# REGULATION OF NEURONAL CELL NUMBER IN THE DEVELOPING CILIARY GANGLION

by

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CERTIFICATE OF APPROVAL

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## Dedication

I would like to dedicate this thesis to my family.

My parents: Thomas and Christina Hung Sau Lai

My siblings: Alberta, Dawn, Benjamin, and Nicholas

Even though it has been a number of years since I left home, we are closer than before.

I thank my parents for the opportunities they have provided for all their children.

My family have never questioned why I want to work the long hours and get the small pay. They have always given me all their blessings, love, and relentless support.

I also want to express my gratitude to the Margolis family: Ellie, Sue, and Bill.

You have been my family here in Portland,

I have missed you so much during the last couple of years.

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Last but not the least, I want to give the biggest "Thank you" to Niki Larson. She has helped me in every way imaginable. I could not have finished my thesis without her.

# I. Abstract

The overall objective of this thesis is to explore mechanisms that can regulate cell number during development. During the formation of the nervous system, neural precursor cells undergo successive division, differentiation, and eventually cell death to achieve their final number. Throughout this thesis, I have mainly focused on questions related to neuronal differentiation and cell death.

Cell death is one of the major mechanisms that can control cell number and plays an important role during neuronal development. To date, the best characterization has come from studies examining neuronal cell death that occurs during synaptogenesis. Most of the cell death during this time has been attributed to the availability of target-derived trophic factors. When developing neurons innervate their targets, those that obtain sufficient amount of trophic support will survive. On the other hand, those that do not will die. Not only can extracellular signals keep cells alive, they can also cause them to die. Increasing evidence has pointed to the control of neuronal apoptosis by death factors. During the first part of my thesis project, I addressed the possibility that a death activity in the developing eye can induce cell death in ciliary ganglion neurons. Experiments in Chapter 1 described the purification and characterization of this activity. The purified molecule was identified as chick diferric transferrin and I demonstrated that

cell death mediated by diferric transferrin involved iron and binding to the chick transferrin receptor. More importantly, I found that the circulating levels of diferric transferrin in the embryos were higher than concentrations required to kill neurons. These results suggest that developmental cell death can be regulated by death factors and that the source of these factors may be target-derived, locally produced, or systemic.

Not only have previous studies that examine neuronal cell death focused largely on the period of synaptogenesis, they have also mainly relied on counting total number of neurons. In order to explore mechanisms that regulate cell death, I sought to determine when neurons are committed to a death program. Using the TUNEL assay, I discovered that the period of cell death in the developing ciliary ganglion did not strictly coincide with target innervation. Instead, cell death occurred as soon as the ciliary ganglion was formed and a large peak of cell death was observed two days prior to synapse formation at St. 29. These observations suggest that multiple mechanisms can be operating to control cell death in developing neurons and the possibility of target tissue regulating an early phase of cell death was addressed in Chapter 2.

Finally, I also found that dying neurons seemed to be replenished after the early peak of cell death at St. 29. Because ciliary ganglion neurons are post-mitotic by St. 29, I

hypothesize that non-neuronal cells in the ganglion may have the capability to differentiate into neurons. Part of Chapter 2 and most of Chapter 3 presented experiments that addressed this question and results obtained from those studies support the notion that non-neuronal cells in the ciliary ganglion may indeed have neurogenic potential.

Overall, results from my thesis have demonstrated that the establishment of neuronal number is a complex process that can involve multiple rounds of neurogenesis, cell differentiation, and cell death. Some of the mechanisms that govern these steps are addressed in this thesis.

# II. Introduction

One of the most intriguing problems in developing biology is how the size of an organism is controlled. The overall size of an animal or an organ depends on the number of cells and the size of the cells. The total cell number is in turn a function of cell proliferation, differentiation, and cell death. In the developing nervous system, for example, neural precursors divide to form neuroblasts, differentiate into neurons, and then their final number is stabilized to match their target fields by cell death.

## Neural crest cells

Studies in the neural crest have shed light on many important issues in the control of cell number. The neural crest is a transient structure that is induced to form at the dorsal neural tube (LaBonne and Bronner-Fraser, 1999). Shortly after neural closure, multipotent neural crest cells migrate extensively to various destinations. In addition to giving rise to most of the peripheral nervous system, neural crest cells also generate melanocytes, smooth muscle cells, skeletal structures, connective tissues, chromaffin cells, etc. (Le Douarin and Kalcheim, 1999). Because neural crest cells can generate a wide diversity of cell types, they offer a unique system to study the process of differentiation as well as the control of cell number.

Neural crest cells divide while they are migrating and they seem to be multipotent at this stage. By injecting a lineage tracer into individual neural crest cells before their exit from the neural tube, Bronner-Fraser and Fraser first showed that at least some premigratory crest cells can give rise to all neural crest derivatives (Bronner-Fraser and Fraser, 1989; Bronner-Fraser and Fraser, 1988); they then showed that early migrating neural crest cells are also multipotential by using a similar technique (Fraser and Bronner-

Fraser, 1991). These results suggested that migrating neural crest cells have equal potential and can generate all neural crest derivatives. However, other studies have also shown that migrating neural crest cells are heterogeneous in terms of lineage segregation. *In vitro* clonal analyses have been performed extensively in neural crest cells to examine the developmental potential of an individual cell (Baroffio et al., 1988; Ito and Sieber-Blum, 1991). When single neural crest cells are placed in environments that can presumably promote differentiation of all cell types, the sizes and types of clones that are generated varied greatly. Some clones contained many cells, suggesting that the crest cells in those clones are able to divide many times. In addition, multiple cell types are seen in some clones whereas in some others, only neurons or glia (but not both) are observed. The general conclusion is that migrating neural crest cells are asynchronously specified and precursor cells that vary in their ability to expand and differentiate can be observed (Henion and Weston, 1997; Sieber-Blum and Cohen, 1980; Stemple and Anderson, 1992). In support of this view, Anderson and co-workers have shown that Neurogenin is expressed as soon as neural crest cells emigrate from the neural tube, thereby committing those cells to a sensory neuronal fate (Ma et al., 1998; Perez et al., 1999). Taken together, neural crest cells are multipotent precursors and their fates are restricted at different times during development.

Although some of the neural crest cells may be specified to a particular lineage by the time they leave the neural tube, the final differentiation of these precursor cells also largely depends on the local environment to which they migrate. Transplantation studies have shown that neural crest cells that have arrived at their final destination can still differentiate into other cell types. For example, when E4 to E6 dorsal root ganglia are backtransplanted into the trunk, cells in that ganglia can give rise to both sensory and sympathetic neurons (Le Lievre et al., 1980; Rohrer et al., 1986). Similarly, by inserting whole pieces of peripheral ganglia, Le Douarin and co-workers have shown that neural

crest cells migrate out from the transplanted tissues and give rise to a wide variety of neural crest derivatives (Ayer-Le Lievre and Le Douarin, 1982; Fontaine-Perus et al., 1988; Le Douarin et al., 1978; Le Lievre, et al., 1980). Cell culture studies have also shown that extracellular signals such as growth factors can bias the differentiation of neural crest cells to neurons, glia or smooth muscle cells (Schneider et al., 1999; Shah et al., 1996; Shah et al., 1994; White et al., 2001). Furthermore, neural crest derived neurons can change their phenotypes after they have differentiated. For instance, when cholinergic ciliary ganglion neurons are transplanted into the trunk, they take on phenotypes that are characteristic of the sympathetic neurons in this location—they express catecholamine and can even re-enter the cell cycle (Sechrist et al., 1998). These data suggest that differentiation of neural crest cells into neurons or other cell types is influenced by their lineages as well as by extrinsic factors.

In the neural crest, the number of neural crest cells produced and the size of the peripheral ganglia seem to be tightly regulated. When the number of neural crest cells is altered by ablating the neural folds, the remaining neuroepithelium is able to fill in the depleted crest cells (Sechrist et al., 1995). On the other hand, when extra neural crest cells are made by overexpressing Noelin-1 (Barembaum et al., 2000), all of the resulting ganglia are of similar size to their control counterparts. These results suggest that there are mechanisms in place to ensure the homeostasis of the neural crest cell number and this can conceivably be achieved by changing the rate of cell proliferation, cell death, or both.

**Neuronal cell death**

Cell death plays an important role in the developing nervous system, with both predetermined and environmental factors contributing vitally to the regulation of neuronal cell death. For instance, studies in C. elegans suggest that cell death in that organism is pre-programmed, i.e., the number and type of cells that die are predictable and

invariant. In contrast, in the vertebrate nervous system, neurons are generated in excess and then trimmed down to their final number to match their targets by programmed cell death. The magnitude of cell death can be influenced by interactions with others tissues (reviewed in Oppenheim, 1991). For example, when the developing limb bud was removed, fewer neurons were observed in the sensory ganglia (Hamburger and Levi-Montalcini, 1949). On the other hand, when extra target tissues were grafted, more neurons survived (Hollyday and Hamburger, 1976). These results, together with the observation that cell death typically occurs when neurons are reaching their targets, suggest that cell death in developing neurons can be modified by interactions with their target tissues and that neurons may be competing for factors supplied by target tissues that are crucial to their survival. Some of the factors that neurons may be competing for are target-derived trophic factors. Trophic factors are present in neuronal targets in limiting amounts, hence, only neurons that have reached their targets and formed functional synapses will obtain a sufficient amount to enhance survival (Barde, 1989). Thus, target-dependent cell death ensures that neurons that have navigated to the incorrect target are eliminated so that appropriate neural connections can be maintained.

In addition to extracellular signals that can mediate neuronal survival, some factors have been demonstrated to induce neuronal apoptosis. Even though nerve growth factor (NGF) is best known as the first bonafide target-derived trophic factor (Purves and Lichtman, 1985), it can also mediate cell death by interacting with the neurotrophin receptor p75 (p75). In the retina, Frade and co-workers showed that NGF can cause cell death of the developing ganglion cells at a time when ganglion cells only express p75 but not the signaling receptor for NGF, Trk A. Since then p75 has been implicated in causing apoptosis in different cell types by interacting with NGF, other neurotrophins, or even by itself (Bunone et al., 1997; Casaccia-Bonnefil et al., 1996; Cotrina et al., 2000; Frade et al., 1996; Rabizadeh et al., 1993). In p75 deficient mice, a decrease in cell death can be

observed in different structures (Bamji et al., 1998; Frade and Barde, 1999), supporting the notion that p75 normally functions to induce cell death. Bone morphogenetic protein 4 (BMP-4) has also been characterized as a death factor. In the hindbrain neural crest, the odd-numbered rhombomere crest cells are eliminated by signals from even-numbered rhombomeres and BMP-4 has been shown to be responsible for this process (Graham et al., 1994; Graham et al., 1993). It is conceivable that some growth factors can control cell number by controlling both cell survival and cell death, depending on the cellular contexts.

**The avian ciliary ganglion as a model system**

The avian ciliary ganglion (CG) has been a useful model to study questions that address the regulation of neuronal number and differentiation. The CG is part of the parasympathetic system and is derived from the mesencephalic neural crest (Hammond and Yntema, 1958; Narayanan and Narayanan, 1978b; Noden, 1978). Mesencephalic crest cells start migrating towards the eye prior to St. 12 and a recognizable ganglion can be identified behind the eye by St. 24. CG neurons are born by St. 28 (D'Amico-Martel, 1982; Dupin, 1984; Rohrer and Thoenen, 1987; Sechrist et al., 1998) and there are only two types of neurons in this structure. The ciliary neurons innervate the iris and ciliary body in the anterior chamber of the eye, while the choroid neurons innervate the smooth muscle of the choroid coat (Marwitt et al., 1971). Both types of neurons are cholinergic but the choroid neurons also express the neuropeptide, somatostatin (Epstein et al., 1988). Studies in our laboratory have shown that the induction of somatostatin can be modulated by activin A and follistatin which are expressed in the target tissues (Coulombe et al., 1993; Darland et al., 1995; Darland and Nishi, 1998). Synapse formation begins around St. 34 and is complete by St. 40 (Landmesser and Pilar, 1978). Concomitant with synaptogenesis, several other developmental processes take place—including the upregulation of neurotransmitter, glial development and neuronal cell death

(Dryer, 1994).

Developmental cell death in the CG has been well documented (Landmesser and Pilar, 1974b). Between St. 34 and St. 40, CG neurons undergo cell death which results in a 50% reduction in neuronal number. This cell death has been shown to be target-dependent because removal of the eye further reduces the surviving neurons to 10-15% of their original number (Landmesser and Pilar, 1974b). Chick ciliary neurotrophic factor (chCNTF) has been shown to be a target-derived trophic factor for CG neurons (Finn et al., 1998; Finn and Nishi, 1996a), and glial cell line-derived neurotrophic factor and its family members, have also recently been demonstrated to support neuronal survival and to induce neurite outgrowth in CG cultures (Hashino et al., 1999; Hashino et al., 2000). Overexpression of chCNTF can reduce CG neuronal cell death, suggesting that targets control cell death, at least in part, by limiting the availability of trophic support (Finn et al., 1998; Finn and Nishi, 1996a). In addition, removal of the preganglionic input to the CG (Furber et al., 1987) can exacerbate cell death and blockade of synaptic activity by alpha bungarotoxin (Meriney et al., 1987) can decrease cell death. These data suggest that multiple mechanisms can be at work to control neuronal cell death in the developing CG.

## Questions addressed in this thesis

Based on what is known about neuronal cell death, I was interested in different mechanisms that can regulate cell death. I wondered if CG cell death can be regulated by a death factor and if so, how? In addition, I wanted to determine if CG cell death also occurs prior to synapse formation and if so, do target tissues or target-derived neurotrophic factors play any roles in this early death? I have attempted to address these

questions in this thesis and the outline of my experiments are as summarized below:

1. Mechanisms that control cell death in the CG

a. The initial goal of my thesis was to test the hypothesis that a death-inducing activity regulates CG neuronal death. An increasing amount of evidence has accumulated to suggest that neuronal cell death can be actively triggered. NGF, for example, has been implicated in causing cell death by interacting with the neurotrophin receptor p75. We have observed a death activity in embryonic chick eye extracts and have ventured to characterize and purify this activity. We identified this activity as (diferric transferrin) $Fe_2Tf$. Results in Chapter 1 describe in detail our characterization and purification.

b. By examining the pattern of dying cells in the CG, I have discovered another phase of cell death that occurs two days earlier than that described previously (St. 29 compared to St. 33/34). I have also found that cell death can be observed throughout the CG development, not only during synaptogenesis, suggesting that in addition to neuronal-target interactions, other processes may govern developmental cell death. I then addressed whether cell death at St. 29 can be altered by removing the optic vesicle or by overexpressing chCNTF. Experiments presented in Chapter 2 are designed to answer these questions and I have obtained results to suggest that cell death at St. 29 can be affected by both manipulations.

2. Regulation of neuronal number

Upon re-examination of neuronal number in the developing CG, I observed several changes in the number of neurons prior to St. 33/34. Cell death at St. 29 is followed by a depletion of neurons by St. 31, however, the neuronal number recovers by St. 33/34. How are the dying neurons being replaced? Part of Chapter 2 and most of Chapter 3 are

devoted to addressing this question. I first confirmed previous findings that CG neurons are post-mitotic by St. 29 (D'Amico-Martel, 1982; Dupin, 1984; Rohrer and Thoenen, 1987; Sechrist et al., 1998) and then I began to characterize potential precursor cells in the developing CG. Results from my immunolabeling studies, together with my *in vivo* transplantations, suggest that a subpopulation of non-neuronal cells in the CG have the potential to become neurons and they may do so without cell division.

# III. Materials and Methods

## Avian embryos used

White Leghorn X New Hampshire Red (Animal Sciences Dept., Oregon State University, Corvallis, OR), White Leghorn (Featherland Farms, Eugene, OR), and pathogen-free White Leghorn (SPAFAS, used for retroviral studies; Charles River, CT) fertile eggs were incubated at 38°C. Chick embryos were staged according to Hamburger and Hamilton (Hamburger and Hamilton, 1951). Japanese quail eggs were obtained from Boyd's Birds (Pullman, WA).

## Tissue preparation and Islet-1 immunocytochemisty for stereology

Ciliary ganglia were dissected as previously described (Nishi, 1996) and were immediately fixed in 4% paraformaldehyde in phosphate buffer saline (PBS; 150 mM NaCl, 20 mM sodium phosphate, pH 7.4) for 2 to 3 hours at room temperature (RT) or overnight at 4°C. After rinsing three times in PBS, tissues were allowed to equilibrate in 30% sucrose in PBS overnight at 4°C. Serial sections were cut at 30 µm on a Leica Jung cryostat at -25°C and collected on gelatin/poly-L-lysine subbed slides. Slides were air dried for 15 minutes, post fixed for 15 minutes in 4% paraformaldehyde vapors and then were completely immersed for an additional 15-30 minutes.

Slides were washed 3 times in PBS and incubated overnight at 4°C in a blocking solution of (2% (w/v) bovine serum albumin (BSA), 2% (v/v) normal goat serum, 0.5% (v/v) triton X-100 and 0.2% (w/v) sodium azide in PBS). Sections were then incubated with anti-islet-1 (1:250 hybridoma supernatant, clone 39.405, which recognizes both islet-1 and islet-2, Developmental Studies Hybridoma Bank, University of Iowa) in blocking solution overnight at 4°C. Following 3 washes with PBS and 0.5% (v/v)

TX-100 (PBST) and inactivation of endogenous peroxidase with 0.5% $H_2O_2$, slides were incubated for 1.5 hours at room temperature with a biotinylated goat anti-mouse antibody (1:500, Vector Labs) and then washed 3 times with PBST. Finally, sections were incubated for 1.5 hours at RT in a Vectastain ABC-HRP solution (1:500, Vector Labs). Islet-1 immunoreactivity was visualized by nickel/cobalt enhanced diaminobenzidine (NiDAB) solution (0.5 mg/ml DAB (Sigma), 0.1% $NiCl_2$, 0.1% $CoCl_2$ and 0.01% $H_2O_2$ in PBS). After color development, slides were rinsed three times in PBS and incubated in blocking solution for an hour at RT. Sections were then incubated in anti-HuC/D (1:250, Molecular Probes) in blocking buffer overnight at 4°C. Slides were treated with secondary antibody and ABC-HRP as described above. Anti-HuC/D immunoreactivity was detected by reaction with 0.5 mg/ml DAB without nickel/cobalt enhancement.

**Detection of dying cells with TUNEL**

CG were fixed in 4% paraformaldehyde in PBS containing 1mM EDTA to inhibit DNase activity. After fixation, slides were blocked in 2% BSA, 2% sheep serum in PBST overnight at 4°C. TUNEL assays were performed using the In Situ Cell Death Detection POD kit (Roche Biochemicals) which incorporates fluorescein isothiocyanate (FITC) labeled nucleotides into the free 3'OH ends of DNA and amplifies the labeled DNA with an antibody against FITC that is conjugated to horseradish peroxidase (POD). The following modifications were made to the manufacturer's protocol: 1) the nucleotide labeling mix was diluted 1:1 and the reaction was run for 3 hours; 2) the anti-fluorescein POD was diluted 1:5 and incubated for 2.5-3 hours; 3) TUNEL labeling was visualized by incubating tissue sections in NiDAB solution. All sections were then processed for anti-HuC/D immunocytochemistry as described above. The TUNEL assay was performed in cultured cells as described above except that anti-HuC/D immunocytochemistry was not routinely carried out as neurons could usually be

identified by their rounded cell bodies and extended processes.

**Cell counting using design based stereology**

We counted islet-1- and TUNEL- positive cells with design-based stereology using an optical fractionator probe (West et al., 1991). In order to use this technique to its full efficiency, tissue collapse during processing had to be minimized. We mounted 6-10 CG together and cut 30 μm serial cryostat sections, which were collected on slides and processed for immunohistochemistry for islet-1 and HuC/D. When we measured our sections after staining and mounting, their thickness was consistently 26-28 μm, demonstrating that our processing method could preserve tissue integrity. In addition, islet-1+ nuclei were observed throughout the entire z-axis of the sections, confirming that our immunocytochemical reagents penetrated the entire thickness of the tissue.

Stereology software (Stereo Investigator, Microbrightfield) and the optical fractionator probe were used in conjunction with a Nikon Optiphot microscope with an X, Y, Z stage drive and a position transducer. Spacing between sampling sites (grid size) was set such that 10-12 sampling sites per section were counted according to the manufacturer's instructions. Each sampling site was optimized to contain 0-5 counting objects so that 100-300 cells were counted for each ganglion. Because CG increased in volume as the embryo developed, grid sizes were adjusted in order to normalize the sampling criteria across the embryonic ages. Counting frame and grid size were determined for each age by counting each section at least three times with or without the same sampling sites; conditions that yielded cell counts within 5% of error were chosen for subsequent quantification. In addition, an upper guard zone of 4 μm and a lower guard zone of 7 μm were used to restrict counting to the volume of tissue sections that was devoid of cutting artifacts.

To establish the reliability of our cell counting method, we counted the same sections across various developmental ages at least three times using the same sampling sites, then repeated with different sampling sites. The margin of error of these counts was within 5%. When we quantified the number of TUNEL+ cells, all counting was done with a grid size of 40x40 and grid spacing of 100x100 regardless of the age of CG examined. For counting islet-1+ cells, different grid sizes and spacing were used depending on the age of CG; they are given in table format as follows:

| Embryonic age | Grid size | Grid spacing |
|---|---|---|
| 5 | 20x20 | 120x120 |
| 6 | 20x20 | 120x120 |
| 7 | 25x25 | 125x125 |
| 8 | 25x25 | 125x125 |
| 10 | 30x30 | 130x130 |
| 12 | 35x35 | 135x135 |
| 14 | 35x35 | 140x140 |

**BrDU treatment and immunocytochemistry**

BrDU (10-25 µg; Roche Biochemicals) was injected into the amnion of embryos between St. 24-31. Treated embryos were collected at St. 33/34 and processed as described above. Cryostat sections (25 µm) were cut and treated with 50 µg/ml DNase I (Roche) for 30-45 minutes at 37°C after fixation, followed by three rinses in PBS+1 mM EDTA and one rinse in PBS. Slides were blocked and incubated in both anti-HuC/D (1:250) and anti-BrDU (1:500, Harlan SeraLab) overnight at 4°C. Anti-HuC/D and anti-BrDU immunoreactivity was visualized by incubating sections in Alexa™ 488-conjugated goat anti-mouse IgG and Cy3™- or Rhodamine Red-X- conjugated donkey anti-rat IgG (Jackson ImmunoResearch), respectively. Fluorescent images were acquired using a Leica inverted microscope equipped with a digital camera.

**HuA and HuC/D immunohistochemistry**

St. 29 CG were collected and fixed as described (see section for *Tissue preparation and islet-1 immunocytochemistry for stereology*). 10 μm cryostat sections were cut, air-dried, and post-fixed in 4% paraformaldehyde in PBS for 10-20 minutes at RT. Slides were incubated in anti-HuA (gift from Dr. Marusich and Dr. Weston, University of Oregon) and anti-HuC/D together overnight at 4°C. Anti-HuA immunoreactivity was visualized by using an Alexa™488-conjugated goat anti-mouse IgG1 and anti-HuC/D by an Alexa™568-conjugated goat anti-mouse IgG2b. Fluorescent images were taken on a BioRad 1024 laser scanning confocal microscope and optical sections were collected at 1-2 μm intervals.

**Optic vesicle ablation**

Surgeries were performed on St. 11-13 embryos as previously described (Landmesser and Pilar, 1974b). Briefly, embryos were exposed by windowing the eggs and neutral red was used to visualize the embryos. Sharpened tungsten needles were used to remove one of the optic vesicles in each embryo; the eggs were sealed with scotch tape and returned to the incubator until St. 29.

**Production of stable virus producing cells and high titer viral inoculum**

Stable virus producer cells were generated by calcium phosphate transfection with pRCASBP(A)-chCNTF (Finn et al., 1998) and pRCASBP(A)-GFP (kindly provided by C. Cepko) as previously described (Morgan and Fekete, 1996), with the exception that the continuous chicken fibroblast cell line DF-1 (Himly et al., 1998) was used rather than primary chicken embryo fibroblasts. Infection by RCASBP(A) and transgene expression (chCNTF) in DF-1 cells were verified by p27gag and chCNTF immunocytochemistry, respectively. The DF-1 cells continued to show complete viral infection with no loss in transgene expression after 20+ passages.

Culture supernatants containing viruses were concentrated approximately 100-fold by ultracentrifugation at 70,000 g. The concentrated viral stocks used throughout these experiments were from the same lot and had titers of approximately 4.3 X $10^8$ infectious units (IU)/mL (RCASBP(A)-GFP) and 2.0 X $10^8$ IU/mL (RCASBP(A)-chCNTF). Concentrated virus (250-350 nL) was injected into the lumen of the developing mesencephalon in 8 somites embryos using a Nanoject II positive displacement microinjector (Drummond) mounted to a micromanipulator (Marhauser model MM33). Embryos were returned to the incubator until they were harvested at St. 29.

### *In situ* hybridization

A 745 base pair chCNTF receptor alpha (chCNTFRα) fragment was cloned into the Nco I site of pGEM5z (Promega) and anti-sense and sense digoxigenin-labeled riboprobes were generated using MegaScript SP6 and T7 kits (Ambion). Hybridization was carried out at 65°C followed by washes at the same temperature. Tissue sections were then incubated in alkaline phosphatase conjugated anti-digoxigenin (Roche) overnight at 4°C. In situ hybridization product was visualized using a NBT/BCIP substrate.

### Preparation and immunostaining of acutely dissociated cells

CG were dissociated as described (Nishi, 1996) but instead of being plated onto coated tissue culture wells, cell suspensions were subjected to centrifugation on the Cytospin (Shandon-Lipshaw). A total volume of 200 µl was loaded into each cytofunnel and spun at 1, 000 x g for 10 minutes. Cells were collected onto gelatin-subbed slides and air-dried before post-fixing with 4% paraformaldehyde. Immunostaining was performed using the same protocol for the CG cryostat sections described above.

**Cell Culture and immunocytochemistry**

CG from St. 29 embryos were dissociated and plated on polylysine-laminin coated tissue culture coverslips (Fisher) as previously described (Nishi, 1996), with the modification that the basal medium used consisted of DMEM, 10% heat inactivated horse serum, 2% fetal calf serum, and 2% chick embryo extract. Recombinant [his]6-chCNTFyyy (Finn and Nishi, 1996a) and recombinant fibroblast growth factor 2 (FGF-2 or bFGF) were used at 20 ng/ml. Cultures were assessed for neuronal survival and photographed 24 hours after plating for a chCNTF survival assay.

For live labeling of antibodies (anti-O4, used at 1:250, generous gifts from Dr. S. Back and Dr. S. Pfeiffer, and anti-chCNTFRα, used at 1:100, generous gift from Dr. H. Rohrer), cultures were incubated in primary antibodies at 37°C for 30-45 minutes. After fixation in 4% paraformaldehyde in PBS, cultures were rinsed in PBS three times and blocked in PBS with 0.2% Tween 20, 2%BSA and 2% normal goat serum for an hour RT. CG cultures were then incubated in anti-p75 (1:3,000) or anti-HuC/D overnight at 4°C. Anti-chCNTFRα and anti-p75 were visualized by using an Alexa™488-conjugated goat anti-rabbit secondary antibody (Molecular Probes), anti-O4 was visualized by a Rhodamine Red-X-conjugated goat anti-mouse μ chain specific secondary antibody (Jackson ImmunoResearch), and anti-HuC/D was visualized by a Rhodamine Red-X-conjugated goat anti-mouse secondary antibody (Jackson ImmunoResearch). Other primary antibodies used were: anti-7B3 (generous gift from Dr. J. Weston, University of Oregon), anti-Thy1 (generous gift from Dr. Peter Jeffrey), and anti-QCPN (Developmental Studies Hybridoma Bank, University of Iowa).

**Cell counting of dissociated CG cultures**

Dissociated CG that were cytocentrifuged or cultured were both processed for immunocytochemistry and counterstained with a nuclear dye (Hoechst). Cell counting

was done using a Nikon Eclipse 800 Fluorescent microscope with a 60X oil objective. 10 non-overlapping fields of view were counted; total cell number was obtained by counting Hoechst+ nuclei with the UV filter. Percent of neuronal number was obtained by dividing total number of neurons (typically HuC/D+ cells) by the total cell number (Hoechst+ nuclei).

**Enrichment of non-neuronal cells from E5 and E10 quail ciliary ganglia**

CG were dissected and dissociated as described previously (Nishi, 1996). CG obtained from E10 quail embryos were incubated in trypsin for 45 minutes to an hour. After trituration, cell suspensions from E10 CG were passed through a 70- or 100- μm nylon mesh to get rid tissue clumps. Both E5 and E10 CG cells were plated onto polylysine/laminin coated T-25 or T-75 tissue culture flasks (Nunc), about 45 E5 CG and 15 E10 CG were typically seeded onto a T-25 flask. After 4 hours to overnight culture, neurons were manually shaken off while being monitored under a microscope. Culture medium was replaced by 1xMPG during the shaking and MPG was changed several times to rinse off neurons. Non-neuronal cells that remained in the flasks were recovered by incubating in 0.1-0.25% trypsin in MPG for 15-20 minutes, followed by centrifugation. Non-neuronal cell pellets were then resuspended in DMEM with 10% heat inactivated horse serum, 2% fetal calf serum, and 2% chick embryo extract supplemented with 20 ng/ml recombinant chCNTF and 20 ng/ml recombinant FGF-2. The resulting non-neuronal cells were usually 90-95% pure as judged by morphology under phase-contrast microscopes and/or immunostaining with anti-HuC/D. Because there were not many non-neuronal cells in E5 to E6 CG and many of them were also lost during shaking, we typically had to dissect 200-300 E5 quail CG for one transplantation experiment. For E10 or older ganglia, there were more non-neuronal cells and their recovery rate was better compared to E5. We usually isolated about 120-150 E10 quail CG for injections.

**Transplantation of quail cells into chick embryos and tissue processing**

Quail cells derived from E5 or E10 CG were either used as mixed cell suspensions or as enriched non-neuronal donor cell materials. E2 (St. 10-St. 15) embryos were as hosts used for backtransplantation into the head mesenchyme. Quail cell suspensions were concentrated in an eppendorf tube and loaded into a glass needle. Cells were then injected by air pressure in a setup equipped with a micromanipulator. Transplanted embryos were returned to the incubator until the day of harvest when they were fixed in 4% paraformaldehyde in PBS for 4 hours RT or overnight at 4°C. Embryos were then rinsed 2-3 times in PBS and infiltrated with 5% and 15% sucrose sequentially. Finally embryos were equilibrated in 7.5% gelatin for embedding. Gelatin-embedded embryos were frozen in liquid nitrogen and 10 µm serial cryostat sections were cut in a Microm cryostat. Immunohistochemistry for anti-QCPN and anti-HuC/D was preformed as described above. Fluorescent images were taken on a BioRad 1024 laser scanning confocal microscope and optical sections were collected at 1-2 µm intervals.

**E8 ciliary ganglion neurons cell survival bioassay**

Dissociated CG neurons were generated from E8 embryos as described (Nishi, 1996) and plated on 48-well tissue culture plates (Falcon). Cultures were maintained in Eagle's Minimum Essential (MEM) with 25 mM KCl (HiK+) supplemented with 10% heat inactivated horse serum, 100u penicillin, 100u streptomycin, and 10 ng/ml chCNTF (Finn and Nishi, 1996a). Three days after plating, reagents to be tested (e.g. chick diferric transferrin and dihydropyridine, etc.) were added to culture with a change in medium. Neuronal survival was scored by counting 15 fields of view on a Nikon inverted microscope at 20X two days after treatment (5 days *in vitro* total).

**Embryonic eye extracts and chromatographies**

Whole eyes, excluding the vitreous humor, were collected from E15-17 chicken embryos.

**Protein A purification of DA 14 IgG**

DA14 IgG was purified using the HiTrap protein A affinity column (1ml or 5ml, Pharmacia) according to manufacturer's protocol with Pierce® gentle binding and elution buffers (Pierce). Protein A purified DA14 IgG was collected and checked for protein concentration.

**Affinity purification of DA**

Protein A purified DA14 IgG was immobilized onto an affinity column by following manufacturer's instructions (DA14 affinity column; the affinity column was purchased as part of the AminoLink Immunobilization Kit from Pierce). DEAE eluate was loaded onto the DA14 affinity column at 4°C. Affinity purified DA was eluted with 5 ml of 100 mM glycine, pH 2.5 into 0.7 ml of 1 M Tris buffer, pH 8.6, to bring the eluate back to a neutral pH. Samples were then dialyzed against 2 changes of PBS, 2 hours each, and concentrated to approximately 1ml with CentriPrep. Affinity purified DA was tested for activity on E8 CG bioassay, run on protein gels to check for purity, and sent to be sequenced.

***In vivo* application of hybridoma cells**

Hybridoma cells secreting blocking antibody to DA (DA14) or control hybridoma cells (48G) were collected and centrifuged at 1,000 x g for 10 minutes. The cell pellets were resuspended in sterile PBS, pH 7.2. Chicken eggs were windowed at E3 and sealed with Scotch tape before returning to egg incubators. Hybridoma cells were applied on top of the chorioallantoic membrane at 5-6 x $10^6$ cells/50 µl of PBS at E4. Control groups received either hybridoma cells secreting an irrelevant IgG or PBS only. Embryos were then returned to incubators and allowed to develop without further disruptions until the desired age of collection.

# IV. Results

## Chapter 1

## Induction of neuronal apoptosis by transferrin-mediated iron uptake

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**Abstract**

Programmed cell death is widespread throughout the developing nervous system. However, little is known about factors that induce death. We have purified an activity that causes apoptosis of embryonic ciliary ganglion (CG) neurons in culture. We identified the active molecule as chicken diferric transferrin ($cFe_2Tf$), which kills via iron uptake through the transferrin-receptor mediated pathway. Physiological concentrations of $cFe_2Tf$ are sufficient to induce apoptosis, and neuronal susceptibility in the CG is age-dependent. Besides playing a role in normal developmental cell death, deprotection of neurons from the deleterious effects of transferrin-mediated iron uptake may be involved in causing neurodegeneration in adults.

## Introduction

The development of the nervous system is characterized by specific periods of programmed cell death. During neurogenesis, cell death occurs throughout the ventricular layer of the neural tube (Blaschke et al., 1998; Homma et al., 1994), and neural hypertrophy is observed when this cell death is prevented (Kuida et al., 1996). In the developing spinal cord, three distinct zones of pyknotic profiles have been observed; these have been postulated to play a role in early phenotypic selection (Homma et al., 1994). Massive cell death has also been observed in virtually all neural tissues after differentiated neurons have extended their processes and initiated synapse formation. Cell death that occurs during synaptogenesis has been best characterized, roughly 50% of the neurons that are generated are eliminated during this phase of cell death (Oppenheim, 1991).

Extensive studies of cell death during synaptogenesis have suggested that cell death is regulated by multiple mechanisms (Oppenheim, 1991). This later phase of cell death is target-dependent and is influenced by the availability of molecules necessary to promote neuronal survival (neurotrophic factors). For instance, when target tissues are removed, more cell death is resulted in the neurons that innervated them. On the other hand, when extra target tissues are grafted, more neurons will survive (Barde, 1989). Afferent input can also affect cell number: removal of presynaptic neurons increases cell death (Furber et al., 1987); this is thought to be regulated by synaptic activity as well as electrical activity in the neurons themselves (Oppenheim, 1991).

Recently, specific ligands that trigger cell death in the developing nervous system have also been reported. For example, nerve growth factor (NGF) can induce cell death through the p75 neurotrophin receptor (p75NTR) during neurogenesis of the retina and in cultured oligodendrocytes (Casaccia-Bonnefil et al., 1996; Frade et al., 1996). It is not

clear whether other endogenously expressed molecules may induce developmental neuronal cell death.

The avian ciliary ganglion (CG) has been widely used to characterize regulators of target-dependent neuronal cell death (Dryer, 1994; Nishi, 1994). By E8, neurons in the CG have completely withdrawn from the mitotic cycle and have extended processes to their peripheral targets (Landmesser and Pilar, 1974b). More than half of the initial population of neurons dies between E9 and E14 (Finn et al., 1998; Landmesser and Pilar, 1974b). Processes known to influence this cell death include preganglionic inputs (Furber et al., 1987; Meriney et al., 1985) as well as competition of neurons for synaptic targets (Pilar et al., 1980), and chick ciliary neurotrophic factor (chCNTF). chCNTF has been shown to be a target-derived neurotrophic factor that supports survival of CG neurons *in vitro* (Barbin et al., 1984; Leung et al., 1992) and *in vivo* (Finn et al., 1998).

**Results**

We have observed a death activity (DA) in the embryonic eye extract that could induce cell death in dissociated E8 CG neurons. When we added DA into the culture medium as we seeded the CG neuronal cultures, all neurons were dead even in the presence of saturating amounts of chCNTF. Several lines of evidence suggested that the effect of the DA was specific. First, DA induced apoptosis selectively in neurons; non-neuronal cells in the same culture remained alive (Figure 1). Second, when DA was added at the time of plating, we consistently observed cell death 4-5 days after neurons were seeded, suggesting that DA was not a rapidly acting cell poison or lytic agent (Figure 2). Third, we determined that all neurons could be induced to die within 48 hours if DA was applied 3 days after plating, indicating that CG neurons had to develop sensitivity to DA, possibly by expressing a specific receptor (Figure 3). Finally, although E8 and E9 CG neurons are sensitive to DA, neurons isolated from ganglia that have proceeded through cell death in vivo (E14) were insensitive (Lee and Nishi, unpublished results).

We purified DA by generating a function-blocking monoclonal antibody (mAb DA14). The mAb DA14 was used to purify a protein with an apparent molecular weight of 70 kD by affinity chromatography (Figure 4). This protein was sequenced, and the N-terminal 13 residues matched that of chicken transferrin (cTf). The partially purified DA used for generating mAb DA14 was chromatographed on a high-resolution anion-exchange column (Poros QE/M), and DA resolved as a single peak that co-eluted with a chicken diferric transferrin ($cFe_2Tf$) standard (Figure 5). The death activity of recombinant $cFe_2Tf$ (Mason et al., 1996) was compared to the purified DA, and the effective concentration at which half the neurons were killed ($EC_{50}$) of the purified DA from embryonic chicken eyes was 8-10nM (300-800 ng/ml, Figure 6), closely matching the $EC_{50}$ of recombinant $cFe_2Tf$ (5 nM or 400 ng/ml, Figure 6). Taken together, these results identified DA as $cFe_2Tf$.

We next tested if $cFe_2Tf$ induced apoptosis by interacting with a novel receptor signaling system or by binding to the transferrin receptor. Cell death was blocked by incubating $cFe_2Tf$ with a monoclonal antibody which had been shown to inhibit $cFe_2Tf$ binding to the chicken transferrin receptor (Mason et al., 1995; Mason et al., 1996). Furthermore, human $cFe_2Tf$, which does not bind to the chicken transferrin receptor (Buchegger et al., 1996; Sorokin and Morgan, 1988), did not induce cell death even when 250 µg/ml (500-fold more than $EC_{50}$ of $cFe_2Tf$, which was 400 ng/ml) was added (Table 1). We also found that apo-transferrin was approximately 100-fold less potent at inducing cell death than iron-loaded transferrin, indicating that iron was important for the death activity. Cell death required the release of free iron inside CG neurons because 50 µM of deferoxamine mesylate, an intracellular iron chelator, prevented cell death (Table 1). These data indicated that CG cell death is mediated by iron uptake via the transferrin receptor pathway.

To test if CG neurons *in vivo* would normally be exposed to levels of $cFe_2Tf$ sufficient to induce cell death, we compared the protein concentration and death-inducing activity of $cFe_2Tf$ found in chicken embryos. We prepared soluble extracts from eyes and heads isolated at E8, E10, E12, and E14, embryonic stages during which CG neurons *in vivo* are dying. We quantified the level of $cFe_2Tf$ with a competitive radioimmunoassay (Mason et al., 1995) and the death activity of $cFe_2Tf$ with a bioassay using the E8 CG neurons (Table 2). At least 10 µg/ml $cFe_2Tf$ was found in the chicken embryo at all ages examined, constituting roughly 0.3% of the total protein (Table 2). These levels agree with the amount of transferrin reported in human cerebrospinal fluid (20-80 µg/ml), all of which is bound to iron (Bradbury, 1997). All of the calculated $EC_{50}$s of the death activity due to $cFe_2Tf$ were between 300-800ng/ml (Table 2); these values were comparable to the $EC_{50}$ of recombinant $cFe_2Tf$ (400 ng/ml), demonstrating that nearly all of the death-inducing activity in the embryonic extracts could be attributed to $cFe_2Tf$.

These results show that the death activity of $cFe_2Tf$ observed in culture is physiologically significant because the concentration at which CG neurons is killed is well below the circulating level of $cFe_2Tf$ in the animal.

Finally, we tested whether other neuronal populations were susceptible to $cFe_2Tf$. Neurons dissociated from neural retinae from E8, E10, or E12 were largely unaffected by $cFe_2Tf$, although effects on a minor subpopulation such as ganglion cells were not determined with certainty (Nishi, unpublished observations). Depending on the type of growth factor added to the culture medium, lumbar sympathetic neurons in cultures were differentially affected by $cFe_2Tf$. We found that sympathetic neurons that were grown in NGF were insensitive to $cFe_2Tf$; on the other hand, $cFe_2Tf$ killed those that were supported by CNTF (Figure 7). When sympathetic neurons were grown in medium containing NGF and CNTF, half the neurons died after $cFe_2Tf$ was added.

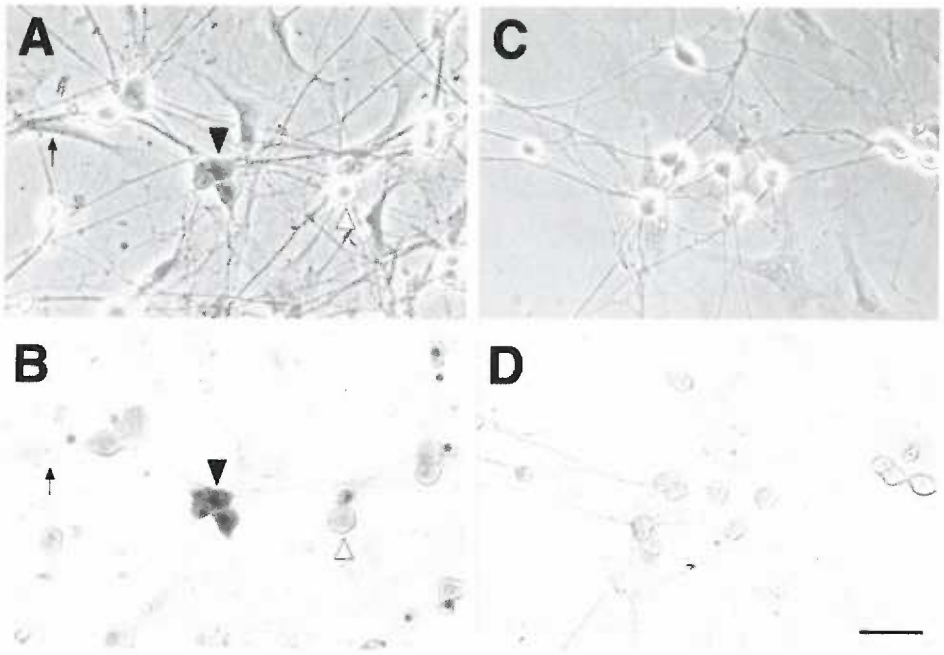


**Figure 1. TdT-mediated dUTP nucleotide end-labeling (TUNEL) of CG neurons after DA addition.**

(A) Phase-contrast photomicrograph of E8 CG neurons in culture fixed 24 hrs after DA addition. Three neurons in the center of the field were beginning to lose their phase brightness (arrowhead); they were dead a day later. (B) Bright-field photomicrograph corresponding to the same field of view as (A). The three neurons that showed early signs of cell death were darkly stained by TUNEL (arrowhead). Non-neuronal cells remained healthy and were unstained (small arrow in A and B); live neurons were phase-bright and unstained (white arrowhead in A and B). (C) Phase-contrast photomicrograph of untreated E8 CG neurons showing that all neurons were healthy and phase-bright. (D) Bright-field photomicrograph corresponding to the same field of view as (C). Neurons in control cultures were unstained. E8 CG cultures were generated as previously described and grown in 20 ng/ml of recombinant chicken [his]6-CNTFyyy (bacterially expressed in pQE8 and purified by nickel affinity chromatography according to vendor's instructions (Qiagen) in our laboratory. Three days after plating, DA was added in fresh medium. DA also induced apoptosis of CG neurons grown in 20ng/ml of fibroblast growthfactor 2 (FGF-2). Phase-contrast and bright-field photomicrographs were taken with a Zeiss inverted microscope with a 32x objective. Scale bar = 40 $\mu$m.

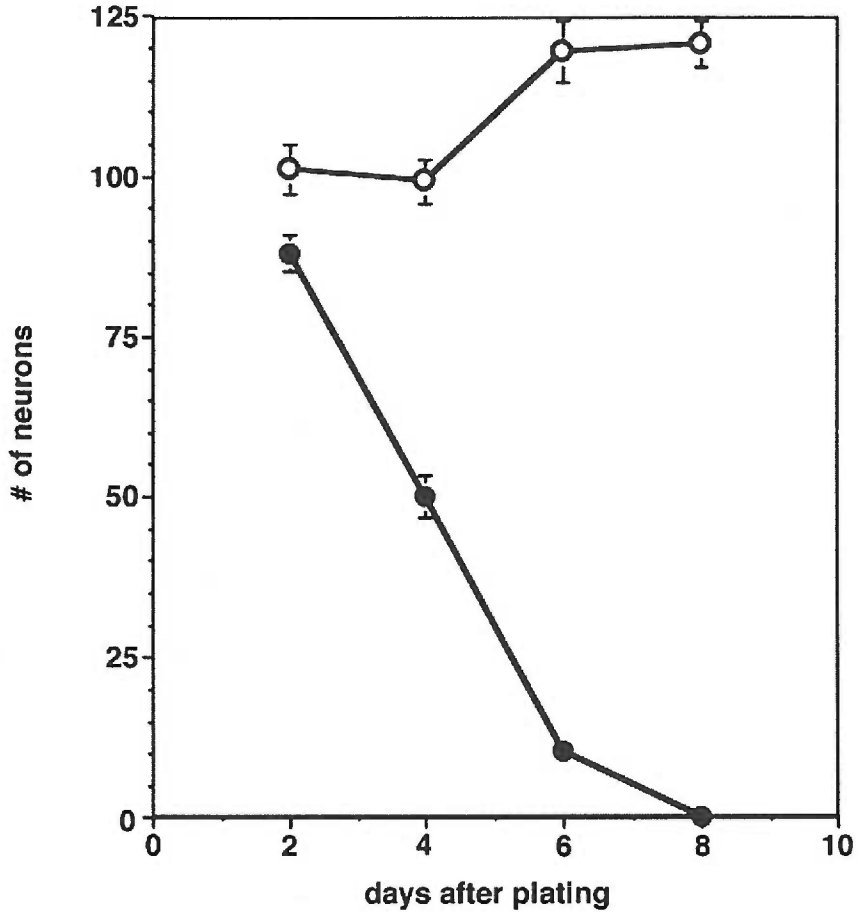


**Figure 2. DA induced E8 CG neuron cell death in culture.**
E8 CG neurons were dissociated and plated onto polylysine/laminin coated tissue culture plates. DA (typically 5% E15-E17 eye extracts) was added in the culture medium at the time of plating and changed every third day. Almost 50% of CG neurons were dead after 4 days and after one week, all the neurons were dead. (open circles = control cultures; closed circles = DA-treated cultures; y-error bars = stanadard error of means)

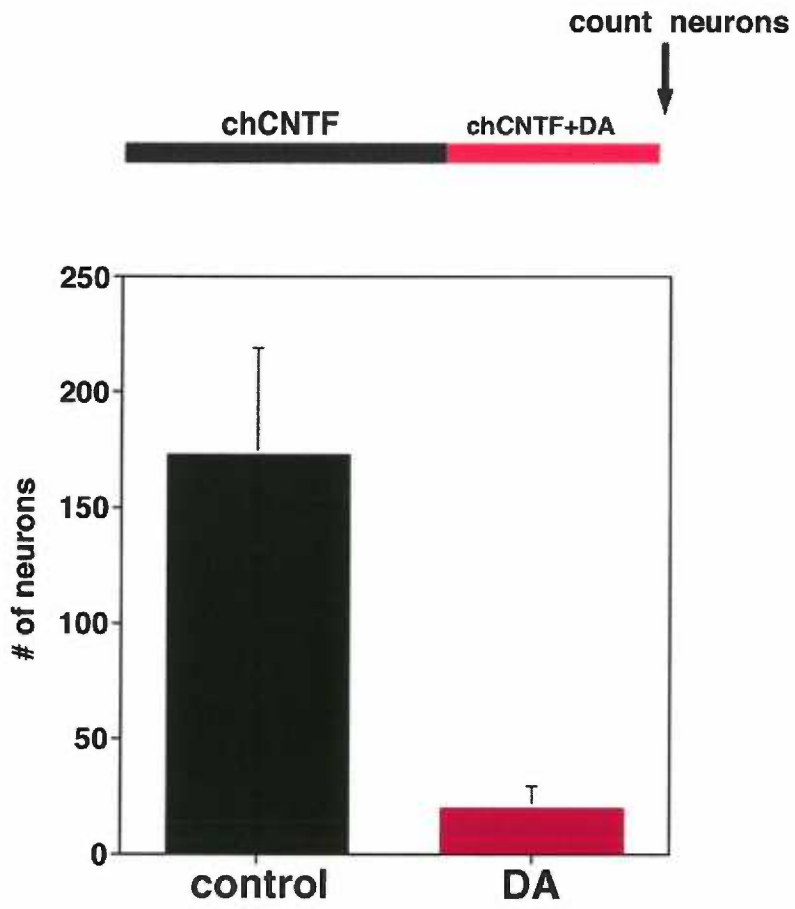


**Figure 3. CG neurons have to develop sensitivity to DA.**
E8 CG neurons were plated in High K+ MEM with 10 ng/ml chCNTF. DA was added in some cultures at the time of plating, one day, or two days. At day three, cultures that had received DA were replaced with fresh medium containing chCNTF, and DA was also added to 3-day old cultures that had not been exposed to DA before. When neuronal survival was assayed at day 5, we found that CG neurons did not die if DA was added during the first 48 hours after plating. On the hand, all the neurons were dead when we applied DA to 3-day old cultures, suggesting that CG neurons are not sensitive to DA within 2 days after dissociation.

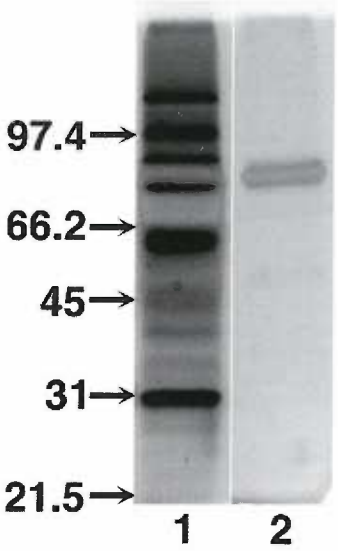


**Figure 4. Affinity purification of DA.**
Proteins from partially purified DA before (lane 1) and after (lane 2) affinity purification that were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) under non-reducing conditions and silver stained. Affinity chromatography using mAb DA14 yielded a single band that migrated at an apparent molecular weight of 70kDa (lane 2).

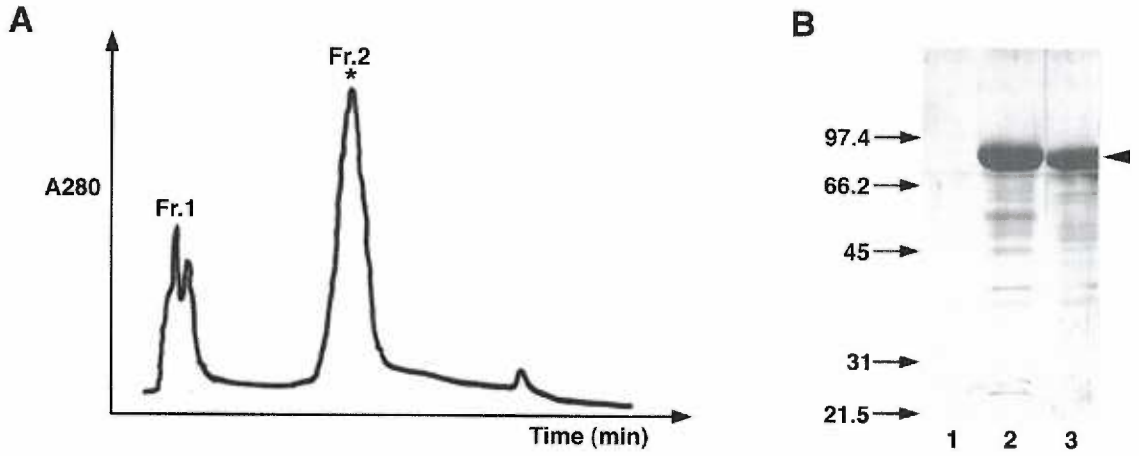


**Figure 5. Purification of DA by high-resolution anion-exchange chromatography.**
(A) Two major peaks were resolved when partially purified DA was loaded onto the Poros QE/M column (4.6/100mm, 1.7ml) and run on a PerSeptive Biosystems Sprint chromatography system as described. All death activity was contained in fraction 2 (Fr.2, *). (B) Coomassie-blue stain of the protein gel when fractions 1 and 2 were subjected to SDS PAGE. Fraction 1 (lane 1) contained no protein; fraction 2 (lane 2) migrated at the same molecular weight as purified $cFe_2Tf$ (lane 3 and arrowhead).

**Table 1. Cell death requires transferrin-mediated iron uptake.**

| Treatment | # of neurons |
|---|---|
| Control | 100±11.1(15) |
| $cFe_2Tf$ | 1.6±1.7(15) |
| $cFe_2Tf$+anti-DA | 99.4±12.9(3) |
| $cFe_2Tf$+anti-Tf | 115.7±11.2(3) |
| $cFe_2Tf$+deferoxamine | 84.8±13.5(6) |
| $hFe_2Tf$ | 94±14.8(9) |

E8 CG neurons were generated as previously described (Nishi, 1996) in 48-well tissue culture plates (Falcon). Samples to be tested for bioactivity were added to cultures three days after plating. Live cells were counted two days after sample addition under a Nikon inverted microscope with a 20x objective. 15 fields of views were counted per well, and counts were added together to give the total number of cells for each well.

The number of live cells in treated cultures were normalized to controls, with controls as 100%.

Values given as # of neurons are % of control values ± standard deviation (n = # of cultures

$cFe_2Tf$ = 1 µg/ml purified chicken diferric transferrin

anti-DA = mAb DA14, a function-blocking monoclonal antibody raised against partially purified DA, used at 40-50 µg/ml

anti-Tf = a monoclonal antibody raised against one of the receptor binding domains of chicken transferrin generated and characterized by Mason et al. (Mason et al., 1995), used at 7.5 µg/ml

deferoxamine = 50 µM deferoxamine mesylate (Ciba-Geigy)

$hFe_2Tf$ = 250 µg/ml human diferric transferrin (Boerhinger-Mannheim)

**Table 2. CG neurons undergoing cell death *in vivo* are exposed to saturating concentrations of $cFe_2Tf$.**

| Source | [extract] (μg/ml) | [$cFe_2Tf$] (μg/ml) | % total protein | $EC_{50}$ death activity in crude extract (μg/ml) | Calculated $EC_{50}$ of $cFe_2Tf$ (μg/ml) |
|---|---|---|---|---|---|
| E8 eyes | 6460 | 23.9 | 0.4 | 120 | 0.5 |
| E8 heads | 4620 | 18.6 | 0.4 | 120 | 0.5 |
| E10 eyes | 4890 | 10.0 | 0.2 | 270 | 0.5 |
| E10 heads | 5850 | 25.1 | 0.4 | 200 | 0.8 |
| E12 eyes | 6530 | 11.1 | 0.2 | 200 | 0.4 |
| E12 heads | 6000 | 22.8 | 0.4 | 80 | 0.3 |
| E14 eyes | 5600 | 15.9 | 0.3 | 150 | 0.5 |
| E14 heads | 4640 | 23.4 | 0.5 | 100 | 0.5 |

Crude extracts were prepared by homogenizing embryonic chicken tissues with 20 mM Tris buffer including a cocktail of protease inhibitors (aprotinin, leupeptin ad pepstatin A, 1 mg/ml each, Sigma Chemical). Homogenates were then centrifuged at 30,000 x g for 30 minutes at 4°C. The $EC_{50}$ of the death activity in the crude extract was determined by CG bioassays as described in Table 1. Protein concentrations of the extracts were determined by a protein assay (BioRad). Concentrations of $cFe_2Tf$ in the embryos were measured by a competitive radioimmunoassay as previously described (Mason et al., 1995), using purified diferric ovotransferrin as a standard.

$EC_{50}$=concentration necessary to kill 50% of neurons compared to control cultures

% total protein = [$cFe_2Tf$]/[extract]

calculated $EC_{50}$ of $cFe_2Tf$ = ($EC_{50}$ death activity in crude extract) X (% total protein).

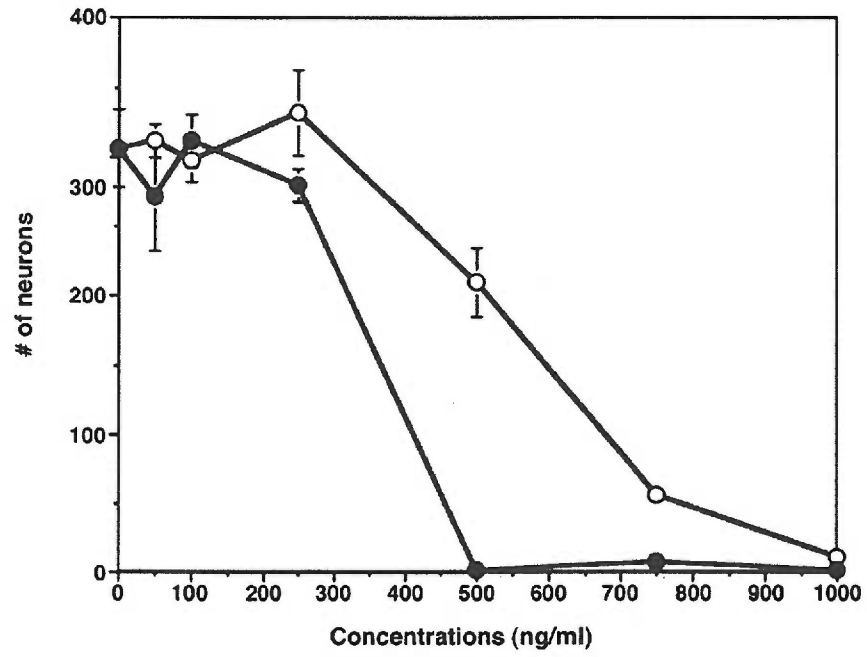


**Figure 6. Recombinant and purified $cFe_2Tf$ had similar biological activities.** The ability of recombinant and purified $cFe_2Tf$ to induce cell death in E8 ciliary ganglion neurons was compared by using different concentrations of $cFe_2Tf$ and their respective dose response curve generated. The $EC_{50}$ recombinant $cFe_2Tf$ was 400 ng/ml (5 nM) while that of purified $cFe_2Tf$ was about 300-800 ng/ml (8-10 nM). This demonstrated that the activities of recombinant and purified $cFe_2Tf$ were very similar and confirmed that purified DA was indeed $cFe_2Tf$. (open circles = purified $cFe_2Tf$; closed circles = recombinant $cFe_2Tf$; y-errors bar = standard error of means; each data point was generated from 3 sister cultures in one representative experiment and the experiment was repeated at least 3 times).

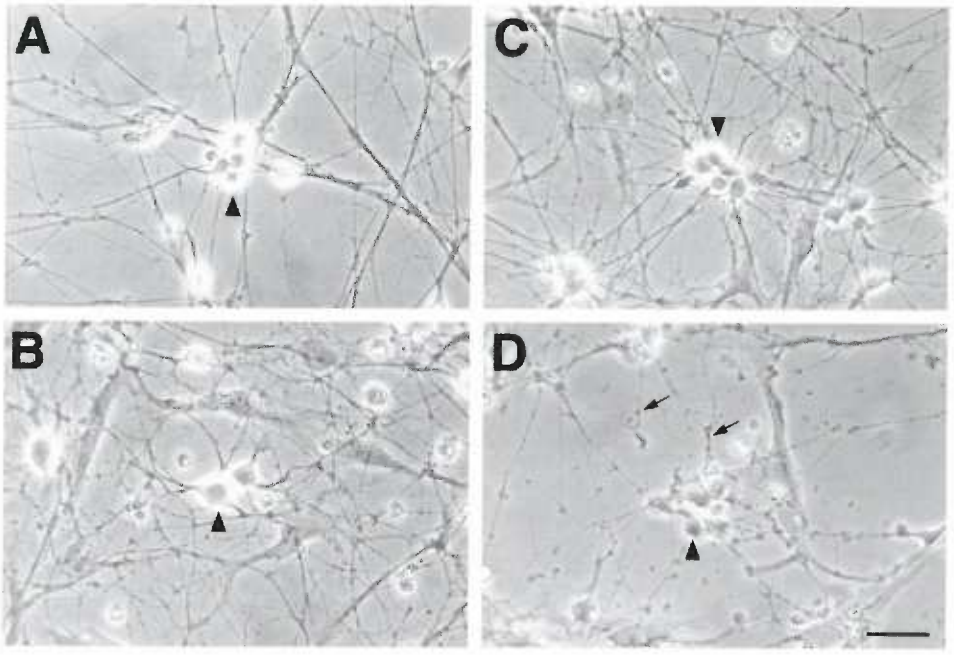


**Figure 7. Differential sensitivity of E13 lumbar sympathetic neurons to $cFe_2Tf$.** (A) Chick lumbar sympathetic neurons grown in NGF. Note that the cell bodies are phase bright and processes are abundant, indicating that the neurons are healthy. (B) Sympathetic neurons plated in NGF as in (A), but treated with $cFe_2Tf$. Neurons in NGF-supported cultures remained healthy regardless of $cFe_2Tf$ addition (arrowheads in A and B). (C) Sympathetic neurons grown in CNTF, these neurons are healthy, as seen in (A). (D) Sympathetic neurons grown in CNTF as in (C), but treated with $cFe_2Tf$. In contrast to sympathetic neurons in NGF, sympathetic neurons grown in CNTF were all dead two days after exposure to $cFe_2Tf$. Note that neurons are no longer phase-bright (arrowhead) and their processes appear blebby (small arrows). Lumbar sympathetic neurons were isolated as previously described and grown in complete medium containing either 20 ng/ml 2.5s mouse NGF (Alomone Labs) or 20 ng/ml recombinant chick poly-his CNTF prepared in our lab. All cultures were fed on the third day of plating; control cultures received fresh medium containing NGF or CNTF; $cFe_2Tf$ treated cultures received medium containing 1 $\mu$g/ml recombinant $cFe_2Tf$ plus either NGF or CNTF. Phase contrast photomicrographs were taken 2 days after medium change (a total of 5 days after plating). Scale bar = 40 $\mu$m.

**Discussion**

These studies demonstrate that transferrin-mediated iron uptake can induce cell death in select populations of developing neurons, suggesting a novel function for $cFe_2Tf$. Importantly, the effect of $cFe_2Tf$ was not an artifact of exposing neurons to non-physiological conditions because the concentration of $cFe_2Tf$ necessary for killing activity is considerably lower than the levels normally found in the chicken embryos. Because CG neurons display an age-dependent sensitivity to $cFe_2Tf$ that corresponds to the normal phase of cell death *in vivo*, we suggest that systemic molecules, such as $cFe_2Tf$, can be utilized to induce neuronal cell death. Even though neurons are normally protected from iron, developmentally regulated processes can render specific populations of neurons vulnerable to iron-induced apoptosis. We hypothesize that interactions with target tissues facilitated by neurotrophic factors are necessary to prevent neurons from dying by restoring their protection against iron.

Iron is essential for cell function because it is a transition metal that readily donates electrons; however, it can also be dangerous if allowed to exist as free $Fe^{2+}$. Appropriate delivery of iron for metabolic needs is assured by transferrin, a major constituent of blood and cerebrospinal fluid. Transferrin also carries iron across the blood brain barrier. When bound to transferrin, iron is in its oxidized form, $Fe^{3+}$; once inside cells, iron is displaced from transferrin in the acidic environment of the endosomes and transported into the cytosol as $Fe^{2+}$. $Fe^{2+}$ is highly reactive and forms free radicals that can cause cellular damage; therefore, cells are normally protected against the deleterious effects of $Fe^{2+}$ by multiple mechanisms. Iron homeostasis is maintained by several processes (Connor et al., 1992a; Connor et al., 1992b; Hirsch and Faucheux, 1998; Jenner and Olanow, 1996; Olanow, 1992): 1) tight regulation of iron entry by controlling the expression of the transferrin receptor, 2) iron chelation by controlling the expression of the intracellular iron-binding protein ferritin, and

3) protection against free radicals by proteins such as superoxide dismutase and glutathione oxidase. Our results suggest that dysregulation of one or more protective mechanisms against iron overload can control neuronal cell number.

Oxidative cell death mediated by iron has been implicated in neurodegenerative diseases such as Parkinson's Disease and Alzheimer's Disease (Beal, 1996; Jenner and Olanow, 1996). The central nervous system contains a large amount of iron (Gerlach et al., 1994), and iron accumulation has been observed in post-mortem brains obtained from Parkinson's and Alzheimer's patients. Altered iron transport in mitochondria has also been implicated as a cause of Friedreich's ataxia (Babcock et al., 1997). We find that dysfunction of normal protective mechanisms against iron overload causes cell death of neurons, but this sensitivity can be regulated. Thus, in addition to playing a role in normal developmental cell death, de-protection of cells against transferrin-mediated iron may also be involved in causing neurodegeneration in adults.

## Acknowledgments

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Note: The manuscript that comprises this chapter was originally written for submission to *Science*. It was not accepted, and due to unexplained reasons, our ciliary ganglion neurons have become unresponsive to the death-inducing activity of $Fe_2Tf$. Therefore, we were unable to conduct additional experiments and resubmit the manuscript at the time when this thesis was written.

# Chapter 2

## Cell Death and Neuronal Replacement During Formation of the Avian Ciliary Ganglion

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## ABSTRACT

Programmed cell death is a prominent feature of embryonic development, and is essential in matching the number of neurons to the target tissues that are innervated. Although a decrease in neuronal number which coincides with peripheral synaptogenesis has been well documented in the avian ciliary ganglion, it has not been clear whether cell death also occurs earlier. We observed TUNEL-positive neurons as early as stage 24, with a large peak at stage 29. This cell death at stage 29 was followed by a statistically significant ($p<0.0001$) decrease in total neuron number at stage 31. The total number of neurons recovered by stage 33/34. This suggested that dying neurons were replaced by new neurons. This replacement process did not involve proliferation because bromodeoxyuridine applied at stages 29 and 31 was unable to label neurons harvested at stage 33/34. The peak of cell death at stage 29 was increased 2.3-fold by removal of the optic vesicle and was reduced by 50% when chCNTF was overexpressed. Taken together, these results suggest that the regulation of neuron number in the ciliary ganglion is a dynamic process involving both cell death and neural replacement from post-mitotic precursors prior to differentiation and innervation of target tissues.

**Keywords: parasympathetic, apoptosis, Hu, TUNEL, cranial neural crest, peripheral nervous system, ciliary neurotrophic factor**

## INTRODUCTION

The normal development of multicellular organisms requires cell death. Cell death is used to eliminate superfluous cells, sculpt tissues, regulate organ size, and to eliminate damaged cells [reviewed in (Vaux and Korsmeyer, 1999)]. Much of the cell death that occurs during development is highly predictable and appears to be "programmed". For example, the development of the nematode C. elegans occurs through a precise, stereotypic pattern of cell divisions and cell deaths of embryonic cells (Ellis and Horvitz, 1986). The term "programmed cell death" was coined to describe the massive loss of intersegmental muscles during insect metamorphosis (Lockshin and Williams, 1965), and is now used to describe most cases of cell death that are actively induced by the expression of specific genes.

In the nervous system, programmed cell death is prominent throughout normal development [for review, see (Nijhawan et al., 2000)]. Cell death occurs during neurogenesis in the ventricular layer of the neural tube (Blaschke et al., 1996; Homma et al., 1994), and neural hypertrophy is observed when this cell death is prevented (Kuida et al., 1996). In the developing spinal cord, three distinct zones of pyknotic nuclear profiles have been observed and these have been postulated to play a role in early phenotypic selection (Homma et al., 1994). By far the best studied cell death coincides with synaptogenesis in post mitotic neurons (Oppenheim, 1991). After neurons have differentiated and extended axons to their targets, 40-60% of the original number are eliminated. The number of neurons that die is not preprogrammed but is affected by the target. For example, fewer neurons survive when the target of innervation is removed, and more neurons are found when extra target is added. Such studies have led to the formulation of the trophic hypothesis, which states that cell death is induced by a limiting amount of neurotrophic factor produced by the target tissues (Barde, 1989). A great deal of support for the trophic hypothesis comes from studies of

nerve growth factor (NGF) and its relatives, the neurotrophins.

One system in which target-dependent neuronal death can be readily studied is the avian ciliary ganglion (CG). The CG is formed by cells that migrate from the neural crest of the caudal mesencephalon and rostral metencephalon (Noden, 1975). The neural precursors withdraw from the mitotic cycle at E5.5 (D'Amico-Martel, 1982) and the CG is numerically complete by St. 31 (Landmesser and Pilar, 1974b). Two types of neurons differentiate within the CG, ciliary and choroid (Marwitt et al., 1971). Both types of CG neurons innervate tissues in the eye, which can be readily removed prior to the formation of the CG. Between St. 35 to St. 40 (E9 to E14), the number of neurons in the CG is reduced by 50% (Landmesser and Pilar, 1974b). This reduction in number coincides with peripheral synaptogenesis, changes in neuronal ion channels, induction of choline acetyltransferase activity, expression of somatostatin in choroid neurons, and gliogenesis (Dryer, 1994; Nishi, 1994). When the optic cup is ablated more than 90% of the neurons die (Landmesser and Pilar, 1974b). In contrast, when an extra eye is transplanted, thereby making more target tissue available, fewer CG neurons die (Narayanan and Narayanan, 1978a). Furthermore, when a portion of the ciliary and iris muscle is denervated by cutting one branch of the ciliary nerve, the resulting free synaptic space is taken over by neighboring ciliary neurons, resulting in a higher degree of survival (Pilar et al., 1980). Neurotrophic factors that support CG neuron survival in cell culture are ciliary neurotrophic factor (CNTF) and glial cell line derived neurotrophic factor (GDNF). Chicken CNTF is expressed in target tissues of ciliary ganglion neurons during and after the period of cell death (Finn and Nishi, 1996a), and CNTF also rescues CG neurons from cell death when overexpressed in the eye (Finn et al., 1998).

In considering the possible mechanisms of target-dependent cell death we sought to

determine the timing of commitment to death with the disappearance of viable neurons. Because previous studies in the CG have usually monitored changes in total neuron number, we focused on identifying dying neurons by using an in situ labeling method for fragmented DNA. To our surprise, we discovered that neurons in the CG were dying at much earlier stages than expected– while neuronal cell loss occurred between St. 35 and St. 40, we observed neurons dying as early as St. 24, with a large peak representing 13% of the total at St. 29. The peak of cell death was followed by a decrement in total neuron number at St. 31, which recovered by St. 33/34 to the number found at St. 29. The peak of cell death observed at St. 29 could be modified by target extirpation as well as overexpression of chCNTF.

## RESULTS

### Cell death of CG neurons occurs during early embryonic development

Dying cells identified by TUNEL, which labeled fragmented DNA, could be seen throughout development in the CG (Figure 1). TUNEL+ nuclei were observed as early as St. 24. To test whether the labeled cells were neuronal, we also stained the same tissue sections with an antibody against HuC/D, a RNA binding protein that is expressed only in neurons (Marusich et al., 1994; Wakamatsu and Weston, 1997). Many of the TUNEL labeled nuclei between St. 26 and St. 33/34 were surrounded by HuC/D+ cytoplasm (Figures 1A-C; E). At these early stages of CG development, we observed many TUNEL+ nuclei that were comparable in diameter to the nuclei of healthy neurons, indicating that dying neurons could be detected prior to the formation of a pyknotic nucleus (Figure 1E).

We quantified the total number of TUNEL+ cells between St. 26 and St. 40 using design-based stereology (Figure 2). The largest peak of cell death was seen at St. 29 [(3,043±113 (n=9); Figure 1A; Figure 2]. The large peak at St. 29 was followed by another one at St. 33/34 [1,703±120 (n=7)], and a third, smaller peak at St. 38 [1,319±165 (n=9)]. Each peak of TUNEL+ nuclei was followed by a significant decrease the following day (Figures 1A-C; Figure 2), suggesting that cells committed to dying were cleared within 24 hours. In addition, the large peak of cell death at St. 29 was followed by a decrease in total neuron number at St. 31 (compare Figures 2 and 4). Thus, the TUNEL reaction most likely labeled cells committed to dying, rather than cells in the process of DNA repair. Many TUNEL+ nuclei were associated with HuC/D+ cytoplasm between St. 24 and St. 34 (Figure 1D and data not shown), indicating that the majority of the dying cells at early stages were neurons. After St. 36, some TUNEL-labeled cells appeared neuronal but were small, and often not associated with HuC/D immunoreactivity, suggesting that they represented apoptotic nuclei

contributed by both neurons and non-neuronal cells in the CG (Figure 1F). At St. 40, there were relatively fewer TUNEL labeled nuclei (422±65, n=8; Figure 2) compared to St. 38, consistent with the observation that developmental cell death in CG ceases by St. 40.

**A re-examination of the CG using islet-1 immunoreactivity confirms that cell number declines between St. 33/34-St. 40**

To determine when cell death occurred relative to a reduction in overall survival, we re-examined neuronal number in the CG. Accordingly, we characterized the staining pattern of a transcription factor, islet-1, which had previously been shown to identify motor neurons (Ericson et al., 1992; Tsuchida et al., 1994) and other peripheral neurons (Avivi and Goldstein, 1999). As early as St. 24 (Figure 3A) we observed islet-1 immunoreactivity in the CG. This suggested that CG neurons expressed islet-1 very early, perhaps as soon as the mesencephalic neural crest cells commenced differentiating as CG neurons. When we examined other embryonic ages, we found that virtually all CG neurons co-expressed islet-1 and HuC/D from St. 24-40, confirming that islet-1 is a valid marker for all CG neurons. In addition, the nuclear localization of islet-1 greatly facilitated the counting of CG neurons, especially at early stages (St. 26-34; Figures 3A, B and data not shown) when neuronal profiles were small and the ganglia were tightly packed with both neuronal and non-neuronal nuclei. At St. 29, both neurons and non-neuronal cells were of similar sizes (6-10 μm) and they could not be distinguished morphologically (Dupin, 1984). We were able to obtain accurate neuronal counts in CG that were younger than St. 33 by using neuronal markers such as HuC/D and islet-1. At later ages, the volume of the CG expanded considerably and neurons were spaced further apart (compare Figures 3A, 3B to 3C, 3D), presumably because of the proliferation and differentiation of the non-neuronal cells in the CG.

We quantified the total number of CG neurons from staged embryos (Hamburger and

Hamilton, 1951) between St. 26 and St. 40 (Figure 4). The number of neurons in the CG increased from 10,344±731 (n=11) at St. 26/27 (E5) to 17,502±462 (n=8) at St. 29. This increase presumably represented the differentiation of neurons from neural crest cell committed to the neural lineage (D'Amico-Martel, 1982). The peak in neuronal number at St. 29 was followed by a decrease in neuronal number to 12,701±331 (n=8) at St. 31, but the number returned to 17,714±1193 ( n=10) by St. 33/34. The lowered number of neurons at St. 31 was statistically significant from the number observed at St. 29 and St. 33/34 by using the Student t-test ($p<0.0001$). In confirmation of previous observations (Finn et al., 1998; Furber et al., 1987; Landmesser and Pilar, 1974b), we saw a 50% reduction in the number of neurons between St. 33/34 and St. 40.

**Dying neurons are replaced by post-mitotic precursors**

The cell death we observed at St. 29 together with the statistically significant reduction in total neuron number from St. 29 to St. 31, which recovered by St. 33/34, suggested that dying neurons could be replaced by newly differentiated cells. Previous studies by several groups have shown that CG neurons were not labeled if [$^3$H]thymidine is injected into embryos after E5.5 (D'Amico-Martel, 1982; Rohrer, 1987; Sechrist, 1998). It was possible that some newly generated neurons were not detected due to the limited ability of [$^3$H]thymidine to penetrate photographic emulsion; thus we tested whether dying neurons were replaced from a proliferating neural precursor by applying bromodeoxyuridine (BrDU) into chick embryos at various stages of development and examined St. 33/34 CG for co-localization of BrDU with HuC/D-immunoreactivity. When BrDU was applied at St. 24, many neuronal nuclei were BrDU positive (Figure 5A); application of BrDU at St. 26/27 resulted in a mixture of labeling in neurons and non-neuronal cells (Figure 5B). In contrast, when BrDU was injected at St. 29 or St. 31, many small, non-neuronal nuclei could be seen labeled (Figures 5C and D), but none of the BrDU immunoreactivity was co-localized with HuC/D immunoreactivity.

Thus, the neurons that are formed between St. 31 and St. 33/34 do not arise from a proliferating cell population.

As a first step towards identifying the precursor population from which the CG neurons are generated, we double-stained St. 29 CG with antibodies against HuA and HuC/D. Previous studies have shown that HuA is expressed in uncommitted neural crest cells and differentiating neurons, but not in glia of the developing avian dorsal root ganglion (Wakamatsu and Weston, 1997). We found that a subpopulation of the non-neuronal cells in the CG express HuA, while neurons express both HuA and HuC/D (Figure 6). Interestingly, the HuA+ /HuC/D- cells are often adjacent to neurons, suggesting that direct cell/cell interactions may regulate the replacement process. The spaces lacking immunoreactivity are filled with nuclei, presumably of glial cells or glial precursors (data not shown).

**Cell death at St. 29 is increased by ablation of the optic vesicle and reduced by chCNTF**

In order to test whether target deprivation caused an increase in CG neurons dying at St. 29, we ablated the developing optic vesicle at St. 11-13 and counted the number of TUNEL+ neurons at St. 29. Operated embryos developed normally with the exception that little or no eye formed on one side. CG that were processed looked normal and could be identified in the tissue by HuC/D immunoreactivity. We found that there were 2.3-fold more dying cells on the operated side compared to the contralateral side (Figure 7). TUNEL+ nuclei on the operated side were often found associated with HuC/D+ cytoplasm, indicating that the additional dying cells were neuronal.

We next asked if the cell death at St. 29 could be modified by availability of trophic factor. We have previously purified and cloned a chicken ciliary neurotrophic factor

(chCNTF; (Leung et al., 1992)). St. 29 CG neurons expressed chCNTF receptor a (chCNTFRa) in vitro (Figures 8A and 8B) and in vivo (Figures 8C and 8D). We then tested whether chCNTF could support the survival of St. 29 CG in cell culture. In the absence of chCNTF, all St. 29 neurons were dead 24 hours after plating, but when recombinant chCNTF (10 ng/ml) was added, many neurons survived and they appeared phase-bright and healthy (Figure 8E and 8F). These neurons were identified by their morphology as well as immunoreactivity for the neuron-specific markers HuC/D and islet-1 (Figure 7C and data not shown).

We overexpressed chCNTF in chick embryos by using the replication competent retroviral vector RCASBP(A). Control embryos received injections of RCASBP(A) encoding green fluorescent protein (GFP). We then harvested CG at St. 29 and quantified the TUNEL+ cells. RCASBP(A)-chCNTF infected embryos had a 50% decrease in the number of TUNEL+ cells compared to the RCASBP(A)-GFP controls (Figure 9).

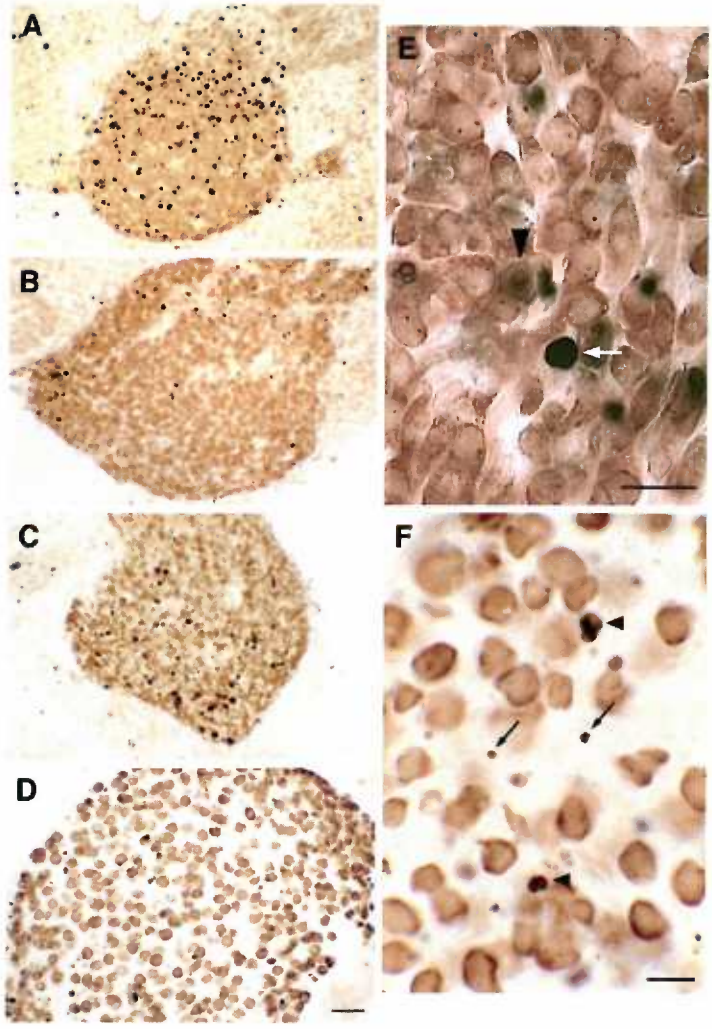


**Figure 1. Pattern of dying cells in the ciliary ganglion.**
CG were collected and processed for TUNEL and anti-HuC/D immunocytochemistry various embryonic ages. TUNEL labels are black and nuclear; HuC/D immunoreactivity is brown, cytoplasmic, and neuron-specific. (A-D) Low magnification photomicrographs St. 29, 31, 33/34, and 38 CG that were processed for TUNEL and HuC/D immunocytochemistry. (A) At St. 29, a large number of TUNEL+ cells could be seen throughout the CG. (B) At St. 31, the number of dying cells was low compared to other stages (A, C, D). (C) There are more dying cells at St. 33/34 compared to St. 31 (B) but less than St. 29 (A). (D) TUNEL+ nuclei of varying sizes could be seen at St. 38 CG. (E) Higher magnification of TUNEL- and HuC/D- labeled St. 29 CG showing that some TUNEL-labeled nuclei were as large as intact neuronal nuclei (white arrow) and some of them could be observed within a HuC/D-positive cytoplasm (black arrowhead). (F)Higher magnification of TUNEL- and HuC/D- labeled CG at St. 38 (E12) showing some TUNEL-labeled nuclei close to other neurons with nuclei roughly the same size as a neuron (arrowheads). There were also a number of TUNEL-labeled nuclei that were much smaller and located in areas that were devoid of neurons (small arrows).

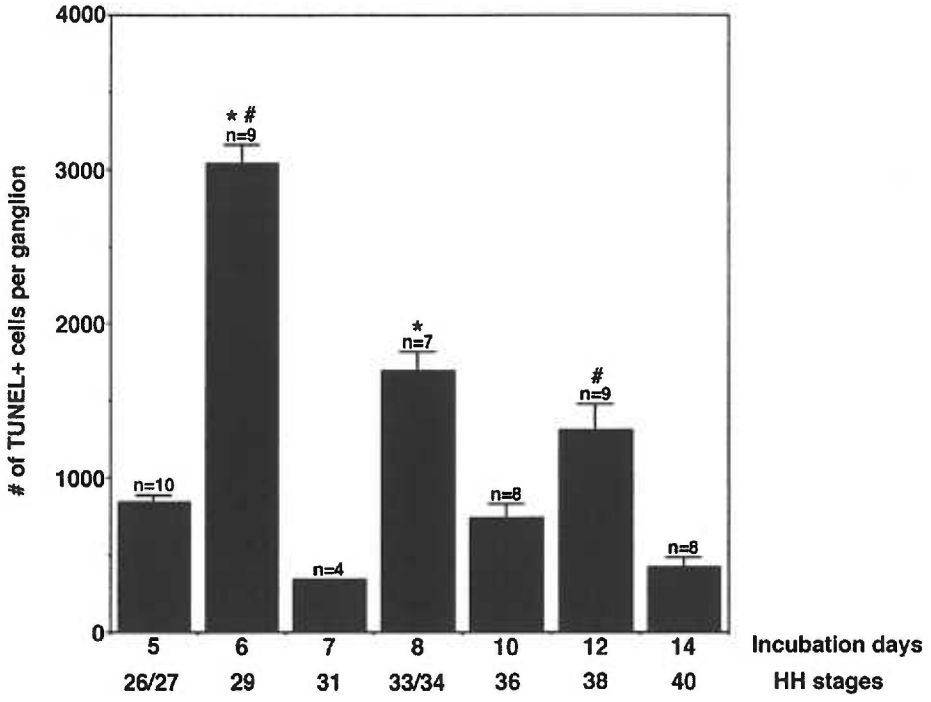


**Figure 2. Quantification of TUNEL labeled cells in the developing CG.**
The total number of TUNEL+ cells in St. 26-40 CG were counted using design-based stereology and their values represented as histograms (means ± s.e.m.). Cell death could be seen throughout CG development. The largest peak of TUNEL+ cells was observed at St. 29. A second peak appeared at St. 33/34 (value between St. 29 and St. 33/34; *$p<0.0001$, Student t-test). At St. 31, there were many fewer dying cells compared to St. 29 and St. 33/34. At St. 38 there was another wave of dying cells, but the value was smaller compared to St. 29 (#$p<0.0001$, Student t-test). The number of CG counted for each time point (n) is listed on top of each bar; y-error bars = standard error of means (s.e.m.).

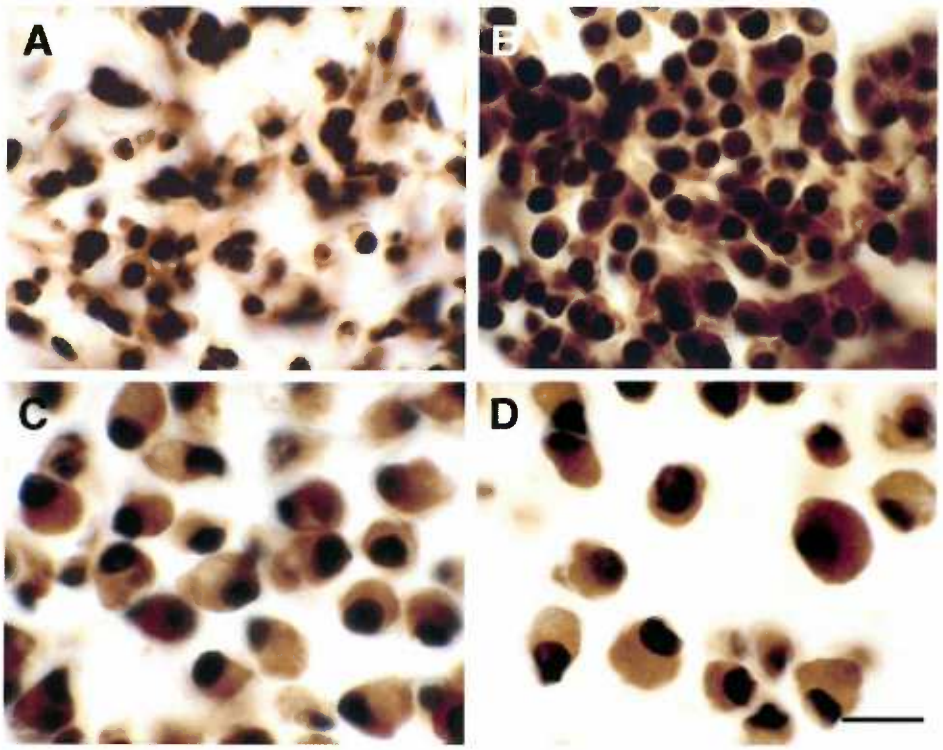


**Figure 3. CG neurons express islet-1 throughout development.**
CG neurons expressed both islet-1 and HuC/D as early as St. 24 and persisted until St. 40. CG sections were processed for anti-islet-1 (black) and anti-HuC/D (brown) immunoreactivity. Anti-islet-1 immunoreactivity was nuclear while anti-HuC/D was cytoplasmic. At all stages examined virtually all of the islet-1 positive nuclei were located within cell bodies expressing HuC/D immunoreactivity. (A) At St. 24, anti-islet-1 and HuC/D identified neurons as soon as they commenced differentiation. (B) At St. 29, CG neurons were tightly packed together, but the dark, nuclear staining of anti-islet-1 allowed easy identification and greatly facilitated counting individual neurons. (C) St. 36 CG (D) St. 38 CG. After St. 36, the volume of CG increased, with a corresponding increase in the size and separation of islet-1+ neurons (compare D with B). Panels A-D were taken at the same magnification, scalebar = 20 $\mu$m.

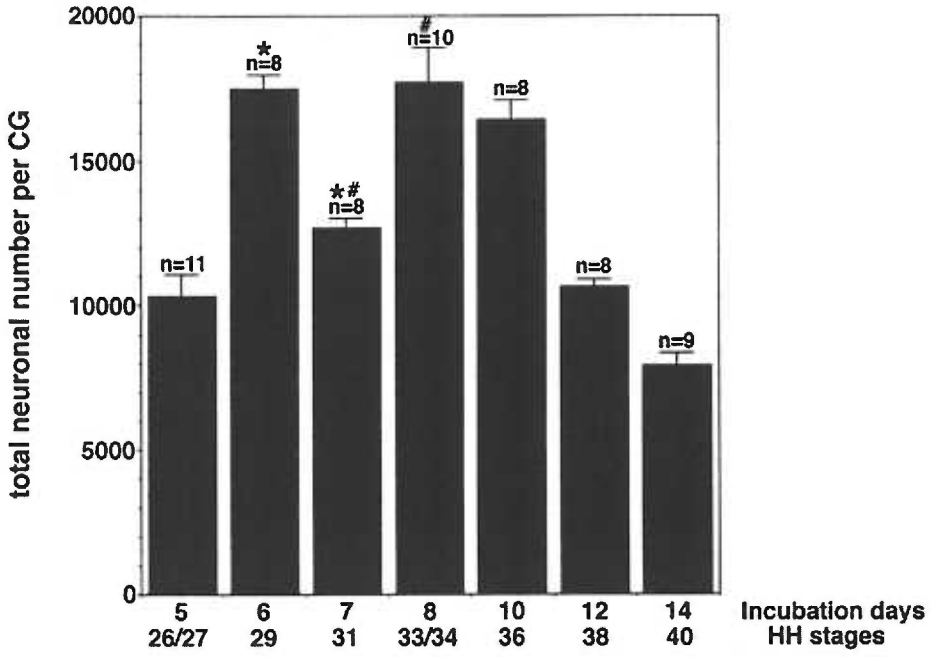


**Figure 4. Quantification of neuronal number in the developing CG.**
Total number of islet-1 positive neurons between St. 26-40 were counted using design-based stereology. The maximum number of neurons was observed at St. 29 (17,502±462) followed by a decrease at St. 31 (12,701±331; value between St. 29 and St. 31; *$p<0.0001$, Student t-test); however, by St. 33/34 the neuronal number returned to a level comparable to St. 29 (17,714±1193; value between St. 31 and St. 33/34; #$p<0.001$, Student t-test). Between St. 33/34 to St. 40 the number of neurons decreased by 55%. The number of CG counted for each time point (n) is listed on top of each bar; y-error bars = s.e.m..

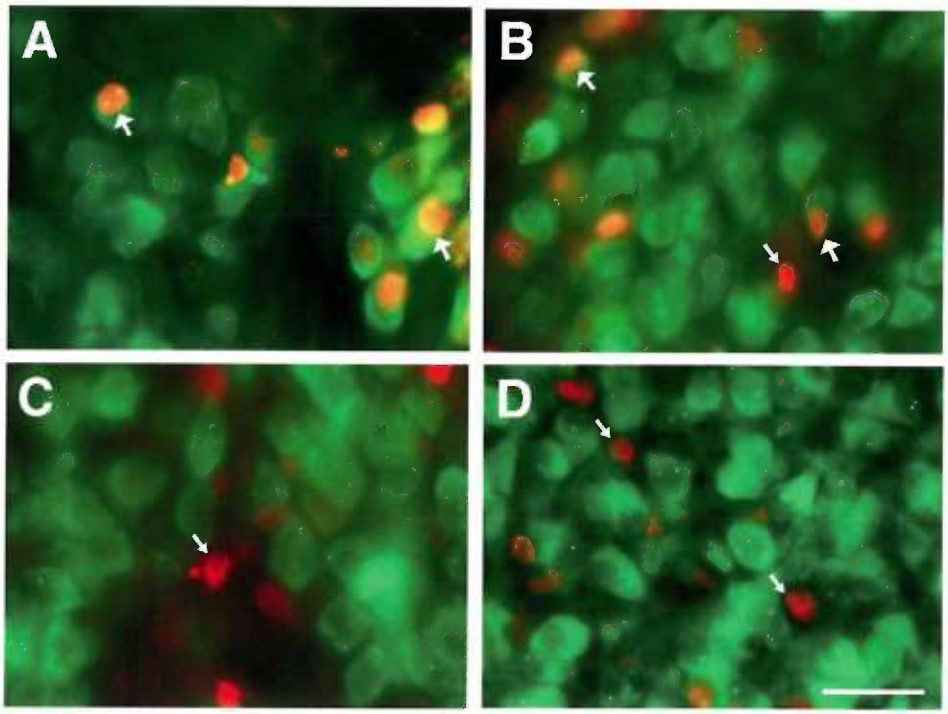


**Figure 5. No CG neurons were generated by proliferation after St. 29.**
Chick embryos were treated with BrDU at St. 24 (A), St. 26/27 (B), St. 29 (C), and St. 31 (D) and CG harvested at St. 33/34. (A-B) BrDU-positive nuclei (red) could be seen within HuC/D-positive neurons (green) when embryos were pulsed with BrDU at St. 24 and St. 26/27 (A and B, big white arrows). (A) When BrDU was added onto St. 24 embryos, BrDU+ neurons (HuC/D+) could be observed at St. 33/34 (big white arrows). (B) When BrDU was added onto St. 26/27 embryos, BrDU+/HuC/D+ neurons (big white arrows) as wells as BrDU+ only nuclei (small white arrow) could be observed, suggesting that some neurons and non-neuronal cells were dividing at that age. (C-D) When we pulsed embryos with BrDU at or after St. 29, no BrDU+/HuC/D+ neurons could be labeled. Small arrows in C and D pointed to BrDU+ only nuclei, suggesting that no CG neurons underwent cell division after St. 29. (C) BrDU added onto St. 29 embryos yielded no BrDU+ neurons at St. 33/34. (D) BrDU applied at St. 31 also did not result in BrDU+ neurons. Red = BrDU, green =.HuC/D, scalebar = 20 $\mu$m.

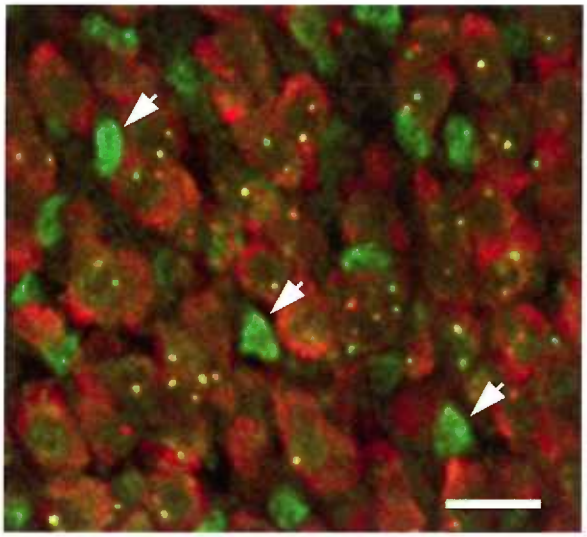

**Figure 6. St. 29 CG contains a subpopulation of cells that express only HuA, a marker of undifferentiated cells.**
St. 29 chick CG were fixed, cryostat sectioned, and double stained for HuA (green) and HuC/D (red). Sections were imaged by confocal microscopy. While all differentiated neurons expressed both HuA and HuC/D antigens, many singly labeled HuA cells could be observed in the ganglion (white arrows). These HuA+, HuC/D- cells may be the cells that replace CG neurons that die at St. 29. Scalebar = 10 $\mu$m.

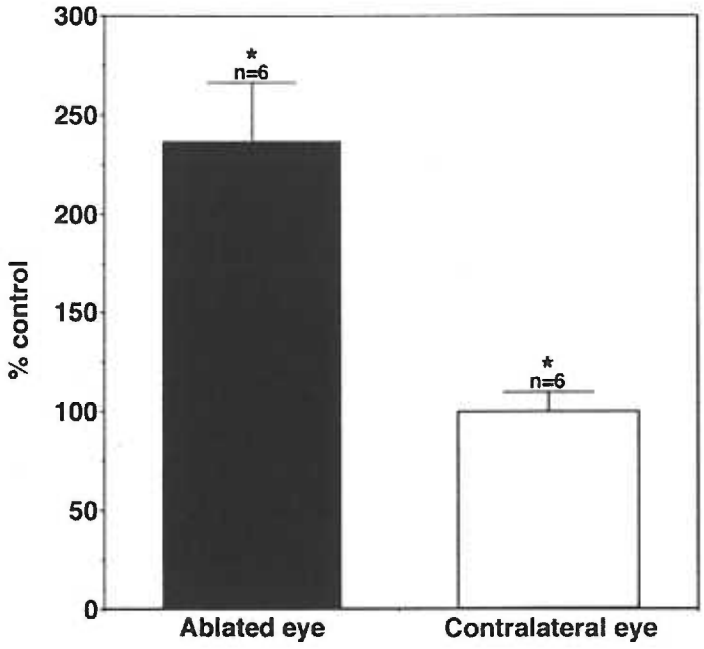


**Figure 7. Eye ablation exacerbated cell death at St. 29.**
Optic vesicles were removed from one eye of E2 (St. 11-13) chick embryos and the other side was left unperturbed as controls. Embryos were collected at St. 29 and their CG processed for the TUNEL assay and anti-HuC/D immunocytochemistry. The total number of TUNEL+ cells was counted in CG from ablated eyes and contralateral controls. The number of TUNEL+ cells in the ablated eye was represented as a percentage of controls. There were 2.3-fold more dying cells in the ablated side compared to the control side (*$p<0.001$, Student t-test). The number of CG counted for each time point (n) is listed on top of each bar; y-error bars = s.e.m..

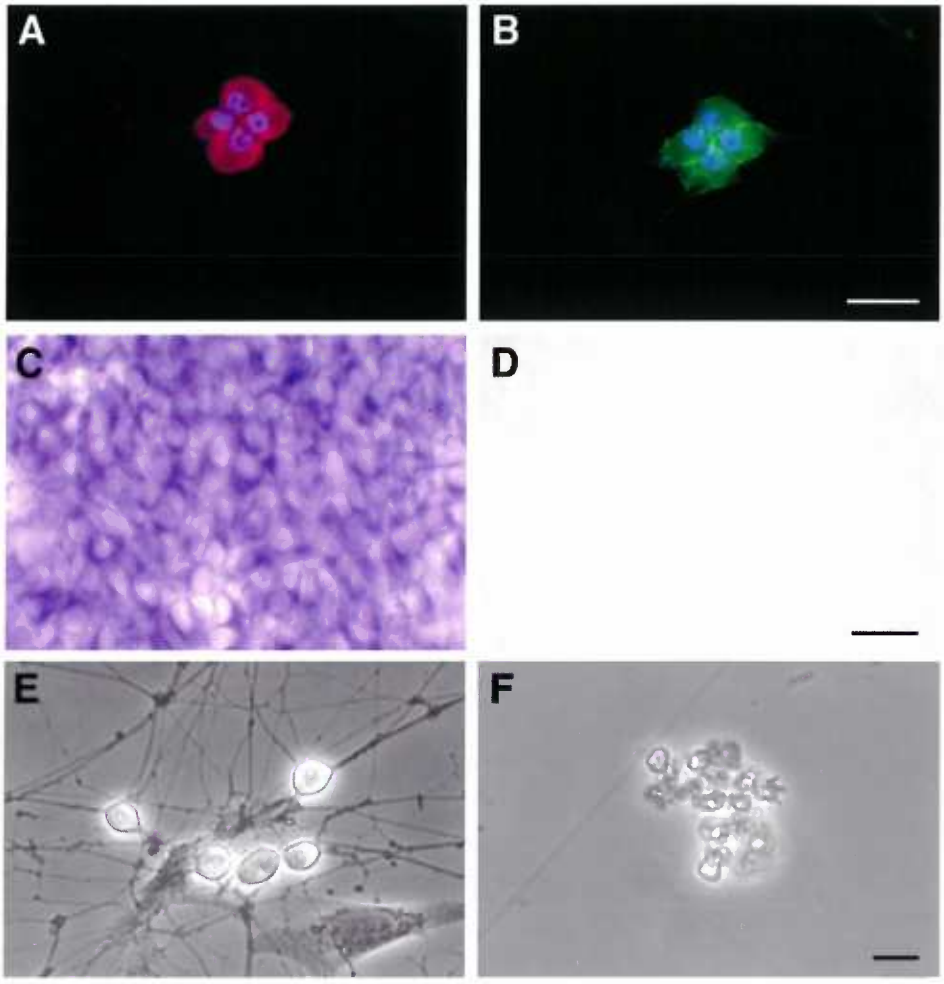


**Figure 8. St. 29 CG neurons express CNTFRα and are dependent upon CNTF for survival in cell culture.**

St. 29 CG neurons expressed chCNTF receptor α (chCNTFRα) in vitro. (A) CG neurons identified by HuC/D immunoreactivity (red). (B) Same field of view showing neurons expressing chCNTFRα immunoreactivity (green). Transcripts for chCNTF receptor α could be observed in vivo by in situ hybridization with digoxigenin-labeled riboprobe for chCNTFRα. (C) Hybridization with the anti-sense probe yielded cytoplasmic staining in St. 29 CG neurons. (D) No signal was obtained when sense probe was used in the hybridization. Expression of chCNTFRα is functional- when St. 29 cultures were seeded with (E) or without (F) 10 ng/ml of recombinant chCNTF, neuronal survival was supported. In CG cultures where chCNTF was omitted all neurons were dead the following day. Scalebar in A and B = 20 $\mu$m; Scalebar in C and D = 20 $\mu$m.; Scalebar in E and F = 50 $\mu$m.

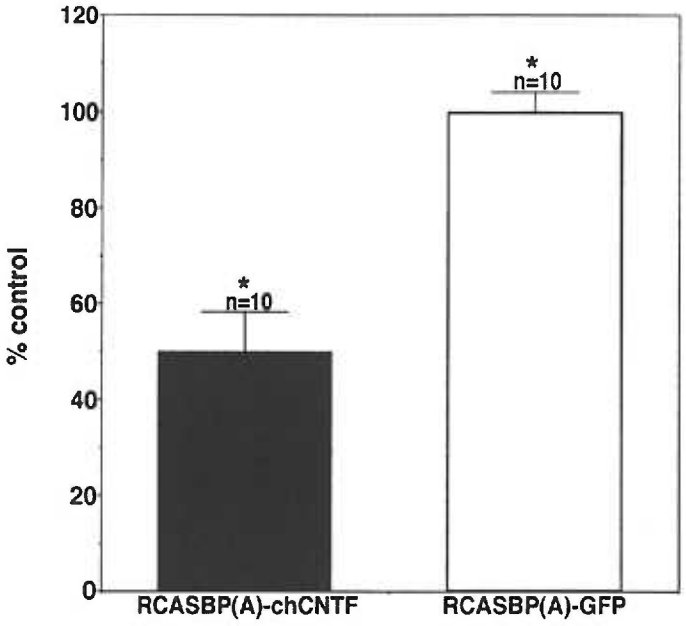


**Figure 9. Overexpression of chCNTF reduced cell death at St. 29.**
Retroviruses were injected into the neural tube at stage 8-9 in chick embryos. CG were harvested and processed for TUNEL and anti-HuC/D immunocytochemistry at St. 29. Total number of TUNEL+ cells in the CG from RCASBP(A)-chCNTF and RCASBP(A)-GFP injected embryos were quantified and represented as percent control in the histogram. The number of dying cells at St. 29 decreased by about 50% in embryos where chCNTF was overexpressed compared to the GFP-infected controls (*$p<0.0001$, Student t-test). The number of CG counted (n) is listed on top of each column, error bars = s.e.m..

## DISCUSSION

Our studies reveal new and unexpected data on the regulation of neuron number in the avian ciliary ganglion. First, we have discovered that there are significant numbers of dying neurons in the CG at St. 29, several days prior to the period of time that an actual decrement in cell number had been previously described (Furber, 1987; Landmesser, 1974b). Second, while there is a transient decrease in neuron number at St. 31 following the large amount of cell death at St. 29, neuron number recovers by St. 33/34, suggesting that there is a homeostatic mechanism regulating cell number in the CG. Third, the large amount of cell death at St. 29 is increased if the optic vesicle is removed, and decreased by the overexpression of chCNTF, a trophic molecule for CG neurons.

The peak of cell death that we observed in the CG did not coincide with the greatest period of neuronal cell loss, nor with functional innervation of CG targets. This differs significantly from observations in other peripheral neurons. In spinal ganglia, the number of pyknotic nuclei peaked between St. 26-29, which coincides with functional innervation of the limb muscle by proprioceptors located in the ventrolateral region (Hamburger and Levi-Montalcini, 1949). A similar coincidence in the peak of neural degeneration with innervation of muscle has been observed in spinal cord motorneurons (Chu-Wang and Oppenheim, 1978). In part, our ability to detect cell death in the CG prior to peripheral synaptogenesis may have been due to the greater sensitivity of the TUNEL method; however, our quantification clearly shows that cell death in the CG is highest at this earlier time (St. 29). It may be possible that degenerating cell bodies persist slightly longer at St. 29, leading to a larger number that are counted; but our quantification also shows that the peaks of TUNEL labeling are followed by significant decreases in the number of TUNEL labeled cells within 24 hours, suggesting that changes in clearance rate must be relatively subtle.

The peak of CG cell death at St. 29 also does not coincide with a period of rapid proliferation and neurogenesis, as it does in the developing central nervous system (CNS). In the CNS, an amplified TUNEL method, ISEL+, revealed a startlingly high number of dying cells during early neurogenesis (Blaschke et al., 1996; Blaschke et al., 1998). As a consequence, it was estimated that >70% of the neurons generated in the neural tube could be undergoing cell death during differentiation. Although this observation remains controversial, subsequent studies of transgenic mice lacking genes encoding caspases –3 or –9 have shown that morphological development of the CNS is disrupted by a marked hyperplasia (Kuida et al., 1998; Kuida et al., 1996). In contrast, the cell death we observe is highest after all CG neurons have undergone their last cell division, as we have confirmed with our BrDU labeling (D'Amico-Martel, 1982; Rohrer, 1987; Sechrist, 1998). In dorsal root ganglia (Hamburger and Levi-Montalcini, 1949), as well as in sympathetic ganglia, proliferation and differentiation of neuroblasts is concurrent with target-dependent cell death. This differs significantly with our observations in a parasympathetic ganglion, as well as with observations in spinal cord motorneurons.

Our data suggest a homeostatic replacement of dying neurons from post-mitotic precursors. The large amount of cell death at St. 29 was followed by a significant decrease in total neuron number at St. 31, which rebounded at St. 33/34 to the total neuron number observed at St. 29. Similar observations of homeostasis in final ganglion size were reported in previous studies that manipulated neural crest cell number. For example, when an excess of neural crest cells are introduced by injection (Bronner-Fraser and Cohen, 1980) or induced by overexpressing Noelin-1 (Barembaum et al., 2000), the overall range and size of neural crest derivatives is unchanged. A likely source of the post-mitotic precursors are cells from the St. 29 CG that express both neuronal and non-neuronal markers (Rohrer and Sommer, 1983). Another possibility is

that the CG neurons are replaced by multipotent stem cells (Shah and Anderson, 1997) or undifferentiated precursors that are quiescent at St. 29. Intriguingly, a subpopulation of non-neuronal cells at St. 29 expresses HuA. In previous studies, (Wakamatsu and Weston, 1997) showed that HuA was expressed at high levels in undifferentiated cells of the embryonic avian dorsal root ganglion, but not expressed in glia. Once cells commenced differentiation into neurons by upregulating HuC/D, HuA was also expressed. Thus, the HuA immunoreactivity in the non-neuronal (lacking expression of HuC/D) population of the St. 29 CG may represent cells that have the capacity to replace dying neurons. Regardless of identity, the number of replacement cells is apparently limited because dying cells out-number replacement cells between St. 34- 40, resulting in a net decrease of neurons in the CG. This is reminscent of the restrictions in plasticity of non-neuronal crest cells in the CG observed in backtransplant experiments (Dupin, 1984; Sechrist, 1998).

If neurons are dying at St. 29, several days before target tissues have differentiated and functional synapses have been established, then what causes this early phase of cell death? One possibility is that neural crest derivatives that migrate to the wrong location may be induced to die by the local environment, as suggested for neurogenic precursors that aberrantly migrate along the lateral pathway normally occupied by melanocytes (Wakamatsu et al., 1998). We think this is unlikely because many TUNEL+ nuclei at St. 26/27 are found in HuC/D+ positive cells, indicating that neural differentiation has commenced, an appropriate fate for the crest destined to become the CG. Another possibility is that neurogenic precursors that differentiate into an inappropriate subtype of neuron are eliminated. For example, if cranial neural crest cells differentiate as sensory or sympathetic neurons instead of parasympathetic neurons, they may die because they lack an appropriate neurotrophic factor. This is an interesting possibility that can be tested when transcription factors that distinguish between parasympathetic and

sympathetic neurons are identified. Islet-1, the marker we used to identify neurons in the CG is also expressed in sensory and sympathetic ganglia (Avivi and Goldstein, 1999). In preliminary studies, we find that CG neurons express phox-2, a transcription factor once thought to be selective for sympathetic neurons. A final possibility is that choroid or ciliary neurons that have failed to project axons through the appropriate nerves at St. 29 die. However, there is no independent early marker for the choroid or ciliary subpopulations at St. 29; thus, there is no way to determine by retrograde labeling whether the dying cells have actually projected axons out the "incorrect" nerve. In addition, cell death is not used as a correction mechanism in the motor neuron system (Oppenheim, 1981).

Consistent with previous studies (Furber et al., 1987; Landmesser and Pilar, 1974b), we saw a 50% decrease in neuron between St. 33/34 to St. 40, although our cell counts differed. The number of neurons we obtained at St. 33/34 was considerably higher than previous estimates (17,000 prior to cell death versus 6500); however, other studies incorporated a correction factor (Abercrombie, 1946) that could have underestimated the neuronal number. The larger number we found corroborates the higher numbers of St. 33/34 CG neurons recovered in cell culture (Nishi and Berg, 1979; Tuttle et al., 1980). We also found that the absolute number of neurons within the CG differed according to the strain of chicken from which the fertilized eggs were taken. We consistently saw >50% more neurons per CG from the New Hampshire Red X White Leghorn hybrids in comparison to embryos from a pure White Leghorn stock.

It is unclear if the control of cell death at St. 29 is similar to that between St. 33/34 to St. 40. Our experiments revealed that the large amount of cell death at St. 29 was influenced by the environment—target ablation at St. 11-13 (E2) exacerbated cell death and overexpression of chCNTF reduced cell death. These are the hallmarks of target-

dependent cell death, yet it is unlikely to be regulated by interactions involving synaptogenesis, since few peripheral synapses have formed at St. 29 (Meriney and Pilar, 1987; Pilar et al., 1987). However, the neurons dying at St. 29 are likely to have extended neurites into the optic cup towards their future target tissues, since Landmesser observed that ciliary and choroid nerves could conduct action potentials as early as St. 25 (Landmesser and Pilar, 1972). Thus, the removal of the optic vesicle at E2 could have disrupted the environment in which CG axons pathfind. For example, GDNF produced by the optic vesicle has been proposed to promote neurite outgrowth (Hashino et al., 2000). Disruption of local GDNF concentrations may have exacerbated cell death. Alternatively, we have shown that St. 29 CG neurons are CNTF-dependent; thus, the developing optic cup may express chCNTF, which is necessary for supporting CG neuron survival. Consistent with this hypothesis, we have observed chCNTF expression in the eye at St. 29 (Finn and Nishi, unpublished results) and overexpressing chCNTF decreased cell death significantly at St. 29. Interestingly, both optic vesicle ablation (Landmesser and Pilar, 1974a) and overexpression of chCNTF (Finn et al., 1998) do not alter the total number of neurons at St. 34, but result in significant changes by St. 40. The latency observed in total neuronal cell number could be due to the compensation for neuronal loss by the precursor population.

In summary, these studies reveal a complex pattern of cell death during CG development and suggest that an internal homeostatic mechanism controls neuron number early in development. This dynamic process of cell death and replacement prior to peripheral synaptogenesis should be taken into consideration in future studies of cell death in the CG. For example, manipulations that exacerbate early cell death could deplete the number of postmitotic precursors, leading to changes in final neuron number in the mature CG. It will be of interest to determine whether similar mechanisms are in operation in other nervous tissues.

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# Chapter 3

## Non-neuronal cells in the ciliary ganglion have the potential to differentiate into neurons

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**Abstract**

Neural crest cells are multipotent precursor cells that can give rise to a wide variety of cell types including peripheral neurons and glia. We have previously shown that neuronal cell death in St. 29 to St. 34 ciliary ganglia is offset by cell replacement from post-mitotic precursors. The present study characterizes the cellular populations in St. 29 CG and establishes that non-neuronal cells in the ganglion are able to differentiate into neurons. A subpopulation of the non-neuronal cells do not express either glial- or neuron- specific markers. Instead they express a neural crest marker, p75 and some of them also express HuA, a neural precursor marker. We isolated and purified non-neuronal cells from St. 29 quail ciliary ganglion and backtransplanted them into the migratory pathway of the ciliary ganglion neural crest cells. The injected cells homed to the ciliary ganglion and some differentiated into neurons. Taken together, our culture and transplantation studies suggest that non-neuronal cells in the ciliary ganglion have the potential to become neurons given the appropriate environment.

## Introduction

The neural crest is a transient structure originating from the dorsal neural tube. Neural crest cells migrate along precise pathways to different targets and give rise to a wide range of tissue including the peripheral nervous system (PNS). Through successive lineage restrictions, neural crest cells that have homed to their target organs will differentiate into different cell types depending on their surrounding environments (reviewed in Garcia-Castro and Bronner-Fraser, 1999; Le Douarin et al., 1993).

One of most fascinating aspects of neural crest cells is how a seemingly homogeneous population of cells can give rise to such a wide range of cell types. Importantly, when does the neural crest segregate into different lineages? It is unclear whether all crest cells that leave the neural tube are pluripotent or if some have already committed to a particular fate. By injecting a lineage tracer in the neural tube and into neural crest cells shortly after they have emigrated, *in vivo* lineage analysis showed that many premigratory and migratory crest cells are multipotent ( Bronner-Fraser and Fraser, 1988; Bronner-Fraser and Fraser, 1989; Fraser and Bronner-Fraser, 1991). On the other hand, other studies suggest that migrating crest cells are heterogeneous in nature. Byperforming *in vitro* clonal analysis where the fate of a single neural crest can be followed, several studies have shown that individual crest cells can generate clones that contain different cell number and cell types (Baroffio et al., 1988; Duff et al., 1991; Ito and Sieber-Blum, 1991). Recent evidence reveals that some neural crest cells are committed very shortly after emigration from the neural tube. Neurogenin, which specifies neural crest cells into sensory neurons only, is expressed as soon as crest cells starts migrating. Taken together, these data suggest that neural crest cells begin as multipotent precursors but their developmental potential may be progressively restricted during migration until they finally commit to one cell type upon arrival to their target destination. On the other hand,

even though some migrating crest cells may be specified, there is evidence to suggest that environmental factors can influence their cell fate decisions. In zebrafish, the Wnt signaling pathway has been shown to promote melanocyte production (Dorsky et al., 1998). When they activated the Wnt pathway in lineage restricted crest cells, they demonstrated that more pigment cells were generated at the expense of neurons. These experiment suggest some crest cells that have been specified are still plastic and extrinsic factors can be sufficient in the instruction of cell fates. Finally, differences in neural crest cell fates can also be determined by their origin. Even though neurons, glia and melanocytes can be derived from all trunk levels, some cell types are unique to certain axial levels. For example, cranial crests can form cartilage, and in fish and amphibians, trunk crest cells can form the dorsal fin (Dorsky et al., 2000).

Not only do the migrating neural crests have diverse developmental potential, the cells that have reached the peripheral ganglia are also heterogeneous. Neural crest cells that have incorporated into their respective ganglia may not express all their possible phenotypes but instead will display characteristics that are promoted by their local environments. However, alternative cell fates may be evoked when challenged in a new environment. This has been demonstrated by *in vivo* transplantations where quail peripheral ganglia isolated from different ages were backtransplanted into the neural crest migration pathway of younger chick host embryos (Ayer-Le Lievre and Le Douarin, 1982; Fontaine-Perus et al., 1988; Le Douarin et al., 1978; Le Lievre et al., 1980; Rohrer et al., 1986; Schweizer et al., 1983). The quail-chick chimera system has been particularly useful because donor and host cells can be distinguished easily. Quail nuclei are denser than those of the chicken and can be identified readily by common nuclear dyes such as hematoxylin and Hoechst. Furthermore, a specific antibody, anti-QCPN, has been generated which recognizes all quail, but not chick cells (Le Douarin et al., 1996).

The avian ciliary ganglion (CG) is a parasympathetic ganglion where both neurons and non-neuronal cells are derived from the mesencephalic crest (Hammond and Yntema, 1958; Narayanan and Narayanan, 1978b; Noden, 1978). By St. 24, the CG has coalesced behind the eye and formed a discrete ganglion. All the CG neurons are born by St. 28 (D'Amico-Martel, 1982; Dupin, 1984; Rohrer and Thoenen, 1987; Sechrist et al., 1998) and they undergo cell death beginning at St. 33/34 and by St. 40, only 50% of the neurons generated remain in the ganglion. We have shown previously that CG neurons also undergo a wave of cell death at St. 29 followed by a drop in neuronal number by St. 31. Interestingly, by St. 33/34 the number of neurons returns to a level that is similar to St. 29 (Lee et al., 2001). Because there are no CG neurons generated by neuroblast division after St. 28, we hypothesize that a post-mitotic population of non-neuronal cells in the CG can differentiate into neurons. We have examined both neuronsand non-neuronal cells carefully in culture and found that neural precursors may exist, consistent with previous studies which suggest that non-neuronal cells in the CG are likely to be multipotent. However, those studies implanted pieces of CG that contained neurons and non-neuronal cells. Eeven though CG neurons were not expected to survive, this possibility cannot be eliminated (Dupin, 1984; Le Douarin et al., 1978; Le Lievre et al., 1980). By isolating non-neuronal cells from St. 29 quail CG and injecting them into E2 chick hosts, we were able to observe neurons that were of quail origin. This suggests that a subpopulation of the non-neuronal cells in the CG are neurogenic and can differentiate into neurons when they are placed in permissive environments.

## Results

### Characterization of cellular markers in St. 29 ciliary ganglion

One of the difficulties in identifying precursor cells is that they may not express many unique antigens. To characterize different cell types and define their marker repertoires in St. 29 CG, we generated dissociated cell cultures and labeled them with various cellular markers. When we immunostained St. 29 CG cultures with an antibody that is specific to the chick p75 neurotrophin receptor (p75, Weskamp and Reichardt, 1991), we found that virtually all cells, neurons and non-neuronal, were p75+ (Table 1). This is consistent with previous reports that the p75 receptor is expressed in neural crest cells and their derivatives (Heuer et al., 1990). To identify non-neuronal cells in St. 29 CG cultures, we labeled cultures with a glial cell marker, anti-O4 (Rohrer and Thoenen, 1987; Sommer and Schachner, 1981). A subset of p75+ cells was O4+ (Table 1) and no O4+ cells were neuronal. O4 identifies cells that have committed to a glial lineage and a small number of cells in St. 29 CG belong to this category. When we labeled St. 38 CG cultures, there were many more O4+ cells compared to St. 29 (data not shown), consistent with the notion that glial development begins in the CG later in gangliogenesis than does neural differentiation. To further characterize the non-neuronal cells in the CG, we used another glial cell marker, 7B3. 7B3 has been shown to localize neural crest cells that have been restricted to a glial cell fate (Henion et al., 2000). Consistent with the reported findings, anti-7B3 did not label any neurons in St. 29 CG cultures, rather it stained a subset of p75+ cells that were flat and non-neuronal (Table 1). The number of 7B3+ cells was also greater in St. 38 than in St. 29, similar to the expression of anti-O4. We could not determine, however, if the O4+ cells are the same as 7B3+ cells because both antibodies are IgM, making double-labeling unfeasible.

All neurons in St. 29 CG were labeled by the pan-neuronal marker, anti-HuC/D. When

we double-labeled CG cultures with anti-p75 and anti-HuC/D, all HuC/D+ neurons were also p75+. CG neurons were also immunoreactive for other common neuronal markers such as neurofilament and neuron-specific tubulin (βIII tubulin, data not shown). We chose to use HuC/D because it stains the neuronal somata clearly and intensely, allowing unambiguous assessment of co-localization with other markers. Anti-HuC/D stained all neurons in CG cultures that had distinct neuronal morphology—rounded cell bodies and elongated processes. Interestingly a small subset of HuC/D+ cells were flat and did not have any extended neurites (see below; Figure 2, Table 2), suggesting that they might be immature neurons that have not yet acquired all of the neuronal characteristics.

To identify potential neuronal precursor cells in St. 29, we labeled CG cultures with an antibody against HuA. Previous studies have shown that HuA is expressed in neural crest cells before neural differentiation has commenced and is then upregulated again in differentiated neurons. HuA immunoreactivity is not associated with glia (Wakamatsu and Weston, 1997), suggesting that it may be a useful marker for neural precursors. We have shown that HuA is expressed in all neurons (HuC/D+) and as well as in a subset of non-neuronal cells in St. 29 CG (Figure 1A and Lee et al., 2001). We obtained similar results when we examined the staining pattern of HuA in culture—all HuC/D+ neurons were HuA+ and there were HuC/D–, HuA+ cells. Moreover, many HuC/D–, HuA+ cells were p75+ as well, suggesting that they may be undifferentiated neural crest cells that have neurogenic potential. We also found that the staining pattern of HuA in St. 33/34 CG is very similar to that in St. 29; that is, all HuC/D+ cells (neurons) are HuA+ and there is population of HuC/D–, HuA+ cells juxtaposed to the neurons (Figure 1B).

To follow the development of CG neurons in culture, we examined the staining pattern of anti-islet-1 and anti-HuC/D of CG immediately after dissociation, as well as after they had

been plated for several days in vitro (DIV). Consistent with our previous results, all HuC/D+ cells were also islet-1+ when acutely isolated from the CG (Figure 2A-2C, Table 2; Lee et al., 2001). After 1 DIV, all HuC/D+ neurons with round cell bodies and extended processes were islet-1+ (Figures 2D-2F, Table 2). However, we also saw a minor population of HuC/D+ cells that were flat with no discernible processes. These HuC/D+ flat cells had very faint, diffused, or no islet-1 immunoreactivity in their nuclei (Figure 2D-2I). We quantified the proportion of HuC/D+ and islet-1+ cells in cultures and found that in acutely dissociated CG, 100% of the HuC/D+ cells are islet-1+ (Table 2). By 1DIV, about 18% of the HuC/D+ cells had no significant nuclear islet-1 staining (Table 2) and these HuC/D+, islet-1– cells could be observed up to 5 DIV (Figures 2G-2I and data not shown).

The observation that there are HuC/D–, HuA+ cells in St. 29 CG, as well as a small population of HuC/D+, islet-1– cells present in culture, suggests that there are neuronal precursor cells in St. 29 CG and our culture conditions permit differentiation of the precursors into neurons. The newly differentiated neurons that are still immature may lack some of the CG neuron characteristics such as morphology and expression of islet-1. We also found that no neuroblasts were dividing and giving rise to neurons because when we pulsed St. 29 CG cultures with bromodeoxyuridine (BrDU) and harvested the cultures 3 days later, no neurons had taken up BrDU (Table 3). Another interesting observation is that the proportion of neurons in St. 29 CG cultures remained relatively constant (about 50%) even though the non-neuronal cells were dividing and the total number of cells increased (Table 4). This implies that there were more neurons in St. 29 CG cultures because if the neuronal number was unchanged, one would expect to see a drop in the percentage of neurons when the overall cell number was expanding. Our results indicated that neurons in St. 29 CG cultures constituted about 50% of the total population among proliferating cells, supporting the idea that there is a

concomitant increase in neurons.

**Enrichment of non-neuronal cells from ciliary ganglion**

To test the developmental potential of the non-neuronal cells in the ciliary ganglion, we sought to purify these cells and backtransplant them into younger hosts. From our marker analysis experiments, we were unable to find a cell surface marker that would label all the non-neuronal cells specifically and therefore we could not sort out all of the non-neuronal cells by immunopanning techniques. We first reasoned that if we omitted a trophic factor for CG neurons, chCNTF, the cultured neurons would be dead after overnight incubation and then we could collect the non-neuronal cells that were left behind. To our surprise, not only did the neurons die but most of the non-neuronal cells did not survive either when there was no chCNTF. It is possible that some non-neuronal cells in the CG also depend on chCNTF for survival. We then pre-plated dissociated CG on uncoated tissue culture dishes in order to test if non-neuronal cells, but not neurons, would preferentially adhere to plastic surfaces. We found that the majority of the non-neuronal cells also preferred the polylysine/laminin substrate and pre-plating yielded very few cells, even after 2-3 hours of incubation. Next, we used an antibody to the neuron-specific cell surface antigen, Thy-1, to pan all the neurons to see if we could separate the neurons from the non-neuronal cells. Our experiments showed that anti-Thy-1 indeed bound to and panned CG neurons but that there was still a fair number of unbound neurons left after 3 hours of incubation. These results determined this purification scheme to be unsatisfactory.

Finally, we utilized the differential adhesion properties of neurons vs. non-neuronal cells to separate the two cell types. Neurons can be shaken off tissue culture surfaces more easily than non-neuronal cells. We plated CG cell suspensions onto polylysine/laminin coated tissue culture flasks until all cells had attached (about 3-4 hours) and then

mechanically shook off neurons from tissue culture flasks (Figure 3). Non-neuronal cells that were about 95% pure could be obtained with this method (Figure 4). This mechanical shaking technique gives us the purest population compared to other methods.

**Purified non-neuronal cells from quail CG as donor cells for backtransplantation**

In order to distinguish donor vs. host cells after transplantation, we used the quail-chick chimera system for our experiments. After transplantation, quail cells can be identified by staining with anti-QCPN—an antibody that recognizes a nuclear antigen found in quail but not chick nuclei. In addition, the concentration of heterochromatin in the nucleoli of quail cells can also be observed by nuclear dyes such as Hoechst or hematoxylin (Le Douarin et al., 1996). We used E5 and E10 quail CG (roughly equivalent to HH St. 29 and St. 38 in chick) as sources of donor cells and purified the non-neuronal cells by mechanically shaking off the neurons. As with chick cells, the enriched non-neuronal cells were about 95% pure and were HuC/D– (Figures 4A and 4B, and data not shown). When we examined their marker expression, the purified non-neuronal cells were p75+ and some of them were HuA+, confirming that our non-neuronal donor cells were neural crest cells and some of them may be neurogenic precursors.

**Transplantation of quail CG non-neuronal cells into younger chick hosts**

We purified non-neuronal cells from quail CG and injected them into the head mesenchyme of E2 chicken embryos (Figure 3). We typically collected chick embryos 3 days after transplantation, around St. 26, because by that age the CG is well-formed and CG neurons express markers such as HuC/D to allow for the assessment of neuronal differentiation. We immunostained serial tissue sections with anti-QCPN and anti-HuC/D. Any QCPN+, HuC/D+ cells observed would suggest that the injected

non-neuronal quail cells had differentiated into neurons.

When we transplanted E5 quail CG non-neuronal cells into chick hosts, we saw QCPN+ neurons (HuC/D+) in all embryos examined (n=6). Quail cells homed to the CG, some of them incorporated into the ganglion and differentiated into neurons (Figure 5). Some QCPN+ cells were within the CG but were not associated with a HuC/D+ cytoplasm; it was not clear if those cells would eventually become neurons or if they had become part of the glial constituents in the chick CG. Moreover, we saw QCPN+ cells that were along the migratory path of the mesencephalic crest; their elongated morphology suggested that they might be migrating, possibly towards the CG and/or towards other destinations.

Many transplanted cells could be found within or around the vicinity of the CG and were not seen in the central nervous system. Nonetheless, at least some of the injected cells survived, migrated to the CG and differentiated into neurons, suggesting that E5 non-neuronal cells from CG had the ability to become neurons. This is in agreement with previous studies where neural crest derived non-neuronal cells could become other cell types, including neurons, when challenged in a new environment (Ayer-Le Lievre and Le Douarin, 1982; Fontaine-Perus et al., 1988; Le Lievre et al., 1980). Although we have shown that the non-neuronal donor cells are p75+ and HuA+, we have not verified other marker expression in E5 quail CG non-neuronal cells. Therefore, the non-neuronal cells used in our transplantation studies were defined as HuC/D– but we did not know whether they were O4+ or 7B3+. Our results suggest that non-neuronal cells in the young CG (E5 quail or E6 chick) could differentiate into neurons. They could either be neurogenic precursors and/or neural crest cells that are fated to be glia but could still become neurons when placed in a permissive environment.

To ask if the developmental potential of CG non-neuronal cells decreases with age, we employed the same transplant paradigm as above except we used non-neuronal cells isolated from E10 quail (roughly equivalent to E12 chick) CG as donor cells (Figures 4C and 4D). Similar to the E5 quail donors, E10 QCPN+ cells could be found within and around the CG. We usually saw more QCPN+ cells in chick embryos when E10 quail cells were used, probably because we could obtain purified non-neuronal cells from E10 quail CG in larger number and more easily than at E5. The ability of E5 and E10 non-neuronal cells to divide has yet to be examined. Despite the numerous E10 QCPN+ cells that could be seen in St. 25/26 CG, none of them were neurons as they were not associated with any HuC/D+ cytoplasm (Figures 6A and 6B). We have not determined if these E10 donor cells express any glial-specific marker such as O4 or 7B3 before and after transplantation. These data suggest that the non-neuronal population from older CG may not have any neurogenic precursors, or that the non-neuronal cells have irreversibly committed to a glial lineage and their fate cannot be changed even in a new environment.

To determine if the non-neuronal cells from the CG can also give rise to other neural crest derived cell types, we injected E5 and E10 quail CG non-neuronal cells into somites 18/19 or 23/24 of St. 16-19 chick embryos. Previous studies showed that cells from the CG could populate sympathetic ganglia and other neural crest derivatives when transplanted into the trunk level (Dupin, 1984; Le Douarin et al., 1978; Le Lievre et al., 1980). Our preliminary results of these studies indicate that non-neuronal cells from E10 CG homed to the sympathetic ganglia, periphery nerve and other structures around the adrenal gland. Some quail cells incorporated into sympathetic ganglia but were not found to be HuC/D+ or tyrosine hydroxylase + (Figures 6C and 6D). The embryos that received E5 quail cells have yet to be analyzed carefully.

**Table 1. Marker expression in St. 29 CG cultures**

| Antibody | neurons | non-neuronal cells[a] |
|---|---|---|
| p75 | + | + |
| O4 | – | +[b] |
| 7B3 | – | +[c] |
| Thy-1 | + | – |
| HuC/D | + | – |
| HuA | + | +[d] |
| islet-1 | + | – |

St. 29 CG cultures were plated onto polylysine/laminin coated coverslips. Anti-O4 (1:500) was incubated at 37°C for 30-45 minutes before fixing in 4% paraformaldehyde. Anti-Thy-1 immunocytochemistry was performed in both live and fixed CG cultures, and yielded similar results.

[a] Non-neuronal cells in these cultures were defined as HuC/D- and neurofilament-negative cells.

[b] O4+ cells constituted a subpopulation of p75+ non-neuronal cells (<20%)

[c] 7B3+ cells constituted a subpopulation of p75+ non-neuronal cells (<20%)

[d] All HuC/D+ cells were HuA+. Even though many non-neuronal cells in St. 29 CG were HuA+, it was not clear if all of them were also p75+.

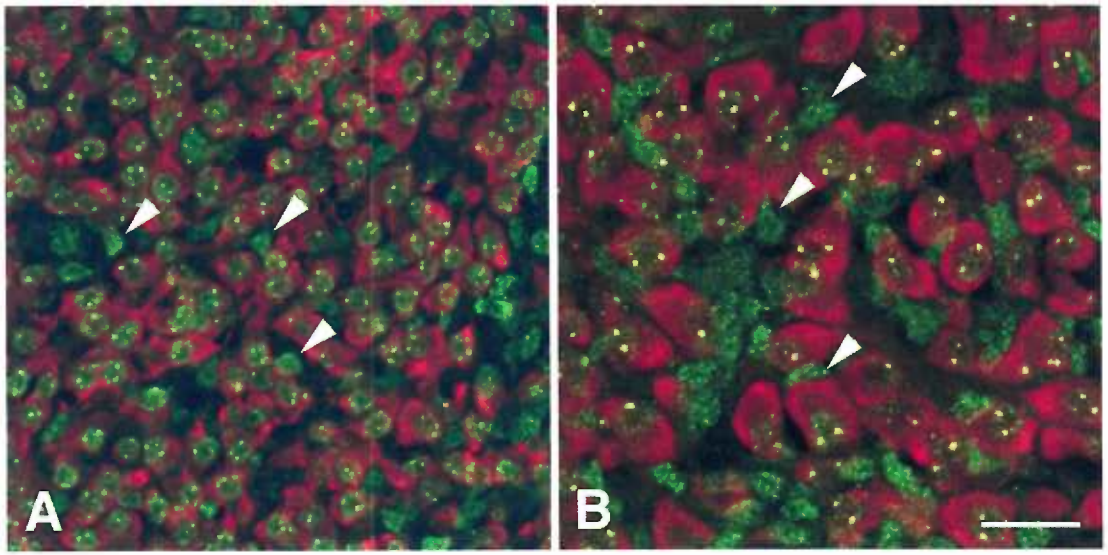


**Figure 1. Non-neuronal cells in St. 29 and St. 33/34 ciliary ganglion express a marker for neuronal precursor, HuA.** St. 29 and St. 33/34 CG were processed and immunostained with anti-HuA(green) and anti-HuC/D (red) as described. All CG neurons express HuC/D and HuA. Many cells that are HuA+ only can be seen in St. 29 and St. 33/34 CG. These may be neural precursor cells that are resident in the CG. Images were taken by laser confocal microscopy and optical sections were captured at 1-2 $\mu$m intervals. Scalebar = 20 $\mu$m.

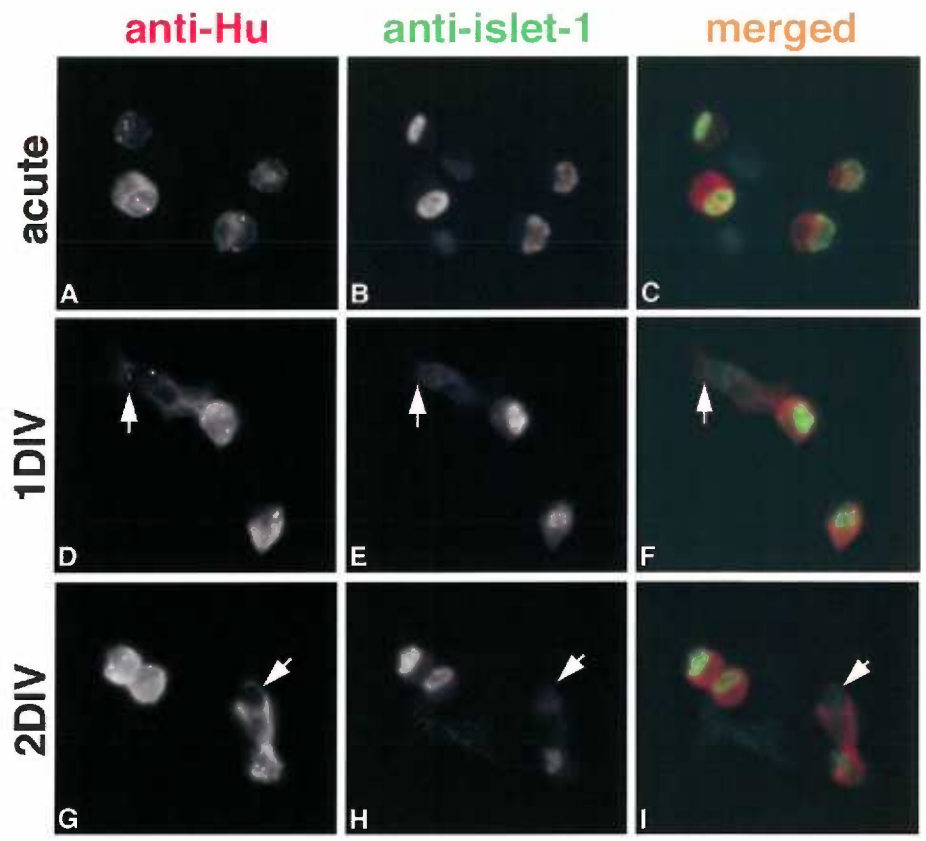


**Figure 2. Some HuC/D+ CG neurons in St. 29 cultures do not express islet-1.** CG were isolated and dissociated as described. (A-C) CG cell suspensions were centrifuged onto microscope slides and immunostained immediately after dissociation (A-C, acute). All CG neurons that were fixed right away expressed both HuC/D (red, cytoplasmic) and islet-1 (green, nuclear). (D-F) After one day in culture (1DIV), we saw a small population of HuC/D+ cells that were flat and they had very faint to no islet-1 immunoreactivity (arrows). These might be newly differentiated neurons that had not begun to express islet-1. (G-I) HuC/D+ and islet-1– cells could be observed after 2DIV (arrows).

**Table 2. A subset of cells in St. 29 CG cultures becomes HuC/D+ after 1 day in vitro**

| days in vitro | % HuC/D+ cells[a] | % islet-1+ cells[b] | % of cells that were HuC/D+ and islet-1+[c] | % of HuC/D+ cells that were islet-1+[d] | % of HuC/D+ cells that were islet-1–[e] |
|---|---|---|---|---|---|
| 0 | 51 | 51 | 51 | 100 | 0 |
| 1 | 67 | 55 | 55 | 82 | 18 |

St. 29 CG were isolated and dissociated as described in the text. Acutely dissociated CG were fixed without plating (0 days in vitro). Cultures were processed for anti-HuC/D and anti-islet-1 immunocytochemistry followed by staining of all nuclei with a Hoechst dye. 10 non-overlapping field of views were counted with a 60x objective on the Nikon Eclipse 800 microscope. Percentages were calculated based on cell counts from 3 sister cultures. When CG cultures were immunostained with anti-islet-1 and anti-HuC/D right after they were isolated from embryos (0 days in vitro), all HuC/D+ cells were islet-1+. When dissociated CG were seeded in culture, a subpopulation of HuC/D+ that were islet-1– could be observed after 1 day in vitro.

[a]Percent of HuC/D+ cells= number of HuC/D+ cells divided by the total number of cells (Hoechst stained nuclei)

[b]Percent of islet-1+ cells=number of islet-1+ cells divided by the total of cells

[c]Percent of cells that were HuC/D+ and islet-1+= number of cells that were double-labeled for HuC/D and islet-1 divided by the total number of cells

[d]Percent of HuC/D+ cells that were islet-1+= number of islet-1+ divided by the number of HuC/D+ cells

[e]Percent of HuC/D+ cells that were islet-1– = number of islet-1–; HuC/D+ cells divided by the number of HuC/D+ cells

**Table 3. No neurons were generated by division of neuroblasts in St. 29 CG cultures**

| | total cell # | BrDU+ cells | HuC/D+ cells | BrDU+ & HuC/D+ cells |
|---|---|---|---|---|
| **average±sem** | 500±36 | 413±42 | 305±18 | 0 |
| **% of total cell** | 100 | 82 | 61 | 0 |

St. 29 CG cultures were seeded onto polylysine/laminin coated coverslips in DMEM with 10% horse serum, 2% fetal calf serum, and 2% chick embryo extract supplemented with 20 ng/ml recombinant chCNTF and 20ng/ml recombinant FGF-2. After 1 day in vitro (DIV), 10 μM of BrDU was applied to cultures for 5 hours. CG cultures were fixed 3 days after BrDU addition (4 DIV total) and processed for anti-BrDU and anti-HuC/D immunocytochemistry. All coverslips were counterstained with a Hoechst dye to locate all nuclei before mounting on glass slides. 10 non-overlapping fields of view were counted on a Nikon Eclipse 800 microscope with a 40X objective.

Total cell number was obtained by counting Hoechst-stained nuclei. Cell counts were performed in 3 sister cultures (n=3), and averages given with standard error of means (sem). No BrDU+ neurons (HuC/D+) were observed in St. 29 after 4 days in cultures. Similar results were obtained when St. 29 were pulsed with BrDU after 2 DIV and collected 3 days afterwards (5 DIV total).

**Table 4. The proportion of neurons in St. 29 remained relatively constant even though the overall cell number increased by 40%**

| Days in vitro | total cell number ± sem[a] | Total HuC/D+ cells ± sem | % HuC/D+ cells | % increase in cell number |
|---|---|---|---|---|
| 1 | 333±20 | 184±3 | 55 | n/a[b] |
| 2 | 466±35 | 235±26 | 50 | 40[c] |

St. 29 CG cultures were plated at the same density and fixed after 1 or 2 days in vitro. They were then immunostained with anti-HuC/D followed by labeling with a Hoechst dye. Cell counts were performed in 3 sister cultures for each experiments (n=3) where 10 non-overlapping fields of view were examined under a Nikon Eclipse 800 microscope with a 60X objective.

Between day 1 and day 2 in vitro, the total cell number in St. 29 CG culture increased by about 40%. In our previous experiment, we showed that there were dividing cells in these cultures but that no new neurons were born (Table 3); the increase in total cell number was likely contributed by non-neuronal cells that were proliferative. The percentage of neurons stayed relatively unchanged despite an increase in overall cell population, suggesting that there must be more neurons in the cultures after 2 days in vitro.

[a] Hoechst-stained nuclei were counted as total cell number and average values given with standard error of means (sem).

[b] n/a = not applicable

[c] % increase in cell number after 2 days in vitro = difference in total cell number between 1 and 2 days in vitro divided by the total number of cells after 1 day in vitro

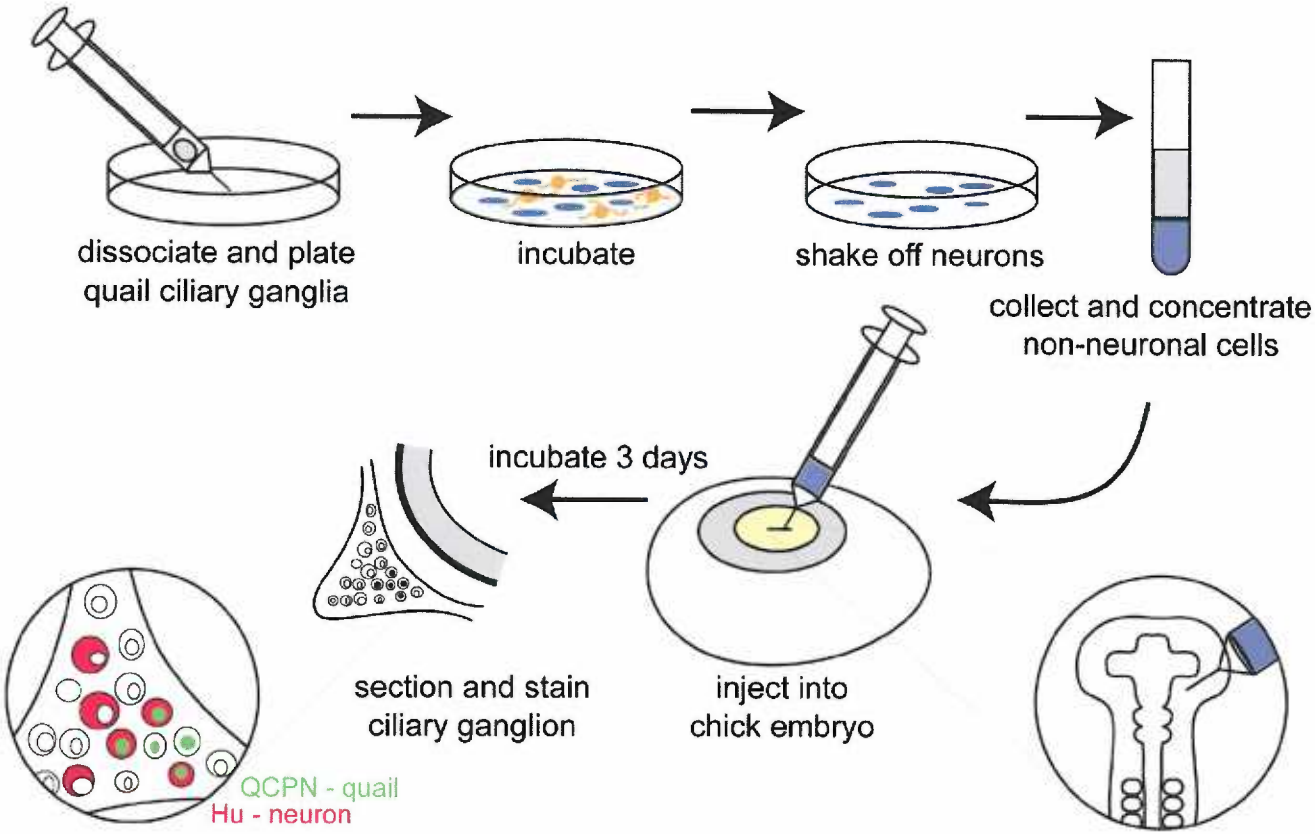


**Figure 3. Experimental design for transplantation of quail ciliary ganglion non-neuronal cells into young chick hosts**

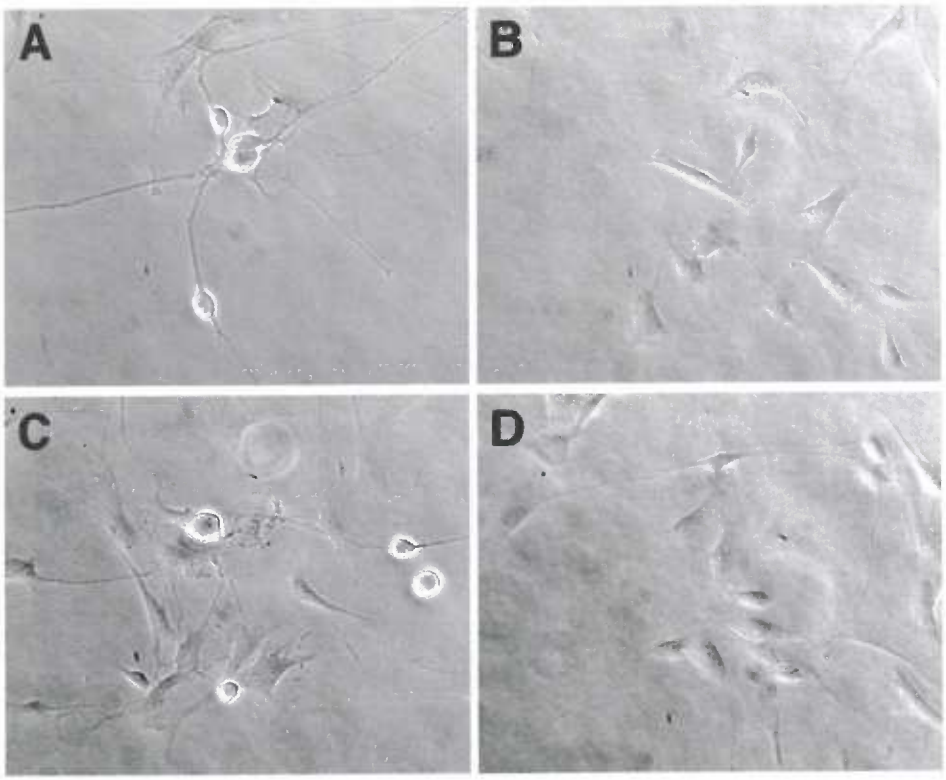


**Figure 4. Pure non-neuronal cells can be obtained by shaking neurons off of tissue culture flasks.**

(A) St. 29 quail cultures that contained both neurons and non-neuronal cells. (B) After sequential shaking and rinsing of St. 29 CG cultures, all neurons had detached. (C) St. 38 quail CG culture with neurons and non-neuronal cells. (D) St. 38 non-neuronal cells after neurons had been shaken off. (A-B) Quail CG cultures derived from St. 29 embryos; (C-D) Quail CG cultures derived from St. 38 embryos.

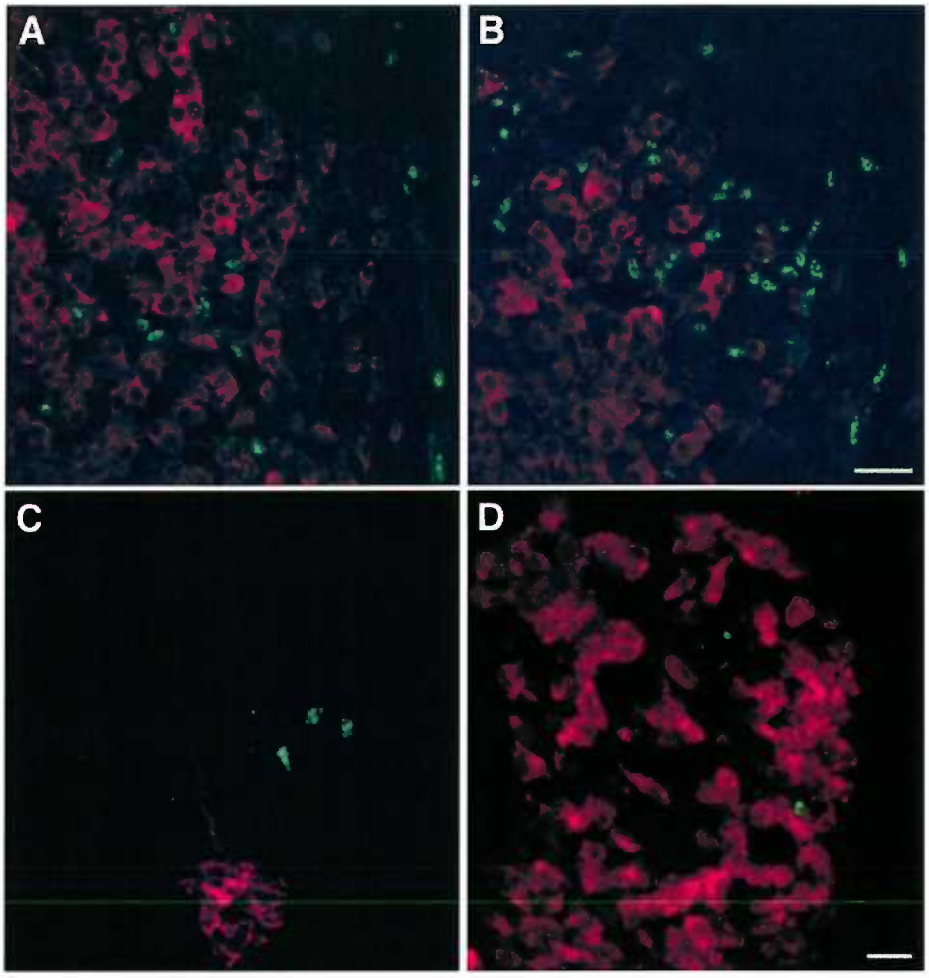


**Figure 6. Many St. 38 quail non-neuronal cells homed to ciliary and sympathetic ganglia but do not differentiate into neurons.**
(A-B) CG from St. 25/26 embryos that received St. 38 quail CG non-neuronal cells. Numerous quail nuclei could be seen throughout the CG but none of them are HuC/D+, indicating that they did not turn into neurons. (C-D) Sympathetic ganglia from St. 29 embryos that were injected with St. 38 CG non-neuronal cells in the trunk. Similar to cranial transplants, no quail neurons could be observed when donor cells were taken from St. 38 CG. Green = anti-QCPN; Red = anti-HuC/D; scalebar in B (for panels A and B) = 20 $\mu$m; scalebar in D (for panels C and D) = 20 $\mu$m.

## Discussion

The results of the present study supports the conclusion that St. 29 ciliary ganglion contains non-neuronal cells that can differentiate into neurons. First of all, our *in vitro* analyses show that a subpopulation of cells in CG culture express neither glia- nor neuron- specific markers. Instead they express neural crest markers such as p75 or the neural precursor marker HuA. Secondly, newly differentiated neurons appear in St. 29 CG cultures without cell division, suggesting that non-proliferating precursors may exist in the CG and can differentiate into neurons. Thirdly, when we purify non-neuronal cells from St. 29 quail CG and backtransplant them into young chick hosts, neurons that are of quail origin can be observed, suggesting that non-neuronal cells in CG still have neurogenic potential. This potential may, however, be progressively restricted with age because when we inject non-neuronal cells from older quail CG (≥E10), none of them become neurons.

### Characterization of neurons vs. non-neuronal cells in St. 29 CG

Results from our marker analyses show that at St. 29, virtually all cells in the CG express the neurotrophin receptor p75. This is consistent with previous reports that embryonic expression of p75 is widespread (Heuer et al., 1990; Raivich et al., 1985; Raivich et al., 1987); however, functional significance of this broad expression of p75 is not clear. In neural crest cells, p75 expression in migrating cells has been postulated to reflect the dependence on neurotrophins of neural crest derivatives such as sensory and sympathetic neurons (Bernd, 1985; Bernd, 1986). In non-neuronal cells of the nervous system, p75 has been implicated in mediating cell death in Schwann cells (Syroid et al., 2000) and in oligodendrocytes (Casaccia-Bonnefil et al., 1996). Moreover, a recent study has further demonstrated that p75 is expressed in the somites and can induce cell death in mesoderm-derived cell types as well (Cotrina et al., 2000). Because CG neurons do not require any neurotrophin for survival, nor do they express any of the

signaling receptors Trk A, B, and C (Appendix), it is unclear what the function of p75 is in this cell population.

Some non-neuronal cells in St. 29 CG express glial markers. Anti-O4 (Rohrer and Thoenen, 1987; Sommer and Schachner, 1981) and anti-7B3 (Henion et al., 2000) recognize cells that have committed to the glial lineage. O4+ or 7B3+ cells constitute a small population of the p75+ non-neuronal cells in St. 29 CG. Their number increases as the ganglion develops, consistent with the idea that glia differentiation occurs later in gangliogenesis compared to neurons. However, because a majority of the non-neuronal cells in St. 29 CG are p75+, O4–, and 7B3–, it is conceivable that these cells have not been committed as either neurons or glia and that they only express neural crest types of markers such as p75. A previous report has shown that about 80% of the cells isolated from E6 CG are O4+ and a large proportion of these O4+ cells also have NGF receptors (Rohrer and Sommer, 1983). The presence of NGF receptors was interpreted as a marker for neuronal trait. Our study shows that all neural crest cells are p75+ which is in agreement with their results that the majority of the cells in E6 CG express this receptor. We know from double-labeling experiments that p75 does not distinguish neurons from non-neuronal cells and should not be considered as a neuronal marker. We also did not observe as large a proportion of O4+ cells (80%) as Rohrer and Sommer did in E6 CG cultures. This difference may be due to the differences in our immunocytochemistry protocols; we always incubate anti-O4 in live cultures because we find that anti-O4 will label neurons non-specifically in fixed cells. Some of the anti-O4 labeling experiments by Rohrer and Sommer were performed in fixed cultures and it is possible that this might have induced a wider staining pattern than in live labeling protocols. Nonetheless, both of our results suggest that multipotent neural crest cells may still be present in St. 29 CG.

HuC/D+, islet-1- cells may be newly differentiated, immature neurons. We have observed a small population of flat cells that are HuC/D+, islet-1– in St. 29 CG cultures after 1-5 days *in vitro*. These HuC/D+, islet-1- cells are not seen in tissue sections (Lee et al., 2001), nor in CG stained immediately after dissociation. HuC/D is one of the first markers that are expressed after neuroblasts have commenced differentiation (Marusich et al., 1994). These HuC/D+, islet-1– flat cells may thus be newly differentiated neurons that have just begun to express HuC/D and have yet to display other neuronal markers such as islet-1. It will be interesting to test if these cells have ion channels or receptors that are unique to neurons. We do not think the HuC/D+, islet-1– cells are neurons that have de-differentiated because the proportion of HuC/D+ neurons in St. 29 cultures does not decrease with time. As the non-neuronal cells are proliferative, one would predict that the percentage of neurons should decrease with time in vitro, however, our results reveal that the percentage of neurons remains relatively constant. We have also shown by pulsing St. 29 cultures with BrDU that no dividing cells differentiate into neurons, suggesting that any newly differentiated neurons will come from non-dividing precursors (Tables 3 and 4).

In contrast to our findings, another group found that dissociated CG from E7 or E8 embryos in culture contained BrDU+ neurons after several days. They attributed the survival and proliferation of these precursor cells to the activity of fibroblast growth factor 2 (FGF-2 or bFGF) but not to CNTF (Gilardino et al., 2000). The difference in our results may be due to differences in our culture conditions. Gilardino and co-workers grow their cultures in a chemically defined medium with N2 supplement and FGF-2; our culture medium contains horse serum, fetal calf serum, and chick embryo extract in addition to FGF-2 and chCNTF. Because we do not fully understand the growth requirement for all cell types in the CG, we plate our culture in a rich medium so that we would not select against the survival of any cell type. It is possible that sera in our

medium promote proliferation, but not differentiation, of neural precursors. Consistent with this idea, culture medium which contains a high level of serum triggers neural crest cell division but not differentiation (Le Douarin, 1986; Ziller and Le Douarin, 1983). However, the observation that we do see new neurons in our cultures weakens this argument. Another possibility is that some of the precursor cells in our cultures are arrested in the G2 phase and in response to appropriate signals, they will undergo their final mitoses before differentiating into neurons. Because BrDU only labels cells in S-phase, we would not have detected mitosis. One possible way to test this is by immunostaining our cultures with an antibody that labels mitotic cells (anti-phospho-histone H3) and to ask if any cells undergoing M-phase are also HuC/D+.

**Non-neuronal cells purified from developing ciliary ganglion can differentiate into neurons**

When we backtransplanted non-neuronal cells from E5 quail CG, we were able to observe quail neurons in the chick CG. This supports the idea that a sub-population of the non-neuronal cells in St. 29 still have the ability to differentiate into neurons. The developmental potential of the non-neuronal cells from the peripheral ganglia has been examined previously. Le Douarin and co-workers created chimeric sensory ganglia in which non-neuronal cells were of quail neural crest origin (Ayer-Le Lievre and Le Douarin, 1982). They then backtransplanted these chimeric ganglia into the trunk of chick hosts and found that the quail non-neuronal cells from the chimeric ganglia could turn into neurons and other trunk crest derivatives. Dupin showed that when she backtransplanted E4 or E5 quail CG into the trunk of E2 hosts, she was able to see quail cells in the sympathetic ganglia and some of them were neuronal (Dupin, 1984). In addition, some of the donor cells undergo cell division, suggesting that cells from the CG have the ability to give rise to other cell types in the autonomic lineage. Similar studies

using quail CG as host tissues implanted entire pieces or portions of the ganglia (Dupin, 1984; Le Douarin et al., 1978; Le Lievre et al., 1980). Because unsorted CG were transplanted, the quail neurons observed could be derived from either neurons or non-neuronal cells of the donor ganglia, even though the number of CG neurons that survived was estimated to be very small. However, when purified CG neurons were injected to the trunk, they could express catecholamine and some of them even re-entered the cell cycle, adopting the characteristics of sympathetic neurons (Sechrist et al., 1998). By purifying the non-neuronal cells instead of transplanting pieces of CG that contained neurons, we extended the findings of Dupin and others, and confirmed that non-neuronal cells from the CG can differentiate into neurons. This strengthens the notion that undifferentiated neural crest cells that are multipotent persist even after gangliogenesis. As mentioned earlier, the non-neuronal cells used in our transplants are HuC/D– but we do not know if they are glial cells or undifferentiated neural crest cells. When we injected non-neuronal cells from older CG (≥E10), we did not see any quail neurons. Because there are more glial cells in the older ganglia, this suggests that the precursor cells, but not glia, have the ability to become neurons. We can address this issue more closely by: 1) examining the glia composition of E5 vs. E10 non-neuronal cells before injecting them into embryos, and 2) using anti-O4 to sort glial cells from E5 or E6 CG before transplanting them into younger hosts to test their potentials.

Results from our studies and others support the idea that non-neuronal cells in neural crest-derived ganglia contain precursor cells that can give rise to a variety of cell types (Ayer-Le Lievre and Le Douarin, 1982; Fontaine-Perus et al., 1988; Le Lievre et al., 1980; Rohrer et al., 1986). Because no definitive marker exists for these precursors, their presence is often inferred from experimental data or defined by the absence of cell-type specific markers. In our search for the precursor cells in the CG, we have examined the

staining pattern of HuA, which has been described as a marker for neural precursor cells in the neural crest (Wakamatsu and Weston, 1997). In St. 29 CG tissue sections, all neurons are HuA and HuC/D positive and there are also cells that are HuA+ (HuC/D–) adjacent to the neurons throughout the ganglion. We have consistently observed many cells in culture that are HuA+ and p75+. It is difficult to quantify the number of HuA+/p75+ cells accurately because the staining pattern of HuA is very diffuse in the cytoplasm and because the cultures are usually so tightly packed with p75+ cells that the borders between cells are almost impossible to discern. Qualitatively, the staining pattern of p75 and HuA largely overlap. It is possible that the cells that are HuA+/p75+ represent precursor cells that are neurogenic but whether they can only give rise to neurons or if they are multipotent precursors is still not clear at this point.

Results from our previous studies suggest that neuronal replacement seems to occur prior to St. 33/34 in the CG, so we asked if there is a difference in the expression pattern of HuA in St. 29 and St. 33/34. Our preliminary results suggest that HuA expression is very similar in both ages and there appears to be as many HuA+ cells in St. 33/34 as in St. 29 CG (Figure 2). It is possible that St. 33/34 CG also have neural precursors (HuA+ cells) and we can test this by transplanting non-neuronal cells from this age into younger embryos and ask if we can see any donor-derived neurons. *In vitro* studies have demonstrated that CG cultures dissociated from E8 embryos consist of dividing cells that will give rise to neurons (Gilardino et al., 2000). Because quail neurons were not seen when we injected E10 CG non-neuronal cells, it will be interesting to examine E10 or older CG to see if the ability to differentiate into neurons correlates with the presence of HuA+ cells. It will also be interesting to see if HuA and p75 are always co-expressed in undifferentiated neural crest cells. It is possible that only one type of precursor cell exists in the CG or other peripheral ganglia after St. 29 which could give rise to all CG cells types and that they express HuA and p75. One scenario is that the number of these HuA+,

p75+ multipotent precursor cells decreases dramatically after E10 and that no neurons can be generated either *in vivo* or *in vitro*. Another is that the potential of these multipotent precursors may decrease with age, i.e., they can give rise to both neurons and glia at St. 29 (HuA+p75+) but later on can only produce glia and down-regulate HuA expression. Another possibility is that precursor cells in St. 29 CG are already heterogeneous, for example, some are strictly neurogenic and they express HuA and p75, while others can only give rise to glial cells and express p75+ but not HuA. To address the precise composition of the precursor cells and their potentials will require clonal analysis experiments.

# V. Discussion

Studies presented in this thesis have addressed several basic questions in neuronal development with an emphasis on the regulation of cell number. I will summarize some of the key findings and then discuss new issues and questions that have emerged.

**Summary of results and conclusions:**

**1. Purification and characterization of a death-inducing factor in the chick eye.** I had characterized a death-inducing activity (DA) in the eye extracts that caused apoptosis of cultured E8 CG neurons and identified it as chick diferric transferrin ($cFe_2Tf$). The death inducing activity of $cFe_2Tf$ required iron, as well as the binding of $cFe_2Tf$ to transferrin receptor. In addition, we found that the concentration of $cFe_2Tf$ at which E8 CG neurons were induced to die was far lower than that found in the developing chick embryos. I also showed that CG neurons expressed transferrin receptor *in vitro* and *in vivo*. In a pilot study, I found that by applying hybridoma cells that produce DA14 to chick embryos, about 35% more CG neurons were observed compared to saline controls. These data suggest that $cFe_2Tf$ can induce cell death in the developing neurons. Unfortunately, I was unable to demonstrate the physiological significance of this death-inducing activity as this project was cut short because the biological activity of $cFe_2Tf$ disappeared, i.e., CG neurons did not die in response to $cFe_2Tf$. I have tested the activity of $cFe_2Tf$ on E8 CG cultures derived from different strains of chickens and to date, we have yet to reproduce the death inducing activity of $cFe_2Tf$. Nonetheless, results presented in Chapter 1 suggest that in addition to target-derived trophic factor, a systemic factor can also regulate cell death during neuronal development.

It is increasingly clear that in addition to signals that are required to maintain neuronal survival, factors that can induce cell death are also important. The characterization that $cFe_2Tf$ can be a death factor is interesting because proliferating cells need iron yet too much iron can be toxic. Thus iron uptake is normally tightly regulated. This again demonstrates that molecules that are crucial for normal cellular processes can also induce cell death depending on the cellular context. NGF has been well-documented in causing apoptosis, yet its presence is also critical for survival, therefore, whether a cell dies upon receiving a death signal may depend on other modifiers inside or outside cell. This point can be highlighted by our observation that CG neurons are no longer responsive to $cFe_2Tf$. The disappearance of this response seems to have coincided with a change in chicken flocks by our supplier and it is possible that different strains of chicken are differentially resistant to iron. I have generated CG cultures from different strains of chickens and tested the ability of $cFe_2Tf$ and $Fe^{3+}$ to induce cell death in these neurons. I found that the extent of cell death from these cultures varied greatly (data not shown). Furthermore, I also showed that sympathetic neurons display differential sensitivity to $cFe_2Tf$ depending on whether they are supported by NGF or CNTF, even though CG neurons would die in the presence of saturating amounts of CNTF. These observations strengthen the notion that there may be subtle qualities that can confer different cellular responses to a given stimulus. It will be of interest to determine what causes cells to be susceptible to $cFe_2Tf$. The sensitivity of neurons to the death-inducing ability of $cFe_2Tf$ may be regulated by the expression of transferrin receptor and/or an intracellular iron binding protein ferritin because their expressions are usually tightly controlled by the intracellular level of iron. Thus determining the "iron-sensing" mechanisms inside cells and the levels of transferrin receptors and ferritin may help us understand why some neurons are more sensitive to $Fe_2Tf$ than others.

Even though I could not carry this study to completion, the characterization of $cFe_2Tf$ as

a death-inducing molecule can potentially be interesting and important. Previous studies on neuronal cell death have largely focused on the role of neurotrophic factors and how their availability can regulate neuronal survival. Other recently described death factors such as NGF, BMP2 or TNF alpha are diffusible factors that are expressed in low abundance. Their ability to induce cell death not only depends on the expression of specific receptors on the receiving cells but also the spatial and temporal expression of the factors themselves. In contrast, the expression of transferrin receptor is ubiquitous (although the level of its expression is exquisitely control by the level of intracellular iron) and the circulating concentrations of $Fe_2Tf$ are sufficient to cause neuronal cell death. The key issue is to understand under what conditions are neurons killed by $Fe_2Tf$. Interestingly, an overabundance of iron level has been correlated with Parkinson's disease where dopaminergic neurons in the substantia nigra degenerate. Understanding how $Fe_2Tf$ can mediate cell death will likely shed light on how neurons may die under pathological situations.

**2. Early cell death in the avian ciliary ganglion.** I have examined cell death by an *in situ* cell death assay (TUNEL) and total cell number by characterizing a transcription factor islet-1, which labels all CG neurons. I quantified changes in cell number in the CG from St. 24 to St. 40. In addition to the neuronal cell death that takes place during synaptogenesis between St. 34 to St. 40, I have discovered that cell death takes place as soon as the CG is formed at St. 24 and that there is a large amount of apoptosis at St. 29. These results suggest that cell death in the CG is a dynamic process and occurs throughout development. I have investigated how cell death at St. 29 can be regulated and found that ablating the CG neuronal targets exacerbated cell death, while overexpressing a trophic factor for CG neurons, chCNTF, decreased cell death.

Two conclusions can be drawn from the results on the early cell death of the CG

neurons. Firstly, cell death occurs earlier than we used to think. Until now we have always assumed that CG neurons undergo cell death between St. 33 to St. 40 when they are innervating their targets, however, my studies have shown that cell death in this ganglion commences before synaptogenesis and prior to the differentiation of target tissues. Secondly, the magnitude of cell death is much higher than previously characterized. Cell death occurs throughout the developing of the CG, demonstrating the total number of neurons that die is far more than 50% of the total.

The early cell death that I observe does not coincide with synaptogenesis and it occurs as soon as the ganglion are formed. These data suggest that some other mechanisms are likely to be involved in regulating cell death prior to synapse formation. In the central nervous system, massive cell death has been documented in the ventricular zone where neuroblasts are proliferating. This is probably different from the cell death I have seen in the CG because the highest peak of cell death occurs in post-mitotic neurons. It is also possible that postmitotic neurons die because they engage in abortive cell division (Yoshikawa, 2000). Several reports have shown that cell cycle proteins are upregulated before the onset of apoptosis (Frade, 2000), although this possibility remains to be explored in the CG.

The significance of this early cell death is still unclear. In the central nervous system, neuroblasts and newly born neurons undergo cell death in the ventricular zone (Blaschke et al., 1996; Blaschke et al., 1998) and it has been suggested that appropriate types of neurons are selected prior to differentiation. In contrast, dying neurons in St. 29 CG are post-mitotic and differentiated; therefore the regulation of this early CG cell death is likely to be different from that in the developing CNS. It will be of interest to determine if the cell death at St. 29 and the later phase of cell death during synaptogenesis are inter-related—what will happen later on if we block or enhance cell death at St. 29?

Previous studies have shown that ablating the eye at E2 can greatly reduce the number of neurons at St. 40 even though neuronal numbers are the same in control vs. eyes ablated CG at St. 33/34 (Furber et al., 1987; Landmesser and Pilar, 1974b). I have demonstrated that eye extirpation can exacerbate cell death at St. 29. It is possible that the precursor cells that can replace dying neurons are limited in number. If dying cells at St. 29 can be replenished by precursor cells, then eye removal will use up more of the precursor cells earlier than during normal CG development, thereby reducing the number of neurons that can potentially be generated later on. It will be interesting to block cell death prior to St. 29 to see how neuronal number is affected.

**3. Dying neurons are replaced after cell death at St. 29.** I also discovered that even though there is a decline in neuronal number following the cell death at St. 29, the number of neurons rebound to a level similar to that found before the cell death by St. 33/34. This suggests that the establishment of neuronal number can be a combination of cell death and replacement and that this process may be tightly regulated. The recovery of neuronal number is not achieved by proliferation and differentiation of CG neuroblasts because several studies have shown that the birth date of CG neurons is between St. 28 to St. 29 (D'Amico-Martel, 1982; Dupin, 1984; Lee et al., 2001; Sechrist et al., 1998).

These results support the idea that the neuron can undergo multiple rounds of neurogenesis and cell death to attain their final number. In the sympathetic ganglia, the period of neurogenesis and cell death overlap and sympathetic neuroblasts express neural markers before they exit the cell cycle. This is different from CG neurons, which begin to express neuronal antigens after their final round of mitosis. One key questions is: why do CG neurons undergo cell death and cell replacement before St. 34 only to go through another phase of cell death? In the CNS, neuroblasts die without leaving the

ventricular zone, in the CG, however, majority of the dying cells are post-mitotic neurons. Is it possible that neurons that are born first undergo cell death earlier than those that are born later? Based on studies in our laboratory, we know that the expression of chCNTF increases as the embryos develop, is it possible that prior to target innervation, neurotrophic factors are always in limited amount such that neurons are dying steadily? Our observation that there is a large peak of cell death at St. 29 relative to other stages weakens this argument. It is possible that cell death at St. 29 is actively induced instead of a result of a lack of trophic factors. What will happen if we can rescue all the neurons that may have died during development? In caspase -3 and -9 deficient mice, cell death fails to occur and there are more neurons in the brain. Do those "extra" neurons make appropriate connections? Furthermore, it is not clear if neuronal replacement by post-mitotic precursors is unique to CG, although this is not likely. Studies in the neural crest have shown that non-neuronal cells can retain their potential until very late in development (Ayer-Le Lievre and Le Douarin, 1982; Fontaine-Perus et al., 1988) and that similar mechanisms can take place at least in other peripheral ganglia. It is also possible that the precursor cells in the CG divide at such a slow rate that I have not been able to detect it with my BrDU experiments. Indeed, recent studies have demonstrated that neural stem cells that are found in the adult nervous system have very a slow rate of division (Gage, 2000).

**4. Neuronal precursors in the developing ciliary ganglion.** Several lines of evidence suggest that neuronal precursors are present in <St. 33/34 CG. First of all, neuronal cell death prior to St. 33/34 appears to be followed by neuronal replacement, but not after St. 34. Secondly, data from my culture experiments suggest that there may be newly differentiated neurons in St. 29 CG cultures. Thirdly, transplanting St. 29 quail CG non-neuronal cells into E2 embryos yielded CG neurons that were of quail origin.

The control of cell number is complicated by the presence of precursor cells that can replenish dying populations of neurons. In addition to initial neurogenesis and target-dependent cell death, we now have to consider compensatory mechanisms that can also contribute to changes in neuronal number. If we can identify a precursor population and learn how they self-renew and differentiate into neurons, we will gain interesting information which would have broad applications in neuronal development and neurodegeneration. Neural stem cells have been the subjects of intense studies because of their potential therapeutic applications in treating neurodegenerative diseases. In the CNS, several studies have confirmed the presence of neural stem cells in the adult animals ranging from rodents to human. These stem cells have been demonstrated to generate neurons and glia but whether they can give rise to all cell types in the brain remains to be studied (Gage, 2000; Anderson, 2001). Stem cells have also been isolated and characterized from the rodent neural crest (Morrison, 1999). These neural crest stem cells have been shown to produce multiple cell types such as neurons, glia and smooth muscles. When these cells were transplanted, they were also able to migrate and differentiate into different neural crest derivatives (Morrison, 1999; White, 2001). In light of these studies, further characterizations of the precursor cell population in the ciliary ganglion may contribute important information into the study of stem cells and their roles in development and regeneration.

Our transplantation results showed that non-neuronal cells in St. 29 CG could take on a neuronal fate given the appropriate signals. This is consistent with previous studies in the peripheral ganglia and underscore the importance of environment signals in cell fate decisions. The relative importance of lineage vs. extrinsic factors in determining cell fate have been studied extensively in neural crest cells. One idea is that neural crest cells are all specified early on to produce specific derivatives, an opposite view is that all crest cells are multipotent and can turn into different cell types depending on the local cues that they

encounter. In an less extreme point of view, neural crest cells can be specified to become a certain cell type prior to or shortly after migration but they still retain the potential to take on alternate phenotypes when new signals are presented. For example, in zebrafish, medial crest cells are destined to become pigment cells whereas the lateral cells will become neurons (Dorsky et al., 1998). This specification takes place in the pre-migratory crest and pigment cells are promoted by the Wnt signaling pathway. On the other hand, neural crest stem cells isolated from rodent embryonic sciatic nerve have been shown to give rise to neurons, glia, and smooth muscle cells depending on the extracellular signals that they receive (Morrison et al., 1999; Shah et al., 1996; Shah et al., 1994). At present we only know that non-neuronal cells from St. 29 CG can become neurons, whether they resemble neural crest stem cells or fate-restricted precursor cells have yet to be explored.

Taken together, results presented in this thesis suggest that the regulation of neuronal number in the ciliary ganglion is more complex than was previously appreciated. I have provided evidence to suggest that CG neurons may undergo multiple waves of neurogenesis and cell death to achieve their final number. Although I have addressed some of the mechanisms that can regulate cell death and have begun to dissect the nature of precursor cells in the CG, these findings have also raised new issues. I will discuss some of the major questions that have emerged from my thesis briefly, as well as some of their future directions.

**A. Is regulation of the early (St. 29) cell death similar to that which occurs between St. 33-St. 40?** By St. 29, some of the CG neurons have reached their targets and cell death at this age seems to be target-dependent. Functional synapses are not formed until E8.5 in the iris or until E10 in the choroid (Pilar, 1987), therefore, even though eye tissues and chCNTF may control CG neuronal survival, it is unlikely to involve competition for synaptic sites. Is chCNTF present only in limiting amounts at this age as well? If that is

the case, how are the neurons supported when their number at St. 33/34 is the same as St. 29 prior to cell death? To dissect the role of chCNTF more carefully, our laboratory has constructed a chCNTFRα ribozyme in order to reduce the endogenous expression of chCNTFRα. We have preliminary data to suggest that the chCNTFRα ribozyme can cut chCNTFRα in an *in vitro* cleavage assay. However, we did not detect a significant reduction of chCNTFRα in CG neurons when we overexpressed the ribozyme *in vivo* (Smiley, Lee, and Nishi, unpublished observations). Efforts are underway to optimize the expression and activity of the chCNTFRα ribozyme to facilitate future experiments. Alternatively, we have obtained an antagonist to human CNTF in a retroviral construct that has been shown to block chCNTF activity as well. This antagonist can bind to CNTFRα but does not evoke downstream signals; it has also been engineered to be secreted. We infected fibroblasts with this virus and saw a high expression of this antagonist. Both the chCNTFRα ribozyme and CNTF antagonist may be useful reagents for disrupting CNTF signaling in chick embryos once their activities are confirmed. Is chCNTF the only trophic factor for CG neurons? Hashino and co-workers have demonstrated that GDNF and its family members may regulate neurite outgrowth and even survival of CG neurons (Hashino et al., 1999; Hashino et al., 2000). It may be useful to determine the role of these growth factors during CG development *in vivo*.

**B. Is there any death-inducing molecule in the developing CG?** Although I have demonstrated that $Fe_2Tf$ can induce cell death in cultured CG neurons, its physiological role in the embryo is still not clear. Another candidate molecule is the neurotrophin receptor, p75. p75, unlike the Trk receptors, lacks tyrosine kinase motifs and is unable to mediate neurotrophin signals alone. It contains a death domain and has recently been implicated in the cell death of neurons and other cell types (Casaccia-Bonnefil et al., 1996; Cotrina et al., 2000; Frade and Barde, 1999; Frade et al., 1996; Syroid et al., 2000). I have shown that developing CG neurons express p75 yet not Trk receptors (Appendix, part B).

It will be interesting to test if p75 can also induce the death of CG neurons.

**C. Precursor cells in the ciliary ganglion.** Based on my *in vitro* and *in vivo* results, it is likely that some sort of multipotent precursor cells exist in the St. 29 CG. My analyses on the transplant embryos are not complete and further examination will yield additional information on the nature of these cells. For example, I have pulsed several transplant embryos with BrDU and it will be interesting to see if I observe any donor cells that have divided and become neurons (QCPN+, BrDU+, HuC/D+). Other approaches will also help to address this issue from different angles, I have briefly described some of the experiments that logically followed from findings presented in this thesis:

*In vitro differentiation of CG non-neuronal into neurons*

I have tested the differentiation capability of CG non-neuronal cells mainly by *in vivo* backtransplantation because all of the signals required for fate determination will be present in the embryo. A complimentary experiment is to take the enriched non-neuronal cells in culture and ask if they can differentiate into neurons. If this approach is successful, it will provide additional proof that non-neuronal cells with neurogenic potential indeed exist in the developing CG. However, a negative result will not be informative because either the non-neuronal cells are unable to become neurons or because we do not have the appropriate culture conditions to permit differentiation. See also *Clonal analysis* below. Stem cells derived from neural crests have been demonstrated *in vivo* and *in vitro* to give rise to multiple cell types and several secreted factors have been implicated to play important roles. For example, BMP2 could promote neuronal differentiation; on the other hand, neuregulin could promote Schwann cell differentiation. However, there may also be intrinsic differences in cell fate choices among neural crest stem cells as these cells take 2-3 days to lose their competence in responding to BMP2 in the presence of neuregulin (Shah and Anderson, 1997). In

contrast, neural crest stem cells express MASH1 only 6-12 hours after exposure to BMP2; these data suggest that both lineage restriction and environment factors can affect the differentiation program that neural crest cells take on.

*Clonal analysis*

A clonal analysis can be performed to further examine the developmental potential of various cell types in the CG. E5-E6 vs. E10-E11 quail CG can be dissociated, diluted into clonal density, and plated onto a chick fibroblast feeder layer as previous described (Link, 1997). Non-neuronal cells can be unequivocally separated from neurons in this experiment and their capabilities examined. If there are indeed multipotent progenitors in the young CG (E5-E6), these cells should be able to give rise to all cellular derivatives of the CG. Neurons can be identified by markers such as HuC/D, neurofilament, Thy1, etc. Glial cells in the CG are not as well characterized but can be tested with O4, 7B3, or even P0 and SMP. This experiment can also address whether the developmental potential of the non-neuronal cells in the CG declines with age or if the number of progenitors is reduced. When E10-E11 quail CG are used as starting materials, one can ask if some clones will give rise to only one cell type, or if some clones have the capability to produce all derivatives, even though the number of these multipotent clones is much lower compared to clones generated from younger CG. Aside from being labor intensive, one of the difficulties of this type of experiment is that the required signals for the development of different cell types may not be present in our culture conditions. However, based on other clonal analysis studies performed in neural crest cells, several molecules have been implicated to promote different cell fates. For instance, bone morphogenic protein 2 (BMP-2) has been shown by several groups to promote neurogenesis and adrenergic differentiation (Schneider et al., 1999; White et al., 2001), while TGFβ and neuregulins have been shown to promote smooth muscle and glial phenotypes, respectively (Shah et al., 1996; Shah et al., 1994).

**D. The role of Notch signaling in CG development**. One attractive model to explain the cell differentiation and replacement in the CG is lateral inhibition by Notch signaling. One can hypothesize that during gangliogenesis, neurons, which are the first to develop, express higher levels of Notch ligands (Delta, Serrate-1, Serrate-2), and Numb, an intracellular antagonist of Notch. These Numb+, Delta+ neurons suppress the differentiation of their neighboring cells into neurons. However, when some of the neurons undergo cell death, the Notch-mediated inhibition may be released and the non-neuronal cells can thus turn into neurons. Notch-Delta signaling is utilized in multiple systems to determine cell fate decisions (Artavanis-Tsakonas et al., 1999; Bray, 1998; Lewis, 1998). In the neural crest, several groups have reported the significance of this pathway in the choice between neuron vs. glia differentiation (Morrison et al., 2000; Wakamatsu et al., 1999; Wakamatsu et al., 2000), and it is therefore plausible that similar mechanisms are at work in the CG. Two observations support this notion in the developing CG: 1) I have shown by RT-PCR that Notch, Numb, Delta, Serrate-1, and Serrate-2 transcripts are present in St. 29 CG, and I have performed in situ hybridizations with Numb, Delta, and Serrate-2 in St. 29 tissue sections (Appendix, part C). 2) Using an antibody against HuA, I have identified a population of HuA+ non-neuronal cells that are located right next to CG neurons. These cells are in a position that would allow them to respond to changes in local signals.

It would be important to determine the expression pattern of the Notch-Delta signaling component in the CG. Because CG are so tightly packed with both neurons and non-neuronal cells (especially CG that are younger than E10), it may be easier and more informative to perform localization studies in dissociated CG by immunostaining or by *in situ* hybridization so that the staining pattern in individual cells can be examined. Furthermore, we have obtained a constitutively active form of the human Notch (Tan-1) in a retrovirus expression vector [RCASBP(A)-RATANIC] which allows us to

misexpress Notch in the developing CG to ask if the cellular constituents will be altered. One may predict that the number of neurons may be decreased if there is an overabundance of the Notch signal.

Alternatively, it is possible that upon cell death, neurons release growth factors to trigger differentiation of their neighboring cells. Candidate molecules such as FGF-1 and FGF-2 are not normally secreted, but may leak out when membrane integrity is compromised during apoptosis. This hypothesis is not mutually exclusive with the possibility that Notch signaling may play a role in neuronal differentiation and merits further consideration.

# VI. Appendix

## A. Pattern of cell death in the developing ciliary ganglion and the effects of eye ablation and chCNTF overexpression on CG neuronal survival

Results in Chapter 2 revealed that cell death occurs a few days earlier than previously reported. I have quantified dying cells from St. 25 to St. 40 but have observed TUNEL+ cells as early as St. 24, shortly after the CG is formed (Figure 1). In addition, I have compiled montages of TUNEL- and HuC/D- stained CG from various ages (Figures 1 to 5) where the distributions of TUNEL+ can be seen throughout tissue sections. I have shown that ablation of the developing optic vesicle exacerbates cell death in St. 29 as indicated by a 2.3-fold increase of TUNEL+ cells in the CG from the ablated eye compared to the contralateral side. I have included the actual cell counts of the dying cells in St. 29 CG from eye-ablated embryos in Table 1. The dying cells were previously presented as percent of control (Chapter 2). Even though there are at least twice as many dying cells in the ablated side compared to contralateral side, the overall number of dying cells is lower than that of St. 29 CG taken from unmanipulated embryos (3,043 ± 113). This suggests the affect of eye ablation may be more complicated than merely causing an increase in cell death.

Previous studies have shown that when the eye cup is extirpated at E2 and cell number examined from St. 33/34 to St. 40, the number of neurons is the same at St. 33/34 in both the injured side and the control side (Furber et al., 1987; Landmesser and Pilar, 1974b). When I examined the total neuronal number of CG neurons from embryos thathad their optic cup removed, I found that the total cell number at St. 29 was affected as well. If neuronal numbers are the same at St. 33/34 regardless of eye ablation, this suggests that a massive neuronal replacement must have taken place between St. 29 and St. 33/34. It is possible that precursor cells in the CG (described in chapter 2) are involved in this

process. If we have a reliable marker for the precursor cells in the CG it will then be possible to see if their number is greatly depleted in St. 33/34 CG where the optic vesicles have been removed compared to the control CG. It will also be important to examine total CG neuronal number in St. 31 and St. 33/34 embryos with eye ablation to gain insights in the control of neuronal survival in those ages.

When I ablated the eye at St. 24 (E4) instead of at St. 11-13 (E2), there were less dying cells (Table 2) on the injured side compared to the contralateral side. It is also interesting that all CG from embryos that have underdone this surgery have fewer TUNEL+ compared to normal CG, regardless of the age when operated on. This suggests that the ablation may have elicited injury responses, such as the release of growth factors thatcan prevent neuronal cell death. When I examined the total neuronal number in the St. 24-operated embryos, CG from the ablated side had fewer neurons than did the controls, which is similar to those operated on at St. 11-13. In considering the differences of cell death resulting from the timing of operations, it is interesting to note that by St. 24 the CG has formed, as compared to St. 11-13. It will be interesting to perform sham eye removal operations on St. 11-13 and St. 24 embryos to examine the number of dying cells as well as total neuronal numbers. This will help us to understand the impact of surgery alone (without target removal) on neuronal cell death.

Finally, when I overexpressed chCNTF, there was roughly a 50% decrease of CG cell death in St. 29 embryos that received exogenous chCNTF compared to GFP control embryos. I have also examined the total neuronal number and found that there are more CG neurons in embryos that were infected with RCASBP(A)-chCNTF as compared to GFP controls (Table 3). However, when I quantified the number of dying cells and total number of neurons in RCASBP(A)-chCNTF injected embryos at St. 33/34, I found that there were more dying cells and less neurons in these embryos compared to GFP control.

This is in contrast with the results obtained previously in our laboratory—there was no difference in neuronal number at St. 33/34 CG when chCNTF was overexpressed with the same retroviral construct (Finn et al., 1998). It is possible that I observed different results because I injected 7 to 8 somites chick embryos in the neural tube instead of E2.5 to E3.5 embryos in the eye. Therefore more CG neurons are infected with my injection paradigm compared to infection of eye tissues. It is conceivable that overexpression of chCNTF in CG neurons themselves, instead of their target tissues, may induce neuronal cell death.

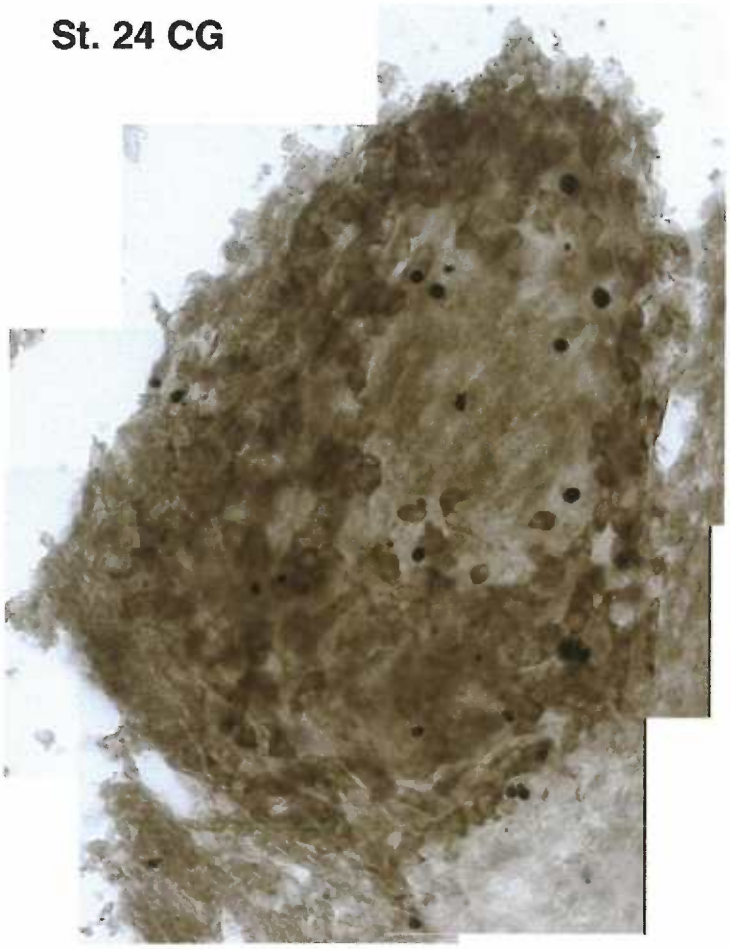


**Figure 1. Pattern of dying cells in St. 24 ciliary ganglion.**
Cell death can be observed as early as St. 24, soon after CG formation. This image was generated by combining 20 pictures taken at 40x and reconstructing the entire ganglion in Adobe PhotoShop.

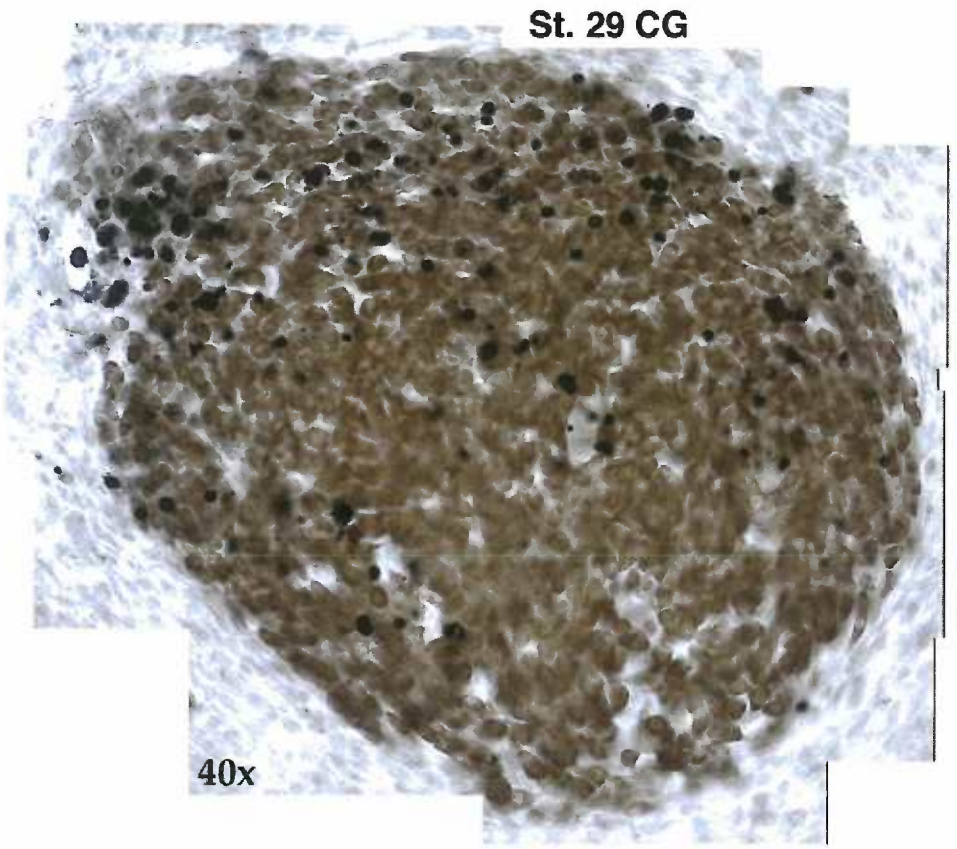


**Figure 2. Pattern of cell death in St. 29 ciliary ganglion.**
Many darkly stained TUNEL+ (black, nuclear) cells can be observed throughout the ganglion.

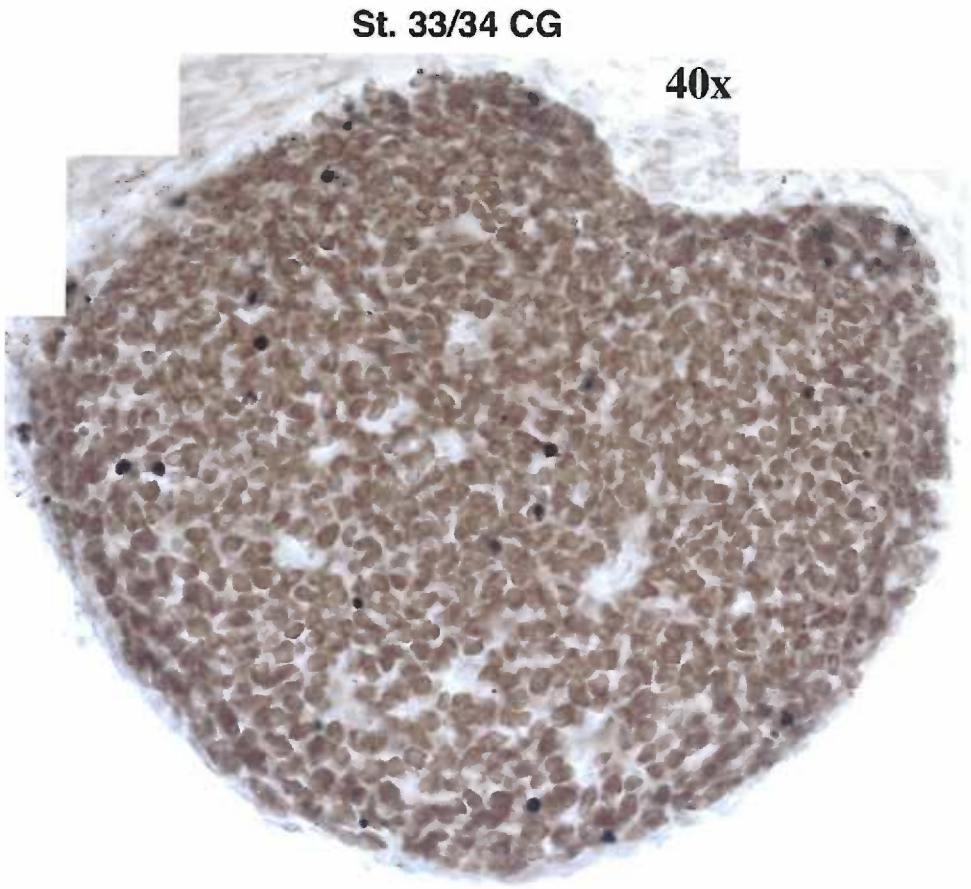


**Figure 3. Pattern of cell death in St. 33/34 ciliary ganglion.**

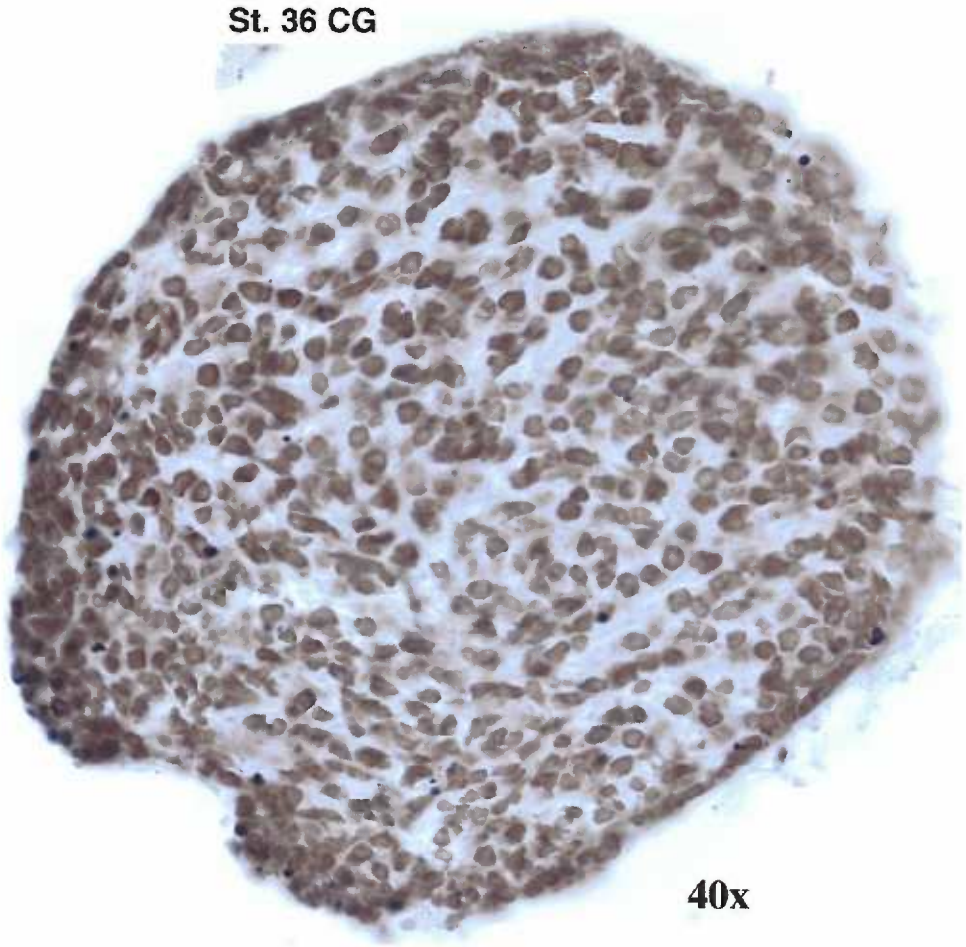


**Figure 4. Pattern of cell death in St. 36 ciliary ganglion**

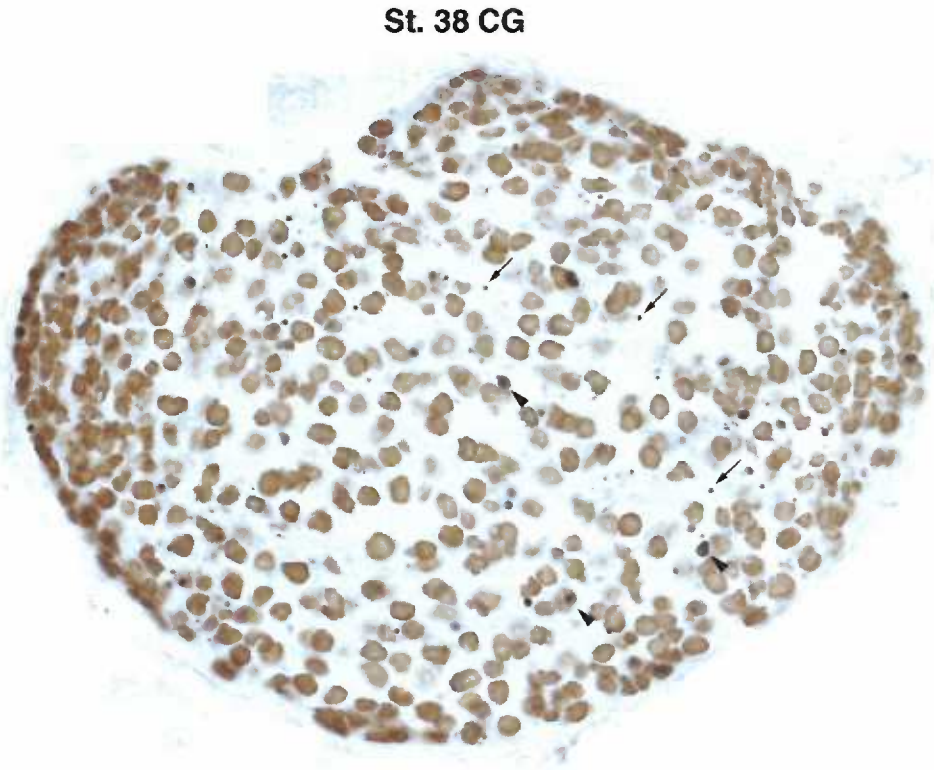


**Figure 5. Pattern of cell death in St. 38 ciliary ganglion.**
By this stage, neurons are spaced further apart compared to younger CG. This is due, at least in part to, the development of glial cells in the ganglion. TUNEL+ nuclei (black) could clearly be seen in HuC/D+ cytoplasm (brown), indicating that these are dying neurons (arrowheads). In addition, many small TUNEL+ cells that were also labeled (small, thin arrows), they were probably glial cells that were undergoing apoptosis as they were usually located in neuron-poor areas and their nuclei were much smaller than neuronal nuclei.

**Table 1. There were fewer neurons in St. 29 CG when the optic vesicle was ablated at St. 11-13**

| Treatment | Total # of dying cells | Total # of neurons |
|---|---|---|
| Ablated side (n=6) | 1,064 ± 139 | 12,500 ± 1,635 |
| Contra. control (n=6) | 453 ± 44 | 15,557 ± 557 |

Optic vesicles were removed as described in Materials and Methods at St. 11-13. Embryos were harvested at St. 29 and CG processed for either TUNEL or islet-1 immunohistochemistry.

Total # of dying cells = total average number of TUNEL+ cells ± standard error of means (sem)

Total # of neurons = total average number of islet-1+ cells ± standard error of means (sem)

Ablated side = CG taken from the side where the eye cups were extirpated

Contralateral side = CG taken from the same embryos that received eye surgeries but on opposite side where the eyes developed normally

n = number of CG counted

**Table 2. Total neuronal number and dying cells in St. 29 CG were different when optic vesicles were removed at St. 24 compared to St. 11-13**

| Treatment | Total # of dying cells (n) | Total # of neurons (n) |
|---|---|---|
| Ablated | 890 ± 68 (7) | 11,553 ± 1,514 (7) |
| Contra. control | 1,228 ± 245 (7) | 14,000 ± 1,653 (7) |

Optic vesicles were removed at St. 24 (E4). Embryos were harvested at St. 29 and CG processed for either TUNEL or islet-1 immunohistochemistry.

Total # of dying cells = total average number of TUNEL+ cells ± standard error of means (sem)

Total # of neurons = total average number of islet-1+ cells ± standard error of means (sem)

Ablated side = CG taken from the side where the eye cups were extirpated

Contralateral side = CG taken from the same embryos that received eye surgeries but on the opposite side where the eyes developed normally

n = number of CG counted

**Table 3. Effects of chCNTF overexpression on cell death and survival of St. 29 and St. 33/34 CG neurons**

| St. 29 CG | total # dying cells (n) | total # neurons (n) |
|---|---|---|
| RCASBP(A)-chCNTF | 299 ± 49 (10) | 15,651 ± 1234 (11) |
| RCASBP(A)-GFP | 593 ± 24 (10) | 12,844 ± 701 (10) |

| St. 33/34 CG | total # dying cells (n) | total # neurons (n) |
|---|---|---|
| RCASBP(A)-chCNTF | 838 ± 122 (9) | 9,256 ± 989 (9) |
| RCASBP(A)-GFP | 541 ± 71 (4) | 14,107 ± 475 (5) |

Concentrated viruses were injected in the neural tubes of 7-8 somites embryos and then embryos were returned to the incubator until they reached St. 29.

Total # of dying cells = total average number of TUNEL+ cells ± standard error of means (sem)

Total # of neurons = total average number of islet-1+ cells ± standard error of means (sem)

Ablated side = CG taken from the side where the eye cups were extirpated

Contralateral side = CG taken from the same embryos that received eye surgeries but on the opposite side where the eyes developed normally

n = number of CG counted

**B. Ciliary ganglion neurons express p75 but do not express Trk receptors**

Because CG neurons do not depend on neurotrophins for survival, yet express high levels of p75 receptor, I sought to determine if they express any Trk receptors. I found that CG neurons do not express Trk A, B, or C receptors by immunostaining live CG cultures with antibodies specific to each receptor (Figure 1). Multiple reports have demonstrated that p75 can induce apoptosis in cells that express only p75 but no Trks (Casaccia-Bonnefil et al., 1996; Cotrina et al., 2000; Frade et al., 1996; Syroid et al., 2000). It will be interesting to test if p75 can cause cell death of CG neurons.

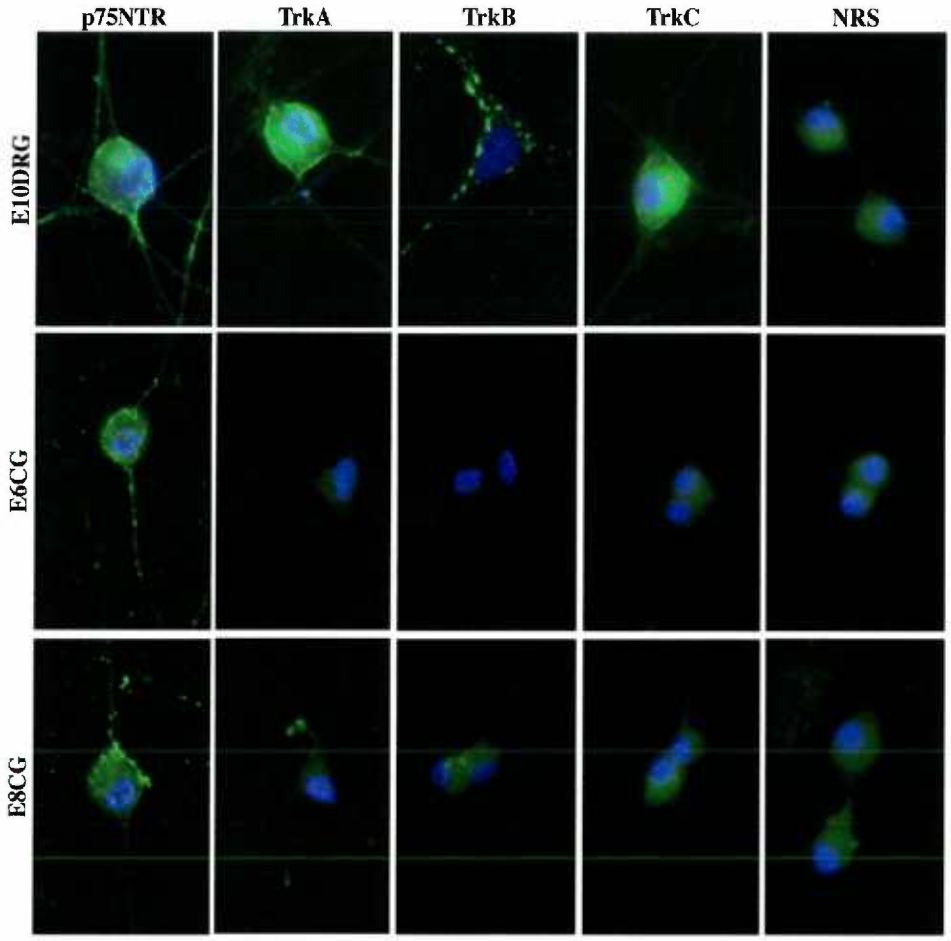


**Figure 6. Ciliary ganglion neurons express the neurotrophin receptor p75 but do not express Trk receptors.**

Live neuronal cultures were incubated with antibodies against each receptor for 30 minutes and then fixed. Alexa-488 conjugated goat anti-rabbit IgG was used to visualizereceptor immunoreactivity. E10 dorsal root ganglion neurons were used as positive controls and incubation with normal goat serum (NRS) served as the negative control.

### C. Components of the Notch signaling pathway are expressed in St. 29 CG

We are interested in exploring the role of Notch signaling in the differentiation of CG neurons. Previous studies have shown that differentiation of neural crest derived neurons is regulated by the activity of Notch and its ligands. In the avian DRG, neurons express Delta and Numb and seem to inhibit neighboring cells from becoming neurons. Following cell death, a decrease in neuronal number in the CG between St. 29-31 occurs. By St. 33/34, the number of neurons recovers to the level equivalent to that of St. 29. One possibility is that a population of non-neuronal cells residing in the CG has the ability to differentiate. We hypothesize that a subset of CG non-neuronal cells located adjacent to the neurons (expressing Delta or other Notch ligands such as Serrate-1 or Serrate-2) are usually prevented from taking up a neuronal fate by Notch activity. When massive neuronal cell death occurs at St. 29, releasing the activation of Notch, non-neuronal cells can then adopt a neuron identity. As a first step to test this hypothesis, we have started to characterize the expression pattern of Notch and its signaling components in St. 29 CG. I have previously shown by RT-PCR that Notch, Delta, Serrate-1, Serrate-2, and Numb mRNA can be detected in St. 29 CG. To ask which cell types in the CG express each of these transcripts, I performed *in situ* hybridization in St. 29 tissue sections. My preliminary results show that Numb, Delta, and Serrate-2 transcripts are present in St. 29 CG (Figure 1). Confirmation of the expression of Notch and Serrate-1 will require further analysis by *in situ* hybridization in tissue sections or dissociated CG.

Based on the data that I have obtained so far, it is likely that differentiation of neurons and glia in the CG is regulated by the Notch signaling pathway.

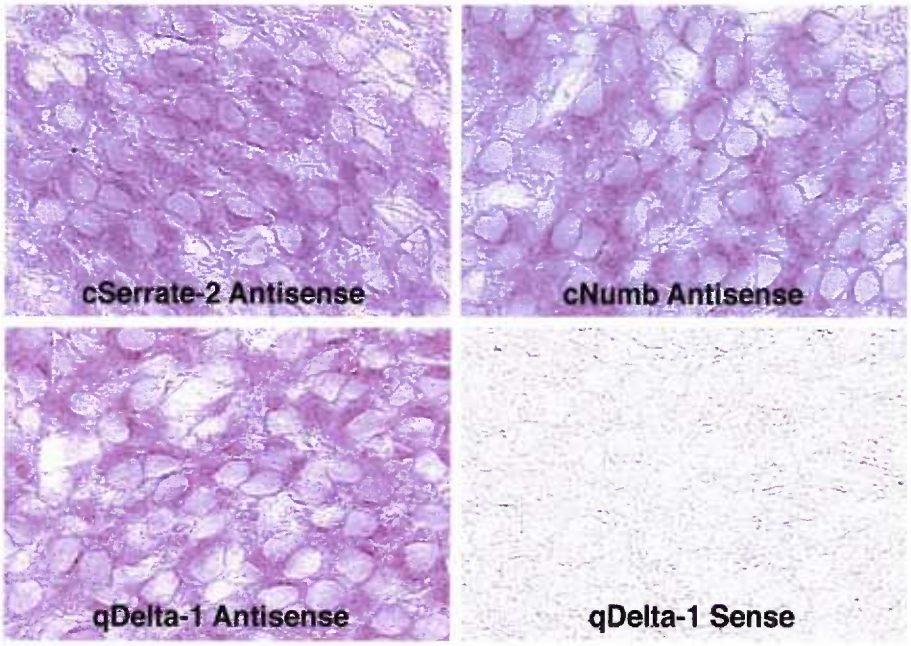


**Figure 7. E6 ciliary ganglion neurons express the Notch ligands Delta-1 and Serrate-2, and the intracellular inhibitor of Notch, Numb.**
E6 CG were collected and fixed in 4% paraformaldehyde, 20 $\mu$m frozen sections were cut on a cryostat after equilibration in 30% sucrose. The digoxigenin-labeled chick Numb and Serrate-2 probes were generated by PCR and subcloned into TOPO-PCRII). The quail Delta-1 probe was made from a full length sequence cloned (generously provided by Dr. Wakamatsu) in pBluescript, and has been shown to give identical staining pattern in chick and quail tissues (Wakamatsu 2000, Development 127: 2811-2821).

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