6-2001

Apoptosis Following Peripheral Sensory Deafferentation in the Olfactory Blub of Adult Zebrafish, *Danio Rerio*

Akiko M. VanKirk
Western Michigan University

APOPTOSIS FOLLOWING PERIPHERAL SENSORY DEAFFERENTATION IN THE OLFACTORY BULB OF ADULT ZEBRAFISH, *DANIO RERIO*

by

Akiko M. VanKirk

A Thesis
Submitted to the
Faculty of The Graduate College
in partial fulfillment of the
requirements for the
Degree of Master of Science
Department of Biological Sciences

Western Michigan University
Kalamazoo, Michigan
June 2001

ACKNOWLEDGMENTS

This work could not have been possible without the help of my committee, family, and friends. First, I would like to thank my major advisor, Dr. Christine Byrd, for excellent mentorship, generosity, patience, and friendship. I especially appreciate what I have learned from her and for having confidence and faith in my abilities. I also thank my committee members, Dr. John Jellies and Dr. John Spitsbergen, for their support and guidance. Thank-you to my wonderful friends, Lynn Shooks and Jean Hsiao, for always being there for coffee breaks, lunch dates, and general mental health.

Finally, I would like to thank my parents for helping me to keep things in perspective and for their lifelong encouragement and belief in me that allowed me to achieve my goals, and my sisters for their support. And to Jim, my source of energy and strength, for tolerating and supporting me, and trying to understand what I was going through, thank-you.

Portions of this work were supported by Western Michigan University College of Arts and Sciences (CAB), NIH-NIDCD (CAB), The Graduate College (AV) and Sigma Xi (AV).

Akiko M. VanKirk

APOPTOSIS FOLLOWING PERIPHERAL SENSORY DEAFFERENTATION IN THE OLFACTORY BULB OF ADULS ZEBRAFISH, *DANIO RERIO*

Akiko M. VanKirk, M.S.

Western Michigan University, 2000

Removal of the olfactory organ in the adult zebrafish results in a significant decrease in volume of the ipsilateral olfactory bulb. Our lab has been investigating the potential role of apoptosis in this phenomenon. My hypothesis is that cells in the adult olfactory bulb normally undergo minimal apoptosis and that apoptosis will increase when sensory stimulation is removed. The TUNEL method allowed detection of cells undergoing DNA-fragmentation, which indicates an apoptotic response. Double-label immunohistochemistry was used to mark which apoptotic cells are neurons by using antibody to Hu, a neuron specific protein. Triple-labeling with bis-benzimide confirmed the apoptotic nature of the response. In the normal adult olfactory bulb, TUNEL+ profiles are few and appear to be localized to the outer layers of the bulb. In deafferented animals, there is a significant increase in the number of TUNEL+ profiles. The apoptotic response occurs in two waves and is confined to the rostral half of the bulb. The first wave of cell death occurs 1 hour post-surgery. These apoptotic profiles appear to be primarily glial or immune in nature since they do not label with Hu. The second wave of cell death takes place at 24 hours and appears to decline to normal levels by 1 week. At the 24 hour time point some profiles are TUNEL+/Hu+ suggesting that there is a small population of neurons undergoing apoptosis.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

onl = olfactory nerve layer

gl = glomerular layer

icl = internal cell layer

OB = olfactory bulb

PFA = paraformaldehyde

RT = room temperature

PBS = phosphate buffered saline

TUNEL = TdT mediated deoxyuridine triphosphate dUTP Nick End Labeling

TdT = terminal deoxynucleotidyl tranferase

dUTP = deoxyuridine triphosphate

TH = tyrosine hydroxylase

CHAPTER I

INTRODUCTION

Literature Review

It is widely accepted that programmed cell death, apoptosis, is a key factor in normal development and aging of the CNS. In addition, it appears to be involved in neurodegenerative disorders such as Parkinson's disease (Mochizuki, et al., 1996), Alzheimer's disease (Mattson, et al., 1999), as well as following acute injury-related neuronal damage: lesion (Borsello, et al., 2000), spinal cord trauma (Nakahara, et al., 1999) and stroke (Isenmann, et al., 1998). Apoptosis is a genetically regulated process that is characterized by several hallmark morphological changes. Among those are preservation of membrane integrity, cell shrinkage, nuclear condensation, and the generation of internucleosomal DNA fragments (Kerr, Wyllie, and Currie, 1972; Wyllie, Kerr, and Currie, 1980; Schwartzman and Cidlowski, 1993; Surh and Sprent, 1994). There are four main phases of apoptosis: (1) activation of the death program by several apoptotic triggers such as removal of nerve growth factors (Du, et al., 1997); (2) metabolic changes such as an increase in glutamate (Banasiak, et al., 2000);

(3) a commitment step which involves caspases and DNA fragmentation (Bobba, et al., 1999); and the final phase is cell lysis (Kerr, et al., 1972).

One experimental technique commonly used to identify apoptotic cells is by terminal transferase (TdT)-mediated deoxyuridine triphosphate (d-UTP)-biotin nick end labeling, the TUNEL method. This method incorporates a deoxynucleotide conjugated to a chromagen or fluorophore to the 3' ends of DNA breaks (Gavrieli, et al., 1992). DNA fragmentation is one of the characteristic signs of apoptosis (Kerr, Wyllie, and Currie, 1972), thus, TUNEL has become a common method used to detect apoptotic events.

This form of cell death occurs normally in the adult gynmotiform fish posterior/prepacemaker nucleus (Soutschek and Zupanc, 1995), after removal of target in rat dentate gyrus (Cameron and Gould, 1996), after injuries in the cerebellum (Zupanc, et al., 1998), and in the rat cerebellar cortex following deafferentation (Borsello, et al., 2000).

Since apoptotic cell death is genetically regulated, it occurs by a different mechanism than necrotic cell death. In response to injury, necrosis is morphologically characterized by a loss of membrane integrity, cellular swelling, and cell lysis (Kerr, et al., 1972; Wyllie, 1997). These morphological characteristics are accompanied by inflammation which leads to scar formation and subsequent inhibition of reinnervation in the injured area (Anders and Hurlock, 1996). Inflammation is not observed with apoptosis, therefore, scars are prevented from being formed in the injured area so new cells may migrate into the area of damage and restore neural function (Zychlinshky, et

al., 1991). This quality of apoptosis may present some clue to a potential cure for neurodegenerative disorders.

During the formation of the nervous system, naturally occurring cell death plays an integral role in directing numbers of neurons and their connectivity (Clarke, 1990). The process is typically characterized by an excess of neurons being produced, with the final number being determined by a phase of apoptosis that occurs shortly after innervation of the target fields (Oppenheim, 1991). Targets rather than afferents have generally been accepted as playing a critical role in this phase of the development of the CNS. Removal of the target organ during development reveals that the neurons intended to innervate that organ are prevented from maturing (Dibner, et al., 1977). For example, dorsal root ganglion cells are stimulated to undergo apoptosis when the developing right wing bud is removed from chick embryos (Straznicky and Rush, 1985). When the thalamus of rat is destroyed at birth, a selective removal of neurons in the trigeminal nucleus principalis is observed (Jacquin, et al., 1996). Similarly, removal of the brain during metamorphosis of the frog, *Xenopus laevis,* results in improper development and changes in neurons of the olfactory epithelium (Higgs and Burd, 1999). Therefore, the target organ indeed plays an important role in neuronal survival.

There is also experimental evidence for the initiation of apoptosis following removal of afferent structures or activity during development. Following tetrodotoxin blockage of intraocular action potentials in developing chicks, neurons in the target tectum immediately undergo degradation (Catsicas, et al., 1992). Deafferentation by

eye removal in developing rats results in apoptosis of neurons in the superior colliculus of the brain (Guimaraes and Linden, 2000).

Many studies have revealed that removal of afferent input to the developing olfactory bulb (OB) will result in dramatic anatomical and neurochemical changes. External naris occlusion of developing rat pups shows that the density and number of cells in the ipsilateral OB is reduced by about twenty-five percent when compared to the contralateral control bulb (Brunjes, 1994; Frazier-Cierpial and Brunjes, 1989). Furthermore, removal of olfactory sensory stimulation in developing rats results in notable increases in cell death in the glomerular and granule cell layers of the OB (Najbauer and Leon, 1994). Sensory deprivation in the developing mouse olfactory system results in a decrease in the number of granule cells, the most abundant and latest forming interneuron type in the bulb (Benson, et al., 1984). In addition, reciprocal synaptic activity between the relay neurons (mitral cells) and granule cells is also reduced (Benson, et al., 1984).

Changes in afferent activity affect expression of a number of proteins in the OB. Tyrosine hydroxylase (TH) is the first rate-limiting enzyme in the synthesis of catecholamines such as dopamine and norepinephrine. TH is expressed by dopaminergic neurons of the OB and plays an important role in their physiology (Brunjes, et al., 1985), and dopamine and gamma-aminobutyric acid (GABA) are known to co-localize in TH-immunoreactive neurons (Kosaka, et al., 1995; Kosaka et al., 1985). With the removal of olfactory stimulation in developing rats, there is a reduced expression of TH in the ipsilateral OB (Baker, 1990). On the contrary,

peripheral deafferentation in the developing mouse shows that there is no observable change in GABA and glutamic acid decarboxylase (GAD) activity (Baker et al., 1988; Baker, 1990). GAD is the enzyme that removes a carbon dioxide group from glutamic acid to form GABA. Thus, in the developing animal, TH expression is down-regulated following olfactory deafferentation, but the cells do not disappear and they continue to express neurochemicals. Another example of activity being affected by removal of afferent input to the target organ is a significant reduction in sodium-potassium-ATPase activity in the ipsilateral bulb after removal of olfactory stimulation to the OB (Meisami and Mousavi, 1981).

Although there is much evidence to support the roles of targets and afferents in the control of apoptosis in development of the CNS (Pinon and Linden, 1996), there has not been much investigation of their role in adult systems. Apoptosis has been well characterized in the developing nervous system, and until recently, there was little evidence of apoptosis in the adult central nervous system. In fact, early studies of adult fish presumed apoptosis to be absent from the central nervous system. This was shown in the nucleus olfacto-retinalis of cichlid fish (Crapon de Caprona and Fritzsch, 1983), the sonic neuromuscular system of the oyster toadfish (Fine, 1989), and the electromotor system of Torpedo (Fox and Richardson, 1982). However, recent work using various techniques demonstrate that apoptosis is indeed involved in postembryonic maintenance of the brain in teleosts and other animals. For example, the high proliferative activity of the central posterior/prepacemaker nucleus in the adult gymnotiform fish, *Apteronotus leptorhynchus,* is accompanied by

apoptosis (Soutschek and Zupanc, 1995). In the adult male canary, projection neurons in the motor pathway controlling song learning and production undergo apoptosis correlating with the seasonal decrease in daylight (Kim and Schwab, 1997). Germ cells, which may provide nutrients to developing oocytes in the adult nematode *Caenorhabditis elegans,* normally undergo genetically controlled cell death (Gumienny, et al., 1999). Similarly, interneurons in the central olfactory pathway of adult lobster undergo cell death in parallel with neurogenesis (Harzsch, et al., 1999). Hence, there are several examples that show the existence of apoptosis in adults.

It has been the ongoing goal of our lab to examine how sensory afferent input affects the maintenance of the OB following postnatal development in the zebrafish, *Danio rerio.* Some studies of adult systems reveal removal of afferent input can significantly alter the morphology and activity of the target organ. There is a significant decrease in the number of receptor cell endings and trans-synaptic response in the olfactory lobe of crayfish with amputation of the olfactory sensory neurons (Sandeman, et al, 1998). Deafferentation studies of the effects of olfactory organ ablation in the adult zebrafish have shown that there is a marked decrease in the volume of the ipsilateral deafferented OB with a change in volume becoming apparent 3-6 weeks following olfactory organ removal (Byrd, 2000). Therefore, it is evident that the afferent structure plays an important role in maintenance of the target organ throughout life.

There are several logical alternatives that may account for the observed reduction in bulb volume following deafferentation: (1) cells may become

"compacted" due to a reduction in intercellular space, (2) cells may become reduced in size, or (3) cells may die resulting in fewer numbers of cells. The purpose of this study is to examine the possibility that the reduced volume of the deafferented OB is a result of a decrease in number of cells caused by cellular death. Specifically, these experiments will address my hypothesis that cell death occurs normally in the adult zebrafish OB and that the levels of apoptosis will increase following olfactory organ ablation.

Animal Model and Experimental Background

The zebrafish, *Danio rerio,* has quickly become a common model system for a variety of studies. They are commonly used for isolation of molecules involved in development, and some zebrafish genes show homology to those found in the human genome (Ardouin, et al., 2000). Therefore, the information resulting from research on this model can be valuable to research regarding human diseases such as Parkinson's disease , Alzheimer's disease, and multiple sclerosis. The larvae of zebrafish are transparent which makes them an opportune system in which to study neuronal circuitry (Fetcho and Liu, 1998), experimental embryology (Brown, et al., 2000), and cell biology (Kim, et al., 1997).

The olfactory system is ideal for investigations into adult neural plasticity. One unique feature of the olfactory system is that new neurons are born throughout life in the periphery (Moulton, 1970; Byrd and Brunjes, 1998) and centrally (Altman,

1969; Kaplan and Hinds, 1977). In addition, numerous reports have examined experimentally induced changes in the developing and adult olfactory system.

There are several reasons why the zebrafish olfactory system is a good model. Unlike the mammalian olfactory system, which has a bony nasal cavity and makes complete deafferentation difficult, the olfactory organ of zebrafish is easily accessible for experimental manipulation, and these fish are able to recover quickly from surgeries, which makes this animal an ideal model for these experiments.

The zebrafish OB is similar in organization to catfish (Finger, 1975), carp (Fujita, et al., 1988), and goldfish (Kosaka and Hama, 1982, 1983). The OB is connected to the olfactory organ, which houses the olfactory epithelium, by a short olfactory nerve. The OB is a bilaterally paired structure, approximately 0.01 mm^3 in volume, and diffusely organized into 3 main laminae: the olfactory nerve layer (onl), the glomerular layer (gl), and the internal cell layer (icl) (Byrd and Brunjes, 1995; Figure 1). The onl is a thin layer covering the OB, consisting of sensory axon projections from the olfactory epithelium. The few cells located in this layer are glia intermingled with the sensory axons. The gl is the middle lamina and contains identifiable glomeruli that consist of tufts and branches from olfactory axons (Byrd and Brunjes, 1995) and the dendrites of bulb neurons as well as the cell bodies of juxtaglomerular neurons, output neurons, and glia. Granule cells and glia are scattered throughout the lamina of the icl, which is the innermost core of the OB. The characterization of the bulb layers, in addition to its small size, allows for rigorous analysis of the bulb in response to manipulations involving the olfactory system.

Since the OBs of zebrafish possess many of the same general types of cells that are found in other organisms (Byrd and Brunjes, 1995), information from studies on this animal may prove useful in increasing our understanding of processes that occur in other species.

For this study, the right olfactory organ of adult zebrafish was removed by cauterization and the OBs of normal, deafferented, and sham-operated animals were examined at various time points in order to determine: (1) if apoptosis occurs normally in the adult OB, and if apoptosis increases following deafferentation (2) if apoptosis is evident in the deafferented bulb, what is the general time course of apoptotic events, and (3) if apoptotic cells are neurons. Preliminary results have been reported previously in abstract form (VanKirk and Byrd, 1999).

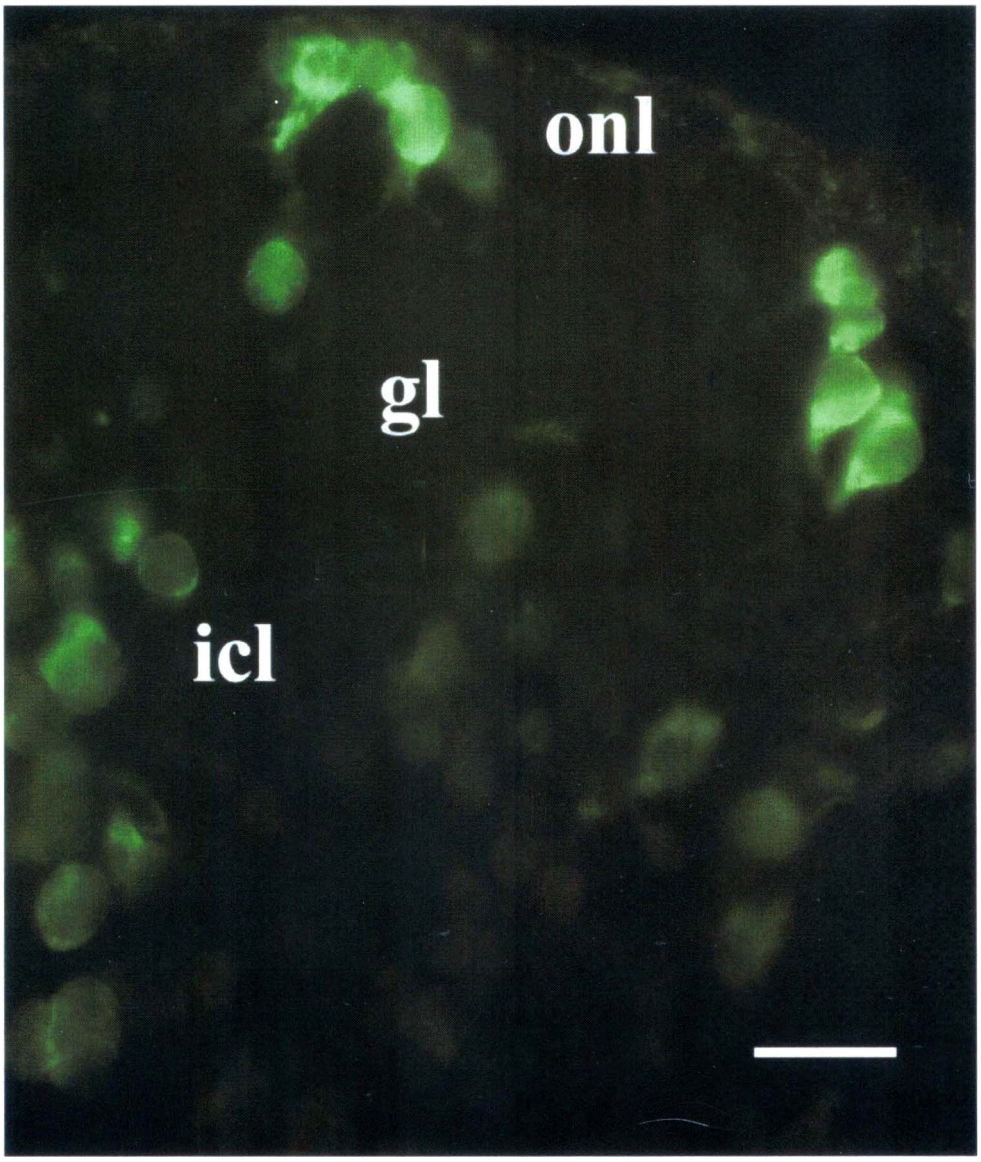

Figure 1. The Olfactory Bulb is a Laminated Structure. 11u-labeling shows the distribution of neurons in the olfactory bulb in adult zebrafish. onl = olfactory nerve layer (no 11u labeling), gl =glomerular layer, and icl = internal cell layer. Scale bar = 25 microns.

CHAPTER II

MATERIALS AND METHODS

Animals

Adult male and female zebrafish, *Danio rerio*, were obtained from a local commercial source (Pet Supplies Plus). Fish were maintained in 28.5°C aerated, conditioned water in 10-gallon aquaria with a 14 hour light:10 hour dark cycle and fed freshwater flake food (Ocean Star International, Snowville, UT) twice daily. All procedures were approved by the Institutional Animal Care and Use Committee.

Deafferentation Procedure

Ninety-one male and female zebrafish over 4 months of age, ranging in size from 0.4 – 1.0 grams and 2.5 – 4.0 centimeters (cm), were anesthetized with 0.03% MS222 (3-aminobenzoic acid ethyl ester, Sigma, St. Louis, MO). Proper anesthetization was determined by lack of reaction to tail pinch. The right olfactory organ was removed via ablation with a small-vessel cautery iron; the left olfactory organ was left intact for use as an internal control. Sham-operated control fish received a wound to the skin between the olfactory organs that was similar in

diameter and depth to that of the deafferented fish. Fish were then transferred to a recovery tank that contained one-half capsule (75 mg) kanacyn, a full spectrum skin absorbing fish antibiotic used to aid in wound healing and to prevent infection, for approximately 10 minutes. Fish were then moved to a larger tank that contained 1/2 capsule of kanacyn and allowed to survive for 1, 6, 12, 24 hours or 3 weeks.

Tissue Preparation

Following the designated survival period, deafferented (n = 50), sham-operated (n = 10), or unoperated (n = 5) fish were over-anesthetized in 0.03% MS222 until cessation of opercular movement then perfused transcardially with 0.01 M phosphate buffered saline (PBS), pH 7.4, followed by 4% paraformaldehyde (PFA) in PBS. Fish were then post-fixed in PFA for 24 hours at room temperature (RT) on a rotary mixer set at 50. Brains were removed, dehydrated through a series of ethanol and toluene rinses, then embedded in paraffin, and sectioned serially at 10 μm. Alternating sections were adhered to Superfrost Plus slides (Fisher, Fairlawn, NJ) or silanized slides (Sigma, St. Louis, MO) then dried at 37°C overnight.

In addition, whole heads of some fish were processed. Following perfusion, the head was cut off and post-fixed overnight in 4% PFA in PBS at 4°C. Heads were extensively rinsed with dH_2O then placed in a 50% solution of rapid bone decalcifyer (RDO, Apex, Plainfield, IL) and water for 2 hours, then rinsed again with dH_2O and further processed for paraffin embedding as described above.

TUNEL Staining

DNA fragmentation was detected in the adult zebrafish OB by (TdT)-mediated deoxyuridine triphosphate (d-UTP)-biotin nick end labeling (TUNEL) method (Gavrieli, et al., 1992) using the ApopTag ® Plus Peroxidase *In Situ* Apoptosis Detection Kit and ApopTag ® Red *In Situ* Apoptosis Detection Kit (Intergen, Purchase, NY). Paraffin sections were deparaffinized and hydrated through xylene, graded ethanols, and PBS then subjected to TUNEL visualized with peroxidase – diaminobenzidine (DAB) or with fluorescence.

The peroxidase staining protocol was followed to establish a time course of apoptotic events following surgery. Sections were permeabilized with 20 μg/ml proteinase K (PK) in 1M Tris-HCl (pH 7.4) for 7.5 minutes on ice then rinsed with PBS. Following blocking of endogenous peroxidases with 3% H_2O_2 for 5 minutes at RT, sections were treated with an equilibration buffer that was applied directly on specimens and covered with plastic cover slips to ensure even distribution of fluid and to prevent specimens from drying out. Working strength TdT enzyme (77 μl Reaction Buffer/33 μl TdT Enzyme) was applied to sections then incubated in a humidified chamber at 37°C for 1 hour. The reaction was terminated by washing in a stop/wash buffer (0.5 ml in 17.5 ml dH_2O) for 10 minutes at RT. Sections were rinsed, and anti-digoxigenin-peroxidase (56 μl Blocking Solution:49 μl Anti-Digoxigenin Conjugate) was applied to each slide before coverslipping and

incubating in a humidified chamber for 30 minutes at 37°C. The sections were washed in PBS and then reacted with diaminobenzidine (147 µl DAB Dilution Buffer and 3 µl of DAB Substrate) at RT to visualize apoptotic profiles. Specimens were then washed in dH_2O followed by dehydration with 100% N-butanol, dipping the slides 10 times each in the first and second wash, followed by 30 seconds in the third wash. Slides were cleared with xylenes and coverslipped with DPX mounting medium.

Several controls were employed for these experiments. Internal non-deafferented bulbs were used to control for variations in staining between fish. Sections of normal female rodent mammary gland tissue (3-5 days post weaning) were provided with the kit and used as positive controls for the staining technique. DNAse positive controls were used to ensure that the technique labeled DNA fragmentation. DNAse control specimens were removed after the PK step, pretreated with DN Buffer (30 mM Trizma base, pH 7.2, 4 mM $MgCl_2$, 0.1 mM DTT) at RT for 5 minutes, immersed in DNAse I dissolved in DN Buffer diluted 1.0 µg/ml for 10 minutes at RT, rinsed extensively in dH_2O, then subjected to the same protocol as above. Negative controls were subjected to the same TUNEL protocol minus the TdT step.

Immunohistochemistry

To determine which cells in the OB were dying, ApopTag-Red ® *In Situ* Apoptosis Detection kit was used because it is amenable to double-label

immunohistochemistry. Double labeling was carried out with specimens from deafferented animals [1 hour (n = 5), 24 hours (n = 5), or 1 week (n = 5)], sham-operated (n = 6), and unoperated (n = 5) control fish. The only changes in the TUNEL assay described above are in the H_2O_2 step, which is left out, and the anti-digoxigenin step. Anti-digoxigenin is conjugated to rhodamine in a working solution of 68 µl Blocking Solution/62 µl Anti-Digoxigenin-Rhodamine Conjugate. At this point, the remainder of the experiment is carried out in the dark, avoiding any excess exposure to light. After TUNEL labeling, the slides were rinsed in several changes of PBS over the course of 3 hours to overnight before being subjected to Hu labeling.

Antigen retrieval was performed by submersing slides in a coplin jar containing 50 mM Tris (pH 8.0) in a boiling water bath for 5 minutes, then cooled to RT. Specimens were washed in PBS plus 0.1% tween followed by PBS rinse, then incubated in a blocking solution of PBS with 3% normal goat serum and 0.1% tween for 1 hour to minimize non-specific staining. Slides were then incubated for 1 hour at RT or 20 hours at 4°C with anti-human neuronal protein HuC/HuD (anti-Hu, Molecular Probes, Eugene, OR) 10 µg/ml in PBS plus 1% BSA (bovine serum albumin, Fisher Scientific, Fair lawn, NJ) to label neurons. Next, specimens were rinsed in PBS plus 1% BSA and immersed in biotin-SP-conjugated affinipure goat anti-mouse IgG (Jackson Laboratories, West Grove, PA) at a concentration of 1:500 in blocking solution for 1 hour at RT. Sections were rinsed in PBS then incubated

with AlexaFluor 488-conjugated avidin (Molecular Probes, Eugene, OR) diluted 1:1000 in PBS for 1 hour at RT, rinsed extensively with PBS and coverslipped with PPD mounting medium [0.1% paraphenylenediamine, 10% 0.01 M phosphate buffer (v/v) with 0.15 M sodium chloride, and 90% glycerol (v/v) pH 9.0 with 0.5 M sodium carbonate buffer] to retard fading of fluorescent label (Burd and Beltz, 1989).

Some specimens were triple labeled, using bis-benzimide (Sigma, St. Louis, MO) nuclear stain. Slides were immersed in bis-benzimide 0.5 μg/ml in PBS for 15 minutes at RT, then rinsed extensively in PBS before mounting with PPD. All slides were viewed with a Nikon type 120 fluorescence microscope. Digital images were collected either by scanning photographic slides or by collecting with a digital camera and further processed with Adobe Photoshop 5.5.

Quantitative Analysis

The distribution of TUNEL-positive profiles in the OB was compared in serial sections of the brains of fish sacrificed 1 hour (n = 5), 24 hours (n = 5), 1 week (n = 3) following deafferentation, and in unoperated (n = 3) and sham-operated (n = 6) control fish. TUNEL-positive profiles were counted in every other serial section of both bulbs in deafferented and control animals and summed. Statistical significance was determined using the two-tailed paired T-test, with a P value set at 0.05.

CHAPTER III

RESULTS

Controls

Bulb sections from normal unoperated and sham-operated animals, DNAse-treated sections from normal animals, and sections of the internal control bulb contralateral to the wound were used as controls. There were very few TUNEL-positive profiles labeled in both the right and left OBs of unoperated control animals and no significant difference in number of profiles stained between right and left bulbs ($P = 0.67$, Table 1, Figure 3). Labeled profiles were found primarily in the onl, which contains numerous glia (Figure 2A). Examination of sham-operated control animals, which receive3d a cautery injury to the skin between the two olfactory organs similar in size to that of deafferented animals, did not show a significant difference in number of positive profiles between right and left bulbs ($P = 0.38$, for 24 hour, and $P = 0.53$ for 1 hour, Table 1, Figure 3) and regularly revealed staining patterns that were equivalent to normal, unoperated animals (Figure 2B). Similarly, analysis of the internal control bulb contralateral to the wound consistently showed levels and

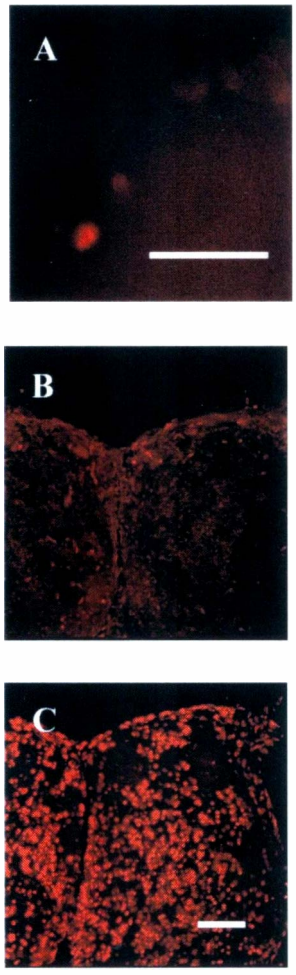

Figure 2. ApopTag-Rhodamine Labeling of Olfactory Bulbs in Control Animals. A) Very minimal TUNEL + labeling was in normal, unoperated animals as seen in this view of the onl/gl region. B) Sham-operated bulbs showed staining patterns similar to unoperated controls with little to no labeling. Maximal intense labeling through all layers of the bulb in DNAse positive controls.
Scale bar = 50 microns in A, 100 microns in B and C.

Treatment	Left OB	Right OB
Unoperated		
1	11	12
2	6	7
3	13	12
1 hour sham-operated		
1	17	16
2	17	18
3	18	20
1 hour deafferented		
1	1091	19
2	315	8
3	491	4
4	313	24
5	299	7
24 hour sham-operated		
1	10	8
2	5	6
3	7	4
24 hour deafferented		
1	1053	24
2	2753	70
3	1610	206
4	2494	78
5	1913	17
1 week deafferented		
1	3	3
2	7	8
3	4	6

Table 1. Total Number of TUNEL-Positive Profiles Counted in Right and Left OBs of Unoperated, Sham-Operated, and Deafferented Zebrafish.

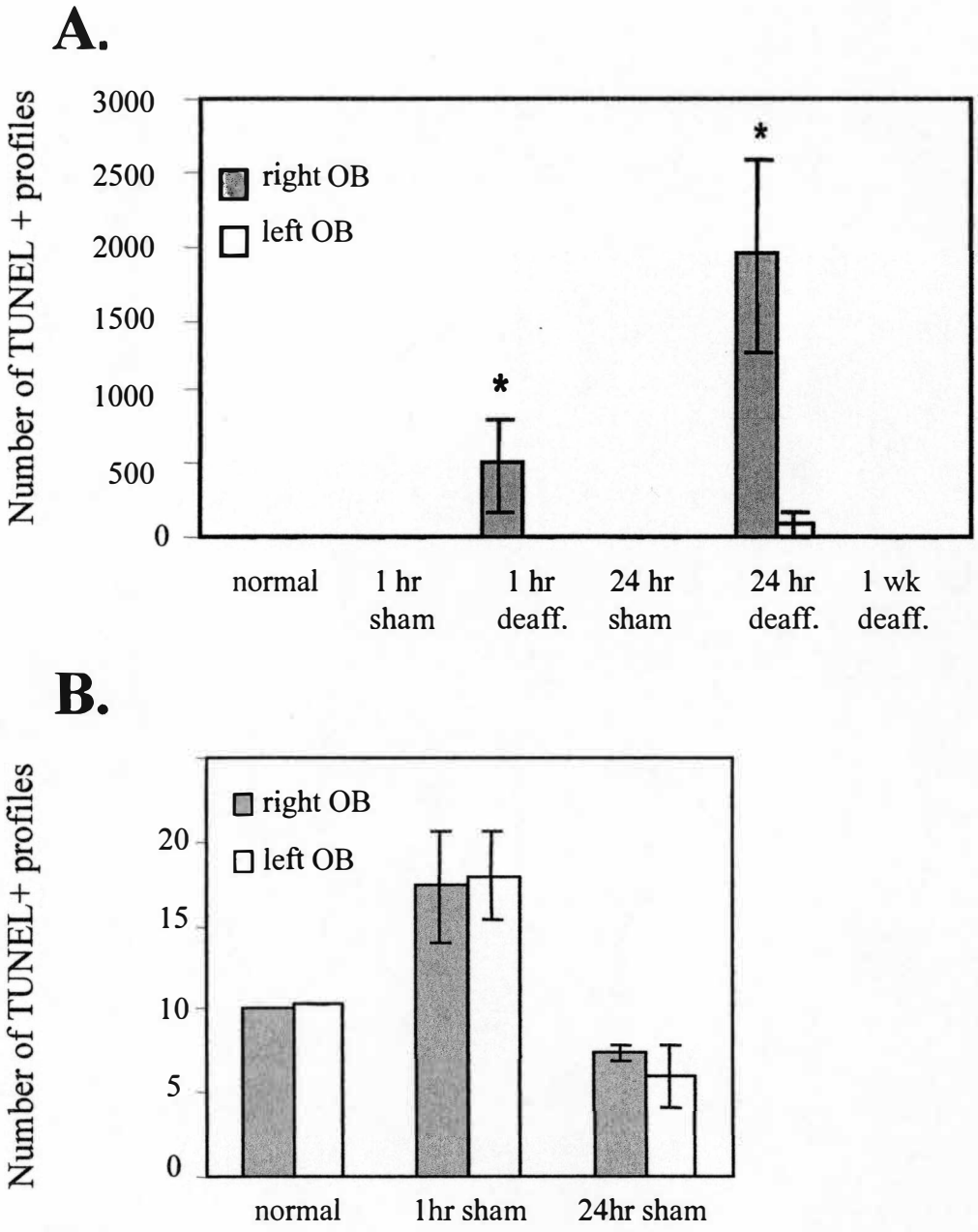

Figure 3. Quantitative Analysis of TUNEL-Positive Profiles in Right and Left Olfactory Bulbs. Graphs show the mean percent difference (+/- S.E.M.) of total number of profiles in (A) normal, sham-operated, and deafferented zebrafish and (B) smaller scale graph to show difference in normal and sham-operated. There is a significant difference in number between the two bulbs at 1 hour and 24 hour post-deafferentation (*$P < 0.05$).

intensity of TUNEL-positive profile labeling comparable to the normal, unoperated animals (Figure 4A). DNAse positive controls showed maximum levels and intensity of TUNEL-positive labeling throughout all layers of the OBs with all cells being stained (Figure 2C).

Time Course of Apoptotic Events in the OB Following Deafferentation

Complete, permanent deafferentation of the OB in adult zebrafish is achieved by cauterization of the olfactory organ (Byrd, 2000). To test the hypothesis that reduction in OB volume following ablation of the olfactory organ is due to a reduction in cell number by apoptosis, the TUNEL method was used.

In order to establish a brief time course of apoptotic events in the deafferented OB, a qualitative analysis of TUNEL-positive profiles was performed. A dramatic increase in levels of apoptosis in the OB of adult zebrafish was observed following ablation of the olfactory organ. The time course of apoptotic events follows a 2-wave trend with peaks occurring at 1 hour and 24 hours.

One hour after deafferentation, investigations of the deafferented bulb revealed a large number of intensely stained TUNEL-positive profiles located primarily in the rostral half of the ipsilateral bulb in the onl and gl (Figure 4A, B). The difference in staining between the deafferented and control bulbs is significant ($P = 0.03$, Table 1, Figure 3). The TUNEL-positive profiles in the onl were oblong or flattened, which is indicative of glia. TUNEL-positive profiles in the gl exhibited several different morphologies (round, ovoid, or irregular).

Examination of deafferented bulbs at the six-hour survival time had an intensity and level of TUNEL-positive profiles that appeared noticeable diminished when compared to the 1-hour survival time. The few positively labeled profiles were

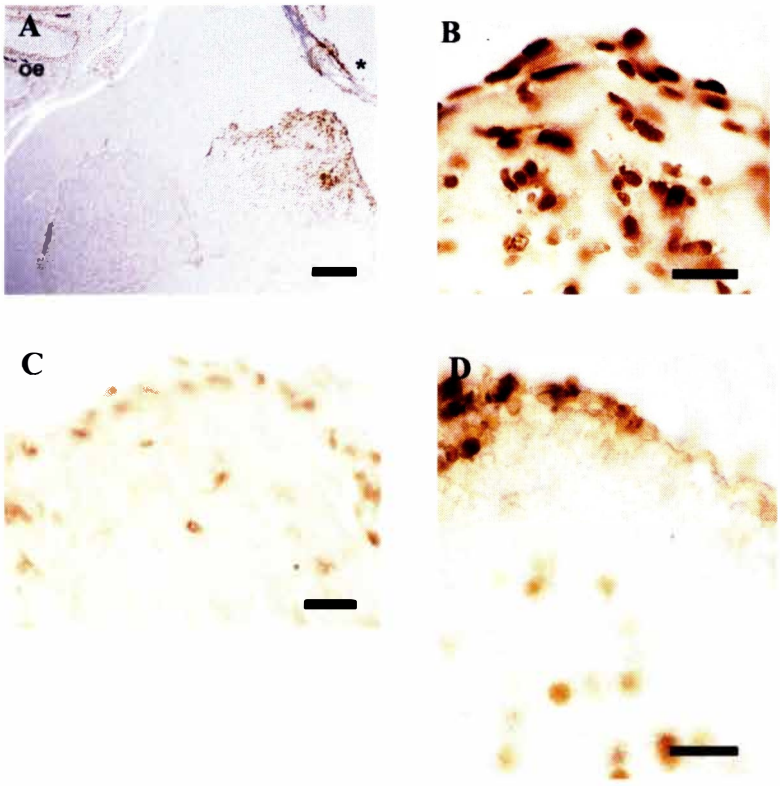

Figure 4. Photomicrographs of TUNEL-Peroxidase Labeled Olfactory Bulbs Taken With Differential Interference Contrast (DIC) Optics. There was a significant number of profiles intensely labeled in the deafferented bulb at 1 hour (A). B) Higher magnification of the onl/gl region in A. The number of positive profiles diminished greatly at 6 hours (C) and increased to moderate labeling at 12 hours (D). oe = olfactory epithelium, * = deafferented side. Scale bar = 100 microns in A, 50 microns in B, 10 microns in C, and 25 microns in D.

localized to the onl and had flattened or oblong morphologies, others were located in the gl and displayed round, ovoid, or irregular morphologies (Figure 4C).

At 12 hours post-surgery, it appeared that there were more TUNEL-positive labeled profiles than at the 6-hour time point, but less than that observed at the 1-hour time point. Profiles were moderately labeled at 12 hours and distributed throughout the bulb; however, most profiles were concentrated around the center of the bulb. Profiles in the onl were characteristically flattened, profiles in the gl showed several different morphologies, and profiles in the icl, which contains an abundance of interneurons, had a round morphology (Figure 4D).

The greatest amount of cell death appeared to take place 24 hours after olfactory organ ablation. There were an extremely large number of intensely stained TUNEL-positive profiles distributed throughout the depth of the OB in the onl, gl, and deep into the icl, the majority of which were concentrated in the icl (Figure 5A, B). It appeared that the extent of staining was comparable to, or even exceeded the number of labeled profiles at the 1-hour survival time. The internal control bulb in most animals shows similar staining patterns to that of normal, unoperated animals and was statistically different from profiles labeled in the deafferented bulb ($P = 0.004$, Table 1, Figure 3). However, a couple of animals showed an increase in the number and intensity of TUNEL-positive profiles in the internal control bulb. This observed increase might be attributed to variations in the extremity of cauterization.

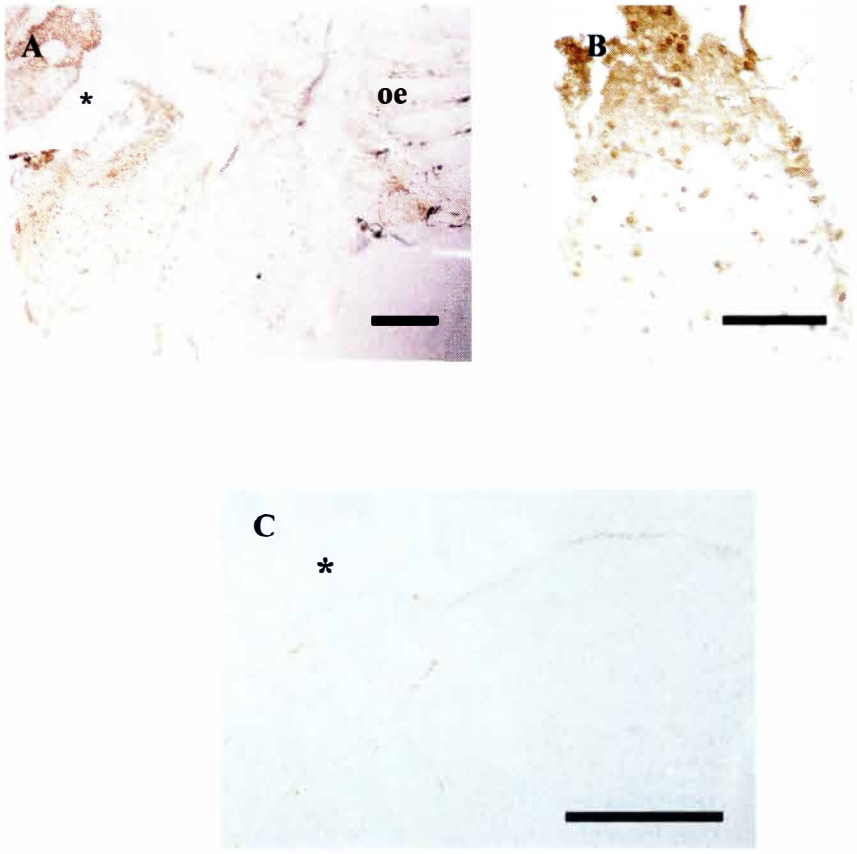

Figure 5. Examination of TUNEL-Positive Profiles at 24 Hours (A, B) and 3 Weeks (C) Post-Deafferentation. Photomicrographs of TUNEL-peroxidase labeling in olfactory bulbs taken with DIC. The most abundant, intense labeling is at 24 hours (A, B). Labeling returns to normal levels 3 weeks after surgery (C). Note the decrease in size of the deafferented bulb (C). oe = olfactory epithelium, * = deafferented side. Scale bar = 200 microns in A, 100 microns in B and C.

At 1 week post-deafferentation, the levels and intensity of TUNEL-positive labeling Appeared to return to that which was observed in normal, unoperated animals ($P = 0.23$, Table 1, Figure 3). The longest survival time evaluated was 3 weeks. By that time, there was a visual confirmation of the decrease in size of the bulb ipsilateral to deafferentation (Figure 5C).

Immunohistochemistry

In order to determine if some of the cells undergoing apoptosis were neurons, double-label immunohistochemistry was performed on unoperated control, sham-operated and deafferented animals using an antibody to Hu, an RNA-binding protein specific for neurons (Yao, et al., 1993). Both normal unoperated and sham-operated control animals had similar staining patterns as described previously. There was very little apoptosis in the OBs, and the amount of staining between right and left bulbs was not significantly different. The TUNEL-positive profiles were located in the onl and were not co-labeled with Hu (data not shown). TUNEL-positive profiles labeled at 1-hour post-deafferentation were also not co-labeled with Hu (Figure 6A-F). In contrast, there were several TUNEL-positive profiles that co-labeled with Hu at the 24-hour survival time (Figure 6G-L). This suggests that at that time point, some of the dying cells were neurons. Most of the profiles positively labeled with TUNEL and Hu were small in size and did not resemble the morphology of the large output neurons, implying that the cells dying at 24 hours were interneurons. However, not all TUNEL-positive profiles were also Hu-positive, which may indicate that most of the population of dying cells was glia (Figure 6G-L).

Further examination of the morphology of TUNEL-positive cells was carried out using bis-benzimide, which is specific for nucleic acids. A protocol for confirming apoptosis by triple labeling with TUNEL, an antibody, and Hoechst has been

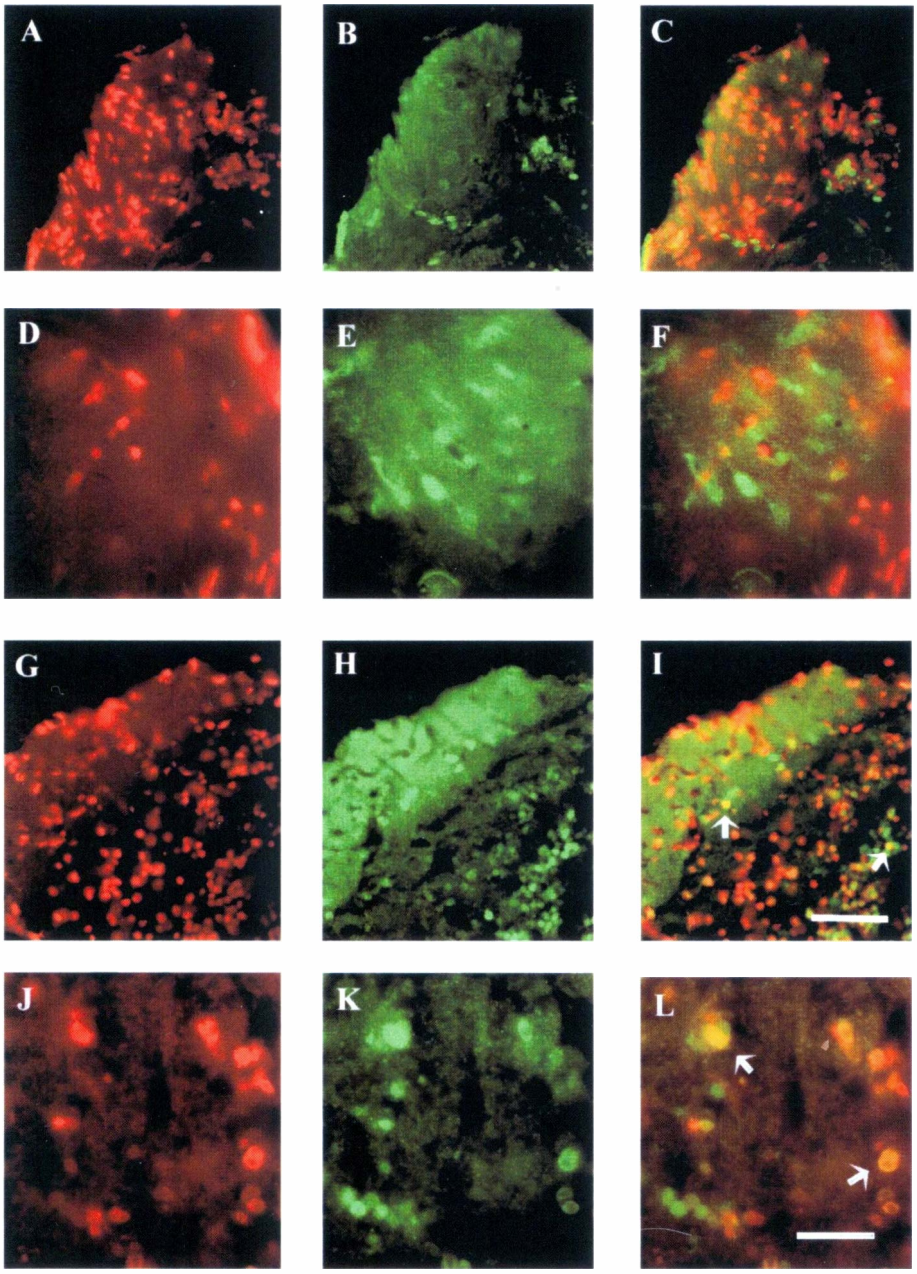

Figure 6. Double-Labeling Reveals Identity of Dying Cells. Photomicrographs show double-label with Apoptag-Rhodamine and Hu. A, D, G, J) TUNEL + profiles. B, E, H, K) Hu labeling in the same region. C, F, I, L) Overlap of the 2 labels. A-F) At 1 hour, TUNEL + profiles were located primarily in the onl and gl, and were not Hu +. G-L) Some TUNEL + profiles are co-labeled with Hu in the gl and icl. Arrows indicate double-labeled profiles. Scale bar in I = 100 microns (A-C, G-I) and scale bar in L = 10 microns (D-F, J-L).

described by Whiteside et al., 1998. Utilization of Hoechst in addition to TUNEL and Hu antibody allowed for confirmation of apoptotic events. TUNEL-positive profiles at both 1 and 24 hours were oblong or flattened in the onl, had various morphologies in the gl, and the positive profiles of 24 hour animals that were located in the icl were round and small, with some exhibiting a "halo" morphology. The pyknotic morphology of some TUNEL-positive profiles at both 1 hour (data not shown) and 24 hour (Figure 7) post-deafferentation, confirmed the apoptotic nature of the response in the OB following deafferentation.

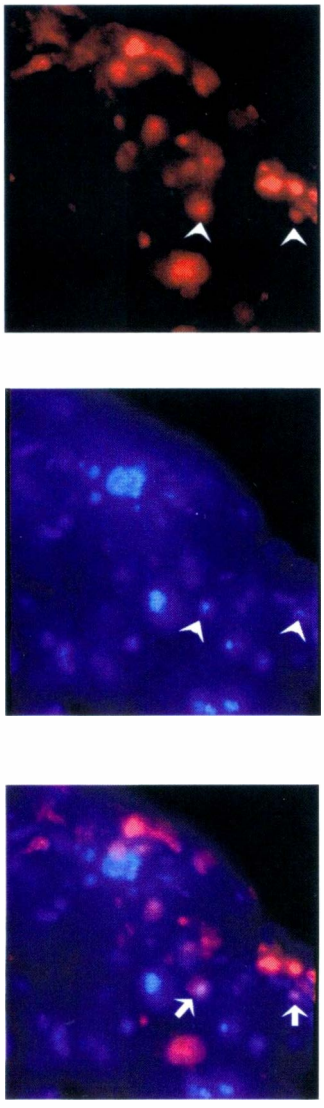

Figure 7. Hoechst Labeling Confirms Apoptosis. Photomicrographs show labeling with ApopTag-Rhodamine and Hoechst. A) TUNEL + profiles (arrowhead). B) Hoecsht labeling of nuclei in the same region (arrowhead). C) Overlap of the 2 labels. Arrows indicate pyknotic TUNEL+ profiles. Scale bar = 10 microns

CHAPTER IV

DISCUSSION

The purpose of this study was to determine if the reduction in OB volume seen following removal of the adult zebrafish olfactory organ is a result of reduced numbers of cells by apoptosis in the target OB. I hypothesized that apoptosis occurs to a minimal extent normally in the adult zebrafish OB and that deafferentation would lead to an increase in the amount of cell death.

To test the hypothesis, control unoperated, sham-operated, and deafferented animals were allowed to survive for various lengths of time and then processed with TUNEL, which labels 3' ends of DNA breaks. In order to determine if cell death was occurring, several protocols for labeling dying cells were attempted in an effort to find a method that produced the most consistent results (see Appendix A). The ApopTag kit from Intergen gave the most reliable results. Normal unoperated animals showed very little labeling, indicating that indeed, cell death is a rare phenomenon in the normal OB.

A brief time course of apoptotic events post-deafferentation was established utilizing a peroxidase protocol. The OBs of animals surviving 1h, 6h, 12h, 24h, and 3 weeks post-deafferentation were labeled with TUNEL and examined. There were 2

waves of cell death observed succeeding the injury. The first peak takes place at 1 hour with strongly labeled TUNEL-positive profiles concentrated in the onl with a small number in the gl being labeled. The morphology of TUNEL-positive profiles in the onl are oval or flattened. These cells appear to be glia due to their morphology and location in the bulb. The second peak of cell death occurs at 24 hours. The TUNEL-positive profiles are very intensely labeled and distributed throughout the depth of the bulb with the highest number being localized to the icl. It seems that most of the positive labeled profiles in the icl are interneurons based upon their round shape and location in the bulb. Analysis of peroxidase TUNEL labeling of profiles in the ipsilateral OB following deafferentation has revealed cell death and provided us with a general time course of events, establishing 2 peaks of activity at 1 and 24 hours. Based upon the morphology and location of positive labeled profiles, we can infer the nature of cell types dying after ablation of the olfactory organ. However, we cannot definitively say that these cells are glial or neuronal simply based upon their morphology and location in the OB. Therefore, tissue samples were further processed with double-label immunohistochemistry. Fluorescent TUNEL labeling was performed, followed by staining with the Hu antibody to determine what types of cells were dying.

Hu is a neuronal marker that was used to allow me to make distinctions between neuronal and non-neuronal cells. Hu genes are autoantigens in humans that are targeted by autoantibodies. These antigens are involved in an autoimmune paraneoplastic neurodegenerative disorder that is associated with a small cell lung

cancer (Graus et al., 1985, 1986; Dalmau, et al., 190; Posner, 1995). Graus et al., reported that Hu antigens are distributed in both nuclear and cytoplasmic regions of most neurons. This type of distribution suggests that Hu may shuttle between the nucleus and the cytoplasm, as has been reported for some nucleolar proteins (Borer et al., 1989; Meier and Blobel, 1992). Hu genes encode neuron-specific RNA binding proteins (Yao, et al., 1993). Studies have shown that Hu is expressed in neuronal cells during neuronal development and maintenance (Perrson, et al., 1997; Park, et al., 2000), and some forms of Hu mRNA and protein continue to be expressed into adulthood for maintenance (Okano and Darnell, 1995). Previous work has revealed that Hu A and Hu G genes are expressed weakly and ubiquitously, while Hu C and Hu D genes are expressed specifically in neuronal cells in zebrafish neurogenesis (Park, et al., 2000). Hu D may be involved with neuronal differentiation or maintenance of a neuronal phenotype because it is usually observed with terminal differentiation of all neuronal structures (Clayton, et al., 1998).

In my endeavor to determine if dying cells were neurons, Hu antibody was used to label TUNEL-positive tissue, specifically anti human neuronal protein HuC/HuD. Cells dying 1 hour after deafferentation do not appear to stain with the neuronal marker Hu, revealing no TUNEL-positive, Hu-positive double-labeling of cells. Their location, morphology, and lack of Hu staining in the onl implies that the cells dying at 1 hour are not neurons, and may likely be glia. However, it has been difficult to find a glial marker that works well in zebrafish, so confirming that these cells are glia is not possible at this time. Most of the TUNEL-positive profiles at 24

hour were concentrated in the icl, which contains an abundance of interneurons. Double labeling of tissue samples with TUNEL and Hu at this time revealed that some of the TUNEL-positive profiles were also Hu positive. It would appear that these cells are neurons because of their location in the icl, their round morphology and Hu-positive staining. There were also many TUNEL-positive profiles that were not Hu-positive. Again, this may represent a population of glia undergoing cell death.

The 2-wave response of cell death seen in adult zebrafish is similar to observations in other models of injury-induced apoptosis. In the rat brain, following a single intraperitoneal injection of kainic acid to induce seizure and neurodegeneration, a model of convulsive status epilepticus, 2 waves of apoptosis take place. The 2 waves correlate with the expression of interleukin (IL)-1β at 5 hours and caspase-1 at 24 hours, respectively (Eriksson, et al., 1999). IL-1 is a proinflammatory cytokine involved in immune response during infection and inflammation (Kuby, 1997 3rd ed.) and expression has been shown to increase in neurodegenerative conditions such as Alzheimer's disease (Griffin, et al., 1989). IL-1β converting enzyme (ICE)/caspase-1 is responsible for converting pro-IL-1β to biologically active IL-1β (Black, et al., 1988), and has been shown to be involved in the cell death process (Miura, et al., 1993). Caspase-1 and other proteases have been implicated in neuronal death (Gagliardini, et al., 1994; Li, et al., 2000). In the newborn rat eye, there are 2 waves of cell death following gamma irradiation. The early time, 6 hour, reveals death of non-dividing cells and active cells out of S phase. The late time, 24 hours, encompasses dying cells in S phase (Borges and Linden, 1999). Similarly, cerebellar lesions in the

cerebellum of the adult teleost fish, *Apteronotus leptorhynchus,* result in peaks of apoptosis at 30 minutes and 1 day (Zupanc, et al., 1998).

An alternative hypothesis to explain the decrease in OB size after rosette ablation is that the reduction is due to a loss of cells by necrosis. Despite the fact that TUNEL-positive labeling has regularly been used to report apoptosis and that there is a foundation supporting 2 waves of apoptotic cell death following injuries, there have also been similar reports of necrotic death occurring in 2 waves. Following spinal cord lesions in the rat, there is an initial wave of necrotic cell death followed by a secondary wave (Dusart and Schwab, 1994). Cerebellar granule cells in culture were deprived of oxygen-glucose and induced to undergo apoptotic cell death that was accompanied by 2 waves of necrotic cell death (Kalda, et al., 1998).

Although the TUNEL method is commonly used to report apoptotic responses, the use of TUNEL alone has come under some scrutiny (Charriaut-Marlangue and Ben-Ari, 1995; Grasl-Kraupp, et al., 1995). Therefore, confirmation of the apoptotic nature of the response was attempted using anti-PARP p85 Fragment pAb, a polyclonal antibody directed against the 85 kDa caspase-cleaved fragment (p85) of human poly (ADP-ribose) polymerase. Inconsistent results were obtained using this method (Appendix 2). Because of the lack of success with anti-PARP, a different method was employed to confirm apoptosis. This was done by triple labeling with bis-benzimide, Hoechst 33258, which is specific for nucleic acids and is utilized to label nuclear profiles. Using Hoechst, I was able to visualize TUNEL-positive cell nuclei at the light microscopic level. Results of triple-labeling

and subsequent analysis of cells revealed that many of the TUNEL-positive dying cells had pyknotic nuclei (Whiteside, et al., 1998; Whiteside and Munglani, 1998), a characteristic of cells undergoing apoptosis (Tammariello et al., 2000). Hence, there is confirmation of my hypothesis, and I conclude that the reduction in OB volume following deafferentation is a result of apoptotic cell death. However, this does not rule out the possibility of other types of cell death, like necrosis, being involved in the degradation of neural tissue following ablation.

Future Directions

We have established that deafferentation induces apoptosis of neurons in the OB of adult zebrafish. However, there is a large population of dying cells examined that were TUNEL-positive but not Hu-positive. It would be beneficial to find a good macrophage and glial marker for adult zebrafish. At present, OX-42, which is used in many systems, may be useful, but preliminary attempts to label macrophages in adult zebrafish yielded variable results. One macrophage marker that may be promising is alpha-Napthyl-butyrate esterase. If a label that successfully labels macrophages in our model is found, the marker may provide an opportunity to confirm the identity of some TUNEL-positive non-neuronal cells.

Another question for future study is what might be the age of the dying cells. We would like to examine whether dying cells are newly generated or terminally differentiated. Investigations of bromodeoxyuridine (BrdU) labeling in the adult zebrafish OB have shown that 3 weeks after exposure to BrdU, newly generated cells

are found throughout the layers of the bulb (Byrd and Brunjes, submitted). To assess whether newly generated cells are undergoing apoptosis, we may expose fish to BrdU following deafferentation, and follow up with immunohistochemical analysis involving double labeling with BrdU and TUNEL. If newly generated cells were dying, then one would expect to see profiles that are both TUNEL-positive and BrdU-positive.

In order to understand the molecular basis of events, it would be necessary to determine which signaling factors might be involved in stimulating / regulating this process. It might be possible to investigate this question using a method for labeling caspases (CaspaTag). ICE and other proteases have been renamed caspases. Activation of caspases is one of the final steps in commitment of a neuron to die by apoptosis. Caspases are cysteine proteases (c) with cleavage sites after aspartic acid residues (aspase). Caspases are synthesized as inactive proenzymes in the cytoplasm and are activated by cleavage at internally specified conserved aspartate residues in cells undergoing apoptosis. The caspases then initiate a cascade of proteolytic cleavage leading to the activation of downstream caspases with cellular substrates, like PARP, poly (ADP-ribose) polymerase.

This reported work begins to answer some questions about cell death following peripheral deafferentation in the OB. However, it is necessary to further examine the apoptosis phenomenon in adult zebrafish. Answers to the posed questions might provide some clue to potential cures for neurodegenerative and acute-injury related disorders.

APPENDIX A

Materials and Methods and Results From Other TUNEL Techniques

Alternative TUNEL Techniques

In our attempts to find a method to consistently label dying cells in the OB, several protocols were investigated. We determined that ApopTag (Intergen) provided the most promising results because of consistent labeling.

The first protocol followed was the original TUNEL assay (Gavrieli, et al., 1992) for labeling fragmented DNA. This method is similar to the ApopTag protocol except that supplies come from various companies. Animals were deafferented and tissue specimens were prepared as previously described in the deafferentation procedure and tissue processing sections in Materials and Methods.

Sections were dewaxed by heating in a 60°C oven, and then cooled to RT. Slides were incubated in Terminal TdT buffer (30 mM Tris HCl, pH 7.2 140 mM sodium cacodylate, and 1 mM cobalt chloride) mixed 6:1 with biotin-16-dUTP (Boehringer Mannheim, Germany) at 37°C for 2 hours. Sections were washed in PBS and biotin labeling was detected with avidin ABC peroxidase reagents (Vectastain elite, Vector Laboratories, Burlingame, CA) for 1.5 hours and visualized with diaminobenzidine. Sections were washed with water and mounted with DPX following dehydration with ethanol.

This protocol resulted in all tissue sections falling off from the slides, and no staining. This might have been due to the harsh treatment of the tissue by incubating

it in the oven at a high temperature for a prolonged amount of time. Another adjustment was made and a second protocol was followed.

This method was altered slightly due to complications with tissue samples. The same protocol was followed except for the dewaxing step. Instead of incubating samples in the oven, they were dewaxed with xylene, rehydrated with ethanols, rinsed in PBS, and incubated with PK (20 μg/ml) for 20 minutes to digest proteins. Then specimens were rinsed extensively with PBS and incubated with 3% H_2O_2 for 10 minutes at RT to quench any endogenous peroxidases, and rinsed with PBS. After adjusting the dewaxing step, some of the tissue remained on the slides and was labeled, but the background staining was very high.

The second protocol was a slight variation of the Gavrieli TUNEL assay. Animals were deafferented and tissue specimens were prepared as described in the deafferentation procedure and tissue processing sections in Materials and Methods. Specimens were dewaxed, rehydrated, and washed with PBS. Tissue sections were then treated with PK (20 μg/ml) for 20 minutes, rinsed extensively, incubated with 3% H_2O_2 at RT, then rinsed. Terminal transferase reaction solution (200 mM potassium cacodylate, 25 mM Tris-HCl, BSA, at 0.55 mg/ml, pH 6.6, 5 mM cobalt chloride, mixed at 6:1 with Biotin-16-dUTP; Boehringer Mannheim, Germany) was added to the samples for 2 hours. Sections were washed and biotin labeling was detected with avidin ABC peroxidase reagents (Vectastain, Burlingame, CA) for 30 minutes. Sections were washed and mounted with DPX following dehydration.

This alternative TUNEL assay also had a poor outcome. We maintained the same protocol but decreased the time in ABC to prevent high background. However, there was still a problem with some tissue falling apart and falling off from the slides. We concluded that the time and temperature of PK treatment was deteriorating the tissue. We tried another protocol that would not be as harsh to the delicate brains.

Next, an antibody to single-stranded DNA, Apostain (Alexis Corporation, San Diego, CA), was examined. Animals were deafferented and some were processed for paraffin embedding as described in the Materials and Methods section. A second group of animals was processed for frozen sections. These fish were processed the same through the anesthetization step then perfused with cold methanol-PBS (6:1), post-fixed overnight in methanol-PBS at 4°C, and then brains were dissected. Brains were embedded in gelatin and infiltrated through an increasing concentration of sucrose solution up to 30%. Gelatin cubes were mounted with OCT medium (Tissue Tek) and 10 μm sections were cut on a cryostat. Paraffin sections were deparaffinized in 2 changes of xylene then incubated in 3 changes of methanol-PBS immersed in tubes containing 9.2 ml PBS + 0.8 ml $MgCl_2$ 6 mg/ml, immersed into a jar with boiling water for 5 minutes, and immediately transferred to new tubes containing ice cold PBS for 10 minutes. Both paraffin and cryostat sections were incubated in 3% H_2O_2, rinsed, treated with 0.1% BSA at RT for 30 minutes, then rinsed with PBS. Anti-single-stranded DNA MabF7-26 (1:1000 in 5% fetal bovine serum) was applied to the tissue sections, incubated at RT for 15 minutes, then rinsed with PBS. Biotin-conjugated goat anti-mouse Ig (1:100) was applied to sections for 15 minutes. Sections were rinsed,

incubated in ABC (Vectastain, Burlingame, CA), reacted with DAB, dehydrated, and coverslipped.

The Apostain method was not as harsh on the tissue, so sections remained on the slides throughout the procedure. However, very little, if any labeling was observed at any time point, which was not the expected outcome. Difficulties might have been attributed to the tissue processing or antibody dilution.

The TUNEL-POD kit (Boehringer Mannheim, Germany) was used for procedure number 4. Fish were deafferented and processed according to previously described protocols. Sections were deparaffinized, rehydrated, rinsed, and treated with PK in Trish HCl pH 7.4, for 2 minutes on ice. Sections were washed, incubated with 3% H_2O_2, washed, then incubated with TUNEL solution (900 µl label solution and 100 µl enzyme solution) in 37°C humid chamber for 1 hour. Specimens were washed, converter-POD was applied, then slides were incubated at 37°C for 30 minutes, washed, reacted with DAB, dehydrated, and coverslipped with DPX.

We had some success with the TUNEL-POD method. The tissue was staying adhered to the slides, but staining was quite variable among animals and between different time points. The variability in staining might have been due to differences between animals, or with some step of the kit.

After reading numerous apoptosis articles, it was evident that several different protocols existed for studying and labeling dying cells. What I found is that the TUNEL method described by Gavrieli appeared to be the most commonly used protocol. Great efforts were made in our attempt to find an effective protocol for our system. When speaking with other investigators, we discovered that our inconsistent

results were similar to their outcomes and that it was quite difficult to find a quality TUNEL labeling method that gave consistent results. We did however; succeed in finding a good TUNEL labeling method (ApopTag) that gave consistent results.

APPENDIX B

Materials and Methods and Results From Anti-PARP Staining

Anti-PARP Staining

Poly (ADP-ribose) polymerase (PARP) is a nuclear DNA-binding protein that detects DNA strand breaks and functions in base excision repair (Trucco, et al., 1998) and might be involved in the commitment step of apoptosis (Simbulan-Rosenthal, et al., 1998; Trucco, et al., 1998). Caspase cascade activation results in cleavage of PARP into 2 characteristic fragments of molecular mass 85 kDa and 25 kDa (Lazebnik, et al., 1994). Anti-PARP p85 fragment pAb is a polyclonal antibody directed against the p85 fragment of PARP.

Fish were deafferented and tissue processed for paraffin sections as described in Materials and Methods. Sections were dewaxed, rinsed in methanol-PBS (6:1), washed in PBS, and permeabilized with 0.2% Triton X-100 in PBS for 5 minutes at RT. Specimens were washed, incubated in 3% H_2O_2, then rinsed. Non-specific staining was inhibited by immersing slides in a blocking solution (PBS with 3% normal goat serum and 0.4% Triton X-100) for 1 hour at RT. Slides were rinsed and incubated for 24 hours at 4°C in a polyclonal antibody to PARP, poly (ADP-ribose) polymerase (Promega, Madison, WI) diluted 1:100 with blocking solution. Slides were washed in PBS, PBS with 0.1% Tween 20, PBS, then immersed in biotinylated goat anti-mouse secondary antibody (Sigma, Saint Louis, MO) diluted 1:100 with blocking solution for 1 hour at RT. Slides were rinsed, placed in avidin-biotin peroxidase solution (Vectastain) for 1.5 hours at RT, rinsed, and reacted with DAB. Slides were rinsed, dehydrated and coverslipped with DPX.

We used anti-PARP in an attempt to confirm the results from TUNEL labeling and had limited success. Labeled nuclei in OBs labeled with anti-PARP were very light and had no distinct morphology. The labeling was diffuse, concentrated in the gl and icl of 24-hour deafferented fish. Perhaps some modification would produce success with this method of staining. In order to use this label legitimately, it would be necessary to prove that this protein is found in zebrafish.

APPENDIX C

Animal Care and Use Committee Approval Form

10/91

Date Received _____
IACUC Number 97-08-01
First Renewal Request 07/20/98
Second Renewal Request 07/15/99

WESTERN MICHIGAN UNIVERSITY
YEARLY RENEWAL FORM APPLICATION TO USE
VERTEBRATE ANIMALS FOR RESEARCH OR TEACHING

GENERAL INFORMATION: Fill in all appropriate information

Christine Byrd	BIOS	7-5843
Principal Investigator/Instructor	Department	Campus Phone
Co-Principal/Student Investigator	Department	Campus Phone

Title of Project/Course Effects of Deafferenation on the Olfactory Bulb of Adult Zebrafish

PRINCIPAL INVESTIGATOR/INSTRUCTOR DECLARATION

I assure that I have obtained IACUC approval prior to implementing this project and that there are no changes in the protocol submitted in the original application to use vertebrate animals for research or teaching. I understand that if at any time changes are made in the use of animals as described in the original application, a letter or amended protocol must be filed for review. I assure that the activities do not unnecessarily duplicate previous experiments.

Signatures:

Principal Investigator/Instructor — Date: 9/9/99

Co-Principal/Student Investigator (If PI not a faculty member) — Date

INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE APPROVAL

IACUC Chairperson — Date: 10/13/99

PLEASE MAIL COMPLETED APPLICATION TO:
Research Compliance Coordinator
Western Michigan University
327E Walwood Hall
Kalamazoo, MI 49008
(616) 387-8293

***NOTE**: It is the responsibility of the Principal Investigator to obtain the signature of any Co-Principal/Student Investigators.*

REFERENCES

Anders, J.J. and J.A. Hurlock (1996) Transplanted glial scar impedes olfactory bulb reinnervation. *Exp. Neurology.* 142:144-150.

Ardouin O., Legouis R., Fasano L., David-Watin B., Korn H., Hardelin J., and Petit C. (2000) Characterization of the two zebrafish orthologues of the KAL-1 gene underlying X chromosome-linked Kallmann syndrome. *Mechanisms of Development.* 90:1:89-94.

Baier, H. and S. Korsching (1994) Olfactory glomeruli in the zebrafish form and invariant pattern are identifiable across animal. *J. Neurosci.* 114:219-230.

Baker, H. (1990) Unilteral, neonatal olfactory deprivation alters tyrosine hydroxylase expression but not aromatic amino acid decarboxylase of GABA immunoreactivity. *Neuroscience.* 36:761-771.

Baker H., Towle A.C., and Margolis F.L. 91988) Differential afferent regulation of dopaminergic and GABAergic neurons in the mouse main olfactory bulb. *Brain Res.* 450:69-80.

Banasiak K.J., Xia Y., and Haddad G.G. (2000) Mechanisms underlying hypoxia-induced neuronal apoptosis. *Prog. in Neurobiology.* 62:3:215-249.

Benson T.E., Ryugo D.K., and Hinds J.W. (1984) Effects of sensory deprivation on the developing mouse olfactory system: A light and electron microscopic, morphometric analysis. *J. Neurosci.* 4:638-653.

Black R.A., Kronheim S.R., Cantrell M., Deeley M.C., March C.J., Prickett K.S., Wignall J., Conlon P.J., Cosman D., Hopp T.P., and Mochizuki D.Y. (1988) Generation of biologically active interleukin-1 β by proteoltic cleavage of the inactive precursor. *J. Biol. Chem.* 263:9437-9442.

Bobba A., Atlante A., Giannattasio S., Sgaramella G., Calissano P., and Marra E. (1999) Early release and subsequent caspase-mediated degradation of cytochrome c in apoptotic cerebellar granule cells. *FEBS Lett.*457:1:126-130.

Borer R.A., Lehner C.F., Eppenberger H.M., and Nigg E.A. (1989) Major nucleolar proteins shuttle between the nucleus and cytoplasm. *Cell.* 56: 379-390.

Borges H.L. and R. Linden (1999) Gamma irradiation leads to two waves of cell death in distinct cell populations of the retina of newborn rats. *J. Cell Sci.* 112:23:4315-4324.

Borsello T., DiLuzio A., Ciotti M.R., Calissano P., and Galli C. (2000) Granule neuron DNA damage following deafferentation in adult rats cerebellar cortex: a lesion model. *Neuroscience.* 95:1:163-171.

Brown L.A., Rodaway A.R.F., Schilling T.F., Jowett T., Ingham P.W., Patient R.K., and Sharrocks A.D. (2000) Insights into early vasculogenesis revealed by the expression of the ETS-domain transcription factor Fli-1 in wild-type and mutant zebrafish embryos. *Mechanisms of Development.* 90:2:237-252.

Brunjes, P.C. (1985) Unilateral odor deprivation : time course of changes in laminar volume. *Brain Res. Bull.* 14:233-237.

Brunjes, P.C. (1994) Unilateral closure and olfactory system development. *Brain Res.Rev.* 19:146-160.

Brunjes P.C., Smith-Crafts L.K., and McCarty R. (1985) Unilateral odor deprivation: effects on development of olfactory bulb catecholamines and behavior. *Dev. Brain Res.* 22:1-6.

Burd, G.D. and B.S. Beltz. (1989) *Immunocytochemical techniques: principles and practice.* Blackwell Scientific Publications, Inc., MA. P. 142.

Byrd, C.A. (2000) Deafferentation-induced changes in the olfactory bulb of adult zebrafish. *Brain Research.* 866:92-100.

Byrd, C.A. and P.C. Brunjes (1995) Organization of the olfactory system in the adult zebrafish: histological, immunohistochemical, and quantitative analysis. *J. Comp. Neurol.* 358:247-259.

Byrd, C.A. and P.C. Brunjes. Neurogenesis in the olfactory bulb of adult zebrafish. Submitted.

Cameron, H.A. and E. Gould (1996) Distinct populations of cells in the adult dentate gyrus undergo mitosis or apoptosis in response to adrenalectomy. *J. Comp. Neurol.* 369:56-63.

Catsicas M., Pequignot Y., and Clarke P.G.H. (1992) Rapid onset of neuronal death induced by blockade of either axoplasmic transport or action potentials in afferent fibers during brain development. *J. Neurosci.* 12:4642-4650.

Charriaut-Marlangue, C. and Y. Ben-Ari (1995) A cautionary note on the use of the TUNEL stain to determine apoptosis. *Neuroreport.* 7:61-64.

Clarke, P.G.H. (1985) Neuronal death in the development of the vertebrate nervous system. *Trends Neurosci.* 8:345-349.

Clayton G.H., Perez G.M., Smith R.L., and Owens G.C. (1998) Expression of mRNA for the elav-like neural-specific RNA binding protein, HuD, during nervous system development. *Dev. Brain Res.* 109:2:271-280.

Conti A.C., Raghupathi R., Trojanowski J.Q., and McIntosh T.K. (1998) Experimental brain injury induces reguionally distinct apoptosis during the acute and delayed post-traumatic period. *J. Neuroscience.* 18:15:1553-1572.

Crapon de Caprona, M.D. and B. Fritzsch (1983) The development of the retinopetal nucleus olfacto-retinalis of two cichlid fish as revealed by horseradish peroxidase. *Dev. Brain Res.* 11:281-301.

Dalmau J., Furneaux H.M., Gralla R.J., Kris M.G., and Posner J.B. (1990) Detection of the anti-Hu antibody in the serum of patients with small cell lung cancer-a quantitative Western blot analysis. *Ann. Neurol.* 27:544—552.

Dibner M.D., Mytilineou C., and Black I.B. (1977) Target organ regulation of sympathetic neuron development. *Brain Res.* 123:2301-10.

Du Y., Dodel R.C., Bales K.R., Jemmerson R., Hamilton-Byrd E., and Paul S.M (1997) Involvement of a caspase-3-like cysteine protease in 1-methyl-4-phenylpyridinium-mediated apoptosis of cultured cerebellar granule neurons. *J. Neurochem.* 69:1382-1388.

Dusart, I. and M.E. Schwab (1994) Secondary cell death and the inflammatory reaction after dorsal hemisection of the rat spinal cord. *Eur. J. Neurocsci.* 6:712-724.

Erikkson C., Van Dam A.M., Lucasssen P.J., Bol J.G.J.M., Winblad B., and Schultzberg M. (1999) Immunihistochemical localizatoin of interleukin-1β, interleukin-1 receptor antagonist and interleukin -1β converting enzyme/ caspase-1 in the rat brain after peripheral administration of kaininc acid. *Neuroscience.*93:3:915-930.

Fetcho, J.R. and K.S. Liu (1998) Zebrafish as a model system for studying neuronal circuits and behavior. *Ann N.Y. Acad Sci.* 860:333:345.

Fine, M.L. (1989) Embryonic, larval and adult development of the sonic neuromuscular system in the oyster toadfish. *Brain Behav. Evol.* 34:13-24.

Finger, T.E. (1975) The distribution of the olfactory tracts in the bullhead catfish, *Ictalurus nebulosus. J. Comp. Neurol.* 267:2:125-141.

Finlay, G.L. and S.L. Pallas 91989) Control of cell number in the developing mammalian visual system. *Prog. Neurobiol.* 32:207-234.

Fox, G.Q. and G.P. Richardson (1982) The developmental morphology of *Torpedo marmorata:* Electric lobe-electromotorneuron proliferation and cell death. *J. Comp. Neurol.* 207:183-190.

Frazier, L.L. and P.C. Brunjes (1988) Unilateral odor deprivation: early postnatal changes in olfactory bulb cell density and number. *J. Comp. Neurol.* 269:355-370.

Frazier-Cierpial, L. and P.C. Brunjes (1989) Early postnatal differentiation of granule cell dendrites in the olfactory bulbs of normal and unilaterally odor-deprived rats. *Dev. Brain Res.* 47:129:136.

Fujita I., Satou M., and Ueda K. (1988) Morphology of physiologically identified mitral cells in the carp olfactory bulb: a light microscopic study after intracellular stining with horseradish peroxidase. *J. Comp. Neurol.* 267:2:253-268.

Galeo A.J., Fine M.L., and Stevenson J.A. (1987) Embryonic and larval development of the sonic motor nucleus in the oyster toadfish. *J. Neurobiol.* 18:359-373.

Gavrieli Y., Sherman Y., and Ben-Sasson S.A. (1992) Identification of programmed cell death in situ via specific labeling of nuclear DNA fragmentation. *J. Cell. Biol.* 119:498-501.

Grasl-Kraupp B., Ruttkay-Nedecky B., Koudelka H. Bukowska K., Bursch W., Schulte-Hermann R. (1995) In situ detection of fragmented DNA (TUNEL assay) fails to discriminate among apoptosis, necrosis, and autolytic cell death: a cautionary note. *Hepatology.* 21:1465-1468.

Graus F., Cordon-Cardo C., and Posner J.B. (1985) Neuronal antinuclear antibody in sensory neuronaopathy from lunc cancer. *Neurology.* 35: 538-543.

Graus F., Elkon K.B., Cordon-Cardo C., and Posner J.B. (1986) Sensory neuronaopathy and small cell lung cancer. *Am. J. Med.* 80:45-52.

Griffin W.S.T III, Stanley L.C., Ling C., White L., MacLeod V., Perrot L.J., White C.L., and Araoz c. (1989) Brain interleukin 1 and S-100 immunoreactivity are elevated in Down syndrome and Alzheimer disease. *Proc. Nat. Acad. Sci.* 86:7611-7615.

Guimaraes C.A. and R. Linden (2000) Chloramphenicol induces apoptosis in developing brain. *Neuropharmacology.*39:9:1673-1679.

Gumienny T.L., Lambie E., Hartwieg E., Horvitz H.R., and Hengartner M.O. (1999) genetic control of programmed cell death in the C. elegans hermaphrodite germline. *Development.* 126:51011-51022.

Harzsch S., Miller J., Benson J., and Beltz B. (1999) From embryo to adult: persistent neurogenesis and apoptotis cell death shape the lobster deuterocerebrum. *J. Neuroscience.* 19:9:472-3485.

Higgs D.M. and G.D. Burd (1999) The role of the brain in metamorphosis of the olfactory epithelium in the frog, *Xenopus laevis. Developmental Brain Reserarch.* 816:2:544-553.

Isenmann S., Stoll g., Schroeter M., Kajewski S., Reed J.C., and Bahr M. (1998) Differential regulation of Bax, Bcl-2 and Bcl-X proteins in focal cortical ischemia in the rat. *Brain Pathol.* 8;49-62.

Jacquin M.F., Rana J.Z., Miller M.W., Chiaia N.L., and Rhoades R.W. (1996) Development of trigeminal nucleus principalis in the rat: effects of target removal at birth. *Eur. J. Neurosci.* 8:81641-57.

Kalda A., Eriste e., Vassiljev v., and Zharkovsky A. (1998) Medium transitory oxygen-glucose deprivation induced both apoptosis and necrosis in cerebellar granule cells. *Neurosci. Lett.* 240:1:21-24.

Kerr J.F.R., Wyllie A.J., and Currie A.R. (1972) Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. *Br. J. Cancer.* 26:239-257.

Kim C.H., Bae Y.K., Yamanaka Y., Shimizu T., Fujii R., Park H.C., Yeo S.H., Huh T.L., Hibi M., and Hirano T. (1997) Overexpression of neurogenin induces ectopic expression of HuC in zebrafish. *Neurosci. Lett.* 239:2:3113-3116.

Kosaka K., Aika Y., Toida K., Heizmann C.W., Hunziker W., Jacobowitz D.M., Nagatsu I., Streit P., Visser T.J., and Kosaka T. (1995) Chemically defined neuron groups and their subpopulations in the glomerular layer of the rat main olfactory bulb. *Neurosci. Res.* 23:73-88.

Kosaka K. and K. Hama (1982-1983) Synaptic organization in the teleost olfactory bulb. *J. Physiol (Paris)* 78:8:707-719.

Kosaka K., Hataguchi Y., Hama K., Nagatsu I., and Wu J.Y. (1985) Coexistence of immunoreactivities for glutamate decarboxylase and tyrosine hydroxylase in some neurons in the periglomerular region of the rat main olfactory bulb: Possible coexistence of gamma-aminobutyric acid (GABA) and dopamine. *Brain Res.* 343:166-171.

Kuby, Janis (1997) *Immunology* 3rd ed. W.H. Freeman and Company, N.Y. pp. 67, 368-371, 386-387.

Lazebnik Y., Kaufmann S., Desnoyers S., Poirier G., and Earnshaw W. (1994) Cleavage of poly (ADP-ribose) polymerase by a proteinase with properties like ICE. *Nature.* 371:346-347.

Li M., Ona V.O., Chen M., Kaul M., Tenneti L., Zhang X., Stieg P.E., Lipton S.A., and Friedlander R.M. (2000) Functional role and therapeutic implications of neuronal caspase-1 and –3 in a mouse model of traumatic spinal cold injury. *Neuroscience.* 99:2:333-342.

Li X., Melamed M.R., and Darzynkiewicz A. (1996) Detection of apoptosis and DNA replication by differential labeling of DNA strand breaks with fluorochromes of different color. *Exp. Cell Res.* 222:28-37.

Li Y.B., Kaur C., and Ling E.A. (1998) Neuronal degerneration and microglial reaction in the fetal and postnatal rat brain after transient maternal hypoxia. *Elsevier.* 32:137-148.

Mattson M.P., Duan W., Chan S.L., and Camandola S. (1999) Par 4: an emerging pivotal player in neuronal apoptosis and neurodegenerative disorders. *J. Mol. Neurosi.* 13:1:217-30.

Meier U.T. and G. Blobel (1992) Nopp 14p shuttles on tracks between nucleolus and cytoplasm. *Cell.* 70:127-138.

Meisami E. and R. Mousavi (1981) Lasting effects of early olfactory deprivation on the growth, DNA, RNA, and protein content, and Na-K-ATPase and AchE activity of the rat olfactory bulb. *Brain Res.* 254:2217-2229.

Miura M., Zhu H., Rotello R., Hartwieg E.A., and Yuan J. (1993) Induction of apoptosis in fibroblasts by IL-1β-converting enzyme, a mammalian homolog of the *C. elegans* cell death gene ced-3. *Cell.* 75:653-660.

Mochizuki H.,Goto K., Mori H., and Mizuno Y. (1996) Histochemical detection of apoptosis in Parkinson's disease. *J. Neurol. Sci.* 137;120-123.

Najbauer J. and M. Leon 91995) Olfactory experience modulates apoptosis in the developing olfactory bulb. *Brain Res.* 674:245-251.

Nakahara S., Kazunori Y., Sakou T., Shouichi W., Nagamine T., Nitayama T., and Ichijo H. (1999) Induction of apoptosis signal regulatin kinase 1 (ASK 1) after spinal cord injury in rats: possible involvement of ASK1-JNK and p38 pathways in neuronal apoptosis. *J.Neuropathology and Exp. Neurology.* 58:5:442-450.

Okano H.J. and R.B. Darnell (1995) A hierarchy of Hu RNA binding proteins in developing and adult neurons. *J. Neurosci.* 17:93024-93037.

Oppenheim R.W. (1991) Cell death during development of the nervous system. *Annu. Rev. Neurosci.* 14:453-501.

Pinon L.G.P. and R. Linden (1996) Target and afferents interact to control developmental cell death in the mesencephalic parabigeminal nucleus of the rat. *J. Neuroscience Research.* 45:174-182.

Posner J.B. (1995) Anti-Hu autoantibody associated sensory neuropathy encephalomyelitis: A model of paraneoplastic syndrome. *Perspect. Biol. Med.* 38:167-181.

Sandeman R., Clarke D., Sandeman D., and Manly M. (1998) Growth-related and antennular amputation – induced changes in the olfactory centers of crayfish brain. *J. Neurosci.* 18:16:6195-6206.

Schwartzman R.A. and J.A. Cidlowski (1993) Apoptosis: the biochemistry and molecular biology of programmed cell death. *Endocrine Rev.* 14:133-151.

Simbulan-Rosenthal C.M., Rosenthal D.S., Iyer S., Boulares A.H., and Smulson M.E. (1998) Transient poly (ADP-ribosyl)ation of nuclear proteins and role of poly (ADP-ribose) polymerase in the early stages of apoptosis. *J. Biol. Chem.* 273:13702-13712.

Soutschek J. and G.K.H. Zupanc (1995) Apoptosis as a regulator of cell proliferation in the central posterior/prepacemaker nucleus of adult gymnotiform fish, *Apteronotus leptorhynchus. Neurosci. Lett.* 202:133-136.

Surh C.D. and J. Sprent (1994) T-cell apoptosis detected in situ during positive and negative selection in the thymus. *Nature.* 372:100-103.

Straznicky C. and R.A. Rush (1985) Nerve growth factor treatment does not prevent dorsal root ganglion cell death induced by target removal in chick embryos. *Anat. Embryol (Berl).* 171:3357-63.

Tammariello S.P., Quinn M.T., and Estus S. (2000) NADPH oxidase contributes directly to oxidative stress and apoptosis in nerve growth factor-deprived sympathetic neurons. *J. Neurosci.* 20:1:R53.

Trucco C., Oliver F.J., de Murcia G., and Menissier-de Murcia J. (1998) DNA repair defect in poly (ADP-ribose) polymerase-deficient cell lines. *Nucl. Acids Res.*

VanKirk, A.V. and C.A. Byrd (1999) *Soc. Neurosci. Abstr.* 25:130.

Whiteside G., Cougnon N., Hunt S.P., and Munglani R. (1998) An improved method for detection of apoptosis in tissue sections and cell culture, using the TUNEL technique combined with Hoechst stain. *Brain Res. Protocols.* 2:160-164.

Whiteside G. and R. Munglani (1998) TUNEL, Hoechst, and immunohistochemistry triple-labeling: an improved method for detection of apoptosis in tissue sections – an update. *Brain Res. Protocols.* 3:1:52-53.

Wyllie A.H., Kerr J.F.R., and Currie A.R. (1980) Cell death: the significance of apoptosis. *Int. Rev. Cytol.* 68:251-306.

Wyllie A.H. (1997) Apoptosis: an overview. *Br. Med. Bull.* 53:451-465.

Zupanc G.K.H., Kompass K.S., Horschke I., Ott R., and Schwarz H. (1998)

Apoptosis after injuries in the cerebellum of adult teleost fish. *Exp. Neurology.* 153:221-230.