown a lack of glial scarring after direct injury (März et al., 2011; Baumgart et al., 2012; Skaggs, Goldman, & Parent, 2014).

The adult zebrafish olfactory system is an excellent model to study neuroplasticity due to its particularly highly plastic nature. The system consists of both peripheral and central nervous system structures. The peripheral component of the

zebrafish olfactory system consists of olfactory sensory neurons (OSNs) held in an olfactory organ in the nasal cavity covered by a nasal flap. The olfactory organ is directly exposed to the environment and easily accessible for experimental manipulation. OSNs send processes to the central component, the olfactory bulb (OB), and communicate with central nervous system (CNS) cells in distinct structures called glomeruli. Glomeruli are recognized by an OSN axon bundle terminating in a circular structure. Their number and locations are conserved, enabling constant recognition of a particular glomerulus is possible among several zebrafish (Baier & Korsching, 1994; Braubach, Fine, & Croll, 2012). This is useful, as glomeruli may differ in their normal biochemical function and may have different activity under experimental conditions. Analyzing a commonly found glomerulus throughout all experimental samples eliminates subtle differences amongst separate glomeruli.

The Dr. Byrd-Jacobs lab has previously shown that the zebrafish olfactory organ is capable of dynamic neuroplasticity (Hentig & Byrd-Jacobs, 2016; Iqbal & Byrd-Jacobs, 2010) and CNS cells can detect alterations in the periphery and change accordingly (Fuller, Villanueva, & Byrd, 2005; Fuller, Yettaw, & Byrd, 2006; Trimpe & Byrd-Jacobs, 2016). However, analysis of astroglia and their roles in neuroplasticity in this system remain unexplored. Since OSNs constantly turn over, synapses between OSNs and CNS cells are constantly formed and degraded, a process which is likely mediated by astrocytes. Astrogliosis has the potential to affect this process and, if it does, can lead to the olfactory system circuitry being affected. However, since zebrafish are extremely regenerative by nature, novel molecular processes may be elucidated by studying astrogliosis in the olfactory system.

Repetitive Injury and Downstream Effects

Astrogliosis is a major contributor to traumatic brain injuries (TBIs) by causing secondary injury to nervous tissue (Burda et al., 2016; Karve, Taylor, & Crack, 2016; Stoica & Faden, 2010). Neurons that are under the focal point of insult may lyse, releasing debris into the surrounding tissue. This debris will activate glia and cause astrogliosis. Insults are typically of sufficient severity to cause moderate to severe astrogliosis which itself causes scarring and secondary neuronal. The lysed neurons may have projections to areas of the brain a vast distance away, leading to the possibility of global astrogliosis and global neuronal damage.

Global astrogliosis is commonly seen in repetitive TBIs. Repetitive insults to the brain cause persisting cell death, buildup of neuronal debris, and chronic global astrogliosis with persisting glial scars (Kulbe & Hall, 2017; Luo et al., 2014; Ojo et al., 2016; Sullan, Asken, Jaffee, DeKosky, & Bauer, 2018). Global astrogliosis could be due to either a variation of the location of insults, or debris from axons spanning vast distances of the brain. Further exploration of this is needed to determine the molecular mechanisms of global astrogliosis.

Study Design

The overall goal of this of this project is to study astrogliosis during repetitive periphery damage. The first objective of this thesis is to develop a method of damage to

the peripheral olfactory organ. To do this, a method will be modified from Kishimoto et al., 2013, where a wax plug will be inserted into the nasal cavity of adult zebrafish repeatedly throughout a 7-day period. Analysis of the pattern of olfactory organ degradation and OSN projections to the olfactory bulb will be performed. Because a characteristic of TBIs is mechanically induced neuronal death, and this method is likely to cause OSN death due to the pressure of the wax plug, this model may be useful to study TBIs.

This thesis will use the terms “astroglia” and “astrocytes” throughout. The definitions of these terms for this thesis are as follows: astroglia are a broad categorization of cells that express GFAP, and astrocytes are cells that influence the activity of several structures such as synapses, neurons, and capillaries. To do that, astrocytes must have processes that terminate on a synapse, neuronal cell body, or a capillary. Therefore, astroglia are determined by expressing GFAP, and astrocytes are determined by a GFAP-positive profile that forms a contact with synapses, neuronal cell bodies, or capillaries.

This distinction is important because this study focuses on astrocytes and will be using α-GFAP antibodies to label them, but may observe and notice effects of other astroglia, such as radial glia and olfactory ensheathing cells (OECs). The typical morphology for radial glia consists of a cell body near the glial limitans with a linear process that extends to the opposite side of the CNS structure (in this case the olfactory bulb). OECs are notoriously difficult to identify individually and, therefore, will be identified based on α-GFAP labeling in their typical location, being the olfactory nerve and nerve layer of the olfactory bulb. However, it is unknown if astrocytes in the adult

zebrafish occupy the olfactory nerve and nerve layer of the olfactory bulb. Furthermore, astrocytes have not been sufficiently identified in the olfactory bulb of adult zebrafish. Therefore, the second objective of this study is to find evidence supporting the claim that α-GFAP antibodies label astrocytes in the olfactory bulb of adult zebrafish.

Once astrocytes have been identified, the other questions of this thesis are “will astrogliosis occur in the olfactory bulb during repetitive damage to the periphery organ” and if this occurs “what is the pattern of astrogliosis during repetitive peripheral injury”. My hypothesis is *repetitive damage to the olfactory organ will cause astrogliosis in the olfactory bulb and it will not result in a glial scar*. To investigate this, the peripheral component of the zebrafish olfactory system will be repeatedly damaged and astroglia will be labeled in the olfactory bulb. Analysis of overall α-GFAP labeling will determine if there is a change in astrocyte branching or size, which are indicative of astrogliosis. Astroglial proliferation will also be examined to determine if they proliferate during repetitive peripheral damage, which is typical of scar forming astrocytes in mammals (Wanner et al., 2013).

The main goal of this study is to explore astrogliosis during repetitive injury to a peripheral structure. Since zebrafish are more neuroplastic than humans, the astrogliosis pattern may be different. If it is, key functional differences can be elucidated from them. These key differences may lead to novel medical interventions for patients of TBIs and neurological diseases.

CHAPTER II

REPETITIVE PERIPHERAL DAMAGE OF THE OLFACTORY ORGAN

Introduction

A model of olfactory bulb deafferentation was previously established by Kishimoto et al., 2013. We used this model to cause mechanical damage to the olfactory organ and have previously published data showing this model causes progressive changes in olfactory organ morphology and size, and causes a decrease in OSN projections to the olfactory bulb (Scheib, Pozzuto, & Byrd-Jacobs, 2019). This led me to believe this would be a good model to cause repetitive damage to the periphery for analysis of astrogliosis in the olfactory bulb.

Methods

Animals

Adult male and female zebrafish, *Danio rerio*, over 6 months of age were obtained from local commercial sources. The fish were maintained in 15-gallon aquaria filled with aerated, conditioned water at 28.5ºC and fed commercial flake food (Tetra)

twice daily; each morning and afternoon. All protocols on animal care and experimental procedures were approved by the Institutional Animal Care and Use Committee (project number 16-04-01). An n of 3-5 was used for every time point.

Repetitive Damage to the Olfactory Organ

A method for deafferentation was developed utilizing the insertion of a wax plug into the rosette, similar to the method used by Kishimoto et al. 2013. Zebrafish (*n*=30) were anesthetized with 0.03% MS222 (3-amino benzoic acid ethyl ester, Sigma) until unresponsive to a tail pinch. Fish were placed on a chilled putty dish and covered with a chilled kim wipe to support them and keep them anesthetized during the procedure. A small ball of medical-grade paraffin orthodontic wax mixed with Methylene Blue powder (for visualization) was inserted into the right naris; the left naris remained unplugged for use as an internal control. The plugs were checked every 12h and reinserted if lost.

Tissue Processing

After appropriate survival times, untreated control fish and treated fish (a minimum of 3 fish per group for each survival time) were anesthetized with 0.03% MS222 and perfused transcardially with PBS before immersion in 4% paraformaldehyde for 24 h at 4 °C. Either dissected brains or decalcified whole heads were used for analysis. Both whole head and brain tissues were rinsed, dehydrated in an ascending ethanol and xylene series, and embedded in paraffin for sectioning. 10µm semi-serial horizontal sections were obtained and mounted on positively charged slides.

Morphologies of whole-head mounted sections were observed following hematoxylin and eosin (H&E) staining.

Immunohistochemistry

An antibody to keyhole limpet hemocyanin (KLH) was used to label olfactory sensory neuron axons in the olfactory bulb. Mounted sections were dewaxed, rehydrated, and subjected to antigen retrieval for 10min in a 10mM sodium citrate solution at 100°C. Endogenous peroxidases were inactivated by treatment with a 3% hydrogen peroxide solution. Slides were immersed in a blocking solution of 3% normal goat serum and 0.4% Triton X-100 in PBS for 1h at room temperature. Sections were incubated for 2 h at room temperature with the primary antibody anti-KLH (Sigma; 1:1000 in blocking solution). Slides were rinsed in PBS and incubated in biotinylated secondary antibody (Vector; goat anti-rabbit IgG, 1:100 in blocking solution) for 1 h at room temperature or 24 h at 4 °C. Following rinses in PBS, sections were incubated in avidin-biotin complex (Vectastain Elite ABC Kit, Vector) and visualized using diaminobenzadine (DAB substrate kit, Vector). All sections were dehydrated and coverslipped with DPX mounting medium (Sigma-Aldrich). Slides were viewed on a Nikon Eclipse E600 brightfield microscope using Spot Advance software.

Quantitative Analysis of Antibody Labeling

To estimate the amount of antibody labeling, microscopy images of labeled sections were converted to grayscale. In at least 3 fish per group, two representative

sections, 40 μm apart, were selected from the right and left olfactory bulbs of each animal. Using ImageJ (Schneider et al., 2012), the mean gray level of the olfactory bulbs of each section was measured and converted to optical density (OD) using the formula: OD = log (intensity of background/intensity of area of interest), with the mean gray level of a similarly-sized, non-stained region of the telencephalon for each section used for background intensity. The average OD values were determined for the olfactory bulbs of each fish, and the percent difference between the right and left bulbs was calculated using the formula: percent difference = (average OD right bulb – average OD left bulb)/average OD left bulb x 100%. Data from right and left olfactory bulbs were compared within groups using paired t-tests and between groups using ANOVA with Tukey's test for multiple comparisons. P values less than 0.05 were considered significant.

Quantitative Analysis of Olfactory Organ Size

Slides of H&E-stained whole-head sections were examined to quantify differences in rosette structure. Estimates of the height of the rosette were made by comparing the number of sections in which each rosette appeared on the slide as a means of approximating the organ height. To obtain the height estimate, 40 μm (section thickness of 10 μm plus 30 μm between each section) was multiplied by the number of appearances on the slide for individual rosettes.

To further investigate the change in size of the rosette, volume estimations were made using images of H&E-labeled sections. Using ImageJ (Schneider et al., 2012), the

perimeter of the rosette was traced to obtain an area for every section. Knowing the size between sections, the area was multiplied by 40 μm to estimate the volume for that section. The volume estimates of every section were summed to obtain an overall volume estimation of the whole rosette. Quantifications were made by using percent difference to the internal control epithelium using the formula: percent difference = (damaged epithelium volume – internal control epithelium volume)/ internal control epithelium volume x 100%.

Results

A wax plug was inserted under the nasal flap into the nasal cavity to crush the olfactory organ of adult zebrafish (Fig 1). Because the wax plug tended to fall out as the fish swam, so it was checked every 12 hours and replaced if needed. This was done for 7-days, after which wax plug insertions stopped and the olfactory system was allowed to recover.

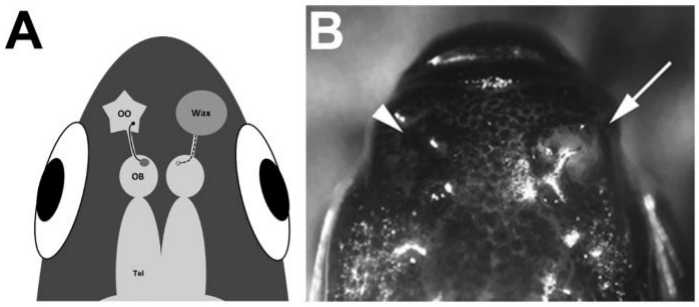

Figure 1. Wax plug insertion**.** Dorsal view of the adult zebrafish head. The wax plug was inserted into the right nasal cavity (arrow) under the nasal flap. The left naris (arrowhead) was unoccluded and served as an internal control.

First, we analyzed the effects of the wax plug insertions on the olfactory organ. Typical olfactory organ morphology consists of a central raphe with several lamellae

projecting toward either side of the nasal cavity consisting of OSN and other supporting cells (Fig 2A). After 1d of wax plug insertions the olfactory organ appeared to swell slightly (Fig 2B). When analyzing sections of tissue from these animals, it was apparent that the affected olfactory organ lost its typical size. Similar observations were seen at 3d of wax plug insertions with an apparent swelling of the olfactory organ yet a loss of olfactory size (Fig 2C). These observations suggest that, due to the olfactory organ's jelly-like consistency, the wax plug put pressure on the organ which caused it to flatten and widen to conserve its volume. After 7d of wax plug insertions the olfactory organ lost its typical morphology with a loss of the central raphe (Fig 2D). Throughout the damaging period, there was also an apparent decrease of olfactory epithelium (Fig 2B-D). One week after the cessation of wax plug insertions the olfactory organ appeared to regain its normal morphology, though some swelling remained (Fig 2E). A similar observation was also made after three weeks of recovery from wax plug insertions (Fig 2F).

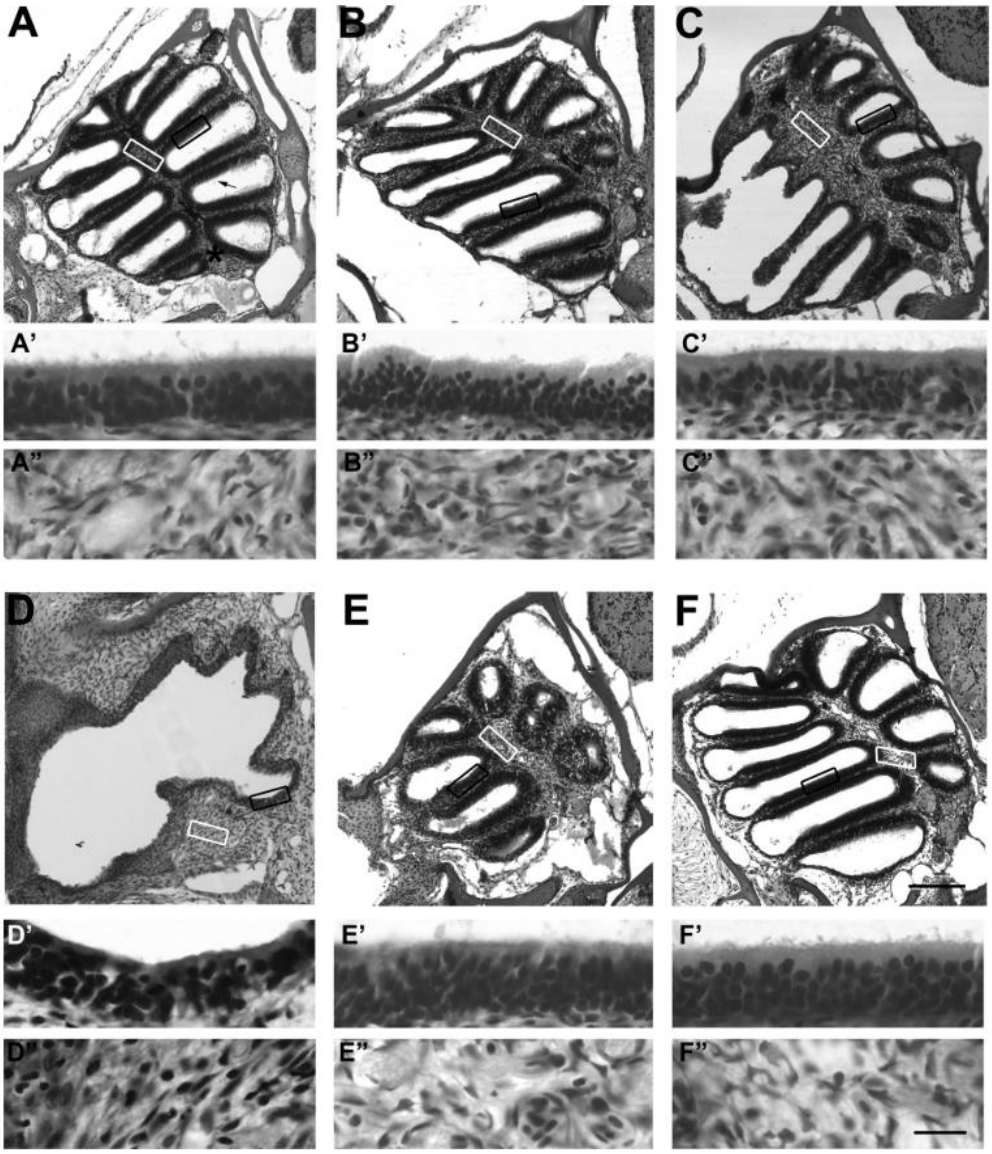

Figure 2. The effects of wax plug insertions on olfactory organ morphology. (A) Morphology of the control rosette showed several lamellae bearing sensory epithelium (arrow) and a central raphe consisting primarily of connective tissue (asterisk) (*n*= 4). (A') Higher magnification morphology of control sensory epithelium from boxed region showed typical pseudostratified columnar epithelium with dense cell nuclei and a rough apical surface indicative of cilia. (A") Lamina propria from the boxed region of the central raphe shown at higher magnification shows the typical connective tissue morphology. (B) With 1 day of wax plug insertion, the lamina propria in the central raphe and lamellae appeared to increase in thickness, but the sensory epithelium at low magnification looked unchanged (*n*=4). (B') The sensory epithelium at higher magnification appeared to lack cilia on the surface and had less cell density. (B") The lamina propria at higher magnification appeared paler and included more round cells than control connective tissue. (C) After 3 days of wax plug insertions the lamina propria appeared to continue expanding, but lamellae remained covered in sensory epithelium (*n*=4). (C') Higher magnification of the sensory epithelium revealed a smooth apical layer and diffuse cell nuclei. (C") The lamina propria in the central raphe had more extracellular matrix between cells than control connective tissue. (D) One week of wax plug insertions caused the rosette to lose its typical rosette morphology and showed no evidence of lamellae (*n*= 3). (D') The cell nuclei of the remaining epithelium seemed disorganized and the apical surface continued to show a smooth morphology. (D") The expansive connective tissue beneath the epithelium lacked the pale round cells noted at the earlier time points. (E) After allowing the rosette to recover for 1 week following wax plug insertions, lamellae had returned although the lamina propria still seemed enlarged (*n*=4). (E') The sensory epithelium seemed to regain its nuclear density and pseudostratified appearance, but the apical surface remained smooth. (E") The connective tissue of the lamina propria remained expansive. (F) Three weeks of recovery after wax plug insertions allowed the rosette to return to typical morphology (*n*=4) (F') The sensory epithelium appeared recovered in nuclear density and ciliated surface. (F") The lamina propria remained paler than controls, but the cell shapes were similar to the control connective tissue. Scale bar = 100µm for A-F and 10µm for A'-F".

To quantify our observations, measurements of the height of the affected olfactory organ were performed and compared to the internal control (Fig 3A). After 7d of wax plug insertions there was a significant decrease in the height of the olfactory organ and it was able to regain its normal size after one week of recovery. To investigate if the volume was affected as well, measurements were taken of olfactory organs and compared as above (Fig 3B). After 7d of wax plug insertions there was a significant decrease in the volume of the affected olfactory organ. However, the organ was able to regain its normal size after one week of recovery. These data show that the wax plug insertions cause dramatic morphological and size changes, yet the organ recovers both of these aspects by one week after the cessation of treatment.

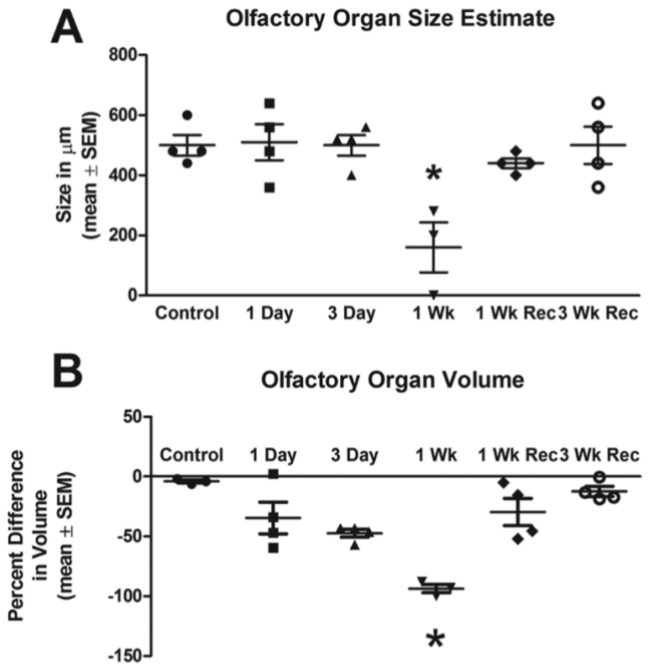

Figure 3. Effects of wax plug insertions on olfactory organ size. Quantitative analysis of effects of wax plug insertions on rosette size and volume. (A) The size of the rosette was significantly reduced after 1 week of wax plug insertions and recovered to control size after 1 week of recovery. (B) Analysis of the percent difference between the treated side and the internal control side revealed that rosette volume loss was significant after 1 week and recovered to control levels by 1 week after cessation of wax plug insertions. *=P<0.05.

Since there was an apparent olfactory epithelium loss, and OSNs are a major constituent of the olfactory epithelium, OSNs may have been lost during wax plug insertions. If they were lost, there would be a decrease of OSN innervation to the olfactory bulb. To see if this was the case, antibodies against KLH, a typical marker of OSN axons in teleost fish, were used to label OSNs in the olfactory bulb during wax plug insertions (Fig 4). In control bulbs, α-KLH labels the nerve layer and glomeruli in the glomerular layer of the olfactory bulb (Fig 4A). After 7d of wax plug insertions there was a noticeable loss of labeling in the affected bulb compared to the internal control (Fig 4B). Three weeks of recovery from wax plug insertions allowed α-KLH labeling to return to typical morphology in the affected bulb (Fig 4C). To quantify this, optical density measurements were taken of each bulb and compared (Fig 4D). There was a significant decrease in the optical density of α-KLH labeling after 7d of wax plug insertions, however the affected olfactory bulb recovered its typical α-KLH labeling after one week of recovery.

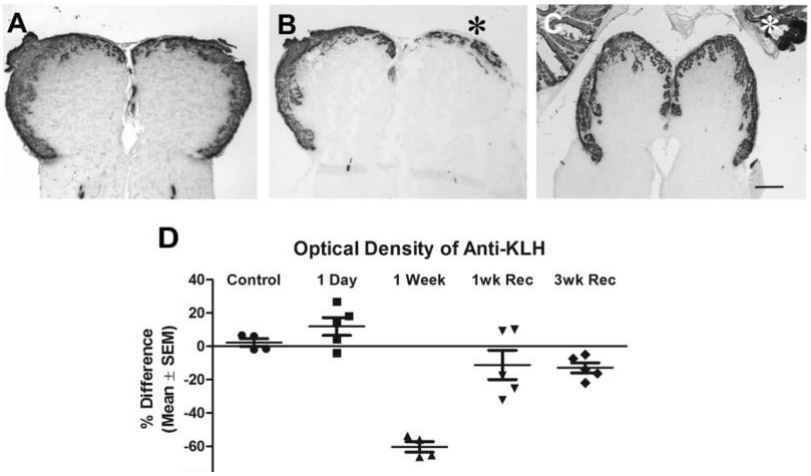

Figure 4. Effects of wax plug insertions on olfactory sensory neuron axons. Anti-KLH immunoreactivity in the olfactory bulb revealed alterations in innervation. (A) Axonal labeling in control olfactory bulbs showed typical patterns with equal innervation on both sides (*n*=4). (B) The affected olfactory bulb (asterisk) had less anti-KLH labeling after 1 week of wax plug insertions, and the label was restricted to the superficial region of the rostral portion of the bulb (*n*=4). (C) Three weeks of recovery allowed the affected bulb (asterisk) to regain similar anti-KLH labeling as the internal control bulb and untreated control bulbs. Scale bar = 100μm for all. (D) Anti-KLH immunoreactivity was significantly reduced in the affected bulb compared to the internal control bulb after 1 week of damage, but staining returned to control levels after 1 week of recovery (*n*=5).

Summary

Inserting wax plugs into the nasal cavity of adult zebrafish every 12 hours for seven days cause dramatic morphological and size differences that were recoverable. The olfactory epithelium appeared to decrease as well, which is indicative of a loss of OSNs. This observation was confirmed by a decrease in the optical density of OSN axon labeling in the olfactory bulb.

These data lead me to conclude that this model destroys the OSNs progressively up to 7d, and their projections are lost from the olfactory bulb. However, the OSNs and their projections can recover, which lead me to believe that this is an excellent model to study astrogliosis with repetitive peripheral damage. Since this model also allows the olfactory system to regain its typical morphology after the cessation of treatments, it would also be interesting to see if a glial scar persists or not.

CHAPTER III

ASTROGLIA AND ASTROGLIOSIS IN THE OLFACTORY BULB OF ADULT ZEBRAFISH

Introduction

Astroglial structures in adult zebrafish have previously been identified but have not been sufficiently examined specifically in the olfactory bulb, and astrocytes in particular have been poorly explored (Grupp et al., 2010). To identify zebrafish astroglia in the olfactory bulb, antibodies against GFAP, a typical marker of astrocytes, will be used. Astrocytes will be identified if a GFAP+ process is observed to contact a synapse or capillary.

After astroglial structures in the olfactory bulb have been identified, zebrafish will be subject to wax plug insertions as described in Chapter 2. To investigate if astrogliosis occurs during repetitive peripheral damage, optical density measurements of olfactory bulbs and glomeruli will be taken in addition to qualitative analysis of astrocyte morphology. Astroglial proliferation will also be examined to determine the extent of astrogliosis and the possibility of a glial scar, since newly proliferated astrocytes typically contribute to the glial scar (Pekny et al., 2014; Burda et al., 2016; Pekny & Pekna, 2016). My hypothesis is that astrogliosis will occur in the presence of repetitive

peripheral damage. This may be supported by an increase in α-GFAP labeling and by changes in astrocyte branching and size. Since zebrafish have previously been shown to lack glial scarring, I also hypothesize that there will be little to no astrocyte proliferation and no evidence of a glial scar, which would be supported if the increase in α-GFAP labeling persists.

Methods

Animals

Adult male and female zebrafish, *Danio rerio*, over 6 months of age were obtained from local commercial sources. The fish were maintained in 15-gallon aquaria filled with aerated, conditioned water at 28.5^{o}C and fed commercial flake food (Tetra) twice daily; each morning and afternoon. All protocols on animal care and experimental procedures were approved by the Institutional Animal Care and Use Committee (project number 16-04-01). An n of 3-5 was used for every time point.

Repetitive Damage to the Olfactory Organ

A method for deafferentation was developed utilizing the insertion of a wax plug into the rosette, similar to the method used by Kishimoto et al. (2013). Zebrafish (*n*=30) were anesthetized with 0.03% MS222 (3-amino benzoic acid ethyl ester, Sigma) until unresponsive to a tail pinch. Fish were placed on a chilled putty dish and covered with a

chilled kim wipe to support them and keep them anesthetized during the procedure. A small ball of medical-grade paraffin orthodontic wax mixed with Methylene Blue powder (for visualization) was inserted into the right naris; the left naris remained unplugged for use as an internal control for comparison. The plugs were checked every 12h and reinserted if lost.

Tissue Processing

After appropriate survival times, untreated control fish and treated fish (a minimum of 3 fish per group for each survival time were used) were over anesthetized with 0.03% MS222 and perfused transcardially with PBS before immersion in 4% paraformaldehyde for 24 h at 4^{o}C. Dissected brains were then rinsed in PBS and mounted in a gelatin and sucrose mixture that was fixed in 4% paraformaldehyde for 24 h at 4°C. The gelatin mixture was then cryoprotected by taking the block through a gradient of sucrose solutions up to 30% sucrose. Brains were then flash frozen in 2-methylbutane and embedded in OCT (Tissue-Tek) and dissected on a cryostat (Leica CM1860) in 30µm sections for whole bulb and glomerular analysis or 50µm sections for proliferation analysis. Sectioned brains were mounted on Colorfrost Plus (Fisherbrand) positively charged slides or gelatin covered neutral slides (CEL Associates, Inc.) and vacuum sealed overnight.

Immunohistochemistry

To investigate changes in astroglial morphology on a gross scale and around glomeruli, an antibody to keyhole limpet hemocyanin (KLH) was used to label olfactory sensory neuron axons and an antibody to glial fibrillary acidic protein (GFAP) was used to label astroglia in the olfactory bulb. Mounted sections were rinsed in PBS and immersed in a blocking solution of 3% normal goat serum and 0.4% Triton X-100 in PBS for 1h at room temperature. Sections were incubated for 2 h at room temperature with the primary antibody anti-GFAP (Dako; 1:1000 in blocking solution). Slides were rinsed in PBS and incubated in Alexa Fluor 488 secondary antibody (Invitrogen; goat anti-rabbit IgG; 1:200 in blocking solution) for 1 h at room temperature or 24 h at 4 °C. Following rinses in PBS, sections were incubated in a 3% normal rabbit serum for 1h at room temperature, rinsed in PBS, then incubated in 30µg/mL Fab fragments (Jackson ImmunoResearch Laboratories Inc.) for 1h at room temperature to block any α-rabbit binding sites. Slides were then rinsed in PBS and incubated with the second primary antibody α-KLH (Sigma; 1:1000 in blocking solution) overnight at 4°C. Sections were then rinsed in PBS and incubated in Alexa Fluor 563 secondary antibody (Invitrogen; goat anti-rabbit IgG; 1:200 in blocking solution) for 1h at room temperature. After this, slides were rinse in PBS and coverslipped using a PVA-DABCO mounting solution.

Antibodies against GFAP and proliferating nuclear cell antigen (PCNA) were used to label proliferating astroglia. Mounted sections were rinsed in PBS and immersed in a 2N HCl for 30 minutes to enhance α-PCNA labeling. Sections were rinsed and incubated in a blocking solution of 3% normal goat serum and 0.4% Triton X-

100 in PBS for 1h at room temperature. Sections were incubated for 2 h at room temperature with both primary antibodies (α-GFAP, Dako, 1:1000 in blocking solution; α-PCNA, Sigma, 1:1000 in blocking solution) for 2h at room temperature. Sections were rinsed in PBS and incubated in both secondary antibodies (Goat α-Rabbit Alexa Fluor 488, Invitrogen, 1:200 in blocking solution; Goat α-Mouse Alexa Fluor 594, Invitrogen, 1:200 in blocking solution) for 1h at room temperature. It was also noticed that Goat α-Mouse Alexa Fluor 594 labeled capillaries, allowing us to view astrocyte processes contacting them. Slides were then rinsed and incubated in Hoescht dye (2'-[4-ethoxyphenyl]-5-[4-methyl-1-piperazinyl]-2,5'-bi-1H-benzimidazole trihydrochloride trihydrate; (1:15000) for 10 min at room temperature before being rinsed and coverslipped with PVA-DABCO mounting solution.

Quantitative Analysis of Astroglial Branching and Hypertrophy

Whole olfactory bulbs and a ventral medial glomerulus that was consistently found in all samples were viewed using a confocal microscope (Nikon Ti Eclipse). Z stacks were taken using Nikon C2 Elements software and the full intensity image was for quantifications. The image was viewed using ImageJ software and the channels split individually into grayscale. To obtain a mean gray value of GFAP labeling of whole bulbs, the bulb was traced on the GFAP channel and measured. To obtain a mean grey value of GFAP labeling associated with the glomerulus, the glomerulus was traced on the KLH channel, the size and position of the trace was maintained when switched to the GFAP channel, and a mean grey value was taken within the trace. These data were

converted to optical density (OD) using the following formula: OD = (intensity of the background/ intensity of area of interest). Then, the ODs were compared using a percent difference formula: % Dif = (OD of experimental - OD of control)/ OD of control. These data were compared within groups using pared t-tests and between groups using ANOVA with Tukey's test for multiple comparisons on GraphPad software. P values less than 0.05 were considered significant.

Quantitative Analysis of Astroglial Proliferation

Images were obtained of tissue labeled with antibodies against GFAP and PCNA and Hoechst dye similar to above. These images were viewed using ImageJ software, the channels split and converted to grayscale. First, the PCNA channel was viewed and any labeled profile was marked using the cell marker tool. Then, the profiles were confirmed as cells if they were positive for Hoechst on the next channel. Finally, these cells were confirmed as glia if GFAP positive on the next channel. Any cell marker that was not positive for all three of these channels was not counted. These data were compared within groups using pared t-tests and between groups using ANOVA with Tukey's test for multiple comparisons on GraphPad software. P values less than 0.05 were considered significant.

Results

Astrocyte Identification

First, I analyzed astroglial morphology in sections labeled with α-KLH to identify glomeruli. If astroglial processes are associated with glomeruli, which are areas with a high density of synapses, it can be inferred that these are astrocyte processes communicating with synapses in that area. Indeed, GFAP+ profiles were seen associated with glomeruli (Fig 5). Every glomerulus observed seemed to have astrocyte processes associated with it. This is evidence supporting that α-GFAP antibodies in fact label astrocytes in the olfactory bulb of adult zebrafish.

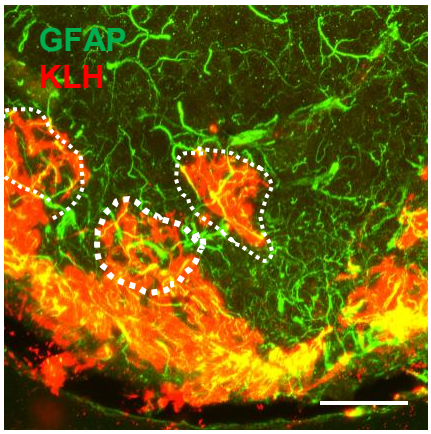

Figure 5. Astrocyte branches in the olfactory bulb in glomeruli. Antibodies to GFAP labeled many processes in the olfactory bulb, though no clear cell bodies were seen. Some α-GFAP-labeled processes entered into glomeruli (circled), identified by α-KLH. Scale bar = 20μm.

To further confirm that astrocytes are found in the olfactory bulb of adult zebrafish and can be labeled using α-GFAP antibodies, analysis was done with sections in which capillaries were labeled. GFAP-positive profiles were seen to terminate on

capillaries, and the termination seemed to enlarge immediately before the capillary which is typical of an astrocyte endfoot (Fig 6).

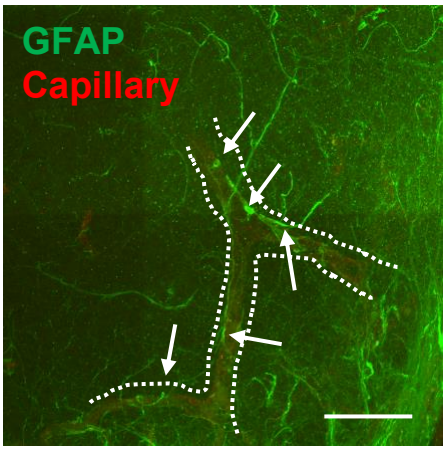

Figure 6. Astrocyte branches in the olfactory bulb contact capillaries. Some anti-GFAP labeled processes terminated on capillaries (outlined) in an endfoot (arrows). Scale bar = 20μm.

α-GFAP antibodies were confirmed to label processes that are categorically astrocyte processes. Interestingly, no clear cell body was found for these astrocyte processes. This is in contrast to typical mammalian astrocytes, which are commonly found to have a distinct cell body with several branches emerging from it.

Astrocyte Branching and Hypertrophy

To investigate if astrogliosis occurs in the presence of repetitive peripheral damage, zebrafish were subject to wax plug insertions every 12h for 7d and their olfactory bulbs labeled with α-GFAP and α-KLH antibodies. Gross analysis of whole bulbs showed an apparent increase in overall α-GFAP labeling in both bulbs at 4h, 12h, and 1d of wax plug insertions, with the affected bulb appearing more labeled than the

internal control bulb (Fig 7). This apparent increase in α-GFAP labeling was not seen at 3d and 7d of treatment. Optical density measurements of α-GFAP labeling in the entire bulb confirmed my observation that the affected bulb had a higher amount of α-GFAP labeling at 1d, and this effect was not present at the later time points.

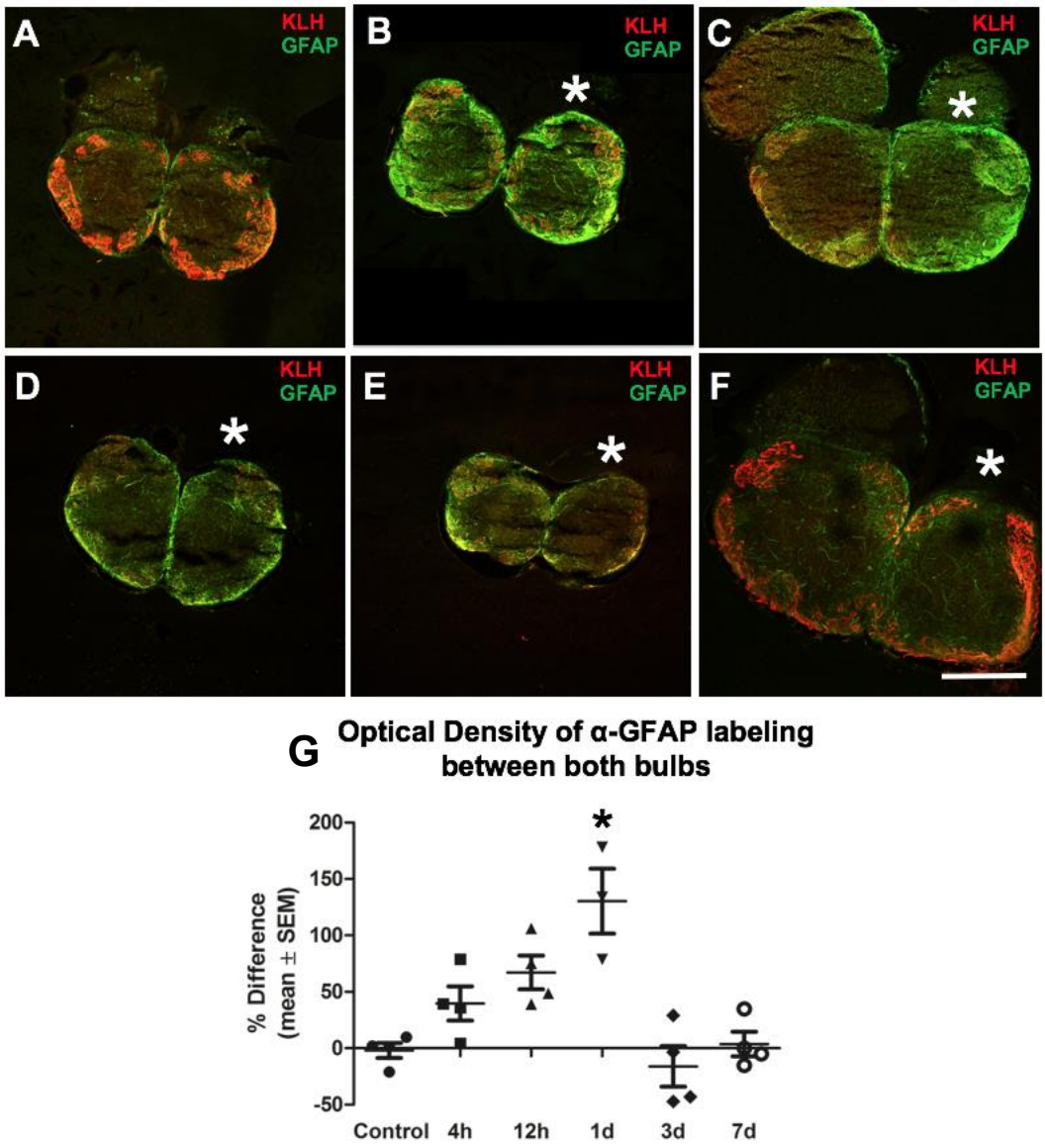

Figure 7. Anti-GFAP labeling of the whole bulb during repetitive peripheral damage. (A) Untreated control bulbs show sparse GFAP labeling (green) and KLH labeling (red) of olfactory sensory neuron axons in the nerve layer and glomerular layer. (B) 4h after wax plug insertions there was an apparent increase of GFAP labeling in the affected bulb (asterisk). This affect seemed to remain at 12h (C) and 1d (D) after wax plug treatments as well. (E) After 3d of wax plug treatments GFAP labeling in the affected bulb appeared to return to control morphology. (F) GFAP labeling in the affected bulb (asterisk) was similar to control levels after 7d. Scale bar = 100μm for all. (G) There was significant increase in the affected bulb of the optical density of GFAP labeling at 1d of repetitive peripheral damage when compared to the internal control. However, this effect was not apparent as wax plug insertions persisted at 3d and 7d.

Since astrocytes communicate with synapses, and glomeruli are areas of a high density of synapses, astrocytes likely detect neuronal damage by their synaptic processes. Therefore, higher magnification analysis of a commonly found ventral medial glomerulus was performed to further explore astrogliosis. Qualitative analysis revealed an apparent increase in the amount and size of branches associated with the glomerulus at 4h, 12h, and 1d (Fig 8). This effect was not apparent at the later time points. To quantify this, optical density measurements of the α-GFAP labeling within the glomerulus were performed. There was a significant difference in the optical density of α-GFAP labeling at 4h, 12h, and 1d of wax plug insertions in the affected bulb when compared to the internal control. However, this effect was not apparent at 3d and 7d of treatment.

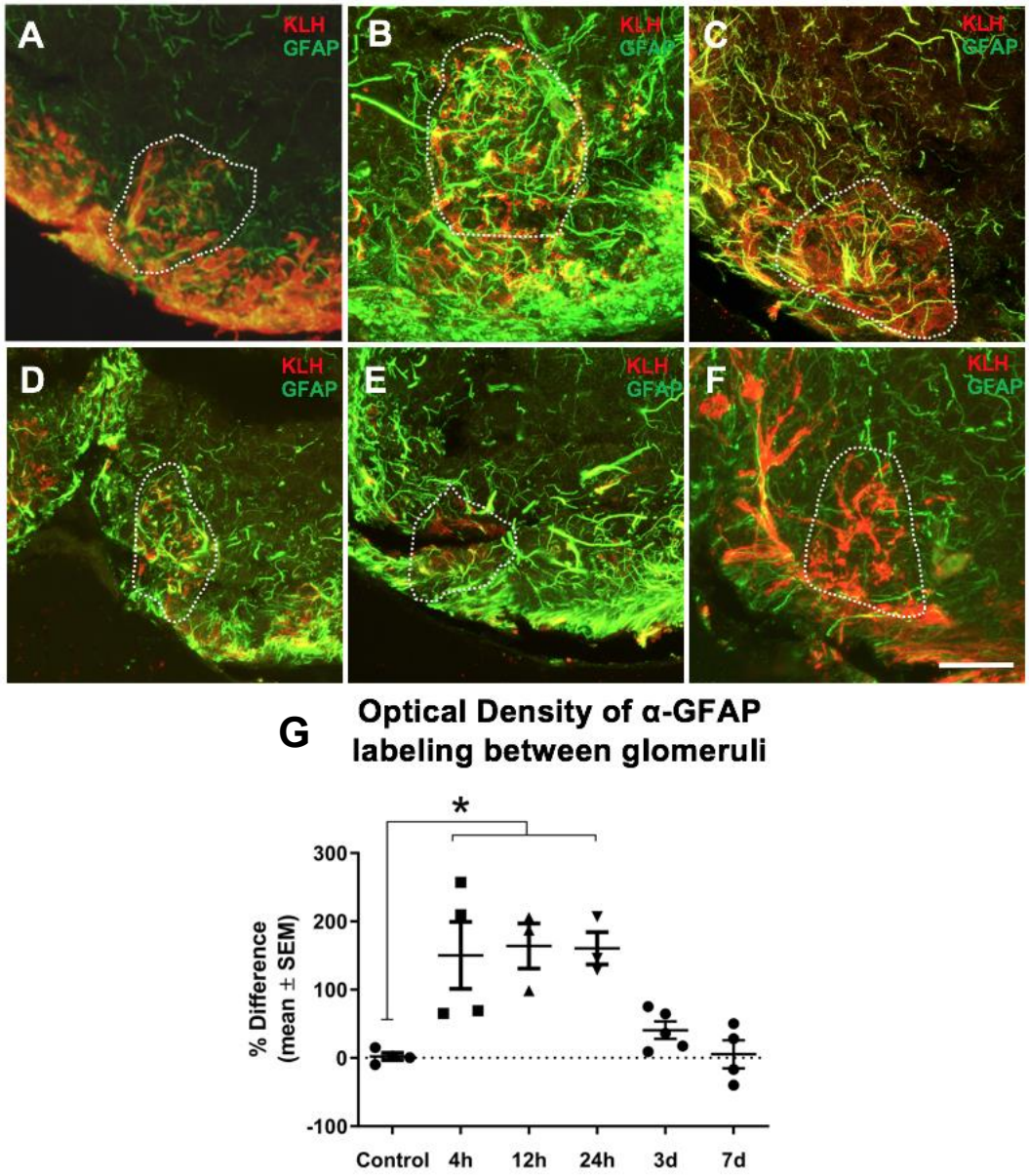

Figure 8. Anti-GFAP labeling of glomeruli during repetitive peripheral damage. (A) Untreated control glomeruli (circled) have a roughly circular appearance with several KLH labeled axons (red) and thin GFAP labeled glial processes (green). (B) After 4h of wax plug insertions affected glomeruli (circled) appeared to have many GFAP labeled processes associated with it that seemed larger than in controls. This affect continued at 12h (C) and 1d (D) of wax plug insertions. (E) After 3d of wax plug insertions, although the surrounding GFAP labeled processes seemed enlarged, processes that were associated with glomeruli (circled) seemed to be reduced when compared to controls. (F) After 7d of wax plug insertions GFAP labeled processes within glomeruli (circled) appeared to be similar to their control morphology. Scale bar = 20μm for all. (G) The optical density of GFAP labeling was significantly higher in the affected glomeruli when compared to the internal control at 4h, 12h, and 24h of wax plug insertions, though this effect was not seen at 3d and 7d.

Astroglial Proliferation

Moderate to severe astrogliosis consists of newly proliferated astrocytes, which typically for a scar in mammals. To investigate if repetitive peripheral damage causes astrocytes to proliferate, olfactory bulbs from zebrafish that were exposed to wax plug insertions were labeled with α-GFAP and α-PCNA antibodies (Fig 9). Since the secondary antibody used for the α-PCNA primary antibody also cross reacted with capillaries, cells were confirmed using a Hoechst dye. Triple labeled cells appeared to be circular, which are indicative of astrocytes withdrawing their processes to divide, or, could possibly be OECs.

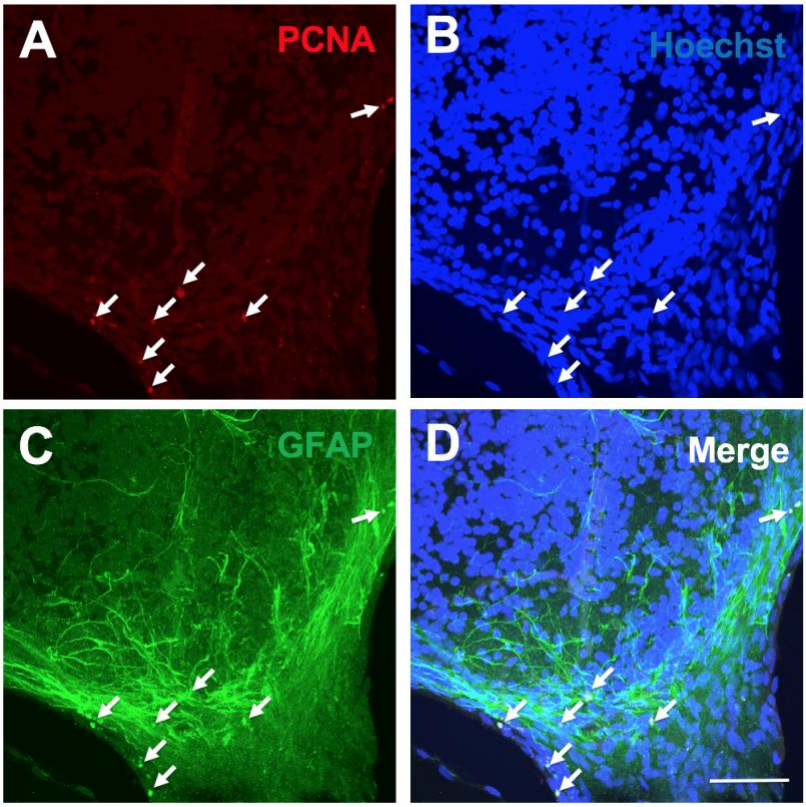

Figure 9. Examples of proliferating profiles. (A) Several PCNA+ profiles were seen in the olfactory bulb (arrows). (B) These profiles (arrows) were confirmed as cells if labeled with Hoechst dye. (C) These cells (arrows) were then confirmed as astroglia if labeled with antibodies against GFAP. (D) Merged image showing all three labels. Proliferating astroglia (arrows) appear as triple-labeled profiles and appear white. Scale bar = 20μm for all.

Cells that were positive for all three labels were counted in both bulbs. A significant increase in these cells was seen in the affected bulb at 1d when compared to control bulbs (Fig 10A). Proliferating profiles were found in the glomerular layer and nerve layer of the olfactory bulb. Since OECs are typically found in the nerve layer, proliferating profiles in the glomerular layer may be due to astrocytes. To further investigate this, the number proliferating profiles specifically in the glomerular layer were compared, and there was a significant increase in the number of these profiles in the glomerular layer of the affected bulb at 1d and 3d (Fig 10C). Since OECs are not typically found in the glomerular and internal cell layer of the olfactory bulb, this observation may be due to other proliferating astroglia, such as astrocytes.

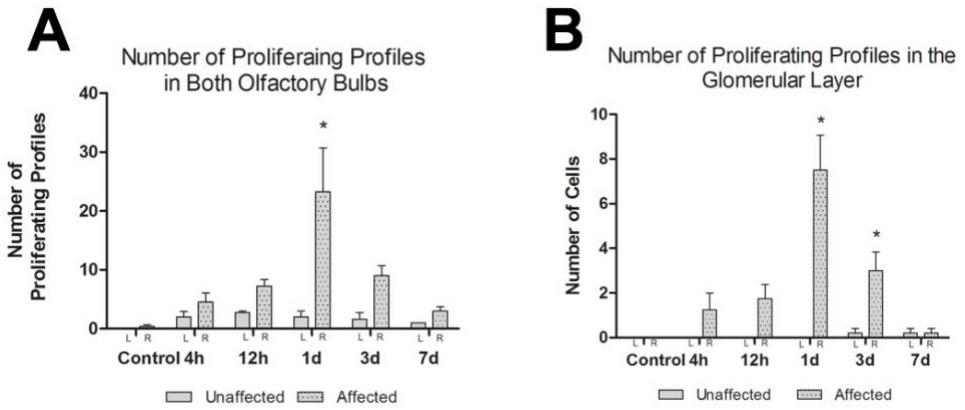

Figure 10. Quantifications of Proliferating Astroglia During Repetitive Peripheral Damage. (A) There was a significant increase of the overall amount of proliferating profiles after 1d of wax plug insertions. (B) In the unaffected bulb, proliferation was seen only in the nerve layer until 3d. The affected bulb had proliferating profiles in both layers during every experimental timepoint. There seemed to be a large amount of proliferating profiles in the glomerular layer when compared to the nerve layer at 1d and 3d. (C) There was a significant increase of the number of proliferating profiles in the glomerular layer of the affected bulb after 24 hours of wax plug insertions.

Glial Scar

Optical density measurements were made similar as above to determine if a residual amount of α-GFAP labeling remained in the affected olfactory after the

cessation of repetitive peripheral damage (Fig 11). The percent difference of optical density in α-GFAP labeling was measured in whole bulbs and glomeruli of fish that were allowed 1 week to recover after 7 days of wax plug treatments. There was no significant difference in the percent difference in optical density of α-GFAP labeling between these bulbs and control bulbs. Since the glial scar is defined by a persisting large amount of α-GFAP labeling, no evidence of a glial scar was found.

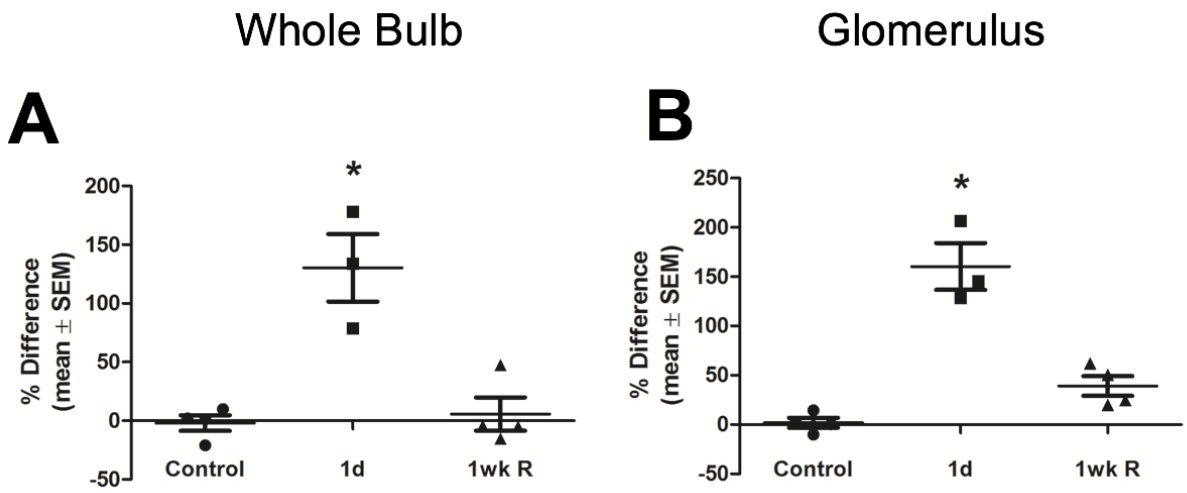

Figure 11. Percent difference in the optical density of anti-GFAP labeling in the whole olfactory bulb and in a glomerulus. (A) There was no significant difference between control bulbs and bulbs that were allowed to recovery from 7d of wax plug insertions for 1 week. (B) there was no significant difference between control glomeruli and glomeruli that were allowed to recovery from 7d of wax plug insertions for 1 week.

Summary

Astrocytes were observed in the olfactory bulb; however, no clear cell body was found and therefore the full morphology of astrocytes in the zebrafish olfactory bulb was

not found. However, astrocyte processes were seen in glomeruli and observed to contact capillaries.

Astrogliosis did occur in the presence of repetitive peripheral damage. There was an apparent increase in number and size of astrocyte branches that were associated with glomeruli. Anti-GFAP labeling significantly increased in affected bulbs and glomeruli when compared to internal controls. Interestingly, this effect was seen only at earlier time points. Since there was no residual increase in α-GFAP labeling in the affected bulb, a glial scar was not formed. However, since after 24 hours of treatments there was an increase of proliferating profiles found glomerular layer of the olfactory bulb, possibly due to astrocyte proliferation. Evidence of astrocyte proliferation and lack of glial scarring is in contrast to astrocyte activity in mammals, where newly proliferated astrocytes build the glial scar.

CHAPTER IV

DISCUSSION

Wax Plug Insertions as a Model for the Effects of Peripheral Damage on the CNS

Repeated wax plug insertions into the nasal cavity of adult zebrafish caused dramatic changes in both size and morphology of the olfactory organ. The organ appeared to flatten and widen during the duration of wax plug insertions and was morphologically unrecognizable after 7 days of insertions. The pressure of the plug in the nasal cavity most likely crushed the organ throughout the damaging period.

The loss of organ volume indicates a loss in cell bodies. Since OSN cell bodies constitute a large portion of the olfactory epithelium, and the epithelium appeared to decrease in volume in proportion to overall organ volume, it's likely that OSNs died during wax plug insertions. If OSN cell bodies were lost during wax plug insertions, their axons would have been degraded and not present in the olfactory bulb. Indeed, it was shown through α-KLH labeling that OSN axons were significantly reduced in the affected bulb after 7 days of wax plug insertions.

These data support the claim that wax plug insertions cause mechanical stress on OSNs which results in their degradation and death. This is similar to TBIs, where mechanical or kinetic stress causes neurons to die. Necrosis is largely the method of

cell death during the initial insult in TBI, after which apoptosis becomes the secondary mechanism of cell death (Stoica & Faden, 2010; Yakovlev & Faden, 2001). Necrosis releases neuronal debris which then can activate astrocytes in TBIs (Burda et al., 2016). Wax plug insertions likely cause necrosis of OSNs due to the pressure of the plug. When OSNs die, their axons in the olfactory bulb degrade, and the debris likely activate glia in the olfactory bulb.

This wax plug model is also useful to study the global effects of focal damage, since the damage is localized in peripheral structures. Many previous studies of TBIs have focused on the area of insult to the brain. However, global astrogliosis is seen in patients who have suffered repetitive TBIs (Fehily & Fitzgerald, 2017; Kulbe & Hall, 2017). Clearly, astrocytes in brain areas other than the area of insult become activated, but it is unclear by what mechanism. The model described have allowed examination of glial activation that is not due to direct mechanical stress, since the insult is localized to the peripheral structures.

Interestingly, the cessation of wax plug insertions allowed the olfactory organ and OSN axons to recover their typical morphology. This allows study of tissue remodeling of the periphery and reinnervation from virtually complete deafferentation. Further study using this method may elucidate unique molecular mechanisms of zebrafish neuroplasticity.

Astrocytes in the Olfactory Bulb of Adult Zebrafish

Antibodies against GFAP labeled processes throughout the olfactory bulb. The olfactory nerve had extensive labeling with little to no clear cell bodies or cellular branches apparent. This is most likely due to OECs in the nerve layer. The glomerular layer had extensive branches that were also seen within glomeruli. Astrocytes in mammalian olfactory bulbs that associate with glomeruli envelop them and separate synapses (Valverde and Lopez-Mascaraque, 1991; Bailey and Shipley, 1993; Chao et al., 1997). Therefore, our observations show similarities between zebrafish and mammalian olfactory astrocytes.

Glial processes were also seen to contact capillaries with enlarged terminations. This is typical of mammalian astrocytes that communicate with capillaries to form the blood brain barrier and maintain homeostasis within the brain. Therefore, our observation of glial processes contacting capillaries in zebrafish is consistent with mammalian astrocytes

Long branches were seen in the internal cell layer of the olfactory bulb. Some of these branches contacted capillaries and others had no clear termination. GFAP-positive profiles that did not have a clear termination could be due to a process entering and exiting the tissue section to terminate elsewhere. Therefore, since this is likely a long process, and the origin and termination are unknown, it could either be the branch of a radial glia or astrocyte.

Interestingly, no clear astrocyte cell bodies were seen, only branches that by definition had to come from an astrocyte. It is possible that GFAP is not present in the

cell body of zebrafish astrocytes. If this is the case, further exploration with other astrocyte markers is needed to fully elucidate normal astrocyte morphology in zebrafish.

Astrogliosis in the Presence of Repetitive Peripheral Damage

An increase in the optical density of α-GFAP labeling in affected olfactory bulbs and glomeruli was observed. Qualitative analysis showed an apparent increase in the size and number of branches in and around glomeruli. Increases of the amount of GFAP is likely due to the apparent increase of number and size of branches and is typical of astrogliosis. Therefore, astrogliosis was seen in the presence of repetitive peripheral damage via wax plug insertions into the nasal cavity. As stated above, the wax plug likely caused necrosis of OSNs, and since OSNs send axons to glomeruli where astrocyte processes are seen, the debris likely existed in the olfactory nerve and glomerular layer of the olfactory bulb to activate astrocytes.

One other possibility for astrocyte activation is the presence of activated microglia (Karve et al., 2016; Liddelow et al., 2017). Microglia become activated in the presence of cellular debris as well. Activated microglia are known to activate astrocytes (Liddelow et al., 2017). Microglia-activated astrocytes, in mammals, have been shown to become neurotoxic. Microglia-activated astrocytes have yet to be explored in zebrafish, but due to their neuroplastic nature, they may not be neurotoxic.

While the mechanism of astrocyte activation after repetitive peripheral damage is unclear, this study showed that astrogliosis was apparent at early time points then was attenuated by the third day of damage. This is not typical of mammalian astrocytes,

which would have retained their astrogliosis morphology and formed astrocyte scars (Kulbe & Hall, 2017; Luo et al., 2014; Sullan et al., 2018). Insults to the zebrafish nervous system have been reported previously as insufficient to cause a glial scar formation (Kroehne et al., 2011; Baumgart et al., 2012; Noorimotlagh et al., 2017). However, this is the first study on repetitive mechanical insults to the zebrafish nervous system. Therefore, while zebrafish astrogliosis attenuation is a novel finding, and similar mechanisms may exist for the lack of glial scarring and astrogliosis attenuation.

There was no noticeable difference in the amount of α-GFAP labeling at 1 week of recovery from wax plug insertions. Therefore, this study showed that repetitive peripheral damage is insufficient to cause a glial scar. Interestingly, astroglial proliferation was observed. Proliferating cells that were positive for α-GFAP were observed in the nerve layer and glomerular layer of the olfactory bulb. These cells also appeared to be round, which is indicative of both proliferating astrocytes and OECs. Since zebrafish olfactory astrocyte and OEC literature is severely lacking, it is unknown if OECs are seen only in the nerve layer or if they are also seen in the glomerular layer. Furthermore, it is unknown if astrocytes can migrate to the nerve layer and divide there. Astrocytes may need to migrate to the nerve layer to support growing axons, though OECs could also be performing this task (Khankan, Wanner, & Phelps, 2015; Roet & Verhaagen, 2014). Clearly, further exploration is needed of astroglia in the olfactory system of adult zebrafish with astrocyte-specific markers that do not label OECs.

This study suggests the wax plug insertion model as a method to study the downstream effects of mechanical neuronal stress, identified astrocytes in the olfactory bulb of adult zebrafish, and showed that they respond to repetitive peripheral damage

with proliferation and an apparent increase in cell branching and size but attenuate their response. Further exploration of astrocytes in zebrafish, in particular this apparent astrogliosis attenuation, has the potential to elucidate key differences in astrocyte function. These key differences have the potential to be exploited for medical intervention in brain trauma and disease in humans.

Future Directions

Clearly, further exploration is needed to find the molecular mechanisms of astrocyte activation, astroglial attenuation, and lack of scarring in this model. To determine the method of astrocyte activation, the olfactory organ may need to be labeled with an apoptosis marker such as α-caspase antibodies and a nuclear marker such as Hoechst dye. Since necrotic cells have a swollen nucleus, Hoechst dye can be used to identify cells undergoing necrosis. Identifying the pattern of cell death will be useful, perhaps necrosis and therefore cellular debris is only apparent at earlier time points. Since astroglia are activated by cellular debris, the reduction in astroglial activation may be explained. However, microglia can also activate astrocytes. A transgenic mouse with Csfr1 knocked out lacks microglia (Anderson et al., 2016). Perhaps a transgenic zebrafish knockout is possible; if not, microglia can be ablated using other methods (Van Rooijen, 1989; Ponzoni et al., 2018). These techniques would allow us to explore astroglia activity in the absence of microglia and may show that astrocytes need microglia to perform astrogliosis properly. Furthermore, if astrogliosis does occur, the process of attenuation may be affected.

The novel finding of astrogliosis attenuation needs to be fully explored. Other methods of repetitive injury to the olfactory organ, such as repeated chemical ablation with detergent (Paskin, Iqbal, & Byrd-Jacobs, 2011) should be used to add rigor to this observation. If this observation is indeed consistent, the molecular mechanisms of this needs to be elucidated. Understanding the full glial involvement in this process might help explain this. Microglia and oligodendrocytes are known to communicate with astrocytes during gliosis (Burda & Sofroniew, 2014; Pekny & Pekna, 2016). It is likely that they are key players in astrogliosis attenuation.

Astroglial proliferation during repetitive peripheral damage needs to be addressed further. Newly proliferated astrocytes are known to contribute to the glial scar and are likely neurotoxic in mammals. Our observation of astroglial proliferation, yet a lack of scarring, is in contrast to literature of mammalian astrocytes. Using astrocyte-specific markers, antibodies that recognize proteins that provide the astrocyte's functional definition, we can eliminate any doubt of false positives such as OEC proliferation. However, since zebrafish astrocytes have may have functional differences and OECs are relatively unexplored, it is unclear if these markers will be useful in this system.

Summary

This thesis has developed a method of repetitive injury to the peripheral organ, identified astroglial structures in the olfactory bulb of adult zebrafish, and examined astrogliosis during repetitive peripheral injury. I found that astrogliosis appears to

attenuate during repetitive injury to the periphery, which is a novel finding. Mammalian astrocytes would have likely retained their astrogliosis morphology and contributed to a glial scar, causing secondary damage to the brain. This suggests that astrocyte function between the two species may be a key difference in neuroplasticity abilities, and therefore the ability to recover from trauma. Fully exploring astrocytes in the adult zebrafish and their process of astrogliosis may elucidate key physiological differences. Once these differences have been identified they may be used to develop novel medical interventions for patients of TBIs and neurological diseases.

Appendix A

Institutional Animal Care and Use Committee

WESTERN MICHIGAN UNIVERSITY

Institutional Animal Care and Use Committee

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Western Michigan University

ANNUAL REVIEW OF VERTEBRATE ANIMAL USE

PROJECT OR COURSE TITLE: Analysis Of Olfactory-Mediated Behavior In Zebrafish
IACUC Protocol Number: 16-04-02
Date of Review Request: 05/03/18 Date of Last Approval: 4/12/17
Purpose of project (select one): ☐Teaching ☒Research ☐Other (specify):

PRINCIPAL INVESTIGATOR OR ADVISOR
Name: Christine Byrd-Jacobs Title: Acting Dean, Graduate College
Department: BIOS Electronic Mail Address: christine.byrd@wmich.edu
CO-PRINCIPAL OR STUDENT INVESTIGATOR
Name: Jackson Scheib Title: Undergraduate student
Department: BIOS Electronic Mail Address: jackson.j.scheib@wmich.edu

1. The research, as approved by the IACUC, is completed:
☐Yes (Continue with items 4-5 below.) ☒No (Continue with items 2-5 below.)

If the answer to any of the following questions (items 2-4) is "Yes," please provide a detailed explanation on an attached sheet of paper. Include details of any modifications made to the protocol based on new findings or publications, adverse events or mortalities.

2. Have there been any changes in Principal or Co-Principal Investigators? ☐Yes ☒No
3. Have there been any new findings or publications relative to this research that require you to alter your study? ☐Yes ☒No
   Describe the sources used to determine the availability of new findings or publications:
   ☐No search conducted (Please provide a justification on an attached sheet.)
   ☐Animal Welfare Information Center (AWIC)
   ☐Search of literature databases (select all applicable)
   ☐AGRICOLA ☐Current Research Information Service (CRIS)
   ☐Biological Abstracts ☒Medline
   ☐Other (please specify):
   Date of search: 04/08/18 Years covered by the search: no limits
   Key words: zebrafish, olfactory behavior, odorant-mediated behavior and combinations of these terms
   ☐Additional search strategy narrative:
4. Are there any adverse events, in terms of animal well-being, or mortalities to report as a result of this research? ☐Yes ☒No
   Cumulative number of mortalities: 0
5. Animal usage: Number of animals used during this quarter (3 months): 0 Cumulative number of animals used to date: 0

/ _____ 4/10/18
Principal Investigator/Faculty Advisor Signature Date

/ _____ 4/10/18
Co-Principal or Student Investigator Signature Date

IACUC REVIEW AND APPROVAL
Upon review of the relevant information regarding this protocol, the IACUC approval for this project has been extended for one year from the date of this signature.

/ Kathryn Echelmeyer, DVM 4-11-18
IACUC CHAIR SIGNATURE DATE

Revised 01/2012 WMU IACUC All other copies obsolete.

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