Control of Embryonic Motoneuron Survival in Vivo by Ciliary Neurotrophic Factor

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During development of the nervous system, neurons in many regions are overproduced by proliferation, after which the excess cells are eliminated by cell death. The survival of only a proportion of neurons during normal development is thought to be regulated by the limited availability of neurotrophic agents. One such putative trophic agent is ciliary neurotrophic factor (CNTF), a polypeptide that promotes the survival of ciliary, sensory, and sympathetic neurons in vitro. In contrast to the results of in vitro studies, however, the daily treatment of chick embryos in vivo with purified human recombinant CNTF failed to rescue any of these cell populations from cell death, whereas CNTF did promote the in vivo survival of spinal motoneurons. Thus, CNTF may not act as a neurotrophic agent in vivo for those embryonic neurons (especially ciliary neurons) on which it acts in vitro. Rather, CNTF may be required for in vivo survival of motoneurons.

NATURALLY OCCURRING OR PROgrammed neuronal death probably serves many different biological functions and is mediated by different cellular and molecular mechanisms (*1*). A current view of neuronal death is that neurons compete for trophic molecules that are supplied by targets, afferents, or glia (*2*). Because the availability of trophic agents is thought to be limited, only a proportion of all post-mitotic neurons in a population would be able to attain amounts sufficient for their survival. This view is known as the trophic or neurotrophic hypothesis, and it has guided most studies in this field since the discovery of the prototypical trophic agent, nerve growth factor (NGF) (*3*). The identification and characterization of putative neurotrophic agents other than NGF has been slow, and only recently have a number of additional molecules been identified that promote the survival of specific populations of neurons in vitro (*4*). One of these molecules is CNTF, a small polypeptide that was purified from chick ocular tissue, bovine heart, and rat and rabbit sciatic nerve, and which has recently been sequenced and cloned (*5, 6*). Although the most common bioassay for CNTF activity has been its ability to promote the survival of avian embryo ciliary ganglion neurons in vitro (*5, 6*), it also (i) promotes the survival of developing sensory, sympathetic, and retinal neurons in vitro (*5–7*), (ii) prevents the death of axotomized facial motoneurons and preganglionic sympathetic neurons in vivo (*8, 9*), (iii) promotes cholinergic differentiation in vitro (*10*), and (iv) regulates the phenotypic expression of astrocytes in vitro (*11*). To test the role of CNTF in neuronal survival in vivo, we administered CNTF to chick embryos in ovo during those periods when a number of different neuronal populations are undergoing naturally occurring cell death (*1*).

Purified human recombinant CNTF (*12*) was diluted in 50 μl of a solution containing sodium phosphate (10 mM), NaCl (50 mM), EGTA (0.1 mM), and EDTA (0.1 mM) and administered daily at different doses from embryonic day 6 (E6) to E9 or from E9 to E14. Control embryos were treated daily with an equal volume of the solution used for dilution. Both CNTF and control materials were administered by injection onto the vascularized chorio-allantoic membrane (CAM) through a small window in the shell, a method that is effective for delivering trophic and other agents to the avian embryo (*13, 14*). At the end of the treatment period, the embryos were killed by decapitation and the tissues processed for histology (*14, 15*).

Treatment of chick embryos in ovo from E6 to E9 with various doses of CNTF had no statistically significant effect on the survival of sensory, sympathetic, nodose, or sympathetic preganglionic neurons (Table 1). Treatment of embryos with CNTF during the period of naturally occurring ciliary neuron death (E9 to E14) also did not promote their survival and, in fact, at high concentrations appeared to reduce survival (Fig. 1). All of these neuronal populations undergo a period of cell death in which 30 to 50% of the original population degenerates (*1*), yet treatment with CNTF during the cell death period failed to prevent these losses. It is unlikely that these negative results were due to inaccessibility of CNTF to the neurons. Other putative trophic agents, drugs, and neurotoxins administered in vivo in the same manner rescue a variety of different types of neurons from cell death in the avian embryo (*13, 14*). Moreover, injections of CNTF into the eye also failed to promote ciliary ganglion (CG) survival (*16*). Furthermore, we found one population of cells (motoneurons) that did, in fact, respond to CNTF administered onto the CAM.

Table 1. The number (mean ± SD) of sensory (DRG), sympathetic (SG), and nodose (ND) ganglion cells and sympathetic preganglionic cells (PG) on E10 after daily treatment from E6 to E9 with 10 μg of CNTF. Similar negative results were also obtained for all of these cell types after treatment with 0.05, 0.5, 2, and 20 μg of CNTF from E6 to E9 (*23*). Cell numbers were determined by individuals blind as to treatment. Cell numbers in DRG and SG were determined only in the third lumbar (L3) ganglion. Preganglionic cells were only counted in the last thoracic segment (T6), as defined by the last adjacent thoracic DRG before the beginning of the lumbar lateral motor column (*14*). None of the differences shown were statistically significant (*t* test).

Cells	Control (*n*)	CNTF (10 μg) (*n*)
DRG	9,507 ± 1,140 (5)	9,770 ± 960 (5)
SG	12,178 ± 2,154 (4)	10,461 ± 2,797 (4)
PG	1,713 ± 159 (4)	1,808 ± 158 (4)
ND	5,810 ± 589 (5)	5,385 ± 544 (7)

Between E6 and E10, about 40 to 50% of the lumbar spinal motoneurons degenerate (*17*). Treatment with appropriate doses of CNTF from E6 to E9 rescued one-half of the motoneurons that normally die (Fig. 2). Over the range of doses used here (0.05 to 20 μg per injection), there was a dose-response relation; 2 μg was a saturating

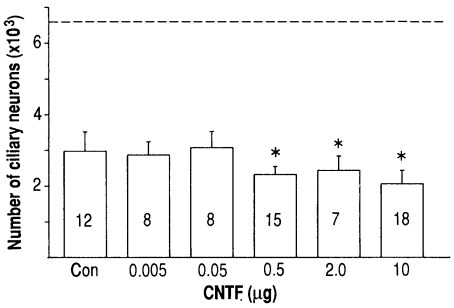

Fig. 1. The number (mean ± SD) of surviving ciliary ganglion neurons on E15 after daily treatment from E9 to E14 with different doses of CNTF. Numbers in bars are the sample sizes. Embryos were killed and the stage determined (*24*); the ciliary ganglia were dissected and processed, and the ciliary neurons were counted (*25*). Cell numbers were determined by individuals blind as to treatment (control or CNTF). The dashed line indicates the average number of cells present on E8 to E9 before treatment. *$P < 0.007$, *t* test between CNTF and control with Bonferroni correction. Similar results were obtained when CNTF (2 μg) was administered from E8 to E14; CG cell numbers in controls were 2796 ± 417 ($n = 7$) and 2332 ± 414 ($n = 9$) for E8 to E14 CNTF-treated embryos. This is a statistically significant difference ($P < 0.05$; *t* test).

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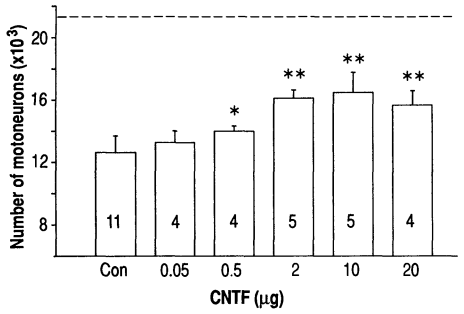

Fig. 2. The number (mean ± SD) of surviving lumbar motoneurons on E10 after daily treatment from E5 to E9 with different doses of CNTF. Numbers in bars are the sample sizes. Embryos were killed, staged (*24*), processed (*14, 15*), and motoneurons counted (*26*). Cell counts were made blind as to treatment (control or CNTF). The dashed line indicates the average number of neurons present on E6 before treatment. $^{**}P < 0.001$, $^{*}P < 0.03$, *t* test between CNTF and control with Bonferroni correction.

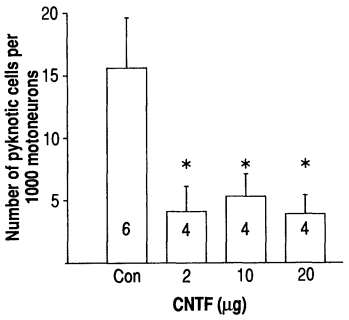

Fig. 3. The number (mean ± SD) of pyknotic motoneuron profiles in the lumbar spinal cord per 1000 healthy motoneurons on E8 after daily treatment with CNTF from E6 to E8. Total motoneuron numbers in these E8 control embryos were 16,231 ± 879 ($n = 4$) and 18,698 ± 1,011 ($n = 4$) for CNTF-treated embryos. This is a statistically significant difference ($P < 0.001$; *t* test). Pyknotic motoneurons were identified by established criteria (*17*). All pyknotic counts were done blind as to treatment. $^{*}P < 0.001$, *t* test with Bonferroni correction.

dose. Although the effect of CNTF on motoneuron numbers could be because of mechanisms other than the inhibition of cell death (for example, mitogenic, differentiation, or metabolic effects), this seems unlikely. The number of pyknotic motoneurons present on E8 (the peak time of cell death) was significantly reduced by CNTF treatment (Fig. 3), indicating that CNTF prevented the degeneration of cells.

Treatment of embryos in vivo with NGF prevents the death of sensory and sympathetic neurons and enhances their growth and differentiation (*18*). On the basis of cytological features and cell size, CNTF had no effect on the growth and differentiation of either spinal motoneurons or ciliary neurons in vivo (*19*). Although an effect of CNTF on differentiation cannot be excluded, these data indicate that CNTF may act specifically in vivo to promote cell survival.

The failure of CNTF to promote the in vivo survival of the major cell type (ciliary neurons) that has been used in bioassays for its purification is unexpected. Because it is unlikely that this failure is due to the mode of administration or inappropriate doses, it seems reasonable to conclude that in vitro bioassays for trophic activity may not always reflect the situation during development in vivo. Tissue culture may create conditions (for example, injury, altered cell-cell interactions, changed metabolic requirements) that are not normally operative in vivo. However, our data do not exclude the possibility that CNTF may act as a trophic agent for ciliary or other types of neurons in the postnatal and adult animal or that treatment with exogenous CNTF after nerve injury can prevent neuronal degeneration (*8, 9*). The reason for the apparent inhibitory effects of CNTF on ciliary neurons in vivo at high doses is not clear. None of the other neuronal populations examined here exhibited decreased survival after CNTF treatment and the same preparation of CNTF is not toxic to ciliary neurons in vitro (*12*).

Motoneurons may be a major target of CNTF. The survival of enriched populations of developing avian spinal motoneurons in vitro is promoted by CNTF, and CNTF can rescue neonatal rat facial motoneurons from axotomy-induced cell death (*9, 20*). Other purified proteins have also been shown to promote the survival of spinal motoneurons both in vitro and in vivo (*13, 20, 21*). It is possible that some of these putative motoneuron trophic agents are members of a family of molecules that share a common receptor. Alternatively, during development, motoneuron survival may be regulated by two or more distinct molecules, one of which is CNTF (*20*). The failure of CNTF to rescue all of the motoneurons that normally die is consistent with this idea. CNTF is a cytosolic protein that lacks a leader or signal sequence characteristic of most secreted molecules, and its tissue distribution and developmental regulation in the rat do not appear to be consistent with its being involved in naturally occurring cell death (*6, 9*). However, until more is known about alternative modes of protein secretion and release (*22*), and until the tissue distribution and developmental regulation of CNTF in the central and peripheral nervous system are more thoroughly examined, the possibility that CNTF is a trophic agent for developing motoneurons in vivo cannot be excluded. Our observations suggest that CNTF may be involved in regulating the widespread phenomenon of naturally occurring motoneuron death in vertebrates.

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12. Human recombinant CNTF was expressed in *Escherichia coli* strain BL21 (DE3). CNTF was purified from the bacterial cell lysate with ion-exchange chromatography. Purified CNTF had <0.05% contaminating *E. coli* proteins as determined by reversed-phase high-performance liquid chromatography and had 0.00048 endotoxin units (USP) per microgram. Purified recombinant CNTF promoted the survival of E8 chicken embryo ciliary ganglion neurons in culture [L.-F. H. Lin, L. G. Armes, A. Sommer, D. J. Smith, F. Collins, *J. Biol. Chem.* **265**, 8942 (1990)] with half-maximal survival occurring at 0.47 ± 0.04 ng/ml ($n = 5$).
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15. After fixation in Carnoy's solution, the thoracolumbar spinal cord (including the attached dorsal root and sympathetic ganglia), as well as the nodose ganglia and the ciliary ganglia were dissected, processed, embedded in paraffin, serially sectioned (10 μm), and stained with thionin.
16. Embryos (E10) were treated with 2 μg of CNTF in 4-μl solution injected into the vitreous fluid of the right eye, killed on E11.5, and the number of CG neurons was determined (*25, 26*). Control embryos were injected with 4 μl of vehicle. Normally, approximately 1100 ± 321 (mean ± SD, $n = 12$) CG cells degenerate between E10 and E11.5 to E12.0. Because there was not a significant difference (by *t* test) in cell numbers between the right and left CG of either CNTF- or vehicle-injected embryos on E11.5, data from the two sides were pooled. Vehi-

cle-treated embryos had 3667 ± 398 CG cells (mean ± SD, n = 11), whereas CNTF-treated embryos had 3784 ± 501 (n = 12). This difference is not statistically significant (by a t test).
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19. Cell size was estimated by measuring the nuclear diameter at its widest extent. Cells were drawn at ×1100 with a drawing tube. Spinal motoneurons in all regions of the ventral horn in lumbar (L) segment L4 and ciliary neurons in all regions of the ganglion were included. Nuclear diameters were calculated with a morphometric program with the Zeiss Videoplan. Motoneuron nuclear diameter (mean ± SD) was 8.98 ± 0.83 μm (n = 150) for E10 controls and 8.89 ± 0.63 μm (n = 150) for embryos treated from E6 to E9 with 10 μg of CNTF. Similar results were obtained for embryos treated with 0.5 μg of CNTF (R. W. Oppenheim, D. Prevette, Y. Qin-Wei, F. Collins, J. MacDonald, unpublished data). Ciliary neuron nuclear diameter was 8.52 ± 0.86 μm (n = 125) for E15 controls and 8.59 ± 1.09 μm (n = 125) for embryos treated with 0.5, 2, or 10 μg of CNTF from E9 to E14. These differences are not statistically significant (t test).
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" Is it really a great discovery or just Pankhurst making damn fools of us?"