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A Quantitative Analysis of Morphological Changes in Specific Laminae of the Olfactory Bulb of Adult Zebrafish Following Peripheral Sensory Deafferentation

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A QUANTITATIVE ANALYSIS OF MORPHOLOGICAL CHANGES IN SPECIFIC LAMINAE OF THE OLFACTORY BULB OF ADULT ZEBRAFISH FOLLOWING PERIPHERAL SENSORY DEAFFERENTATION

by

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Permanent removal of the olfactory organ in adult zebrafish has been shown to result in a significant reduction in the total volume of the ipsilateral olfactory bulb. The overall objective of the current project was to investigate the hypothesis that contact between the axons of the olfactory sensory neurons and the olfactory bulb is necessary for the maintenance of the normal post-synaptic organization of the olfactory bulb by quantifying the morphological changes that take place within specific laminae of the olfactory bulb following peripheral deafferentation. Complete, unilateral ablation of the olfactory organ resulted in permanent deafferentation of the ipsilateral olfactory bulb and caused a significant reduction in total bulb volume that corresponded to a reduction in the laminar volume of the olfactory nerve layer, the glomerular layer, and the internal cell layer of the deafferented olfactory bulb. Although the deafferentation procedure did not appear to affect the size distribution of nuclear profiles within the deafferented olfactory bulb, changes in both cell density and cell number were observed within the glomerular layer and internal cell layer following peripheral deafferentation. These results support the conclusion that contact with olfactory sensory neurons is required to maintain the normal morphology of the ipsilateral olfactory bulb and that several different populations of neurons throughout the olfactory bulb are likely to be affected by the loss of afferent input.

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CHAPTER I

INTRODUCTION

The Vertebrate Olfactory System

Overview of the Vertebrate Olfactory System

The olfactory system is common to all animals and allows an organism to detect chemosensory cues from the surrounding environment. The paired olfactory organs are generally the most anteriorly-located structures within the olfactory system and are in direct contact with the external environment. Odorant molecules in the environment bind to and activate olfactory sensory neurons in the olfactory epithelia of the olfactory organs. For most vertebrate species, the axons of all olfactory sensory neurons within the same olfactory organ come together to form one of the olfactory nerves, which connects that olfactory organ to the ipsilateral olfactory bulb in the brain of the organism. As odorant molecules bind to receptors on the olfactory sensory neurons, action potentials travel along the axons that make up the olfactory nerves and provide sensory input to the olfactory bulbs, which are a part of the central nervous system. The paired olfactory bulbs are typically laminated structures, although the degree to which the specific laminae can be distinguished can vary among vertebrates. In mammals, for example, each olfactory bulb is typically divided into six layers, including the olfactory nerve layer, the glomerular layer, the external plexiform layer, the mitral cell layer, the internal plexiform layer, and the granule cell layer (Brunjes, 1994). Neurons in the olfactory bulb generally can be divided into two main classes: (1) principal (output) neurons that are under the influence of both

peripheral and central neural pathways and produce output signals directed to more central areas of the brain, and (2) local circuit neurons (interneurons) that contribute to the processing of olfactory information within and between the peripheral and central neural pathways (Halász, 1990).

The primary type of output neuron in the olfactory bulb of most vertebrates is the mitral cell, although additional types of output neurons exist in some species. In general, primary dendrites from mitral cells extend peripherally to make contact with the axon terminals of the olfactory sensory neurons in spherical structures known as glomeruli. The axons of most mitral cells project centrally and leave the olfactory bulb through the olfactory tracts, with axon terminals located in higher processing regions of the brain.

While the actual number of output neurons in the olfactory bulb is relatively small, there are many interneurons found in close proximity to these output neurons that are believed to play an important role in the processing and transmission of olfactory signals, perhaps even relaying information from more central regions of the brain back to the output neurons. Unlike the output neurons, the unifying characteristic of most interneurons is that they do not have projections that extend outside of the olfactory bulb. As a result, the various types of interneurons are distinguished based upon cell size, location, arborization patterns, and synaptic relationships (Halász, 1990).

For example, the vast majority of interneurons located in more peripheral regions of the olfactory bulb are generally classified as periglomerular or juxtaglomerular cells. As their name suggests, these cells are typically found in close proximity to the glomeruli, often in contact with the peripheral dendrites of the output neurons (usually near the point where the axon terminals of the olfactory sensory

neurons contact the mitral cells). More specifically, the primary dendrites of mitral cells are believed to receive postsynaptic input from the axon terminals of the periglomerular cells from neighboring glomeruli (Pinching, 1970), suggesting that inhibitory signals from neighboring glomeruli may modify the response of mitral cells to impulses received from the olfactory epithelium, providing one mechanism for second-order processing of olfactory information. The likelihood that such mechanisms exist is further supported by evidence that the dendrites of some periglomerular cells may also receive direct synaptic contact from the axons of the olfactory sensory neurons (Pinching and Powell, 1971), although this contact may not be characteristic of all vertebrates.

In contrast to periglomerular cells, the processes of interneurons that are found in more central regions of the olfactory bulb generally do not extend far enough peripherally to enter the glomeruli and, as a result, do not form connections with the primary dendrites of the mitral cells (Halász, 1990). Most of these centrally located interneurons are classified as granule cells, and they comprise the most numerous population of neurons within the olfactory bulb. Granule cells do not have true axons, but send out dendrites that synapse onto the axons and secondary or basal (non-glomerular) dendrites of the output neurons of the bulb. Although some of these interneurons are interspersed in the neuropil among the mitral cells, the majority of granule cells are relatively small neurons that are found within the internal or granule cell layer deep within the olfactory bulb. Some of the cell processes of these centrally-located granule cells receive unidirectional input from what appear to be central fibers from other regions of the brain, suggesting that granule cells may participate in the integration of both peripheral and central signals received by the mitral cells that determine which olfactory signals are relayed to the higher processing

regions of the brain (Halász, 1990).

Importance of the Vertebrate Olfactory System

Olfactory cues are known to elicit many effects, including the ability to modify the behavior and physiology of either a single organism or a group of organisms (Christensen and Sorensen, 1996). As a result, many species depend upon olfactory signals as a critically important mode of communication, with olfaction playing a fundamental role in both survival and reproduction. For example, an organism may depend upon its sense of smell, at least in part, to find food or avoid being killed by a potential predator, sometimes even communicating this information to other members of the same species through the release of pheromones. In addition to simple survival, the ability to discriminate olfactory signals can be a critical prerequisite to successful reproduction, allowing an organism to locate a mate in a sparsely populated area or, in areas where several similar species co-exist, providing a necessary means of species recognition required for successful mating.

In addition to its important contribution to both survival and reproduction, there are several characteristics of the olfactory system that make it unique among the other sensory systems. While significant morphological changes induced by sensory deprivation in other sensory systems (such as the visual and auditory system) are only believed to occur within a defined period of time during development (Wiesel and Hubel, 1963; Maruniak et al., 1989; Sie and Rubel, 1992), this does not appear to be the case for the olfactory system. The absence of such a critical period in the olfactory system may be related to the observation that new neurons are born in both the peripheral and central structures of the olfactory system throughout the life of an organism. Olfactory sensory neurons are continually produced from basal cells in the

olfactory epithelium, even in adults. Due to the importance of olfaction for survival and reproduction, it is easy to understand how the ability to replace these neurons would be selected for, especially when viewed in the context that the olfactory sensory neurons come into direct contact with the external environment. As a result, olfactory sensory neurons have a much higher probability of being exposed to potentially toxic stimuli that could result in the death of these cells.

In addition to the birth of olfactory sensory neurons in the periphery, the olfactory bulbs are one of the few regions of the brain where neurogenesis continues beyond development, as some populations of interneurons are generated throughout the life of an adult organism. One implication of the constantly changing nature of the olfactory epithelium as neurons die and are replaced is that the presence of axon terminals within the olfactory bulb is likely quite transient. As a result, it makes sense that selection would favor the ability of the central nervous system to respond to changes in innervation patterns as dying olfactory sensory neurons within the olfactory epithelium are replaced by newly differentiated cells. Continued neurogenesis in the olfactory bulb also could be a result of selective pressure to increase the ability of neuronal circuits within the olfactory bulb to discriminate and maximize differences between odor representations. For example, the activity-dependent survival of newly generated interneurons previously reported in the olfactory bulb (Petreanu and Alvarez-Buylla, 2002) may allow for the construction of transient inhibitory circuits that better enable discrimination of different types of odorants encountered by an organism during different periods of its life.

The continuous turnover of olfactory sensory neurons in the olfactory epithelium and the production of new central neurons during adulthood may induce more plastic changes in the olfactory bulb than in any other brain region (Halász,

1990), making the olfactory system an interesting model for studying neuronal plasticity and activity-dependent cell death and proliferation. As a result, research using this model system has the potential to increase scientific understanding in the areas of brain injury and neurodegenerative disorders, memory and learning, and the normal physiology of neurons, specifically, how neurons in the central nervous system respond to loss of afferent contact and/or activity.

Manipulation of the Vertebrate Olfactory System

One way to investigate the extent and mechanisms of neuronal plasticity within the vertebrate olfactory system is to explore how the loss of peripheral afferent input affects the downstream neurons in the olfactory bulb and other regions of the brain. Although the foundational principle is the same: prevent the olfactory sensory neurons in the olfactory epithelium from relaying odorant information from the external environment to the output neurons in the olfactory bulb, there are a variety of experimental techniques that are used to investigate the effects of peripheral sensory neurons on their central targets.

One technique is to inhibit olfactory sensory neuron function, thereby preventing electrical impulses from carrying odorant information to the brain. This is usually accomplished by depriving the organism of odorant contact by occluding or blocking the olfactory epithelium so that it is no longer in direct contact with the external environment (Meisami and Safari, 1981; Maruniak et al., 1989; Baker et al., 1993). If odorant molecules are unable to stimulate the olfactory sensory neurons, then no activity-dependent electrical impulses are delivered to the olfactory bulb. Similarly, certain neurotoxins such as tetrodotoxin (TTX) have been used to block membrane depolarization of olfactory sensory neurons (Miyamoto et al., 1992). The

important characteristic of occlusion/deprivation experiments is that only the function of the olfactory sensory neurons is inhibited. Physical contact between the olfactory organ and the ipsilateral olfactory bulb is not affected.

In contrast, the second category of experimental manipulation encompasses procedures that interfere with physical contact between the olfactory organs and the olfactory bulbs, thereby affecting both structure *and* function. Experiments within this category can be further classified by the specific mechanisms used to disrupt the normal structure and function of the olfactory system. For example, chemicals such as zinc sulfate and Triton X-100 have been used to irrigate the nasal cavity, thereby killing existing olfactory sensory neurons and resulting in axon degeneration that deprives the olfactory bulb of both contact and activity (Nadi et al., 1981; Casabona et al., 1998; Cummings et al., 2000). A second technique involves the physical transection of the olfactory nerve (axotomy), in which the axons of the olfactory sensory neurons that make up the olfactory nerve are severed, preventing signals from traveling between the olfactory epithelium and the olfactory bulb and resulting in the complete degeneration of mature olfactory sensory neurons (Mandairon et al., 2003; Veyrac et al., 2005). Depending upon the chemical used, both chemical deafferentation and axotomy are generally reversible, however, due to the previously-mentioned capacity of the olfactory epithelium to generate new neurons. As basal cells replace the degenerating olfactory sensory neurons, axons from the newly-differentiated neurons reinnervate the deafferented olfactory bulb (Costanzo, 1991). In contrast to these reversible deafferentation procedures, complete removal or destruction of the olfactory organ is capable of resulting in permanent deafferentation of the olfactory bulb by eliminating both physical contact and neuronal activity. The removal/destruction of basal cells as well as mature olfactory sensory neurons

prevents the reinnervation of the olfactory bulb, resulting in an irreversible type of deafferentation that has been the focus of research in our laboratory.

In addition to its inherent neuronal plasticity, another reason that the olfactory system is such an excellent model system for experimental manipulation has to do with its paired nature. Most species have two olfactory organs, which are connected to separate olfactory bulbs. As a result, one olfactory organ/bulb can be experimentally manipulated while the contralateral bulb can be left in place as an internal control. This allows researchers to compare the effects of the experimental manipulation within the same individual, thereby reducing potential variability that could otherwise be introduced by using different individuals.

The Zebrafish as a Model Organism

The ubiquitous use of the zebrafish (*Danio rerio*) for developmental and genetic studies, as well as a completely sequenced genome and the existence of a wide variety of mutant strains, have helped to establish this species as an important model organism in experimental research. The zebrafish was selected for use on the experiments described within this manuscript for a variety of reasons. The morphology and neural circuitry of the olfactory system in this teleost has been previously described, with general cell types that are similar to those found in other animals (Byrd and Brunjes, 1995). In addition, the zebrafish olfactory organ can be easily manipulated and removed, enabling complete and permanent deafferentation of the olfactory bulb (Byrd, 2000). The small size of the zebrafish allows for rigorous measurement and quantification of the olfactory bulbs, and evidence of previously demonstrated neuroplasticity in teleosts (Raymond, 1991; Otteson and Hitchcock, 2003) makes this species an excellent model organism with which to investigate the

effects of permanent peripheral deafferentation on the post-synaptic organization and structural maintenance of targets within the central nervous system.

The zebrafish olfactory system shares many similarities with the olfactory system found in many other vertebrate species. The paired olfactory organs in zebrafish are located in the dorsal part of the snout and are comprised of olfactory epithelia that are arranged into rosette structures that extend upward from the floor of the nasal capsules. Incurrent and excurrent nares that are located just anterior and posterior to the olfactory rosette allow water to flow over the olfactory epithelium as the fish swims (Hansen and Zeiske, 1993). Odorants that are dissolved in the water stimulate the olfactory sensory neurons in the olfactory epithelium, resulting in the generation of action potentials that travel to the olfactory bulb along the axons of the olfactory sensory neurons that make up the olfactory nerve.

The paired olfactory bulbs of zebrafish are located rostral to the rest of the brain and sessile to the telencephalon. Each olfactory bulb is a diffusely laminated structure that can be roughly divided into three primary layers: (1) the olfactory nerve layer, (2) the glomerular layer, and (3) the internal cell layer, although the delineation between each layer is not as clear as that seen in most mammalian olfactory bulbs. The olfactory nerve layer in zebrafish is primarily comprised of glia and the axons from the olfactory sensory neurons that surround the surface of the olfactory bulb. As its name suggests, the glomerular layer is an intermediate region within the olfactory bulb that houses the glomeruli, where axon terminals of the olfactory sensory neurons make synaptic contact with the primary dendrites of the mitral cells. Although there are several different types of cells found within the glomerular layer, including mitral cells, juxtaglomerular cells, and glia, these cells are widely dispersed throughout the neuropil and relatively few in number. The primary interneurons found within the

glomerular layer of the olfactory bulb in zebrafish are often referred to as juxtaglomerular cells because they have not yet been characterized to the extent that they have been shown to be equivalent to the periglomerular cells described in other species. The innermost layer of the olfactory bulb is known as the internal cell layer, and is comprised of a large number of densely packed granule cells and glia. The olfactory bulb is connected to the rest of the brain by the lateral and medial olfactory tracts, which are comprised of neuronal fibers that enter and leave the olfactory bulb on its ventral surface.

Experimental Background

While experiments designed to investigate how removal of afferent input affects the olfactory bulb during development have been relatively common, there have been far fewer studies conducted to determine how peripheral deafferentation affects the olfactory bulb in adult organisms. Although the results of experiments using neonatal or juvenile animals have provided fundamental information about changes that take place in the olfactory bulb following removal of sensory input, these studies relate more to afferent influence on the developing olfactory bulb. The potential differences that exist between adult and developing individuals have required that additional experiments using adult organisms be conducted in order to confirm whether the changes seen during development differ from the effects of deafferentation on the morphology and physiology of the olfactory bulbs in mature animals. Experiments that have been completed using adult organisms have shown that naris closure in adult mice leads to a reduction in the size of the ipsilateral olfactory bulb (Maruniak et al., 1989), a decrease in the expression of tyrosine hydroxylase within the olfactory bulb (Baker et al., 1993), and reduced neurogenesis

and survival of neural progenitors (Corotto et al., 1994).

One of the primary focuses of our laboratory has been an ongoing investigation of how the olfactory bulb responds to permanent, peripheral deafferentation in the adult zebrafish. We have previously shown that unilateral ablation of the olfactory epithelium results in a significant reduction in both cell number and total volume of the ipsilateral olfactory bulb several weeks following the deafferentation procedure (Byrd, 2000). In addition, we also have observed a reduction in the expression of tyrosine hydroxylase (Byrd, 2000) and ionotropic glutamate receptor subunit 4 (Fuller et al., 2005) within the deafferented olfactory bulb, demonstrating that the neurochemistry of the bulb is affected by deafferentation. Although similar changes have been observed in other species, the specific mechanisms responsible for these changes have not yet been determined.

The overall objective of the current project was to investigate further the hypothesis that contact between the axons of the olfactory sensory neurons and the olfactory bulb is necessary for the maintenance of the post-synaptic organization of the olfactory bulb in adult zebrafish. A detailed investigation of the morphological changes taking place within the olfactory bulb following peripheral deafferentation was conducted in an attempt to further explain the cause of the reduction in total bulb volume previously observed. After confirming our previous results that the total volume of the ipsilateral olfactory bulb was significantly smaller following removal of afferent input, the first question addressed was whether this overall decrease in volume was a result of the loss or shrinkage of one or more specific layers of the bulb. Based upon the observation that the olfactory nerve layer contains the axons of the olfactory sensory neurons destroyed by the deafferentation procedure, we reasoned that the total volume of the olfactory bulb was decreasing, at least in part, due to the

loss of the olfactory nerve layer. We also reasoned that there would likely be changes in the laminar volume of the glomerular layer, since the axon terminals of the olfactory sensory neurons make up a substantial part of the neuropil of the glomerular layer and the majority of the output neurons most likely to be directly affected by the loss of contact and/or activity from the axon terminals of the olfactory sensory neurons also are found in this layer.

Following this investigation of whether the loss or size reduction of one or more specific laminae might contribute to the decrease in total bulb volume, our next goal was to explore two other possible changes that might explain how removal of primary afferent axons causes the volume reduction observed in the ipsilateral olfactory bulb. We attempted to determine: (1) whether specific populations of cells were disappearing from the deafferented bulb, and (2) whether cells within the glomerular and internal cell layers of the olfactory bulb were more densely packed following deafferentation. We anticipated that, once these questions had been addressed and the specific morphological changes taking place in the deafferented olfactory bulb had been quantified and described in greater detail, the door would be opened for future experiments to determine the mechanisms responsible for those changes, thereby allowing us to better investigate the neuronal plasticity of the olfactory bulbs and the factors responsible for the maintenance of post-synaptic organization within the adult brain.

CHAPTER II

MATERIALS AND METHODS

Subjects

Adult zebrafish (*Danio rerio*) were purchased from a local supplier and housed in aquaria containing aerated conditioned fish water maintained at approximately 25-28°C. Fish flakes (Ocean Star International, Snowville, UT) were provided daily. Both male and female zebrafish were used for this study and ranged in size from 3.4 to 4.7 cm in length and 0.23 to 0.91 grams in weight. All procedures were approved by the Institutional Animal Care and Use Committee of Western Michigan University.

Deafferentation Procedure

The animals were randomly selected and divided into unoperated control, sham-operated control, and experimental groups. Animals in the experimental group were anesthetized using 0.03% MS222 (3-aminobenzoic acid ethyl ester, Sigma, St. Louis, MO) until they were unresponsive to a tail pinch and viewed under a dissecting microscope. A small-vessel cautery iron was used to completely remove either the right or left olfactory organ from the experimental animals. The contralateral olfactory organ was left in place to serve as an internal control. Control animals were anesthetized using 0.03% MS222 but were not subjected to subsequent manipulation (unoperated control) or received a wound to the skin from the small-vessel cautery iron between the two olfactory organs, leaving both olfactory organs in place (sham-

operated control). Following each procedure, the animals were allowed to recover in a beaker containing conditioned fish water before being returned to aquaria containing kanamycin, a full spectrum fish antibiotic used to aid in wound healing and prevent infection. The animals were allowed to survive for 1, 3, or 6 weeks before they were over-anesthetized using 0.03% MS222 and perfused transcardially with phosphate buffered saline (PBS) followed by 4% paraformaldehyde. Following perfusion, the animals were post-fixed overnight at 4-8°C using 4% paraformaldehyde.

Histological Preparation

Following overnight fixation, the animals were rinsed in PBS and the brains were removed with the aid of a dissecting microscope. The dissected brains were rinsed in PBS, followed by 50% ethanol, before being placed into 70% ethanol for storage at 4-8°C until embedding. The brains were embedded in paraffin following dehydration in an ascending series of ethanols (80%, 90%, 95%, 100%) and xylenes. A microtome was used to section each brain at 10 μm in the horizontal plane, and every third section was placed onto a positively charged slide. The slides were stained using hematoxylin and eosin (Richard Allen Scientific, Kalamazoo, MI) following standard protocols and coverslipped using DPX mounting medium (Aldrich, Milwaukee, WI).

Quantitative Analyses

All slides were viewed on a Nikon E600 Eclipse microscope, and volume measurements of the olfactory bulbs were collected using SPOT image analysis software (Diagnostic Instruments, Version 3.2.4). For unoperated control and sham-operated control animals, all data collected for the right olfactory bulb were compared

to the data collected for the left olfactory bulb. For experimental animals in which the right olfactory organ was ablated, the right olfactory bulb was designated as the deafferented bulb and the left olfactory bulb was designated as the contralateral control bulb. Similarly, the left olfactory bulb was considered to be the deafferented bulb and the right olfactory bulb was designated as the contralateral control bulb for experimental animals in which the left olfactory organ was removed. For quantification purposes, the data collected for both the left and right deafferented animals were used and comparisons were made between deafferented and control bulbs. Total bulb volume, laminar volume, size distribution of nuclear profiles, cell density, and cell number were determined for unoperated control (n=6), sham-operated control (n=6), and experimental animals at survival periods of 1 week (n=6), 3 weeks (n=6), and 6 weeks (n=6) post-deafferentation.

Total Bulb Volume

To investigate whether there was a difference in total bulb volume between the deafferented olfactory bulb and the contralateral control bulb, the area of the right and left olfactory bulbs was measured in every third section through the entire bulb. Three independent measurements were obtained and the mean area for each section was multiplied by 30 μm to provide a volume estimate that included the sections immediately preceding and immediately following the measured section. The sum of these volume calculations was used as the estimate of the total bulb volume. The percent difference in total bulb volume for the unoperated control and sham-operated control animals was calculated as [(total volume of right bulb – total volume of left bulb) / total volume of left bulb] x 100. The percent difference in total bulb volume for experimental animals within each survival period was calculated as [(total volume

of deafferented bulb – total volume of control bulb) / total volume of control bulb] x 100. The results were compared within each control and experimental group using one-tailed, paired sample *t*-tests. *P* values less than 0.05 were considered significant.

Laminar Volume

For the purposes of this analysis, each olfactory bulb was divided into three layers (the olfactory nerve layer, the glomerular layer, and the internal cell layer) based upon morphological/histological differences observed under a brightfield microscope (Figure 1). While some previous studies (Byrd and Brunjes, 1995; Poling and Brunjes, 2000) have divided the bulb into four layers (olfactory nerve layer, glomerular layer, mitral cell/plexiform layer, and granule cell layer), the diffuse organization of the mitral cells and lack of clear differentiation between the layers of the olfactory bulb in zebrafish led us to combine the mitral cell/plexiform and granule cell layers into a single measurement for the internal cell layer, as previously reported (Byrd, 2000; Byrd and Brunjes, 2001).

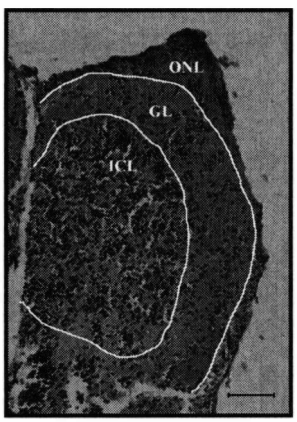

Figure 1. Hematoxylin and eosin-stained horizontal section demonstrating the laminar organization of the adult zebrafish olfactory bulb (ONL = olfactory nerve layer, GL = glomerular layer, ICL = internal cell layer). Scale bar = 50 μm.

The area of each layer was measured in every third section through the extent of both left and right olfactory bulbs. Three independent measurements were obtained for each layer and the mean laminar area for each section was multiplied by 30 μm to provide a laminar volume estimate that included the sections immediately preceding and immediately following the measured section. For each layer, the sum of these volume calculations was used as the estimate of laminar volume. The mean percent difference in the volume of each layer for the unoperated control and sham-operated control animals was calculated as [(laminar volume of right bulb – laminar volume of left bulb) / laminar volume of left bulb] x 100. The mean percent difference in the volume of each olfactory bulb layer for experimental animals within each survival period was calculated as [(laminar volume of deafferented bulb – laminar volume of control bulb) / laminar volume of control bulb] x 100. The results were compared within each control and experimental group using one-tailed, paired sample *t*-tests. *P* values less than 0.05 were considered significant.

In an attempt to gain further insight into whether the relative proportion of total bulb volume occupied by each layer was changing following deafferentation, for each animal the laminar volumes of all three layers were summed to provide a calculated estimate of total bulb volume. For both left and right olfactory bulbs, each laminar volume was then divided by the calculated total bulb volume and multiplied by 100 to yield a percentage of the total bulb volume comprised by that specific layer. Results were averaged across each control or experimental group and the relative proportion of the total olfactory bulb volume comprised by each layer was plotted and compared between the left and right bulbs of control animals and the control and deafferented bulbs of experimental animals.

Size Distribution of Nuclear Profiles

In an attempt to explore the potential effects of deafferentation on specific populations of cells within the olfactory bulb, the sizes of nuclear profiles were measured in three regions from the median section of each bulb: (1) the internal cell layer, (2) the rostral glomerular layer, and (3) the lateral glomerular layer. The olfactory nerve layer was not included in this analysis because there are no neuronal nuclei in this layer. A total of 10 random nuclei were measured in the internal cell layer of each olfactory bulb. Fifteen random nuclei were measured in each of the rostral and lateral regions of the glomerular layer and combined to provide measurements for a total of 30 nuclear profiles in the glomerular layer as a whole. Nuclear profiles were viewed with an oil-immersion lens at a magnification of 1000X and the diameter of each randomly-selected nuclear profile was measured to the nearest micrometer using a scale bar in the ocular of the microscope. Within each layer, nuclear profiles were assigned to one of ten size categories ranging from 0 μm to 10 μm. The number of nuclear profiles within each category was divided by the total number of nuclei measured for that layer of the bulb and multiplied by 100 to yield percentages of cells that were plotted as a function of nuclear size.

Cell Density

To estimate cell density, a 10x5 grid reticle was used to count all nuclear profiles within a 5,000 μm^2 area. The area was multiplied by 10 μm to account for the thickness of each section and cell densities were calculated for three 50,000 μm^3 regions of each olfactory bulb: (1) the internal cell layer, (2) the rostral area of the glomerular layer, and (3) the lateral area of the glomerular layer. Counts of nuclear profiles within the rostral and lateral areas of the glomerular layer were combined to

yield a single value for the combined glomerular layer of that bulb. All measurements were collected from the median section of each bulb and the cell density for each region was calculated for each animal. Mean cell density values were compared within each control and experimental group using a two-tailed, paired sample *t*-test; *P* values less than 0.05 were considered significant. Once again, cell density measurements were not conducted for the olfactory nerve layer because the olfactory nerve layer is comprised primarily of the axons of the olfactory sensory neurons and glia, with no neuronal nuclear profiles.

Estimation of Laminar Cell Number

For each animal, cell numbers within the glomerular layer and internal cell layer of both olfactory bulbs were estimated by multiplying the average laminar cell density obtained from the median section of each bulb by the laminar volume of the appropriate layer for the same bulb. For unoperated control and sham-operated control animals, the percent difference in the estimated number of cells within each layer was calculated using the equation [(estimated cell number of right bulb – estimated cell number of left bulb) / estimated cell number of left bulb] x 100. Similarly, the percent difference in the estimated number of cells for experimental animals within each survival period was calculated using the equation [(estimated cell number of deafferented bulb – estimated cell number of control bulb) / estimated cell number of control bulb] x 100. The mean cell number within each layer of both olfactory bulbs was compared within each control and experimental group using two-tailed, paired sample *t*-tests. *P* values less than 0.05 were considered significant.

CHAPTER III

RESULTS

General Morphological Changes in the Olfactory Bulb

As previously reported (Byrd, 2000), the overall morphology of the ipsilateral olfactory bulb in adult zebrafish was clearly affected by removal of afferent input following ablation of the olfactory organ (Figure 2). While there was expected variation in olfactory bulb size among different unoperated control and sham-operated control animals, there was not a noticeable difference in size between the paired bulbs within the same individual. In addition, the intact, contralateral control bulb of experimental animals did not appear to differ in size when compared to the olfactory bulbs of unoperated control or sham-operated control animals. In contrast, the deafferented olfactory bulb did appear to be smaller than the contralateral control bulb at all survival intervals, with the reduction in size increasing in magnitude with a longer survival time post-deafferentation. There were no apparent differences in the morphological changes observed in the ipsilateral olfactory bulb following removal of either the left or right olfactory organ, suggesting that the response to removal of afferent input is the same in either olfactory bulb. The olfactory nerve layer appeared to be affected the most by ablation of the olfactory organ and was clearly diminished in the ipsilateral olfactory bulb at 3 and 6 weeks post-deafferentation. Although not as apparent, the extent of the glomerular layer and internal cell layer also appeared to be decreased with longer survival time post-deafferentation.

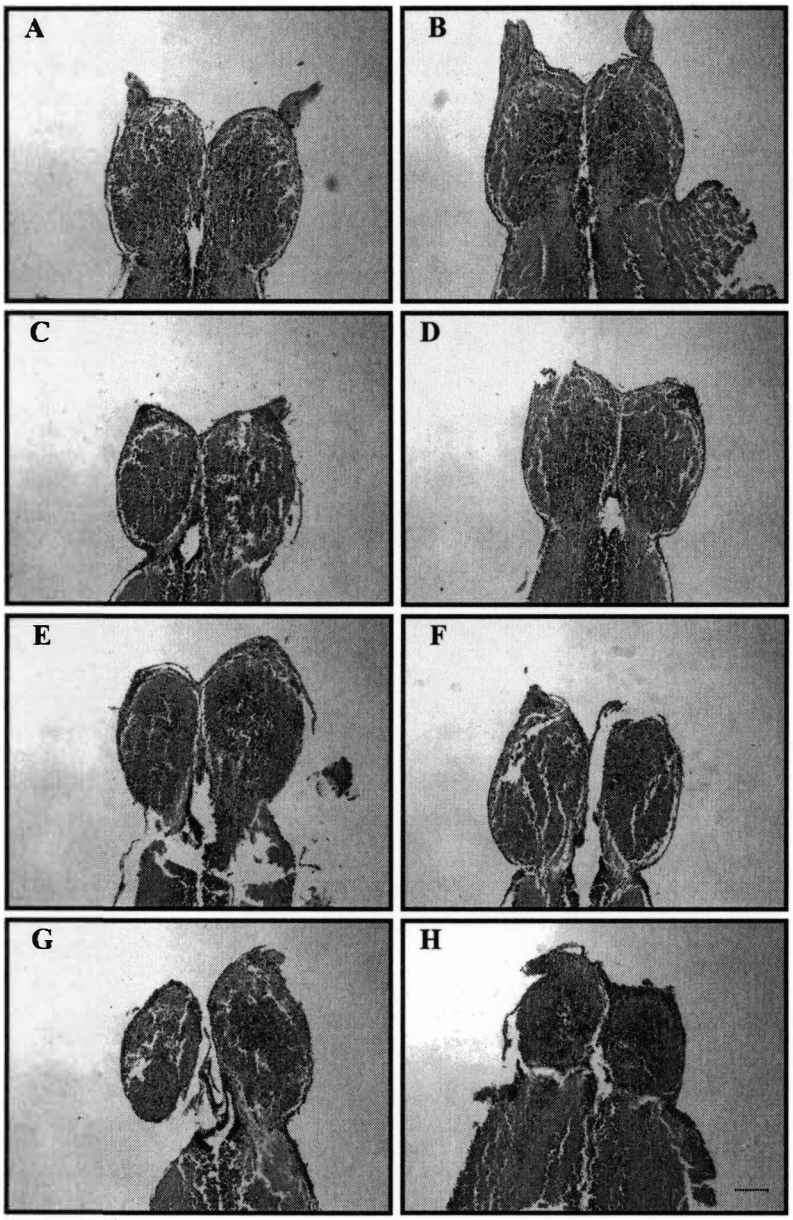

Figure 2. Hematoxylin and eosin-stained horizontal sections through the olfactory bulbs of unoperated control (**A**), sham-operated control (**B**), and experimental animals. The olfactory bulbs of animals in which the left olfactory organ was removed are shown at 1 week (**C**), 3 weeks (**E**), and 6 weeks (**G**) post-deafferentation. The olfactory bulbs of animals in which the right olfactory organ was removed are also shown at 1 week (**D**), 3 weeks (**F**), and 6 weeks (**H**) post-deafferentation. Scale bar = 100 μm.

Effect of Deafferentation on Total Bulb Volume

The quantitative effects of permanent removal of the olfactory organ on the total volume of the ipsilateral olfactory bulb in adult zebrafish are presented in Table 1 and Figure 3. No significant difference in total bulb volume was observed between the left and right olfactory bulb in either unoperated control ($P = 0.48$) or sham-operated control animals ($P = 0.20$). In contrast, the total bulb volume of the deafferented olfactory bulb in experimental animals was significantly smaller than the contralateral control olfactory bulb at 1 week ($P < 0.01$), 3 weeks ($P < 0.03$), and 6 weeks ($P < 0.01$) post-deafferentation. The magnitude of the reduction in total volume of the deafferented olfactory bulb was correlated with the length of the survival time following deafferentation, with a mean percent difference of -6.14%, -15.86%, and -21.64% occurring at 1, 3, and 6 weeks post-deafferentation, respectively.

In order to confirm that there was no difference in the response to the deafferentation procedure depending upon which olfactory organ was removed, the mean percent difference in the total volume of the ipsilateral olfactory bulb of experimental animals having their right olfactory organ removed was compared to experimental animals having their left olfactory organ removed. The results of this comparison are presented in Table 2 and Figure 4. In general, there were no differences in the response of the ipsilateral olfactory bulb following ablation of either the right or left olfactory organ. Although there was minor variation with respect to the mean percent difference for each bulb, deafferentation of either the right or left olfactory bulb resulted in a similar decrease in total bulb volume at each survival interval. Animals in which the right olfactory organ had been removed showed a progressive decrease in the volume of the right olfactory bulb of -7.32% ($P < 0.04$),

-14.07% ($P = 0.07$), and -18.14% ($P < 0.01$) at 1, 3, and 6 weeks post-deafferentation, respectively. Similarly, animals in which the left olfactory organ had been removed showed a decrease in total volume of the left olfactory bulb of -4.96% at 1 week post-deafferentation ($P < 0.01$), -17.66% at 3 weeks post-deafferentation ($P = 0.14$), and -25.15% at 6 weeks post-deafferentation ($P < 0.03$). Although the decrease in the total bulb volume of both the left and right olfactory bulbs at 3 weeks post-deafferentation was not statistically significant, this can most likely be attributed to a higher variability in the volumes measured at this interval and does not necessarily reflect a lack of scientific importance.

Table 1

Effect of Deafferentation on Total Bulb Volume

		Total Bulb Volume[a] *(mm^3)*		
	n	*Control Bulb*[b]	*Deafferented Bulb*[b]	*% Difference*
Unoperated Control	6	0.0292 ± 0.0030	0.0293 ± 0.0033	↓ 0.08 ± 2.00
Sham-Operated Control	6	0.0315 ± 0.0029	0.0310 ± 0.0024	↓ 1.04 ± 1.70
1 Week Post-Deafferentation	6	0.0331 ± 0.0030	0.0310 ± 0.0025	↓ 6.14 ± 0.77*
3 Weeks Post-Deafferentation	6	0.0327 ± 0.0048	0.0267 ± 0.0034	↓ 15.86 ± 5.04*
6 Weeks Post-Deafferentation	6	0.0237 ± 0.0029	0.0184 ± 0.0020	↓ 21.64 ± 3.01*

[a] Mean ± S.E.M.; $^*P < 0.05$

[b] For unoperated control and sham-operated control animals (throughout all tables), the designation "*Control Bulb*" is used for the left olfactory bulb and "*Deafferented Bulb*" is used the right olfactory bulb.

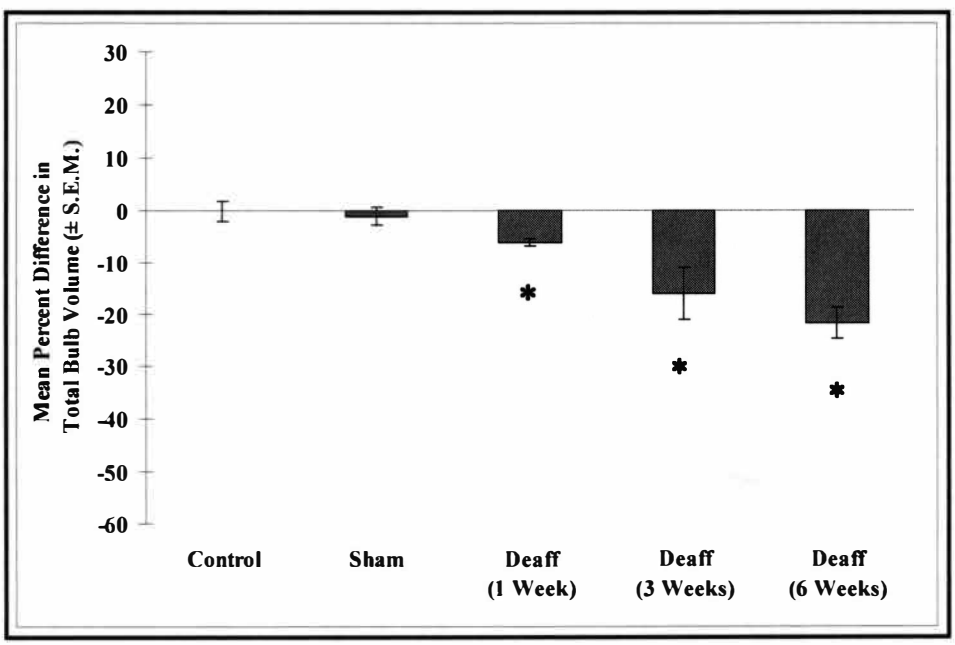

Figure 3. Mean percent difference in total bulb volume following peripheral deafferentation ($^*P < 0.05$).

Table 2

Comparison of the Effect of Deafferentation on Total Bulb Volume Following Removal of the Right or Left Olfactory Organ

		Total Bulb Volume[a] *(mm^3)*		
	n	*Control Bulb*	*Deafferented Bulb*	*% Difference*
Deaff (Right[b]) – 1 Week	3	0.0378 ± 0.0045	0.0349 ± 0.0038	↓ 7.32 ± 1.20*
Deaff (Left[c]) – 1 Week	3	0.0285 ± 0.0015	0.0271 ± 0.0013	↓ 4.96 ± 0.35*
Deaff (Right[b]) – 3 Weeks	3	0.0333 ± 0.0084	0.0281 ± 0.0065	↓ 14.07 ± 4.50
Deaff (Left[c]) – 3 Weeks	3	0.0321 ± 0.0067	0.0253 ± 0.0036	↓ 17.66 ± 10.17
Deaff (Right[b]) – 6 Weeks	3	0.0176 ± 0.0015	0.0145 ± 0.0017	↓ 18.14 ± 3.24*
Deaff (Left[c]) – 6 Weeks	3	0.0298 ± 0.0020	0.0222 ± 0.0017	↓ 25.15 ± 4.74*

[a] Mean ± S.E.M.; [b] Right olfactory organ removed; [c] Left olfactory organ removed
*$P < 0.05$

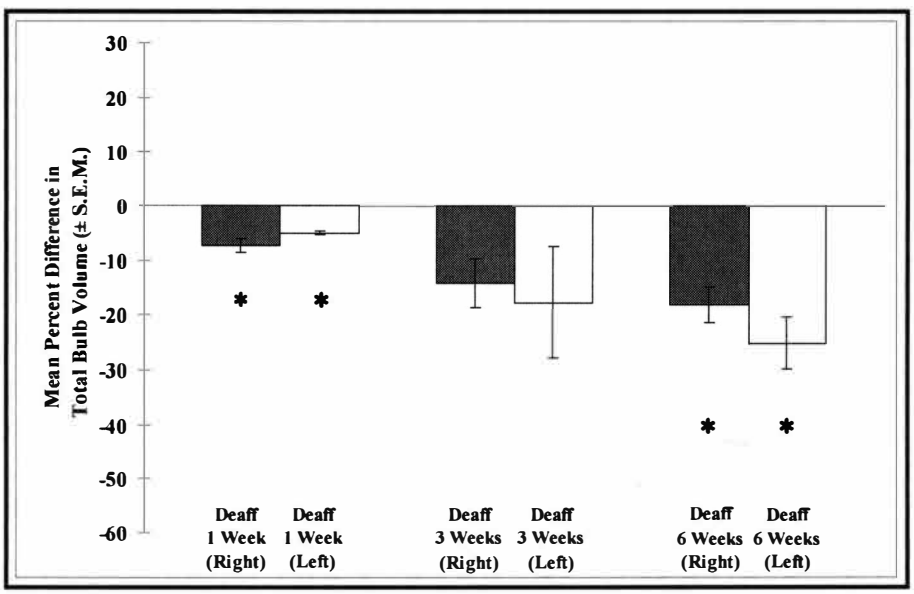

Figure 4. Mean percent difference in total volume following removal of the right or left olfactory organ (*$P < 0.05$).

Effect of Deafferentation on Laminar Volume

Olfactory Nerve Layer

The laminar volume of the olfactory nerve layer was significantly reduced at all survival intervals following ablation of the olfactory organ, as presented in Table 3 and Figure 5. Similar to the pattern observed for total bulb volume, the magnitude of the decrease in the laminar volume of the olfactory nerve layer in the deafferented bulb of experimental animals was strongly correlated with the length of the survival time following the deafferentation procedure, with mean percent differences of -13.26% ($P < 0.02$), -19.59% ($P < 0.04$), and -35.65% ($P < 0.02$) at 1, 3, and 6 weeks post-deafferentation, respectively. It is important to note that these significant decreases were strongly evident despite a relatively high degree of variability in volume measurements of the olfactory nerve layer resulting from differences in the extent to which the olfactory nerve and its connection to the olfactory bulb was preserved during dissection and processing. No significant difference in the volume of the olfactory nerve layer was observed between the left and right olfactory bulbs in unoperated control ($P = 0.29$) or sham-operated control ($P = 0.30$) animals.

Table 3

Effect of Deafferentation on the Volume of the Olfactory Nerve Layer

		Olfactory Nerve Layer Volume[a] *(mm^3)*		
	n	*Control Bulb*	*Deafferented Bulb*	*% Difference*
Unoperated Control	6	0.0049 ± 0.0006	0.0047 ± 0.0007	↓ 3.87 ± 6.27
Sham-Operated Control	6	0.0047 ± 0.0003	0.0046 ± 0.0002	↓ 1.45 ± 2.81
1 Week Post-Deafferentation	6	0.0043 ± 0.0005	0.0038 ± 0.0005	↓ 13.26 ± 4.97*
3 Weeks Post-Deafferentation	6	0.0039 ± 0.0005	0.0030 ± 0.0003	↓ 19.59 ± 8.69*
6 Weeks Post-Deafferentation	6	0.0029 ± 0.0004	0.0018 ± 0.0002	↓ 35.65 ± 7.08*

[a] Mean ± S.E.M.; $^{*}P < 0.05$

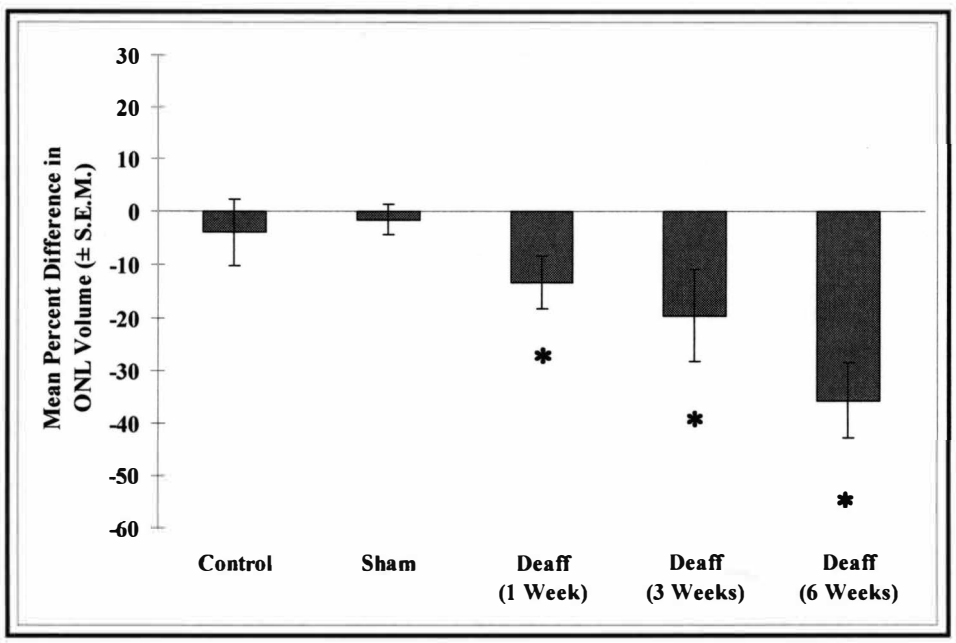

Figure 5. Mean percent difference in the volume of the olfactory nerve layer (ONL) following peripheral deafferentation ($^{*}P < 0.05$).

Glomerular Layer

The effects of the loss of sensory input on the volume of the glomerular layer are presented in Table 4 and Figure 6. As expected, there was no difference in the laminar volume of the glomerular layer when compared between the left and right olfactory bulbs of unoperated control ($P = 0.38$) or sham-operated control ($P = 0.15$) animals. Interestingly, no significant difference in the volume of the glomerular layer was observed between the deafferented bulb and contralateral control bulb 1 week following deafferentation ($P = 0.11$). However, between 1 and 3 weeks post-deafferentation there appeared to be a delayed but substantial decrease (-20.34%, $P < 0.03$) in the volume of the glomerular layer, which was similar to the volume reduction of -19.59% observed in the olfactory nerve layer at 3 weeks post-deafferentation (Figure 8). However, unlike the subsequent decrease in laminar volume seen in the olfactory nerve layer, the magnitude of the volume reduction observed in the glomerular layer did not appear to increase with a longer survival time, based upon a similar decrease in the volume of the glomerular layer observed at 6 weeks post-deafferentation (-19.96%, $P < 0.01$).

Table 4

Effect of Deafferentation on the Volume of the Glomerular Layer

		Glomerular Layer Volume[a] *(mm^3)*		
	n	*Control Bulb*	*Deafferented Bulb*	*% Difference*
Unoperated Control	6	0.0135 ± 0.0025	0.0137 ± 0.0026	↑ 1.00 ± 3.58
Sham-Operated Control	6	0.0182 ± 0.0016	0.0176 ± 0.0012	↓ 2.30 ± 2.65
1 Week Post-Deafferentation	6	0.0160 ± 0.0010	0.0153 ± 0.0082	↓ 3.78 ± 2.75
3 Weeks Post-Deafferentation	6	0.0176 ± 0.0028	0.0134 ± 0.0019	↓ 20.34 ± 5.82*
6 Weeks Post-Deafferentation	6	0.0124 ± 0.0017	0.0099 ± 0.0013	↓ 19.96 ± 4.01*

[a] Mean ± S.E.M.; $^{*}P < 0.05$

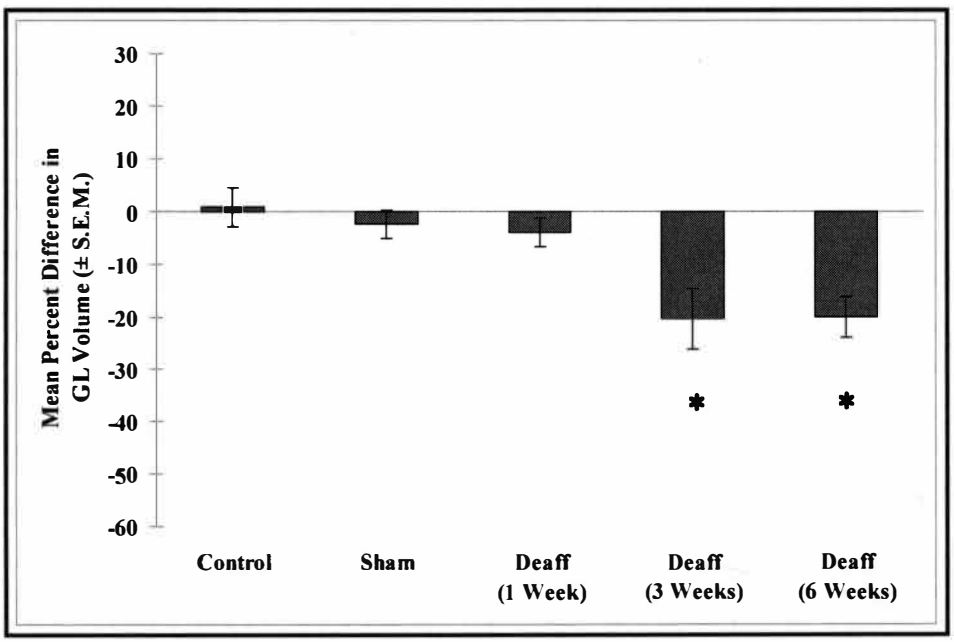

Figure 6. Mean percent difference in the volume of the glomerular layer (GL) following peripheral deafferentation ($^{*}P < 0.05$).

Internal Cell Layer

Changes in the laminar volume of the internal cell layer followed a similar pattern to that observed for total bulb volume and the laminar volume of the olfactory nerve layer and are presented in Table 5 and Figure 7. Once again, no significant difference in internal cell layer volume was observed between the left and right olfactory bulbs in unoperated control ($P = 0.18$) or sham-operated control ($P = 0.17$) animals. In contrast, a significant decrease in the laminar volume of the internal cell layer of the deafferented olfactory bulb was observed when compared to the contralateral control bulb at all survival intervals. The internal cell layer of the deafferented bulb was 8.23% smaller at 1 week post-deafferentation ($P < 0.02$), 14.14% smaller at 3 weeks post-deafferentation ($P < 0.05$), and 20.72% smaller at 6 weeks post-deafferentation ($P < 0.01$), again demonstrating a progressive decrease in laminar volume with increasing length of the survival period post-deafferentation. The overall decrease of 20.72% seen in the volume of the internal cell layer observed at the longest survival interval was similar in magnitude to the size reduction of 19.96% observed for the glomerular layer at 6 weeks post-deafferentation (Figure 8).

Table 5

Effect of Deafferentation on the Volume of the Internal Cell Layer

		Internal Cell Layer Volume[a] *(mm^3)*		
	n	*Control Bulb*	*Deafferented Bulb*	*% Difference*
Unoperated Control	6	0.0104 ± 0.0014	0.0106 ± 0.0013	↑ 2.00 ± 1.59
Sham-Operated Control	6	0.0091 ± 0.0008	0.0094 ± 0.0008	↑ 3.31 ± 2.65
1 Week Post-Deafferentation	6	0.0117 ± 0.0019	0.0110 ± 0.0020	↓ 8.23 ± 3.07*
3 Weeks Post-Deafferentation	6	0.0113 ± 0.0015	0.0095 ± 0.0012	↓ 14.14 ± 5.18*
6 Weeks Post-Deafferentation	6	0.0082 ± 0.0008	0.0063 ± 0.0004	↓ 20.72 ± 4.04*

[a] Mean ± S.E.M.; $^{*}P < 0.05$

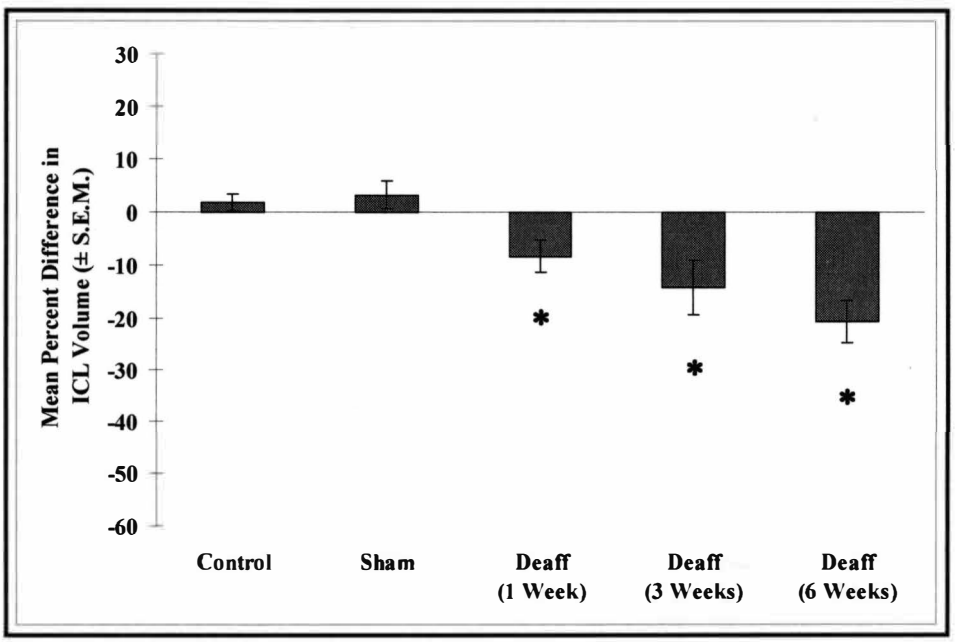

Figure 7. Mean percent difference in the volume of the internal cell layer (ICL) following peripheral deafferentation ($^{*}P < 0.05$).

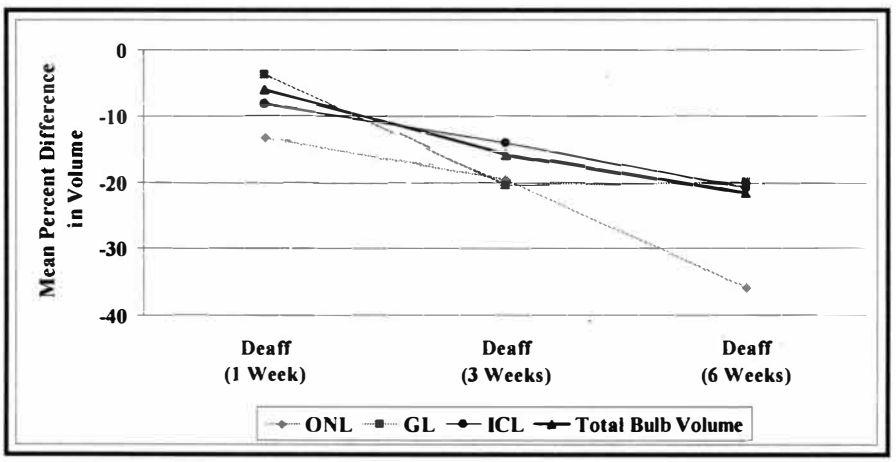

Figure 8. Summary of laminar volume changes observed following peripheral deafferentation.

Laminar Volume as a Percentage of Total Bulb Volume

To explore further the possible changes taking place in the deafferented olfactory bulb, general comparisons were made with respect to the percentage of total bulb volume comprised by each layer, as shown in Figure 9. Overall, the proportion of the total bulb volume occupied by each layer did not appear to differ between unoperated control, sham-operated control, or experimental animals. For all groups, the olfactory nerve layer occupied the smallest proportion of the olfactory bulb at 12-18% of the total bulb volume in the left/control bulb and 10-17% of the total bulb volume in the right/deafferented bulb. In contrast, the glomerular layer occupied the largest proportion of the olfactory bulb, ranging from 46-57% of the total bulb volume in the left/control bulb and 46-56% of the total bulb volume in the right/deafferented bulb. The mean percentage of total bulb volume occupied by the internal cell layer in the left/control bulb ranged from 28-36%, compared to a range of 29-37% in the right/deafferented bulb.

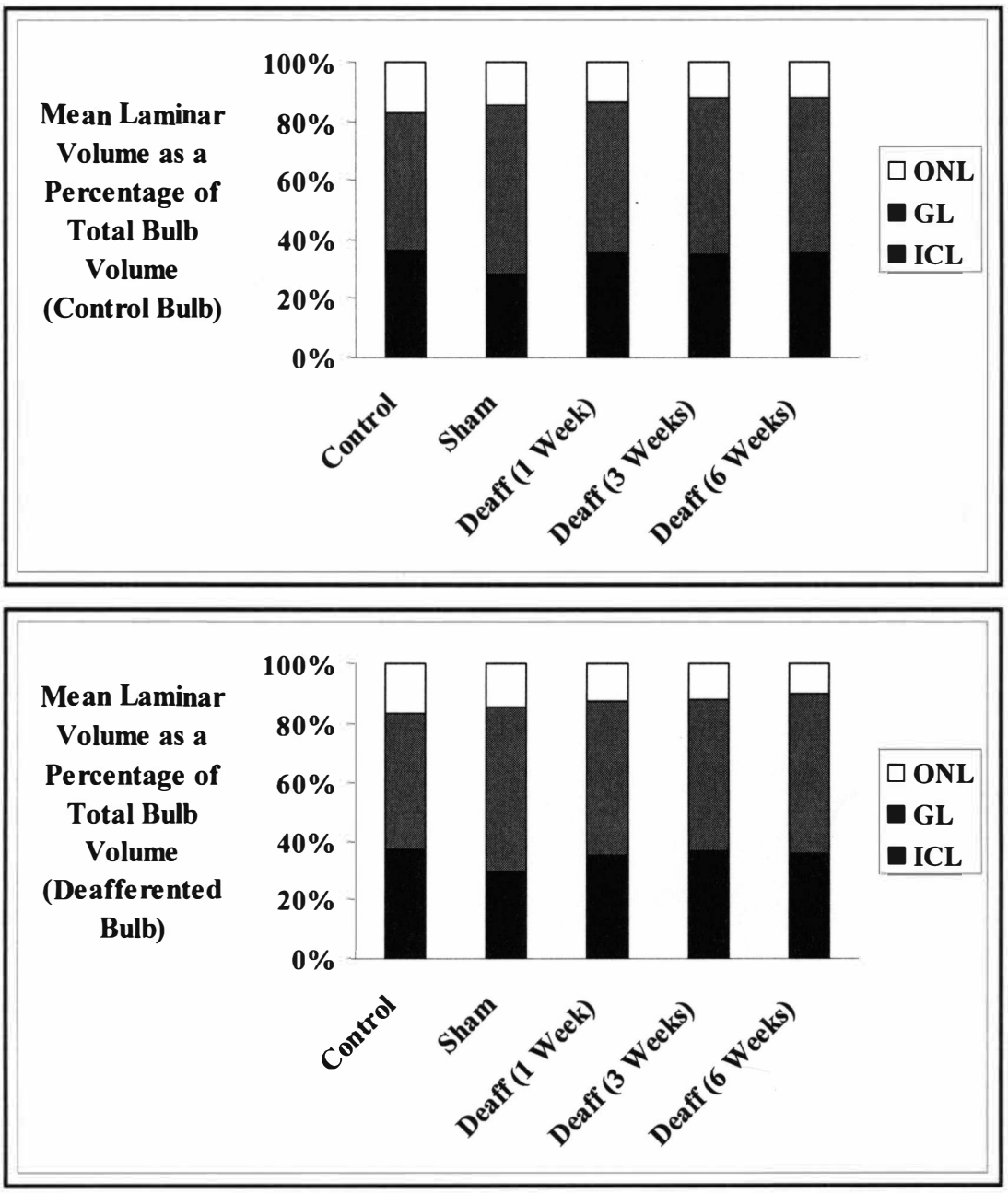

Figure 9. Percent composition of control and deafferented olfactory bulbs in adult zebrafish.

Effect of Deafferentation on the Size Distribution of Nuclear Profiles within the Glomerular and Internal Cell Layers of the Olfactory Bulb

There were relatively few differences between the size distribution of nuclear profiles within either the glomerular layer or the internal cell layer when comparisons were made between the control and deafferented bulbs. Nuclear profiles within the glomerular layer ranged from approximately 2 to 7 μm in diameter, with approximately 58-66% of nuclear profiles measuring only 3 μm in diameter and the vast majority (92-98%) of nuclear profiles measuring between 3 and 5 μm in diameter (Figure 10). Although the sizes of nuclear profiles in the internal cell layer also ranged from approximately 2 to 6 μm, a larger percentage of nuclear profiles within the internal cell layer tended to be smaller, with approximately 80-90% of nuclear profiles measuring only 3 μm in diameter (Figure 11). In experimental animals at all survival intervals, the percentage of nuclear profiles falling under each size category remained relatively consistent between the control and deafferented bulbs for both the glomerular layer and the internal cell layer, as represented in Figure 12 and Figure 13, respectively.

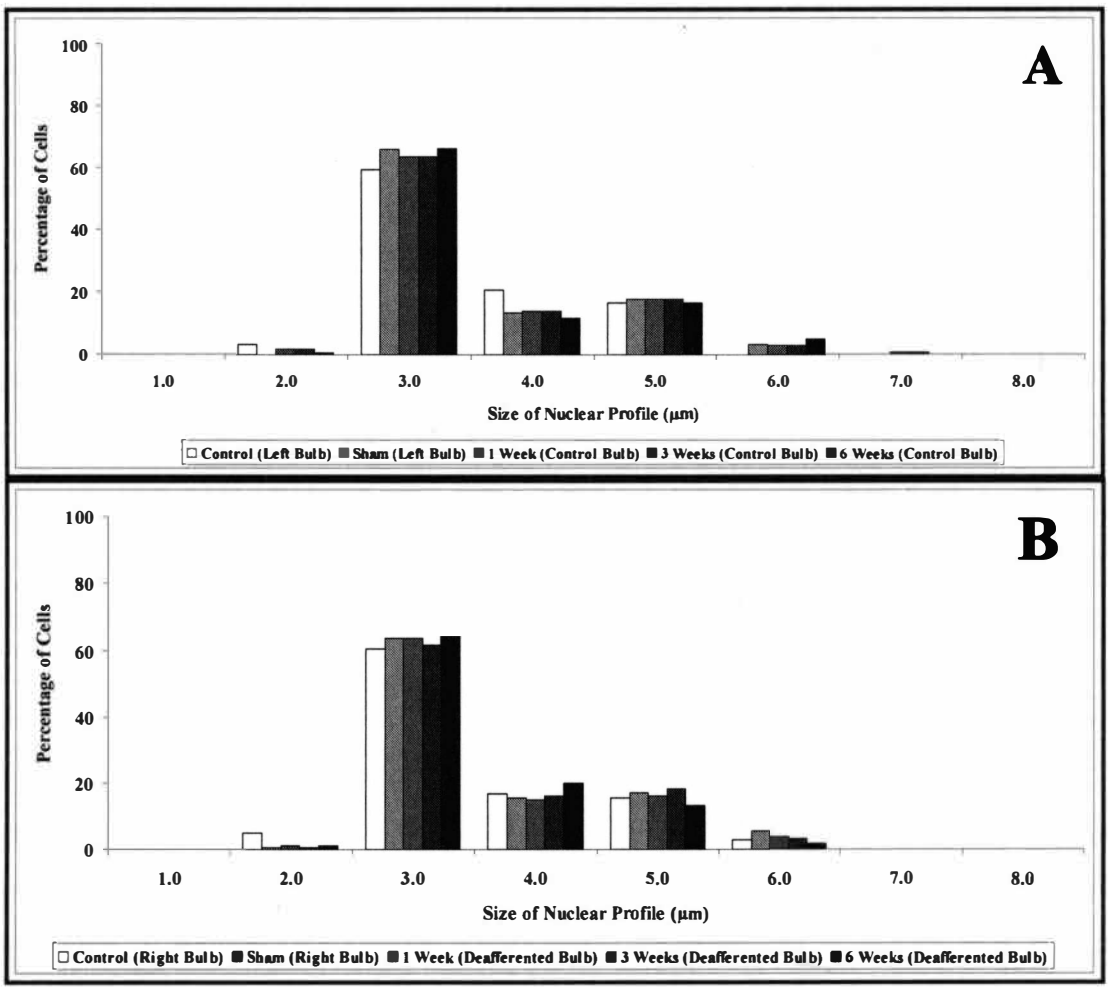

Figure 10. Comparison of the size distribution of nuclear profiles within the glomerular layer of control (**A**) and deafferented (**B**) olfactory bulbs.

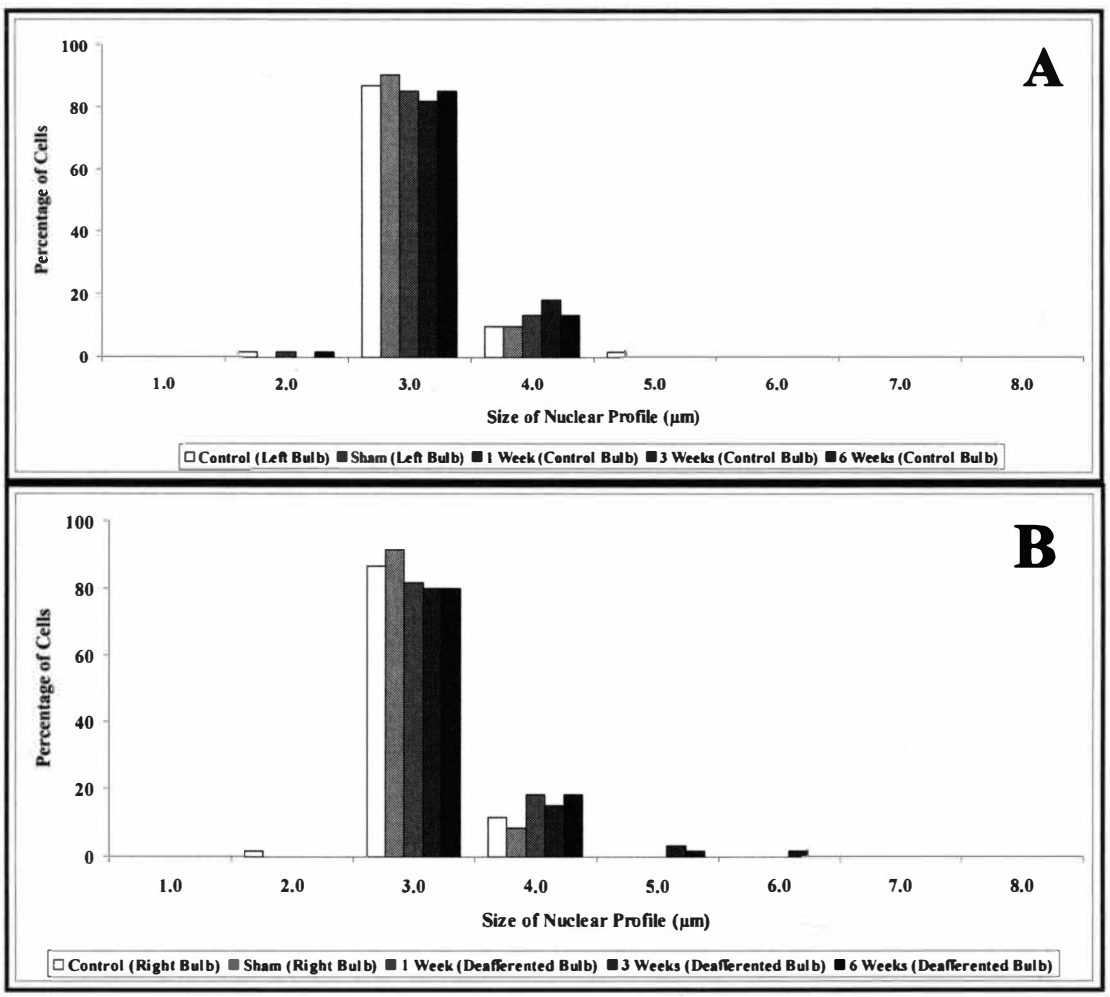

Figure 11. Comparison of the size distribution of nuclear profiles within the internal cell layer of control (**A**) and deafferented (**B**) olfactory bulbs.

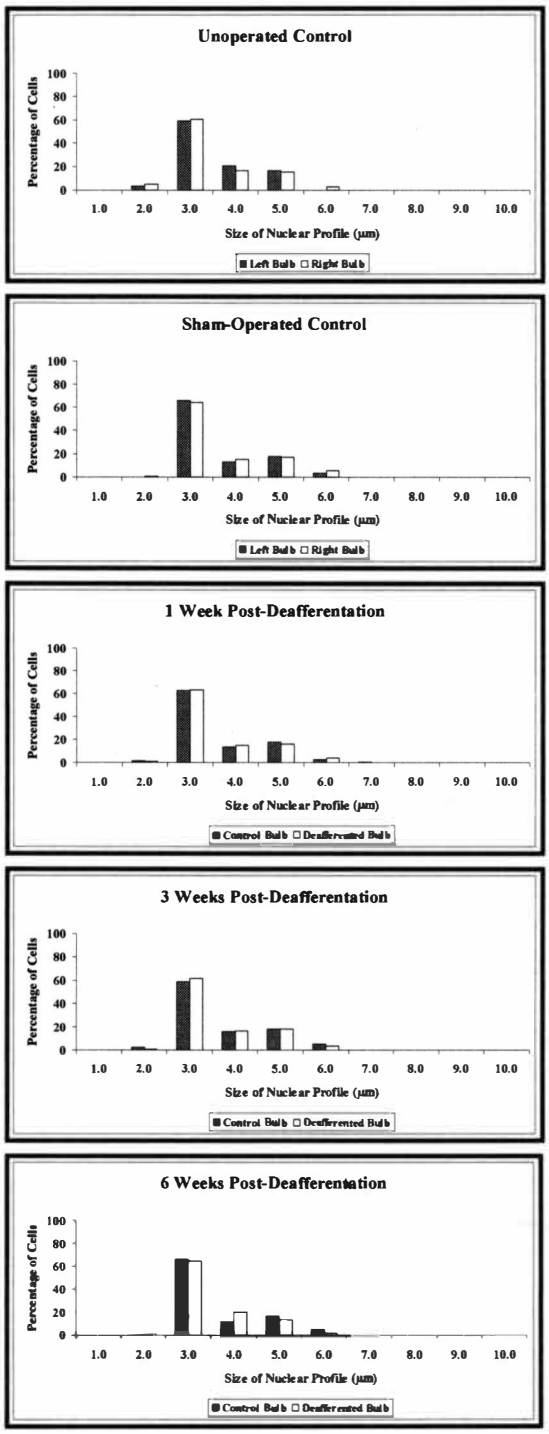

Figure 12. Size distribution of nuclear profiles within the glomerular layer following peripheral deafferentation.

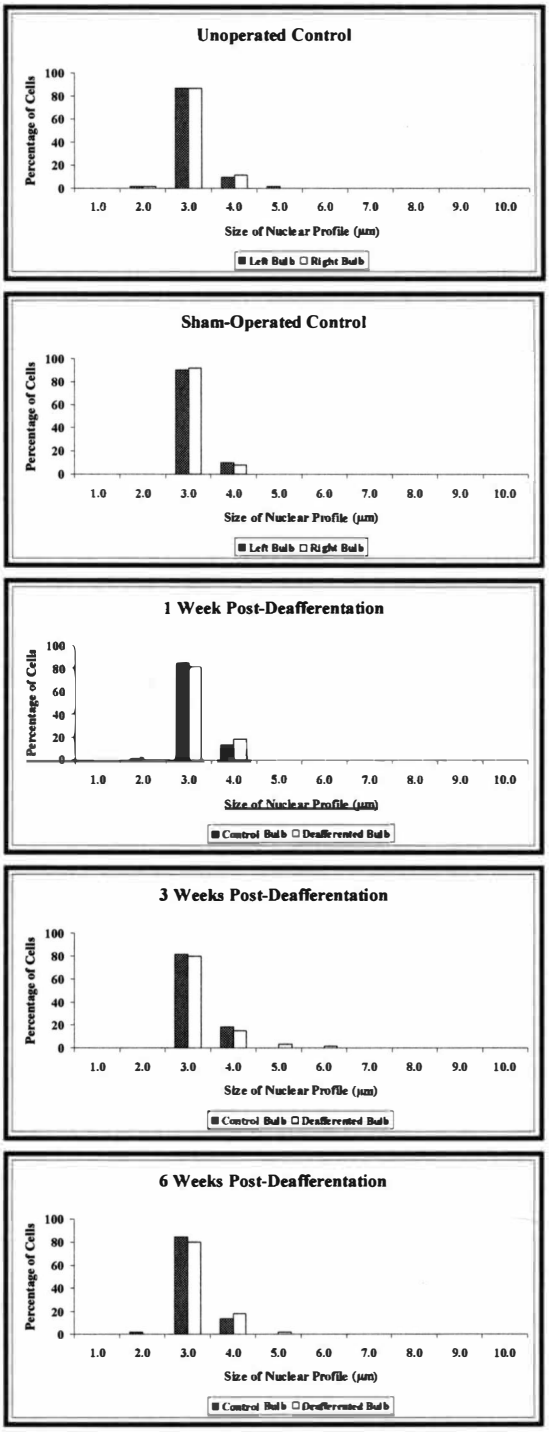

Figure 13. Size distribution of nuclear profiles within the internal cell layer following peripheral deafferentation.

Effect of Deafferentation on Cell Density

Cell Density Changes within the Glomerular Layer

Cell density measurements for the glomerular layer are presented in Table 6 and Figure 14. While there were fluctuations in cell density within the glomerular layer among the experimental animals at the various survival intervals, there did not appear to be a consistent pattern reflected in the cell density differences observed between the control and deafferented olfactory bulbs. As expected, there was no significant difference between the cell density of the glomerular layer in the left and right olfactory bulbs of unoperated control ($P = 0.22$) or sham-operated control ($P = 0.13$) animals. There was little change in glomerular layer cell density observed within the deafferented bulb at 1 week and 6 weeks post-deafferentation ($P = 0.79$ and $P = 0.30$, respectively). Indeed, the magnitude of the percent difference in glomerular layer cell density of the experimental animals at 1 and 6 weeks post-deafferentation was actually less than the magnitude of the percent difference observed between the cell density of the glomerular layer in the left and right olfactory bulbs of unoperated control and sham-operated control animals, respectively. Although the increase in glomerular layer cell density within the deafferented olfactory bulb observed at 3 weeks post-deafferentation was statistically significant ($P < 0.03$), this increase appeared to be a transient effect since there was no consistent pattern of cell density change with increasing survival time post-deafferentation. However, it may be of interest to note that the increase in glomerular layer cell density at 3 weeks post-deafferentation occurred at approximately the same time that the drastic reduction in the laminar volume of the glomerular layer was observed between 1 and 3 weeks post-deafferentation.

Table 6

Effect of Deafferentation on Cell Density within the Glomerular Layer

		Glomerular Layer Cell Density[a] (cells/μm³)		
	n	*Control Bulb*	*Deafferented Bulb*	*% Difference*
Unoperated Control	6	0.00184 ± 0.00023	0.00163 ± 0.00013	↓ 6.58 ± 9.14
Sham-Operated Control	6	0.00135 ± 0.00008	0.00150 ± 0.00004	↑ 12.94 ± 6.86
1 Week Post-Deafferentation	6	0.00144 ± 0.00013	0.00140 ± 0.00018	↓ 1.57 ± 12.18
3 Weeks Post-Deafferentation	6	0.00149 ± 0.00017	0.00188 ± 0.00024	↑ 27.23 ± 8.24*
6 Weeks Post-Deafferentation	6	0.00202 ± 0.00024	0.00219 ± 0.00024	↑ 9.93 ± 6.86

[a] Mean ± S.E.M.; $^{*}P < 0.05$

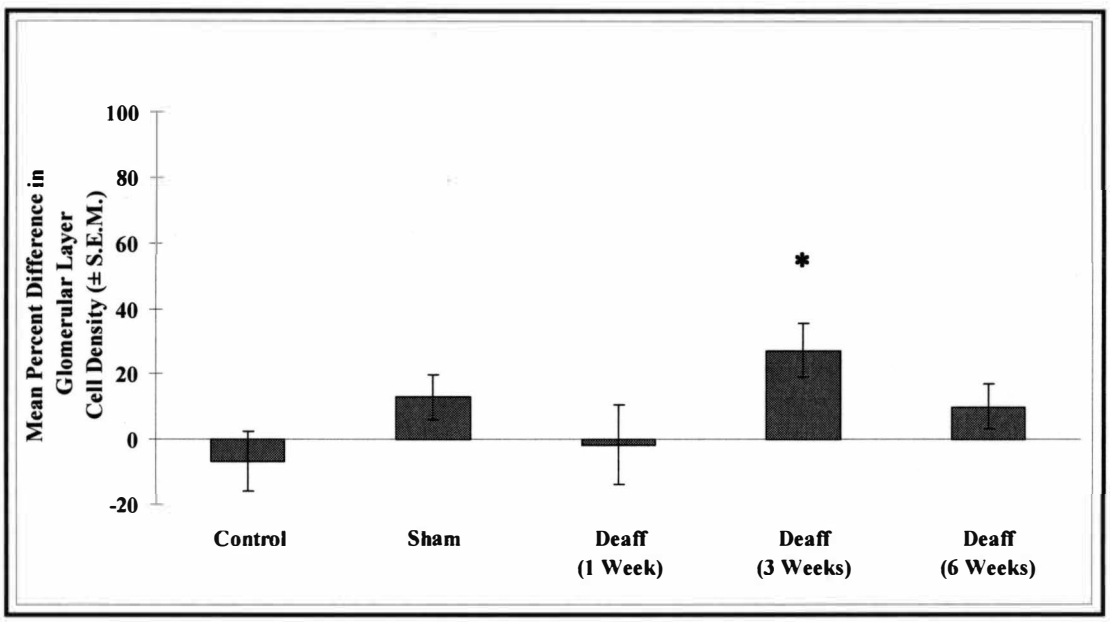

Figure 14. Mean percent difference in cell density within the glomerular layer following peripheral deafferentation ($^{*}P < 0.05$).

Cell Density Changes within the Internal Cell Layer

Cell density measurements for the internal cell layer are presented in Table 7 and Figure 15. Overall, cells were much more densely packed within the internal cell layer compared to the glomerular layer, with cell density values at least 2-3 times as high as those observed in the glomerular layer. As with the glomerular layer, no significant differences were observed in the cell density of the internal cell layer between the left and right olfactory bulbs in unoperated control ($P = 0.84$) or sham-operated control ($P = 0.36$) animals. Although the cell density of the deafferented bulb in experimental animals tended to be slightly higher than the contralateral control bulb at 1, 3, and 6 weeks post-deafferentation, none of the increases were statistically significant ($P = 0.56$, 0.10, and 0.52, respectively). However, it is interesting to note that the smaller fluctuations in cell density observed in the internal cell layer still resembled the pattern observed in the glomerular layer, with the largest change in cell density in both layers occurring at 3 weeks post-deafferentation.

Table 7

Effect of Deafferentation on Cell Density within the Internal Cell Layer

		Internal Cell Layer Cell Density[a] *(cells/μm³)*		
	n	*Control Bulb*	*Deafferented Bulb*	*% Difference*
Unoperated Control	6	0.00517 ± 0.00024	0.00522 ± 0.00021	↑ 1.55 ± 4.32
Sham-Operated Control	6	0.00515 ± 0.00018	0.00500 ± 0.00027	↓ 3.10 ± 2.85
1 Week Post-Deafferentation	6	0.00469 ± 0.00031	0.00491 ± 0.00022	↑ 6.94 ± 8.68
3 Weeks Post-Deafferentation	6	0.00472 ± 0.00039	0.00511 ± 0.00029	↑ 9.78 ± 4.35
6 Weeks Post-Deafferentation	6	0.00582 ± 0.00033	0.00600 ± 0.00032	↑ 3.66 ± 4.33

[a] Mean ± S.E.M.

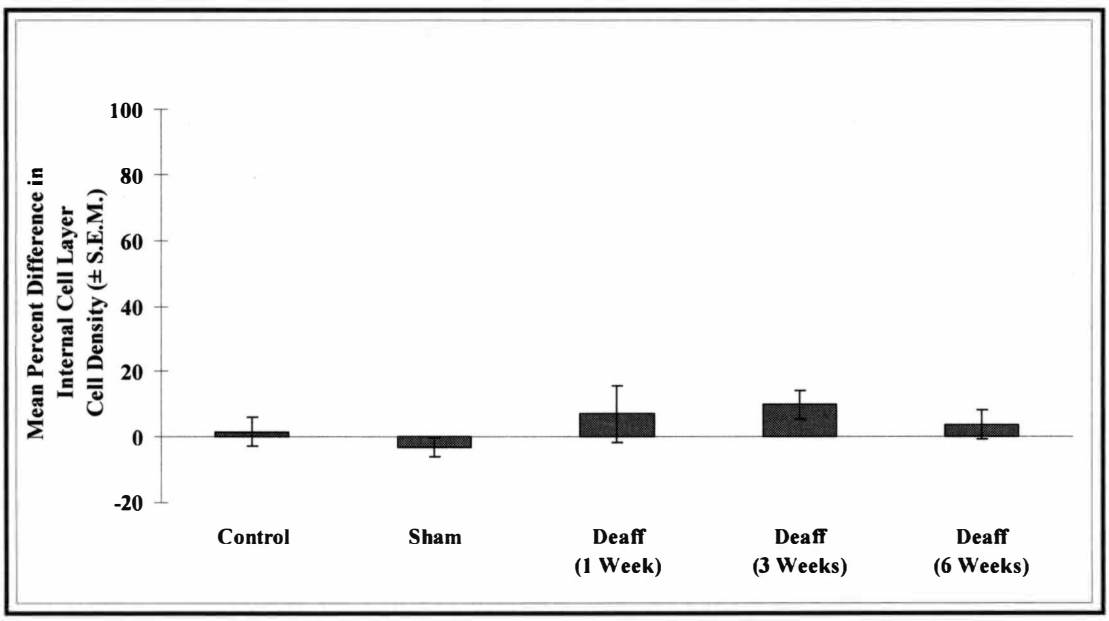

Figure 15. Mean percent difference in cell density within the internal cell layer following peripheral deafferentation ($^{*}P < 0.05$).

Effect of Deafferentation on Cell Number within the Glomerular and Internal Cell Layers of the Olfactory Bulb

Glomerular Layer

Changes in the estimated number of cells within the glomerular layer are summarized in Figure 16. There was no significant difference in estimated cell number within the glomerular layer when comparisons were made between the left and right olfactory bulbs of unoperated control ($P = 0.63$) or sham-operated control ($P = 0.19$) animals. Neither were there statistically significant differences in estimated glomerular layer cell numbers between the contralateral control bulb and deafferented bulb of experimental animals at 1 week ($P = 0.72$), 3 weeks ($P = 0.74$), or 6 weeks ($P = 0.12$) post-deafferentation.

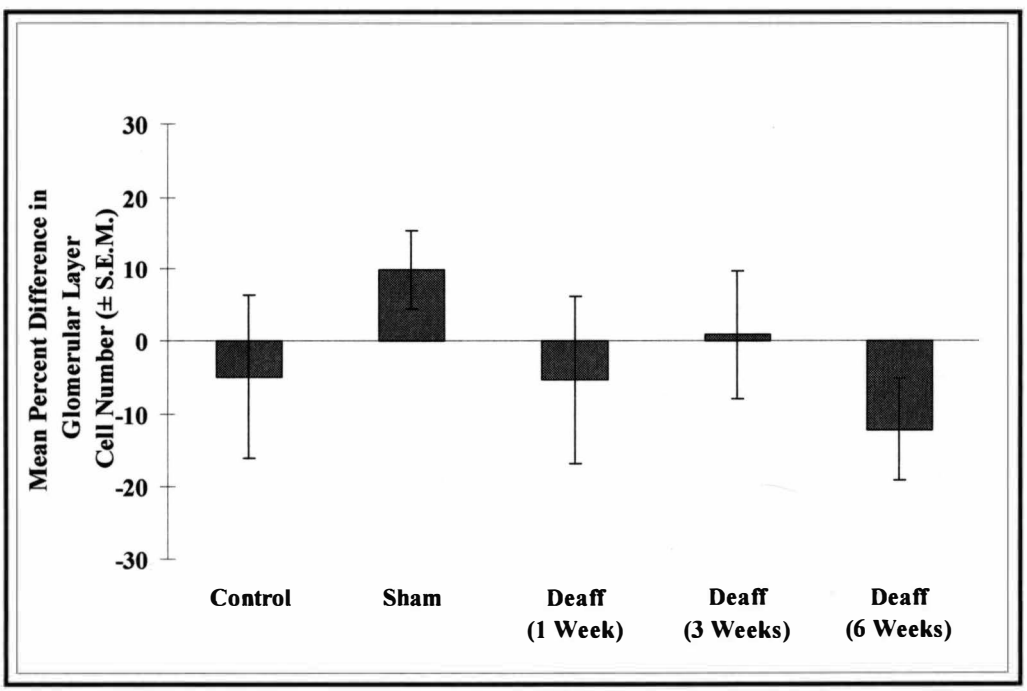

Figure 16. Mean percent difference in estimated cell number within the glomerular layer following peripheral deafferentation.

Internal Cell Layer

As with the glomerular layer, estimated cell numbers within the internal cell layer did not differ significantly between the left and right olfactory bulbs of unoperated control ($P = 0.28$) or sham-operated control ($P = 0.78$) animals (Figure 17). However, the estimated number of cells within the internal cell layer of the deafferented bulb of experimental animals tended to decrease with increasing survival time when compared to the contralateral control bulb within the same animal (Figure 17). Although the decrease was not statistically significant at 1 week ($P = 0.80$) or 3 weeks ($P = 0.54$) post-deafferentation, there were significantly fewer cells within the internal cell layer of the deafferented bulb of experimental animals at 6 weeks post-deafferentation ($P < 0.02$).

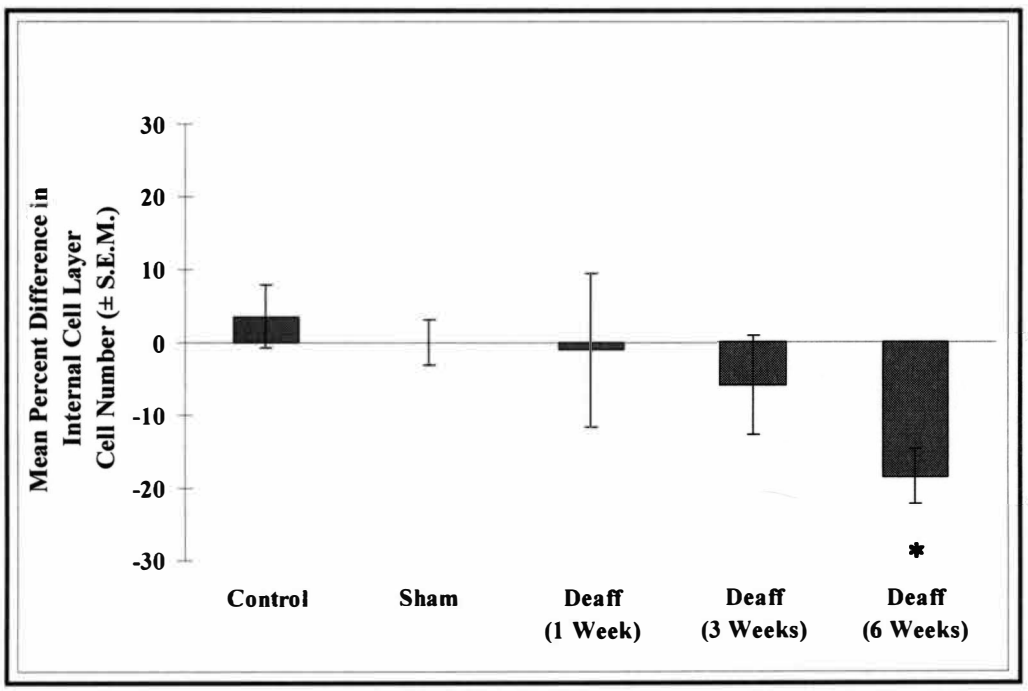

Figure 17. Mean percent difference in estimated cell number within the internal cell layer following peripheral deafferentation ($^{*}P < 0.05$).

Comparison of Control Values

To confirm that the contralateral control bulb of experimental animals was not changing in response to deafferentation, the mean values for total bulb volume, laminar volume, laminar cell density, and estimated laminar cell number obtained for the contralateral control bulb of experimental animals were compared to the mean values obtained for the same parameters in unoperated control animals using two-tailed, unpaired *t* tests, with a P value set at 0.05. With one exception, no significant differences were found between the left bulb of unoperated control animals, the left bulb of sham-operated control animals, or the contralateral control bulb of experimental animals at any survival interval. Although a statistically significant difference ($P < 0.03$) was observed between the laminar volume of the olfactory nerve layer in the control bulb of experimental animals at 6 weeks post-deafferentation when compared to unoperated controls, this could have been a result of differences in the degree to which the olfactory nerve layer remained intact and connected to the olfactory bulb following dissection and processing of the brain tissue, rather than an actual difference between the two laminar volumes as a result of the deafferentation procedure.

CHAPTER IV

DISCUSSION

Complete, unilateral ablation of the olfactory organ and surrounding tissues in adult zebrafish resulted in permanent, peripheral deafferentation of the ipsilateral olfactory bulb. The effects of this deafferentation procedure caused significant morphological changes within the olfactory bulb, including a reduction in total bulb volume and a decrease in the laminar volume of all three layers of the olfactory bulb. Although no significant changes were seen in the size distribution of nuclear profiles in the deafferented olfactory bulb, variations in cell density and cell number within the glomerular layer and internal cell layer were observed. These results provide additional evidence that normal contact between the axons of the olfactory sensory neurons and the olfactory bulb is necessary for the maintenance of the normal morphology of the olfactory bulb in the brain of adult zebrafish.

Effect of Deafferentation on Total Bulb Volume

The significant decrease in total bulb volume observed at 1, 3, and 6 weeks post-deafferentation was consistent with the results of previous experiments using adult zebrafish reported by our laboratory (Byrd, 2000) and other researchers (Poling and Brunjes, 2000), with a trend toward a greater decrease in volume with longer survival time post-deafferentation. Similar results have been reported in mammals following chemical deafferentation (Margolis et al., 1974; Harding et al., 1978) and naris closure (Maruniak et al., 1989). For example, unilateral naris closure for approximately 4 weeks in adult mice resulted in a 17% reduction in the weight of the

ipsilateral olfactory bulb (Maruniak et al., 1989), a decrease similar in magnitude to the 16% reduction in total bulb volume reported here at 3 weeks post-deafferentation.

Interestingly, the reduction in total bulb volume reported here appeared to be a result of a decrease in size among all three layers of the olfactory bulb, rather than the complete loss of one or more specific layers. As expected, however, the greatest reduction in laminar volume was observed in the olfactory nerve layer, which was approximately 36% smaller in the deafferented bulb at 6 weeks post-deafferentation. In comparison, both the glomerular layer and the internal cell layer of the deafferented bulb were approximately 20% smaller than the contralateral control bulb after 6 weeks. While the volume of the internal cell layer decreased gradually and at a relatively constant rate between each survival interval, the volume of the glomerular layer was not significantly smaller at 1 week post-deafferentation but rapidly decreased in size between 1 and 3 weeks post-deafferentation, with little additional change between 3 and 6 weeks post-deafferentation. Therefore, it is clear that even though the end result of the deafferentation procedure was a reduction in size among each of the three layers of the olfactory bulb, not all layers responded to the loss of afferent input in the same way.

Effects of Deafferentation on Specific Laminae within the Olfactory Bulb

Olfactory Nerve Layer

The olfactory nerve layer consists primarily of the axons of the olfactory sensory neurons and glial cells that ensheath the olfactory nerve (Perroteau et al., 1999). The significant reduction in the volume of the olfactory nerve layer observed following ablation of the olfactory epithelium is probably a direct result of the degeneration of the axons of the olfactory sensory neurons that innervate the olfactory

bulb. This explanation is supported by the results of experiments conducted using adult mice, in which peripheral deafferentation following intranasal irrigation with a high concentration of zinc sulfate was reported to result in a gradual but complete loss of olfactory marker protein in the ipsilateral olfactory bulb (Margolis et al., 1974; Perroteau et al., 1999). This protein is a marker for olfactory sensory neurons and its disappearance corresponds to the loss of contact between the olfactory epithelium and the olfactory bulb resulting from the degeneration of mature olfactory sensory neurons following injury. Although the results were not quantified, Perroteau and colleagues (1999) also observed that the thickness of the olfactory nerve layer was remarkably reduced in size with respect to control animals following zinc sulfate deafferentation in adult mice. Other researchers (Harding et al., 1978) have reported the complete absence of the olfactory nerve layer of the deafferented olfactory bulb 3 weeks after zinc sulfate treatment in adult mice.

Glomerular Layer

Similar reductions in the volume of the glomerular layer of the olfactory bulb have also been described following deafferentation in other species. For example, microscopic examination of the olfactory bulb following chemical deafferentation in adult mice revealed a decrease in the gross size of the glomeruli and reduced overall thickness of the glomerular layer (Margolis et al., 1974; Harding et al., 1978; Perroteau et al., 1999). As with the olfactory nerve layer, the degeneration of olfactory sensory neuron axon terminals that make synaptic contact with the dendrites of output neurons in the glomeruli could be one possible explanation for the decrease in laminar volume and glomerular size. Baker and colleagues (1984) observed a 30% reduction in olfactory bulb weight in adult mice 4 weeks after unilateral surgical

deafferentation and attributed this size reduction to the loss of the olfactory nerve layer and attendant glomerular shrinkage. Indeed, electron microscopy of the glomeruli following surgical removal of the olfactory mucosa in adult rats has confirmed that the axon terminals of some olfactory sensory neurons demonstrate signs of degeneration as early as 2 days following the deafferentation procedure, with collective degeneration of large groups of axon terminals within 5 days (Estable-Puig and De Estable, 1969). Interestingly, these degenerating axon terminals appear to be completely engulfed by the processes of astrocytes within the glomeruli, suggesting that one possible explanation for the transient increase in cell density observed within the glomerular layer at 3 weeks post-deafferentation that may warrant further investigation is that there is an influx or proliferation of glia within the glomerular layer in response to the degeneration of olfactory sensory axon terminals. Glial cells that surround the olfactory nerve have been shown to migrate towards the olfactory bulb when axonal contacts are lost following intranasal irrigation with zinc sulfate (Chuah et al., 1995). Anders and Johnson (1990) have also reported an increase in one indicator of astrocytic reactivity within both the olfactory nerve layer and the glomerular layer following transection of the olfactory nerve in adult rats, proposing that the increase in reactivity could have resulted from increased proliferation of astrocytes within the olfactory bulb.

While evidence supports the possibility that the decrease in the volume of the glomerular layer can be attributed, at least in part, to the degeneration of olfactory sensory axon terminals, the extent of the reduction in laminar volume that might be attributed to the effects of deafferentation on the primary output neurons within the olfactory bulb following loss of synaptic contact is not as clear. In mammals, the cell bodies of mitral cells are found primarily in the mitral cell layer and these cells extend

dendrites peripherally through the external plexiform layer to make contact with the axons of olfactory sensory neurons within the glomerular layer. In zebrafish, where the organization of the olfactory bulb is not as clearly delineated, the majority of cell bodies and primary dendrites of mitral cells are found within the glomerular layer of this teleost. There have been conflicting reports with respect to how removal of afferent input affects the survival of mitral cells within the olfactory bulb. While Meisami and Noushinfar (1986) have reported a relative decrease in mitral cell numbers following naris closure in neonatal rats, the same procedure in adult (Henegar and Maruniak, 1991) and neonatal (Benson et al., 1984) mice did not result in a significant reduction in mitral cell numbers within the deafferented olfactory bulb. Although non-specific labeling did not allow for a direct quantification of mitral cell numbers in the present experiment, no change in the size distribution of nuclear profiles within the glomerular layer was observed, suggesting that no single population of cells was preferentially lost following removal of afferent input.

However, even if mitral cell numbers are not affected by loss of sensory input in adult zebrafish, it is still possible that mitral cell morphological changes could contribute to a decrease in the volume of the glomerular layer. Such morphological changes have been reported in these output neurons in the developing olfactory bulb of neonatal rats following naris closure (Meisami and Noushinfar, 1986), including decreased soma size and reduced length of both primary dendrites (that normally make contact with the axons of olfactory sensory neurons) and basal dendrites (that are one of the principle sites of interaction between the mitral cells and granule cells). Shorter primary dendrites as well as a decrease in the extent of mitral cell dendritic arborization within the glomeruli have also been reported in neonatal mice following naris closure (Benson et al., 1984).

In addition to mitral cells, the potential effects of deafferentation on interneurons within the glomerular layer should also be considered. As mentioned previously, the juxtaglomerular cells in zebrafish closely resemble the periglomerular cells found in mammals. A subpopulation of these interneurons are dopaminergic neurons that are believed to modify the response of mitral cells to incoming olfactory signals. Previous experiments within our laboratory have revealed a decrease in the expression of tyrosine hydroxylase in the deafferented olfactory bulb of adult zebrafish (Byrd, 2000). While naris closure in adult mice also has been shown to reduce the expression of tyrosine hydroxylase in these inhibitory neurons (Baker et al., 1984; Baker et al., 1993), other enzymes in the dopamine biosynthetic pathway are still expressed, suggesting that the reduction in the expression of tyrosine hydroxylase is not due to the death of these cells (Baker et al., 1984). Similarly, no deafferentation-induced increase in cell death was observed in the periglomerular regions of the olfactory bulb in adult mice following axotomy of the olfactory nerve (Mandairon et al., 1993). In comparison, chemical deafferentation in adult mice has been shown to result in an increase in the degree to which periglomerular cells were compacted (Margolis et al., 1974; Harding et al., 1978; Perroteau et al., 1999) and Baker and colleagues (1993) hypothesized that the decrease in the size of the glomeruli observed in adult mice following naris closure resulted in a higher density among periglomerular cells. Therefore, the continued survival of juxtaglomerular interneurons in combination with the reduction in laminar volume and glomerular size following peripheral deafferentation might provide another possible explanation for the increase in cell density observed within the glomerular layer of adult zebrafish at 3 weeks post-deafferentation.

Internal Cell Layer

In mammals, unilateral naris closure has been shown to result in a significant reduction in the size of the granule cell layer as well as decreased granule cell numbers in the deprived olfactory bulb of both newborn (Skeen et al., 1985) and adult (Henegar and Maruniak, 1991; Mandairon et al., 2003) mice, with no significant change in cell density. Similar changes were seen here in the internal cell layer of adult zebrafish. Despite the significant reduction in laminar volume observed at 1, 3, and 6 weeks post-deafferentation, there was not a significant increase in cell density at any survival interval, suggesting that one possible explanation for the volume reduction in the internal cell layer is that granule cells or glia within this layer are being lost following removal of sensory input. Increased levels of cell death have been observed within the granule cell layer of deprived olfactory bulbs in both postnatal and juvenile rats following 4 weeks of unilateral naris occlusion (Fiske and Brunjes, 2001). Similarly, increased cell death among granule cells, specifically, has been reported in the odor-deprived bulbs of adult mice 4 weeks following unilateral naris closure (Corotto et al., 1994). In addition, Henegar and Maruniak (1991) found that, following naris closure in adult mice, the closed-side granule cell layer had 30% fewer cells than the open-side granule cell layer, suggesting that overall shrinkage in the closed-side olfactory bulb was due, at least in part, to the loss of granule cells. Although the magnitude of the decrease in estimated cell number within the internal cell layer observed at 6 weeks post-deafferentation in the current project was only about 18%, similar mechanisms could be causing the reduction in cell number observed in this layer of the adult zebrafish olfactory bulb following peripheral deafferentation.

In addition to increased levels of cell death among granule cells already present in the olfactory bulb at the time of deafferentation, another possibility that may warrant further investigation is that the decrease in the laminar volume of the internal cell layer may be a result of reduced proliferation or survival of adult-born granule cells. Adult-born cells labeled with BrdU (a thymidine analog taken up by actively dividing cells during mitosis) have been reported in the internal cell layer of adult zebrafish at 4 weeks following BrdU exposure (Byrd and Brunjes, 2001). In addition to their round nuclear morphology, the co-labeling of a portion of these BrdU-positive nuclei with the Hu antibody (a general neuronal marker) suggests that at least some of the newly formed cells are granule cells (Byrd and Brunjes, 2001). Studies in mice have revealed that newly generated neurons born in the subventricular zone of the adult brain migrate into the olfactory bulb, where a majority of the neuronal precursors differentiate into granule cells (Petreanu and Alvarez-Buylla, 2002). Following an initial period of maturation, however, there is a sharp decline in the number of newly formed neurons in the granule cell layer of anosmic mice (a knock-out strain of mouse that lacks electrical input to the olfactory bulb), suggesting that the survival of adult-born granule cells is dependent upon incoming activity from the olfactory epithelium (Petreanu and Alvarez-Buylla, 2002). Similarly, the reduction in granule cell number reported in the olfactory bulb of adult, wild-type mice following naris occlusion (Henegar and Maruniak, 1991; Corotto et al., 1994; Mandairon et al., 2003) was believed to be due, at least in part, to reduced neurogenesis and reduced survival of these adult-generated neurons (Corotto et al., 1994). Similarly, analysis of newly generated cells in the adult rat olfactory bulb also has revealed that approximately 50% of neural progenitors and young neurons are eliminated by apoptosis unless they receive synaptic input or trophic support (Winner

et al., 2002). Ablation of the olfactory epithelium in our study effectively eliminates electrical activity to the deafferented olfactory bulb, suggesting that the survival of the adult-born granule cells previously observed in the internal cell layer of zebrafish could be affected by removal of sensory input. Evidence that younger neurons are more susceptible to the effects of deafferentation (Skeen et al., 1985) may provide an explanation of why granule cell numbers decrease following removal of primary afferent input, since these cells are believed to be the last cells born in the olfactory bulb.

In addition to possible increases in levels of granule cell death, another factor that might contribute to the reduction in the volume of the internal cell layer could be morphological changes taking place within granule cell populations that remain in the deafferented olfactory bulb. For example, a 20% reduction in the length of apical dendrites of granule cells has been observed following the loss of mitral cells in a mutant strain of adult mice (Greer, 1987). While there is evidence that mitral cell numbers are not affected by deafferentation (Benson et al., 1984; Henegar and Maruniak, 1991), if the reduction in granule cell dendritic length reported by Greer (1987) was a result of the loss of mitral cell activity rather than physical contact, the possibility that changes in granule cell morphology occur as a result of the effect of the deafferentation procedure on mitral cell activity should not be ruled out until additional experiments are conducted.

Future Directions

The results presented here support the conclusion that the overall volume reduction observed in the adult zebrafish olfactory bulb following peripheral deafferentation is due to a reduction in the volume of all three laminae of the olfactory

bulb. The volume reduction observed in each layer suggests that several different populations of cells respond to the loss of sensory input and that these responses may affect the neural relays between olfactory sensory neurons, output neurons, and interneurons within the olfactory bulb. While the scope of the current project did not allow us to address the specific mechanisms that might be responsible for these changes, the results presented here will hopefully provide direction and focus to future investigations into these mechanisms.

There is also much information that may still be learned by refining and extending the experimental techniques utilized in the current project. Peripheral deafferentation resulting from ablation of the olfactory epithelium eliminates both physical contact and activity between the olfactory organ and the olfactory bulb. As a result, one important question that we would like to address in future experiments is whether the changes observed in the deafferented olfactory bulb are due primarily to the degeneration of the axons of olfactory sensory neurons and subsequent loss of physical contact, or whether it is the cessation of electrical activity from the olfactory sensory neurons that affects the postsynaptic neurons within the olfactory bulb. In addition, the non-specific staining techniques utilized here did not allow for comparison of potential changes in specific cell populations between the control and deafferented olfactory bulbs. It would be valuable to use cell-specific labeling techniques to confirm the results presented here as well as determine if there are unique changes taking place among specific neuronal and non-neuronal cell populations in the deafferented olfactory bulb. Finally, it should be noted that the results presented here with respect to cell density, estimated cell number, and the size distribution of nuclear profiles within the glomerular and internal cell layers were based upon counts and measurements obtained from the median section of each bulb,

which may or may not be an accurate reflection of changes that could be taking place in other regions of the olfactory bulb.

Further quantification may provide additional insight into the changes taking place within the olfactory bulb following peripheral deafferentation that will allow us to expand our current understanding of the neuronal plasticity observed in the olfactory system and reveal the possible implications this increased understanding might have on research currently being conducted in the areas of brain injury and neurodegenerative disorders, memory and learning, and the normal physiological and functional relationships that exist between populations of neurons in the peripheral and central nervous system.

Appendix

Institutional Animal Care and Use Committee Protocol Approval Forms

WESTERN MICHIGAN UNIVERSITY

Institutional Animal Care and Use Committee

Centennial 1903-2003 Celebration

Date: June 15, 2004

To: Christine Byrd, Principal Investigator

From: Robert Eversole, Chair

Re: IACUC Protocol No. 04-06-01

Your protocol entitled "Physical and Chemical Deafferentiation of the Olfactory Bulb of Adult Zebrafish" has received approval from the Institutional Animal Care and Use Committee. The conditions and duration of this approval are specified in the Policies of Western Michigan University. You may now begin to implement the research as described in the application.

The Board wishes you success in the pursuit of your research goals.

Approval Termination: June 15, 2005

Walwood Hall, Kalamazoo MI 49008-5456
Phone: (616) 387-8293 Fax: (616) 387-8276

WESTERN MICHIGAN UNIVERSITY

Institutional Animal Care and Use Committee

ANNUAL REVIEW OF VERTEBRATE ANIMAL USE

PROJECT OR COURSE TITLE: Physical And Chemical Deafferentation Of The Olfactory Bulb Of Adult Zebrafish

IACUC Protocol Number: 04-06-01

Date of Review Request: 05/03/05 Date of Last Approval: 06/15/04

Purpose of project (select one): ☐Teaching ☒Research ☐Other (specify):

PRINCIPAL INVESTIGATOR OR ADVISOR

Name: Christine Byrd Title: Assoc./Assist. Professor

Department: BIOS Electronic Mail Address: christine.byrd@wmich.edu

CO-PRINCIPAL OR STUDENT INVESTIGATOR

Name: Cynthia Fuller Title: Student / Travis Devlin title: student

Department: BIOS Electronic Mail Address: cynthia.deyoung@wmich.edu / BIOS travis.devlin@msn.com

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? ☐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: 05/01/05 Years covered by the search: 1980-present

     Key words: zebrafish, olfactory, deafferentation, denervation, ablation
   - ☐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): 30 Cumulative number of animals used to date: 100

Christine Byrd 05/03/05
Principal Investigator/Faculty Advisor Signature Date

Cynthia Fuller 05/03/05 [signature] 5-2-05
Co-Principal or Student Investigator Signature Date Student 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.

[signature] 5/5/05
IACUC Chair Signature Date

Revised 10/01 WMU IACUC
All other copies obsolete.

WESTERN MICHIGAN UNIVERSITY

Institutional Animal Care and Use Committee

ANNUAL REVIEW OF VERTEBRATE ANIMAL USE

PROJECT OR COURSE TITLE: Physical And Chemical Deafferentation Of The Olfactory Bulb Of Adult Zebrafish

IACUC Protocol Number: 04-06-01

Date of Review Request: 05/03/06 Date of Last Approval: 05/03/05

Purpose of project (select one): ☐ Teaching ☒ Research ☐ Other (specify):

PRINCIPAL INVESTIGATOR OR ADVISOR

Name: Christine Byrd Title: Assoc./Assist. Professor

Department: BIOS Electronic Mail Address: christine.byrd@wmich.edu

CO-PRINCIPAL OR STUDENT INVESTIGATOR

Name: Travis Devlin Title: Student

Department: BIOS Electronic Mail Address: travis_devlin@msn.com

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? ☐ 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
     - ☐ Biological Abstracts
     - ☐ Current Research Information Service (CRIS)
     - ☒ Medline
     - ☐ Other (please specify):

     Date of search: 05/01/06 Years covered by the search: 1980-present

     Key words:
   - ☒ Additional search strategy narrative: personal communication with other researchers in this field
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): 30 Cumulative number of animals used to date: 200

/ [signature] 5/2/06
Principal Investigator/Faculty Advisor Signature Date

/ [signature] 5.2.06
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.

/ [signature] 5/3/06
IACUC Chair Signature Date

Revised 10/01 WMU IACUC
All other copies obsolete.

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