

# Overexpression of Ciliary Neurotrophic Factor *In Vivo* Rescues Chick Ciliary Ganglion Neurons from Cell Death

Tom P. Finn, Songte Kim, Rae Nishi

Department of Cell and Developmental Biology, L-215, Oregon Health Sciences University, 3181 SW Sam Jackson Park Rd., Portland, Oregon 97201

Received 23 August 1997; accepted 24 September 1997

**ABSTRACT:** Ciliary ganglion (CG) neurons undergo target-dependent cell death during embryonic development. Although ciliary neurotrophic factor (CNTF) was identified *in vitro* by its ability to support the survival of chick CG neurons, its function as a target-derived neurotrophic factor has been questioned by those working on mammalian-derived forms of CNTF. We have purified and cloned a chicken CNTF [chCNTF; formerly growth-promoting activity (GPA)] that is expressed in CG targets during the period of cell death and is secreted by cells transfected with chCNTF. In the present study we used a retroviral vector, RCASBP(A), to overexpress chCNTF in

CG target tissues. Elevation of chCNTF biological activity three- to fourfold in the embryonic eye rescued an average of 31% of the neurons that would have normally died *in vivo*. In some individuals, nearly all of the neurons were rescued. ChCNTF had no effect on the number of neurons observed prior to cell death, nor were there any deleterious effects of either viral infection or overexpression of CNTF. These results show that chCNTF is able to function *in vivo* as a trophic factor for CG neurons, and suggest that limited availability of trophic support is one of the factors regulating CG neuron survival during development.

© 1998 John Wiley & Sons, Inc. *J Neurobiol* 34: 283–293, 1998

## INTRODUCTION

The process by which cell death during development is controlled in many neuronal populations is not well understood. In many instances, neuronal death is exacerbated by removing neuronal target tissues and reduced by adding more targets. The neurotrophic theory is a model that attempts to explain how cell death is controlled by target tissues. According to the neurotrophic theory, neurons require a neurotrophic factor produced by the target for survival. Because availability of the target-derived factor is limited, neurons compete for an adequate supply of the factor to survive cell death. How the amount and availability of neurotrophic factor

is precisely regulated so that the size of the neuronal population is matched to its target is not clear, but possible mechanisms include limited synthesis, neuronal activity, and synapse formation (Oppenheim, 1989).

The avian ciliary ganglion (CG) undergoes cell death during development and is particularly well suited for the study of trophic dependency and target-cell interactions. The CG contains only two populations of neurons: choroid neurons that innervate smooth-muscle cells in arteries of the choroid layer, and ciliary neurons that innervate the iris and ciliary muscle. Cell death occurs between E9 and E14, when the number of neurons is reduced by approximately 50% (Landmesser and Pilar, 1974b). Cell death is exacerbated by removal of the eye before it is innervated by the CG (Landmesser and Pilar, 1974a; Furber et al., 1987), while addition of an extra eye enhances CG survival (Narayanan and Narayanan, 1978).

Correspondence to: R. Nishi

Contract grant sponsor: NIH; contract grant number: NS25767

© 1998 John Wiley & Sons, Inc. CCC 0022-3034/98/030283-11

**Table 1 Infection of CG Targets *In Vitro* and *In Vivo* with RCASBP(A)-chCNTF Elevates chCNTF Levels**

| Sample  | Bioassay (Trophic Units) |                    |
|---------|--------------------------|--------------------|
|         | Choroid Cultures (CM)    | Infected Eyes (XT) |
| Control | 112 ± 61                 | 455 ± 35           |
| chCNTF  | 9136 ± 1086              | 1280 ± 54          |

Conditioned medium (CM) was collected from three 100-mm plates 8 days after infection of choroid cells with RCASBP(A)-vector (control) and RCASBP(A)-chCNTF (chCNTF) and extracts (XT) prepared from nine eyes of infected embryos. The total amount of trophic activity present in each was determined by bioassay on E8 CG neurons. The numbers represent the mean and standard deviation obtained from three independent determinations. Most of this activity could be immunodepleted with anti-chCNTF antibodies.

Importantly, the period of neurogenesis in the CG is separated temporally from the period of cell death, with CG neurons becoming postmitotic by embryonic day 6 (E6). Studies in cell culture demonstrating that CG neurons destined to die *in vivo* are rescued by the addition of soluble factors suggest that the neurotrophic theory may be applicable to CG neurons (Nishi and Berg, 1979).

Chicken CNTF (chCNTF) is a target-derived molecule that may regulate cell death of CG neurons. ChCNTF supports the survival of CG neurons in culture and was first reported in chick eye extracts as ciliary neuronotrophic factor (Adler et al., 1979) and growth-promoting activity (GPA) (Nishi and Berg, 1981). GPA was cloned and sequenced in 1992 and found to share 48% amino acid identity with rat CNTF (Leung et al., 1992). Because of similarities in structure and biological activity, we have chosen to rename GPA as chicken CNTF. ChCNTF is a potent neurotrophic factor *in vitro* with an ED<sub>50</sub> of about 1 pM (Eckenstein et al., 1990). ChCNTF message and protein are present in the target tissue at the time the neurons are dependent upon their targets for survival (Leung et al., 1992; Finn and Nishi, 1996), and high-affinity receptors for chCNTF are also present in the CG during the same time period (Heller et al., 1995; Koshlukova et al., 1996). While rat, rabbit, mouse, and human CNTF are generally considered to be nonsecreted or very poorly secreted proteins (Lin et al., 1989; Stöckli et al., 1989; Sendtner et al., 1990), chCNTF can readily be detected in choroid smooth-muscle-conditioned medium (R. Nishi, unpublished observation) or in cell lines transfected with chCNTF (Leung et al., 1992). The secretion of chCNTF appears to be due to the pre-

sence of an internal signal sequence between amino acids 40 and 60 (G. Reiness et al., 1995). Taken together, these data support the involvement of chCNTF in the regulation of developmental cell death in the CG.

If chCNTF regulates cell death in the CG, then it should rescue CG neurons from cell death when supplied exogenously. Although CG neurons are known to express a receptor that binds chCNTF (GPAR $\alpha$ ) (Heller et al., 1995), there has never been a direct demonstration that CG neuron cell death can be modified by increasing the availability of CNTF. Indeed, one attempt to demonstrate such an effect on the CG by adding CNTF to the chorioallantoic membrane (Oppenheim et al., 1991) was unsuccessful, although an effect on motor neurons was observed. The lack of an effect in these experiments underscores the limitations of exogenous addition experiments, even under conditions where a huge excess of factor is used. Simple application of factors cannot control for protein turnover, lack of diffusion into target tissues, and binding to cell types other than CG neurons. It also does not allow for the presence of continuously elevated levels of factor—a condition CG neurons may require *in vivo*. To adequately test the ability of chCNTF to affect CG neuronal survival, a delivery system is needed that can continuously supply the factor from within the target itself. In the present study, we report the results of infecting CG target tissues with a retroviral vector to stably overexpress a transgene encoding chCNTF.

## MATERIALS AND METHODS

### ChCNTF Bioassays

Short-term (2–5 days) CG neuron cultures were prepared as previously described (Nishi, 1996) and short-term survival assays were performed according to Eckenstein et al. (1990), using E8 (Hamburger–Hamilton Stage 34) ciliary ganglia, except that ganglia were plated at a density of 0.75 ganglia/well in 48-well plates. Neuronal survival was determined by counting the number of large phase-bright cells with processes > 3 cell diameters in 4-day cultures. Sixteen fields of view were counted per well, and all samples were tested in duplicate or triplicate. One trophic unit was defined as the amount of protein necessary to achieve half maximal stimulation.

### Choroid Smooth-Muscle Cultures

Choroid cultures were prepared according to Coulombe and Nishi (1991) with the following modifications: Cells from one E14 choroid layer were plated per 100-mm

collagen-coated plate and infected with concentrated viral stock on the third day after plating. Choroid smooth-muscle cultures were maintained up to 2 weeks after infection.

### Preparation of Extracts

Fibroblast or choroid smooth-muscle cultures were collected from two 100-mm plates after 8 days of incubation [5 days after infection with RCASBP(A)], pelleted by centrifugation, then resuspended in a small volume of 10 mM sodium 3-(N-morpholino)-propane-sulfonate (Na-MOPS), 1 mM ethylenediaminetetraacetic acid (EDTA), pH 7.4, with a cocktail of protease inhibitors. Extracts were prepared by sonication for 30 s on ice with a probe-type sonicator and the lysate was centrifuged at  $30,000 \times g$  for 20 min at 4°C. Eye extracts were prepared by a similar protocol, except samples were adjusted to 0.5 mL buffer for each eye used and homogenized with a Tekmar tissue homogenizer for 45 s on ice prior to sonication. All lysate supernatants were concentrated by ultrafiltration 10-fold to remove molecules < 10 kDa and incubated with heparin-agarose (BioRad) for 1 h at 4°C. Conditioned medium from infected fibroblast and choroid smooth-muscle cultures was concentrated 10-fold by ultracentrifugation before use. All samples were adjusted for equal protein concentration and 0.2- $\mu$ m-filter-sterilized before addition to the cultures. Some samples were immunodepleted of chCNTF by incubating with rabbit anti-chCNTF antibodies bound to protein-A agarose beads (Gibco/BRL) for 4 h at 4°C. Identical aliquots were incubated with normal rabbit serum and protein-A agarose.



**Figure 1** Embryos grown in shell-less cultures and infected with RCASBP(A)-vector develop normally. The embryo on the left was placed into a sterile shell-less tripod at E2.75, infected with RCASBP(A)-vector at E4, and incubated under high-humidity conditions until E14, when it was collected and compared to an uninfected embryo grown in an intact egg (right). Embryos incubated by the two methods are indistinguishable, and infection with the virus does not alter viability or development of the embryo.

### Shell-Less Chicken Cultures

To allow for more precise injections near the eye, most experiments were performed using shell-less chick embryo cultures. Plastic tripods for holding cultures were constructed as described by Dunn (1981). Briefly, 3-inch plastic PVC pipe was cut into the shape of a tripod. A well was made in the tripod using plastic wrap (Fisher) and held in place by a plastic ring made from 3-inch PVC pipe, and the whole assembly was ultraviolet (UV)-sterilized for 2 h and covered with a sterile 100-mm petri dish bottom. E2–E3.5 chick embryos were placed into sterile tripods and incubated at 38.5°C under high humidity conditions. No antibiotics or calcium were added to the cultures. Embryos cultured in this way were healthy and viable and could be maintained through E14.

### Preparation of Windowed Eggs

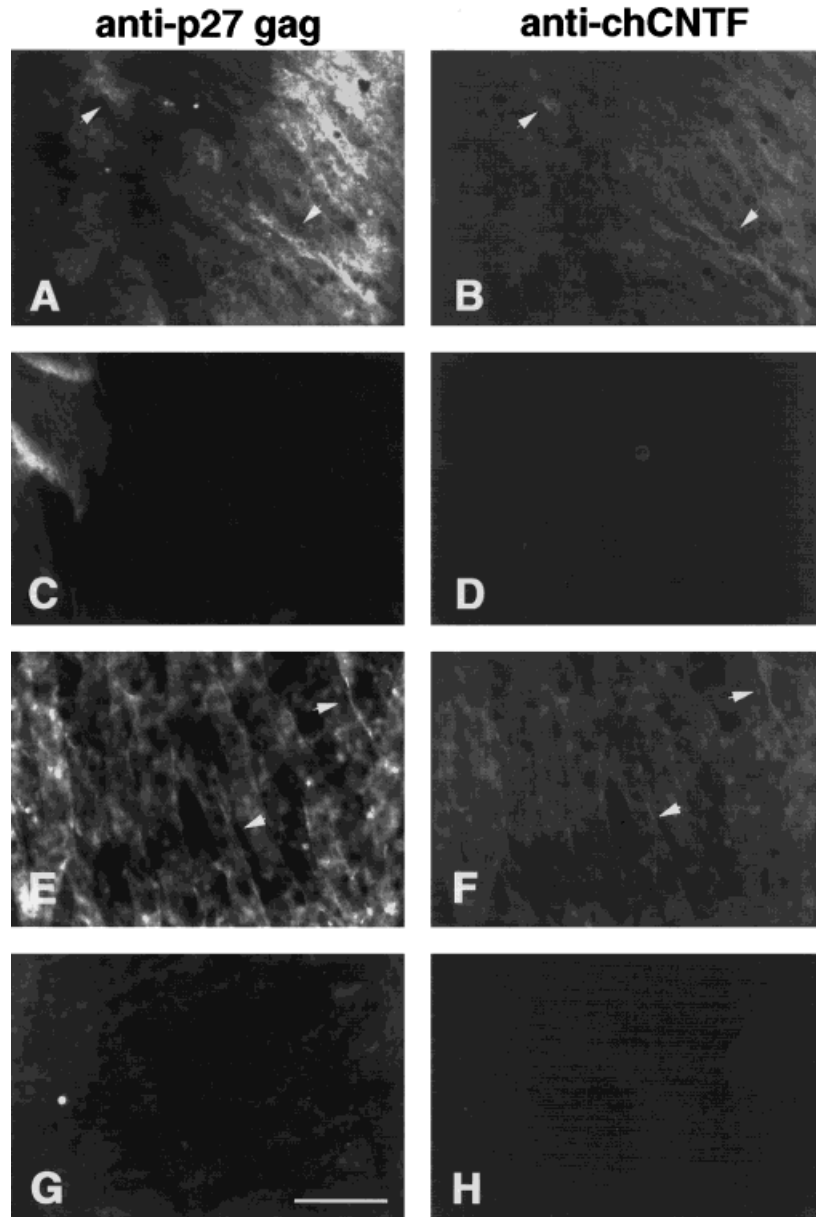
In two sets of experiments, virus was delivered to embryos developing in windowed eggs. Eggs to be windowed were set on their side 12 h prior to opening and the position of the embryo marked by candling. A small window was cut out of the top of the shell of E3–E4 eggs with the aid of a small hacksaw blade and the opening sealed with Blendoderm surgical tape (3M Corp.) and incubated for the duration of the experiment on its side at 38.5°C.

### RCASBP(A) Gene Constructs and Virus Production

The coding region of chCNTF was inserted into RCASBP(A) (permission obtained from S. Hughes) through the SLAX 12 shuttle vector (kindly provided by D. Fekete), which adds the Kozak consensus sequence of the src gene as well as the necessary *cl*1 sites (Morgan and Fekete, 1996). Viral stocks were generated by transfecting fibroblast cultures prepared from pathogen-free eggs (Hyvac Corp.; Adel, IA) with 1  $\mu$ g RCASBP(A) plasmid. Fibroblast cultures were maintained in modified L-15CO<sub>2</sub> (Mains and Patterson, 1973) supplemented with 10% (v/v) fetal calf serum (Hyclone), 33 mM glucose, 50 U/mL penicillin, and 50  $\mu$ g/mL streptomycin. Viral stocks were concentrated approximately 20-fold by ultracentrifugation at  $90,000 \times g$  at 4°C for 3 h and stored at –80°C. Concentrated stocks were titered by limiting dilution and infectivity of cells measured by staining for p27 gag protein. Only those stocks with titers > 10<sup>8</sup> infectious particles/mL were used in this study.

### Viral Infection

Two to four microliters of concentrated viral stock was injected directly at multiple sites into the region of the eye of E2.5–3.5 embryos using narrow glass tubing pulled to a fine point. In some instances, a dilute solution of Fast green dye was used to assess the efficiency of injection. Concentrated viral stocks were thawed immediately be-



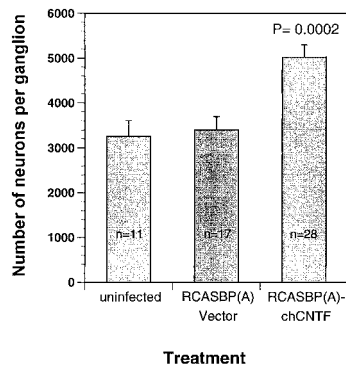
**Figure 2** RCASBP(A)-chCNTF-infected embryos overexpress chCNTF in CG targets. Shell-less embryos were either left uninfected (C,D,G,H) or infected at E4 with RCASBP(A)-chCNTF (A,B,E,F). Isolated iris ciliary muscle (A–D) and choroid layer whole mounts (E–H) were stained for both p27 gag (A,C,E,G) and for chCNTF (B,D,F,H). Infected embryos showed very strong p27 immunoreactivity throughout both tissues (A,E), while uninfected embryos displayed little or no specific signal [the high signal in (C) is nonspecific fluorescence in the iris]. Endogenous chCNTF (D,F) is barely detectable at E14, but in RCASBP(A)-ChCNTF-infected embryos chCNTF-like immunoreactivity is clearly present (B,F). Scale bar = 200  $\mu$ m.

fore injecting. For infection of cultured cells, 1  $\mu$ L of concentrated viral stock ( $1-7 \times 10^8$  infectious particles/mL) was added to each 35-mm or 100-mm plate 2 days after the original plating of cells.

### Counting Methods

Ciliary ganglia were isolated from E14 embryos and fixed in 4% paraformaldehyde for at least 72 h, prestained in

small amounts of thionin, dehydrated in an ethanol/xylene series, and embedded in paraffin. Eight-micrometer sections were cut and floated onto gelatin-coated slides. Sections were cleared of paraffin, rehydrated, and subsequently stained with 0.25% thionin, dehydrated, and coverslipped. Neuronal profiles were counted by determining the number of neurons with distinct nuclei in every fifth section at  $\times 600$  in the case of E8 and E11 ganglia, and



**Figure 3** Overexpression of chCNTF in CG targets enhances CG neuronal survival. E3 chick embryos were infected with concentrated viral stock of either RCASBP(A)-vector or RCASBP(A)-chCNTF and allowed to develop to E14. The number of neurons present in each E14 CG was determined from profile counts of thionin-stained CG sections and compared to E14 CG from uninfected embryos. Embryos overexpressing chCNTF had a significantly higher number of neurons than either uninfected controls or those infected with viral vector alone. Error bars indicate the standard error of the mean. The  $p$  value from Student  $t$  test comparing RCASBP(A)-vector and RCASBP(A)-chCNTF-infected embryos is shown.

at  $\times 400$  in every 10th section for E14. Mean cell diameters were calculated by acquiring images with a digital camera and traced within the NIH-Image software package. The total number of neurons present in the ganglia was calculated by dividing the sum of profiles counted by the number of sections counted, then multiplied by the total number of sections in each ganglion. No correction factor for double counting was made, as the total distance between sections was always larger than the largest profile diameter.

### Western Blot Analysis

All samples were run on 16% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels in  $\beta$ -mercaptoethanol-containing sample buffer, transferred to nitrocellulose (Schleicher and Schuell, Keene, NA), or PVDF (MSI, Westboro, MA) for 30 min at 150 mA using a semidry blotting apparatus, and blocked for at least 24 h at 4°C in 10% horse serum/2% powdered milk/0.1% Triton-X100 in phosphate-buffered saline (PBS). The blots were incubated with primary antibodies diluted in 10% horse serum, 0.1% Triton-X100 for 60 min at 25°C, washed three times in 0.5 M NaCl/0.1% Triton-X100/PBS and three times in PBS, then incubated for 60 min with a biotinylated secondary antibody (Vector Labs), washed, and incubated with alkaline phosphatase-coupled avidin (Gibco-BRL) for an additional 60 min. Blots were developed using NBT/BCIP (Promega) as a substrate.

### Immunocytochemistry

Choroid layers used for staining were prepared according to Finn and Nishi (1996). Choroid layers were fixed in 1% paraformaldehyde–15% picric acid at room temperature for 1 h, then blocked overnight in 10% horse serum. Choroid layers were stained floating with a 1:500 dilution of either rabbit anti-P27 (Spafas) or mouse anti-chCNTF (1:8) for 8 h at room temperature. Rhodamine-conjugated goat anti-rabbit (Sigma) and fluorescein isothiocyanate-conjugated goat anti-mouse secondary antibodies (Sigma) were used at a final concentration of 1:500 and stained for 4 h at 4°C. All steps were followed by washing six times in 0.5% Triton-X100, 5% horse serum in PBS. A similar protocol was followed for choroid smooth-muscle cultures, except incubation times for primary and secondary antibodies were 2 h and 1 h, respectively, and all incubations were performed at room temperature. Eyes used for cryostat sectioning were fixed for at least 2 days in 4% paraformaldehyde at 4°C, incubated overnight in 15% sucrose in PBS, and embedded in Tissue-Tek (OCT; Miles). Ten-micron sections were collected onto subbed slides, air-dried, then stained for 1–4 min with 0.125% thionin.

### RESULTS

#### RCASBP(A)-chCNTF Expression *In Vitro*

For the present study, we chose to overexpress chCNTF in chicken embryos with a Rous sarcoma-based replication competent retroviral vector, RCASBP(A), which produces high-level expression of a transgene (Petropoulos and Hughes, 1991; Boyer et al., 1993). Retroviral vectors have been shown to be an excellent means of stably expressing a transgene of choice in chick embryos at different stages of development (Morgan and Fekete, 1995).

To assess the ability of RCASBP(A)-chCNTF to infect CG target cells, we exposed smooth-muscle cells from the choroid layer of the eye to infective viral particles carrying RCASBP(A)-chCNTF in culture. Virtually every cell became infected within 3 days. Infected choroid cells showed strong p27 viral gag and chCNTF-like immunofluorescence (data not shown). By comparison, endogenous chCNTF in uninfected choroid cells or cells infected with vector alone was undetectable by immunofluorescence.

Bioassays based upon measuring CG neuronal survival confirmed that much higher levels of chCNTF-like activity were expressed in RCASBP(A)-chCNTF-infected choroid cells in culture, with typically 82-fold more biological activity present in conditioned medium (Table 1). Greater than 80% of this biological activity could be immunode-

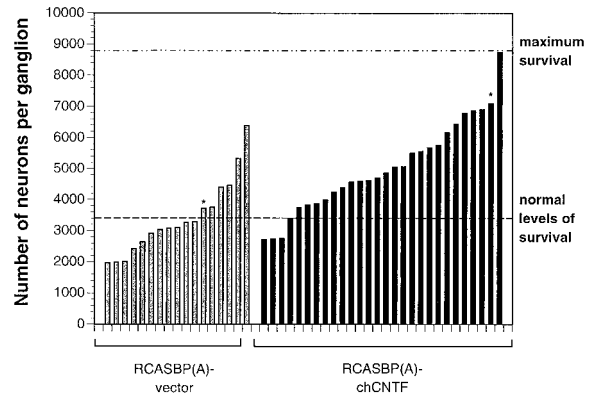
pleted with anti-chCNTF antibodies, verifying that the majority of neurotrophic activity being measured was due to chCNTF. Western blots of extracts from RCASBP(A)-chCNTF-infected choroid cultures confirmed that the full-length chCNTF protein was being made (data not shown).

### RCASBP(A) chCNTF Expression *In Vivo*

To maximize the chance that RCAS retrovirus would spread throughout CG targets in the eye, we injected viruses into the vicinity of the developing eye in E3–E4 chick embryos. Chicken embryos grown in culture outside of the shell (shell-less cultures) were used for the majority of viral infections because more precise injections could be made. To be certain that culture or viral infection did not slow the development of chick embryos or CG target tissues, we compared infected embryos grown in shell-less cultures with unmanipulated embryos incubated *in ovo* (Fig. 1). No differences in size or development could be seen between the two embryos, and the eyes appeared to be completely normal in terms of size, shape, and appearance.

We next sought to determine the ability of the virus to spread throughout the eye *in vivo*. The extent of infection and spread of the virus throughout the targets was measured by staining choroid layers and iris from RCASBP(A)-chCNTF-infected embryos for viral p27 gag and chCNTF. Viral gag p27 immunoreactivity was found throughout both the ciliary muscle and choroid layer of the eye from the infected side of the embryo [Fig. 2(A,E)]. Expression was uniform throughout the target tissues examined. ChCNTF-like immunoreactivity was detectable in a similar pattern, although the immunoreactivity levels were lower compared to p27 staining [Fig. 2(B,F)]. Virtually every cell that was immunopositive for p27 also showed immunoreactivity for chCNTF. ChCNTF immunoreactivity was substantially higher than endogenous levels of chCNTF, which is undetectable by immunofluorescence in E14 embryos under the conditions used here [Fig. 2(D,H)]. Uninfected embryos showed little or no specific p27 immunoreactivity, as would be expected for embryos developing from pathogen-free eggs [Fig. 2(C,G)].

The amount of chCNTF-like activity present in extracts of eyes pooled from either RCASBP(A)-chCNTF- or vector alone-infected embryos was quantified by bioassay on CG neurons in cell culture. Embryos infected at E4 with virus and collected at E14 showed an average increase of 2.8-fold in chCNTF-like activity (Table 1). In one case, a 21-fold increase in activity was found.



**Figure 4** Extent of rescue of CG neurons by RCASBP(A)-chCNTF infection varies considerably across individuals. A comparison of counts from individual CG was made to determine the overall effectiveness of elevating chCNTF levels *in vivo* on neuronal survival and the variation between different infected embryos. Data were from the same set of experiments as shown in Figure 3. Asterisks indicates ganglia used for comparison of neuronal density in chCNTF-infected embryos versus vector infected embryos (see Fig. 5).

### Effects of chCNTF Overexpression on Neuronal Survival

To test if elevation of chCNTF levels was able to affect CG neuronal survival, we infected E2.5–E4 shell-less chick embryos with either RCASBP(A)-chCNTF or RCASBP(A)-vector and performed neuronal counts on E14 CG. We chose E14 as an end point for several reasons. First, cell death in the avian CG is largely complete by E14 (Landmesser and Pilar, 1974); second, by E14 CG neurons have little or no dependence for target cell-conditioned medium (Alcain, 1988) or purified chCNTF for survival (Finn, unpublished observations); third, the viability of shell-less cultures is greatly reduced after E14.

Neuronal profile counts showed a 47% average increase in the number of neurons in RCASBP(A)-chCNTF infected embryos compared to controls (Fig. 3). Therefore, an additional 31% (1624 neurons) of the approximately 5234 neurons that would have died as part of naturally occurring cell death survived. An examination of E11 CG from embryos infected with RCASBP(A)-chCNTF showed a similar effect, with a rescue of 34% of the neurons that would have died by E11 compared to RCASBP(A) vector controls. Comparisons of individual ganglia from RCASBP(A)-chCNTF-infected embryos illustrated the wide range in neuronal survival (Fig. 4), possibly owing to differences in the extent of infection between embryos. Seven embryos showed more than a 50% rescue of those neurons that would

**Table 2 Overexpression of chCNTF Does Not Increase Neuronal Proliferation or Affect Size of Neurons**

| Property                            | Age         |             |             |             |
|-------------------------------------|-------------|-------------|-------------|-------------|
|                                     | E8          |             | E14         |             |
|                                     | Control     | chCNTF      | Control     | chCNTF      |
| Biological activity (trophic units) | 56          | 220         | 280         | 520         |
| No. profiles/CG                     | 8221 ± 926  | 9047 ± 1528 | 2854 ± 285  | 4069 ± 78   |
| Average cell diameter               | 18 ± 3.7 μm | 19 ± 1.4 μm | 31 ± 1.4 μm | 33 ± 1.3 μm |
| Average diameter of nucleus         | 10 ± 0.9 μm | 11 ± 1.0 μm | 17 ± 1.3 μm | 19 ± 1.3 μm |

Chick embryos were infected at E3 with either RCASBP(A)-vector (control) or RCASBP(A)-chCNTF (chCNTF) and collected at either E8 or allowed to continue incubating through E14. The amount of chCNTF-like trophic activity was measured in extracts of pooled eyes by bioassay. Ganglia or eyes were collected from the injected side of three embryos for each condition. At least 100 neuronal or nuclear profiles were measured in each set of ganglia assessed for neuronal survival.

have otherwise died, and one embryo contained nearly as many CG neurons as E8 controls. Embryos infected with concentrated viral DNA containing the chCNTF-coding region also showed an increase in CG neuronal survival, compared to embryos receiving vector DNA alone.

The difference in neuronal counts could not be explained by the presence of larger neuronal profiles in chCNTF-infected embryos, since both average neuronal and nuclear profile diameters were the same in RCASBP(A)-vector and RCASBP(A)-chCNTF CG (Table 2). A comparison of the two groups of ganglia showed that the CG appeared to be normal and of about equal size. The density of neurons within the ganglia, however, was greater in RCASBP(A)-chCNTF-infected embryos, as would be predicted if average neuronal size and ganglion size were the same but the number of neurons was increased (Fig. 5).

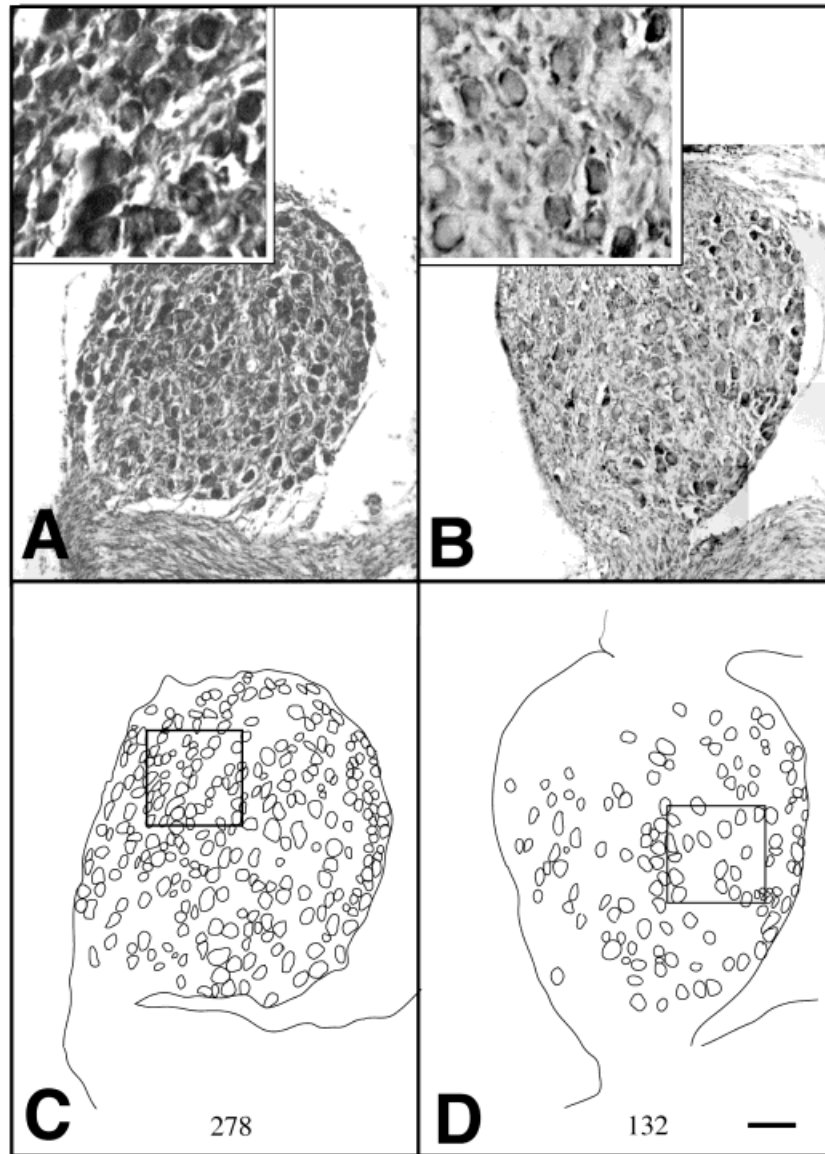
To test whether increased numbers of CG neurons at E11 and E14 could be attributed to an increase in formation of neurons prior to cell death, embryos were infected at E3 and collected at either E8 or allowed to continue to E14. ChCNTF-like activity from E8 eye extracts of infected embryos was fourfold higher in RCASBP(A)-chCNTF-infected embryos than RCASBP(A)-vector-infected embryos, demonstrating that elevated levels of chCNTF were present at the time when CG neurons become dependent upon their targets for survival (Table 2). Ciliary ganglia from E8 RCASBP(A)-chCNTF-infected embryos showed normal levels of CG neurons, while E14 CG had elevated levels compared to controls (Table 2). The CG is numerically complete by E7, and therefore, the neurons present at E8 represent those neurons that have become target dependent but have not yet begun cell death.

Ciliary neurotrophic factor has a wide range of biological properties in addition to its neurotrophic

activity (Sendtner et al., 1994). Since it is possible that chCNTF could play multiple roles *in vivo*, it was important to verify that CG targets developed normally. An examination of whole eyes from uninfected, RCASBP(A)-vector- and RCASBP(A)-chCNTF-infected embryos showed that both the overall appearance of the eye and the iris appeared to be identical [Fig. 6(A–D)]. No morphological differences could be seen in cross sections of iris [Fig. 6(E,F)] or the choroid layer of the eye [Fig. 6(G,H)]. Whole brain, including the cerebellum, which is known to express relatively high levels of chCNTFRa (Heller et al., 1995), appeared to be unaffected. The lack of a profound effect could be an indication that chCNTF plays very specific roles or that the levels being expressed were too low to have noticeable effects. The possibility of more subtle effects, such as enhanced neuronal survival in central nervous system (CNS) populations, was not examined in the context of the present study.

## DISCUSSION

Our previous studies have shown that chCNTF has many properties consistent with its being a target-derived neurotrophic factor for CG neurons: (a) It is secreted by virtue of an internal signal peptide (Leung et al., 1992; Reiness et al., 1995); (b) it is expressed in cells innervated by CG neurons (Finn and Nishi, 1996a); and (c) its expression coincides with the onset of the period of CG cell death (Leung et al., 1992; Finn and Nishi, 1996a). We now offer evidence that overexpressing chCNTF rescues CG neurons from cell death—neuron number is significantly increased after cell death (E14) in the CG of chCNTF-overexpressing embryos. Importantly, the overexpression of chCNTF during early development of the chick did not affect the number of neurons in found in the CG prior to the period of



**Figure 5** Ciliary ganglia from RCASBP(A)-chCNTF-infected embryos have a higher neuronal density. The 8- $\mu$ m paraffin section representing the numerical center of either an RCASBP(A)-chCNTF-infected embryo (A) or RCASBP(A)-vector (B) was scanned and each neuronal profile traced (C,D). Insets of (A) and (B) show the relative neuronal density of each ganglion. The number of profiles identified in each whole section is given below the outline of the ganglion. The area outlined in (C) and (D) represents the magnified views of (A) and (B). Scale bar = 100  $\mu$ m.

cell death (E8). All CG neurons are normally post-mitotic by E6 (Pilar et al., 1980), and the CG is numerically complete by E7 (Landmesser and Pilar, 1974b). Thus, the changes in neuronal number that occur after E8 are most likely to be due to differences in cell death.

It is essential to demonstrate a direct effect of a neurotrophic factor on developmental cell death, because neurotrophic molecules isolated by *in vitro* assays may have entirely different effects *in vivo*.

For example, fibroblast growth factor (FGF) initially generated a great deal of interest for its properties *in vitro* as a neurotrophic factor for spinal cord motor neurons. However, when injected *in vivo*, FGF failed to rescue motor neurons from cell death despite significant elevation of mitogenic activity in the embryo (Oppenheim et al., 1992). In another example, brain-derived neurotrophic factor (BDNF) supported the survival of spinal cord motor neurons *in vitro*; however, motor neurons were largely unaf-

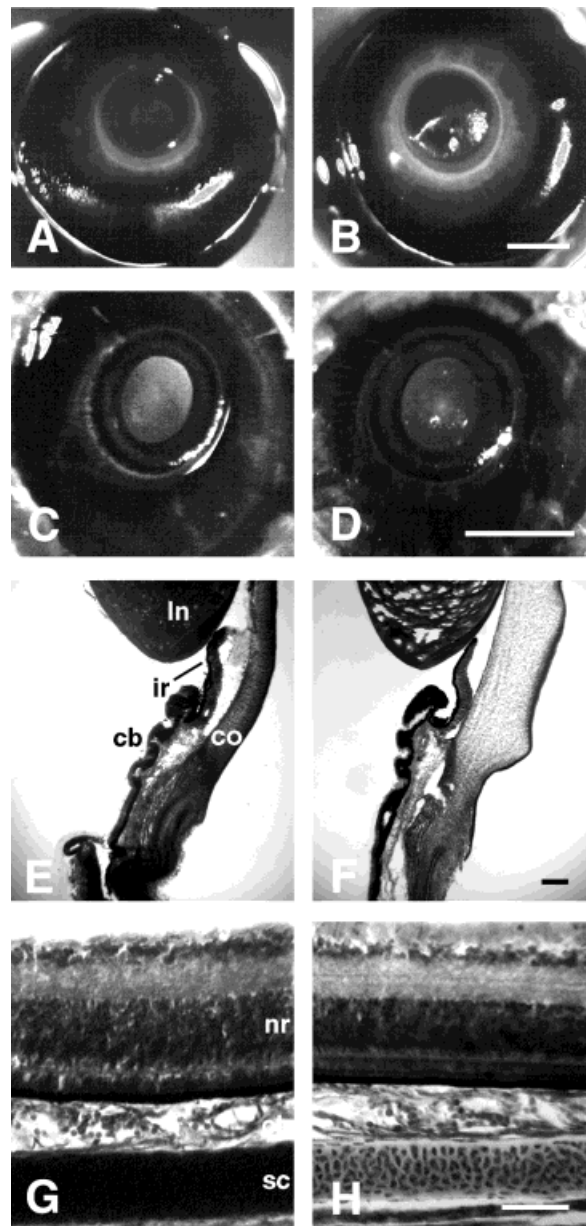
ected in the transgenic BDNF knockout mouse (Jones et al., 1994).

The ability to rescue neurons from cell death by application of a trophic factor also suggests that the availability of endogenous neurotrophic factor is limiting, and, therefore, controlling the degree of cell death that occurs, as would be predicted by the neurotrophic theory. This does not exclude other mechanisms, such as neuronal activity, in regulating cell death, but is an important confirmation that the neurotrophic theory is still valid for the CG.

Our results differ from experiments where infusion or injection of human CNTF rescued chick motor neurons but did not alter the survival of CG (Oppenheim et al., 1991). In preliminary attempts at infusion of chCNTF on the chorioallantoic membrane, we were also unable to rescue CG neurons from cell death. The most likely explanation for this discrepancy is that CG neurons require a more continual supply of chCNTF than would be provided by the pulsatile nature of infusions or injections. For example, rat CNTF injected intravenously has a half life of only a few minutes (Sendtner et al., 1992). In contrast, the retroviral approach we used here ensures a continual supply of chCNTF through stable incorporation, and expression of chCNTF-encoding cDNA into target cells is critical for demonstrating an effect of chCNTF on CG neurons. Continuously supplied chCNTF is less likely to be degraded or sequestered by surrounding tissues. Alternatively, chick motor neurons may be more sensitive to human or rat CNTF than to chick CNTF. Such properties have been observed in chick sympathetic neurons, which are more sensitive to rat CNTF than chick CNTF for survival (R. Nishi, unpublished observations), and on whose receptors rat CNTF competes more effectively for binding than chick CNTF (Heller et al., 1993).

An important counterpart to the present study will be to block or knock out the expression of chCNTF *in vivo* to test the extent to which endogenously expressed CNTF regulates CG neuron survival. Although a CNTF gene knockout mouse has been created (Masu et al., 1992), the murine ciliary ganglion is sufficiently diffuse and difficult to locate and isolate that it has not been possible to examine developmental cell death (Jackson, 1986). At present, it is not possible to obtain gene knockouts in chickens; thus, these studies will rely on obtaining appropriate reagents for blocking chCNTF synthesis or activity over a prolonged period of time.

The properties that we have found for chicken CNTF are in contrast to the mammalian forms of CNTF, which are considered to be lesion-induced regeneration factors (Richardson, 1994; Sendtner et



**Figure 6** Development of eyes and target tissues in RCASBP(A)-chCNTF-infected embryos is unaffected. Both whole eyes (A–D) and sections of either the anterior chamber (E,F) or the three eye layers (G,H) from infected eyes were compared from RCASBP(A)-vector (A,C,E,G) or RCASBP(A)-chCNTF-infected embryos (B,D,F,H). Morphologically, all tissues appeared to be identical. Embryos were infected at E3 and incubated until E14 when they were collected. All tissues were immediately placed in fixative after harvesting. (B,C) A closeup of the iris and pupil after the overlying cornea has been removed. In some sections the cornea appeared to be thicker than in viral vector controls, but it is unclear if this is a consequence of chCNTF. ln = lens; ir = iris; ch = choroid layer; cb = ciliary body; co = cornea. Scale bar = 5 mm for (A–D) and 150  $\mu$ m for (E–H).

al., 1994). There is very little expression of rat CNTF during embryonic development, and cells transfected with rat CNTF cDNA are unable to release a biologically active molecule. It is still controversial as to whether a secreted form of CNTF exists in mammals, although this possibility has been reinforced by the recent report that the CNTFR $\alpha$  knockout mouse has a more severe phenotype than the CNTF knockout (DeChiara et al., 1995; Finn and Nishi, 1996b).

The control of neuronal survival is a complex process that appears to involve the interaction of a variety of factors. Although our studies indicate that chCNTF is likely to be a target-derived trophic factor for both ciliary and choroid neurons of the CG, other studies have shown that presynaptic elements (Meriney et al., 1985, 1991; Furber et al., 1987), synaptic transmission (Meriney et al., 1987), and factors produced by non-target-related structures may also be important in regulating neuronal survival (Pilar et al., 1988; Oppenheim, 1989). It is not known whether these influences each act directly upon neurons by altering signal transduction or indirectly by altering the availability of trophic support. The challenge now is to study the relative role of these influences in regulating target-dependent cell death.

The authors thank Brian Boucher for moral and technical support. They are also grateful to Drs. C. Cepko, D. Fekete, and S. Hughes for their technical advice regarding the use of RCAS. Drs. S. Matsumoto and G. Reiness provided helpful comments on the manuscript. This study was funded by NIH Grant NS25767.

## REFERENCES

- ADLER, R., LANDA, K. B., MANTHORPE, M., and VARON, S. (1979). Cholinergic neurotrophic factors: intraocular distribution of trophic activity for ciliary neurons. *Science* **204**:1434–1436.
- ALCAIN, F. J. (1988). Age-dependent responses of ciliary ganglion neurons to conditioned media on cells at different stages of embryonic development. *Int. J. Neurosci.* **43**:189–193.
- BARDE, Y.-A. (1989). Trophic factors and neuronal survival. *Neuron* **2**:1525–1534.
- BOYER, P. L., COLMENARES, C., STAVNEZER, E., and HUGHES, S. H. (1993). Sequence and biological activity of chicken snoN cDNA clones. *Oncogene* **8**:457–466.
- COULOMBE, J. N. and NISHI, R. (1991). Stimulation of somatostatin expression in developing ciliary ganglion neurons by cells of the choroid layer. *J. Neurosci.* **11**:553–562.
- DECHIARA, T. M., VEJSADA, R., POUYEMIROU, W. T., ACHESON, A., SURI, C., CONOVER, J. C., FRIEDMAN, B., MCCLAIN, J., PAN, L., STAHL, N., IP, N. Y., KATO, A., and YANCOPOULOS, G. D. (1995). Mice lacking the CNTF receptor, unlike mice lacking CNTF, exhibit profound motor neuron deficits at birth. *Cell* **83**:313–322.
- DUNN, B. E., FITZHARRIS, T. P., and BARNETT, B. D. (1981). Effects of varying chamber construction and embryo pre-incubation age on survival and growth of chick embryos in shell-less culture. *Anat. Rec.* **199**:33–43.
- ECKENSTEIN, F. P., ESCH, F., HOLBERT, T., BLACHER, R. W., and NISHI, R. (1990). Purification and characterization of a trophic factor for embryonic peripheral neurons: comparison with fibroblast growth factors. *Neuron* **4**:623–631.
- FINN, T. P. and NISHI, R. (1996a). Expression of a chicken ciliary neurotrophic factor in targets of ciliary ganglion neurons during and after the cell-death phase. *J. Comp. Neurol.* **366**:559–571.
- FINN, T. P. and NISHI, R. (1996b). Does ciliary neurotrophic factor serve different functions in the rat versus the chicken? *Perspect. Devel. Neurobiol.* **4**:91–99.
- FURBER, S., OPPENHEIM, R. W., and PREVETTE, D. (1987). Naturally-occurring neuron death in the ciliary ganglion of the chick embryo following removal of preganglionic input: evidence for the role of afferents in ganglion cell survival. *J. Neurosci.* **7**:1816–1832.
- HELLER, S., FINN, T. P., HUBER, J., NISHI, R., GEISSEN, M., PUSCHEL, A. W., and ROHRER, H. (1995). Analysis of function and expression of the chick GPA receptor (GPAR alpha) suggests multiple roles in neuronal development. *Development* **121**:2681–2693.
- HELLER, S., HUBER, J., FINN, T. P., NISHI, R., and ROHRER, H. (1993). GPA and CNTF produce similar effects in sympathetic neurones but differ in receptor binding. *Neuroreport* **5**:357–360.
- JACKSON, P. C. (1986). Innervation of the iris by individual parasympathetic axons in the adult mouse. *J. Physiol. Lond.* **378**:485–495.
- JONES, K. R., FARINAS, I., BACKUS, C., and REICHARDT, L. F. (1994). Targeted disruption of the BDNF gene perturbs brain and sensory neuron development but not motor neuron development. *Cell* **76**:989–999.
- KOSHLUKOVA, S., FINN, T. P., NISHI, R., and HALVORSEN, S. W. (1996). Identification of functional receptors for ciliary neurotrophic factor on chick ciliary ganglion neurons. *Neuroscience* **72**:821–832.
- LANDMESSER, L. and PILAR, G. (1974a). Synapse formation during embryogenesis on ganglion cells lacking a periphery. *J. Physiol. Lond.* **241**:715–736.
- LANDMESSER, L. and PILAR, G. (1974b). Synaptic transmission and cell death during normal ganglionic development. *J. Physiol. Lond.* **241**:737–749.
- LEUNG, D. W., PARENT, A. S., CACHIANES, G., ESCH, F., COULOMBE, J. N., NIKOLICS, K., ECKENSTEIN, F. P., and NISHI, R. (1992). Cloning, expression during development, and evidence for release of a trophic factor for ciliary ganglion neurons. *Neuron* **8**:1045–1053.
- LIN, L. F., MISMER, D., LILE, J. D., ARMES, L. G., BUT-

- LER, E. T. III, VANNICE, J. L., and COLLINS, F. (1989). Purification, cloning, and expression of ciliary neurotrophic factor (CNTF). *Science* **246**:1023–1025.
- MAINS, R. E. and PATTERSON, P. H. (1973). Primary cultures of dissociated sympathetic neurons. I. Establishment of long-term growth in culture and studies of differentiated properties. *J. Cell Biol.* **59**:329–345.
- MANTHORPE, M., ADLER, R., and VARON, S. (1981). Cholinergic neuronotrophic factors. VI. Age-dependent requirements by chick embryo ciliary ganglionic neurons. *Dev. Biol.* **85**:156–163.
- MASU, Y., WOLF, E., HOLTSMANN, B., SENDTNER, M., BREM, G., and THOENEN, H. (1992). Disruption of the CNTF gene results in motor neuron degeneration. *Nature* **365**:27–32.
- MERINEY, S. D., GRAY, D. B., and PILAR, G. (1985). Morphine-induced delay of normal cell death in the avian ciliary ganglion. *Science* **228**:1451–1453.
- MERINEY, S. D., FORD, M. J., OLIVA, D., and PILAR, G. (1991). Endogenous opioids modulate neuronal survival in the developing avian ciliary ganglion. *J. Neurosci.* **11**:3705–3717.
- MERINEY, S. D., PILAR, G., OGAWA, M., and NUNEZ, R. (1987). Differential neuronal survival in the avian ciliary ganglion after chronic acetylcholine receptor blockade. *J. Neurosci.* **7**:3840–3849.
- MORGAN, B. A. and FEKETE, D. M. (1996). Manipulating gene expression with replication-competent retroviruses. *Methods Cell Biol.* **51**:185–218.
- NARAYANAN, C. H. and NARAYANAN, Y. (1978). Neuronal adjustments in developing nuclear centers of the chick embryo following transplantation of an additional optic primordium. *J. Embryol. Exp. Morphol.* **44**:53–70.
- NISHI, R. (1996) Autonomic and sensory neuron cultures. *Methods Cell Biol.* **51**:249–262.
- NISHI, R. and BERG, D. K. (1981). Two components from eye tissue that differentially stimulate the growth and development of ciliary ganglion neurons in cell culture. *J. Neurosci.* **1**:505–513.
- OPPENHEIM, R. W. (1989) The neurotrophic theory and naturally occurring motoneuron death. *Trends Neurosci.* **12**:252–255.
- OPPENHEIM, R. W., PREVETTE, D., and FULLER, F. (1992). The lack of effect of basic and acidic fibroblast growth factors on the naturally occurring death of neurons in the chick embryo. *J. Neurosci.* **12**:2726–2734.
- OPPENHEIM, R. W., PREVETTE, D., YIN, Q. W., COLLINS, F., and MACDONALD, J. (1991). Control of embryonic motoneuron survival in vivo by ciliary neurotrophic factor. *Science* **251**:1616–1618.
- PETROPOULOS, C. J. and HUGHES, S. H. (1991). Replication-competent retrovirus vectors for the transfer and expression of gene cassettes in avian cells. *J. Virol.* **65**:3728–3737.
- PILAR, G., LANDMESSER, L., and BURSTEIN, L. (1980). Competition for survival among developing ciliary ganglion cells. *J. Neurophysiol.* **43**:233–254.
- PILAR, G. R., MERINEY, S. D., and GRAY, D. B. (1988). Trophic effects of muscle on nerve cell survival and development. In: *Nerve-Muscle Cell Trophic Communication*. H. Fernandez and J. Donsco, Eds. CRC Press, Boca Raton, FL.
- REINISS, C. G., SWEENEY, S., and NISHI, R. (1995). Identification of a structural domain responsible for secretion of a neurotrophic factor for ciliary ganglion neurons. *Soc. Neurosci. Abstr.* **21**:1047.
- RICHARDSON, P. M. (1994). Ciliary neurotrophic factor: a review. *Pharmacol. Therapeu.* **63**:187–198.
- SENDTNER, M., SCHMALBRUCH, H., STÖCKLI, K. A., CARROLL, P., KREUTZBERG, G. W., and THOENEN, H. (1992). Ciliary neurotrophic factor prevents degeneration of motor neurons in mouse mutant progressive motor neuronopathy. *Nature* **358**:502–504.
- SENDTNER, M., CARROLL, P., HOLTSMANN, B., HUGHES, R. A., and THOENEN, H. (1994). Ciliary neurotrophic factor. *J. Neurobiol.* **25**:1436–1453.
- SENDTNER, M., KREUTZBERG, G. W., and THOENEN, H. (1990). Ciliary neurotrophic factor prevents the degeneration of motor neurons after axotomy. *Nature* **345**:440–441.
- STÖCKLI, K. A., LOTTSPEICH, F., SENDTNER, M., MASIAKOWSKI, P., CARROLL, P., GOTZ, R., LINDHOLM, D., and THOENEN, H. (1989). Molecular cloning, expression and regional distribution of rat ciliary neurotrophic factor. *Nature* **342**:920–923.