Olfactory Bulb Neurons of Adult Zebrafish Morphology, Distribution, Cellular Interactions and Structural Stability following Deafferentation

Cynthia L. Fuller
Western Michigan University

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OLFACTORY BULB NEURONS OF ADULT ZEBRAFISH MORPHOLOGY,
DISTRIBUTION, CELLULAR INTERACTIONS AND STRUCTURAL
STABILITY FOLLOWING DEAFFERENTATION

by

Cynthia L. Fuller

A Dissertation
Submitted to the
Faculty of The Graduate College
in partial fulfillment of the
requirements for the
Degree of Philosophy
Department of Biological Sciences
Dr. Christine Byrd, Advisor

Western Michigan University
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The zebrafish is becoming an increasingly popular model for studies involving olfactory function, yet there is still much to be learned about the anatomy and circuitry of different cell types in the olfactory bulb. This study focuses on identifying the morphology and distribution of output neurons and interneurons in the olfactory bulb of adult zebrafish, *Danio rerio*. Furthermore, this investigation examines the cellular interactions of the primary output neuron, the mitral cell, and addresses the issue of neuronal plasticity by considering the structural stability of this cell type following loss of afferent innervation.

Using retrograde tract tracing with various dextran labels and live tissue culture, we were able to label several types of output neurons in the olfactory bulb including mitral cells, ruffled cells, and ganglion cells of the terminal nerve. Mitral cells were the most numerous output neurons in the olfactory bulb. These cells, located primarily in the glomerular layer and superficial internal cell layer, had variable-shaped somata that ranged in size from 5-20μm in diameter and 22-156μm² in surface area. These cells typically had a single dendritic tuft, although some cells possessed multiple primary dendrites. Even mitral cells with multiple dendrites appeared to contact a single glomerulus, a finding that suggests olfactory coding in these teleosts may be more similar to mammals than previously suggested. This information
provides further background for olfactory coding and processing studies in this key model system.

Another focus of this study was to examine the structural integrity of mitral cells following denervation of the olfactory nerve. A comparison of dendritic complexity at 3 to 5 months post-deafferentation showed that mitral cell morphology was affected by loss of afferent input. This investigation demonstrated that innervation was required in order to maintain dendritic complexity by 8 weeks after injury, but did not appear to alter significantly major dendritic processes even 5 months following ablation. This finding suggests that mitral cells are extremely stable structures that may be capable of reforming synaptic contacts if afferent targets are re-established following injury.
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Cynthia L. Fuller
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CHAPTER I
INTRODUCTION

General Introduction

Neurology has become an increasingly recognized discipline in today's world because of the number of degenerative disorders, ailments, and injuries that involve loss of brain function in humans. Whether researchers examine the molecular biology of the mammalian hippocampus, the physiology of the avian auditory system or the anatomy of the fish olfactory bulb, these studies contribute to our general understanding of the brain as a whole. Further, with recent advances in disease models, the scientific community is better able to examine the effects of injury on the anatomy and physiology of specific areas in the brain. Collectively, these studies contribute to our ability to understand the function and, more importantly, the recovery of the human brain following injury or disease. Such foundations continue to lead to advances in treatments for conditions such as Parkinson's, Alzheimer's, stroke, and epilepsy, to name a few.

The Zebrafish

The zebrafish, Danio rerio, is a popular model in the scientific community and has been used in a number of disciplines including: cellular biology (Kim et al., 1997), molecular biology (Weth et al., 1996), genetics (Ardouin et al., 2000), histology (Byrd and Brunjes, 1995), neurobiology (Fetcho and Liu, 1998; Byrd and Brunjes, 2001), developmental biology/embryology (Dynes and Ngai, 1998; Brown et al., 2000), behavior (Fetcho and Liu, 1998), and physiology (Friedrich and Korsching, 1998), to name a few. There are a number of reasons why the zebrafish is
a good model system. For example, this animal is easy to maintain, fairly inexpensive to purchase, and reproduces efficiently. Furthermore, zebrafish eggs and larvae are transparent, which makes them strong candidates for developmental studies. There also is substantial existing knowledge regarding this species and since some zebrafish genes are homologous to human genes (Morizot, 1991; Ardouin et al., 2000), studies suggest that once large numbers of genes have been mapped in zebrafish it will be possible to determine where those genes are located in the human genome (Postlethwait et al., 1999). As a result, the data generated from studies on this fish could have enormous influence on disease research in humans.

The zebrafish is also an exceptional model for studies of the olfactory system (Baier et al., 1994; Byrd, 1995; Dynes and Ngai, 1998; Whitlock and Westerfield, 1998; Friedrich and Laurent, 2001; Edwards and Michel, 2002). First, the olfactory epithelium in this animal is much more accessible than the mammalian olfactory epithelium, which is surrounded by a bony nasal cavity. This allows more ease with experimental manipulations to the nose and is much less invasive than similar procedures in mammals. As a result, fish are able to recover quickly and have high survival rates after injury. In addition, gaining access to the olfactory bulb and brain is less difficult in the zebrafish because the casing surrounding these structures is much thinner and can be removed with forceps, unlike mammals and other larger teleosts, where micro-rongeurs are often required to chip away at this housing and can cause damage to the underlying tissue.

While numerous studies have begun to characterize the anatomy of the olfactory system in this species (Baier and Korsching, 1994; Byrd, 1995; Edwards and Michel, 2002), there is still little known about the structure and function of the different cell types within the olfactory bulb. In order to gain further understanding of
the way zebrafish process olfactory information, many researchers have focused on
the functional aspects of the olfactory system, such as odor representation and
stimulation (Corotto et al, 1996; Freidrich and Korsching, 1998; Friedrich and
Laurent, 2001; Edwards and Michel, 2002; Lipschitz and Michel, 2002; Michel et al,
2003). While some of these studies have touched upon characteristics of zebrafish
bulbar neurons, a complete analysis is lacking. Thus, this study will examine
olfactory bulb neurons in detail to provide further insight into information processing
and potential plasticity in this key model system.

The Olfactory System

The olfactory system is excellent for studies involving plasticity of the
nervous system. The adult olfactory bulb is among the most plastic structures in the
brain. Olfactory sensory neurons in the nose send axons to the olfactory bulb where
they synapse with bulbar neurons. There is normal turnover of these sensory neurons
in the olfactory epithelium, which results in the need for continual identification of
new targets and formation of new synapses within the bulb (for review, see Farbman,
1992). Furthermore, the olfactory bulb itself undergoes continuous neurogenesis
(Altman, 1969; Kaplan and Hinds, 1997; Burd and Sein, 1998; Peretto et al., 1999;
Byrd and Brunjes, 2001; Iwai et al., 2003, Stenman et al., 2003), which adds to the
plasticity of this sensory system. Consequently, the olfactory system is a logical
model for studies involving restructuring and recovery after injury or disease.

The olfactory system has been studied in a variety of animals including
mammals (Farbman and Margolis, 1980; Graziadei et al., 1980; Greer and Shepherd,
1982; Hinds et al., 1984; Shipley and Adamek, 1984; Menco and Farbman, 1985;
Mori et al., 1985; Meredith, 1986; Wilson and Leon, 1988; Guthrie and Leon, 1989;
Baker, 1990; Finger et al., 1990; Buck and Axel, 1991; Brunjes, 1994; Puche et al., 1996; Sullivan et al., 1996; Menco and Jackson, 1997; Mombaerts, 1999), birds (Graziadei and Bannister, 1967; Wenzel and Sieck, 1972; Clark and Smeraski, 1990), reptiles (Halpern and Kubie, 1980; Wang and Halpern, 1988; Lanuza and Halpern, 1998), amphibians (Graziadei and Metcalf, 1971; Getchell, 1974; 1977; Silver et al., 1988; Byrd and Burd, 1991; Burd, 1992; Byrd and Burd, 1993; Firestein et al., 1993; Hansen et al., 1998; Higgs and Burd, 1999), fishes (Byrd and Caprio, 1982; Kosaka and Hama, 1982; Satou, 1990; Hansen and Zeiske, 1993; Ngai et al., 1993; Restrepo et al., 1993; Baier et al., 1994; Byrd and Brunjes, 1995; Kang and Caprio, 1995; Friedrich and Korsching, 1997; Byrd and Brunjes, 2001; Sorensen et al., 1998; Whitlock and Westerfield, 1998; Byrd, 2000; Edwards and Michel, 2002) and a number of invertebrates (Kaissling, 1986; McClintock and Ache, 1989; Tolbert and Oland, 1989; Vogt et al., 1989; Michel et al., 1991; Emery, 1992; Bargmann et al., 1993; Chase and Tolloczko, 1993; Krull et al., 1994; Carlson, 1996; Kaissling, 1996; Christensen and Hildebrand, 1997; Oland et al., 1998).

In vertebrates, this sensory system is generally comprised of a peripheral, receptive olfactory epithelium, the olfactory bulb, the olfactory tracts, and the olfactory cortical areas that process information. In most terrestrial vertebrates, the system also includes a vomeronasal organ and an accessory olfactory bulb (Farbman, 1992). This organ, which is noted for its connections to pheromonal-processing, apparently is absent in fish, birds, and higher primates (Farbman, 1992). The olfactory epithelium is comprised of a mucous membrane that is housed in the nasal cavity and comes into contact with the external environment, as well as the lamina propria, which is an underlying layer made up of connective tissue, blood vessels, and glands (Farbman, 2000).
The major cell type in the olfactory epithelium is the sensory neuron, which comprises about 70-80% of the total cells (Farbman, 2000). These sensory neurons extend their dendrites to the surface of the nasal epithelium where they receive information about odors from the outside environment via olfactory receptors. Thus, they also are termed olfactory receptor neurons. It should be noted that primary sensory neurons vary in dendritic morphology. In most fish, for example, there are at least two morphologies of sensory neurons including ciliated and microvillar (Laberge and Hara, 2001). It has been implied that microvillar neurons respond to pheromones while ciliated neurons detect amino acids (Zielinski and Hara, 1988, Zippel et al., 1997; Hansen et al., 2003).

It also has been suggested that the four different classes of odorants detected by fish, including amino acids, gonadal steroids, bile salts, and prostaglandins (Hara, 1994), are detected by different transduction mechanisms (Laberge and Hara, 2001). Whereas the current consensus is that most receptors transmit odorant-binding information via a second messenger pathway (for review, see figure 1), it also is known that different types of sensory neurons can express different families of receptor molecules and G-proteins (Jones and Reed, 1989; Buck and Axel, 1991; Shinohara et al., 1992; Jia and Halpern, 1996; for review, see Mori et al., 2000; Hansen et al., 2003). Thus, there also is agreement upon the variation that exists between these signal transduction cascades and the mechanisms involved in odorant perception.
Figure 1. A schematic representation summarizes potential intracellular signal transduction pathways used in olfactory processing. There are several suggested pathways for odorant perception, but the most commonly agreed upon pathway includes a receptor protein (seen here docking with an odorant attached to an odorant binding protein), a GTP-binding protein (G1, G0 for example), an adenylyl cyclase (AC) and a gated cation channel that is activated by adenosine 3',5'-cyclic monophosphate (cAMP; Ache and Restrepo, 2000). The other suggested pathway includes a different receptor, a different GTP-binding protein, a phospholipase C (PLC) and a gated cation channel activated by inositol 1,4,5-triphosphate (IP3; Ache and Restrepo, 2000). These initial pathways also can trigger secondary pathways such as calcium-activated chloride channels or calcium-activated potassium channels that carry some of the transduction current (Ache and Restrepo, 2000).

Olfactory Bulb Structure

Another form of variation that exists in the olfactory system is exhibited in the differences between olfactory bulb structures in different animals. One significant distinction between the olfactory systems of lower vertebrates and mammals is in the layering of the olfactory bulb. Mammals have a laminar olfactory bulb with well-characterized cell types in each discrete layer (Shepherd, 1972). The organization of the teleost olfactory bulb, in contrast, is much less obvious (Figure 2). It does, however, contain several diffuse layers: the olfactory nerve layer (ONL), the glomerular layer (GL), the mitral cell layer (MCL), and the granule cell layer (GCL; Oka et al., 1982; Satou, 1990; Byrd and Brunjes, 1995). Because the MCL and GCL
overlap substantially, it is hard to distinguish them. As a result, they are collectively termed the internal cell layer (ICL; Byrd, 2000). Furthermore, unlike mammals fish have both a lateral and medial olfactory tract where axons from mitral cells bundle and project to the brain. It has been suggested that the medial tract processes pheromonal information and mediates reproductive behavior while the lateral tract conveys information about food (Satou et al., 1983; Stacey and Kyle, 1983; Demski and Dulka, 1984; Satou et al., 1984; Kyle et al., 1987; Sorensen et al., 1991; Hamdani, Alexander and Doving, 2001; Hamdani, Kasumyan and Doving, 2001; Weltzien et al., 2003). As a result, differences between the output neurons projecting to the medial or the lateral olfactory tract could provide important information about olfactory coding in the teleost olfactory bulb.

Olfactory Bulb Circuitry

The olfactory receptor neurons project their axons to glomeruli in the olfactory bulb. Glomeruli are spherical regions of neuropil containing numerous neuronal processes and synapses. In glomeruli the axons of these primary olfactory receptor neurons synapse with the dendrites of secondary neurons, such as juxtaglomerular cells or mitral cells (Figure 3). The juxtaglomerular cells are local interneurons, located in the glomerular layer of the olfactory bulb. Mitral cells are projection neurons that carry information out of the olfactory bulb to the main olfactory cortical areas, including the anterior olfactory nucleus, the olfactory tubercle, the posterior pyriform cortex, the amygdala, the entorhinal cortex, as well as parts of the hippocampus and hypothalamus.
Mitral cells can contact a group of cells known as the granule cells, which are interneurons located in the deeper layers of the bulb (Figure 3). Also, mitral cells can contact another type of output neuron, the ruffled cell, through dendro-dendritic synapses (Figure 3). Juxtaglomerular cells, granule cells, and ruffled cells modify the output of the mitral cells. So mitral cells represent a functionally important class of neurons that integrate chemical signals. Consequently, this study will attempt to further examine mitral cells in the teleost system.
Deafferentation

A portion of this project focuses on examining alterations in the morphology and plasticity of neurons following loss of afferent input. Several studies have shown that changes in afferent input from the olfactory organ can result in drastic changes to the olfactory bulb. For example, ablation of the nasal epithelium in zebrafish (Byrd, 2000) and unilateral naris closure in rat pups (Brunjes, 1985) result in reduction of bulb size and loss of volume. Additional studies suggest that unilateral olfactory deprivation results in modification of olfactory bulb anatomy, chemistry, and function in vertebrates (Wilson and Wood, 1992; Brunjes, 1994; Fuller et al., 2005). These findings support the fact that reorganization of the olfactory bulb occurs following activity blockade or removal of the olfactory organ.

Many investigators have used deafferentation to examine injury. Researchers have used chemical deafferentation of the nasal epithelium as a means of testing permanent and temporary injury as well as recovery in mammals (Nadi et al, 1981). Other methods by which loss of afferent input has been analyzed include removal of olfactory epithelium precursors via surgery and transaction of the nerve (Matthew and Powell, 1962; Sanes et al, 1977; Graziadei, 1980; Stout and Graziadei, 1980; Meisami and Hamedi, 1986; Oland and Tolbert, 1987; Byrd and Burd, 1993). In all of the above studies, loss of afferent input has caused drastic changes in the development, the anatomy, or the function of the bulb or antennal lobe.
Figure 3. A schematic representation of the zebrafish olfactory bulb shows the circuitry of cellular interactions. The olfactory sensory neurons, or primary sensory neurons, are located in the olfactory epithelium and project their axons into the olfactory bulb. Here, they make synaptic contacts with the primary output neurons of the bulb, the mitral cells, and the interneurons, juxtaglomerular cells. Other cell types including the ruffled cell and the granule cell can contact mitral cells and modify their output to higher brain centers.

Deafferentation has been used as source for examining injury in other systems as well. For example, it has been noted that lesions to photoreceptor axons have caused changes in the formation of the optic ganglion in invertebrates (Power, 1943; Meyerowitz and Kankel, 1978). In the vertebrate visual system, reduction of afferent input resulted in an alteration of molecular activity (Wong-Riley, 1979; LeVay et al, 1980; Zhang et al, 1995; Pires et al, 1998; Yan and Ribak, 1998). Additionally, investigators have evaluated the role of afferent input and injury in the auditory system (Levi-Montalcini, 1949; Parks, 1979; Garden et al, 1995; Zheng et al, 1998; Edmonds et al, 1999; Russell and Moore, 1999) and motor-cortical region of birds (Johnson et al., 1997). Thus, results from an olfactory deafferentation study may be
applicable to other sensory systems in other models.

**Goals of This Dissertation**

The following dissertation examines the olfactory system in the adult zebrafish, *Danio rerio*. The first goal of this study is to characterize the morphology and distribution of both output neurons and interneurons in the olfactory bulb of untreated animals, employing various methods of visualization. In addition, cellular interactions between the primary and secondary neurons of the olfactory bulb are addressed. Finally, the plasticity of this central nervous system structure is examined by analyzing changes in protein immunoreactivity and cellular morphology following peripheral injury and subsequent loss of afferent innervation.
CHAPTER II

IDENTIFYING THE MORPHOLOGY AND DISTRIBUTION OF JUXTAGLOMERULAR CELLS IN THE OLFACTORY BULB USING A TRANSGENIC ZEBRAFISH LINE

(Collaboration with Drs. Dan Goldman and Steven Suhr, University of Michigan)

Introduction

The cytomegalovirus (CMV) gene promoters are among the most potent mammalian enhancer elements known and often are used as promoters in the development of transgenic animals because of their ubiquitous expression. In this study, a simian CMV promoter was used to drive the expression of a fluorescent reporter protein, dsRED. The construct, which was injected into zebrafish eggs, was comprised of the CMV promoter, a dsRED protein-encoding region, and an SV40 polyA signal that was added to prevent rapid degradation of the transcript (Figure 4). These injections yielded a transgenic population that showed dsRED expression in a highly specific and consistent pattern. The expression was limited primarily to portions of the nervous system including cells in the retina, cerebellum, spinal cord and olfactory bulb. The focus of this study was to establish the identity of the olfactory bulb cells expressing the transgene and to use this information as a basis for determining the morphology and distribution of specific cell types in the olfactory bulb of adult zebrafish.
Figure 4. A schematic diagram shows the steps taken to develop the transgenic zebrafish as well as a diagram of the sCMV-dsRED expression cassette. A) 1. The dsRED expression plasmid was only one of several constructs injected into the zebrafish eggs. 2. The mix of plasmids was introduced into fish eggs at the single-cell stage by microinjection. 3. Fish that had the potential for germ cell transmission were identified by the red fluorescence in F0 animals. 4. Positive F0s were mated and positive F1 progeny also were identified by fluorescence. 5. Positive F1s were screened for the presence of the co-injected plasmid by PCR analysis of tail DNA. These animals were out-crossed to wild-type fish to perpetuate the lines. B) The expression vector was comprised of a 990bp sCMV promoter, a dsRED protein-encoding region, and an SV40 polyA signal.

Materials and Methods

Transgenic Fish

Simian CMV-dsRED transgenic lines were created by the injection of plasmid constructs into fertilized fish eggs by standard methods (Figure 1). Positive F0 fish were identified by the presence of dsRED expression at 10 days post-fertilization. These animals were mated to produce an F1 population, which also was screened at 10 days post-fertilization for dsRED expression. Positive F1s were out-crossed to wild-type fish to perpetuate the transgenic line.

Immunocytochemistry

Adult zebrafish were over-anesthetized with tricaine and perfused transcardially with phosphate-buffered saline (PBS) followed by 4%
paraformaldehyde in PBS. The fish were then incubated overnight in the fixative solution. Following dissection, brains were embedded in gelatin, sectioned at 10-20μm on a cryostat, and mounted on positively charged slides. Olfactory bulb sections were blocked with 3% normal goat serum and 0.4% triton X-100 in PBS and treated with primary antibody (anti-tyrosine hydroxylase, ImmunoStar, 1:1000 in blocker, or anti-HuC/HuD, Molecular Probes, 1:100 in PBS with 1% BSA) for 24 hours. After rinsing in PBS, slides were incubated in biotinylated secondary antibody (goat anti-mouse IgM, Jackson ImmunoResearch, 1:100 in blocking solution) for 1 hour, and antibodies were visualized using fluorescent avidins (Molecular Probes, 1:200 in PBS). Other tissue sections were either treated with DAPI, a DNA-labeling fluorescent dye that serves as a general nuclear marker, or the red/green cone photoreceptor antibody, FRET 43 (Zpr-1, University of Oregon monoclonal bank, 1:200 in PBS). Neuromast staining along the lateral line was completed by immersing whole fish in 5mM 4-(4-diethylaminostyryl)-N-methylpyridinium iodide (4-di-2-Asp, Sigma) for 5 minutes in tank water. Fish were then anesthetized and imaged with a Leica FLUO microscope.

**Olfactory Tract Tracing**

For olfactory tract-tracing, fish were anesthetized with 0.03% MS222 (3-amino benzoic acid ethyl ester, Sigma) and perfused transcardially with phosphate buffered saline (PBS). The brains were dissected, and the fluorescent dextran microemerald (3000 MW, diluted 5mg/ml in PBS, Molecular Probes) was injected into the medial or lateral olfactory tracts or into both tracts of each olfactory bulb using a PV 800 Pneumatic Picopump (World Precision Instruments). The brains were then transferred to a sterilized filter in a six-well culture dish (Costar) and incubated
at 28.5°C and 1.5% CO₂ in artificial fish cerebrospinal fluid (100mM NaCl, 2.46mM KCl, 1mM MgCl₂·6H₂O, 0.44mM NaH₂PO₄·H₂O, 1.13mM CaCl₂·2H₂O, 5mM NaHCO₃) for approximately 4 hours (Tomizawa et al., 2001). Following fixation in 4% paraformaldehyde for 24 hours, horizontal cryostat sections at 20-50μm were obtained and mounted on positively charged slides. The tissue was coverslipped and viewed on a Nikon E600 Eclipse microscope or a Zeiss LSM 510 confocal microscope.

Results

General dsRED Expression

Expression of the dsRED transgene was seen in a number of zebrafish structures including the olfactory bulb, the retina, the spinal cord, the cerebellum, the adrenal gland, and nerve fibers that innervate the lateral line (Figure 5). Expression patterns were first visible in the olfactory bulb at 5 days post-fertilization (dpf), making this the first structure in which cells expressed the transgene. By 10dpf, cells in the eye and spinal cord were apparent and at 15-30dpf, fibers of the lateral line, the adrenal gland and Purkinje cells in the cerebellum were noticed (Figure 5).

Expression in the Olfactory Bulb

Initial screenings of transgene expression in the olfactory bulb showed dsRED-positive cells in the superficial internal cell layer (ICL) and in the glomerular layer (GL; Figure 6A). The labeled cell bodies had teardrop-shaped somata that ranged in size from 5-10μm in diameter (Figure 6B). The processes were visible protruding from the cell bodies and terminated as a tuft in the glomerular layer (Figure 6C-D).
In order to determine the cell type that was being labeled, it was first necessary to establish whether the cells were a neuronal population, a non-neuronal population, or both. Based on labeling experiments with the neuronal marker HuC/HuD, the cells expressing the transgene were neurons (Figure 7). However, retrograde tract tracing experiments with a fluorescent dextran confirmed that the cells were not output neurons (Figure 8). Another neuronal cell type found in the GL and the superficial ICL is a group of interneurons known as juxtaglomerular cells. These cells are commonly labeled with an antibody against tyrosine hydroxylase, which is a potential indicator of dopaminergic neurons. Labeling with anti-tyrosine hydroxylase yielded co-localization of the markers in some, but not all cell bodies (Figure 9).

Discussion

The creation of this transgenic line is interesting in that unlike many other CMV promoters that exhibit universal expression, the expression of this promoter is confined almost exclusively to the nervous system. Further, this particular transgenic line is beneficial in that the transgene is expressed early in development, which allows tracking of specific cell populations from very early stages through adulthood. The focus of this study was to establish the identity of the cells expressing dsRED in the olfactory bulb of adult zebrafish. Based on HuC/HuD labeling, the cells are neurons. While these cells are found in the same location as mitral cells, they are not output neurons since retrograde labeling of the olfactory tracts with a fluorescent dextran labels a different subset of bulbar neurons. Although, it also is possible that not all of the axons were labeled when the tract tracing experiments were being completed.
Figure 5. The dsRED expression pattern was observed in several regions of the fish. A) A fluorescence image of a zebrafish whole mount showed high levels of expression in the olfactory bulb. B) UV/blue subtype cones were dsRED positive. The FRET43 antibody (Zpr-1, University of Oregon), which is specific for the red/green cone subtype, was viewed with FITC (green). C) A low magnification view of the zebrafish flank showed positive cells in the spinal cord (arrows). D) Purkinje cells in the cerebellum also were labeled by the transgene. E) The adrenal gland located just rostral to the dorsal fin showed distinct expression. The fluorescent blue marker, DAPI, showed staining of nuclei. F) Nerve fibers innervating the lateral line were dsRED positive. Green channel fluorescence represented 4-di-2-Asp staining of the lateral line neuromasts, and yellow was indicative of colocalization (arrow).

Additionally, it is possible that the dextran was not allowed ample time to travel and did not label all of the cells bodies. On the other hand, previous work in our laboratory has shown that cells in the most rostral regions of the olfactory bulb can be labeled in the tracing time used for this study (data not shown).

Another population of cells distributed in the same vicinity as mitral cells is the juxtaglomerular cells. These cells, which can range from 5-10μm in diameter, are local interneurons that modify the output of mitral cells. Since these interneurons interact closely with mitral cell dendrites, they also are distributed throughout the superficial internal cell layer and the glomerular layer.
Figure 6. The transgene was expressed in a subset of cells in the olfactory bulb. A) In the olfactory bulb, dsRED-positive cells were observed in the superficial ICL and the GL but not the ONL. B) A high magnification image of dsRED-expressing cell bodies showed that the cells were approximately 5-10 μm in diameter. C and D) Cellular processes also showed the presence of the reporter protein.

Based on immunocytochemistry experiments with the antibody against tyrosine hydroxylase (TH), it is apparent that many of the dsRED positive cells also are TH positive. Because TH is used as a marker for interneurons in this species (Edwards and Michel, 2002), we conclude that the majority of the dsRED expressing cells are interneurons.
Based on the distribution of the cells in the glomerular layer, we conclude that these cells are juxtaglomerular interneurons. Some, but not all, dsRED cells were TH positive. It is possible that some of the dsRED positive cells that are TH negative are a different subpopulation of juxtaglomerular cells that label with a protein other than TH. In some mammalian studies, periglomerular cells (the presumptive equivalent of juxtaglomerular cells in fish) label with calcium binding proteins such as calbendin or calretinin (Crespo et al., 1997). Thus, the dsRED cells that did not label with TH still may be juxtaglomerular cells. Further studies are necessary to establish this possibility.

This study is important in that it begins to reveal the morphology and distribution of juxtaglomerular cells in the adult zebrafish olfactory bulb. This cell type is often considered equivalent to the granule cell interneurons of the internal cell layer, but its morphology has never been established independently in this animal. In addition, this transgenic line may provide an important clue as to the development of juxtaglomerular cells in zebrafish from very early stages following fertilization through adulthood.
Figure 8. Cells that were dsRED+ were not output neurons. Cells showing dsRED expression (A) were seen in the GL and ICL of the olfactory bulb as were output neurons labeled with microemerald applied to the olfactory tracts (B). C) Overlay of the two channels, however, showed that dsRED cells were not output neurons. D) A higher magnification image of dsRED and microemerald again suggests that the dsRED cells are not mitral cells since they do not extend axons to the medial or lateral olfactory tracts although both mitral cells (E) and dsRED cells (F) exhibited a similar size and morphology.
Figure 9. A few dsRED cells were positive for tyrosine hydroxylase. A) Typical tyrosine hydroxylase antibody labeling, shown here with diaminobenzidine, identified numerous cells and processes scattered throughout the bulb. B and C) Some dsRED-positive cells showed tyrosine hydroxylase immunoreactivity (arrows), but others did not label with the antibody. D) Processes of dsRED-positive cells and TH-positive cells were tightly interspersed throughout the glomerular layer.

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CHAPTER III
IONOTROPIC GLUTAMATE RECEPTOR SUBUNIT 4 IN THE OLFATORY BULB OF ADULT ZEBRAFISH: DISTRIBUTION AND EXPRESSION FOLLOWING LOSS OF INNERVATION

Introduction

The adult olfactory bulb is an extremely plastic structure. There is normal replacement of the olfactory sensory neurons in the olfactory epithelium (for review, see Farbman, 1992) as well as continuous neurogenesis in the olfactory bulb (Altman, 1969; Kaplan and Hinds, 1977; Burd and Sein, 1998; Peretto et al, 1999; Byrd and Brunjes, 2001; Iwai et al, 2003, Stenman et al, 2003). Due to this constant turnover, there is a need for continual identification of new targets and formation of new synapses within the bulb (for review, see Farbman, 1992). In addition to the natural plasticity of this region in adult animals, deafferentation studies show the ability of the adult olfactory bulb to respond to changes in afferent input. For example, ablation of the olfactory organ in fish (Byrd, 2000; Poling and Brunjes, 2000), removal of the nasal epithelium in rabbits (Matthews and Powell, 1962) and chemical deafferentation in mice (Margolis et al., 1974; Harding et al., 1978) result in reduction of bulb size. Other studies have shown that olfactory deprivation results in changes in mRNA and protein expression (Ehrlich et al., 1990; Stone et al., 1991; Ferraris et al., 1997; Casabona et al., 1998; Oberto et al., 2001) as well as enzymatic activity (Nadi et al., 1981; Baker et al., 1984). Furthermore, numerous studies suggest that loss of afferent input results in decreased cell number (Henegar and Maruniak, 1991; Meisami and Safari, 1991; Byrd, 2000) and changes in cellular morphology (Pinching and Powell, 1971) in the deprived bulb of adult animals.

Many studies have shown that glutamatergic systems influence synaptic
plasticity (Bliss and Collingridge, 1993; Gean et al., 1993; Bear, 1996; Carroll et al., 1999). Glutamate is the primary excitatory neurotransmitter in the vertebrate central nervous system (for review, see Johnson, 1972) and is the chief excitatory neurotransmitter in the vertebrate olfactory system as well. Glutamate is used by olfactory sensory neurons to communicate with the primary output neurons of the olfactory bulb, the mitral cells, and the interneurons at the first level of lateral interaction, the periglomerular cells (Sassoe-Poggetto et al., 1993; Berkowicz et al., 1994; Ennis et al., 1996). Mitral cells also use glutamate to communicate with granule cells, the interneurons involved in the second level of lateral inhibition in the olfactory bulb (Jacobson et al., 1986; Trombley and Westbrook, 1990). Glutamate can activate both metabotropic (mGluR 1-8) and ionotropic (iGluR 1-7) receptors on the postsynaptic cell. Metabotropic receptors are composed of one polypeptide linked to a G-protein second messenger. The ionotropic receptors are ligand-gated ion channels and mediate fast transmission (Berkowicz et al., 1994) they respond to the agonists, N-methyl-D-aspartic acid (NMDA), a-amino-3-hydroxyl-5-methylisoxazole-4-proprionic acid (AMPA), or kainic acid (KA). It has been shown that iGluRs have a tetrameric structure (Rosenmund et al., 1998; Chen et al., 1999) formed by a dimerization of dimers (Armstrong and Gouaux, 2000; Ayalon and Stern-Bach, 2001). These receptors can be formed by a combination of iGluR1-iGluR4 subunits (for review, see Mansour et al., 2001; Esteban, 2003).

Previous studies in mammals show that iGluRs, specifically AMPA receptors, are expressed in a number of neurons in the olfactory bulb (Petralia and Wenthold, 1992; Molnar et al., 1993; Sato et al., 1993; Montague and Greer, 1999). Montague and Greer (1999) propose that the response of postsynaptic cells to glutamate will vary depending upon the differential distribution of these subunits in the synapse. In
fish, pharmacological examinations also suggest the presence of AMPA/KA receptors in the olfactory bulb (Edwards and Michel, 2002). These analyses reveal that AMPA and KA receptors, activated by kainate, stimulate mitral cells, granule cells, and juxtaglomerular/TH-positive cells (Edwards and Michel, 2002). Periglomerular cells have not been defined in teleosts, but we refer to their presumptive equivalents in zebrafish as juxtaglomerular cells based on their distribution and function. While these findings are significant in that they help to define the role of glutamatergic neurotransmission in the fish olfactory bulb, the AMPA and KA receptors are grouped into a single category. As a result, it is unclear which specific receptor subunits are expressed by individual cell types. In general, AMPA receptors have been widely studied in the brain and spinal cord, but investigations into their precise distribution in the olfactory bulb has been limited.

The ionotropic AMPA receptor, iGluR4, is of particular interest because it is expressed in high levels where cells display fast kinetics and rapid desensitization (Moyner et al., 1991; Raman et al., 1994; Rubio and Wenthold, 1997; Silver et al., 1992). Localization of this subunit has been examined specifically in the central cervical nucleus of rat (Ragnarson, 2003), the outer plexiform layer of goldfish (Schultz, 1997), retinal cells of chick embryos (Cristovao and Carvahlo, 2003), and the human cerebral cortex (Ong and Garey, 1996). It is expressed in auditory cells that need to communicate signals for pinpointing sound (Raman et al., 1994) and is preferentially expressed in motor neurons of the spinal cord (Tomiyama et al., 1996) where prompt neurotransmission has been documented (Smith et al., 1991). In turn, it may be important for specific neurons in the olfactory bulb to express this receptor subunit, where rapid response may prove significant in detection of food, predators, or mates.
The present study examines the distribution of the AMPA-type receptor subunit, iGluR4, in the olfactory bulb of adult zebrafish, Danio rerio. It establishes that olfactory sensory input plays a role in the maintenance of iGluR4 immunoreactivity in this plastic system. Our results demonstrate a specific laminar and cellular distribution of iGluR4 and distinct changes in the localization or expression of this protein following loss of afferent input.

Materials and Methods

Western Blot Analysis

In order to confirm that the ionotropic GluR4 antibody made against rat protein was not crossreactive with multiple proteins in the zebrafish olfactory bulb, Western blot analysis was performed. Adult zebrafish brains (n=10) were dissected out of anesthetized, live animals, frozen on dry ice, and macerated in lysis buffer (Tris-HCl 20mM, Sucrose 10%, EDTA 1mM, SDS 2%, phenylmethanesulfonylfluoride 1mM and Protease Inhibitor Cocktail 1:100, all from Sigma). A rat brain (Sprague Dawley, 4 weeks of age) also was examined, using the above protocol, in order to compare protein size in both animals. The rat was anesthetized by carbon dioxide asphyxiation followed by thoracotomy. It was being used for another study and the brain was a generous gift of Dr. John Spitsbergen. The samples were loaded into a 10% Tris-Glycine gel (Invitrogen) and the iGluR4 blocking peptide (1:1000 in lysis buffer, Chemicon) also was loaded in order to confirm binding specificity of the peptide to the antibody (data not shown). The gel was run at 200V for 1 hour. Proteins were then transferred to an Immobilon-P PVDF transfer membrane (Millipore) for 2 hours and blocked with blocking agent (5% in
distilled water, ECL Western Blot Analysis System, Amersham Biosciences). The membrane was incubated in primary antibody (anti-ionotropic GluR4, Chemicon, 1:1000 in blocking agent) for 12 hours at 4°C and treated with secondary antibody (goat anti-rabbit IgG, Amersham Biosciences, 1:2500 in blocking agent) for 1 hour. The membrane was then treated with a peracid and luminol mixture (1:1, ECL Western Blot Analysis System, Amersham Biosciences), exposed to photographic film (OMAT, Kodak) for 3 minutes and developed.

**Tissue Processing**

Adult male and female zebrafish, at least 2.5cm in length, were obtained commercially from local suppliers. They were maintained in 28.5°C aerated, conditioned fish water and fed freshwater flake food (Wardley Corporation) twice daily. All animal care protocols and experimental procedures were approved by the Institutional Animal Care and Use Committee.

Fish were anesthetized with 0.03% MS222 (3-amino benzoic acid ethyl ester, Sigma) and perfused transcardially with phosphate buffered saline (PBS) followed by 4% paraformaldehyde. The animals were placed in 4% paraformaldehyde overnight for further fixation of the tissue. The brains were dissected, washed in PBS, dehydrated, and embedded in paraffin blocks. Ten-micron horizontal or coronal sections of the olfactory bulbs were obtained and mounted on positively charged slides.

**Immunocytochemistry**

Sections were rehydrated, treated with 3% hydrogen peroxide to remove endogenous peroxidases, and blocked with 3% normal goat serum and 0.4% Triton
X-100 in PBS. They were treated with a polyclonal antibody (anti-ionotropic GluR4, 1:100 in blocking solution, Chemicon) for 24-48 hours at 4°C, rinsed, and incubated in biotinylated secondary antibody (goat anti-rabbit IgG, 1:100 in blocking solution, Jackson ImmunoResearch). They were then treated with avidin-biotin complex (Vectastain Elite ABC Kit, Vector) and visualized using diaminobenzadine (DAB substrate kit, Vector). Intensity profiles were examined using the Metamorph program. For double-label fluorescence techniques, slides were treated sequentially with primary antibody (polyclonal anti-ionotropic GluR4, 1:100 in blocking solution, Chemicon or monoclonal anti-tyrosine hydroxylase, 1:1000 in blocking solution, ImmunoStar) for 24 hours at 4°C, rinsed in PBS, and incubated in a fluorescently labeled secondary antibody (goat anti-rabbit Alexafluor 594, 1:100 in PBS, Molecular Probes or goat anti-mouse Alexafluor 488, 1:100 in PBS, Molecular Probes) for 1 hour. Following staining, the slides were coverslipped with glycerol and PBS (50:50) and viewed on a Zeiss LSM 510 confocal microscope using a multitrack overlay with narrow band filters. A total of five fish were examined in double label experiments.

Controls

Negative controls, in which the primary antibody was omitted in order to account for the possibility of non-specific staining, yielded no staining in both Western blot analysis and immunocytochemistry. Positive controls were completed using anti-keyhole limpet hemocyanin (KLH, 1:100 in blocking solution, Sigma) to confirm the immunocytochemical staining method. In addition, antibody specificity controls were done by pre-absorbing the diluted antibody with the iGluR4 blocking peptide (1:100, Chemicon). This control showed no staining in both Western blot analysis and immunocytochemistry. In order to confirm that the iGluR4 antibody was
not binding to the tyrosine hydroxylase protein, an additional western blot analysis was performed as described above; anti-tyrosine hydroxylase (TH, Immunostar, 1:2000 in blocking agent) was used as the primary antibody and goat anti-mouse (Amersham Biosciences, 1:2500 in blocking agent) served as the secondary antibody (data not shown). Also, to verify the results of the tissue blocking experiment, an additional control was completed whereby anti-KLH was pre-absorbed with the iGluR4 blocking peptide (1:100). This experiment resulted in the standard KLH staining pattern on tissue sections suggesting that the blocking peptide did not prevent labeling in a non-specific manner.

**Deafferentation**

To examine the effects of afferent removal on glutamate receptor immunoreactivity in the bulb, peripheral deafferentation was performed as described previously (Byrd, 2000). The fish were anesthetized using 0.03% MS222 until they no longer responded to a tail pinch, and the right olfactory organ was extirpated using a small-vessel cautery iron. The left olfactory organ remained intact for use as an internal control for comparison. Sham-operated control animals (n=4) received a wound to the tissue between the olfactory organs that was similar in size to that of the deafferented fish in order to control for non-specific wound responses. Fish were returned to aquarium water containing the antibiotic Kanacyn (0.01mg/ml, Aquatronics) and were allowed to survive for 24 hours (n=7), 48 hours (n=5), 1 week (n=5), or 3 weeks (n=5). Control fish (n=5) were maintained in a separate aquarium and also were treated with Kanacyn (0.01mg/ml, Aquatronics). After the appropriate survival period, the deafferented, sham-operated, and control fish were processed as described above.
Quantitative Analysis

In order to quantify ionotropic GluR4-immunoreactivity, digital images were obtained using a Nikon E600 microscope and Spot Advance software. The color images were converted to grayscale images, and the average gray values of 3 midbulbar sections of left and right olfactory bulbs were determined using the ImageJ program provided by NIH. For each animal, the largest sections from right and left bulbs were chosen independently and analyzed in five measures: whole bulb, rostral medial quadrant, caudal medial quadrant, rostral lateral quadrant, and caudal lateral quadrant. In addition, measurements were recorded from a section 30μm to the right and 30μm to the left of the largest section in order to accurately reflect staining in different planes of the olfactory bulb.

To account for minor variations in tissue processing and background staining, gray values for each section were converted to optical density (OD) values by the formula: \( OD = \log \left( \frac{\text{intensity of background}}{\text{intensity of area of interest}} \right) \). The background intensity was established by measuring the average gray level of a non-staining telencephalic region similar in size to the area of interest. The optical densities for the three midbulbar sections were then used to calculate an average OD value for the right and left bulb of each fish. Percentage differences between the right and left bulbs were calculated using the following formula: \( \frac{\text{Average OD Right Bulb} - \text{Average OD Left Bulb}}{\text{Average OD Right Bulb}} \times 100\% \). The percentages for all animals in a given time point were averaged. Paired Two-Sample T-tests were used to examine differences between deafferented and internal control bulbs at each time point. Also, a one-way ANOVA and Fisher’s test for multiple comparisons were used to determine if there was a significant difference in iGluR4-immunorectivity in control and deafferented tissue across and between all time points. P-values less than
0.05 were considered significant.

To determine the size of immunoreactive cell bodies in the olfactory bulb, high magnification digital images were obtained from the olfactory bulbs of adult animals, and the diameters of somata were measured using the Spot Advance Software line measurement tool.

Results

Antibody Specificity and Controls

The specificity of the antibody to the iGluR4 protein in zebrafish and rat brain was analyzed using Western immunoblots (Figure 10A). In zebrafish and rat brain homogenates, the antibody to iGluR4 showed an immunoreactive band with a presumptive molecular mass just above 100kDa. In addition, a labeled band frequently appeared around 50kDa in both the rat and the zebrafish homogenates. On tissue sections, a control in which the primary antibody was omitted (10B) resulted in no staining and, when the iGluR4 antibody (10C) was pre-absorbed with the iGluR4 peptide (10D), the labeling was blocked. In order to confirm that blocking was specific, a positive control was run whereby anti-keyhole limpet hemocyanin (KLH) was incubated with the iGluR4 peptide. In this case, the KLH antibody showed the same pattern of immunoreactivity on tissue sections as the control KLH (compare Figure 10E and 10F). This confirms that the iGluR4 peptide is specific to the iGluR4 antibody and does not cause non-specific blocking. Based on the results of the peptide blocking experiments, consistency of band migration between species, and other investigations that report similar results at both 50kDa and 100kDa (Wenthold et al., 1992), we conclude that the antibody is specific to iGluR4.
Laminar Distribution of iGluR4 in Zebrafish Olfactory Bulbs

Distribution of the AMPA receptor subunit iGluR4 in the adult zebrafish olfactory bulb was examined using immunocytochemistry. Ionotropic GluR4 immunoreactivity displayed a laminar pattern in the olfactory bulb. Antibodies to this receptor subunit showed the greatest amount of labeling in the glomerular layer (GL, Figure 11A, B), with dendrites of several cells showing prominent immunoreactivity (Figure 11B, C). There was lesser staining in the internal cell layer (ICL), and virtually no staining in the olfactory nerve layer (ONL, Figure 11A, B). A 10μm section of the bulb was analyzed using Metamorph Intensity Profile (Figure 11D, E). This program utilizes optical density to establish intensity distributions where the maximum height of the profile is correlated with the lightest area of the tissue, or in this case, reduced immunoreactivity. The highest level of labeling was found in the GL and the lowest level in the ONL (Figure 11D, E). Ionotropic GluR4 immunoreactivity also was examined in coronal sections in order to examine further the laminar distribution seen in the horizontal plane. These analyses showed a similar pattern of iGluR4 immunoreactivity with the most abundant staining seen in the GL (Figure 12A,B) and lesser staining observed in the ICL (Figure 12C).

Cellular Distribution of iGluR4 in the Olfactory Bulb

With regard to cellular distribution, numerous somata were labeled throughout the glomerular region (Figure 11A-C, 12B-C, arrows). Cell bodies ranged in size from 4μm to 8μm in diameter, and their processes were visible in the glomerular layer (Figure 11B, C).
Figure 10. Control experiments were used to confirm the specificity of the antibody to zebrafish tissue. (A): Western blot analysis of zebrafish and rat brain tissue, using an antibody to ionotropic glutamate receptor subunit 4, iGluR4, shows an immunoreactive band at 108kDa in both species. (B): A control in which the primary antibody was excluded yields no staining when treated with goat anti-rabbit secondary antibody and diaminobenzadine. (C): Ionotropic GluR4 (iGluR4) labels somata (arrows) and shows high immunoreactivity in the glomerular layer (GL) of the adult zebrafish olfactory bulb. There is no label in the olfactory nerve layer (ONL). (D): Immunoreactivity is blocked completely when the iGluR4 antibody is pre-absorbed with the iGluR4 peptide (iGluR4 pep). (E): An antibody against keyhole limpet hemocyanin (KLH) is used as a positive control in the adult zebrafish olfactory bulb due to its distinct and consistent labeling of the olfactory nerve layer in fish (arrow, for example). (F): When the KLH antibody is incubated with the iGluR4 peptide at the same concentration used in the anti-iGluR4 blocking experiment, normal anti-KLH staining is observed. Scale bar = 80µm (B-F).
Figure 11. Ionotropic GluR4 is expressed in the adult zebrafish olfactory bulb. (A): A low magnification view shows the heaviest immunoreactivity in the glomerular layer (GL), lighter expression in the internal cell layer (ICL), and very little to no immunoreactivity in the telencephalon (TEL) and olfactory nerve layer (ONL). Even at this magnification, cell bodies are discernable (arrow). (B): At higher magnification, laminar distribution is seen more clearly when staining time is reduced. Numerous cell bodies (arrow) and abundant processes (arrowhead) are labeled in the GL. Some immunopositive cells and processes also are found in deeper regions of the bulb. (C): The labeled soma of a single cell, found in the GL, exhibits iGluR4 immunoreactivity in a primary dendrite and a presumptive axon, as well. (D): This Metamorph Intensity Profile, which corresponds directly to the bulb segment in E, reveals that the greatest iGluR4 immunoreactivity is in the GL and the least iGluR4 immunoreactivity is in the ONL. The lowest points on the profile are representative of the darkest areas of the tissue or areas of highest optical density and greatest immunoreactivity. The highest points on the profile indicate regions that are much lighter and have low optical density and low immunoreactivity. (E): A strip of the adult zebrafish olfactory bulb corresponding to the profile data in D is shown. Scale bar = 100μm (A), or 40μm (B), or 25μm (C) or 15μm (E).
Olfactory bulbs were examined using double-label fluorescence techniques with the iGluR4 antibody as well as an antibody to tyrosine hydroxylase (TH). TH, the rate-limiting enzyme in catecholamine synthesis, is expressed in a subset of neurons found in the glomerular layer of the adult zebrafish olfactory bulb (Byrd and Brunjes, 1995; Edwards and Michel, 2002). TH-positive cells and iGluR4-positive cells, individually, gave similar immunoreactive profiles (compare Figures 13A, B). Also, double-label techniques showed colocalization between TH and iGluR4-expressing cells (Figure 13C-E). However, some iGluR4-immunoreactive cells were TH-negative (Figure 13E, F, arrow) and some TH-immunoreactive cells were iGluR4-negative (Figure 13E, arrowheads). These cells had the same size and morphology as the TH-positive/iGluR4-positive cells and also were distributed in the glomerular region of the bulb. Due to the similarity in the expression patterns of antibodies to the two proteins, Western blot analysis of zebrafish brain was completed using antibodies to iGluR4 and TH. The two proteins each displayed bands of different molecular weight suggesting that they were not crossreactive with one another in the
fluorescence stain (data not shown).

Effects Of Deafferentation On iglur4 Immunoreactivity

Ionotropic GluR4 immunoreactivity in the adult zebrafish olfactory bulb was examined following removal of afferent input. The whole bulb, excluding the olfactory nerve layer, was analyzed by averaging the OD values of three right and left midbulbar sections in at least 3 fish at a given timepoint. In unoperated, control animals, there was no significant difference between the labeling of iGluR4 in the right and left olfactory bulbs (P=0.719, Figure 14A). In sham-operated animals, where a small wound was made to the tissue between the right and left nares, iGluR4-staining intensity remained unchanged between the right and left bulb (P=0.821, Figure 14B). At 24 hours post-deafferentation, however, there was a significant difference in iGluR4 immunoreactivity between the right, unoperated bulb and the left, treated bulb (P=0.018, Figure 14C). At this timepoint, the iGluR4 label was noticeably diminished in the deafferented bulb. This decrease appeared to occur primarily in the processes (compare Figure 14G,H), as iGluR4-positive cell bodies were still apparent; however, overall the number of immunoreactive cells appeared less than in control animals. By 48 hours after surgery, the right bulb still showed a trend of reduced immunoreactivity (Figure 14D), but statistically there was no difference between the treated and untreated bulbs (P=0.054). At 1 and 3 weeks post-deafferentation, iGluR4 staining had returned to normal levels (P=0.343, Figure 14E, F). A one-way ANOVA showed a significant difference in immunostaining between the right and left olfactory bulbs across all time points (P=0.048), and Fisher’s test for multiple comparisons confirmed that the only significant difference was between the 24-hour time point and all others.
Figure 13. Immunocytochemical techniques help to identify iGluR4-expressing cell types. (A): Cell bodies and processes labeled with iGluR4 are found predominantly in the GL and the superficial ICL, a similar distribution pattern to tyrosine hydroxylase (TH)-immunoreactivity (B). Ionotropic GluR4 (C) and TH (D), observed with immunofluorescence, demonstrate the same labeling patterns seen with diaminobenzadine. (E): Double labeling with iGluR4 (red) and TH (green) shows almost complete overlap of the two antibodies. Notice that some iGluR4-positive cells (arrow) are not TH-positive and some TH-positive cells (arrowheads) are not iGluR4-positive. (F): A high magnification image confirms that not all iGluR4 cells are TH-positive (arrow). Scale bar =50μm (A, B) or 40μm (C-E) or 10μm (F).

To analyze further the effects of sensory input loss on iGluR4 immunoreactivity, the distribution of the AMPA receptor subunit was examined in four quadrants of the olfactory bulb in the horizontal plane: rostral medial, rostral...
lateral, caudal medial, caudal lateral. These quadrants were chosen based on glomerular distribution and provided a delineation between the medial and lateral portions of the bulb as well as the rostral and caudal portions of the bulb. Again, no difference was seen between the right and left bulbs of control or sham animals in any quadrant (Figure 15A-D). At 24 hours post-surgery, both the rostral medial and rostral lateral quadrants showed a decreased labeling of iGluR4 (Figure 15A, B). Both caudal quadrants did not show this loss of immunoreactivity at the 24-hour timepoint (Figure 15C, D). Forty-eight hours, 1 week, and 3 weeks after loss of sensory input, iGluR4 staining had returned to initial levels in all quadrants (Figure 15A-D). Fisher’s test for multiple comparisons confirmed that the only significant difference in the rostral areas of the bulb was between the 24-hour time point and all others.

Discussion

These results show the distribution of the AMPA-type receptor subunit iGluR4 in the normal and deafferented adult zebrafish olfactory bulb. The specificity of the antibody and the validity of the staining method were confirmed using several controls including Western blots and pre-absorption with the iGluR4 peptide in tissue sections. Western blots of rat and zebrafish brain homogenates show bands with presumptive molecular weights of 108kDa and 51kDa. Ionotropic GluR4 has an apparent molecular mass of 108kDa in rat (Wenthold et al., 1992) and goldfish (Schultz et al., 1997). The two bands in our immunoblots migrate equally in both rat and zebrafish, and other researchers have found a 51kDa band in addition to the 108kDa band (Wenthold et al., 1992). In our experiments, omission of the primary antibody and pre-absorption of the iGluR4 antibody with the iGluR4 peptide yield no staining on Western blots.
Figure 14. Quantitative analysis illustrates changes in iGluR4 immunoreactivity in the whole olfactory bulb. The graph shows the mean percent difference (± SEM) in optical density values between the left, control bulb and the right, treated bulb. Control (n=5), sham-operated (n=4), and deafferented 24 hour (n=7), 48 hour (n=5), 1 week (n=5), and 3 week (n=5) olfactory bulbs were analyzed. There is a significant difference between the two bulbs at 24 hours post-deafferentation (*P<0.05). Photomicrographs below each bar show iGluR4 immunoreactivity in the right bulbs of control and deafferented animals. Control (A) and sham-operated (B) animals show high levels of iGluR4 label in the olfactory bulb. Twenty-four hours after deafferentation (C) the extent of labeling is noticeably diminished, particularly in the medial bulb regions. At 48 hours (D), 1 week (E), and 3 weeks (F) post-deafferentation, iGluR4 immunoreactivity in the olfactory bulb has returned to normal. (G): In a control animal, the glomerular layer shows heavy labeling in the cell bodies and processes. (H): Twenty-four hours after deafferentation immunoreactivity in the cell bodies is still apparent, but labeling in the processes has significantly decreased. Scale bar = 100μm (A-F) or 15μm (G, H).
Figure 15. Quantitative analysis shows changes in iGluR4 immunoreactivity following deafferentation in four bulb quadrants. There is a significant difference between the control bulbs and the treated bulbs at 24 hours in the rostral medial quadrant (A) and rostral lateral quadrant (B), but not in the caudal medial quadrant (C) or the caudal lateral quadrant (D). The number of animals analyzed is the same as in Figure 5. *P<0.05.

Finally, a GenBank BLAST (Pubmed) query of the protein sequence to which the antibody was made does not generate matches for any other proteins (data not shown). Together, these results suggest that the iGluR4 antibody is specific to iGluR4 in zebrafish and that the smaller band is likely due to proteolytic breakdown of the
antigen that can occur with rat tissue as well.

Ionotropic GluR4-immunoreactivity in tissue sections is highest in the rostral portion of the bulb in the GL, with lesser labeling in the ICL and no labeling in the ONL. Further, iGluR4-positive cells co-localize with TH-positive cells. These data suggest that the expression of iGluR4 occurs primarily in the TH-positive juxtaglomerular (JG) cells of the olfactory bulb and in many processes in the glomerular region. However, some iGluR4-immunoreactive cells located in the glomerular layer were TH-negative. Edwards and Michel (2002) describe two populations of cells in this region that are smaller than mitral cells: one is TH-positive and the other is TH-negative. Both cell types contain low glutamate levels and high GABA levels and are different from the granule cells of the ICL. It is possible that the iGluR4-positive cells that did not show TH-immunoreactivity are equivalent to the TH-negative JG cells observed by Edwards and Michel. The TH-positive cells are likely to be the equivalent of periglomerular cells in other animals. However, since periglomerular cells have not been defined specifically in teleosts, we identify them as JG cells and otherwise define them based on their TH expression pattern. The distribution of this AMPA subunit is consistent with the idea that iGluR4 is preferentially expressed in areas of fast kinetics and rapid desensitization. Juxtaglomerular/periglomerular cells are inhibitory interneurons that receive input from the olfactory nerve and apical dendrites of projection neurons and modify the output of mitral cells through release of inhibitory neurotransmitter (for review, see Farbman, 1992). Rapid activation of interneurons is likely involved in proper localization and identification of odorants as it would prevent the brain from receiving equal input from a large number of mitral cells corresponding to a greater surface area on the olfactory epithelium.
In mammals, iGluR4 is expressed preferentially in the olfactory bulb layers. Previous studies have described its presence in the neuropil of the external plexiform layer (Petralia and Wenthold, 1992; Montague and Greer, 1999) and the glomerular layer (Petralia and Wenthold, 1992; Martin et al., 1993; Montague and Greer, 1999), a distribution similar to that of teleosts. This distribution has further been examined on the ultrastructural level and is defined as secondary dendrites of mitral/tufted cells in the external plexiform layer and periglomerular and mitral/tufted cell processes in the glomerular layer (Montague and Greer, 1999). Due to the diffuse laminar structure of the zebrafish olfactory bulb, distinguishing between mitral cell and JG cell processes is difficult. As a result, the heavy glomerular distribution of the iGluR4 protein seen in this animal could consist of processes from JG cells, mitral cells, and astrocytes. The mammalian olfactory bulb appears to show little iGluR4 immunoreactivity in the mitral cell layer and the granule cell layer (Petralia and Wenthold, 1992; Montague and Greer, 1999), which are equivalent to the zebrafish ICL. Unlike in teleosts, however, iGluR4 in mammals also is expressed in the ONL (Petralia and Wenthold, 1992; Montague and Greer, 1999).

With regard to cellular localization, iGluR4 expression in mammals has been shown in mitral cells (Montague and Greer, 1999), granule cells (Petralia and Wenthold, 1992), astrocytes (Martin et al., 1993; Montague and Greer, 1999), and in periglomerular cells (Petralia and Wenthold, 1992; Montague and Greer, 1999). Martin and colleagues (1993) suggest that minor differences in results could be due to variations in synthetic peptide lengths, immunocytochemical procedures, or data analysis and interpretation. In general, the distribution of the iGluR4 protein in zebrafish is similar to that of mammals.

It is also possible that the minor differences in iGluR4 expression between
mammals and teleosts are due to the differences in olfactory circuitry between the two vertebrate groups. For example, several studies have shown that teleosts exhibit distinct differences from mammals with regard to mitral cells (Oka, 1983; Dryer and Graziadei, 1994). In teleosts such as goldfish (Kosaka and Hama, 1982) and tench (Alonso et al., 1988) mitral cells have far-reaching dendritic projections that reportedly contact multiple glomeruli (Nieuwenhuys, 1967; Dryer and Graziadei, 1994). In mammals, however, each mitral cell sends a single primary dendrite to a solitary glomerulus (for review, see Dryer and Graziadei, 1994) and the axons of olfactory receptor neurons that possess the same type of odorant receptor project to the same glomerulus (for review, see McClintock, 2000). This suggests that odors may be processed differently between the two groups. On the other hand, studies examining the role of glutamatergic neurotransmission in the adult zebrafish olfactory bulb suggest that the synaptology of the fish olfactory bulb is inherently similar to that of mammals (Edwards and Michel, 2002). In fact, preliminary results on the morphology of mitral cells in zebrafish suggest that these cells may be more similar to mammalian mitral cells than previously believed, with most mitral cells, including those with multiple primary dendrites, innervating a single glomerulus (Fuller and Byrd, 2004). While olfactory processing is not yet understood fully, it is possible that differences in olfactory bulb circuitry in fish and mammals call for minor variations in the distribution of the iGluR4 subunit.

In this study, we show that iGluR4 immunoreactivity can be altered by afferent input. Controls were used to confirm that changes in apparent protein levels were due to loss of afferent input rather than a general immune response to the wound. There was no significant difference in antibody labeling between control animals that received no wound and sham-operated animals that received a wound.
between the right and left nares. However, there was a significant difference in iGluR4-immunoreactivity of the deafferented and control bulbs at the 24-hour time point. At this stage, antibody labeling in the deafferented bulb was significantly less than that of the untreated bulb. This suggests reduced protein levels resulting from changes in synthesis and/or degradation. Furthermore, quadrant analyses showed that the rostral portion of the bulb showed a significant reduction of labeling with deafferentation while the caudal region did not. This is likely due to the distribution of the glomeruli, which are located primarily in the rostral portion of the bulb. Since the glomerular layer shows the heaviest iGluR4 immunoreactivity in control animals, loss of staining in the neuronal processes of deafferented animals would be most prevalent where glomeruli are denser. By 48 hours, iGluR4 staining had returned to normal and was maintained through 3 weeks post-surgery.

Previous studies in the adult zebrafish olfactory bulb showed an increased level of apoptosis at 24 hours post-deafferentation (Vankirk and Byrd, 2003). This could account for the decrease in iGluR4-immunoreactivity seen at the 24-hour time point, except that staining intensity returns to normal by 48 hours. If apoptosis were causing the decrease in iGluR4 labeling, it is unlikely that the levels of immunoreactivity would return to normal at 48 hours. It is equally likely that the JG cells do not die off in large quantities at this stage, rather that their expression of iGluR4 is down-regulated as an initial response to the loss of afferent input. Several studies have shown decreased protein expression in response to sensory input loss (Baker et al., 1983, Baker et al., 1993). This is a likely possibility considering a previous deafferentation study in zebrafish that shows 24 hours post-surgery apoptotic cells are found primarily in the internal cell layer, and only a small percentage of these cells are neurons (Vankirk and Byrd, 2003). Therefore, the
majority of the neuronal cells undergoing apoptosis at this time point are interneurons, based on their location and size. However, because the majority of these interneurons are found in the deep internal cell layer it is most likely that they are granule cells, not juxtaglomerular cells. In another zebrafish study, significant loss of TH-expressing cells in the olfactory bulb is seen at 1 week post-deafferentation (Byrd, 2000). This study also suggests that the TH-positive cells are not degenerating, rather they reduce expression of the TH protein. In mammals, when olfactory sensory input is reintroduced following chemical lesion of the olfactory epithelium, TH expression that had been reduced with loss of afferent input returns to normal levels (Baker et al., 1983). These studies suggest that reduced iGluR4 immunoreactivity at 24 hours post-deafferentation could be due to a change in the pattern of protein expression following loss of afferent input rather than a loss of JG cells expressing the protein. However, it also is possible that there is degradation of the iGluR4 protein present in the tissue rather than decreased synthesis of the peptide.

In this study, TH and iGluR4 immunoreactivity co-localize to the same cells. Previous work in the adult zebrafish showed that there was a reduction in the number of TH-positive cells at 1 week and 3 weeks post-deafferentation (Byrd, 2000). Our current work suggests that changes in iGluR4 expression follow a different time course: iGluR4 is reduced at 24 hours and has returned to normal levels by 1 and 3 weeks post-surgery. Once the axons of the olfactory nerve are severed there are at least two possible scenarios to describe the resulting cellular response. First, severed axons release glutamate into the olfactory bulb causing internalization of the iGluR4 receptor subunit. Previous studies showed a higher concentration of extracellular glutamate following brain injury (Katayama et al., 1990, Nilsson et al., 1990), suggesting an increase in the release of this neurotransmitter after trauma. It has
already been established that iGluRs undergo endocytosis as a part of normal synaptic trafficking (Carroll et al., 1999) and other recent studies have shown that iGluRs can be internalized rapidly following activation by increased levels of glutamate and AMPA (Lissin et al., 1999). This redistribution is reversible following removal of the agonist, which could explain why the receptors are once again expressed when the excess glutamate clears. This could result in the return of iGluR4 immunoreactivity seen at 48 hours post-surgery. Several studies have shown an up-regulation of protein receptors in response to deafferentation (Gomez-Pinilla et al., 1989; Casabona et al., 1998).

A second possibility is that loss of afferent input results in an immediate loss of activation, which causes down-regulation of receptors in the juxtaglomerular cells. Olfactory denervation results in the down-regulation of specific proteins (Sashihara et al., 1996) and enzymes (Ehrlich et al., 1990). This correlates to the immediate reduction in immunoreactivity seen at 24 hours post-deafferentation. Once a steady state situation is attained, even though it does not include odorant-activated responses, the glutamate receptors are produced again. This is supported by investigations showing returned expression of mRNAs and proteins in the olfactory bulb following loss and subsequent return of afferent input (Oberto et al., 2001). While a similar initial response can be expected for an enzyme such as TH that aides in dopamine production for juxtaglomerular interneurons, the return to normal levels would not be expected. This is due to the continued loss of sensory input and in turn, continued reduction in the expression of the enzyme. Since the mitral cells are not being activated, the JG neurons do not need to make a neurotransmitter that helps to modify mitral cell output. As a result, the reduction in TH immunoreactivity and the return of iGluR4 immunoreactivity seen at 1 and 3 weeks post-deafferentation is

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expected.

At this time it is unknown whether iGluR4 immunoreactivity is reduced as a result of glutamate excitotoxicity or immediate loss of activation, but distinguishing between the potential mechanisms that result in this change is the focus of future studies. Our goal is to gain a better understanding of the cellular interactions involved in afferent target regulation in this key model system.

This information was presented in a 2005 publication entitled, “Changes in glutamate receptor subunit 4 expression in the deafferented olfactory bulb of zebrafish” in Brain Research.
CHAPTER IV
THE MORPHOLOGY AND DISTRIBUTION OF RUFFED CELLS IN THE
OLFACTORY BULB OF ADULT ZEBRAFISH

Introduction

The ruffed cell is an output neuron that has been identified in the olfactory bulb of select fish, but not in other animal groups. It was first described in goldfish (Kosaka and Hama, 1979a; 1979b; Kosaka, 1980) and later was recognized in at least ten other teleosts (Kosaka and Hama, 1980; Alonso et al., 1987; Arevalo et al., 1991; Matz, 1995). The ruffed cell is defined as a neuron whose axon has a distinct unmyelinated portion at the initial segment with several membranous protrusions arising from this region (Kosaka and Hama, 1979a; 1979b; Kosaka, 1980; Kosaka and Hama, 1980). The remainder of the axon is myelinated (Kosaka and Hama, 1979a; 1979b; 1980). The cell bodies are ovoid or spherical in shape and range in size from 9-30μm in diameter (Kosaka and Hama 1979b; Kosaka, 1980; Kosaka and Hama, 1980; Alonso et al., 1987; Matz, 1995). These cells are located between the olfactory nerve layer and the anterior olfactory nucleus as defined by Kosaka and Hama (1979b; 1980) or throughout the mitral cell and glomerular layers as defined by Alonso and colleagues (1987). The fish olfactory bulb is generally divided into three or four concentric layers. The superficial region of the bulb is always termed the olfactory nerve layer, and it contains the olfactory axons. The innermost layer is termed either the anterior olfactory nucleus (which is poorly defined in fish, see Matz, 1995) or the internal cell or granule cell layer, which is composed, in part, of numerous interneurons. The region between these is considered either a single layer, the glomerular layer including the mitral cells, or is subdivided into two general regions, the glomerular and external plexiform layers or the glomerular and mitral...
cell layers. Thus, the ruffed cells described in other teleost studies are located in regions equivalent to what we describe as the glomerular layer and internal cell layer in zebrafish.

Kosaka and Hama (1980) suggest that the ruffed cell is generally present in all teleost olfactory bulbs; however, until now ruffed cells have not been identified in the olfactory bulb of zebrafish. Previous work has sought to describe the adult zebrafish olfactory bulb (Byrd and Brunjes, 1995; Friedrich and Laurent, 2001; Edwards and Michel, 2002), but none of these studies identified the presence of ruffed cells. In addition, pharmacological studies dealing with neurotransmission in the zebrafish olfactory bulb have questioned the presence of ruffed cells as an additional output neuron (Edwards and Michel, 2002). The goal of the present study was to establish if ruffed cells were present in the adult zebrafish olfactory bulb. The identification of this neuron in zebrafish has implications for olfactory coding studies in this model system.

Materials and Methods

Animals

Adult male and female zebrafish, at least 2.5cm in length, were obtained from local distributors. They were maintained in aquaria containing 28.5°C aerated, conditioned fish water and fed freshwater flake food (Wardley Corporation) twice daily. All animal care protocols and experimental procedures were approved by the Institutional Animal Care and Use Committee.

Olfactory Tract-Tracing

For olfactory tract-tracing, fish were anesthetized with 0.03% MS222 (3-
amino benzoic acid ethyl ester, Sigma) and perfused transcardially with phosphate buffered saline (PBS). The brains were dissected, and biotinylated dextran amine (BDA, 3000 MW, 10% in PBS, Molecular Probes) was injected into the medial or lateral olfactory tracts or into both tracts of each olfactory bulb using a PV 800 Pneumatic Picopump (World Precision Instruments). The brains were then transferred to a sterilized filter in a six-well culture dish (Costar) and incubated at 28.5°C and 1.5% CO$_2$ in artificial fish cerebrospinal fluid (100mM NaCl, 2.46mM KCl, 1mM MgCl$_2$-6H$_2$O, 0.44mM NaH$_2$PO$_4$-H$_2$O, 1.13mM CaCl$_2$-H$_2$O, 5mM NaHCO$_3$) for approximately 4 hours (Tomizawa et al., 2001). Following fixation in 4% paraformaldehyde for 24 hours, horizontal or coronal cryostat sections at 20-50μm were obtained and mounted on positively charged slides. The tissue was treated with a fluorescent avidin (Alexafluor 488 avidin, 1:200 in PBS, Molecular Probes) for 1 hour at room temperature, rinsed, coverslipped, and viewed on a Zeiss LSM 510 confocal microscope.

**Immunocytochemistry**

For immunocytochemistry, BDA-stained sections were blocked with 3% normal goat serum and 0.4% Triton X-100 in PBS. They were treated with a monoclonal antibody (anti-HuC/HuD, 1:100 in blocking solution, Molecular Probes) for 24 hours at 4°C. HuC/HuD is an RNA binding protein that is often used as a general neuronal marker. The sections were then rinsed in PBS and incubated in a fluorescently labeled secondary antibody (Alexafluor 594 goat anti-mouse IgG, 1:100 in PBS, Molecular Probes) for 1 hour. Following staining, the slides were coverslipped with glycerol and PBS (50:50) and viewed on a Zeiss LSM 510 confocal microscope.

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Golgi-Kopsch Technique

The Golgi-Kopsch procedure was adapted from Riley (1979) and optimized for zebrafish olfactory tissue. Adult zebrafish were anesthetized and perfused transcardially with PBS followed by fixative (1% paraformaldehyde, 1% glutaraldehyde in 0.1M PBS). The brain was removed and stored in fixative at 4°C for 48 hours. The tissue was rinsed and stored in fresh dichromate solution (1.19M potassium dichromate, 0.44M sucrose, 37% formaldehyde, all from Fisher Scientific) at room temperature, protected from light, for 72 hours. The tissue was then rinsed multiple times in silver nitrate solution (0.04M, Fisher Scientific) and stored in fresh silver nitrate solution at room temperature, protected from light, for 12 to 32 hours. Following incubation in silver nitrate solution, the tissue was dehydrated in ethanols followed by a 1:1 solution of ether and ethanol and stored in celloidin (6g parlodion, Mallinckrodt; 50% Ethanol, Fisher Scientific; 45% Ethyl Ether, Fisher Scientific) at room temperature, protected from light, overnight. The brains were mounted in celloidin, which was solidified in a chloroform chamber and stored in butanol overnight. Sections were obtained on a sliding microtome at 100μm, dehydrated and mounted on slides using DPX mounting medium (Aldrich Chemical Company). The medium was allowed to dry for at least four days before the slides were analyzed using brightfield microscopy and SPOT advanced software.

Quantitative Analysis

In order to determine the size of labeled cell bodies in the olfactory bulb, high magnification digital images were obtained from the BDA-labeled or Golgi-impregnated olfactory bulbs of adult animals, and the height and width of somata were measured using the Spot Advance Software line measurement tool.
Measurements were made from 33 ruffed cells and 105 mitral cells from 8 animals. Averages and standard errors of the mean were reported. To determine the percentage of ruffed cells in the total output neuron population, cell counts were obtained from the right and left olfactory bulbs of 8 fish. For each fish, the total number of labeled output neurons for the right and left bulb was averaged, and the total number of ruffed cells for the right and left bulbs was averaged. To obtain the percentage of ruffed cells in a single olfactory bulb for a single fish, the average number of ruffed cells was then divided by the average number of labeled output neurons.

Results

Ruffed cells in the adult zebrafish olfactory bulb were identified using retrograde tract-tracing with a biotinylated dextran, and their presence was confirmed with Golgi impregnation analysis. Their cell bodies were generally spherical or ovoid in shape. They were neurons based on HuC/HuD co-labeling (data not shown). The unique feature of this cell type was a significant protrusion of the membrane at the initial segment of the axon (Figure 16A, B, arrow). Mitral cells did not exhibit these elaborate axonal ruffs (Fig 16D). The ruffed cell protrusions usually occurred within 4μm of the cell body and varied in size from 10-30μm along the length of the axon. The ruff typically had a spherical or cylindrical shape whose diameter ranged from 10-25μm around the shaft of the axon. The axons of the ruffed cells, approximately 1μm in diameter, projected to either the medial or the lateral olfactory tracts. The ruffed cells were located deep in the glomerular layer or superficial internal cell layer, and the cell bodies appeared to be evenly distributed throughout these regions in the entire olfactory bulb (Figure 16C). The ruffed cell dendrites projected superficially to the glomerular layer, and the tufts were generally in close proximity to the soma.
The soma height and width of ruffed cells were compared to the soma height and width of the major output neurons, mitral cells, in the same animals (see Table 1). The ruffed cell bodies ranged in size from 7-12\(\mu\)m in width and 8-15\(\mu\)m in height and had a mean height of 11.2\(\mu\)m \(\pm\) 0.6\(\mu\)m and a mean width of 9.9\(\mu\)m \(\pm\) 0.3\(\mu\)m. The mitral cells, on the other hand, had a mean height of 9.0\(\mu\)m \(\pm\) 0.4\(\mu\)m and a mean width of 8.3\(\mu\)m \(\pm\) 0.2\(\mu\)m (Table 1). These data suggest that, on average, zebrafish ruffed cells are larger than zebrafish mitral cells. Ruffed cells, however, appeared to be far less frequent in number than mitral cells. Analysis of 8 fish revealed that ruffed cells make up well less than 5\% of output neurons (Table 2).

Discussion

We have established the presence of ruffed cells in the adult zebrafish olfactory bulb and have described their morphology and distribution in this model system. Because the fish olfactory bulb is not organized in discrete layers like the mammalian olfactory bulb, identification of cell types based on distribution alone is difficult. As a result, morphological, physiological and/or pharmacological analyses of individual cell types are required to make an accurate identification. Our work supports previous suggestions that ruffed cells are present in all teleost olfactory bulbs (Alonso et al., 1987; Kosaka and Hama, 1980). Due to differences in the data reported in previous studies, we were unable to compare all aspects of ruffed cell morphology between zebrafish and other cyprinids.

Based on available comparisons, we find that the ruffed cells of zebrafish are more similar to the ruffed cells of goldfish and catfish rather than sea eel.
For example, like goldfish and catfish the ruff of the zebrafish occurs fairly close to the cell body, unlike the sea eel whose axonal protrusions are exhibited at a much further distance from the soma, generally 30-70μm (Kosaka and Hama, 1979b; 1980). These variations can be expected because the goldfish and the catfish are more closely related to the zebrafish than the sea eel (Figure 17). The goldfish and the zebrafish are close allies and even share a common lineage down to the same order and family, while the catfish is not as closely related but does share the superorder, Ostariophysi, and the subdivision, Euteleostei (Wulliman, 1998).

The ancestors of sea eel, on the other hand, diverged from the teleost line before the Euteleosts. This fish is of a different subdivision, Elopoccephala, and a different superorder, Elopomorpha (Wulliman, 1998).
Table 1. Summary of Ruffed Cell and Mitral Cell Soma Measurements

<table>
<thead>
<tr>
<th>Animal</th>
<th>Ruffed Cells</th>
<th>Mitral Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Height (μm)</td>
<td>Width (μm)</td>
</tr>
<tr>
<td>ZF1</td>
<td>11.4±0.4</td>
<td>9.8±0.3</td>
</tr>
<tr>
<td>ZF2</td>
<td>12.3±0.3</td>
<td>11.3±0.7</td>
</tr>
<tr>
<td>ZF3</td>
<td>12.3±0.8</td>
<td>9.6±1.0</td>
</tr>
<tr>
<td>ZF4</td>
<td>9.3±0.9</td>
<td>8.7±0.3</td>
</tr>
<tr>
<td>ZF5</td>
<td>11.5±0.5</td>
<td>10±2.0</td>
</tr>
<tr>
<td>ZF6</td>
<td>9.0</td>
<td>9.0</td>
</tr>
<tr>
<td>ZF7</td>
<td>10.3±0.9</td>
<td>10.0±1.0</td>
</tr>
<tr>
<td>ZF8</td>
<td>14.0±1.0</td>
<td>10.5±1.5</td>
</tr>
<tr>
<td>Average</td>
<td>11.2±0.6</td>
<td>9.9±0.3</td>
</tr>
</tbody>
</table>

The variations seen in the ruffed cell morphology between the sea eel and the other fish is likely due to the evolutionary divergence between the species. There are similarities between the goldfish, catfish and sea eel with regard to the initial part of the ruff, which forms a spherical field about 20-50μm in diameter (Kosaka and Hama, 1979a; 1979b; Kosaka, 1980; Alonso et al., 1987). This initial segment also is seen in the zebrafish, but its diameter ranges from 10-25μm. Thus, the ruffed cell appears to be unique to fish, but morphologies may vary between types of fish.
We found that ruffed cells are much less common than mitral cells, but it is possible that ruffed cells make up a larger proportion of total output neurons in the zebrafish olfactory bulb than reported here. We applied rigorous standards when identifying the ruffed cells in the zebrafish olfactory bulb. Because the membranous protrusions of a ruff are similar in morphology to some orientations of a dendritic tuft, we included a cell as ruffed only if we could see the axon projecting from the cell body as well as the projection of the axon through the "ruffled" zone. The low number of ruffed cells identified in this study also could be a result of several factors including incubation time of the tracer substance or variability in injection location. However, a large number of output neurons and their dendrites filled with the tracer, so it is unlikely that further incubation would have allowed for a much greater number of ruffed cells to be filled.

<table>
<thead>
<tr>
<th>Animal</th>
<th>Ruffed Cells</th>
<th>Percentage of Output Neurons that are Ruffed Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>ZF1</td>
<td>24/597</td>
<td>4.0%</td>
</tr>
<tr>
<td>ZF2</td>
<td>3/338</td>
<td>0.9%</td>
</tr>
<tr>
<td>ZF3</td>
<td>4/234</td>
<td>1.7%</td>
</tr>
<tr>
<td>ZF4</td>
<td>3/362</td>
<td>0.8%</td>
</tr>
<tr>
<td>ZF5</td>
<td>3/231</td>
<td>1.3%</td>
</tr>
<tr>
<td>ZF6</td>
<td>1/270</td>
<td>0.4%</td>
</tr>
<tr>
<td>ZF7</td>
<td>3/402</td>
<td>0.7%</td>
</tr>
<tr>
<td>ZF8</td>
<td>2/185</td>
<td>1.1%</td>
</tr>
</tbody>
</table>
Figure 17. A phylogenetic tree of the teleost line exhibits the interrelationships of the primary fish discussed in this study: the zebrafish (*), goldfish, catfish and sea eel. This cladogram was modified from M. Wullimann (1998).
In addition, we have injected at various locations in the telencephalon in an attempt to reconcile the possibility that ruffed cells project to a different location than the other output neurons. Based on these strict identification criteria, we propose that ruffed cells make up less than 10% of the population of output neurons.

Edwards and Michel (2002) describe several neuronal phenotypes in the adult zebrafish olfactory bulb based on labeling with \( \gamma \)-aminobutyric acid (GABA), glutamate and tyrosine hydroxylase (TH). They found that mitral cells labeled with glutamate, but not with GABA or TH. They describe two populations of juxtaglomerular cells, both labeled with low levels of glutamate and high levels of GABA, but only one group labeled with TH. It is possible, based on the distribution of these cells, that the TH-negative juxtaglomerular cells are ruffed cells. Most of the ruffed cell bodies were located deep in the glomerular layer or the superficial internal cell layer and their dendrites projected outwardly to the glomerular layer. Also, studies in our laboratory showed that ruffed cells are TH-negative (unpublished observation). On the other hand, Edwards and Michel (2002) reported that the TH-negative cells were approximately \( 6.1 \pm 1.2 \mu \text{m} \) in diameter, and the average width of the ruffed cells in this study measured \( 9.9 \pm 0.3 \mu \text{m} \). So, it is unlikely that the TH-negative juxtaglomerular cells are ruffed cells. However, differences in tissue processing and plane of section between the two studies may account for some of the deviation in the average soma size. Further studies are necessary to resolve this issue.

Ruffed cells, while similar in size and distribution to mitral cells, possess distinct morphological and physiological properties. Previous studies have suggested that ruffed cell axonal protrusions synapse with granule cells and that the dendrites receive no input from mitral cells or olfactory sensory neurons (Kosaka and Hama, 1979b; Kosaka, 1980). Furthermore, physiological studies comparing response patterns of mitral cells and ruffed cells in goldfish show that during application of a
stimulus, ruffled cells and mitral cells showed contrasting interactions (Zippel et al., 1999). For example, when the epithelial activity was blocked, mitral cell responses were reduced whereas ruffled cell activity increased (Zippel et al, 1999). Thus, the ruffed cell represents a functionally significant class of neuron whose identification in the adult zebrafish olfactory bulb could prove important in studies of olfactory coding, olfactory processing, and comparative neurology.

This information was presented in 2005 in a publication entitled, “Ruffed Cells Identified in the Adult Zebrafish Olfactory Bulb” in Neuroscience Letters.
CHAPTER V
THE MORPHOLOGY AND DISTRIBUTION OF MITRAL CELLS IN THE
OLFACTORY BULB OF ADULT ZEBRAFISH

Introduction

Mitral cells are the major output neurons and the primary relay in the olfactory bulb of vertebrates (Allison, 1953; Nieuwenhuys, 1967; Andres, 1970). Their structure differs significantly among mammals and lower vertebrates (Allison, 1953; Shepherd, 1972). In mammalian systems, the olfactory bulb is a laminar structure with well-defined cell types in each layer (Shepherd, 1972). In these species, mitral cells are mitre-shaped macroneurons with a single primary dendrite that contacts a single glomerulus (Meisami and Safari, 1981). They have secondary dendrites that do not form synapses in any glomeruli and have cell bodies ranging in size from 10µm-20µm in diameter (Meisami and Safari, 1981; Dryer and Graziadei, 1994).

In contrast to the mammalian olfactory bulb, the teleost olfactory bulb has a diffusely organized laminar structure composed of four principal layers (Oka et al., 1982; Satou, 1990; Byrd and Brunjes, 1995). These layers include the olfactory nerve layer (ONL), the glomerular layer (GL), the mitral cell layer (MCL), and the granule cell layer (GCL). Due to the ill-defined nature of the MCL and GCL, they often are combined into a single layer termed the internal cell layer (ICL; Byrd, 2000). The morphology of mitral cells within teleost olfactory bulbs has been characterized primarily through the use of classic Golgi staining (Sheldon, 1912; Holmgren, 1920; Kosaka and Hama, 1982; Oka, 1983), although intracellular horseradish peroxidase methods also have been employed (Fujita et al., 1988). These studies suggest that mitral cells in teleosts are large neurons of varying soma and dendritic arborization...
characteristics (Oka, 1983). Cell bodies can be triangular or ovoid and, in contrast to mammalian output neurons, teleost mitral cells reportedly have multiple primary dendrites with glomerular tufts and no secondary dendrites (Oka, 1983; Alonso et al., 1988; Dryer and Graziadei, 1994). In goldfish and carp, mitral cells are generally located 100-200μm from the surface of the bulb and are approximately 10-25μm in diameter (Kosaka and Hama, 1982; Oka, 1983). In general, each cell contains several dendritic projections (2-6μm in diameter) that form tufts at various lengths from the cell body (Kosaka and Hama, 1982).

Mammals have a single olfactory tract per main olfactory bulb, but fish have both a lateral and medial olfactory tract where axons from mitral cells bundle and project to the brain. In zebrafish, these tracts are distinct and can easily be distinguished as they make their way from the olfactory bulb through the adjacent telencephalon. It has been suggested that the medial tract processes pheromonal information and mediates reproductive behavior while the lateral tract conveys information about food (Satou et al., 1983; Stacey and Kyle, 1983; Demski and Dulka, 1984; Satou et al., 1984; Kyle et al., 1987; Sorensen et al., 1991; Hamdani, Alexander and Døving, 2001; Hamdani, Kasumyan and Døving, 2001; Weltzien et al., 2003). As a result, potential differences between the mitral cells projecting to the medial or the lateral olfactory tract could provide important information about olfactory coding in the teleost olfactory bulb.

The zebrafish is an excellent model for studies of the olfactory system (Baier and Korsching, 1994; Byrd and Brunjes, 1995; Dynes and Ngai, 1998; Whitlock and Westerfield, 1998; Friedrich and Laurent, 2001; Edwards and Michel, 2002; Tabor et al., 2004; Miyasaka et al., 2005; Sato et al., 2005) and is becoming an increasingly popular model in the scientific community. While numerous studies have begun to
characterize the anatomy of the olfactory system in this species (Baier et al., 1994; Byrd and Brunjes, 1995; Edwards and Michel, 2002; Sato et al., 2005), there is still little known about the structure and function of the different cell types within the olfactory bulb. In order to gain an understanding of the way zebrafish process information, many researchers have focused on the functional aspects of the olfactory system, such as odor representation and stimulation (Corotto et al., 1996; Freidrich and Korsching, 1998; Friedrich and Laurent, 2001; Edwards and Michel, 2002; Lipschitz and Michel, 2002; Michel et al., 2003; Friedrich et al., 2004; Tabor et al., 2004; Friedrich and Laurent, 2004; Liy et al., 2005). While some of these studies have touched upon characteristics of zebrafish mitral cells, a detailed analysis is lacking. Since the mitral cell is the major output neuron of the olfactory bulb, it is important to understand its structure, distribution, and interactions with other cells. This study will analyze mitral cell morphology and distribution in the adult zebrafish olfactory bulb in order to provide further insight into information processing and olfactory coding in this important model system.

Materials and Methods

Animals

Adult male and female zebrafish, *Danio rerio*, at least 2.5cm in length, were obtained from commercial sources (RJ Ray Distributors, Pontiac, MI). The fish were maintained in 15-gallon aquaria filled with aerated, conditioned fish water at 28.5oC, and they were fed commercial flake food (Wardley Corporation) twice daily. All protocols on animal care and experimental procedures were approved by the Institutional Animal Care and Use Committee.
Olfactory Tract Tracing

Fish were anesthetized with 0.03% MS222 (3-amino benzoic acid ethyl ester, Sigma) and perfused with phosphate buffered saline (PBS). The brains were dissected, and approximately 0.05μl-0.1μl of a 10000MW dextran (biotinylated dextran amine, BDA, 100mg/ml in PBS, Molecular Probes or Texas Red dextran, 5mg/ml in PBS, Molecular Probes) or a 3000MW dextran (micro-ruby, tetramethylrhodamine and biotin dextran, 5mg/ml in PBS, Molecular Probes) was injected either into the medial, lateral, or both olfactory tracts using a PV 800 Pneumatic Picopump (World Precision Instruments). The brains were then placed on a 3μm polycarbonate filter in a sterilized six-well culture dish (Costar) containing artificial fish cerebrospinal fluid (100mM NaCl, 2.46mM KCl, 1mM MgCl·6H2O, 0.44mM NaH2PO4H2O, 1.13mM CaCl2H2O, 5mM NaHCO3) and incubated at 28.5°C and 1.5% CO2 for approximately 4 hours (Tomizawa et al., 2001).

Following fixation in 4% paraformaldehyde for 24 hours, the tissue was either viewed as whole mounts, or brains were cryostat sectioned in the horizontal or coronal plane and mounted on positively charged slides. Brains injected with Texas Red dextran or micro-ruby first were viewed as whole mounts for dendritic analysis and then were serial sectioned at 20μm-100μm for soma analysis and/or immunocytochemistry. The tissue was viewed on a Zeiss LSM 510 confocal microscope using a Texas Red filter. The BDA-treated tissue sections, also taken at 20μm-100μm, were incubated with a fluorescent avidin (Alexafluor 488 avidin, 1:200 in PBS, Molecular Probes) for 1 hour at room temperature, rinsed, coverslipped with PBS and glycerol containing 0.1% para-phenylenediamine (PPD; Beltz and Burd, 1989) and viewed on a Zeiss LSM 510 confocal microscope using a FITC filter. To visualize the BDA-labeled cells with light microscopy, avidin-HRP (1:1000 in PBS,
Molecular Probes BDA Neuronal Tracer Kit, N-7167) treatment was used. The sectioned tissue was incubated overnight at 4°C, rinsed with PBS for 30 minutes, and treated with a 0.05% 3,3'-diaminobenzadine solution (Molecular Probes BDA Neuronal Tracer Kit, N-7167). After rinsing, dehydrating, and coverslipping with DPX mounting medium (Aldrich Chemical Company), the tissue was viewed on a Nikon Eclipse E600 microscope.

**Golgi-Kopsch Technique**

This procedure was modified from Greer (1987) and Riley (1979) and optimized for zebrafish olfactory tissue. Zebrafish were anesthetized and perfused with PBS followed by fixative (1% paraformaldehyde, 1% glutaraldehyde in 0.1M PBS). The brain was dissected, placed in fixative at 4°C for 48 hours, rinsed and incubated in fresh dichromate solution (1.19M potassium dichromate, 0.44M sucrose, 37% formaldehyde, all from Fisher Scientific), in a light-safe container at room temperature for 72 hours. Following a rinse in silver nitrate solution (0.04M, Fisher Scientific), the tissue was stored in this medium, protected from light, at room temperature for 12 to 24 hours. It was then dehydrated in ethanol followed by a 1:1 solution of ether and ethanol and stored in celloidin (5% parlodion, Mallinckrodt; 50% Ethanol, Fisher Scientific; 45% Ethyl Ether, Fisher Scientific), in a light-safe container at room temperature overnight. The brains were mounted in celloidin, solidified in a chloroform chamber and stored in butanol overnight. Sections were obtained in 100μm increments on a sliding microtome, dehydrated, mounted on slides using DPX mounting medium (Aldrich Chemical Company) and viewed on a Nikon Eclipse E600 microscope using brightfield microscopy. The tissue embedded in this manner was used to confirm that the morphologies of mitral cells seen with the
retrograde tract-tracing technique also were present using other methods. These Golgi-impregnated cells were not used for quantitative analysis.

**Immunocytochemistry**

BDA-labeled sections that had already been treated with a fluorescent avidin were blocked with 3% normal goat serum and 0.4% Triton X-100 in PBS. They were treated with a mouse monoclonal antibody [anti-HuC/HuD, 1:100 in PBS, Molecular Probes, CAT#A-21271/LOT# 7201-3, or anti-tyrosine hydroxylase (TH), 1:1000 in PBS, ImmunoStar, CAT#22941/LOT# 148003] or a rabbit polyclonal antibody [anti-FMRFamide, 1:500 in PBS, Chemicon, #AB1917, or anti-keyhole limpet hemocyanin, KLH, 1:500 in PBS, Sigma, #H0892] for 24 hours at 4°C. HuC/HuD, is used as a neuronal marker because it recognizes a neuron-specific RNA-binding protein. The HuC/HuD antibody employed in this study was created against the peptide sequence QAQRFRLDNLLN (Marusich et al., 1994) and has been shown to label specifically neurons in zebrafish (Henion et al., 1996; Byrd and Brunjes, 2001; Zupanc et al., 2005). Tyrosine hydroxylase is the rate-limiting step in catecholamine synthesis, so the antibody to this enzyme is used commonly to identify dopaminergic neurons (usually periglomerular cells) in the olfactory bulb. The TH antibody was made against an epitope in the mid-portion of the molecule (manufacturer’s technical information) and it did not cross react with other proteins when examined with Western Blot analysis (data not shown). This antibody produced a pattern of immunoreactivity in the zebrafish brain that was identical to other studies (Edwards and Michel, 2002; Fuller, Villanueva, and Byrd, 2005). FMRFamide is a neuroactive peptide marker shown to label specifically terminal nerve cells in the zebrafish olfactory bulb (Li and Dowling, 2000; Maaswinkel and Li, 2003). The antibody used
in this study was made against the 4 amino acid sequence FMRF conjugated to neat bovine serum albumin (manufacturer’s technical information). The antibody against KLH labels consistently the olfactory nerve in teleosts (Riddle and Oakley, 1992; Riddle et al., 1993; Jarrard, 1997; Starcevic and Zielinski, 1997), including this species (Fuller, Villanueva, and Byrd, 2005). Following a PBS rinse and incubation in a fluorescently labeled secondary antibody (Alexafluor 594 goat anti-mouse IgG, 1:200 in PBS, Molecular Probes, or Alexafluor 594 goat anti-rabbit IgG, 1:200 in PBS, Molecular Probes) for 1 hour, the slides were coverslipped with glycerol and PBS (50:50) and viewed on a Zeiss LSM 510 confocal microscope using a multitrack overlay with narrow band filters.

**Glomerular Tracing**

Glomeruli were labeled with DiA (4-(4-(dihexadecyamine) styryl)-N-methylpyrindinium iodide, 5mg/ml in dimethyl sulfoxide, (Fisher Scientific), Molecular Probes) injections into the olfactory organs according to the protocol developed by Baier and Korsching (1994). Following this procedure, the animals were returned to a 15-gallon aquarium for recovery and the tank was kept dark in order to prevent bleaching of the photosensitive tracer. Twenty-four hours later, fish were processed for olfactory tract tracing as described above, using BDA (100mg/ml in PBS, Molecular Probes) or Texas Red dextran (5mg/ml in PBS, Molecular Probes) as a tracer. To optimize visualization of mitral cell dendritic arbors and glomeruli, 80μm cryostat sections and whole mounts were examined on a Zeiss LSM 510 confocal microscope using Z stacks and image projections at multiple angles of difference. These images were captured using a multitrack overlay with narrow band pass filters.
Quantitative Analysis

For a variety of morphological and quantitative analyses, over 1000 cells in 30 adult animals were examined. The morphologies of mitral cells in 10 adult zebrafish, whole olfactory bulbs were compared for the presence of multiple versus single primary dendrites. Of these 10 fish, 5 had the medial olfactory tract loaded with tracer and 5 had the lateral olfactory tract loaded with tracer. A total of 100 cells were analyzed for dendritic morphology and the percentage of mitral cells with multiple dendrites versus single dendrites was established for the entire bulb as well as for cells projecting to the medial olfactory tract and cells projecting to the lateral olfactory tract. The percentage of mitral cells with single dendrites was determined by dividing the total number of mitral cells with a single process emanating from the soma by the number of randomly selected cells (n=100), and the percentage of mitral cells with multiple dendrites was determined by dividing the total number of mitral cells with multiple processes emanating from the cell body by the number of randomly selected cells (n=100). Comparisons between uni-dendritic and multi-dendritic percentages were made using a one-way ANOVA. For all quantitative analyses, averages were reported with standard errors of the mean and P values less than 0.05 were considered significant.

To obtain dendritic reconstructions of these cells, whole mounts were examined using confocal microscopy. Once a cellular profile was identified, Z-stack images were used to gather optical sections of the cell at 1µm intervals and the cell was traced using the LSM510 overlay tool. Different colors represent varying planes of focus. The image was converted to a projection to visualize the cell in three dimensions and confirm that all dendritic bifurcations were represented in the z-stack trace.
The average number of mitral cells in a single olfactory bulb for a single fish was determined by obtaining counts of profiles retrogradely labeled with a 3000MW dextran from the right and left olfactory bulbs of 15 fish. For each fish, the total number of mitral cells for the right and left bulbs was averaged. These totals were then combined and divided by the total number of fish evaluated (n=15) to obtain the average number of mitral cells in a single bulb. Only mitral cells were considered in these analyses based on specific morphological criteria (see Results).

In order to ascertain if mitral cells possessed a normal distribution with regard to soma size, cell cross-sectional area measurements from 100 retrogradely labeled cells in 10 fish were obtained using the LSM 510 confocal microscope overlay and measurement tools. Once a cellular profile was identified, Z-stack images were used to gather fine optical sections of the cell. The image was then converted to a projection to visualize the cell in three dimensions and to determine the largest axis of the soma. Cell cross-sectional areas were then obtained by tracing the perimeter of the soma in its largest dimension. The cells were binned based on 10μm² areas and the total number of cells in each category was determined. The Kolmogorov-Smirnov test for normality was used to determine the distribution of all mitral cells as well as TH+ interneurons. Further, the distribution was examined individually for cells with multiple dendrites, cells with a single dendrite, cells on the medial side of the bulb, and cells on the lateral side of the bulb.

Average cross-sectional area comparisons between mitral cells with multiple dendrites and mitral cells with single dendrites were done using two-sample t-tests, as the distribution of these populations was normal (p>0.5). Also, cross-sectional area comparisons were done using the non-parametric Mann-Whitney test to evaluate potential differences in the soma sizes of mitral cells on the lateral side versus the
medial side of the olfactory bulb because these populations did not exhibit a normal distribution (p<0.05). Further, a two-way ANOVA was used to compare soma sizes for all subcategories. For example, the soma sizes of lateral cells with a single dendrite were compared to the soma sizes of lateral cells with multiple dendrites.

To compare the average sizes of mitral cells to other cellular profiles in the olfactory bulb, high magnification digital images were obtained from dextran-loaded or TH-treated olfactory bulbs in similar histological preparations in 20 adult animals. Diameters of somata were measured using the Spot Advance Software line measurement tool. Dimensions including soma cross-sectional area, length and width were used to compare the size of TH-positive interneurons to mitral cells. TH-positive cells on the medial side of the bulb also were compared to TH+ cells on lateral sides of the olfactory bulb using two-sample t-tests to confirm that the two populations were not significantly different in size before grouping them to compare them to mitral cells. Length was defined as the diameter of the cell along the axis from which the major dendrite(s) projected regardless of the orientation of the cell in the olfactory bulb, while width was the diameter of the cell perpendicular to that measure. These measurements were made from a total of 100 presumptive mitral cells and 75 TH-positive interneurons.

Hematoxylin and Eosin Staining of Paraffin Sections

Fish were anesthetized and perfused with Bouin’s fixative solution. Two hours post-fixation, the brains were dissected, dehydrated, and embedded in paraffin. Following serial sectioning in the horizontal plane at 10μm and mounting on gelatin-coated slides, the tissue was dewaxed in xylenes, rehydrated with ethanols, and rinsed in water. Sections were stained with hematoxylin for 1 minute, rinsed in water, and
incubated in clarifying solution for 1 minute. They were then rinsed with water, treated with bluing reagent, rinsed, and washed in 95% ethanol. After incubation in eosin y for 1 minute and three rinses in 100% ethanol, the tissue was rinsed in xylenes several times and coverslipped with DPX mounting medium (Aldrich Chemical Company). All hematoxylin and eosin reagents were purchased from Richard Allen, Inc.

Results

Identification of Mitral Cells

Output cells in the adult zebrafish olfactory bulb were identified using retrograde tract tracing with various dextran and a modified Golgi-Kopsch technique. The labeled profiles were found in the GL and the superficial ICL (Figure 18), and they showed positive immunoreactivity with a neuronal marker, HuC/HuD antibody (data not shown). Based on the location, neuronal identity, and projection of the axon to one of the olfactory tracts, we conclude that the majority of these labeled profiles are mitral cells.

Mitral Cell Morphology and Distribution

The mitral cells exhibited two main types of morphologies with regard to their dendrites: the uni-dendritic morphology was a single primary dendrite with one or more tufts, but multi-dendritic mitral cells also were seen. The uni-dendritic mitral cell somata were generally ovoid or spherical in shape (Figure 19), but there was variability in that some cells had teardrop, fusiform, pear-shaped or elongated somata. The typical morphology of this neuron was a cell body ranging in size from 4-13μm.
in diameter with a mean length of $9.3 \pm 0.2\mu m$ and a mean width of $6.2 \pm 0.1\mu m$ (Table 3). Further, the cell cross-sectional areas at the largest point ranged from 31-70$\mu m^2$ with a mean area of $47.1 \pm 1.1\mu m^2$ (Table 3). These mitral cells had a single dendritic trunk, generally 2-5$\mu m$ in diameter, with a distinct glomerular tuft. Although, in some cases a single trunk would branch at some distance from the cell body and create two or more small tufts. The dendritic tufts generally ranged from 20-50$\mu m$ in diameter and were typically found within close proximity (under 40$\mu m$) away from the cell body, but they also were seen up to 80$\mu m$ away from the soma (Fig. 19). Occasionally, the dendrites projected further; however, this phenomenon was limited as we have seen only a few instances. When the cell body was located in the GL the dendrites projected either laterally or deep to reach their synaptic targets, but when the cell body was located in the superficial ICL the dendrites projected superficially instead. The axons of these cells, typically 1-2$\mu m$ in diameter, did not exhibit elaborate protrusions of the membrane such as ruffed cell possess (Fuller and Byrd, 2005), and they projected to either the medial or the lateral olfactory tract.

A smaller percentage of mitral cells did not exhibit this morphology. Multidendritic mitral cells were similar in shape to the other mitral cells, but they were significantly larger than mitral cells with a single apical dendrite ($P<0.05$). They had a mean length of $11.9 \pm 0.4\mu m$, a mean width of $8.0 \pm 0.3\mu m$, and their soma cross-sectional areas ranged from 46-96$\mu m^2$ with a mean area of $76.8 \pm 2.1\mu m^2$ (Table 3). The distinct feature of the multi-dendritic mitral cells was that they possessed multiple dendritic processes emanating from the cell body (Figure 20). Some of these dendrites had discrete dendritic tufts while others possessed less distinct projections. These dendrites occurred at varying lengths from the cell body and in most cases, regardless of the dendritic morphology, tufts from different dendrites of the same cell
appeared to localize to the same region in the GL (Figure 20A-C). Examination of the
dextran-loaded cells, when the olfactory sensory axons were labeled with an antibody
against KLH (Figure 3D) or traced with DiA (data not shown), showed that even
mitral cells with multiple dendrites can innervate a single glomerulus.

As with the uni-dendritic mitral cells, multi-dendritic mitral cells were
distributed throughout the GL and the superficial ICL, and their dendrites projected
either superficially or deep depending on the location of the soma. Their axons
projected to either the medial olfactory tract (MOT) or the lateral olfactory tract
(LOT) since these morphologies were seen when either the MOT or LOT was
injected with tracer. Analysis of 100 cells in 10 fish revealed that uni-dendritic mitral
cells make up approximately 69% of the total population while multi-dendritic mitral
cells comprise about 31% of the total population.

Mitral Cells and the Olfactory Tracts

Mitral cell distribution was examined based on the projection of the axon to
the medial or the lateral olfactory tract. In most cases, the location of the cell on the
medial or lateral side of the bulb was indicative of the tract to which it would project
(Figure 21). However, it also was apparent that mitral cells in the rostral portion of
the bulb projected primarily to the MOT while mitral cells in the caudal region of the
bulb were more likely to project to the LOT.

There appeared to be no segregation in the morphology of the cells projecting
to the MOT and the LOT, since uni- and multi-dendritic mitral cells were seen on
both the medial and the lateral sides of the olfactory bulb. Uni-dendritic versus multi-
dendritic morphologies can be further analyzed, however, by examining the
percentage of these populations that project to the medial versus the lateral olfactory

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tract. Of the MOT-projecting cells analyzed for dendritic morphology (n=50), 42% were multi-dendritic mitral cells compared to 20% of the LOT-projecting cells analyzed (n=50). Statistical analysis shows that there are significantly more multi-dendritic mitral cells on the medial side of the olfactory bulb than on the lateral side of the olfactory bulb (P<0.05) and there are significantly more uni-dendritic mitral cells on the lateral side of the olfactory bulb than on the medial side (P<0.05). In addition, when examining both uni- and multi-dendritic cells on the lateral side of the olfactory bulb, there are significantly more uni-dendritic cells than multi-dendritic cells (P<0.05). However, there is no significant difference in the number of uni-dendritic versus multi-dendritic cells on the medial side of the olfactory bulb (P>0.05).

The average size of the cell bodies showed significant variation between the lateral and medial sides of the olfactory bulb (P<0.05 for all comparisons). Of 100 cells examined, the average cross-sectional area of uni-dendritic cells on the lateral half was $43.9 \pm 1.5 \mu m^2$, while the average area of uni-dendritic cells on the medial half was $51.4 \pm 1.3 \mu m^2$ (Table 3). Further, the average cross-sectional area of multi-dendritic cells in the lateral half of the bulb was $67.3 \pm 4.0 \mu m^2$, while the average area of multi-dendritic cells on the medial side of the bulb was $81.3 \pm 1.8 \mu m^2$ (Table 3).
Further Examination of Mitral Cells

Area measurements of all mitral cell somata grouped in 10μm² increments did not show a normal distribution (p<0.05). Thus, cells were segregated based on either their morphologies or their tract projections and the total number of cells in each category was reported (Figure 22). Cells segregated based on medial or lateral olfactory tract projection also did not show a normal distribution (P<0.05), unlike cells segregated based on uni-dendritic or multi-dendritic morphology (P>0.05). When the cells are binned according to morphology and tract projection, multi-dendritic cells and medial cells occupy more of the higher area bins than uni-dendritic cells and the lateral cells (Figure 22).
Figure 19. Various methods of labeling revealed the typical structure of an adult zebrafish mitral cell: a soma (*) of variable-shape, a single primary dendrite (arrow) terminating in fairly discrete dendritic tufts near the cell body, and a single axon (arrowhead) projecting toward the medial or lateral olfactory tract. Dextran-labeled cells treated with diaminobenzidine (A) and Alexafluor 488 (B) yielded a similar morphology to a cell labeled with the fluorescent dextran, micro-ruby, (C) and a Golgi-Kopsch impregnated cell (D). Dendritic reconstructions of mitral cells showed dendritic processes with a distinct tufting region in uni-dendritic cells (E, F). Different colors represent varying planes of focus. The majority of the more than 1000 cells examined exhibited this morphology. Scale bar = 10μm (A-F).

The representative number of mitral cells in a single olfactory bulb was estimated. Based on calculations from 15 fish, the total number of labeled mitral cells in an adult zebrafish olfactory bulb ranged from 188-830 and the average number of labeled mitral cells seen with our method was 395 ± 53. We did see as many as 830 cells in a single bulb; therefore we suggest that there are over 750 mitral cells per olfactory bulb in the typical adult zebrafish.
Figure 20. The morphologies of multi-dendritic mitral cells also were seen using the retrograde tract-tracing technique. Mitral cells with multiple dendrites (A, B, D arrows) were less frequent than those with single primary dendrites. Dendritic reconstructions showed that even cells with multiple dendrites exhibited dendritic tufts within close proximity to one another (C). Different colors represent varying planes of focus. Labeling of olfactory sensory axons with anti-KLH (red) showed that mitral cells with multiple dendrites can innervate a single glomerulus (D). Scale bar = 20µm (A,B) or 15µm (C,D).
Table 3. Comparisons of Soma Cross-sectional Areas, Lengths, and Widths for All Populations of Mitral Cells.

<table>
<thead>
<tr>
<th></th>
<th>N</th>
<th>Soma Cross-sectional Area (avg. μm² ± SEM)</th>
<th>Soma Length (avg. μm ± SEM)</th>
<th>Soma Width (avg. μm ± SEM)</th>
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<td></td>
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<td>(All Cells)</td>
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<tr>
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<td>10.2 ± 0.3</td>
<td>6.3 ± 0.2</td>
</tr>
<tr>
<td>Multi</td>
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<td>81.3 ± 1.8</td>
<td>12.6 ± 0.5</td>
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</tr>
<tr>
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<tr>
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<td>All Multi</td>
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<tr>
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<tr>
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<td>67.3 ± 4.0</td>
<td>9.5 ± 0.9</td>
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Comparison of Mitral Cells to Other Cells in the Olfactory Bulb

The retrograde tract-tracing technique also allowed for the identification of two other types of output neurons: the ruffled cell (Figure 23A) and the terminal nerve ganglion cells (Figure 23B). These profiles were identified as non-mitral cell by their unique morphologies. The ruffled cells had an obvious membranous field surrounding the initial part of the axon (Fuller and Byrd, 2005). The terminal nerve cells were large, amorphous clusters that often could be found along the axon bundles that made up the MOT and the LOT or in large groups in the ONL and GL near the most rostral portion of the bulb, similar to that reported by Li and Dowling (2000).
Figure 21. The distribution of MOT-projecting or LOT-projecting mitral cells was examined in the horizontal and coronal planes. A horizontal section, viewed with fluorescence, showed that mitral cells were distributed around the periphery of the bulb when both the MOT and the LOT were labeled (A). Schematic diagrams shown in B and C illustrate general locations of MOT-projecting or LOT-projecting mitral cells based on the dye injection site. In the horizontal plane (B), mitral cells projecting to the MOT (stars) appeared most prominently in the medial and rostral portions of the bulb, while mitral cells projecting to the LOT (triangles) were more abundant in the lateral and caudal regions of the bulb. A similar distribution pattern was seen in the coronal plane of the olfactory bulb (C). Scale bar = 50μm

The identity of the latter cell type was subsequently confirmed by co-labeling with FMRFamide (Figure 23C) and measurements of these output cells established that mitral cells are not the largest cells in the olfactory bulb. The terminal nerve cells were much larger. They had an average soma length of 18.8 ± 2.1μm and an average soma width of 18.3 ± 1.5μm. Thus, even though our tracing technique labeled several types of profiles, we were able to identify specifically the mitral cells for this study.
Figure 22. Mitral cells were binned according to the cross-sectional area of their cell bodies at the largest point. These measurements were further analyzed by grouping them according to tract projection and/or cellular morphology.

Another neuron in the olfactory bulb, the juxtaglomerular cell, also shared a common distribution with the mitral cell and sometimes shared a similar morphology as well (compare Figure 23D and E). This interneuron, identified using an antibody against tyrosine hydroxylase (TH), appeared relatively similar in size to mitral cells (Figure 23F). Given the common distribution pattern and potential for anatomical resemblance, the sizes of TH-positive interneurons and mitral cells also were
compared. Analysis of TH+ soma areas showed a normal distribution (P>0.05), and there was no significant difference between the areas of the cells on the medial side of the olfactory bulb versus the cells in the later side of the olfactory bulb (P>0.05). Thus, all TH+ cells were grouped for comparison to mitral cells. The soma length and width of these two cell types were evaluated and TH+ cells were compared to each subcategory of mitral cell (Figure 24). The mitral cell bodies, including all populations of cells, ranged in size from 6-18\(\mu\)m in length and 4-12\(\mu\)m in width (Table 3). The TH-positive cells, on the other hand, ranged in size from 5-10\(\mu\)m in length and 4-10\(\mu\)m in width. The TH-positive cells had a mean length of 7.0 ± 0.1\(\mu\)m and a mean width of 6.5 ± 0.1\(\mu\)m. The smallest mitral cells, the uni-dendritic cells on the lateral side of the bulb, had a mean length of 8.7 ± 0.2\(\mu\)m and a mean width of 6.2 ± 0.2\(\mu\)m. The largest cells, the multi-dendritic mitral cells on the medial side of the olfactory bulb, had a mean length of 12.6 ± 0.5\(\mu\)m and a mean width of 8.1 ± 0.4\(\mu\)m. This shows substantial overlap between TH+ cells and some, but not all, populations of mitral cells (Figure 24).

Discussion

We have described the morphology and distribution of the major output neuron, the mitral cell, in the adult zebrafish olfactory bulb. This cell type has been described in detail in a number of animals including other teleosts (Kosaka and Hama, 1982; Oka, 1983; Alonso et al., 1988; Fujita et al., 1988; Satou; 1990), cyclostomes (Johnston, 1902; Heier, 1948; Iwahori et al., 1987), elasmobranchs (Iwahori et al., 1992; Dryer and Gaziadei, 1993, 1994), amphibians (Scalia et al., 1991; Jiang and Holley, 1992), reptiles (Garcia-Verdugo et al., 1986; Iwahori et al., 1991; Jiang and Holley, 1992), and mammals.
1989), and mammals (Price and Powell, 1970; Macrides and Schneider, 1982; Mori et al., 1983; Lopez-Mascaraque et al., 1990; Malun and Brunjes, 1996). While mitral cell structure varies between the animal groups studied, in general the morphology remains consistent within the animal groups. For example, in the teleost family mitral cells in carp, goldfish, and tench appear to be similar (Kosaka and Hama, 1982; Oka, 1983; Alonso et al., 1988; Fujita et al., 1988). In carp, the mitral cell soma is generally fusiform, elongated, oval, or triangular in shape, has a mean size of 30\(\mu\)m by 14\(\mu\)m and possesses multiple primary dendrites (Fujita et al., 1988). In goldfish, the mitral cell usually is round or triangular, ranging in size from 10-25\(\mu\)m in diameter, and also has multiple dendrites (Kosaka and Hama, 1982; Oka, 1983). The tench has some variability in that it contains two types of mitral cells. The first type, which is the most prominent, is generally fusiform or triangular in shape and has two or more dendritic trunks that have tufts located at a distance from one another (Alonso et al., 1988). The second type is round or ovoid and possesses one to four primary dendrites that arborize in a single dendritic field (Alonso et al., 1988). Thus, the mitral cells of these teleosts exhibit only minor variations in morphological features such as soma size, which can be accounted for by the difference in the sizes of the fishes being examined; the zebrafish is only 2.5cm in length as an adult.

The shapes of zebrafish mitral cell somata are generally similar to that of other teleosts (ovoid, spherical, fusiform or elongated), although they differ in their dendritic morphology. Some zebrafish mitral cells have multiple dendrites, but we have shown that this number is typically less than 35% of the total population. The typical zebrafish mitral cell has only a single dendritic trunk. Most other teleosts that have been examined have mitral cells with multiple dendritic trunks (Kosaka and Hama, 1982; Oka, 1983; Fujita et al., 1988; Satou; 1990).
Figure 23. The morphology and distribution of mitral cells was compared to other neurons in the olfactory bulb. Ruffled cells (A), which are output neurons that can be identified by a distinct membranous protrusion on their axon (arrowhead), were even fewer in number than the atypical mitral cells. These cells exhibited a single primary dendrite extending from the cell body (arrow). Terminal nerve cells (B) appeared to be the largest neurons in the bulb labeled with our technique, and their cell bodies (*) were often found in tight clusters near the axon bundles of the medial olfactory tract and in large groups in the most rostral portion of the bulb near the ONL (C, *). In panels B and C, rostral is to the right of the image. The identity of these cells was confirmed using an antibody against FMRFamide (C, red). While they shared a similar distribution with mitral cells (green) of the glomerular layer (GL), their morphology was distinct. Mitral cells (E) also resembled tyrosine hydroxylase-positive (TH+) cells (D) with regard to size (D-F), morphology (D, E) and distribution (F). Cells retrogradely-labeled with BDA (green) were found in the GL and the superficial ICL, as were juxtaglomerular cells labeled with TH (red). Scale bar = 20μm (A,B,D,E) or 15μm (C,F).
Figure 24. Mitral cells sizes overlapped somewhat with tyrosine hydroxylase-positive (TH+) cells. For the 75 TH+ cells and 100 mitral cells measured, there was a great deal of overlap in soma length and width of TH+ cells (+) and lateral uni-dendritic mitral cells (triangles) as well as medial uni-dendritic mitral cells (circles). Multi-dendritic mitral cells on the medial (diamond) and lateral (square) side of the olfactory bulb were typically much larger in length and width than TH+ cells.

The exception to this rule is found in the tench, where Type II mitral cells can have from one to four dendrites (Alonso et al., 1988). Even in this case, the Type II mitral cells are less prominent than the Type I mitral cells that have multiple dendrites (Alonso et al., 1988). Furthermore, since Type II mitral cells can have from one to four dendrites, only some of these cells have single primary dendrites. Some teleost studies, even those that suggest mitral cells have multiple primary dendrites, show camera lucida drawings that indicate some of these output neurons may have only a single dendritic trunk. These variations in reporting could result from the fact that cells in the minority are not described in detail or there are differences in the way single dendrites are defined. For example, Oka (1983) states that Golgi-impregnated
mitral cells were identified with very thick dendrites; however, in three of the twenty schematic drawings, the mitral cells appear to have a single dendrite that extends from the cell body (see Oka, 1983, Figure 4, Number 12, for example). In these cases, the dendrite further branches and may innervate different glomeruli, but by our anatomical classification there is only a single dendrite leaving the cell body. We classified a cell as having multiple dendrites if more than one dendritic trunk could be seen leaving the cell body. These minor differences in the definition of a single dendrite verses multiple dendrites could result in variation in the classification of cells. Based on our definitions, it appears that goldfish may have mitral cells with a single dendrite. Regardless, in zebrafish the majority rather than a limited number of mitral cells appear to have a single primary dendrite.

In contrast to the typical teleost mitral cell, which has multiple dendrites, it is generally believed that mitral cells in the mammalian olfactory bulb possess only one primary dendrite (Shepherd, 1972; Meisami and Safari, 1981). However, studies have shown mammalian mitral cells in the main olfactory bulb can have multiple primary dendrites, as well (Mori et al., 1983, Puche, personal communication). In rabbits, for example, about 20% of mitral cells examined had two to three primary dendrites (Mori et al., 1983). In our study, just over 30% of the mitral cell population in zebrafish have multiple dendrites. Mitral cells with multiple primary dendrites are also known to exist in the accessory olfactory bulb of mammals. In mice and rats, mitral cells of the accessory olfactory bulb, a structure that aids in deciphering pheromonal cues, each have up to six primary dendrites (Cajal, 1901; Takami and Graziadei, 1991, 1992; Meisami and Bhatnagar, 1998). In zebrafish, we have shown that the medial olfactory bulb has a larger number of multi-dendritic mitral cells than the lateral olfactory bulb. Interestingly, the medial tract in fish has been suggested to
play a significant role in the processing of pheromonal information while the lateral tract processes other kinds of odorants (Satou et al., 1983; Stacey and Kyle, 1983; Demski and Dulka, 1984; Satou et al., 1984; Kyle et al., 1987; Sorensen et al., 1991; Hamdani, Alexander and Døving, 2001; Hamdani, Kasumyan and Døving, 2001; Weltzien et al., 2003). This lends further support to the idea that olfactory coding in this model system may be similar to mammalian coding. Furthermore, our results suggest that since a large number of mitral cells have only a single primary dendrite with a discrete dendritic tuft, it is likely that these cells innervate only a single glomerulus. We also have shown that cells with multiple dendrites appear to tuft within close proximity to one another and also can innervate a single glomerulus. Based on these anatomical results, we find that the zebrafish mitral cells may be more like mammalian mitral cells than previously believed.

One of the most notable differences between the mitral cells of teleosts and mammals is that teleost mitral cells reportedly lack secondary (basal) dendrites (Kosaka and Hama, 1982; 1982-1983; Alonso et al., 1988; Dryer and Graziadei, 1994). In mammals, secondary dendrites are horizontal or tangential projections from the cell body that are confined to the external plexiform layer, meaning they do not interact with the axons of the olfactory sensory neurons. They are, however, still active in cellular communication and associate with the dendrites of granule cells (Shepherd, 1972; Orona et al., 1984). In teleosts, it has been reported that all dendrites end in a glomerular tuft suggesting that there are no secondary dendrites (Satou, 1990). In our study, we observed several cells that appeared to have projections that did not end in tufts (unpublished data). Based on the location of these projections, extending from the initial portion of the apical dendrite rather than the cell body, they would not be defined as the morphological equivalent of secondary
dendrites in mammals, but could be a functional equivalent. It is possible, however, that these observations were due to incomplete filling of the processes with the dextran. It also is possible that these branches do not interact with glomeruli and are just deep to the GL. Since the olfactory bulb of fish is much more diffuse than that of mammals, it is often difficult to delineate the layers. There is very little segregation between the GL and the ICL and, as a result, dendrites that do not interact with glomeruli would be more difficult to identify. Furthermore, many of the studies that suggest teleosts do not have secondary dendrites examined mitral cells utilizing Golgi technique. According to one of these Golgi studies, some mitral cells did have thin branches without terminal arborizations (Kosaka and Hama, 1982). This may have been a result of partial impregnation and could have resulted in failure to classify secondary dendrites (Kosaka and Hama, 1982). Subsequently, it does not appear that zebrafish possess a morphological equivalent of mammalian secondary dendrites, but it is uncertain whether or not a functional equivalent exists in teleost systems.

Other morphological features, such as soma size, are more easily interpreted. In this study, we found that mitral cells segregate to two means based on their morphologies. Uni-dendritic mitral cells have an average length of 9.3 ± 0.2μm, an average width of 6.2 ± 0.1μm, and an average cross-sectional area of 47.1 ± 1.1μm² (n=50), while multi-dendritic mitral cells have an average length of 11.9 ± 0.4μm, an average width of 8.0 ± 0.3μm, and an average cross-sectional area of 76.8 ± 2.1μm² (n=50). In another zebrafish study, the measurement of 50 cells shows that the average length of the cells is 14.4 ± 1.8μm, the average width is 10.1 ± 1.7μm, and the average area is 105.9 ± 13.8μm (Edwards and Michel, 2002). While these mitral cells were not segregated based on morphologies, the averages were still larger than those found in this study. It is likely that the variation in sizes between the two studies
is due to differences in tissue processing and plane of section. Edwards and Michel (2002) labeled mitral cells with an antibody to glutamate using a three-step immunocytochemistry procedure. This technique may have added additional apparent thickness to the cell.

Studies in cyclostomes and reptiles have grouped mitral cells into types, partially based on soma size (Iwahori et al., 1987, 1989). We binned mitral cells in 10μm² increments using cell cross-sectional areas in order to determine if there was a natural grouping in the size of the cell bodies, for example, one population segregating to a single mean versus more than one population differing in size and number. Our results show that the total population of mitral cells does not follow a normal distribution; rather it appears to be composed of more than one population. Initial segregation of the cells into medial versus lateral olfactory bulb mitral cells also yields distributions that are not normal. This suggests that the cells making up the medial olfactory tract are comprised of two populations of cells, as are the cells of the lateral olfactory tract. We then isolated the cells based on uni-dendritic versus multi-dendritic morphology and found a normal distribution. This suggests that cells with a single dendrite segregate to one mean that is different from cells with more than one dendrite. It also suggests that while comparisons can be made between olfactory bulb mitral cells with specific morphologies regardless of their location, a group of cells on the medial side of the olfactory bulb cannot be compared to a group of cells on the lateral side of the olfactory bulb unless their morphologies are first determined. However, it also is important to note that since the retrograde tract-tracing method used in this study does not label all mitral cells, these results were obtained from a sampling of the total population. Thus, the possibility that mitral cells follow a different pattern of distribution cannot be eliminated.
Many of the mitral cell studies that classify these output neurons into groups use additional morphological features to separate them. In this study, the only major morphological difference in the cells occurs in the number of dendrites extending from the cell body. Our results also suggest that cells with multiple dendrites are significantly larger than those with a single dendrite. In fact, when the results of the soma cross-sectional groupings and soma length and width measurements are examined, a clear picture emerges. The largest cells in the olfactory bulb tend to be cells with multiple dendrites on the medial side and the smallest cells tend to be cells with a single dendrite on the lateral side. It is uncertain, however whether or not these morphological variations represent differences in the function of the cells. We know of no studies that have begun to elucidate the potential differences in the electrical properties of mitral cells with single dendrites versus multiple dendrites in zebrafish. Thus, further physiological studies are required to address the issue of functional differences between these anatomically variant cells.

We found that mitral cells were most commonly distributed on the side of the bulb corresponding to the tract to which their axons would project. Cells in the medial and rostral areas of the bulb most often projected to the MOT, while cells in the lateral and caudal regions of the bulb most often projected to the LOT. We found that there is an apparent difference in the mitral cells of the MOT and the mitral cells of the LOT, with regard to dendritic projections; there are more multi-dendritic mitral cells on the medial side of the olfactory bulb. This may support the functional differences seen between the cells of the MOT versus the cells of the LOT. In teleosts, cellular responses to amino acids and bile components occur in different regions of the olfactory bulb (Thommesen, 1978; Døving et al., 1980; Hara and Zhang, 1998; Nikonov and Caprio, 2001). This suggests that the two classes of
odorants may be processed independently of one another (Hara and Zhang, 1996; 1998) and that spatial organization of output neurons is extremely important in olfactory coding (Nikonov and Caprio, 2001). Previous studies also have shown that the MOT processes information about pheromones while the LOT processes information dealing with amino acids or food (Satou et al., 1983; Stacey and Kyle, 1983; Demski and Dulkà, 1984; Satou et al., 1984; Kyle et al., 1987; Sorensen et al., 1991; Hamdani, Alexander and Døving, 2001; Hamdani, Kasumyan and Døving, 2001; Weltzien et al., 2003). The variation in the number of uni-dendritic versus multi-dendritic mitral cells on the medial and lateral sides of the olfactory bulb may prove significant in understanding the apparent functional differences that exist between the two olfactory tracts.

While examining the distribution of mitral cells in the adult zebrafish olfactory bulb, it was noted that there was a great deal of similarity between these output neurons and TH-positive interneurons. We found that mitral cells, while larger than TH-positive cells on average, show substantial size overlap with the interneurons. In addition, their shapes can be similar and they have a comparable location in the bulb. Some studies suggest that mitral cells are easily identified by their large size and morphology. Our study indicates that size, morphology, and/or distribution would not be sufficient evidence for mitral cell identification. Indeed, we have found that mitral cells are not the largest cells in the olfactory bulb; two other cell types, the ruffed cell and the terminal nerve cell, have larger somata. In a study examining ruffed cells in the adult zebrafish, ruffed cells were shown to have mean length of 11.2μm ± 0.6μm and a mean width of 9.9μm ± 0.3μm compared to mitral cells in the same fish, which had a mean length of 9.0μm ± 0.4μm and a mean width of 8.3μm ± 0.2μm (Fuller and Byrd, 2005). There also is distributional similarity
between mitral cells and ruffed cells (Fuller and Byrd, 2005). The current study shows that terminal nerve cells have larger cell bodies than mitral cells, and evaluation of their average soma size suggests that they are larger than ruffed cells, as well. In other fish, retrograde labeling shows the large size of the ganglion cells of the terminal nerve, approximately 70µm by 80µm in comparison to 30µm by 14µm, the average size of mitral cells in the same species (Fujita et al., 1985). Based on these results, mitral cells appear to be the third largest cell type in the olfactory bulb of zebrafish. It will become increasingly important for those studying this system to use alternative methods including morphological, pharmacological and/or physiological evidence to confirm mitral cell identity in the future.

In summary, we have used a variety of techniques for a rigorous analysis of zebrafish mitral cell morphology and distribution. There are two populations of these output neurons in this teleost. Uni-dendritic cells have a single dendrite, typically with a single dendritic tuft, although more than one tuft can be seen when a dendrite branches at a distance from the cell body. Multi-dendritic cells have multiple dendritic processes emanating from the cell body with several dendritic tufts generally located near each other. Both uni-dendritic and multi-dendritic mitral cells are found throughout the bulb, but the medial side of the olfactory bulb possesses more multi-dendritic cells than the lateral side of the bulb and the lateral side of the bulb has more uni-dendritic cells than the medial side of the bulb. In addition, cells on the medial side of the olfactory bulb tend to project their axons to the MOT while cells on the lateral side of the bulb tend to project to the LOT, regardless of the morphology. Further, it does not appear that innervation of multiple glomeruli is a common feature of zebrafish mitral cells. Because mitral cells are such a vital component in olfactory coding and processing, studies in our laboratory will continue
to focus on this area by examining the cellular interactions of these output neurons as well as activity-dependent cytoarchitecture.
CHAPTER VI
THE EFFECTS OF DEAFFERENTATION ON MITRAL CELLS: AN EXAMINATION OF MORPHOLOGY FOLLOWING LOSS OF AFFERENT INNERVATION

Introduction

Afferent-target interactions continue to be a major focus of study in the scientific community because of the interest in the natural plasticity of the nervous system and its ability, or lack thereof, to recover from disease and injury in specific situations. Many studies have examined the role of afferent innervation on the development and maintenance of central nervous system structures. For example, in the visual system it has been shown that removal of innervation modifies molecular activity in the target (Wong-Riley, 1979; LeVay et al, 1980; Zhang et al, 1995; Pires et al, 1998; Yan and Ribak, 1998). In the auditory system, cochlear removal leads to changes in the dendritic profiles of neurons in the medial superior olivary nucleus (Russell and Moore, 1999) and in the motor-cortical region of birds, afferent lesions result in increased cell death (Johnson et al., 1997).

Deafferentation also has been well studied in the olfactory system. Removal of olfactory innervation leads to loss of olfactory bulb volume in both mammals and teleosts (Matthews and Powell, 1962; Margolis et al., 1974; Harding et al., 1978; Brunjes, 1985; Poling and Brunjes, 2000). In adult rodents, olfactory deprivation results in reduced cell number (Henegar and Maruniak, 1991; Meisami and Safari, 1991; Corotto et al., 1994) and decreased neurogenesis (Corotto et al., 1994). Further, other investigations suggest that loss of innervation results in changes in olfactory bulb morphology (Wilson and Wood, 1992), cellular morphology (Pinching and Powell, 1971), enzymatic activity (Nadi et al., 1981; Baker et al., 1984; Baker et al.,
1993), and mRNA and protein expression (Ehrlich et al., 1990; Stone et al., 1991; Ferraris et al., 1997; Casabona et al., 1998; Oberto et al., 2001) in the deprived bulb. Previous studies in our laboratory support these findings showing that removal of innervation from the nose can have drastic effects on the central nervous system target. For example, in adult zebrafish loss of olfactory input via cauterization of the naris results in reduced bulb volume (Figure 25A; Byrd, 2000), increased apoptosis (Figure 25B; Vankirk and Byrd, 2003) and reduced immunoreactivity of various proteins (Figure 25C; Byrd, 2000; Fuller et al., 2005). In this study, we further examine the role of afferent-target innervation in the maintenance of the adult zebrafish olfactory system.

The teleost olfactory bulb is a diffuse structure when compared to the mammalian olfactory bulb. It is organized into four principal layers (Oka et al., 1982; Satou, 1990; Byrd and Brunjes, 1995) including the olfactory nerve layer (ONL), the glomerular layer (GL), the mitral cell layer (MCL), and the granule cell layer (GCL). Based on previous studies in our laboratory, the distribution of mitral cells in zebrafish does not appear to fall into a single layer as it does in mammals (unpublished data). Thus, the MCL and the GCL are often are combined into a single layer termed the internal cell layer (ICL; Byrd, 2000).

Deafferentation studies in adult zebrafish have suggested that ablation of the olfactory rosette results in significantly reduced volumes of the ONL and the GL, but not of the MCL and the GCL at 4 weeks post-denervation (Poling and Brunjes, 2000). The reduction of volume in the ONL is easily explained by the deterioration and eventual loss of the nerve fibers entering the olfactory bulb. There are other possibilities that could explain the reduction in laminar volume of the GL. There could be a reduction in the number of cells in this layer. A previous study in zebrafish
showed that there is an increase in apoptosis following olfactory denervation, although, the cells undergoing this process were located primarily in the ONL and the ICL, not in the GL (Vankirk and Byrd, 2003). However, this study examined the olfactory bulb at time points varying from 1 hour to 1 week post-deafferentation, so there could be a greater number of cells undergoing apoptosis after 1 week. Another study by Baker and colleagues (1993) suggests that even though enzymatic activity of cells located in the GL of rodents might be reduced following deafferentation, the cells are still present. This supports the idea that even 2 months after removal of afferent innervation, a major cell type in the GL does not undergo apoptosis. Another potential source of reduced GL volume may be loss of the dendritic arborizations of mitral cells that project to this layer. This study will examine the morphology of these major output neurons at various time points following deafferentation in an attempt to address one possible component of volume loss in the deprived bulb.

Materials and Methods

Animals

Adult male and female zebrafish, *Danio rerio*, at least 2.5 cm in length, were obtained from commercial sources (RJ Ray Distributors, Pontiac, MI). The fish were maintained in 15-gallon aquaria filled with aerated, conditioned fish water at 28.5°C, and they were fed commercial flake food (Wardley Corporation) twice daily. All protocols on animal care and experimental procedures were approved by the Institutional Animal Care and Use Committee.
Figure 25. Afferent innervation directly influences the maintenance of the olfactory bulb in adult zebrafish. A) A hematoxylin and eosin stained section of the adult olfactory bulb shows a reduction in the volume of the ipsilateral bulb. B) Twenty-four hours following deafferentation, apoptosis increases in the deafferented bulb (ROB) while there is little cell death in the untreated bulb (LOB). This section of the tissue shows that the olfactory epithelium (oe) remains intact in the internal control. Note the lack of epithelium in the left naris region (*). (C) Tyrosine hydroxylase is markedly reduced in the denervated bulb following loss of innervation from the nose.

**Deafferentation**

Adult zebrafish were anesthetized with tricaine and the right olfactory organ was ablated using a small-vessel cautery iron. The left olfactory organ remained intact for use as an internal control for comparison. Fish were returned to aquarium
water containing the antibiotic Kanacyn (0.01 mg/ml, Aquatronics). For the cellular morphology experiments, fish were allowed to survive for 3 weeks, 6 weeks, 8 weeks, or 20 weeks. For the laminar volume experiments, fish were allowed to survive for 1 week, 3 weeks, or 6 weeks. After the appropriate survival period, the deafferented and control fish were processed as described below.

**Olfactory Tract Tracing**

Fish were anesthetized with 0.03% MS222 (3-amino benzoic acid ethyl ester, Sigma) and perfused with phosphate buffered saline (PBS). The brains were dissected, and approximately 0.05 μl-0.1 μl of Texas Red dextran (10000MW, 5mg/ml in PBS, Molecular Probes) was injected either into both olfactory tracts using a PV 800 Pneumatic Picopump (World Precision Instruments). The brains were then placed on a 3μm polycarbonate filter in a sterilized six-well culture dish (Costar) containing artificial fish cerebrospinal fluid (100mM NaCl, 2.46mM KCl, 1mM MgCl2·6H2O, 0.44mM NaH2PO4·H2O, 1.13mM CaCl2·H2O, 5mM NaHCO3) and incubated at 28.5°C and 1.5% CO2 for approximately 4-6 hours (Tomizawa et al., 2001). Following fixation in 4% paraformaldehyde (PFA) for 24 hours, the tissue was viewed on a Zeiss LSM 510 microscope as whole mounts for dendritic analysis.

**Dendritic Analysis**

To obtain dendritic reconstructions of the dextran-labeled cells, whole mounts were examined using confocal microscopy. Once a cellular profile was identified, z-stack images were used to gather fine optical sections of the cell at 1μm intervals. The image was then converted to a projection to visualize the cell in 3 dimensions (Figure 26A).
Figure 26. A stepwise diagram shows the process of dendritic analysis used to obtain estimates of dendritic complexity in control and deafferented olfactory bulbs. These steps included obtaining a three dimensional image of the mitral cell (A), tracing the dendritic processes (B), and counting the number of branch tips within the area of the tuft (C).

The cell was traced using the LSM510 overlay tool (Figure 26B), and the area of the dendritic arbor was measured by connecting the distal tips of the dendritic branches to form a convex polygon (Figure 26C). The trace was analyzed for complexity by counting the number of dendritic branch tips in the dendritic arbor and this number was reported as dendrites per unit area. Dendritic complexities were compared within survival groups using paired T-tests and between groups using an ANOVA and Tukey's test for multiple comparisons. P values less than 0.05 were considered significant.

Results

Mitral Cell Morphology in Control Animals

In untreated animals, mitral cells exhibited both Type I and Type II morphologies. The Type I morphology, which was the most prominent, had a single apical dendrite with a discrete dendritic tuft (Figure 27A-B). Dendritic reconstructions of these cells verified the localization of the tuft to a limited area (Figure 27C). Even cells with a Type II morphology, which was defined by more than
one dendritic projection, showed dendrites that projected to restricted glomerular
regions and formed a discrete glomerular tuft (Figure 27D). One of the most
noticeable features of these dendritic arbors was the large number of fine processes
they contained. These morphologies were seen in both the right and left olfactory
bulbs.

**Mitral Cell Morphology in Deafferented Animals**

At 3 weeks post deafferentation, the treated, or right olfactory bulb appeared
to show no variation from control animals with regard to the fine processes in the
dendritic tufts (Figure 28, 3wk, ROB). Six weeks after ablation, the fine processes
appeared to diminish and the cellular tufts appeared to be less elaborate (Figure 28, 6
wk, ROB). By 8 weeks after denervation the mitral cells appeared to have few fine
processes, yet the large dendritic branches were still apparent (Figure 28, 8wk, ROB).
These changes were not apparent in the internal control bulb (Figure 28, 8wk, LOB).

**Quantitative Analysis of Mitral Cell Dendrites**

Quantitative analysis of the dendrites showed that there was no significant
difference between the density of the branch tips in the LOB and the ROB at 3 weeks
or 6 weeks following removal of the olfactory organ (p=0.3 and 0.1, consecutively;
Figure 29). By 8 weeks and 20 weeks, however, there were significantly fewer branch
tips per unit area (p<0.01 and p=0.03, consecutively; Figure 29). When comparing
only right bulb densities between groups, an ANOVA showed that there were
significantly fewer branch tips in the 6week, 8 week, and 20 week deafferented
animals than in the control animals. Additionally, there were significantly fewer
branches in the 8-week and 20-week deafferented animals than in the 3-week and 6-
week deafferented animals (p< 0.01 for all comparisons; Figure 29). Left bulb densities also were compared between groups and showed no significant difference at any time point (p= 0.08)

To compare the possibility that primary dendritic processes were more spread out over time, which might result in a reduction of the number of branches per unit area, dendritic tuft areas were compared. Results from these findings showed no significant differences in the tuft areas of right and left bulbs at any single timepoint or between groups at any timepoint (P=0.37; Figure 30).

Discussion

The response of cells to loss of afferent innervation is variable. In some studies involving motoneurons in adult animals, peripheral nerve injury leads to decreased size of the dendritic tree (Vanden Noven et al., 1993; O'Hanlon and Lowrie, 1995). In the auditory system, removal of the cochlea during adulthood generates reduced dendritic density in the target cells, but these cells do not show a reduction in area of the dendrite (Russell and Moore, 1999). In one study, hippocampal lesions result in increased dendritic length and increased dendritic branching of denervated cells (Pyapali and Turner, 1994). Based on this variability, it was uncertain how mitral cells would respond to loss of innervation.

Our results indicate that at 3 weeks post-deafferentation, no change is seen in the dendritic complexity of the mitral cells in the ipsilateral bulb. At 6 weeks after surgery, there also is no significant difference between the mitral cell dendrites in the right and left olfactory bulbs, although when compared to control animals the 6-week time point does show a reduction.
Figure 27. Retrograde labeling revealed both Type I (A-C) and Type II (D) mitral cell morphologies. Type I cells had a soma of variable shape (*) ranging from 6-15\(\mu\)m in diameter, a single primary dendrite (arrow) terminating in discrete dendritic tufts near the cell body, and a single axon (arrowhead) projecting toward the medial or lateral olfactory tract. The majority of more than 1000 cells examined showed this morphology. Fewer cells exhibited the Type II morphology with multiple dendrites arising from the cell body. Both phenotypes showed significant arborizations and fine dendritic processes in control animals. Scale bar = 10\(\mu\)m (A-D).

By 8 weeks and 20 weeks after ablation, however, the fine dendritic branches that are seen in both control animals as well as the internal controls are significantly reduced. Interestingly, the major dendritic processes still are apparent at this 5-month timepoint. These findings suggest that mitral cells are highly stable structures that show little variation in dendritic complexity for extended periods following injury. The olfactory system is inherently plastic, with constant turnover of cells in the nasal epithelium and constant renewal of afferent-target interactions (for review see Farbman, 1992). It is possible that this inherent plasticity results in the highly stable morphology of the dendritic tuft of mitral cells through 6 weeks post-denervation and the major processes of those dendrites through 5-months post-deafferentation. It also is possible that the loss of the fine dendritic processes over time is a result of sensory neuron loss while the large processes are maintained by the continued presence of interactions with other cell types in the olfactory bulb such as granule cells.

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Figure 28. Mitral cell morphology examined in animals at 3 weeks, 6 weeks, 8 weeks, and 20 weeks post-deafferentation showed that elaborate dendritic processes still existed in the animals even 2 months after loss of afferent innervation. The most notable effect was seen at 5 months when the major dendritic arbors were still present, although fine processes appeared to be greatly diminished. LOB is shown for comparison as an internal control.
Figure 29. A comparison of dendritic complexity at 3 to 20 weeks post-deafferentation showed that mitral cell morphology was affected by loss of afferent input. Dendritic densities were compared within survival groups using paired T-tests and between groups using an ANOVA and Tukey's test for multiple comparisons.

In mammals, for example, the dendritic branches of the apical dendrite interact with the olfactory sensory axons while the granule cells interact with the secondary dendrites of the cell, which are more proximal to the cell body (for review see Farbman, 1992).

In this study, we also show that there is no significant difference in the area of the dendritic tufts after deafferentation. This suggests that while the fine dendritic processes that make up the density of the tuft eventually are lost, the cell retains the major processes that form its structure. This also indicates that the cells may be capable of re-innervation even after 5 months of olfactory deprivation.
Figure 30. A comparison of dendritic tuft area at 3 to 20 weeks post-deafferentation showed that mitral cell tuft areas were not affected by loss of afferent input. Glomerular tuft areas were compared within survival groups using paired T-tests and between groups using an ANOVA and Tukey's test for multiple comparisons.

Also, as previous work has shown, the size of the olfactory bulb is significantly reduced at 6 weeks post-deafferentation (Byrd, 2000). This, taken with the stability of the mitral cell major processes at 6 weeks after surgery, supports the idea that major reductions in bulb volume after loss of the olfactory nerve layer also occurs in the internal cell layer. Previous studies have shown that deafferentation results in a significant loss of granule cells (Henegar and Maruniak, 1991; Corotto et al., 1994) and reduction in the volume of the granule cell layer (Henegar and Maruniak, 1991), which is equivalent to the internal cell layer in fish.

Other studies in teleosts suggest that at 4 weeks post-denervation laminar volume is markedly reduced in the ONL and the GL, but not in the MCL and the GCL (Poling and Brunjes, 2000). It is possible that our results vary from those of this study with regard to the MCL and GCL results because of the differences in the delineations of the olfactory bulb laminae. In the study by Poling and Brunjes (2000), the investigators use a 4-layer system including a differentiated MCL and GCL. In
our study, we use a 3-layer system, which groups the MCL and the GCL because previous work in our laboratory has shown that mitral cells are not uniformly distributed in a single layer as they are in mammals. Minor variations in differentiating between the laminar areas could result in significant differences in apparent volume.

In general, the results of this study show that mitral cell morphology is not grossly affected by loss of afferent innervation until 8 weeks following deafferentation. Even then, these output neurons are highly stable structures, retaining major dendritic processes and some fine fibers. We will continue to examine the plasticity of these cells and potential mechanisms by which they remain intact by first determining what kind of interactions remain between mitral cells and other neurons in the olfactory bulb following ablation.
CHAPTER VII
DISCUSSION AND FUTURE DIRECTIONS

Major Contributions of This Work

In this dissertation, my results show that the morphology of mitral cells in adult zebrafish is unlike the morphology of mitral cells proposed for all teleosts. Further, this work describes the presence of two cell types whose morphologies have not yet been addressed in the zebrafish olfactory bulb. Finally, I have examined the adult olfactory bulb following removal of olfactory input in order to establish the role that afferent innervation plays in the maintenance of this central nervous system structure. I believe that these studies will be of significance to individuals in the fields of activity-dependence and comparative anatomy, and that they will be a substantial contribution to sensory systems biologists focusing on olfactory coding and processing.

General Discussion

The original purpose of this study was to examine the activity-dependent maintenance of mitral cells in the adult zebrafish olfactory bulb. As I began to examine the potential methodologies for answering this question, I realized that almost nothing is known about the structure of the cells in the olfactory bulb of this species. This animal has been used in a number of olfactory physiology studies (Corotto et al, 1996; Freidrich and Korsching, 1998; Friedrich and Laurent, 2001; Edwards and Michel, 2002; Lipschitz and Michel, 2002; Michel et al, 2003), yet there is little information about the morphology of the cell types in this system. It seemed to me that before we could fully understand the physiology of these cells and the
olfactory coding mechanisms of the entire olfactory bulb, we needed to first establish the morphology of the cells in zebrafish. For example, in mammalian systems, olfactory sensory neurons expressing the same odorant receptors project to a discrete glomerular region, generally a single glomerulus (for review see McClintock, 2000). Further, mitral cells have a single apical dendrite that innervates a single glomerulus (for review see Dryer and Graziadei, 1994). Physiology studies in mammals suggest that individual mitral cells have the potential to respond to a wide range of related odorants (Yokoi et al., 1995). If we knew that mitral cells responded multiple odorants, but were unaware of their morphology, we might conclude that they innervate multiple glomeruli and thus receive input from multiple types of odorant receptors. Because we know that they only innervate a single glomerulus based on their anatomy, our view of olfactory coding in mammals is drastically changed. Thus, morphology presents a significant contribution to the overall functioning of a system and should not be overlooked. With this in mind, I began examining the morphology of cells in the adult zebrafish olfactory bulb.

A few investigations have examined mitral cell in other teleosts using Golgi techniques (Kosaka and Hama, 1982; Oka, 1983; Alonso et al., 1988), but this method is not a reliable indicator of cell type as it appears to label many kinds of cells. Thus, there are significant issues with using this method as the sole technique in identifying the morphology of a cell type that has not yet been established in another manner. Further, few studies have gone beyond this initial series of Golgi investigations to identify the morphology of mitral cells in their fish. A review of mitral cells in various animals used these Golgi studies as a basis for defining mitral cell morphology in all teleosts (Dryer and Graziadei, 1994). This review then generated a basis for individuals studying the teleost olfactory bulb to define the
The first question I had with regard to this project was how could I confirm the identification of the cell type? Since the majority of commercial antibodies are made in mammalian systems, they often fail to produce results in fish. Further, there have been no studies done to suggest a marker for mitral cells, even in mammals. Many antibodies label mitral cells in addition to other cells, but none have been suggested to label mitral cells only. Interestingly, mitral cell identification in mammals is discerned by the soma location in the mitral cell layer of the olfactory bulb. Thus, if a cell is located in the mitral cell layer, it is termed a mitral cell. Even in mammalian studies some authors acknowledge the sometimes-displaced mitral cell (Mori et al., 1983) or the overlapped distribution of mitral cells and tufted cells (Pinching and Powell, 1971; Orona et al., 1984). If one were doing a physiological study where a single neuron response to an odorant was recorded based on its location in the olfactory bulb, it is quite possible that the cell would be misidentified as a mitral cell when it could be another cell type with an abnormal distribution. In zebrafish, especially, this issue is confounded because laminar distribution is not as defined as in mammalian systems. For example, mitral cells often are intermixed with juxtaglomerular cells and ruffed cells in the glomerular layer of the olfactory bulb.
Because these output neurons share a common distribution, size, and occasional morphology with other cell types in the bulb, their identification based on any one factor is not a certainty (see Figure 31).

In one zebrafish study, investigators labeled neurons with Lucifer Yellow using an intracellular loading technique (Friedrich and Laurent, 2001). In this particular project, cells were identified by their size and location in the olfactory bulb. If a cell in this area responded positively to an odorant, it was filled with the dye. The morphology of the mitral cell was then assumed based on the morphology of the cell filled with the tracer. First, based on my work, mitral cells cannot accurately be identified solely based on their size and distribution in the bulb. Thus it is uncertain that the cells being labeled were mitral cells. The second argument made by the authors was that the cells responded to an odorant. Another type of cell known as the juxtaglomerular cell not only overlaps in size and distribution with these output neurons, but it also receives innervation from olfactory sensory neurons. As a result, these cells also would respond positively to an odorant. Taken together, this information implies the possibility that cells other than mitral cells could have been recorded in this study. In this case, it wasn’t even apparent that the recorded cells were output neurons. Had the investigators taken the readings simultaneously with olfactory tract recordings or confirmed the identity of the cells using cellular backfills, the results would prove more convincing. Thus, it becomes increasingly important to confirm cellular identity prior to making generalizations about how these cells respond to odorants and how they interact with other cell types in the olfactory bulb.
Another study in zebrafish used multiple labels to identify mitral cells, but even in this case, the study was pharmacological and morphology was not addressed because the tissue was processed in thin plastic sections to allow for accurate labeling with the antibodies (Edwards and Michel, 2002). Based on the uncertainty of antibody labeling and the likelihood that general morphology questions could not be addressed using these techniques, I opted to use retrograde tract tracing with either a biotinylated dextran or a fluorescent dextran. Using this method, I injected a tracer into the axons of the output neurons and allowed the tracer to be transported retrogradely into the olfactory bulb. By using this approach, I was able to label output neurons in the olfactory bulb of adult animals. Since the amount of tracer injected directly correlated with the number of cells labeled, it was important to establish the question being addressed prior to loading the tracts. For example, when counting the number of presumptive mitral cells in the olfactory bulb, the maximum amount of dextran was injected into both the medial and the lateral olfactory tracts. When
addressing the issue of cellular morphology, it was important to be able to discern the
dendritic processes of one cell from another cell. In these cases, a lesser amount of
tracer was inserted into a single tract.

Following this method, I was able to establish the presence of two other cell
types whose axons projected to the olfactory tracts: the ruffled cells and ganglion cells
of the terminal nerve. The presence of ruffed cells has been described in other teleosts
(Kosaka and Hama, 1979a; 1979b; Kosaka, 1980), but it has not been identified in
zebrafish. As a result, the work done for this dissertation modifies previous studies
that did not account for this cell type when considering olfactory coding and
processing in this animal. Another cell type, the ganglion cells of the terminal nerve,
have been greatly overlooked in zebrafish olfactory bulb studies. One study addresses
the presence of this cell type in the zebrafish olfactory bulb, but the primary concern
of this study was the association of the terminal nerve cells to the olfactoretinal
centrifugal pathway (Li and Dowling, 2000). Another study discusses the expression
of a neuropeptide gene in neurons of the terminal nerve, but again, morphology was
not a primary component of this investigation (Oehlmann et al., 2002). Few studies
have acknowledged the presence of these cells in the bulb of this species. Because of
the close association of these output cells with the axons of the medial olfactory tract
and the olfactory nerve, their function needs to be established as it may act to
substantially modify olfactory output. Again, anatomy plays a significant underlying
role in the physiology of various central nervous system structures.

Once the morphology of the cells in the olfactory bulb of adult zebrafish had
been established, I was able to return to the initial question of activity-dependent
maintenance of mitral cells. Using the same technique, I loaded the output neurons at
various time points following deafferentation. My results showed that even at 2
months post-ablation, the morphology of the mitral cells was grossly intact. By 5 months after surgery, the fine dendritic fibers of the tuft had been reduced, but the overall structure of the cell with its major processes was present. These findings show that mitral cells are highly stable structures. It is likely that since the olfactory system is inherently plastic by nature, that these cells were simply "waiting" to be re-innervated. It also is possible that the existing connections between mitral cells and interneurons of the olfactory bulb helped to retain the general structure of the neurons even though their major source of innervation was lost. Further studies are required to examine this possibility.

Future Directions

Morphology Studies

Based on the significant variation seen between some species of teleosts and the zebrafish, it might be interesting to do a follow-up study on the morphology of mitral cells in other teleosts. Since the majority of previous studies have used Golgi technique to identify these cells, it would be interesting to examine several of these species using the retrograde tracing technique employed for this dissertation. This series of experiments might further our understanding of output neurons in teleost systems. If, for example, the mitral cells were drastically different than reported by Golgi studies, it might speak to accuracy of cell identification using the Golgi method. If, however, the cells looked inherently similar to the descriptions given by other investigators, it would speak to the variation that exists in the teleost order and would lend more support to the idea that more caution should be taken when making broad generalizations.
Development Studies

Another question that should be addressed involves the development of mitral cells in this system. Several studies suggest that mitral cells in mammals undergo a period where they have multiple dendrites and that during their development these dendrites are retracted (Malun and Brunjes, 1996). It would be interesting to see if the Type I and Type II mitral cells described in this study are two different types of adult cells or if they are simply at different developmental stages. At this point, it is uncertain if two developmental stages of mitral cells exist in adult animals. In fact, there is little information about the development of output neurons in teleost olfactory bulbs when comparing it to existing knowledge of mitral cell development in mammals. Further studies may shed light on this question. On the other hand, the retrograde tracing technique in juvenile zebrafish may prove difficult to master since they have less developed brains. Other methods for labeling mitral cells in this situation may need to be discovered.

Neurogenesis Studies

Another question that needs to be addressed involves the identification of progenitor cells in the olfactory bulb. Previous studies have shown that adult zebrafish undergo continuous neurogenesis and that the cells being formed migrate to several areas of the olfactory bulb including the internal cell layer and the glomerular layer (Byrd and Brunjes, 2001). Retrograde labeling in combination with Brdu treatment, which labels cell in the S-phase of mitosis, might suggest if any of these new cells are mitral cells.

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Molecular Studies

One investigation that would have significant impact on a number of individuals examining this cell type would be the creation of an antibody to identify it. It might be possible to use the retrograde tracing technique combined with cell culture methods to selectively identify output neurons in the olfactory bulb and then examine these cells for potential protein markers. The fact that there is no specific antibody for mitral cells at present suggests that this task may be quite difficult.

Deafferentation Studies

One potential question for future studies is what interactions remain between mitral cells and other cell types in the deafferented olfactory bulb? One suggestion for the maintenance of the cell structure following loss of innervation is that the cell remains in contact with a number of other cell types including juxtaglomerular cells. Further, centrifugal fibers carrying efferent input may influence the morphology of these output neurons. This study could be accomplished several ways. First, retrograde labeling could be employed to identify mitral cells. Once these cells are labeled, an antibody against tyrosine hydroxylase could be used to identify the juxtaglomerular cells and examine their morphology following loss of innervation. If their projections are still intact, it is possible that they are helping to maintain the mitral cell dendritic structure. Another possibility would be to use electron microscopy to examine the potential interactions between mitral cells and other neurons in the olfactory bulb to determine if synaptic contact is retained. It also might be interesting to see if the number of synapses on the major processes is significantly reduced in the deafferented animals. This experiment might suggest the degree to which synaptic input still exists and shapes the cell.
Another possible question for further examination involves the activity of mitral cells in the deafferented olfactory bulb. Are the cells in this bulb spontaneously active? If so, is there a reduction in the amount of spontaneous activity between mitral cells in control olfactory bulbs versus mitral cells in deafferented olfactory bulbs? Do cells that lack afferent innervation from the ipsilateral bulb respond to odorants that are applied to the contralateral bulb? These questions might begin to address the level at which cellular activity is retained in mitral cells following ablation of the nose. This might help us begin to understand their plasticity following injury.

One investigation that should be addressed involves apoptosis following deafferentation. While previous studies have shown that the majority of cells undergoing apoptosis following deafferentation are non-neuronal, some are neurons. Future studies should combine retrograde tracing techniques with antibody labeling against apoptosis to determine if any of the cells undergoing programmed cell death are mitral cells. Further, this study should be completed through the 8-week post-deafferentation timepoint, which corresponds to the 8-week deafferentation timepoint examined in this dissertation.

Final Thoughts

This dissertation begins to answer some of the questions about olfactory bulb morphology and circuitry in the adult zebrafish, findings that seem to contradict much of what is discussed in literature involving teleosts. Since this model system is being more frequently employed for studies involving olfactory coding and physiology, the anatomical foundation for these investigations becomes increasingly important. Further, this dissertation shows the incredible stability of output neurons in the
olfactory bulb following loss of afferent innervation, a finding that supports the inherent plasticity of this system. I have learned a great deal in the process of writing this manuscript; primarily that one of the largest problems in the scientific community involves making unfounded generalizations. Too often researchers take for granted the foundations upon which their scientific claims are made. In turn, conclusions based on these initial generalizations can lead to further setbacks in the field when the commonly held belief must be refuted. As scientists, it is our duty to do what it takes to answer the question. Sometimes this means forgetting everything you’ve learned and starting at the very beginning.
REFERENCES


Li L, Dowling JE. 2000. Disruption of the olfactoretinal centrifugal pathway may relate to the visual system defect in night blindness b mutant zebrafish. J Neurosci 20: 1883-1892.


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APPENDIX A

Results from Additional Examinations of Mitral Cell and Olfactory Sensory Neuron Interactions
Figure 1. Images of olfactory axons labeled with DiA show that both distinct glomeruli (A) and diffuse glomeruli (B) are present in the olfactory bulb of zebrafish. The olfactory nerve forms two distinct bundles, a lateral bundle (LB) and a medial bundle (MB). The LB appears to be thicker as it makes its way into the olfactory bulb. Interestingly, the lateral olfactory tract, which is comprised of mitral cell axons that exit the bulb, is much more diffuse and thinner than the medial olfactory tract. A high magnification image of a single glomerulus (C) supports the idea that mitral cell-glomerular interactions can be examined in this species.
Figure 2. An image of axons traced with the anterograde tracer, DiA, shows the presence of distinct glomeruli (A, arrows) in the olfactory bulb. Mitral cells (B, *) labeled with the retrograde tracer, microruby, also can be seen using fluorescence microscopy. Note the presence of the discrete dendritic tuft associated with the mitral cell, as well as the distinct tuft presumably associated with another unidentified cell (arrows). An overlay image of the two tracers (C) shows that the dendritic tuft associated with the large mitral cell occupies the entire area of the small glomerulus.

These images were obtained in conjunction with Ms. Holly Yettaw, an undergraduate researcher from the Biological Sciences department at Western Michigan University.
APPENDIX B

Results from Mitral Cell Studies in Other Teleosts
Figure 1. Mitral cells were identified in the olfactory bulb of the bullhead catfish, *Ictalurus punctatus*, using the retrograde tracing method employed for this dissertation. While mitral cells have been described by Golgi methods in other catfish species, they have not been described in this species. The cells were variable in size and shape and typically had multiple dendrites although some cells with single apical dendrites were seen. In this series of experiments, the dendritic tufts appeared much more diffuse than in zebrafish; however, since the olfactory bulbs of this species are significantly larger than those of zebrafish, it is possible that these cells still projected to a localized glomerular area. Further studies are required to address this issue.
Figure 2. Ruffed cells also were identified in the goldfish and the catfish using the retrograde tracing method. Studies have previously identified goldfish ruffed cells, but as an additional measure of control I wanted to confirm that our method would label these cells. In bullhead catfish, ruffed cells had not yet been identified. These finding show that retrograde tracing is an adequate method for identifying ruffed cells in the olfactory bulbs of these species. Notice that the ruffed cells in the goldfish and the catfish olfactory bulbs are quite similar in morphology to the ruffed cell identified in zebrafish.

These studies were done in collaboration with Dr. John Caprio at Louisiana State University.
APPENDIX C

Animal Care and Use Committee Approval Form
WESTERN MICHIGAN UNIVERSITY
Institutional Animal Care and Use Committee
ANNUAL REVIEW OF VERTEBRATE ANIMAL USE

PROJECT OR COURSE TITLE: Cell Genesis in the Olfactory Bulb of Adult Zebrafish
IACUC Protocol Number: 00-02-04
Date of Review Request: 04/04/02 Date of Last Approval: 05/09/01

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

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 DeYoung Title: Student
Department: BIOS Electronic Mail Address: cynthia.deyoung@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? ☑ 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: 04/03/02 Years covered by the search: 1991-2002
Key words: [Please specify:

☐ 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:

5. Animal usage: Number of animals used during this quarter (3 months): 0

Cumulative number of animals used to date: 50

Principal Investigator/Faculty Advisor Signature: Christine Byrd Date: 04/04/02
Co-Principal or Student Investigator Signature: Cynthia DeYoung Date: 04/04/02

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.

IACUC Chair Signature: Date: 04/04/02

Revised 10/01 WMU IACUC
All other copies obsolete.
PROJECT OR COURSE TITLE: Effects Of Deafferentation On The Olfactory Bulb Of Adult Zebrafish
IACUC Protocol Number: 01-05-03
Date of Review Request: 06/14/02
Date of Last Approval: 06/21/01

NAME: Christine Byrd
Title: Assoc/Assist. Professor
Department: BIOS
Email Address: byrd@wmich.edu

NAME: Cynthia DeYoung
Title: Student
Department: BIOS
Email Address: cynthia.deyoung@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? □ 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.)
   □ Search of literature databases (select all applicable)
   □ Agricola
   □ Current Research Information Service (CRIS)
   □ Biological Abstracts
   □ Medline
   □ Other (please specify):
   Date of search: 04/03/02
   Years covered by the search: 1991-2002

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:

5. Animal usage:
   Number of animals used during this quarter (3 months): 25
   Cumulative number of animals used to date: 100

   Principal Investigator/Faculty Advisor Signature: 5/1/02
   Date

   Co-Principal or Student Investigator Signature: 5/1/02
   Date

IACUC REVIEW AND APPROVAL

Upon receipt 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.

IACUC Chair Signature: 5/16/02
Date

Revised 5/1/02 - 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: Effects Of Deafferentation On The Olfactory Bulb Of Adult Zebrafish

Zebrafish

IACUC Protocol Number: 01-06-03
Date of Review Request: 05/12/03 Date of Last Approval: 05/14/02

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: byrd@wmich.edu

CO-PRINCIPAL OR STUDENT INVESTIGATOR

Name: Cynthia DeYoung
Title: Student
Department: BIOS
Electronic Mail Address: cynthia.deyoung@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? [ ] 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: 05/09/03
Years covered by the search: 1980-present

Key words: deafferentation, olfactory bulb, zebrafish

[ ] 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:

5. Animal usage:

Number of animals used during this quarter (3 months): 25
Cumulative number of animals used to date: 100

Principal Investigator/Faculty Advisor Signature Date

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.

IACUC Chair Signature Date

Revised 10/01 WMU IACUC
All other copies obsolete.
Date: June 11, 2004

To: Christine Byrd, Principal Investigator
    Jolene Berger, Student Investigator for thesis
    Cynthia DeYoung, Student Investigator for dissertation

From: Robert Eversole, Chair

Re: IACUC Protocol No. 03-05-01

Your protocol entitled “Cell Genesis in 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 11, 2004
PROJECT OR COURSE TITLE: Cell Genesis In The Olfactory Bulb Of Adult Zebrafish
IACUC Protocol Number: 03-05-01
Date of Review Request: 05/17/04 Date of Last Approval: 6-11-03
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: byrd@wmich.edu

CO-PRINCIPAL OR STUDENT INVESTIGATOR
Name: Cynthia Fuller
Title: Student
Department: BIOS
Electronic Mail Address: cynthia.deyoung@wmich.edu

1. The research, as approved by the IACUC, is completed:
   [ ] Yes (Continue with items 4-5 below.) [X] 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? [X] Yes [ ] No

3. Have there been any new findings or publications relative to this research? [ ] Yes [X] 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 [X] Medline
   [ ] Other (please specify):
   Date of search: 05/14/04
   Years covered by the search: 1980-present
   Key words: zebrafish, neurogenesis, infection, host

   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 [X] No
   Cumulative number of mortalities:

5. Animal usage: Number of animals used during this quarter (3 months): 0 Cumulative number of animals used to date: 10
   [ ] Principal Investigator/Faculty Advisor Signature Date
   [ ] 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.

[ ] 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: Cell Genesis In The Olfactory Bulb Of Adult Zebrafish
IACUC Protocol Number: 03-05-01 Date of Review Request: 05/03/05 Date of Last Approval: 06/01/04
Purpose of project (select one): ☐ Teaching ☒ Research ☐ Other (specify):

PRINCIPAL INVESTIGATOR OR ADVISOR
Name: Christine Byrd
Department: BIOS
Title: Assoc/Assist. Professor
Electronic Mail Address: christine.byrd@wmich.edu

CO-PRINCIPAL OR STUDENT INVESTIGATOR
Name: Cynthia Fuller
Department: BIOS
Title: Student
Electronic Mail Address: cynthia.deyoung@wmich.edu

1. The research, as approved by the IACUC, is completed:
☐ Yes (Continue with items 4-5 below.) ☒ No (Continue with items 2-3 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: 06/01/05 Years covered by the search: 1980-present
   Key words: zebrafish, newpapers, olfactory, braingrowth
   ☐ 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: 20

_principal_ Investigator/Faculty Advisor Signature Date

_co-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.

IACUC Chair Signature Date

Revised 10/04 WMU IACUC
All other copies obsolete.
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
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/Asst. Professor
Department: BBS
Electronic Mail Address: christine.byrd@wmich.edu

CO-PRINCIPAL OR STUDENT INVESTIGATOR
Name: Cynthia Fuller
Title: Student
Department: BES
Electronic Mail Address: cynthia.deyoung@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? □ 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 □ Medline □ Biological Abstracts □ Current Research Information Service (CRIS)
   □ Other (please specify):
   Date of search: 05/10/05
   Years covered by the search: 1980-present
   Key words: zebrafish, olfactory bulb, deafferentation, description, conclusion
   □ 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
   Number of animals used during this quarter (3 months): 30
   Cumulative number of animals used to date: 100

Principal Investigator/Faculty Advisor Signature
Date

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:

IACUC Chair Signature
Date

Revised 10/01 WMU IACUC
All other copies obsolete.