NEURONAL MIGRATION AND SURVIVAL IN THE DEVELOPING CHICK OPTIC TECTUM

by

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABSTRACT

In the developing brain, neurons are born in the ventricular zone and migrate outward via radial glia scaffolds. There also supposedly is an early period of widespread programmed cell death in the brain, occurring while these neurons are still migrating from embryonic day 7.5 (E7.5) through E8. We hypothesize there is a relation between these two processes whereby the cells that die do so because of anoikis: loss of contact with the necessary extracellular matrix produced by radial glia. Specifically, we predict that fibronectin secreted by radial glia interacts with the $\alpha_8\beta_1$ integrin receptors on the neuronal membrane, initiating an intracellular cascade which eventually increases expression of Bcl-2, an anti-apoptotic molecule. The developing chick optic tectum (OT) was used as a model. OT cells were immunostained at different ages and show a general trend that there is a period where pro-apoptotic signals are present when Bcl-2 is down-regulated. Propidium Iodide staining experiments support this data with more fragmented DNA appearing during this time period, which is a hallmark of apoptosis. Bcl-2 also was ectopically expressed in the OT using a retroviral vector in hopes of analyzing behavior of neurons kept alive artificially. Additionally, OT cells were plated at different densities on a culture dish to determine whether radial glia contact could suppress neuronal apoptosis and/or increase Bcl-2 levels.

Chapter 1

INTRODUCTION

1.1 Neuronal Migration

Neurons are born in the ventricular zone of the developing brain and migrate outwardly; this forms brain laminae in an "inside-out" manner with the newest neurons positioned most superficially and the eldest neurons located most proximally to the brain ventricle (Bronner-Fraiser and Hatten, 2003). This phenomenon occurs because of continuous migration where neurons migrate outwardly as far as possible, past the older neurons that have already migrated. The main mechanism behind this process is through neurons migrating along radial glial processes which extend from the ventricular zone to the pia mater, the first layer of meninges that covers the central nervous system (Rakic, 1972). Correct migration is essential for proper final neuron position in the developing brain. This positioning determines differentiation processes that alter cell morphology, synaptic connectivity, and ultimately, the function of these neurons (Anton et al, 1996). Thus, it is important that these processes occur correctly to allow the brain to develop normally.

1.2 Neuronal Migration Disorders

When neuronal migration proceeds abnormally, a variety of neuronal migration disorders can result. These disorders include structural abnormalities such as schizencephaly,

porencephaly, lissencephaly, agyria, macrogyria, pachygyria, microgyria, micropolygyria, neuronal heterotopias, agenesis of the corpus callosum, and cranial nerve agenesis (Guerrini and Parrini, 2010). There are many steps in neuronal migration which can go awry; thus, there are many different types of disorders seen in people with abnormal neuronal migration.

One type of neuronal migration disorder, called lissencephaly, meaning "smooth brain" in Greek, is one of the more common neuronal migration disorders (Métin et al., 2008). In these patients, the regular grooves called sulci and gyri in the brain are not well-formed, so much so that the brain appears smooth. These sulci and gyri are important for increasing the surface area of the brain and allowing more neurons to be in contact with cerebral capillaries and the surrounding cerebrospinal fluid. Children that are born with lissencephaly usually have symptoms of severe mental retardation, seizures, failure to thrive, and muscle spasms (Kato and Dobyns, 2003). These children can differ in their symptom severity as some children can have near-normal cerebral function, but most patients die before age 2 and have brain development equivalent to a 3 to 5 month-old child (Kato and Dobyns, 2003).

Presently, the only treatment involves giving medication to alleviate seizures. Brain development is a relatively new field, and elucidating the basic mechanisms behind this development is crucial in hopes for discovery of risk factors and future treatments for diseases of this sort. Interestingly, it has been found that in lissencephalic brains, neurons are unable to migrate along radial glial fibers correctly which may contribute to the absence of sulci and gyri (Métin et al., 2008). In this way, the present study may lead to a further understanding not only for normal brain development, but also for current medical problems.

1.3 Programmed Cell Death

Though some cases of incorrect migration result in disorders, normally some cells do not migrate successfully and many cells in the developing brain undergo programmed cell death (Martin, S.S., and Vuori, K., 2004; Blaschke et al., 1996). We believe these two phenomena are related in a mechanism such that the cells that don't migrate correctly are removed from the brain through an apoptotic signaling cascade. Periods of programmed cell death during embryonic development are understood to occur in multiple fashions, the main example given is the death of cells during digit formation to avoid webbed hands and feet. Any kind of tissue remodeling can occur due to altered proliferation of a certain cell type, altered death rates of a certain cell population, or a combination of the two. In this case, we are focused on how the brain is being formed through increased cell death rates of neurons that migrate unsuccessfully.

Cell death can come about in many different forms, the most notable being necrosis and apoptosis. Necrosis is commonly caused by mechanical trauma which ruptures the cell membrane and releases intracellular contents to the extracellular environment. This is generally accompanied by an inflammatory response in the organism. Apoptosis is a type of programmed cell death which is controlled by various signaling molecules inside the cell. This process is sometimes termed "cell suicide" because the cell makes a decision to die based on unfavorable conditions present inside the cell or in its surrounding environment.

It turns out the default pathway of a cell is to undergo apoptosis; a cell needs survival signals in order to stay alive. One type of survival signal is mediated through extracellular matrix proteins and their associated membrane receptors. This interaction initiates an intracellular signaling cascade which blocks the death pathway and allows the cell to survive. In absence of contact with the extracellular matrix (ECM), many cell types

undergo a type of programmed cell death called anoikis. This happens in multicellular organisms in cells that are meant to form tissues and not exist as individual cells.

The intracellular pathways that determine cell survival and apoptosis regulate levels of pro-apoptotic and anti-apoptotic proteins. At any given time, a cell should have both of these types of proteins present. It is the relative proportions of these factors that determine whether the cell lives or dies. When a cell has relatively higher proportions of anti-apoptotic factors, it is more likely to remain alive. When a cell has more pro-apoptotic factors and activated apoptotic proteins, it is more likely to undergo cell death. The main family of apoptotic proteins is the Bcl-2 family. Of these, Bcl-2 and Bcl-X_L are the main anti-apoptotic proteins and Bax, Bad, and Bid are the main pro-apoptotic proteins. The exact mechanisms of how these molecules cause cell survival and cell death, respectively, is the subject of much active research. Even so, basic mechanisms are generally supported. When there are high levels of pro-apoptotic proteins, they allow cytochrome c to be released from the mitochondrial matrix into the cytoplasm and the anti-apoptotic proteins serve to prevent this from occurring.

Cytochrome c binds to apoptotic protease activating factor 1 (Apaf-1), which binds ATP to form what is called an apoptosome. This binds to procaspase-9, cleaving an inhibitory domain of the pro-enzyme (Li et al., 1997). This activates caspase-9, which, in turn, activates other caspases like caspase-3 and caspase-7 (Porter and Jänicke, 1999). In this way, caspase-9 is known as an initiator caspase. Caspases 3 and 7 are referred to as executioner caspases because they cleave multiple cellular targets which lead to cell death. During this apoptotic process, certain morphological features are prevalent, including DNA fragmentation, nuclear condensation, and membrane blebbing (Roth and D'Sa, 2001). The nuclei of apoptotic cells are called pyknotic, which is the Greek word meaning condensed.

1.4 Proposed Model

Our hypothesis is that programmed cell death occurs during early development in the chick optic tectum. The optic tectum is in the dorsal midbrain and is the equivalent to the mammalian superior colliculus. These structures serve mainly to organizing gaze movements. In vision, it is involved in initiating saccadic eye movements and orienting head movements (Wurtz et al., 2005). It is a highly laminated structure and has been used in animal models as a neocortical analog concerning neuronal development and migration (Grey, Leber, and Sanes, 1990; Galileo et al., 1990). The optic tectum differs from the mammalian neocortex in terms of migration patterns by being more ordered with a higher proportion of cells undergoing radial migration, while neocortical neurons are more likely to migrate tangentially along axon fibers (Gray et al., 1990). However, the vast majority of neocortical neurons undergo radial migration (Ayala et al., 2007). The embryonic chick OT animal model is advantageous for studying neuronal migration for multiple reasons. For one, embryonic development is easier to study when removing embryos from the mother is not an obstacle; chick embryos are in their own egg shell. Also, the tectal structure is highly ordered and laminated, which makes it easier to detect cytoarchitectural changes. Conveniently, the chick incubation period is only 21 days and eggs can be refrigerated for up to a week without any development taking place. Thus, we can start an incubation period on any day and results can be obtained relatively faster than if other animal models were used.

Because this model is so fitting, many researchers have used the chick OT as a model for brain development. Thus, many experimental protocols have been developed for this tissue. All cryosectioning, immunostaining, and retroviral infections in the present study have been based upon previous research methods (Gray et al, 1990; Morgan et al., 2000; Zhang and Galileo, 1998a; Zhang and Galileo 1998b; Stettler and Galileo, 2004). For

example, a lineage marker has been engineered through injection of a retroviral vector containing a marker gene, *lacZ*, which encodes β-galactosidase. This type of vector was constructed in the Rous sarcoma virus and has been injected into the chick OT as early as E3 (Gray et al., 1990). The embryo is still able to develop without any perceived developmental abnormalities and a cell and its progeny are able to be detected through identification of the cells containing the lacZ marker gene. This method has been used to study cell lineage, migration, and survival in the brain (Gray et al., 1990; Galileo et al., 1993; Morgan et al., 2000).

The early period of programmed cell death is hypothesized to occur from E7.5 through E8. This time window is specified in the literature as the period when nicked DNA fragments can be detected in the chick OT (Zhang and Galileo, 1998a). DNA degradation is a hallmark of apoptosis (Roth and D'Sa, 2001). Fluorescent *in situ* end labeling (FISEL+) found that many cells in the ventricular zone of the chick E8 OT had nicked DNA ends; this time period corresponds with radial migration of neurons (Zhang and Galileo, 1998a,b). A similar approach was utilized to find programmed cell death in proliferative zones of the embryonic neocortex (Blaschke et al., 1996). Previously, programmed cell death in the embryonic OT had only been seen later on, during synaptogenesis (Raff et al., 1993; Gohlke et al., 2004).

Since these cells are dying in the ventricular zone during a period of radial migration, we hypothesize that these two phenomena are related such that neurons that are not migrating correctly are undergoing programmed cell death. Neurons attach to radial glia in the ventricular zone, and use the glial processes as a guide for migration to upper laminae (Rakic, 1972). Many neurons are born in the ventricular zone and it is possible that not all neurons have the opportunity to attach to radial glia in order to migrate. We believe this

disconnect is what causes these neurons to die. Evolutionarily, this process would be favored because an excess of neurons would ensure a maximum number of neurons to migrate to form upper laminae, yet this would disallow correct connections to form later in development. Incorrect connections may cause mental deficits that decrease the survival rate of an organism.

The mechanism we propose is that the ECM protein, fibronectin, secreted and aligned by radial glia, binds to the $\alpha_8\beta_1$ integrin on neuronal membranes, initiating an intracellular survival signal which increases Bcl-2 levels. The absence of this signal is what causes the glia-deprived neurons to apoptose. Previous work has shown that the fibronectin ligand is produced and aligned by radial glia in the developing chick OT in a pattern consistent with migratory pathways (Stettler and Galileo, 2004). In the mouse, fibronectin has been found deep in the neuroepithelial proliferative zone with dividing neurons and radial glia, where it is purported that these radial glia are the source of fibronectin (Sheppard et al., 1995). Also, when vectors introduced antisense α_8 or β_1 integrin sequences in the chick OT, there was an increase of cell death during this period accompanied by migration inhibition (Zhang et al., 1998b).

1.5 Research Goals

1.5.1 Cell Death Detection

Since the FISEL+ method is somewhat controversial as to whether it can detect false positives, it is prudent to detect apoptosis in these OT cells using other methods (Lesauskaite et al., 2004). A common way to detect apoptosis in dying cells is through immunostaining of proteins involved in the apoptotic process. Active caspase-3, Bax, and

Bcl-2 are all major components of the cell death pathway. Caspase-3 is an executioner caspase, which, once activated, results in irreversible proteolytic events that cause cell death. Bax is a pro-apoptotic factor that allows cytochrome c release from the mitochondria. Bcl-2 is an anti-apoptotic protein that inhibits the functions of Bax and similar proteins to contribute to cell survival. Thus, an apoptotic cell should be able to be visualized by having active caspase-3 present, higher levels of Bax, and lower levels of Bcl-2.

This immunostaining method can be done on cryosections of OT tissue, freshly dissociated and fixed OT cells, and OT cells plated in culture dishes. A positive control for apoptosis can be performed by inducing the apoptotic pathway through incubation in staurosporine. Staurosporine is a protein kinase inhibitor which acts by competitively inhibiting ATP binding (Rüegg and Burgess, 1989). The mechanism of how this induces apoptosis is unclear but capase-3 is activated and cell death occurs soon after. A negative control condition can be implemented by not incubating some samples in primary antibody, or by comparison with an embryo at a stage outside of the E7.5-E8 apoptotic window.

Other major apoptosis detection systems take the nuclear condensation and degradation into account. The nucleus of an apoptotic cell condenses and fragments. These pyknotic nuclei are easily seen through fluorescence wide-field microscopy when DNA is labeled. DNA can be labeled by incubating fixed cells in bisbenzimide, an organic molecule that intercalates between Adenine-Thymine nucleotide pairs (Pjura et al., 1987). This compound fluoresces blue under ultraviolet light to allow for visualization of the nucleus.

Propidium iodide is another DNA intercalating agent with no sequence preference and fluoresces red when excited with green light at a wavelength of 488nm. This substance is commonly used to detect amounts of DNA in a cell. When cells in suspension have their DNA stained, DNA content can be analyzed by flow cytometry. A flow

cytometer can detect the size of a cell and the amount of fluorescence it emits and can do so for thousands of cells per second. This allows for quantitative analysis of cellular DNA content. Cells in G_1 would be most prevalent, followed by cells in G_2/M and S. A cell in G_1 would have a single copy of its genome. DNA content would increase during S phase before a cell would have two full complements in G_2, just before mitosis takes place. No healthy cell should have DNA content less than what is present in G_1. Thus, when a sub-G_1 amount of DNA is detected in a cell, the cell is most likely undergoing apoptosis as shown through DNA degradation.

All of the previous detection methods require cells to be fixed and permeablized. There is also a new non-cytotoxic method of detecting apoptotic cells. FAM-FLIVO™ (Immunochemistry Technologies; Catalog No. 180) is a green fluorescent probe that can diffuse through cells and preferentially forms irreversible covalent bonds with various active caspases. This type of marker is useful in its ability to detect a variety of cell death cascades, since there is evidence that different apoptotic pathways are present in embryonic neuronal programmed cell death (Roth et al., 1999). This substrate can be delivered to the OT tissue in a living embryo to ensure any cell death seen is not due to the death of the animal causing hypoxic conditions.

1.5.2 Artificially Increased Neuronal Survival Rates

Since we believe a large amount of cells die during this embryonic E7.5-E8 window, an indirect method to detect this phenomenon is to note differences seen when these cells are kept alive artificially. A neural progenitor cell divides multiple times to produce a clonal cohort of progeny which tend to migrate radially along the same radial glial process, supposedly due to its close proximity (Gray et al., 1990). Normal clones have a linear

morphology with cells present in all tectal laminae (Gray et al., 1990; Zhang and Galileo, 1998b). If cells are kept alive artificially, we expect there would be more cells per clone, with a surplus of cells located in the ventricular zone since they could not associate with radial glia and migrate away. One way to keep cells alive is to increase expression of Bcl-2 since this protein normally blocks apoptotic processes from happening. If a cell has high levels of Bcl-2, it should stay alive regardless of whether it has survival signals or not. Bcl-2 overexpression has been shown to increase the survival of embryonic chick neurons in previous studies (Allsopp et al., 1993). The same technique has also been applied with success to mouse models (Martinou et al., 1994; Middleton et al., 1996).

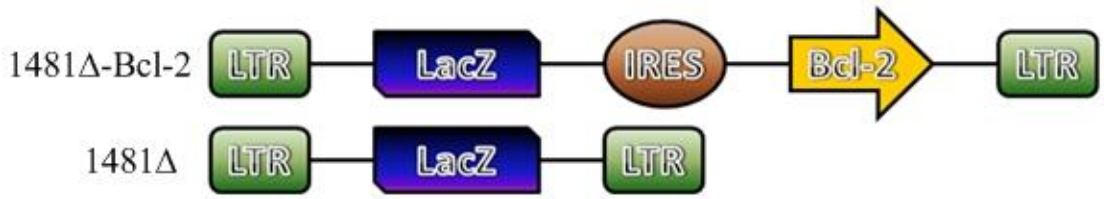

Figure 1. Schematic of Retroviral Vector Compositions.
To study the effect of ectopic Bcl-2 in neuronal migration, a replication-incompetent retroviral vector (1481Δ-Bcl-2) was constructed which contained a reporter lacZ gene, an internal ribosome entry site (IRES), and a bcl-2 gene bounded by two long terminal repeats (LTR). A control vector (1481Δ) included only the lacZ gene between two LTRs.

Since the retroviral vector mentioned before can be used to track neuronal lineage and migration, it is an ideal instrument to use to determine the effect of ectopic Bcl-2 on these clones. A previous undergraduate thesis student in Dr. Galileo's lab, Lindsay Higdon, has constructed a vector called 1481Δ-Bcl-2 (Figure 1). 1481Δ is the vector that encodes solely lacZ as a marker gene. Ms. Higdon altered this retroviral vector by ligating an internal ribosomal entry site and the bcl-2 gene after the lacZ gene with 5' XBaI and 3' SalI ends. The internal ribosomal entry site allows for cap-independent translation of the bcl-2

gene. This vector should be able to cause the cell to express higher levels of Bcl-2 as well as the lacZ gene product, β-galactosidase, which is essential for identifying infected cells.

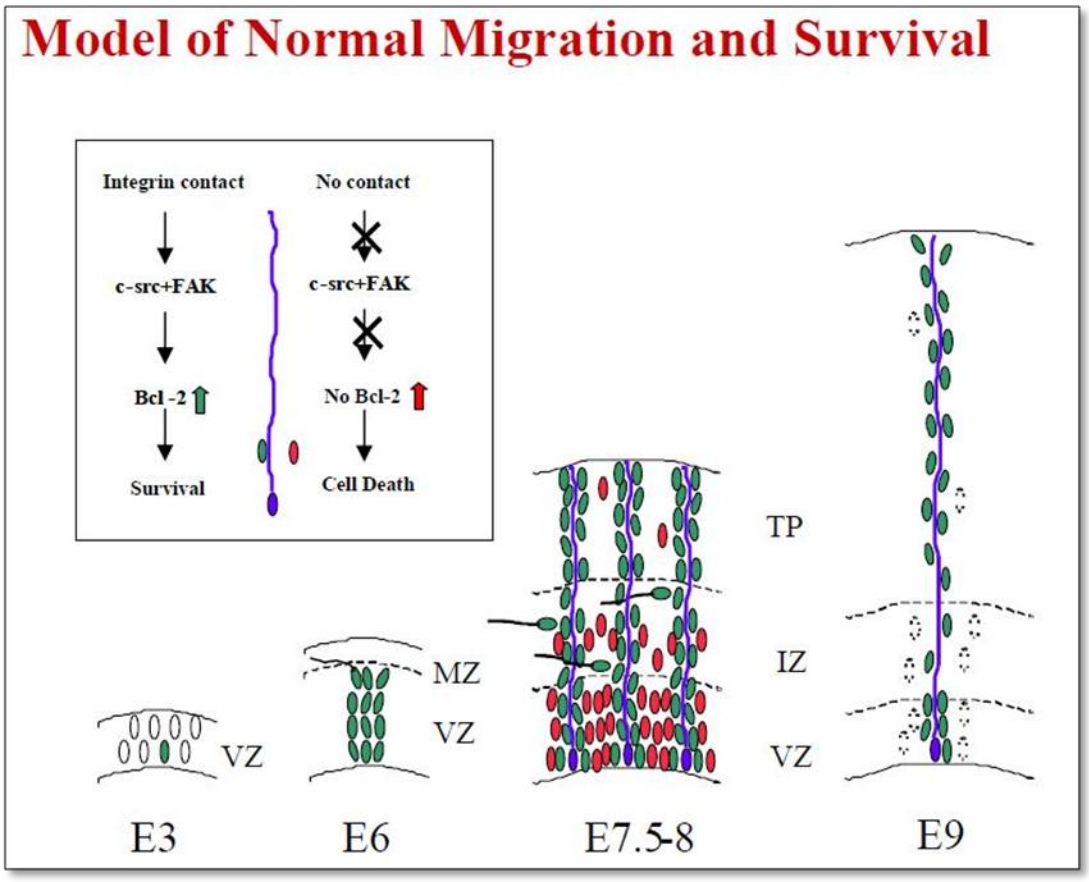

Figure 2. Normal Migration and Survival in the Chick Optic Tectum.
Neurons are first born on E3 in the ventricular zone (VZ) where they divide to form clonal progeny which migrate along nearby radial glia processes during E7 and E8 to produce the intermediate zone (IZ) and tectal plate (TP) of the optic tectum. From E7.5-E8 there is a proposed period of programmed cell death where cells without radial glia contact die via anoikis. Retroviral infection is performed on E3 to track a single progenitor's progeny. (Image courtesy of Dr. Deni Galileo)

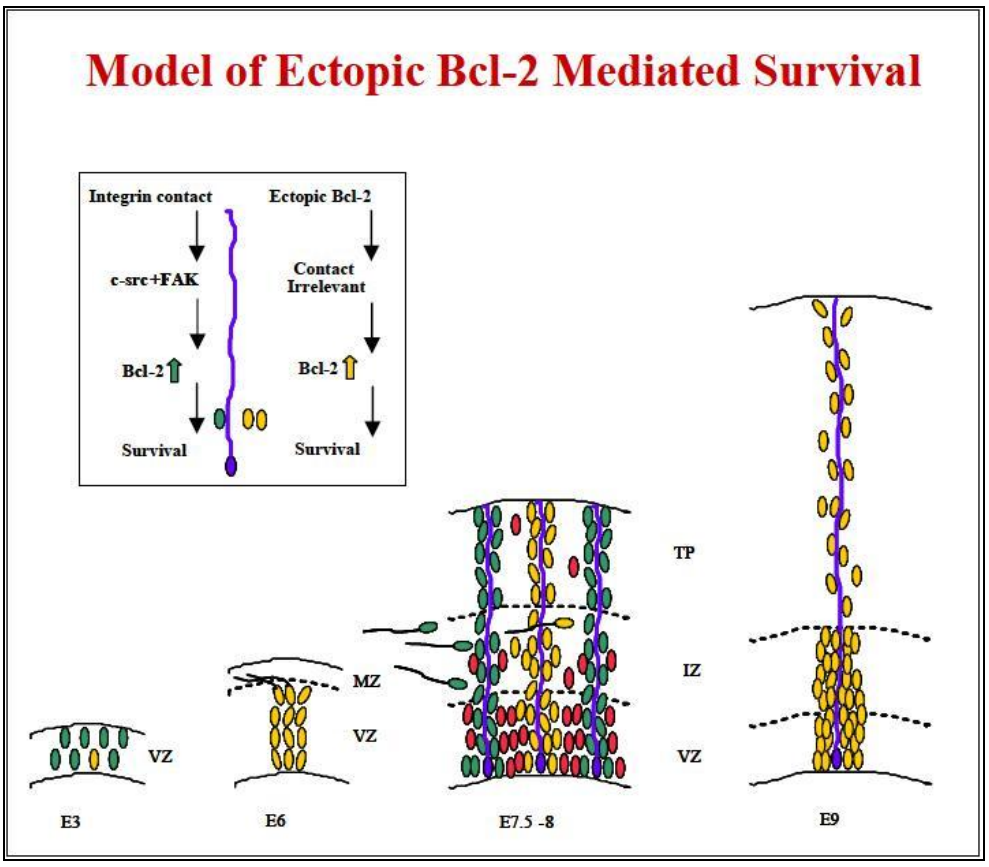

Figure 3. Ectopic Bcl-2 Mediated Survival.
When a progenitor cell is infected with a bcl-2 containing vector, the progeny should stay alive regardless of radial glia contact. We hypothesize this would result in a greater concentration of cells in the lower OT laminae and greater number of cells per clone. (Image courtesy of Dr. Deni Galileo)

This vector can be injected on E3, when neuronal precursors are born, and the embryo can be dissected at E9, after the period of proposed widespread programmed cell death occurs. The clones obtained using this vector can be compared to control clones detected by the normal 1481Δ retroviral vector. It is expected that with Bcl-2, cells that would normally die in the ventricular zone would be kept alive, resulting in a greater proportion of cells in the lower OT laminae (Figure 3). The reasoning behind this is that

without radial glial contact, neurons would be unable to migrate outwardly and this contact provides survival signals to migrating neurons.

1.5.3 Elucidating Survival Factors for OT Cells

In addition to detecting cell death, it is important to determine the factors that keep cells alive. The main trophic factor under consideration is the contact between radial glia and neurons. We think this interaction is due to fibronectin secreted by radial glia interacting with integrins on the neuronal membrane. These interactions are technically difficult to analyze *in vivo*, so our manipulations include cells in culture. There is no chick optic tectum cell line and embryonic timing is important in these experiments. Thus, for valid results, experiments were performed on freshly dissociated embryonic OT cells plated on culture dishes.

The factors to be analyzed included general cell contact, interactions with fibronectin, and radial glia contact. These are three separate but related ways to assess the model that fibronectin secreted by radial glia provides the survival factors for migrating neurons. Increased cell contact in general is hypothesized to decrease cell death; this is a way to see if anoikis is a probable death pathway in this cell type to begin with. To further specify these interactions, fibronectin is assessed to see if this integrin-ECM interaction is what can mediate the survival signals. Furthermore, it is necessary to directly test whether this interaction can be replicated where contact with radial glia is the supposed source of fibronectin.

1.5.4 Integrin Identification

The $\alpha_8\beta_1$ integrin has previously been studied in the neurons of the developing chick optic tectum and is a potential ligand for the fibronectin secreted by radial glia (Zhang and Galileo, 1998b). However, the $\alpha_5\beta_1$ integrin is the prototypical fibronectin receptor but has not been assayed for in the chicken optic tectum at this age. It is important to decipher which integrins are present on neurons at this age because it has been shown that different fibronectin-binding integrins can mediate vastly different cellular responses (Aota, S. et al., 1991).

Additionally, the proposed pathway of fibronectin-integrin interactions increasing Bcl-2 levels has already been studied with the $\alpha_5\beta_1$ integrin; one study indicates a signaling pathway involving Shc, FAK, Ras, PI3K, and Akt (Matter and Ruoslahti, 2001).

Searching for an additional fibronectin ligand does not entail abandoning the hypothesis that the $\alpha_8\beta_1$ integrin plays an integral role. In fact, these two integrin receptors may act synergistically. Cell migration on fibronectin can require binding to an RGD sequence, the major binding site on fibronectin, as well as to a synergy region located nearby on the protein (Nagai et al., 1991). The most important synergy region, containing the amino acid sequence PHSRN, is required for high-affinity binding to fibronectin (Aota et al., 1991; Johansson et al., 1997). While the $\alpha_8\beta_1$ integrin only binds the RGD sequence, the $\alpha_5\beta_1$ integrin can bind either the RGD or PHSRN regions (Johansson et al., 1997).

These two integrins are supposed to be functionally relevant in cell survival. The $\alpha_8\beta_1$ integrin has been shown to increase cell survival via fibronectin interactions through a PI3 kinase pathway (Farias et al., 2005). Similarly, the $\alpha_5\beta_1$ integrin has been shown to increase survival of Chinese Hamster Ovary cells plated on fibronectin by increasing Bcl-2

levels (Zhang et al., 1995). With this in mind, the first step in determining if the $\alpha_5\beta_1$ integrin has any role in this process is to detect if it is present in E8 chicken OT cells.

Chapter 2

MATERIALS AND METHODS

2.1 Chicken Eggs

Fertilized White Leghorn chicken eggs were obtained weekly from the University of Delaware Department of Animal & Food Sciences and stored in a refrigerator at 15°C. These eggs were then incubated at 37.5°C in a humidified forced-air egg incubator until the desired age. The day the eggs are first placed in the incubator is termed embryonic day 0 (E0).

2.2 Cell Death Detection

2.2.1 Cryosection Immunostaining

Eggs were incubated until E8 and then brains were removed and sectioned. Eggs were opened, the embryos were gently removed, and then placed in a Petri dish filled with sterile Calcium/Magnesium-free Tyrode's saline solution (CMF). The embryos were sacrificed via decapitation and the optic tecta (OT) were dissected out, with the overlying meninges removed. The tectal lobes were fixed in 1mL of 2% paraformaldehyde diluted in phosphate-buffered saline (PBS). They were placed in fixative for 2 hours before being

washed with PBS three times, for 15 minutes per wash. The tectal lobes were then incubated in 1mL 30% sucrose in PBS overnight at 4°C.

The next day, the OT lobes were carefully embedded in a small well containing TBS tissue freezing media (Triangle Biomedical Sciences). These cryoblocks were stored in sealed plastic vials with 1mL frozen water at -80°C until sectioning to keep them humidified.

The cryoblocks were cut into 10µm thick sagittal sections using a Leica CM3050 S Cryostat at -20°C. Sections were mounted on Superfrost Plus slides (Fisher Scientific) and air dried. Slides were stored in an airtight desiccator at -20°C until immunostained.

On the day of immunostaining, desiccators with E8 cryosections were removed from the -20°C freezer and thawed at room temperature. A hydrophobic barrier was drawn around each section with a Super Peroxidase-anti-peroxidase (PAP) Pen (Binding Sites, Inc.) to prevent mixing of antibody solutions. All of the following steps occurred in a humidified chamber. The sections were incubated in 30µL of a blocking buffer containing 0.03% Triton-X 100 and 5% Normal Goat Serum (NGS) diluted in PBS for 1 hour.

The primary antibodies used were a rabbit polyclonal anti-active caspase-3 IgG (BD Pharmingen; 557035), a rabbit polyclonal anti-active caspase-3 IgG (Promega; 11551305, 6748A), a mouse monoclonal anti-Bcl-2 IgG (Santa Cruz; sc-7382), and a goat polyclonal anti-Bcl-2 IgG (Santa Cruz; sc-492-G). Each individual experiment used either one of these antibodies, or a combination of an active caspase-3 antibody and a Bcl-2 antibody for double immunostaining. Concentrations of primary antibodies were either 3µg/mL or 10µg/mL, except for the Promega antibody which did not provide a stock concentration. The Promega anti-active caspase-3 antibody was used at the recommended dilution of 1:125. All primary antibodies were diluted in blocking buffer containing 5% serum

(Fetal Bovine Serum (FBS) or NGS), 0.03% Triton-X 100 (Sigma), in PBS. Sections were incubated with a primary antibody mix for 1 hour at room temperature, or alternatively, overnight at 4°C. Some sections served as no primary antibody controls and they were incubated in blocking buffer for the same time period. All sections were then washed twice with PBS for 5 minutes each wash.

The secondary antibodies used were Alexa Fluor 488-conjugated goat-anti-mouse IgG (Invitrogen; A11001), Alexa Fluor 488-conjugated donkey-anti-goat IgG (Invitrogen; A11055), Alexa Fluor 594-conjugated goat-anti-rabbit IgG (Invitrogen; A11037). In some cases, triple-layer immunostaining was performed with the secondary antibodies being biotin-conjugated goat-anti-rabbit IgG (Invitrogen; B2770) or biotin conjugated donkey-anti-goat IgG (Jackson Immunoresearch; 705-06500). In all cases, sections were incubated in 30µL of a secondary antibody solution containing a 1:200 dilution of the secondary antibody in a blocking buffer containing 5% serum (FBS or NGS), 0.03% Triton-X 100, in PBS. This incubation lasted for 1 hour at room temperature before PBS washes took place.

The sections that underwent triple-layer immunostaining were incubated for 1 hour in 30µL of a solution containing a 1:500 dilution of streptavidin-conjugated Alexa Fluor 488 (Invitrogen; S11223) in blocking buffer containing 5% serum (FBS or NGS) and 0.03% Triton-X 100 in PBS. All other sections skipped this step.

Next, all sections were washed twice with PBS for 5 minutes per wash before incubation in bisbenzimide for nuclear staining. 30µL of a 10µg/mL bisbenzimide solution was added to each section for 5 minutes. The sections were then washed with PBS for 5 minutes before coverslips were mounted.

6µL of a solution containing 70% glycerol, 29.95% PBS, and 0.05% p-phenylene diamine (PPD) was added to each cryosection and a glass coverslip was placed down on the slide. The edges of the coverslip were sealed with nail polish before storing slides in -20°C. The cryosections were analyzed using a Nikon Microphot-FX microscope equipped with epi-fluorescence and a Nikon DXM1200 digital camera.

2.2.2 Cell Suspension Immunostaining

Cells were taken from the optic tectum and stained for different pro- and anti-apoptotic markers. Embryos ages E7, E8, E9, and E10 had their optic tecta removed and minced in sterile CMF. This mixture was centrifuged at 143g for 5 minutes in order to have the cells form a pellet so the CMF could be aspirated. With the supernatant aspirated, the cells were resuspended in 1 mL 0.25% trypsin for 20 minutes at 37°C to break cell-cell contacts. Afterwards, cells were put on ice and 1 mL of a mixture of soybean trypsin inhibitor, DNase I, and Medium 199 was added to stop trypsin activity (Stettler and Galileo, 2004). After 5 minutes of incubation on ice, cell solutions were centrifuged at 143g for 5 minutes and the supernatant was aspirated. Cells were resuspended in PBS to wash them and were triturated 10 times to further break up multicellular clumps. The suspension was centrifuged again at 143g for 5 minutes then resuspended in 1% formaldehyde in PBS for 30 minutes for fixation. The fixed cells were again spun down in a centrifuge and the remaining formaldehyde was aspirated. The cell pellet was resuspended in a blocking buffer containing 5% FBS and 0.03% Triton-X 100 in PBS for 1 hour; this was used to wash the cells and to block any nonspecific binding sites.

Vials that were not being used as no primary controls were then centrifuged, the supernatant was aspirated, and the cell pellet was resuspended in a primary antibody

mixture. The primary antibodies used for this purpose were a goat polyclonal anti-Bcl-2 IgG (Santa Cruz; sc-492-G), a rabbit polyclonal anti-active caspase-3 IgG (BD Pharmingen; 557035), and a rabbit anti-Bax IgG (ProteinTech Group, Inc.; 50599-2-Ig). The Bcl-2 and active caspase-3 antibodies were used at a concentration of 3 µg/mL and the Bax antibody was used at a concentration of 1µg/mL. Each sample was only incubated in one of the primary antibodies. All primary antibodies were diluted in the blocking buffer previously described. The samples were incubated in the primary antibody mix overnight at 4°C, except no primary antibody controls were left in blocking buffer for this time at 4°C.

The following day, the samples were washed twice in blocking buffer for 5 minutes each wash. A wash consists of the cells being centrifuged, the old supernatant being aspirated, and the cell pellet being resuspended in blocking buffer. Next, all the samples were incubated in a secondary antibody mix. For the samples stained with the active caspase-3 primary antibody, an Alexa Fluor 488 conjugated goat-anti-rabbit secondary antibody was used. The samples previously stained for Bax were incubated in an Alexa Fluor 488 conjugated goat-anti-rabbit IgG for a secondary antibody. The Bcl-2 samples had an Alexa Fluor 488-conjugated donkey-anti-goat IgG as a secondary antibody. All secondary antibodies were diluted 1:200 in blocking buffer and the cells were incubated in this mixture for 1 hour at room temperature. The samples were washed twice more with blocking buffer before being analyzed by flow cytometry.

2.2.3 Positive Control for Active Caspase-3 Antibody

To make sure the active caspase-3 antibodies were effective, a positive control assay was necessary. Three cell types were analyzed to see if apoptosis could be induced. The cell types used were the QT6 cell line of quail-derived fibroblasts, fresh chicken embryo

fibroblasts (CEFs), and embryonic chicken optic tectum cells. QT6 cells were cultured in Medium 199 containing 20% tryptose phosphate broth, 5% bovine growth serum, 1% dimethyl sulfoxide, 2.92µg/mL L-Glutamate, 10µg/mL Streptomycin, and 10 IU/mL Penicillin. CEF cells were cultured in Dulbecco's Modified Eagle Medium (DMEM), 3% FBS, 2.92µg/mL L-Glutamate, and 1% of an Antibiotic-Antimycotic solution (10,000 IU/mL penicillin, 10,000µg/mL streptomycin, and 25µg/mL amphotericin B). Embryonic OT cells were grown in Medium 199 with 10% FBS, 2.92µg/mL L-Glutamate, 10µg/mL Streptomycin, and 10 IU/mL Penicillin.

CEFs were obtained by cutting out the limbs of E6 chicken embryos and triturating them 10X in SBTI with DNase I with a 16-gauge needle and then a 23-gauge needle. This suspension was centrifuged at 143g for 5 minutes and the supernatant was aspirated. The cell pellet was resuspended in the CEF media as detailed above and plated on a 10cm plate with 10mL media. This plate was washed with 1mL PBS after 1 hour so the only remaining cells stuck to the dish would be fibroblasts. PBS was aspirated and media was replaced.

2.2.3.1 Staurosporine Treatment of QT6 and CEF Cells

The first caspase-3 positive control experiment used QT6 cells and CEFs. QT6 cells and CEFs were incubated at 37°C in a 10cm culture dish and allowed to grow to ~80% confluency and ~65% confluency, respectively. The media was aspirated and the cells were washed with 1mL sterile PBS before being incubated with 1mL 0.05% trypsin at 37°C for 5 minutes. Once the cells were detached from the culture dish, the cell solution was mixed with 11mL of the cells' respective media to stop the trypsinization and dilute the cells for plating in smaller wells. For each well used, 1mL of the cell solution was plated onto a

sterile glass coverslip in a 24-well plate. There were 8 conditions, which are compared in Table 1.

	Condition	
Cell Type	Staurosporine	Primary Antibody
QT6	+	+
QT6	+	-
QT6	-	+
QT6	-	-
CEF	+	+
CEF	+	-
CEF	-	+
CEF	-	-

Table 1. Positive Control for Active Caspase-3: Experimental Conditions.
There were three variables altered in this experiment: cell type, staurosporine presence, and primary antibody incubation. All combinations are presented in this table.

These cells were allowed to grow in these new wells for 3 days before 100nM staurosporine was added to the media of the appropriate wells for 6 hours at 37°C. After staurosporine incubation, media was aspirated and all cell wells were washed with 1mL PBS. Cells were then fixed with 300µL 1% paraformaldehyde in PBS for 30 minutes at room temperature. All wells underwent two 1mL PBS washes. Afterwards, some wells were

incubated in a primary antibody solution containing 3µg/mL rabbit polyclonal anti-active caspase-3 IgG diluted in a blocking buffer of 5% NGS and 0.03% Triton-X 100 in PBS. The no primary controls were incubated in just blocking buffer. Regardless, this incubation lasted overnight at 4°C.

The following day, cells were washed 3 times with 1mL PBS for 5 minutes each wash. Secondary antibody incubation followed with a 1:200 dilution of Alexa Fluor 488-conjugated goat-anti-rabbit IgG (Invitrogen; A11008) in blocking buffer. This incubation lasted 30 minutes before the cells underwent another 3 washes in PBS for 5 minutes each wash. 300µL of a 10µg/mL bisbenzimide solution was added to each well for 5 minutes to stain for nuclei. The sections were then washed with PBS twice for 5 minutes each before coverslips were mounted on a glass slide.

6µL drops of a solution containing 70% glycerol, 29.95% PBS, and 0.05% PPD was added to slides and each cell-containing glass coverslip was placed on top of a drop, with the cell side facing the glass slide. The edges of the coverslips were sealed with nail polish before slides were stored at -20°C. Cell-coated coverslips were analyzed using a Nikon Microphot-FX microscope equipped with epi-fluorescence and a Nikon DXM1200 digital camera.

2.2.3.2 Staurosporine Treatment of E7+1 OT Cells

It was also necessary to see if the active caspase-3 antibodies were able to detect apoptosis in the cells examined in this project, namely, embryonic chicken optic tectum cells. E7 embryos were sacrificed and the optic tectum was dissociated into single cells, as described earlier. However, these cells were not fixed immediately. Samples of these live cells were counted using a hemacytometer and two different samples were

averaged to estimate the number of OT cells present. The E7 OT cells were next plated at $2x10^5$ cells/mL on poly-L-ornithine coated glass coverslips in all wells of a 24-well plate where each well contained 1mL cell-containing solution. The glass coverslips were previously coated with 200µg/mL poly-L-ornithine (high molecular weight) diluted in sterile, distilled, & deionized water then washed in PBS. The poly-L-ornithine coating is required for OT cells to stick to the glass coverslips. The cells were incubated for 24 hours at 37°C in 1mL of the OT media described earlier. After these cells have been in culture, they must be distinguished from embryonic cells. Since these cells were in the embryo for 7 days and *in vitro* for 1 day, they are termed E7+1 OT cells.

The following day, media was replaced on all cells, half of them received normal media, the other half received media containing 100nM staurosporine for 6 hours at 37°C. Afterwards, cells were washed with PBS for 5 minutes then fixed in 1% paraformaldehyde in PBS for 30 minutes at room temperature. The cells were then washed again for 5 minutes with PBS, and, depending on the condition, were incubated overnight in a primary antibody mix or just plain blocking buffer at 4°C. Two different primary antibodies were used: a rabbit polyclonal anti-active caspase-3 IgG from Cell Signaling (9601) and a rabbit polyclonal anti-active Caspase-3 IgG from BD (557035). The Cell Signaling antibody was used at a 1:100 dilution (no concentration given) and the BD antibody was used at 3µg/mL concentration diluted in blocking buffer. The blocking buffer composition was varied to determine optimal Triton-X 100 concentrations. The buffer contained 5% heat-inactivated FBS and either 0.03%, 0.10%, or 0.30% Triton-X 100 in PBS. The cells also had varied conditions depending on whether they were treated with staurosporine or not and whether they were incubated in primary antibody solutions or served as no primary antibody controls. In total, there were 24 different conditions tested. These conditions can be visualized in Figure 4.

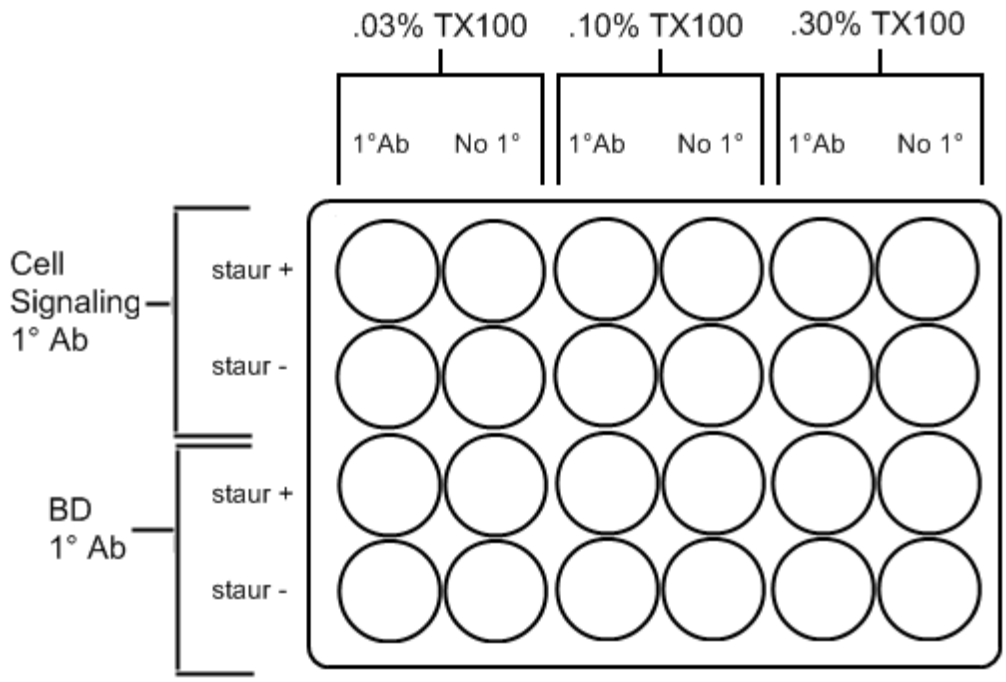

Figure 4. Conditions for Active Caspase-3 Positive Control Using E7+1 OT Cells. Cells were subjected to a total of 24 different conditions with the variables being Triton-X concentration, staurosporine presence, primary antibody incubation, and brand of primary antibody.

All cells were washed 3 times with PBS for 5 minutes each wash before being incubated for 1 hour at room temperature in a secondary antibody solution containing a 1:200 dilution of an Alexa Fluor 488 conjugated goat-anti-rabbit IgG in the appropriate blocking buffer solution. Subsequently, cells were washed 3 times with PBS again before a 5 minute incubation period in 10µg/mL bisbenzimide. The cells were washed again with PBS and the cell-coated coverslips were mounted on slides as described earlier. The slides were

analyzed using a Nikon Microphot-FX microscope equipped with epi-fluorescence and a Nikon DXM1200 digital camera.

2.2.4 DNA Fragmentation

E7, E8, and E9 embryos had OTs removed and dissociated as described previously. However, the cells were not fixed in paraformaldehyde. Instead, they were fixed in a solution with 70% ethanol and 30% distilled, deionized water at -20°C for one to three weeks. When ready to use, vials were centrifuged and the ethanol solution aspirated. The cells were resuspended in PBS to wash them for 5 minutes before again being centrifuged. The cells were resuspended in 1mL of a DNA staining solution including 200µg/mL DNAse-free RNAse A and 20µg/mL propidium iodide in PBS. Cells were incubated in this solution for 30 minutes at room temperature in the dark and were then analyzed with flow cytometry.

2.2.5 Comprehensive *in vivo* Caspase Detection

E8 embryos were also evaluated for cell death markers *in vivo*. Eggs were incubated for 8 days at 37.5°C with the eggs rotated daily to prevent embryo-containing membranes from adhering to the shell wall. On E8, eggs were cracked and the contents were gently poured into a shellless culture hammock which consisted of a piece of cut PVC pipe lined with plastic wrap. In this manner, embryos were able to be manipulated, which would not be possible *in ovo* at this developmental stage.

Embryos were injected into the OT ventricle with 5-10µL of a FAM-FLIVO™ solution. This solution contained 10µL FAM-FLIVO™ (Immunochemistry Technologies; 6218) and 10µL of 1% Fast Green, diluted in 110µL Injection Buffer (Immunochemistry

Technologies; 6220). Fast Green (4,4'-Bis-(N (Ethyl, 3 sulphobenzyl)-amino 2-sulfo-4-hydroxy-fuchsonlum, Disodium salt) is a dye used widely to color foods and was used to visualize if injections entered the OT ventricle (Galileo et al., 1990; Mittal et al., 2008). This FAM-FLIVO™ substrate covalently bonds to active caspases, is excited by 488nm light, and emits photons at 600nm. Thus, the fluorescent substrate can mark apoptotic cells in any pathway involving caspase activation. After injection, embryos were covered with a 10cm culture dish top and returned to the 37.5°C incubator for 1 hour.

Next, the embryos were sacrificed and the OT lobes were minced and dissociated as described earlier. The only difference in this experiment is that these cells were resuspended in Hank's Balanced Salt Solution instead of a fixative, in order to keep these cells alive during flow cytometry analysis. Cells were sorted through a flow cytometer to determine fluorescence levels caused by FAM-FLIVO™ activation. In positive controls, 10μM staurosporine was injected along with the FAM-FLIVO™ solution to induce cell death in some of these embryos. Some embryos underwent all procedures except the injection to serve as negative controls.

2.3 Artificially Increased Neuronal Survival Rates

Bcl-2 levels were ectopically upregulated through infection with a Rous sarcoma virus-derived replication-incompetent retroviral vector. This vector, termed 1481Δ-Bcl-2 contains a marker gene, LacZ, an internal ribosomal entry site, and the bcl-2 gene but not the viral genes *gag*, *pol*, and *env*. This vector was constructed by Lindsay Higdon in the Galileo lab during a previous senior thesis project. A control vector was also used (1481Δ) which only differed by not including the internal ribosomal entry site or the bcl-2 gene.

Eggs were placed in the incubator at 37.5°C in a horizontal manner for 3 days because at this stage, the optimal way to get to embryos is from a side angle. Eggs were cut open above the embryos using sterile surgical scissors. A 30μL aliquot of virus was taken from liquid nitrogen storage and allowed to thaw on ice. This viral concentrate was mixed with 1μL of a 1mg/mL solution of polybrene and 2μL 1% Fast Green solution. Polybrene (hexadimethrine bromide) is a positively-charged polymer that increases retroviral infection rates by neutralizing negative charges between cellular sialic acid residues and virions (Davis et al., 2004).

1-2 μL of this solution was injected into each embryo's OT ventricle using a pulled glass micropipette with a beveled tip and a pressure micro-injector (World Precision Instruments; PV-830). Several drops of 50mg/mL ampicillin were given to the embryo to prevent microbial contamination. The holes in the eggs were covered with clear tape and the embryos were returned to the incubator until E9.

On E9, embryos were sacrificed and the OT lobes were isolated with the overlying meninges removed. The OTs were fixed in a 2% paraformaldehyde solution in PBS for 90 minutes Alternatively, the fixative used was 2% paraformaldehyde and 0.4% glutaraldehyde in PBS and incubation time was 1 hour. In either case, the OTs were then washed twice in PBS for 30 minutes each wash. The tecta were then incubated overnight in X-Gal solution to allow β-galactosidase, the product of the lacZ gene, to enzymatically cleave the X-Gal substrate to form a blue-colored precipitate. This X-Gal solution consisted of 2% 40mg/ml X-Gal stock in dimethyl sulfoxide, 12mM fresh potassium ferricyanide, 13.7mM fresh potassium ferrocyanide, and 0.2% Magnesium Chloride, all diluted in PBS. The overnight incubation was in the dark, at room temperature, and on a shaker to ensure mixing of the solution throughout the tissue.

The tecta were then washed three times for 30 minutes each and then post-fixed in 2% formaldehyde in PBS overnight in the dark, at room temperature, on a shaker table. Tecta were then washed with PBS three times for 5 minutes each before being placed in a preservation solution containing 70% glycerol and 30% distilled, deionized water. Clonal cohorts of infected cells, identified by their blue coloration, were manually cut out of the tissue using forceps and a razor blade fragment. These slices were mounted on glass slides, a drop of 70% glycerol/30% ddH_2O was added, and a coverslip was laid on top. The sections were analyzed using a Nikon Microphot-FX microscope and a Nikon DXM1200 digital camera.

2.4 Elucidating Survival Factors for Tectal Cells

2.4.1 General Contact-Mediated Survival

E7 OT were dissociated and cultured for 1 day *in vitro*, as described earlier. The cells were plated at different densities, however. There was a low contact condition that had cells plated at a density of $3x10^6$ cells/mL and a high contact condition with cells plated at $3x10^7$ cells/mL. Cells were cultured in 10mL OT cell media (described earlier) in 10cm dishes. The difference in cell density caused half of the low density cells to be in contact with another cell while all the cells in the high density condition had contact with at least one other cell.

After incubation for 1 day, cells were trypsinized with 1mL 0.25% trypsin for 5 minutes at 37°C. 1mL Medium 199 containing soybean trypsin inhibitor and DNAse I was added to stop trypsin action. The cell solution was transferred to a microfuge tube and centrifuged at 313g for 5 minutes. The supernatant was aspirated and the cells were

resuspended in 1mL cold 70% ethanol at -20°C for one to two weeks. Cells were washed and stained for DNA with propidium iodide as detailed earlier before being analyzed via flow cytometry.

2.4.1.1 Pyknotic Nuclei

In a separate experiment, the effect of cell density on apoptosis was analyzed by DNA morphological patterns visualized through bisbenzimide staining of nuclei. Cells undergoing apoptosis express pyknotic nuclei which appear as condensed chromatin blebbing into fragments. Instead of a round, intact nucleus, a pyknotic nucleus has small, brightly stained spheres seemingly separating from one another. Optic tectum cells were dissociated from embryonic day 7 (E7) chicken embryos and different concentrations of cells were plated on poly-L-ornithine-coated glass coverslips in a 24-well plate. These cells were incubated at 37°C in CEF media for 1 day. Then, cells were washed with PBS before being fixed for 30 minutes in 1% formaldehyde at room temperature. The coverslips were then immunostained for Bcl-2 and nuclei were counterstained with bisbenzimide as described earlier. There were 3 coverslips per plating density condition, with the density of the two conditions differing by a factor of 5. The high-density condition was expected to have a lower proportion of pyknotic nuclei due to cell-contact mediating survival signals. For each density condition, there were 3 coverslips. For each coverslip, 3 random fields of view were analyzed for the presence of pyknotic nuclei. Thus, in total, there were 6 coverslips used and 18 fields of view assessed. Differentiating between intact and pyknotic nuclei was straightforward and there were no instances where it was unsure whether a cell had a pyknotic nucleus or not.

2.4.2 Fibronectin-Mediated Survival

E7 OT were dissociated and cultured for 1 day *in vitro* in a 24-well plate, as described in earlier experimental protocols. There were six coverslips used in this experiment which were all plated with 200µg/mL poly-L-ornithine for 30 minutes. However, three of these coverslips were then plated with 30µg/mL human plasma fibronectin (Millipore) for 1 hour. Coverslips were washed 3 times for 5 minutes each using distilled water before cells were plated on them. Cells were plated at a density of $1x10^5$cells/mL, a density where the majority of cells have no physical contact with any other cell. After overnight incubation at 37°C, cells were fixed with 1% formaldehyde in PBS for 30 minutes at room temperature. Cells were washed 3 times in PBS, for 5 minutes each wash.

The cells were then immunostained for Bcl-2. All wells not serving as no primary controls were incubated for 1 hour in a primary antibody mixture containing a 3µg/mL concentration of a goat polyclonal anti-Bcl-2 IgG (sc-492G; Santa Cruz), diluted in a blocking buffer containing 5% FBS, 0.03% Triton-X 100, diluted in PBS. No primary antibody controls were incubated in blocking buffer without the primary antibody. Cells were then washed with PBS 3 times, for 5 minutes each. All wells were then incubated in a secondary antibody mix, containing a 1:200 dilution of an Alexa Fluor 488 conjugated donkey-anti-goat IgG diluted in blocking buffer. Cells were washed 3 times in PBS, 5 minutes each. Next, cells were incubated for 5 minutes in bisbenzimide and subsequently washed 3 times in PBS for 5 minutes each. Coverslips were mounted on glass slides with 6µl glycerol/ para-phenylenediamine (PPD) per slide. An immunofluorescence microscope was used to take photos of the cells, and slides were stored in a -20°C freezer. Metamorph software (version 6.1; Molecular Devices, Inc.) was used to quantify differences in Bcl-2

levels between cells grown on coverslips with both fibronectin and poly-ornithine and control cells on coverslips with only poly-ornithine.

2.4.3 Radial Glia-Mediated Neuronal Survival

A similar experiment to the fibronectin-mediated survival was an assay designed to see if contact with radial glia could keep neurons alive. E7 OT were removed from embryos and dissociated as before, and plated at either $1x10^5$ cells/mL, $2x10^5$cells/mL, or $5x10^5$ cells/mL. The cell densities were used at levels whereas intracellular interactions would occur rarely ($1x10^5$ cells/mL), moderately ($2x10^5$ cells/mL), or at high levels ($5x10^5$ cells/mL). These cells were plated on glass coverslips in a 24-well plate, previously coated with 200µg/mL poly-L-ornithine.

After 1 day in the tissue culture incubator at 37°C, cells were fixed with 1% formaldehyde in PBS for 30 minutes, followed by three 5-minute PBS washes. Some wells were then incubated for 1 hour in a primary antibody mix containing a 3µg/mL concentration of a rabbit polyclonal anti-active caspase-3 IgG (BD Biosciences) and a 1:5 dilution of a mouse monoclonal hybridoma supernatant, termed H5 (Developmental Studies Hybridoma Bank), which labels vimentin, a radial glia-specific intermediate filament (Herman et al., 1993). The primary antibodies were diluted in blocking buffer containing 5% heat-inactivated FBS and 0.03% Triton-X 100 in PBS. The other wells served as no primary antibody controls and were incubated in just blocking buffer during this hour. All wells were washed 3 times with PBS for 5 minutes each.

Next, all wells were incubated for 1 hour in a secondary antibody mixture containing a 1:200 dilution of Alexa Fluor 594 conjugated goat-anti-mouse IgG and a 1:200 dilution of Alexa Fluor 488 conjugated goat-anti-rabbit IgG. These were diluted in the same

blocking buffer as used before. These wells were all washed 3 times with PBS for 5 minutes each wash, incubated in bisbenzimide for 5 minutes, then washed again with PBS before coverslips were mounted on glass slides and sealed. In some trials of this experiment, the active caspase-3 antibody detection was performed with triple-layer staining (primary antibody, biotinylated secondary antibody, and fluorescently-labeled streptavidin). In another variation, Bcl-2 was stained for in lieu of active caspase-3 using the goat polyclonal anti-Bcl-2 primary antibody. In the variation that looked at Bcl-2 and vimentin, some wells were stained for only Bcl-2 and others stained for only vimentin because double immunostaining was not possible when using a goat primary antibody when the only secondary antibodies against the mouse vimentin antibody available in the laboratory were made by goat. Cross-reactivity would occur because the secondary antibody against the goat primary antibody would also target the other secondary antibody because it would also have goat antigens. The scientific design employed is summarized in Figure 5.

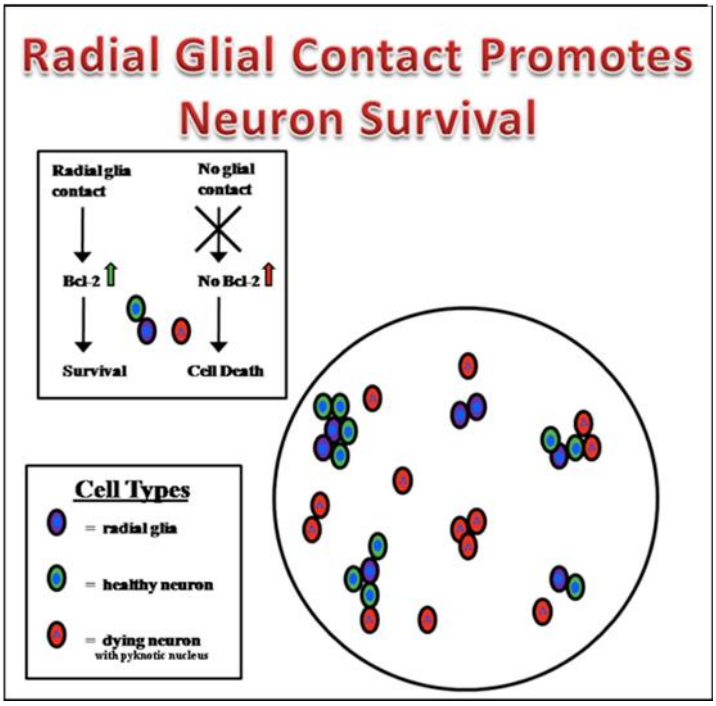

Figure 5. Radial Glia-Mediated Neuronal Survival Model
This diagram shows the anticipated interactions between E7+1 neurons and radial glia *in vitro*. Neurons will either be living (green) or dying (red). We believe the neurons in contact with radial glia will be more likely to survive than those without this contact due to survival signals produced from radial glia (purple).

2.5 α_5 Integrin Detection

The α_5 integrin subunit was also assayed for because it was previously not known whether the prototypical fibronectin receptor, $\alpha_5\beta_1$ integrin, is present in E8 chick OT cells.

2.5.1 Chicken Embryo Fibroblasts as a Positive Control

The positive control for this experiment was to use this primary antibody with chicken embryo fibroblasts (CEFs). These were obtained by collecting limbs from freshly

sacrificed E4 chicken embryos dissected in calcium and magnesium-free solution. This tissue was homogenized through trituration through a 16-gauge, then a 23-gauge hypodermic needle in a solution of soybean trypsin inhibitor/ DNAse I in Medium 199. After trituration, the cell solution was centrifuged at 143g for 5 minutes, the supernatant was aspirated, and the cells were resuspended in Dulbecco's Modified Eagle Media supplemented with 2mM L-glutamine, 3% FBS, and 1% of an Antibiotic-Antimycotic solution (Invitrogen) containing 10,000units/mL penicillin G sodium, 10,000µg/mL streptomycin sulfate, and 25µg/mL amphotericin B as Fungizone in 0.85% saline. The cells were plated on a 10cm culture dish and after 1 hour the old media was replaced with new media, with a PBS wash in between. The only cells that are able to stick to the culture plate that quickly are fibroblasts, all other cells are lost through media removal and the PBS wash.

After a day in culture, these CEFs had their media aspirated and were trypsinized with 1mL 0.05% trypsin for 5 minutes at 37°C. 1mL soybean trypsin inhibitor + DNAse I in Medium 199 was added to stop trypsin activity. These cells were then plated on uncoated sterile glass coverslips in a 24-well culture plate in the CEF Media described earlier. These cells were cultured until about 80% confluence, which took less than a week.

These cells were fixed and immunostained in the same manner as the E7+1 OT cells were immunostained in other experiments. Cells were fixed in 1% paraformaldehyde in PBS for 30 minutes at room temperature. The primary antibody used a 1:200 dilution of hybridoma ascites fluid containing a mouse monoclonal IgG1-κ subunit against the avian α_5 integrin subunit (DSHB; D71E2; Muschler and Horwitz, 1991). The secondary antibody used was a 1:200 dilution of Alexa Fluor 488 conjugated goat-anti-mouse IgG (Invitrogen; A11001). Both antibodies were diluted in a blocking buffer containing 5% NGS and 0.03% Triton-X 100 in PBS. No primary controls were incubated in blocking buffer instead of the

primary antibody mix for the 1 hour room temperature incubation. The secondary antibody incubation also lasted 1 hour at room temperature. Bisbenzimide incubation lasted for 5 minutes at room temperature. There were 2 PBS washes after each incubation step for 5 minutes each. Coverslips were mounted on glass slides using 6µL glycerol/PPD drops, one for each coverslip. Coverslips were sealed with nail polish and analyzed using a Nikon Microphot-FX microscope and a Nikon DXM1200 digital camera.

2.5.2 α_5 Integrins on E8 OT Cryosections

E8 OT cryosections were made as described earlier. The protocol for immunostaining these cryosections is identical to the immunostaining procedure detailed for other E8 OT cryosections, except the antibodies and blocking buffer may slightly differ. The blocking buffer used in this experiment was 5% NGS and 0.03% Triton-X 100 in PBS. The primary antibody used was the same 1:200 dilution of anti-avian α_5 integrin primary antibody used in the positive control. The secondary antibody was also the same antibody used in the positive control experiment using CEFs.

Chapter 3

RESULTS

3.1 Cell Death Detection

3.1.1 Anti-Apoptotic Protein Expression

E8 OT sagittal cryosections were immunostained for Bcl-2 and active caspase-3. Bcl-2 staining was positive in comparison to negative controls which were not incubated in primary antibody. Just about all cells expressed some level of Bcl-2, which was expected, since this protein is essential for cell survival. Even during programmed cell death some Bcl-2 would still be present, though it is expected that the ratio of Bcl-2 to pro-apoptotic factors would change. This may be the reason why we observed heterologous levels of Bcl-2 in E8 cryosections (Figure 6). Some cells were visibly brighter than others, though there was no obvious pattern to which cells were brighter or dimmer. Through using different immunostaining protocols, it was determined that the goat polyclonal IgG was the most effective primary antibody used against Bcl-2. The primary antibody concentrations of 3µg/mL or 10µg/mL did not make a difference, nor did triple-layer immunostaining with biotin and streptavidin.

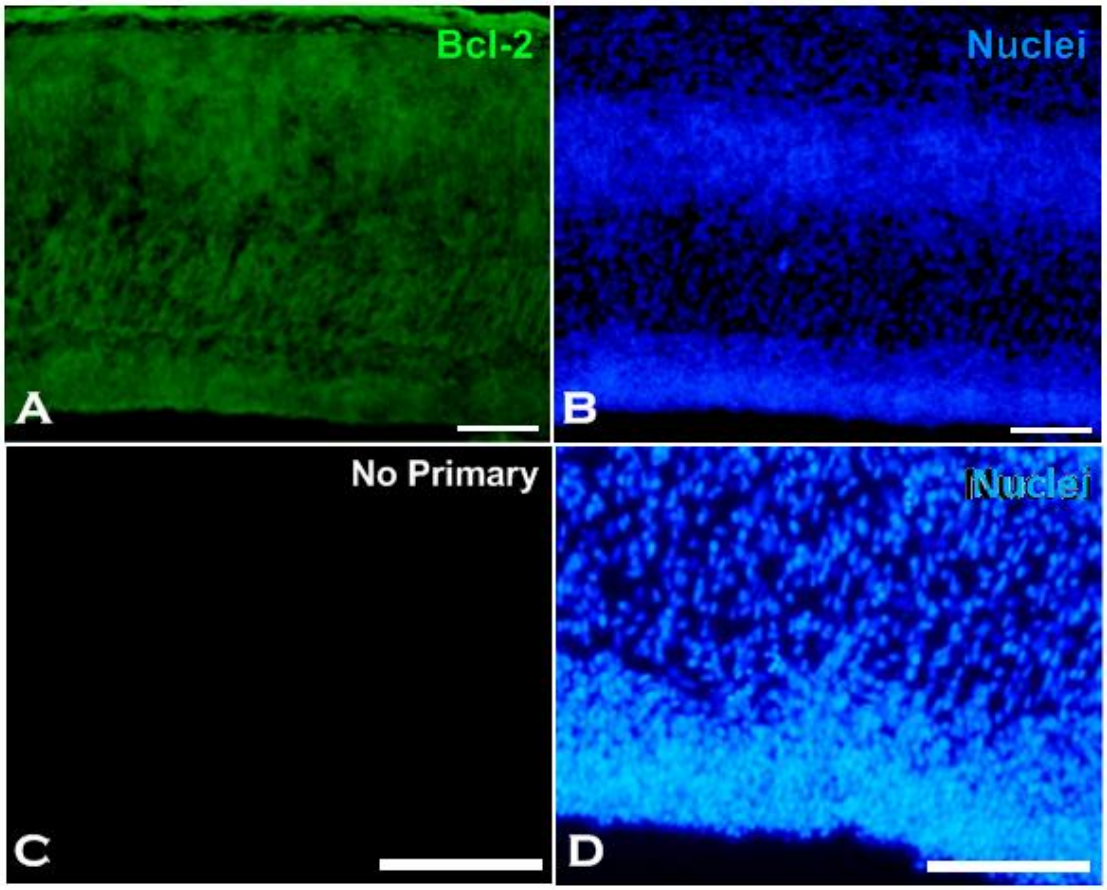

Figure 6. Bcl-2 Presence in the E8 Chicken Optic Tectum.
Bcl-2 was detected at heterologous levels in the E8 OT with no obvious pattern of which cells types have more or less Bcl-2 expression (A). This staining is prevalent compared to no primary control sections (C). Nuclei for both experimental (B) and no primary control (D) sections are visualized by bisbenzimide counterstaining to demonstrate the presence and distribution of OT cells in the fields of view. Scale bars= 100µm.

Bcl-2 levels also seemed to be decreased during E7 and E8 compared to E9 and E10 when cells were analyzed by flow cytometry (Figure 7). This decrease in relative Bcl-2 levels at this period of proposed cell death is important to show because at this time, the OT cells may be more susceptible to apoptotic signals. However, this is indirect evidence supporting the proposed model.

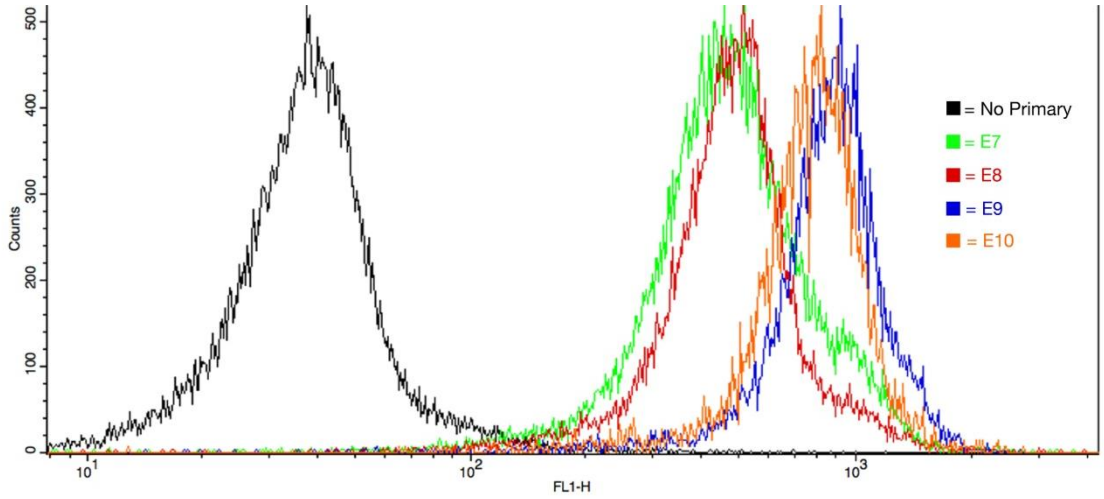

Figure 7. Changes in Bcl-2 Expression During OT Development.
Bcl-2 expression increases after the period of programmed cell death is over. This difference is seen in the peak difference between the lower E7 and E8 common fluorescence peak and the elevated E9 and E10 common fluorescence peak. There is a large difference between the staining compared to no primary control staining, indicating all cells have some Bcl-2 expression. No primary controls were taken from each embryonic age but here a representative no primary antibody control fluorescence distribution is shown for simplicity.

3.1.2 Pro-Apoptotic Protein Expression

Active caspase-3 immunostaining was performed in order to confirm previous reports of programmed cell death in the E8 chick optic tectum (Zhang and Galileo, 1998a). This is an executioner caspase that, when active, is involved in an irreversible apoptotic cascade that results in cell death (Porter and Jänicke, 1999). Active caspase-3 is a common marker used to look for apoptosis and would be a simple way to confirm the previous FISEL+ detection method. However, active caspase-3 was unable to be detected through

immunostaining methods in the optic tectum, even when a triple-layer staining method with biotin and streptavidin was employed (data not shown).

While cryosection immunostaining did not yield any qualitative data, flow cytometry analysis of OT cells from E7 through E10 did not show any caspase-3 activation in the OT as the chick embryo develops during this period (Figure 8). This result is in opposition to our model which predicted that caspase-3 should be active during E7 and E8 but not present thereafter.

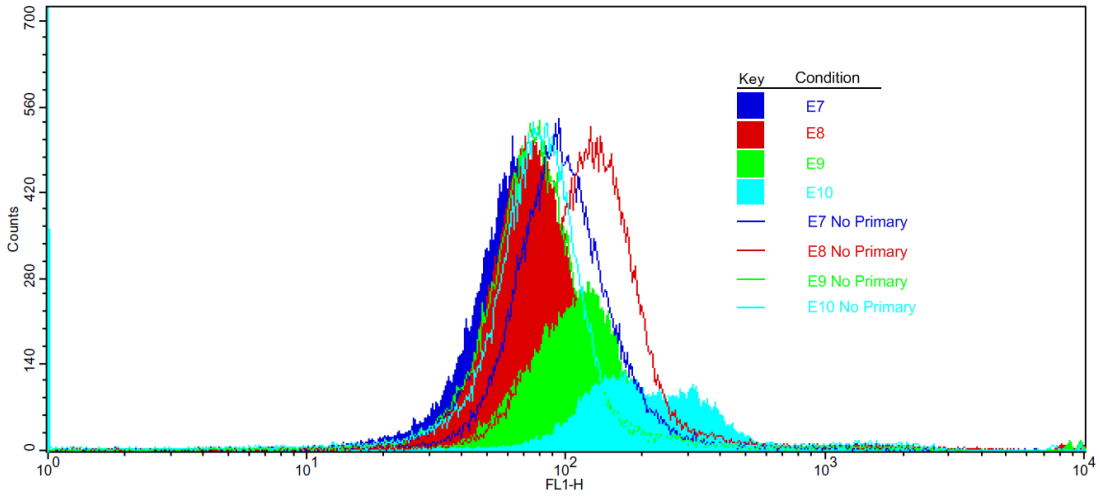

Figure 8. Active Caspase-3 Expression in OT Cells ages E7 through E10.
The active Caspase-3 expression seen through flow cytometry does not seem to differ significantly from no primary antibody controls. Thus, there is variability between samples but at no age from E7 through E10 is active Caspase-3 present at a detectable level in OT cells.

In addition, the pro-apoptotic protein, Bax, was also unable to be detected in the expected pattern in developing chicken OT cells by flow cytometry (Figure 9). It is expected that cells undergoing apoptosis have a higher Bax/Bcl-2 ratio with Bax being concentrated on the mitochondrial membrane (Middleton et al., 1996). Surprisingly, this effect was not seen in our experiments. Instead, like active caspase-3, we saw no Bax

expression from E7 through E10. It was expected that there would be some Bax present in all cells, though at low levels. These low levels may have been masked by the sample variability which overlapped extensively with no primary antibody negative controls.

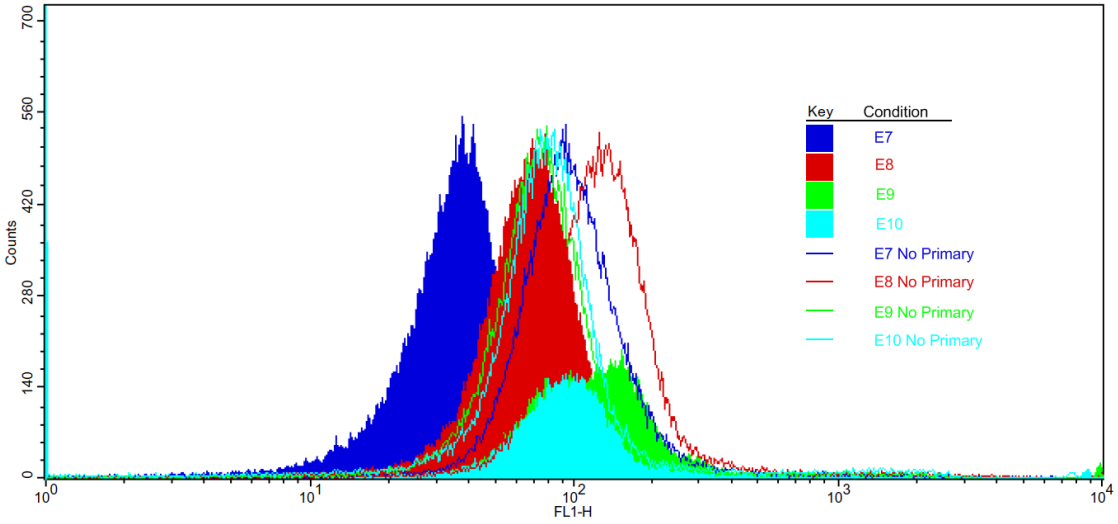

Figure 9. Bax Expression in OT Cells ages E7 through E10.
The Bax expression seen through flow cytometry does not seem to differ significantly from no primary controls. Thus, there is variability between samples, but at no age from E7 through E10 is Bax present at a detectable level in OT cells.

With this in mind, there are a few possibilities. The simplest explanation is that there is no apoptosis occurring in these cells. However, it is also possible that the primary antibodies were unreactive since they had not been tested in chicken before. Alternatively, there may be a Bax or caspase-3-independent pathway of cell death taking place in these cells. It also has been proposed that the developing brain has dying cells phagocytosed within 1 to 4 hours, which makes it difficult to document late-stage apoptosis (Roth and D'Sa, 2001). However, Bax expression and caspase-3 activation are some of the earliest apoptosis indicators and we would expect to detect these death markers.

The possibility that there is no cell death in these cells is a real threat to the proposed model and thus, providing evidence toward cell death was a major goal of this work. The active caspase-3 primary antibody was scrutinized in a positive control where different avian cell types were treated with staurosporine to induce apoptosis. The active caspase-3 primary antibody proved to detect staurosporine-induced apoptosis in QT6 cells and CEFs (Figure 10).

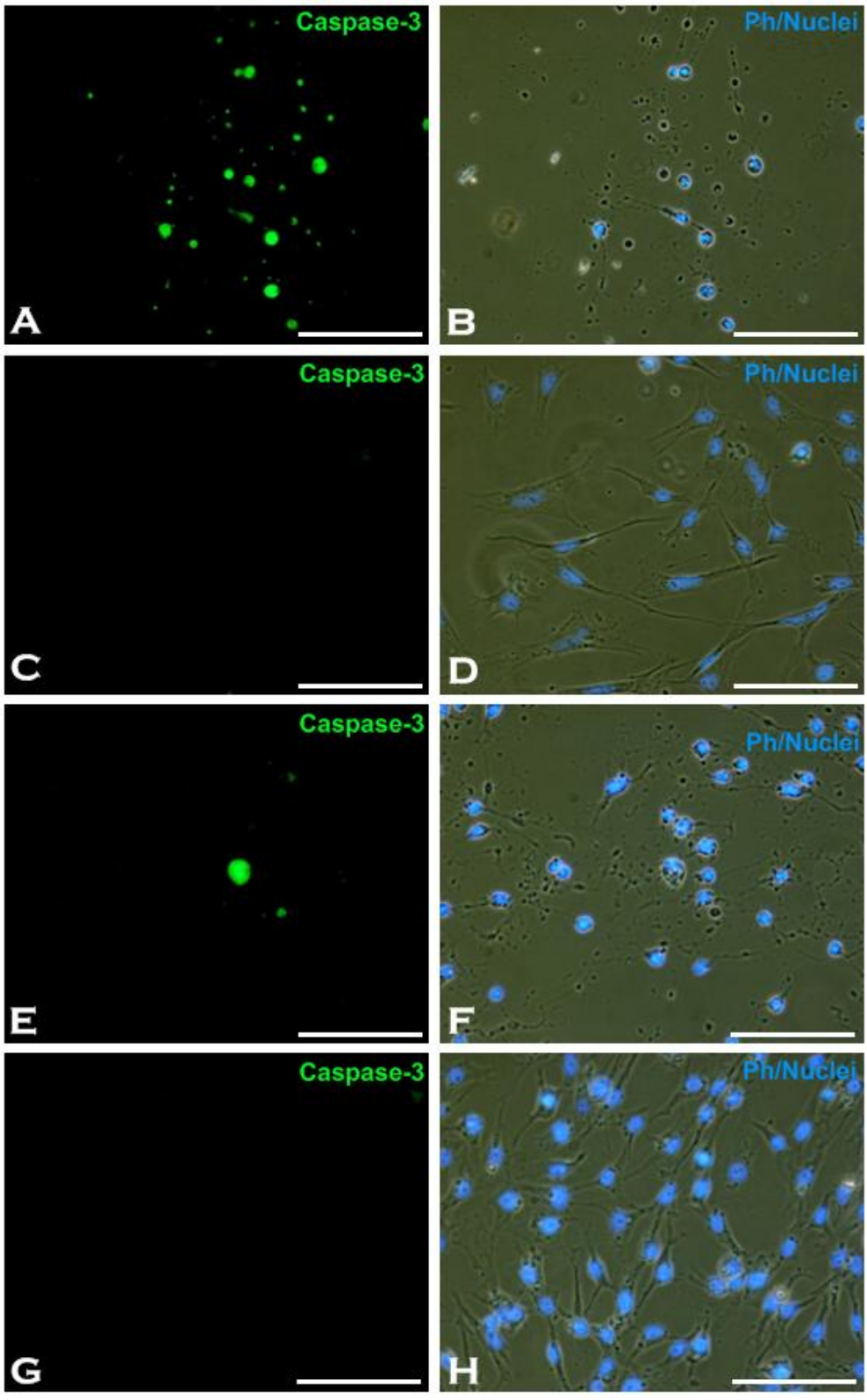
Caspase-3
Ph/Nuclei
A
B
Caspase-3
Ph/Nuclei
C
D
Caspase-3
Ph/Nuclei
E
F
Caspase-3
Ph/Nuclei
G
H

Figure 10. Positive Control for Active Caspase-3 Primary Antibody in CEF and QT6 Cells.
Chicken embryo fibroblasts (A-D) and QT6 cells (E-H) were cultured with 100nM staurosporine in their culture media (A,B,E,F) or in unadulterated media (C,D,G,H). Green fluorescence indicates active caspase-3 staining and blue fluorescence in phase-contrast images indicates nuclear counterstaining. Phase-contrast images were simultaneously illuminated by bright field light to see cell morphology and ultraviolet light to photoelectrically excite the bisbenzimide substrate. A greater proportion of CEFs were caspase positive (A,B) compared to QT6 cells (E,F) when incubated in staurosporine. Membrane blebbing, pyknotic nuclei, and active caspase-3 staining are present and colocalize in cells exposed to staurosporine. Typical fibroblast morphology, intact nuclei, and the absence of active caspase-3 staining are all typical of the cells incubated in unadulterated media. Scale bars= 100μm.

Thus, the antibody appears to detect caspase-3 as it was supposed to. One interesting feature to note is that the immortalized QT6 cell line is less susceptible to the effects of staurosporine compared to CEFs. This can be seen as the proportion of cells with pyknotic nuclei and active caspase-3 staining.

In another experiment, embryonic OT cells were incubated in staurosporine to determine whether active caspase-3 antibodies can detect apoptosis in these specific cells of interest. Two antibodies were used, one from BD and one from Cell Signaling. While the Cell Signaling primary antibody seemed to work better, there were apoptotic cells found with both antibodies (Figure 11). In addition, active caspase-3 positive cells were detected even in cells without staurosporine treatment. Since embryonic tectal cells are hypothesized to be dying around this time anyway, this finding supports our model. However, it seems staurosporine does not have a great effect on these E7+1 OT cells and may not be an ideal positive control method for caspase-3-mediated apoptosis.

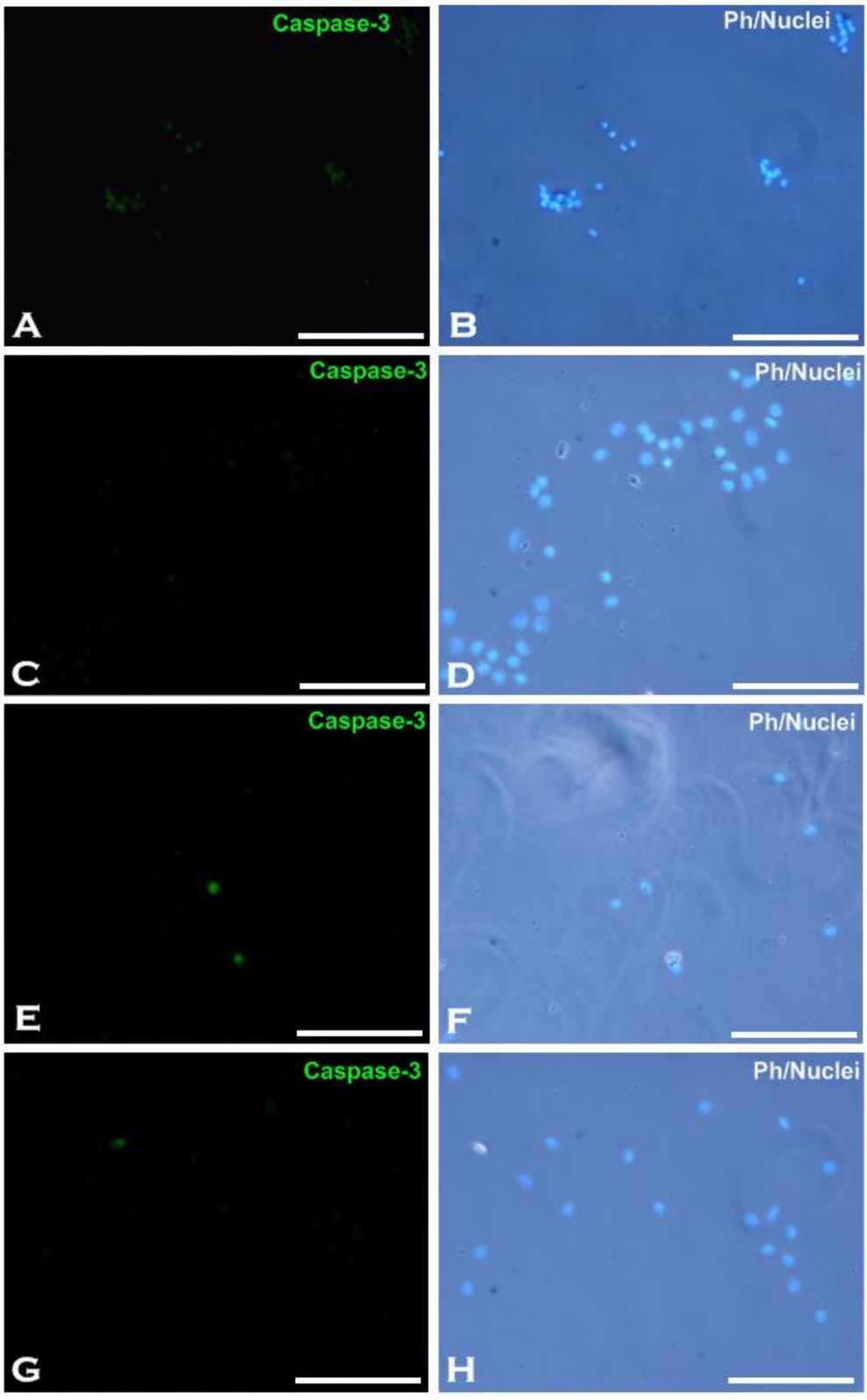
Caspase-3
Ph/Nuclei
A
B
Caspase-3
Ph/Nuclei
C
D
Caspase-3
Ph/Nuclei
E
F
Caspase-3
Ph/Nuclei
G
H

Figure 11. Positive Control for Active Caspase-3 in E7+1 OT Cells.
E7 embryos had OT cells dissociated and plated on poly-L-ornithine-coated glass coverslips for 1 day before being incubated for 6 hours with 100nM staurosporine in their culture media (A,B,E,F) or in unadulterated media (C,D,G,H). Green fluorescence indicates active caspase-3 staining and blue fluorescence in phase-contrast images indicates nuclear counterstaining. Phase-contrast images were simultaneously illuminated by bright field light to see cell morphology and ultraviolet light to excite the bisbenzimide substrate. This experiment also used two different primary antibodies against active caspase-3. The BD primary antibody (A-D) was seen to stain E7+1 OT cells treated with staurosporine (A) though considerable staining was seen even in cells without staurosporine treatment (C). The Cell Signaling primary antibody (E-H) brightly stained some E7+1 OT cells treated with staurosporine (E) but there seemed to be a small amount of fluorescence in cells without staurosporine treatment (G). Pyknotic nuclei and active caspase-3 staining are present and colocalize in cells exposed to staurosporine. However, cellular morphology does not seem to change between conditions. These are small, round cells that have most of their volume dedicated to the nucleus. Intact nuclei and the absence of active caspase-3 staining are typical of the cells incubated in unadulterated media. Scale bars= 100µm.

The Bax primary antibody was not subjected to a positive control test when it was found that there is an apoptosis pathway independent of Bax or active caspase-3 found in mouse brain development (D'Sa-Eipper et al., 2001). On the possibility that the chicken OT did not show the typical apoptosis mechanism, further studies were implemented to look at broader apoptosis detection systems. Other studies had pointed towards a neurodevelopmental apoptosis mechanism involving caspase-9 as opposed to caspase-3 (Kuida et al., 1998; Zaidi et al., 2001; D'Sa-Eipper et al., 2001).

3.1.3 DNA Fragmentation

Optic tectum cells were analyzed at different ages to determine the amount of DNA fragmentation present as the embryo progressed through different stages of development. It was expected that during the period of proposed programmed cell death on E7 and E8 there would be an increased proportion of cells with fragmented DNA, as this is

commonly recognized as a hallmark of apoptosis (Roth and D'Sa, 2001). This type of DNA degradation is visualized as a sub-G_1 peak on a flow cytometry analysis of cellular DNA content (Riccardi and Nicoletti, 2006).

In one experiment, there was a sub-G_1 peak present on E8 and E9 but not E7 (Figure 12). However, this result could not be replicated. On the second iteration of this experiment, there were no sub-G_1 peaks present at any age but the G_1 and G_2 peaks appeared as expected at all ages (Figure 13).

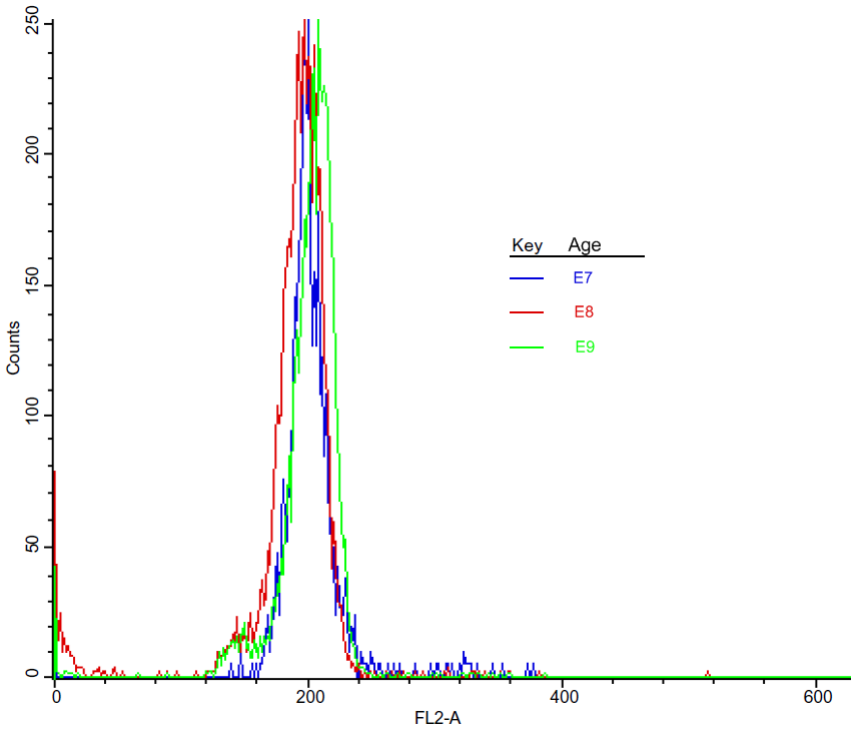

Figure 12. DNA Content of Chick OT Cells Ages E7 through E9: Experiment 1. OT cells were dissociated from embryos, fixed, and stained for DNA with propidium iodide. The G1 peak is the prominent peak seen with few cells in S-phase or G2, indicating that most cells are post-mitotic. E8 and E9 samples had slight shoulders just below the G1 peak. This sub-G1 peak may show apoptosis because of the DNA degradation that occurs during this process. The data points with fluorescence levels close to zero are considered debris. The E7 data is shown in blue, E8 in red, and E9 in green.

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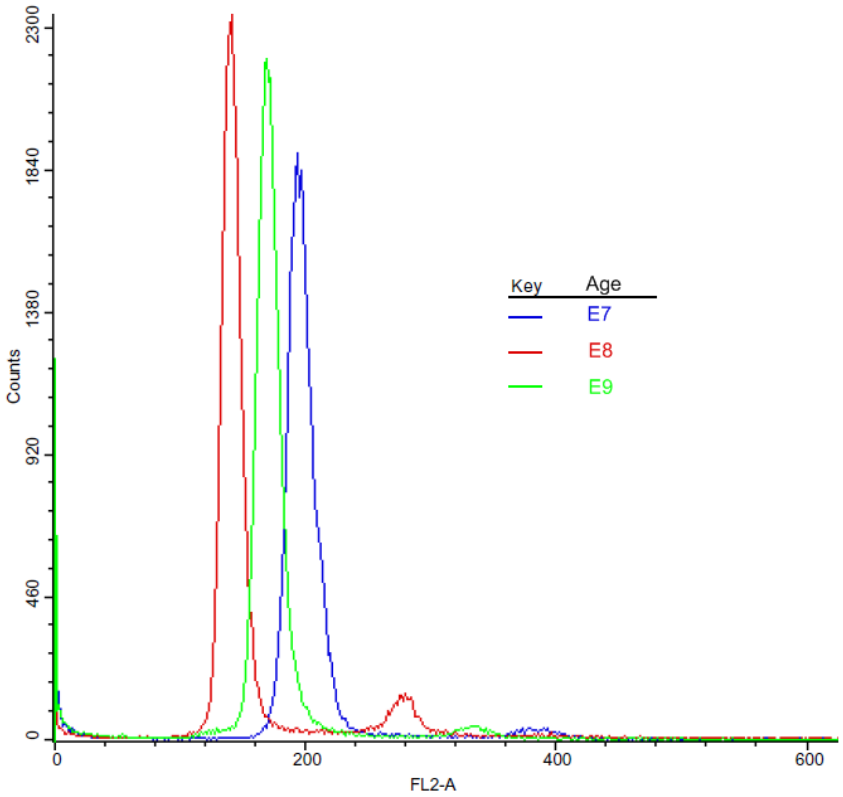

Figure 13. DNA Content of Chick OT Cells Ages E7 through E9: Experiment 2. This figure is a replication of the previous DNA analysis experiment. However, results differed significantly. The different ages have their G1 peaks at slightly different fluorescent levels for unknown reasons. Additionally, E8 OT cells showed a distinct G2 peak in this trial and no ages showed a sub-G1 apoptosis peak. The E7 data is shown in blue, E8 in red, and E9 in green.

These inconsistencies are curious and evade explanation as both experiments were performed in the same manner. There may simply be variation between embryos which may result in inconsistent results when few embryos are used in the experimental design. Although hundreds of thousands of cells were analyzed, there was only one embryo used per age per experiment. One possible difference lies in the fact that Experiment 1 had a fixation time of 3 weeks and Experiment 2 had a fixation time of 1 week.

3.1.4 Comprehensive *in vivo* Caspase Detection

Another way of broadly detecting apoptosis involved the use of a pan-caspase detection system called FAM-FLIVO™. Fluorescence due to this substrate is indicative of caspase activation. In live, freshly dissociated E8 OT cells injected with this substrate, there was a definite increase in fluorescence compared to noninjected controls (Figure 14). This finding is supportive of the model in showing there may be caspase-3 independent cell death in the chicken OT on E8.

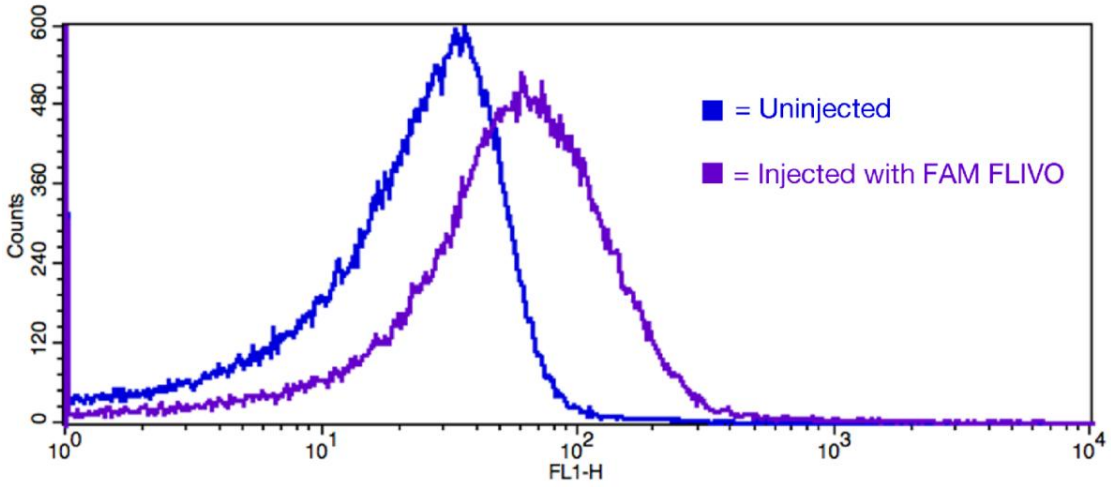

Figure 14. E8 Optic Tectum Caspase Activation.
E8 embryos were injected with a fluorescent caspase marker, FAM-FLIVO™. The OT was dissociated into single cells and these cells' fluorescence levels were analyzed via flow cytometry. The injected embryo had fluorescence levels higher than that of the uninjected embryo, indicating caspase activation in the E8 optic tectum.

However, the control using a different age embryo was necessary to determine that fluorescence is specific for this age. To ensure embryo variability would not pose a major problem, 7 embryos of age E6 and 7 of age E8 were injected with the caspase-detecting substrate. On average, the E8 embryos had greater fluorescence levels

than E6 embryos or their corresponding uninjected control counterpart (Figure 15). While these results support our model that there should be activated caspases at E8 but not E6, there was a large amount of variability seen between samples. Even though many samples were used (n=14), this study is not conclusive due to the high degree of variability witnessed.

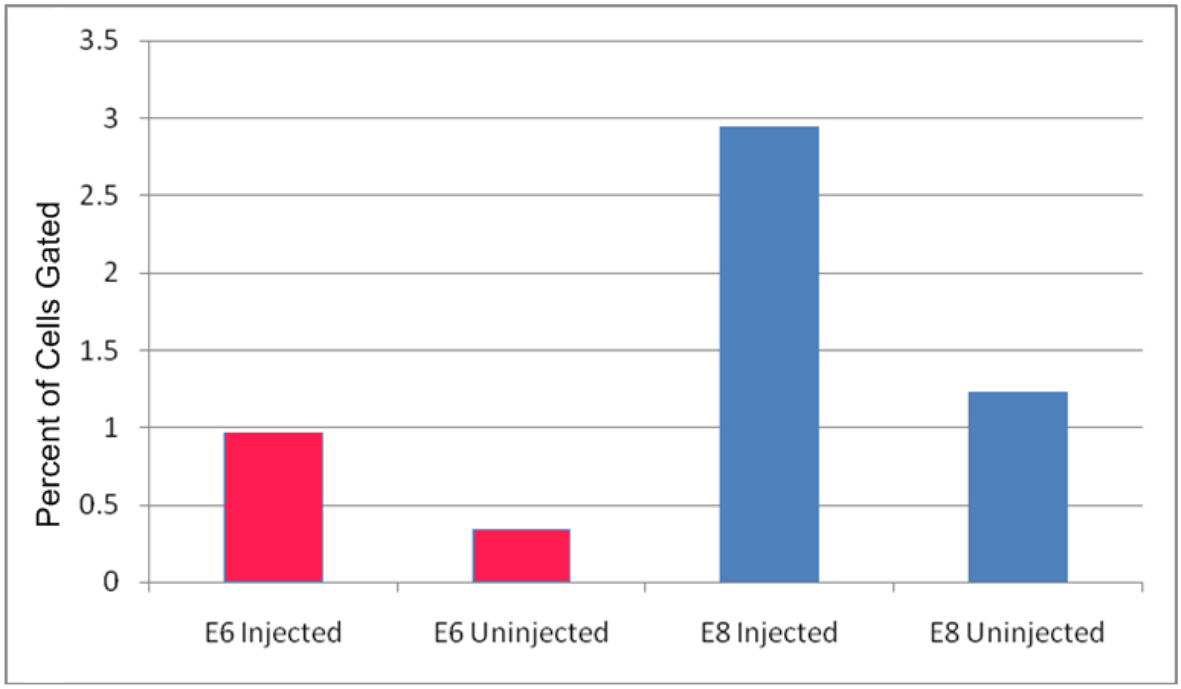

Figure 15. E6 and E8 Optic Tectum Caspase Activation
14 embryos were used, 7 at E6 and 7 at E8. Of these 7, 2 were used as uninjected negative controls per age. OT were injected with a FAM-FLIVO™ -containing solution and later dissociated into single cells for flow cytometry analysis. During data collection, a "gate" was set as a way to measure slight differences in fluorescence levels between samples. This gate only counts cells meeting a minimum fluorescence level. Thus, the more cells gated, the higher the average fluorescent level and the more caspase activation. Displayed are the average percent of cells gated for each condition.

3.2 Artificially Increased Neuronal Survival

Embryos were injected on E3 with either a control vector, 1481Δ, or a Bcl-2 cDNA-containing vector, 1481Δ-Bcl-2. These embryos were allowed to develop until E9, after the proposed period of programmed cell death. It was expected that compared to the control vector, clones of infected cells from the 1481Δ-Bcl-2 vector would have more cells since they would not undergo cell death with ectopic Bcl-2 expression.

However, the 1481Δ-Bcl-2 retroviral vector seemed to be of a low titer, resulting in fewer clones. The clones that were able to be isolated and analyzed seemed to be smaller than those obtained from embryos injected with the 1481Δ vector. This was a surprising result which does not confer with the proposed model. Even with a low titer, the few clones seen should look robust compared to control clones. In addition, all clones analyzed had vague cell boundaries so it was not possible to quantify the number of cells per clone or the proportion of each clone that resides in each lamina. Fixation protocols were altered to include 0.4% glutaraldehyde in addition to using all freshly made solutions-but this was to no avail.

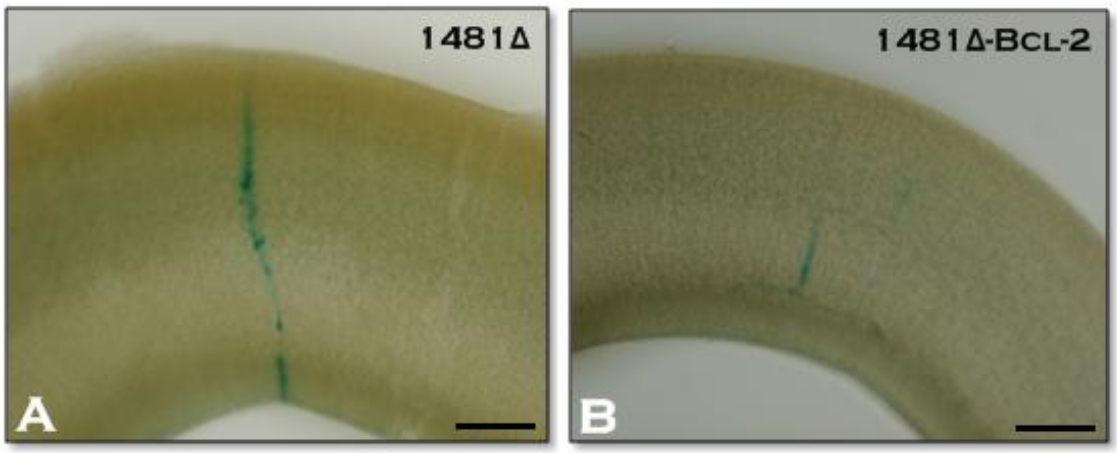

Figure 16. Clonal Cohorts from Retroviral Vector Infections.
The control 1481Δ vector resulted in the formation of many clones with the expected linear

morphology throughout all tectal laminae (A). The 1481Δ-Bcl-2 vector was supposed to ectopically increase Bcl-2 expression in infected cells and result in large clones. However, few 1481Δ-Bcl-2 clones were found and those that were exhibited a poor morphology (B). Scale bars= 100μm.

3.3 Elucidating Survival Factors for Tectal Cells

3.3.1 General Contact-Mediated Survival

Cells dissociated from E7 OT were plated for a day *in vitro* at differing concentrations. The cells that were plated at a low concentration were found to have a greater proportion of cells undergoing programmed cell death, measured by degraded DNA levels by flow cytometry (Figure 17). This effect is likely due to the survival factors presented by neighboring cells. This result aides our proposed model by indirectly showing that embryonic chick OT cells can undergo anoikis.

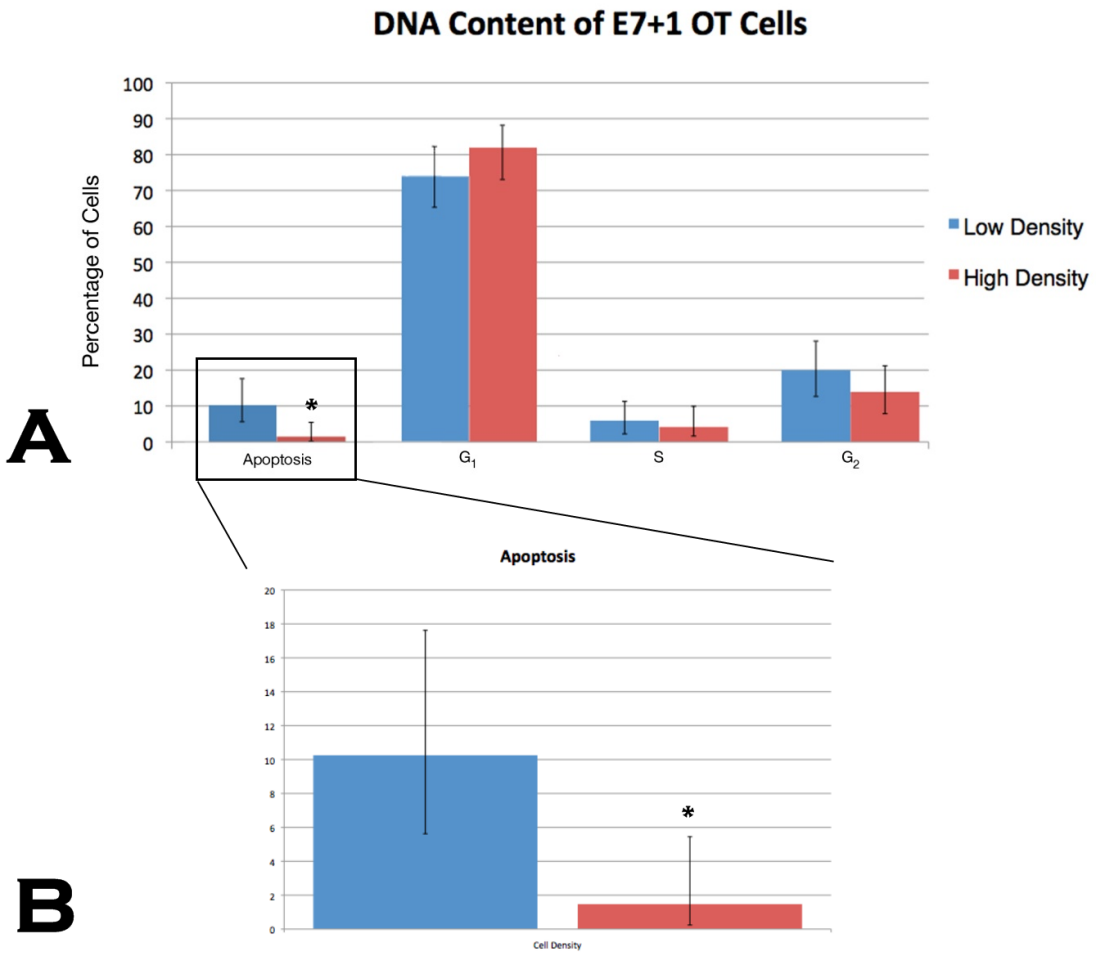

Figure 17. DNA Content of E7+1 OT Cells Plated at Different Densities.
E7 OT cells were plated in a high-density or low-density condition for 1 day before being fixed, treated with propidium iodide, and their DNA content was analyzed via flow cytometry. Flow cytometry data was analyzed using ModFit© and simplified into a bar graph (A). The population of cells plated at a high-density only differed significantly from the low-density by having less apoptosis ($p<0.05$, student's t-test). The apoptosis data is enlarged to show detail (B). 1.47% of the cells plated at the high density were seen to have degraded DNA, as opposed to 10.25% of the cells plated at a low density (B).

3.3.1.1 Pyknotic Nuclei

E7 OT cells were plated at either a high or low density for 1 day *in vitro* and analyzed for pyknotic nuclei as a morphological indicator of apoptosis and the results are shown in Table 2 below.

Density	Total Cells	Apoptotic Cells	Percent of Apoptotic Cells
High	1565	132	8.43%
Low	399	43	10.77%

Table 2. Pyknotic Nuclei Prevalence in E7+1 OT Cells
8.4% of the E7+1 OT cells plated at a high density had pyknotic nuclei as opposed to 10.8% of the cells plated at a low density. This difference is in the direction expected but only yields a p-value of 0.13 (student's t-test).

These results indicate it is likely that increased cell contact keeps OT cells from undergoing apoptosis. Since the results are not considered statistically significant ($p>0.05$), it is worth noting that these cells are taken out of their normal environment in the brain and introduced to a completely different *in vitro* culture where the normal signals are no longer present and 3D structure is lost. This assay was used to see if cell contact can influence the survival of these cells but even in the high-density condition there is only cell contact in two dimensions and there is still a significant percentage of these cells undergoing apoptosis. It is likely that while contact increases survival rates, there is not sufficient contact *in vitro* no matter the density the cells are plated and this phenomenon can mask the effect of cell-contact on apoptosis. Basically, higher plating densities may be able to alleviate *apoptosis*, but will not save all cells from *programmed cell death*.

From this point, it became important to determine what survival factors are present in the developing chick midbrain that protects some cells from apoptosis while a subset of neurons supposedly undergoes programmed cell death.

3.3.2 Fibronectin-Mediated Survival

Fibronectin has been found to be produced and secreted by chicken OT radial glia (Stettler and Galileo, 2004). Also, when cells are plated on a fibronectin substrate, it has been found that this can cause Bcl-2 expression to increase (Matter and Ruoslahti, 2001). Thus, analyzing the effect of fibronectin on embryonic OT cell survival is a worthwhile endeavor. In this case, we focused on whether fibronectin could increase Bcl-2 levels in embryonic OT cells since this is the proposed mechanism of how radial glial contact keeps neurons alive *in vivo*.

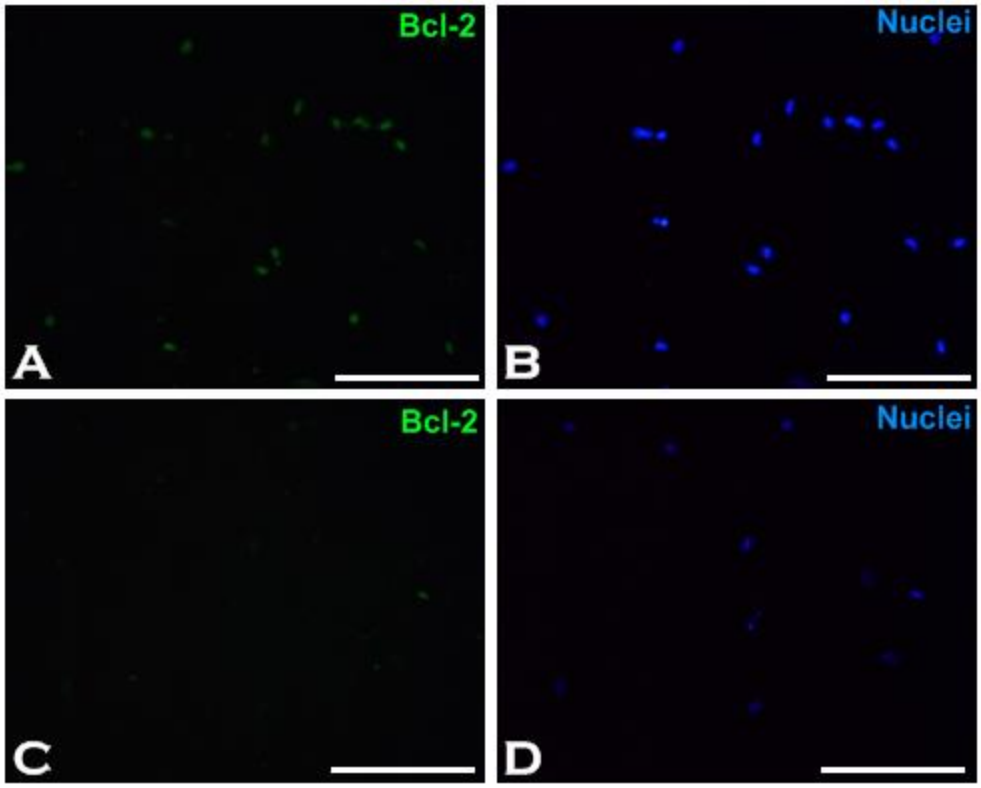

Figure 18. The Effect of Fibronectin on Bcl-2 Expression in E7+1 OT Cells.
OT cells were dissociated from E7 chicken embryos and plated on either a combination of fibronectin and poly-L-ornithine (A, B) or just poly-L-ornithine (C,D). The cells plated on fibronectin had qualitatively higher Bcl-2 expression as visualized through immunostaining techniques. Scale bars= 100µm.

Figure 18 shows one experiment indicating that plating OT cells on fibronectin increases Bcl-2 levels in comparison to poly-L-ornithine. This Bcl-2 staining was above background levels seen in no primary control coverslips. Preliminary quantitation data support this finding (data not shown). However, the poly-L-ornithine coverslips in other experiments were not consistent in low Bcl-2 expression. In fact, some poly-L-ornithine coated coverslips without fibronectin had Bcl-2 staining just as bright as cells on coverslips coated with both ECM substrates (data not shown).

3.3.3 Radial Glia-Mediated Neuronal Survival

To more directly examine the proposed model, the effect of radial glial contact with neurons was analyzed. If radial glial contact normally keeps migrating neurons alive *in vivo*, this contact may have a similar effect on dissociated OT cells *in vitro*. This may explain how general contact decreases apoptosis and how fibronectin may increase Bcl-2 levels in E7+1 OT cells.

Curiously, when OT cells were kept in culture for one day, most of the cells stained positively for vimentin, a marker for radial glia. This may be due to extensive neuronal death during this *in vitro* condition or non-specificity of the primary antibody used. It was expected that neurons in contact with radial glia would remain alive and ones without this contact would express active caspase-3 and have low Bcl-2 levels. However, active caspase-3 staining was not found in any of these cells and Bcl-2 levels appeared relatively uniform among the cell population.

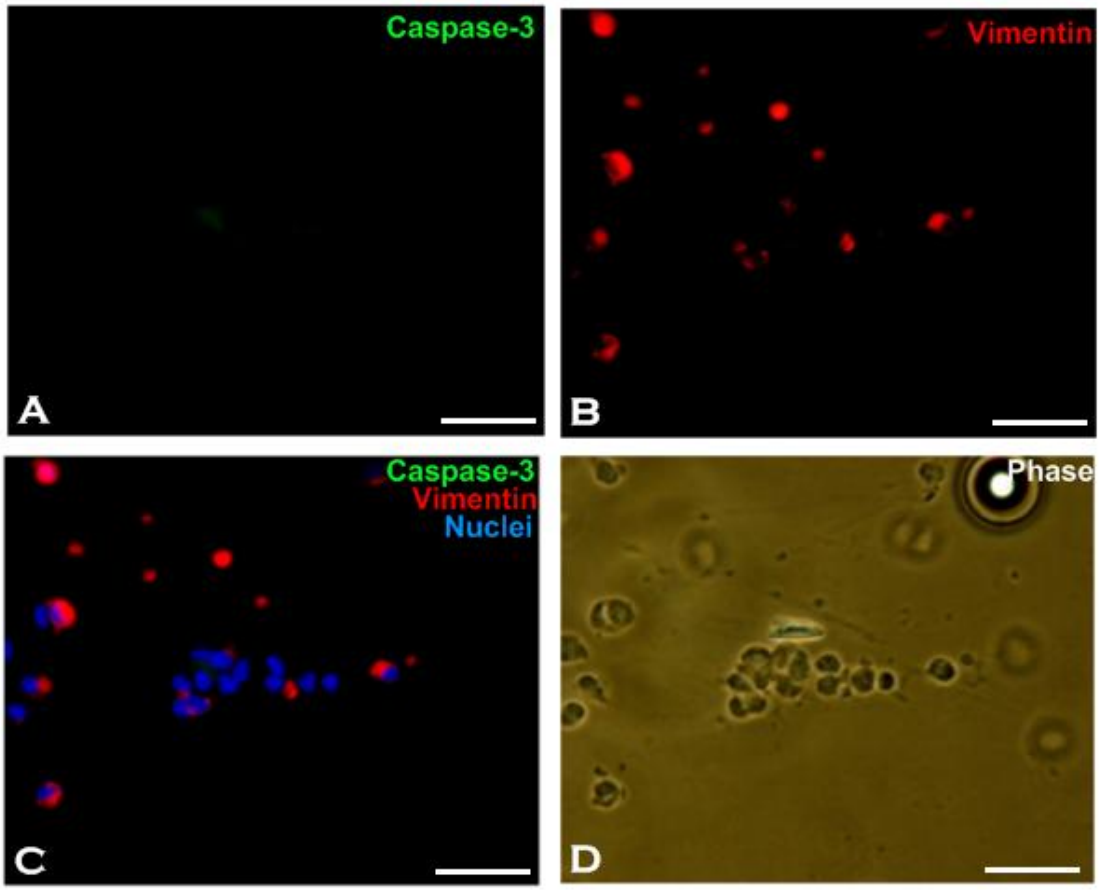

Figure 19. The Effect of Radial Glia Contact on Neuronal Survival
E7 OT cells were dissociated and plated on poly-L-ornithine-coated coverslips for 1 day *in vitro* before being fixed and immunostained for active caspase-3 (green), vimentin (red) and counterstained for DNA with bisbenzimide (blue). There is no active caspase-3 staining seen (A,C) and the majority of cells seen are positive for vimentin (B,C). A phase-contrast image is shown to visualize the cellular morphology (D). Panels A and B are merged with nuclear staining in panel C. Scale bars= 30μm.

3.4 α_5 Integrin Detection

The presence of the prototypical fibronectin receptor, $\alpha_5\beta_1$, is important to determine in order to decipher a possible mechanism for how fibronectin from radial glia may initiate a survival signal in neurons. The avian α5 integrin subunit was detected in E8 OT

cryosections as shown in Figure 21. The positive control for this primary antibody was performed with CEFs which were positively stained (Figure 20).

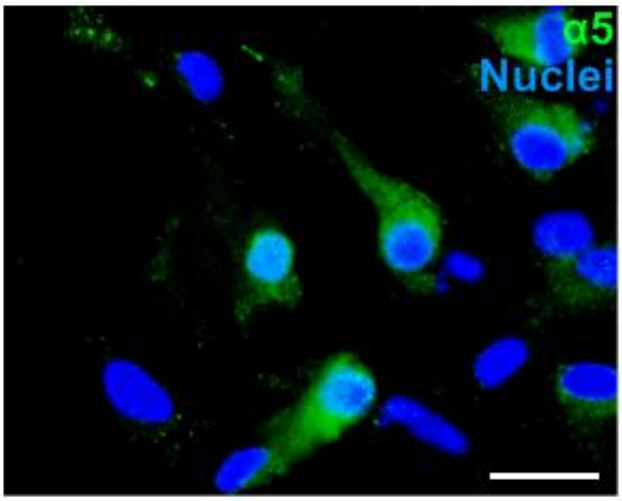

Figure 20. Positive Control for the Avian α5 Integrin Subunit.
Chicken embryo fibroblast cells plated on glass coverslips were also immunostained for the avian α5 integrin subunit as a positive control. These cells were found to express this antigen. α5 is shown in green, DNA is shown in blue. Scale bar= 30μm.

Although both positive and negative controls were used in this experiment, the negative control was just a section not incubated in the primary antibody (Figure 21, panel B). This type of negative control is used to make sure there is no non-specific binding by the secondary antibody used. However, it is still possible that the primary antibody is not specific for the α5 integrin subunit. Since the α5 subunit is found in the majority of cell types, a negative control was not available. Thus, it is not certain that these cells express the $\alpha_5\beta_1$ integrin receptor.

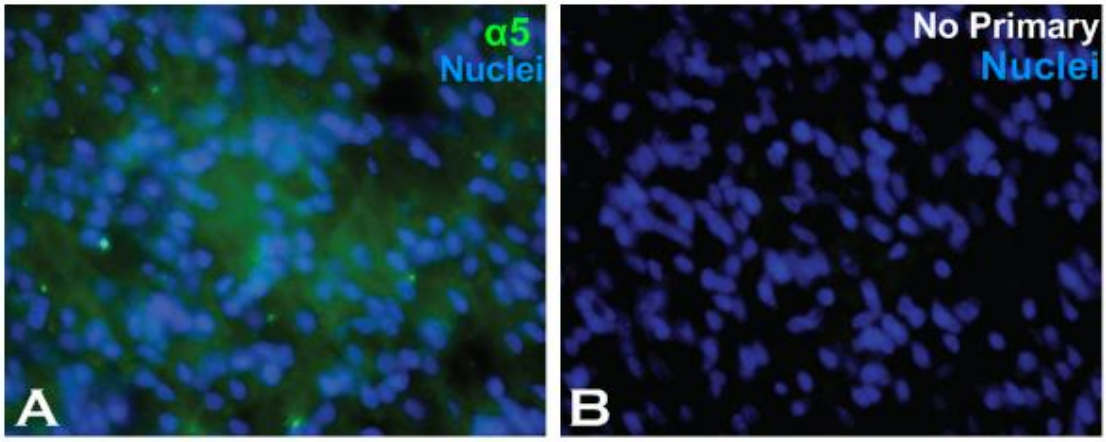

Figure 21. α5 Integrin Presence in E8 OT.
E8 optic tecta were dissected, fixed, and cryosectioned in 10µm slices before being immunostained for the avian α5 integrin subunit. Positive staining was seen (A) in comparison to no primary antibody control sections (B). Scale bars= 30µm.

Chapter 4

DISCUSSION

4.1 Cell Death Detection

Bcl-2 was detected in a heterogeneous pattern in the E8 chicken optic tectum and Bcl-2 levels were increased on E7 and E8 in comparison to E9 and E10. These findings are in support of the proposed model by showing that Bcl-2 is present throughout the OT but some cells have more or less. I believe this heterogeneity is due to the survival signals mediated by radial glia. With higher Bcl-2 levels, neurons would be expected to have meaningful contact with radial glia and be more likely to survive the period of proposed programmed cell death. Also, there is decreased Bcl-2 during E7 and E8 which may be due to a large proportion of dying neurons with lower Bcl-2 levels. When these cells are cleared out by E9, Bcl-2 levels increase.

Active caspase-3 and Bax were not detected in the embryonic OT and thus, if there is a period of early programmed cell death in this tissue, it occurs in a pathway independent of these factors. Since a comprehensive caspase detection system indicated there may be caspase activation on E8, there is some additional evidence that there may be an apoptotic wave present at this age that acts through at least one caspase-activation pathway. One member of interest is caspase-9 due to its role in caspase-3 independent neurodevelopmental apoptosis; however, this has yet to be assessed in the developing chick

optic tectum (Kuida et al., 1998; Zaidi et al., 2001; D'Sa-Eipper et al., 2001). The next logical step to study this model seems to be immunostaining for active caspase-9 to determine its role in this process.

It is also possible that there is a caspase-independent method of cell death occurring in these cells that was seen in nicked DNA labeling studies but not in any methods used here (Zhang and Galileo, 1998a). There is such a potential pathway involving Bit1, a mitochondrial protein, which may be regulated by integrin receptors on the neuronal membrane (Jan et al., 2004). A caspase-independent pathway has been noticed before in developing mammalian neurons with caspase genes deleted (Oppenheim et al., 2001).

Even if there was a caspase-independent mechanism of cell death, DNA must still be degraded during apoptosis. Unfortunately, flow cytometry data of DNA content in embryonic OT cells was inconclusive due to the lack of reproducible results. Since this protocol was recently implemented in our laboratory, it may just take trial and error to optimize the protocol in a way to get valid and reproducible results.

In this study, the presence of widespread programmed cell death in the developing chick optic tectum could not be confirmed nor refuted. However, it has been found that these cells do not undergo a Bax or caspase-3-mediated cell death pathway at this developmental stage. There is preliminary evidence that other caspases may be activated during E8 and this may be combined with other caspase-independent pathways not directly studied in this project.

4.2 Artificially Increasing Neuronal Survival Rates

While the control 1481Δ vector was able to produce ample clones in the expected morphology, the 1481Δ-Bcl-2 vector was less successful. The virus titer may not

have been high enough to produce many clones. However, the ones found were small and did not fit our expectations. It is unclear of whether this vector was able to increase Bcl-2 expression in these cells. However, previous work by Lindsay Higdon had shown increased Bcl-2 expression in QT6 cells with this vector compared to uninfected cells. Overall, the results of this experiment did not agree with our model. However, the aberrant results are most likely due to technical difficulties encountered with the effectiveness of the vector used since Bcl-2 upregulation should not cause a decrease in cell survival.

4.3 Survival Factors for Tectal Cells

General contact was found to increase survival rates of embryonic tectal cells. This finding was found through flow cytometry analysis of DNA and through morphological nuclear analysis. These findings indicate that this cell type is susceptible to anoikis and this supports the model that OT neurons die in this manner. However, this result would be expected for many cell types and the actual mechanism of what extrinsic signals increase survival rates is a more pertinent question.

Assays involving plating OT cells on fibronectin were inconclusive. While cells on fibronectin had high Bcl-2 levels, cells not on fibronectin were found with similarly high levels of Bcl-2. It is possible that growth factors or fibronectin present in serum-containing media could keep cells alive regardless of fibronectin interactions. Also, purified fibronectin was used in this assay and in this purification process, solutions are subjected to non-physiological conditions, such as high levels of urea, which can alter the conformation of fibronectin. These types of changes to fibronectin's structure have been shown to have detrimental effects on the functions of this molecule (Johansson et al., 1997). In this manner, this fibronectin assay is limited. While the purification process of fibronectin may be difficult

to alter, the complications with growth factors may be avoided by serum-depriving the cells and determining whether fibronectin can save these cells from such stress.

Radial glia contact is hypothesized to provide survival signals *in vivo* but this proved to be difficult to study *in vitro*. Vimentin staining was much more widespread than originally thought and contact with vimentin-positive cells did not seem to correlate with cell survival. This assay does not replicate the complex three-dimensional structure in the developing optic tectum and cannot be expected to be representative of the complex interactions that occur in brain development.

4.4 α_5 Integrin Detection

The detection of the α_5 integrin subunit is an important finding. Because this only dimerizes with the β_1 subunit, this finding indicates that the $\alpha_5\beta_1$ integrin receptor is present in these neurons (Johansson et al., 1997). This is the prototypical fibronectin receptor and can possibly interact in a synergistic fashion with the previously studied $\alpha_8\beta_1$ fibronectin receptor to mediate an intracellular signaling cascade to increase Bcl-2 levels.

4.5 Future Directions

During the course of this project, future experiments have been proposed. For detecting cell death, literature points towards the possibility of active caspase-9 being involved in caspase-3-independent cell death so immunostaining for this marker during E7 and E8 would be the next step. Additionally, ligation-mediated polymerase chain reaction experiments (LM-PCR) can be done to detect if small amounts of DNA are being degraded and detected as an apoptotic DNA ladder (Blaschke et al., 1996).

To ectopically increase Bcl-2 levels in embryonic OT neurons, a new virus may be of use. Specifically, a lentiviral vector may prove to be more suitable than the retroviral vector used in this study. If this vector also encoded green fluorescent protein (GFP), more *in vivo* studies would become possible.

The specific mechanism of how radial glial contact may keep neurons alive is an extremely important part of the model that deserves to be studied more. It should be assessed whether fibronectin can save OT cells from undergoing apoptosis due to serum-deprivation by increasing Bcl-2 levels since this was seen in other cell types (Zhang et al., 1995). Specific intracellular factors involved in this process remain elusive. Nevertheless, connecting integrin signaling to altered Bcl-2 expression would be a critical mechanism if there is indeed this early period of widespread programmed cell death.

4.6 Implications

These findings continue an endeavor to search for the mechanisms involved in neuronal migration during early brain development. While results could not prove widespread programmed cell death in the chicken optic tectum, this study provides evidence that a caspase-3 independent mechanism may be involved. This population of cells also has a transient decrease in Bcl-2 expression and survival is dependent on cell-cell contacts.

I am not able to predict all the implications of my basic scientific research but as the field progresses, others will continue to discover the mechanisms of neuronal migration and survival. In turn, this may aide our understanding of neuronal migration disorders to allow for the potential for future treatments.

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