Death of Developing Septal Cholinergic Neurons following NGF Withdrawal *in vitro*: Protection by Protein Synthesis Inhibition

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Fetal septal neurons were grown *in vitro* under glass coverslips. This sandwich culture method significantly increased general neuronal survival, reduced glial proliferation, and permitted the removal of serum from the growth medium after 5 d *in vitro*. Thereafter, a simple, and completely defined, medium was used, and the effects of NGF, NGF withdrawal, and protein synthesis inhibition were examined on septal cholinergic neurons. NGF added to septal cultures at the time of plating resulted in a threefold increase in the number of cholinergic neurons seen at 14 d *in vitro* but had no effect on the survival of non-cholinergic cells. Cholinergic neurons identified by staining for AChE, ChAT, and p75[NGFR] could be maintained in serum-free, NGF-supplemented medium for over 40 d. When NGF was removed and NGF antibodies added to 14-d-old cultures, less than 30% of cholinergic neurons survived a further 4 d, but when NGF was similarly withdrawn from 35-d-old cultures, over 75% of cholinergic neurons survived. Reapplication of NGF after 3 but not after 12 or more hours of NGF withdrawal from 14-d-old cultures prevented the death of most cholinergic neurons. When NGF was withdrawn from 14-d-old cultures in the presence of the protein synthesis inhibitor cycloheximide, over 75% of the cholinergic neurons survived. These findings suggest that septal cholinergic neurons are dependent on NGF for survival only during a critical period of development and that growth factor–regulated developmental cell death may occur in CNS neurons by activation of programmed cell death requiring protein synthesis.

[Key words: NGF, cycloheximide, basal forebrain cholinergic neurons, primary neuronal tissue culture, naturally occurring cell death, apoptosis]

Studies on the PNS suggest that NGF released by target cells regulates the survival and differentiation of developing sympathetic and dorsal root ganglion (DRG) neurons (Levi-Montalcini and Angeletti, 1963). Mature sympathetic neurons appear to remain dependent on NGF for survival (Greene, 1977a;

Gorin and Johnson, 1979), while mature DRG neurons do not (Greene, 1977b; Barde et al., 1980; Schwartz et al., 1982). Within the CNS, cholinergic neurons of the basal forebrain bear low-affinity (p75[NGFR]) and high-affinity (p140[trk]) NGF receptors and retrogradely transport NGF from their target region, the hippocampus (Seiler and Schwab, 1984; Taniuchi et al., 1986; Dawbarn et al., 1988; Mufson et al., 1989; Holtzman et al., 1992), but the role of NGF regarding these neurons is not well understood.

Addition of NGF to tissue cultures of basal forebrain cells (Hartikka and Hefti, 1988), and intraventricular infusion of NGF to neonatal and adult rats (Gnahn et al., 1983; Mobley et al., 1986), increases the size and ChAT activity of basal forebrain cholinergic neurons, indicating that NGF can regulate the chemical and structural phenotype of these neurons both *in vitro* and *in vivo*. Studies assessing the effects of NGF on the survival of basal forebrain cholinergic neurons show that NGF increases the number of developing cholinergic neurons in low-density but not high-density septal cultures (Hartikka and Hefti, 1988; Alderson et al., 1990), and that pharmacologic doses of NGF prevent the disappearance of septal cholinergic neurons after fimbria-fornix lesions in the adult rat (Hefti, 1986; Williams et al., 1986; Kromer, 1987), suggesting that NGF might be an obligatory survival factor for these neurons throughout life. However, infusions of NGF antibody into the ventricles of neonatal rats appear not to cause the death of basal forebrain cholinergic neurons (Gnahn et al., 1983; Vantini et al., 1989), and adult septal cholinergic neurons survive following excitotoxic lesions that deplete the hippocampus of NGF mRNA (Sofroniew et al., 1990, 1993). Thus, the degree to which NGF regulates the survival of basal forebrain cholinergic neurons during development or in the adult is uncertain.

In this study we examined whether septal cholinergic neurons grown in tissue culture in the presence of NGF are dependent on NGF for survival at different stages of maturity. Since dissociated cultures of fetal septal cells undergo massive degeneration over the first 7 d *in vitro* and are difficult to maintain for long periods of time in serum-free conditions (Hartikka and Hefti, 1988; Alderson et al., 1990), we adapted a sandwich culture method previously shown to increase the short- and long-term survival of hippocampal neurons *in vitro* (Brewer and Cotman, 1989). Using this method we examined the reaction of septal cholinergic neurons, maintained in the presence of NGF, to NGF withdrawal at 14 or 35 d *in vitro*. In addition, we investigated whether the cell death observed following NGF withdrawal from septal cholinergic neurons after 14 d *in vitro* could be prevented by inhibition of protein synthesis as previously described for developing peripheral neurons (Martin et al., 1988; Edwards et al., 1991).

Materials and Methods

Basal forebrain cultures. Brains were removed from Wistar rat fetuses harvested at embryonic day 16 (E16) and immersed in Hanks balanced salt solution (HBSS; Sigma) without calcium and magnesium. A longitudinal cut was made in the cortex, exposing the underlying subcortical structures, and the septum, distinguished by its mediorostral position and its characteristic oval shape, was carefully dissected out whole from each side of the brain (Hefti et al., 1989). After removing meningial tissues, 12 pairs of septa were transferred into 0.1% trypsin and incubated at 37°C for 25 min. Following three 7 min washes in 5 ml of HBSS with calcium and magnesium, the septa were gently triturated in 1.2 ml of HBSS using a glass pipette with a narrow polished tip. Various volumes of the resulting cell suspension were added to 1 ml of CO_2-equilibrated Dulbecco's modified Eagle's medium (DMEM; Imperial Labs) supplemented with 100 mM glutamine, 1 μl/ml antibiotic/antimyotic (Sigma), and 10% fetal calf serum (FCS; Imperial Labs) in 24 multiwell dishes (NUNC, GIBCO). The dishes were then placed in a 37°C humidified, CO_2-controlled (5%) incubator and the cells allowed to fall to rest over a 15 min period and settle onto either glass coverslips or the plastic bottom of the well coated with poly-L-lysine (1 mg/ml; Sigma). Using 12 septa in a final trituration volume of 1.2 ml and adding 50 μl of the resulting cell suspension to 1 ml of medium gave a final plating cell density of approximately 50,000 cells/cm². Once cells had attached to either the glass coverslips or bottoms of the plastic wells, three different methods were compared for long-term maintenance of the cultures. Cultures plated onto glass coverslips either were left open-faced or, 1 hr after plating, coverslips were gently lifted and inverted, leaving the neurons sandwiched between the plastic of the NUNC dish and the poly-L-lysine. Cultures plated directly onto plastic either were left open-faced, or 1 hr after plating, were covered with a glass coverslip to form the sandwich culture. A comparison of plating onto glass or plastic revealed that glass coverslips did not sustain neuronal survival as well as the plastic bottom of the NUNC well. Therefore, after a number of initial experiments, all further studies used cultures plated directly onto the bottom of plastic dishes with a glass coverslip on top to form a sandwich culture. At 5 d *in vitro,* all cultures were washed with an identical growth medium but lacking FCS. This completely defined medium (DMEM supplemented only with glutamine and antibiotic) was exchanged for fresh, similar medium every 7 d. For different experiments, NGF (100 ng/ml; Dr. E. M. Johnson, Washington University, St. Louis), NGF antibodies (0.2% goat polyclonal anti-NGF, lot 55-050190, Dr. E. M. Johnson; or 30 μg/ml mouse monoclonal anti-NGF, Dr. H. Rohrer, Rohrer et al., 1988), or cycloheximide (0.01–1.0 μg/ml; Sigma) was added to this medium.

Dorsal root ganglion cultures. Primary dissociated cultures of rat sensory neurons were prepared from E16–E17 Wistar rat fetuses. Between 100 and 150 DRGs were dissected from eight embryos, placed in HBSS, and dispersed by trypsinization and trituration as described above; 40 ml of the resulting suspension was pipetted onto dry coverslips coated with collagen (1 mg/ml; Sigma) and placed in 24-well, 16 mm NUNC dishes. After 60 min in a humidified, CO_2-controlled (5%) incubator, 500 ml of DMEM supplemented with glutamine, antibiotics, and 10% FCS was added to each well. Some cultures were left open-faced while others were inverted, in both cases in the presence or absence of NGF (100 ng/ml). After 48 hr in the 37°C, CO_2-controlled incubator, the cultures were fixed and stained for cytological analysis.

Immunocytochemistry. When cultures were harvested for fixation, coverslips were gently rotated and removed. Without this step some neurons were washed off due to the suction effect of raising the coverslip. Cultures were fixed in 4% paraformaldehyde for 45 min, washed three times in Tris buffer with azide (pH 7.8), and left overnight in primary antibody to either glial fibrillary acidic protein (GFAP; 1:2700; DAKO), neuron-specific enolase (NSE; 1:2000; DAKO), choline acetyltransferase (ChAT; 1:12; Dr. F. Eckenstein, Oregon Health Sciences University), or low-affinity NGF receptor (p75^NGFR; 1.5 μg/ml MC-192; Dr. E. M. Johnson) in Triton/carrageenan solution. Following three 15 min washes in Tris with azide, the cultures were incubated for 1 hr in the appropriate second antibody: anti-rat IgG for ChAT (1:40; Sigma), biotinylated anti-mouse IgG for p75^NGFR (1:200; Vector Labs), and biotinylated anti-rabbit IgG for NSE and GFAP (1:200; Vector Labs). Cultures processed for ChAT were then incubated for another hour in rat peroxidase-antiperoxidase (1:40; Sera Labs) and cultures processed for p75^NGFR, GFAP, and NSE were stained via addition of avidin and biotinylated peroxidase complex (Vector Labs). All staining was developed by exposure to a diaminobenzidine (2 mg/ml; Sigma), H_2O_2 (5 ml/10 ml)

mixture for 10 min before being washed in Tris buffer with azide. Cultures grown on glass coverslips were dehydrated, cleared in xylene, and mounted onto glass slides using DePex mounting medium. Cultures grown on the surface of the NUNC cultures dishes were washed in Tris buffer with azide and mounted in five drops of heated (48°C) liquid gelatin, which then solidifed on cooling.

AChE histochemistry and cytology. Cultures were fixed in 4% paraformaldehyde for 25 min, washed three times in 0.1 M sodium acetate, and incubated in 50 mM acetate buffer (pH 6.0) containing 5 mM sodium citrate, 3 mM cupric sulfate, 10 mM potassium ferricyanide, and 4 mM acetylthiocholine iodide for 1 hr. Cultures were then washed once in acetate buffer (0.1 M), exposed to 1% ammonium sulfate for 1 min, washed again in acetate buffer followed by nitrate buffer (0.1 M), and developed in 0.1% silver nitrate for 1 min. For general cytological staining, luxol fast blue (LFB) at 0.1% in 70% alcohol was added for 30 min to cultures previously stained for AChE, or to unstained cultures fixed in 4% paraformaldehyde and partially dehydrated in 50% alcohol. Cultures were then rinsed twice in 100% alcohol, cleared and mounted in DePex (cultures grown on glass coverslips), or rinsed in water and mounted in gelatin (cultures grown in NUNC wells).

Quantitative analysis. Total neuronal counts and cell size measurements were performed using a semiautomatic, interactive, computerized image analysis system (Seescan, Cambridge). For cell counts, a video image with a final field size of 220 μm × 300 μm was obtained using a 25× objective. Viable neurons were identified on the basis of having a phase-bright cell body with at least two visible processes. Cholinergic neurons were counted as AChE, p75^NGFR, or ChAT positive when over 50% of the cell body was positively stained. In LFB stained cultures, neurons and astrocytes could be differentiated on the basis of cell morphology as compared with neurons and astrocytes identified by immunohistochemical labeling with, respectively, NSE or GFAP (see Fig. 3). The number of cholinergic or non-cholinergic neurons was counted in five random adjacent fields located in the outer 3–4 mm of the cultures and the average number extrapolated to give a total number of neurons per cm². Dead or degenerating neurons were identified by severe shrinkage of the cell body and absence of processes (see Fig. 5) and were not counted. The very center of cultures were avoided for cell counts because after 21 d *in vitro* variable amounts of necrosis and cell death were noted in the center of sandwich as reported also by Brewer and Cotman (1989) using hippocampal cells. All values are expressed as the mean ± standard error of four to six sister wells per experimental group for each experiment. All experiments described were repeated at least twice with the same results. For cross-sectional area measurements, individual fields were captured using a 40× objective, digitally filtered and thresholded manually based on regions of greatest optical density. The first 20 AChE-positive neurons encountered in randomly selected fields in each of six culture wells per experimental group were measured and values expressed as the mean of the means for the group.

Results

Effects of the sandwich technique and NGF on short-term general neuronal survival in septal and DRG cultures

Initial studies were aimed at characterizing the effects of the sandwich culture technique of Brewer and Cotman (1989) on septal cells. In agreement with their findings, neuronal survival was substantially higher during the first 3 d *in vitro* in sandwich cultures in serum-supplemented medium, particularly at low cell densities (Figs. 1; 2A,C). A plating density of 150,000 cells/cm² was selected for subsequent studies using longer survival times and removal of serum from the medium at 5 d *in vitro.*

After 7 or 14 d *in vitro* only 20% or 1%, respectively, of cells plated at a density of 150,000 cells/cm² in open-faced septal cultures in the absence of exogenous NGF remained alive as neurons, and these were growing on top of a rapidly forming glial monolayer (Fig. 3B,D,F; Table 1). In contrast, at these same time points over 90% and 40% of cells survived as neurons in sister sandwich cultures, and glial cells were few in number and well differentiated (Fig. 3A,C,E; Table 1). The morphological appearance of neurons and glia in LFB-stained cultures (Fig. 3A,B) was confirmed by immunohistochemical staining for, respectively, NSE (Fig. 3E,F) and GFAP (Fig. 3C,D). There was
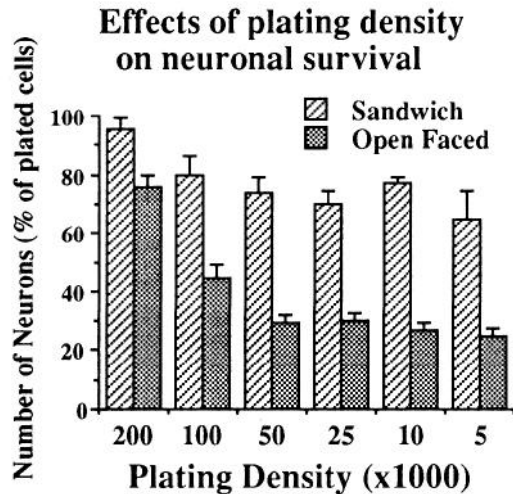
Effects of plating density on neuronal survival

Figure 1. Graph comparing the effects of plating density on general neuronal survival in either open-faced or sandwich sister septal cultures grown in the absence of exogenous NGF. After 3 d *in vitro,* cultures were harvested, and the number of LFB-stained neurons counted in five fields per culture well. Neuronal counts are expressed as a percentage of the number of plated cells (i.e., number of counted neurons/number of plated cells $\times$ 100). $N = 4$ or 6 culture wells per data point ($\pm$SEM) from a typical experiment. This experiment was conducted two times with similar results.

no increase in general neuronal survival when NGF (100 ng/ml) was added at the time of plating to either open-faced or sandwich cultures for 3, 7, or 14 d (Fig. 2, Table 1).

DRG cultures were used to compare the effects of the sandwich culture technique and NGF on a population of neurons known to be dependent on NGF for survival (Johnson et al., 1980). In open-faced DRG cultures plated at 200,000 cells/cm^2 and grown in serum-supplemented medium for 3 d in the absence of exogenous NGF, less than 5% of initial population of plated cells survived as neurons (8095 $\pm$ 5938 neurons/cm^2). In sister sandwich cultures grown under similar conditions there were essentially no viable neurons at 3 d *in vitro.* Addition of NGF (100 ng/ml) to the medium at the time of plating sustained large numbers of neurons in open-faced (175,194 $\pm$ 27,148 neurons/cm^2) cultures and substantially improved neuronal survival in sandwich cultures (69,993 $\pm$ 15,695 neurons/cm^2).

Effects of NGF on cholinergic neurons in maturing sandwich septal cultures

Initial studies used AChE histochemistry to identify presumptive cholinergic neurons (Eckenstein and Sofroniew, 1983) in septal cultures. In the absence of exogenous NGF, a small number of AChE-positive neurons were present in sandwich septal cultures at 7 d and this number had declined by half at 14 d (Table 2), but a few AChE-positive neurons persisted under these conditions for at least 35 d. Addition of NGF (100 ng/
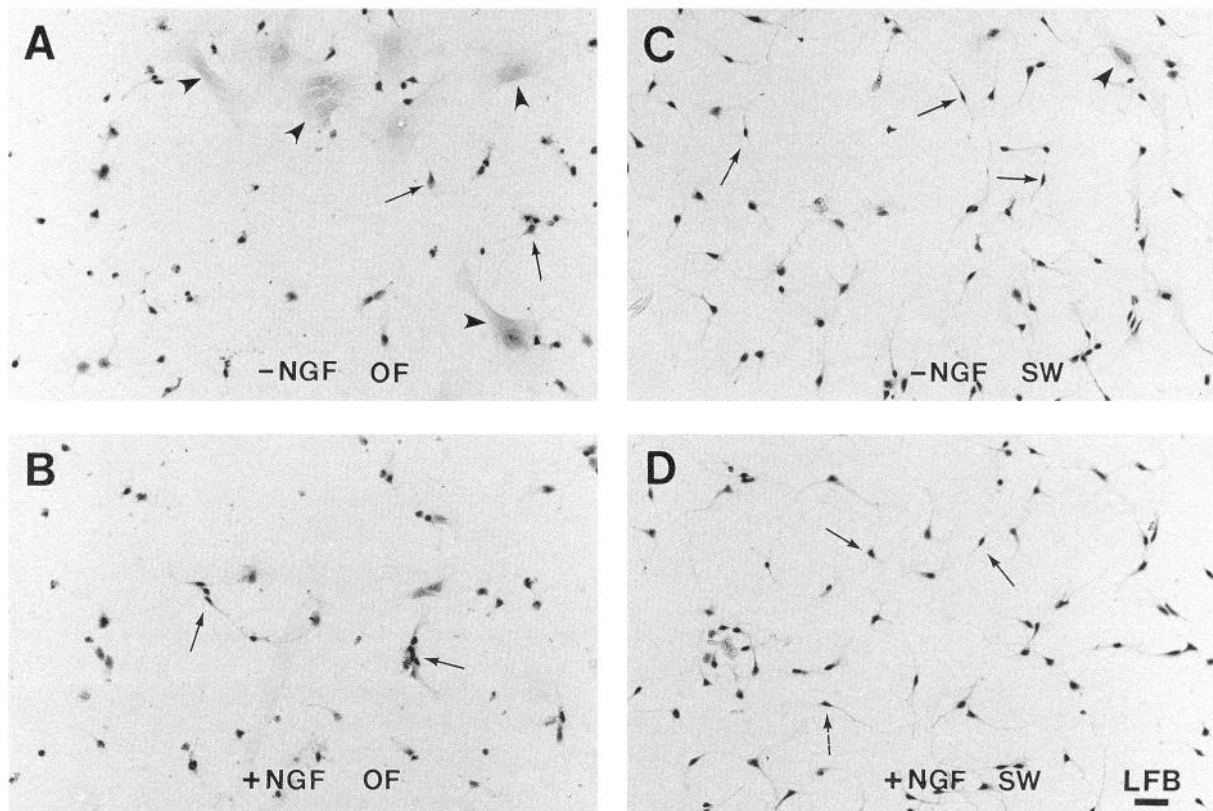

Figure 2. Photomicrographs showing the effects of the sandwich culture technique and NGF treatment on short-term general neuronal survival in septal cultures at 3 d *in vitro.* Cultures were stained with LFB. In open-faced (*OF*) septal cultures (*A*) very few neurons (*arrows*) survived and there were many dividing astrocytes (*arrowheads*). The sandwich (*SW*) culture technique (*C*) significantly increased neuronal (*arrows*) survival and reduced astrocytic (*arrowhead*) proliferation. Addition of NGF (100 ng/ml) to the medium at the time of plating did not increase general neuronal survival or affect the number of astrocytes in either open-faced (*B*) or sandwich (*D*) cultures. Scale bar, 150 μm.
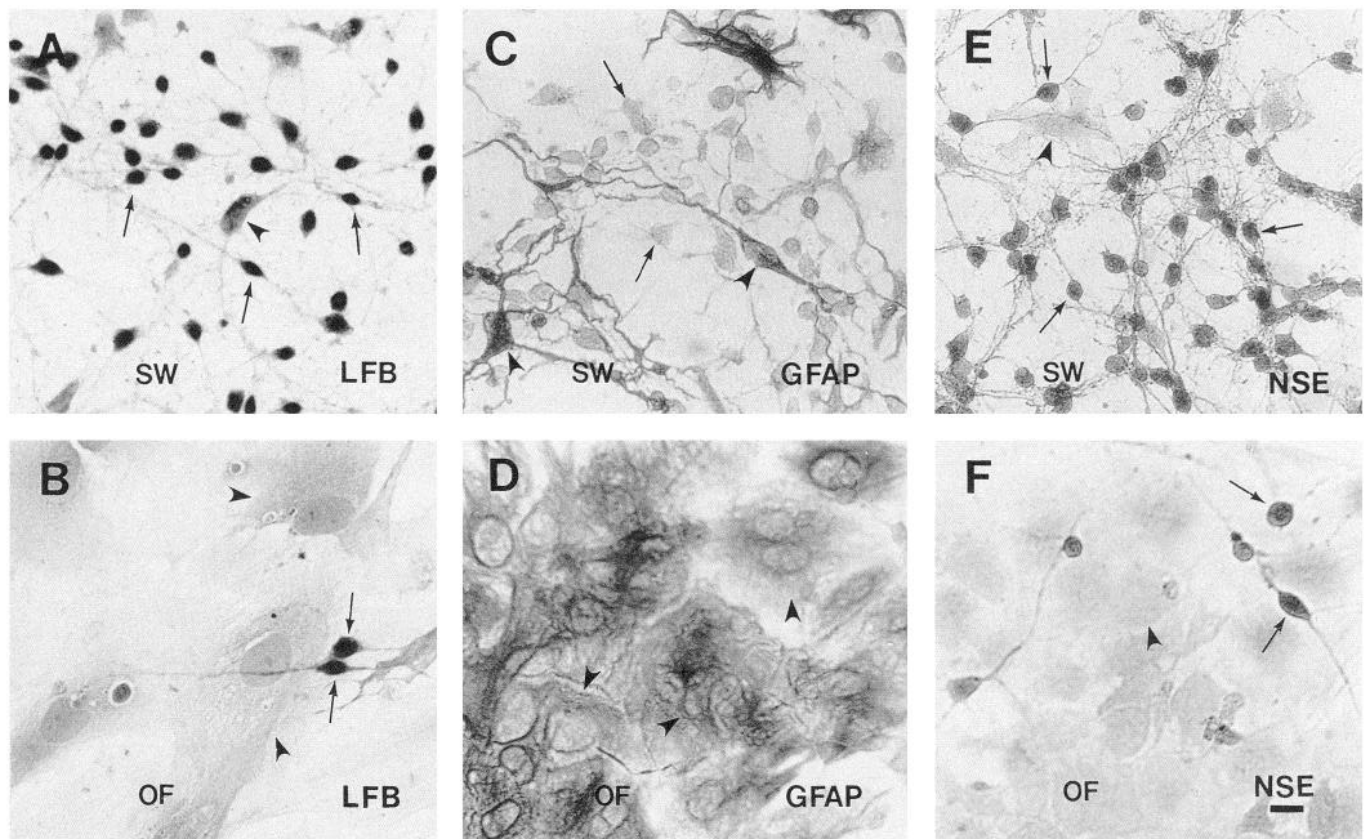
Figure 3. Photomicrographs comparing the density of neurons and glial cells in sister sandwich (*SW*) (*A, C, E*) and open-faced (*OF*) (*B, D, F*) septal cultures at 7 d *in vitro* after plating at a density of 150,000 cells/cm². Cells are stained either with LFB (*A, B*) or immunohistochemically for GFAP (*C, D*) or NSE (*E, F*). Large, GFAP-positive, NSE-negative cells that appear lightly stained with LFB were considered astrocytes (*arrowheads*). Small, NSE-positive, GFAP-negative cells that appear darkly stained with LFB were considered neurons (*arrows*). Note the small number of neurons and confluent mat of glial cells in the open-faced cultures (*B, D, F*) and the large number of neurons and small number of well differentiated astrocytes in the sandwich cultures (*A, C, E*). Scale bar, 30 μm.

ml) to sandwich cultures at the time of plating significantly increased the staining intensity, size, and number of AChE-positive presumptive cholinergic neurons detectable at 7 and 14 d *in vitro* (Table 2). In the continuous presence of NGF, the number of cholinergic neurons remained approximately the same for at least 35 d (992 cholinergic neurons/cm² at 35 d) (Fig. 4). NGF-supplemented cholinergic neurons matured slowly *in vitro*, increasing in size particularly between 7 and 21 d (Fig. 4, Table 2) and gradually elaborating a dense network of AChE-positive fibers (Fig. 4). In contrast, the number of non-cholinergic neurons decreased from nearly 90% of the plating density (140,000

non-cholinergic neurons/cm² at 7 d) to about 10% over this time period (15,625 non-cholinergic neurons/cm² at 35 d) (Fig. 4).

AChE-positive presumptive cholinergic neurons were further characterized by immunohistochemical staining for ChAT and p75^{NGFR}. In the absence of exogenous NGF, no ChAT-positive but some p75^{NGFR}-positive neurons could be detected at 14 d *in vitro* (Table 2). With addition of NGF to the medium from plating, both ChAT- and p75^{NGFR}-positive neurons were detectable at 14 d *in vitro* and were similar in number, size, and appearance to AChE-positive neurons in sister wells (Fig. 5, Table 2). ChAT- and p75^{NGFR}-stained neurons matured with time in the presence of NGF in a manner similar to AChE-positive neurons and were present in about equal numbers for at least 35 d. Delayed addition of NGF for 7 d starting at 14 d *in vitro* did not significantly increase the number of AChE- or p75^{NGFR}-stained neurons, but did significantly increase the number of ChAT-stained neurons, as compared with numbers of neurons detected with these markers at 14 d in the absence of exogenous NGF (Table 2).

Effects of NGF withdrawal on developing cholinergic neurons in sandwich septal cultures

Since NGF increased the number and size of cholinergic neurons in sandwich cultures at 14 d *in vitro*, we assessed whether these neurons were dependent on NGF for survival. NGF was removed from, and antibodies to NGF (0.2% goat polyclonal)

Table 1. Effects of NGF and the sandwich culture method on general neuronal survival at 7 and 14 d *in vitro*

	Treat-ment	Number of surviving neurons	
		7 d *in vitro*	14 d *in vitro*
Open-faced culture	+NGF	32,352 ± 3921	1470 ± 356
	−NGF	34,313 ± 2156	1294 ± 221
Sandwich culture	+NGF	151,960 ± 7058	62,745 ± 5882
	−NGF	144,117 ± 7843	58,823 ± 7647

All values represent mean number of neurons/cm² ± SEM (*n* = 6 cultures per group) from a typical experiment using a plating density of approximately 150,000 cells/cm².

Table 2. Effects of continuous NGF and delayed NGF treatment on septal cholinergic neurons in sandwich cultures

Staining parameters	Treatment	Time *in vitro*		14 d − NGF, 7 d +NGF
		7 d	14 d	
Neuronal counts				
AChE-positive neurons	+NGF	1237 ± 41*	1164 ± 86*	
	−NGF	783 ± 72	360 ± 38	
	−NGF/+NGF			384 ± 22^{ns}
p75^{NGFR}-positive neurons	+NGF		1052 ± 84*	
	−NGF		492 ± 108	
	−NGF/+NGF			464 ± 71^{ns}
ChAT-positive neurons	+NGF		1054 ± 68	
	−NGF		0 ± 0	
	−NGF/+NGF			316 ± 80
Neuronal area				
ACh-E-positive neurons	+NGF	117.6 ± 2.9	220.6 ± 4.7**	
	−NGF	55.8 ± 2.3	58.8 ± 11.7	
	−NGF/+NGF			183.1 ± 16.6**

Sister cultures were grown either in the presence (+NGF) or absence (−NGF) of exogenous NGF for 7 or 14 d, or for 14 d in the absence of NGF followed by 7 d in the presence of NGF (−NGF/+NGF). Different wells were stained either for AChE, p75^{NGFR}, or ChAT. Cell counts represent the mean number of stained neurons/cm^2 ± SEM ($n = 6$ culture wells per group) from a typical experiment using a plating density of approximately 150,000 cells/cm^2. Cell areas are in μm^2 and represent mean of means ± SEM ($n = 6$ culture wells per group with at least 20 positively stained neurons measured from each well) from a typical experiment.

* Significantly different from corresponding values without NGF, $p < 0.01$ (*t* test).

** Significantly different from values without NGF and from value with NGF at 7 d, $p < 0.01$ (ANOVA).

† *Significantly different from corresponding value without NGF at 14 d, $p < 0.01$ (ANOVA).*

^{ns} Not significantly different from corresponding values without NGF at 14 d (ANOVA).
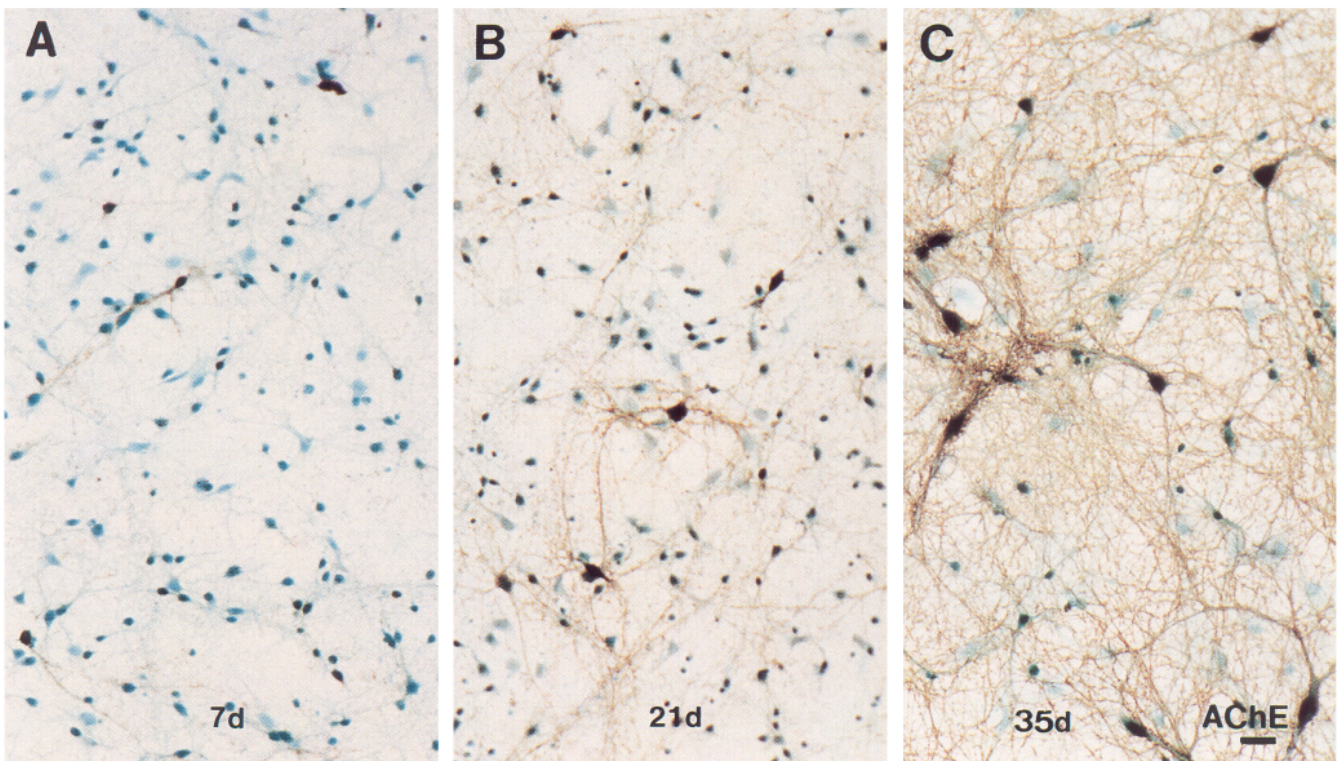

Figure 4. Color photomicrographs showing the gradual maturation of cholinergic neurons and loss of non-cholinergic neurons in septal cultures grown in the continuous presence of exogenous NGF (100 ng/ml). Different wells from sister cultures plated and grown under identical conditions were harvested and stained for both AChE and LFB at 7 d (*A*), 21 d (*B*), and 35 d (*C*) *in vitro*. Over time, the number of the AChE-positive cholinergic neurons (*brown*) did not change, but these neurons gradually increased both their size and extent of neurite outgrowth. In contrast there was a continuous and substantial reduction in the number of LFB-positive non-cholinergic neurons (*dark blue*). Scale bar, 75 μm.
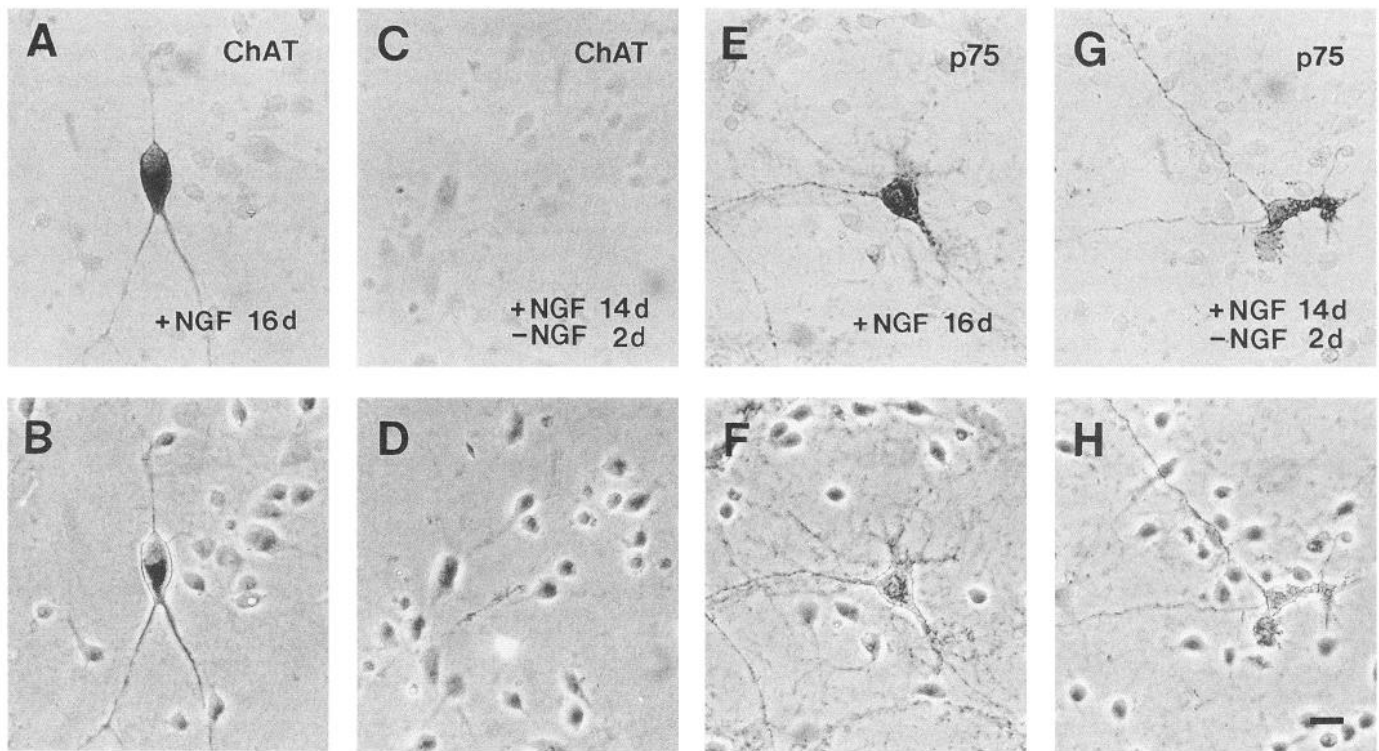
Figure 5. Effects of 2 d of NGF withdrawal and addition of NGF antibodies on cholinergic neurons in 14-d-old sister sandwich septal cultures previously grown in the continuous presence of NGF (100 ng/ml). Representative fields from cultures stained for either ChAT (*A–D*) or p75^{NGFR} (*E, F*) are shown using both bright-field (*A, C, E, G*) and phase-contrast (*B, D, F, H*) microscopy. Cholinergic neurons grown in the presence of NGF for 16 d are large and stain intensely for ChAT (*A, B*) or p75^{NGFR} (*E, F*). Withdrawal of NGF for 2 d from 14-d-old cultures results in the disappearance of nearly all ChAT-stained (*C*) and most p75^{NGFR}-stained neurons. Remaining p75^{NGFR}-stained neurons are somewhat palely stained, shrunken, and atrophic (*G*). Withdrawal of NGF for 2 d has no apparent effect on phase-positive non-cholinergic neurons (*D, H*). Scale bar, 30 μm.

were added to, 14-d-old septal cultures grown continuously in the presence of NGF from plating. At various times following NGF withdrawal in this manner, sister septal cultures were fixed and stained for AChE, ChAT, or p75^{NGFR}. After 1 d, the number of neurons stained for these markers declined substantially, and by 2 d nearly all ChAT-stained and most AChE- or p75^{NGFR}-stained neurons had disappeared (Fig. 6). Most remaining neurons were shrunken and palely stained (Fig. 5G). By 4 d after NGF withdrawal, the numbers of ChAT-, AChE-, and p75^{NGFR}-

Table 3. Effects of NGF reapplication at different time points after NGF withdrawal on the survival of septal cholinergic neurons in sandwich septal cultures

Treatment	Neurons	Percentage of NGF control
NGF control	1380 ± 36	100%
3 hr	1104 ± 64	80%
6 hr	832 ± 48	60%
9 hr	604 ± 56	44%
12 hr	436 ± 44	32%
24 hr	341 ± 28	25%
AB control	256 ± 44	19%

Sister cultures grown in the presence of exogenous NGF for 14 d were exposed either to NGF antibody for 3–24 hr followed by reapplication of NGF, to NGF antibody without reapplication of NGF (AB control), or to the continued presence of NGF without exposure to NGF antibody (NGF control). After 4 d, cultures were harvested and stained for p75^{NGFR}. Cell counts represent the mean number of stained neurons/cm² ± SEM (*n* = 6 culture wells per group) from a typical experiment.

positive neurons were reduced to between 10% and 25% of control values (Fig. 6). A number of small, positively stained rounded structures were observed at 2 or 4 d after NGF withdrawal, which appeared to be cholinergic neurons undergoing a terminal phase of degeneration (Fig. 7A,B). Eight days after NGF withdrawal, most of these degenerating structures had disappeared. The number of non-cholinergic neurons was not obviously decreased following NGF withdrawal (Fig. 7A,B).

To assess whether cholinergic neurons in 14-d-old septal cultures exposed to NGF antibody for 4 d were dead rather than atrophic with downregulated expression of cholinergic marker proteins, NGF was reapplied by removing medium containing NGF antibody, washing cultures two times in fresh medium, and replacing NGF (100 ng/ml) to the final medium. Four days of NGF reapplication did not increase the number of AChE-, p75^{NGFR}-, or ChAT-positive neurons in cultures that had been exposed to NGF antibody for 4 d (Fig. 6). We next determined the duration of NGF withdrawal required for cholinergic neurons to become irreversibly committed to cell death. Fourteen-day-old septal cultures were exposed to NGF antibody for 3–24 hr, followed by reapplication of NGF for 4 d. NGF reapplication rescued most cholinergic neurons after 3 hr of NGF withdrawal, but progressively fewer cholinergic neurons survived after 6 or 9 hr. When NGF reapplication was delayed for 12 or 24 hr the number of neurons surviving was not substantially higher than if NGF was not reapplied at all (Table 3).

To test whether some factor in the polyclonal goat antibody used to neutralize NGF may have had a nonspecific toxic effect on cholinergic neurons, a different anti-NGF was used. A mouse

Progressive effects of NGF withdrawal on cholinergic neurons in 14 day old septal cultures
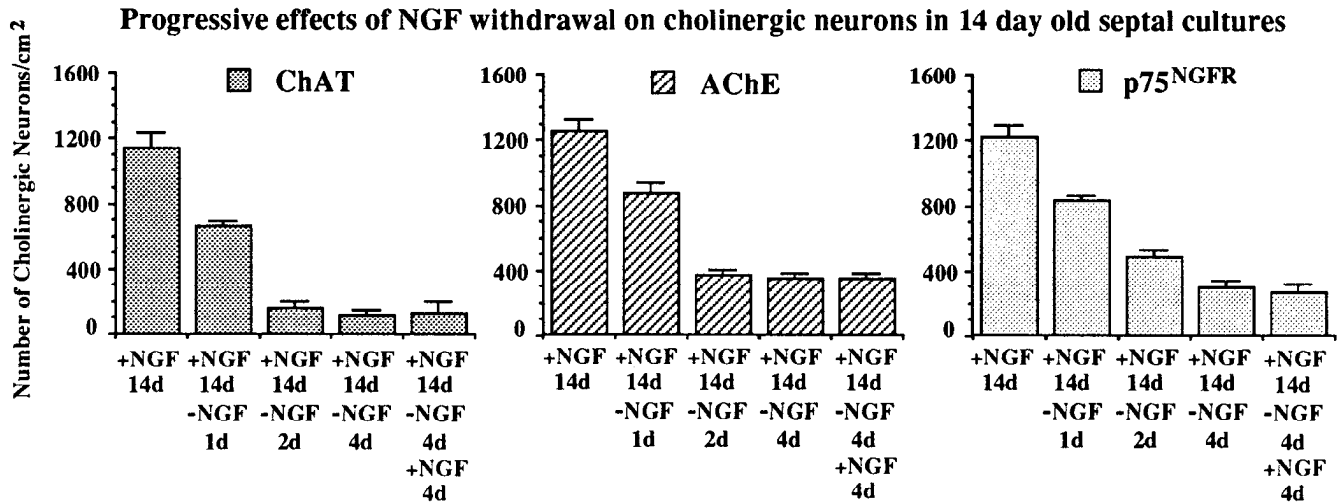
Figure 6. Graph showing the effects of NGF withdrawal on developing cholinergic neurons *in vitro*. Five groups of sandwich septal cultures were grown in the continuous presence of exogenous NGF (100 ng/ml) for 14 d. NGF was then withdrawn and NGF antibodies added to four groups. Cultures were harvested at 14 d (+*NGF*), at 1, 2, and 4 d after NGF withdrawal (−*NGF*), and after 4 d of NGF withdrawal (−*NGF*) followed by reapplication of NGF (100 ng/ml) for a further 4 d (+*NGF*). Cultures were stained for ChAT, p75[NGFR], or AChE, and the number of positive neurons counted in five fields per culture well and extrapolated to neurons/cm². N = 6 culture wells per data point (±SEM) from a typical set of experiments. All values from 2 d −*NGF*, 4 d −*NGF*, and 4 d −NGF followed by 4 d +NGF groups were significantly different from their respective control values of 14 d +NGF (ANOVA, $p < 0.01$). This experiment was conducted three times with similar results.

monoclonal NGF antibody (30 μg/ml) also reduced the number of AChE-positive neurons detectable at 4 d post-NGF withdrawal to approximately 25% of control values (not shown).

Septal cholinergic neurons lose their dependence on NGF as they mature in vitro

Sensory, but not sympathetic, peripheral neurons grown in tissue culture lose their dependence on NGF with increasing time *in vitro* (Greene, 1977a,b; Barde et al., 1980; Schwartz et al., 1982). To test whether cholinergic septal neurons might similarly lose their dependence on NGF as they mature, NGF was removed and NGF antibody (0.2% goat polyclonal) added for 4 d to either 14- or 35-d-old sister cultures and the number of remaining cholinergic neurons was compared with sister cultures continually exposed to NGF. The survival time of 4 d was chosen since most cholinergic neurons in 14-d-old cultures become committed to cell death after 12 hr of NGF-withdrawal and have disappeared by 48 hr. Consistent with the experiments described above, when NGF was withdrawn for 4 d from 14-d-old septal cultures only about 25% the number of ChAT-, AChE-, or p75[NGFR]-stained neurons could be detected relative to the number of cholinergic neurons in cultures maintained in NGF for 18 d (Figs. 7, 8). In contrast, when NGF was withdrawn for 4 d from 35-d-old sister cultures more than 75% of cholinergic neurons could be detected with all three of these markers (Figs. 7, 8). The remaining cholinergic neurons, however, were significantly shrunken (mean cross-sectional area of 169.6 ± 55.4 μm²; $p < 0.001$ vs. control, t test) in comparison to cholinergic neurons in cultures maintained in NGF for 39 d (247.8 ± 64.1 μm²).

Death of immature cholinergic neurons induced by NGF withdrawal is dependent on protein synthesis

Inhibition of protein synthesis significantly attenuates the death of developing sympathetic neurons grown *in vitro* in the absence of NGF (Martin et al., 1988; Edwards et al., 1991). To investigate whether the death of septal cholinergic neurons following NGF withdrawal at 14 d *in vivo* might also depend on protein synthesis, we administered cycloheximide to septal cultures in combination with NGF withdrawal. The concentration of cycloheximide used for cultures of sympathetic neurons (1 μg/ml; Martin et al., 1988) was toxic to 14-d-old septal neurons such that over 80% died within 24 hr (data not shown). The highest concentration of cycloheximide tested that did not kill septal neurons over a 4 d period was 0.1 μg/ml. This concentration of cycloheximide prevented glial division in open-faced high-density septal cultures and was considered effective in preventing protein synthesis. Fourteen-day-old sandwich septal cultures exposed to cycloheximide at this concentration for 4 d in the continuous presence of NGF had about 75% the number of AChE-positive neurons and somewhat reduced numbers of AChE-positive fibers when compared with sister cultures treated with NGF alone (Fig. 9). As expected, 14-d-old cultures deprived of NGF and given NGF antibody for 4 d had only about 25% the number of AChE-positive neurons when compared with NGF-treated cultures (Fig. 9*E,F,I*). In contrast, 14-d-old cultures deprived of NGF and given NGF antibody for 4 d in the presence of cycloheximide had about 75% the number of AChE-positive neurons when compared with NGF-treated cultures, in a manner not significantly different than cultures treated with NGF and cycloheximide (Fig. 9*G–I*). Exposure to NGF antibodies or cycloheximide had no apparent effect on noncholinergic neurons (Fig. 9).

Discussion

In this study, the sandwich culture technique significantly increased the survival of neurons, reduced glial proliferation, and after 5 d *in vitro* allowed the transfer of septal cultures to a serum-free, completely defined growth medium for long-term maintenance of cholinergic neurons. Using this method, we found that withdrawal of NGF from 14-d-old septal cultures grown in the presence of exogenous NGF from plating resulted in the
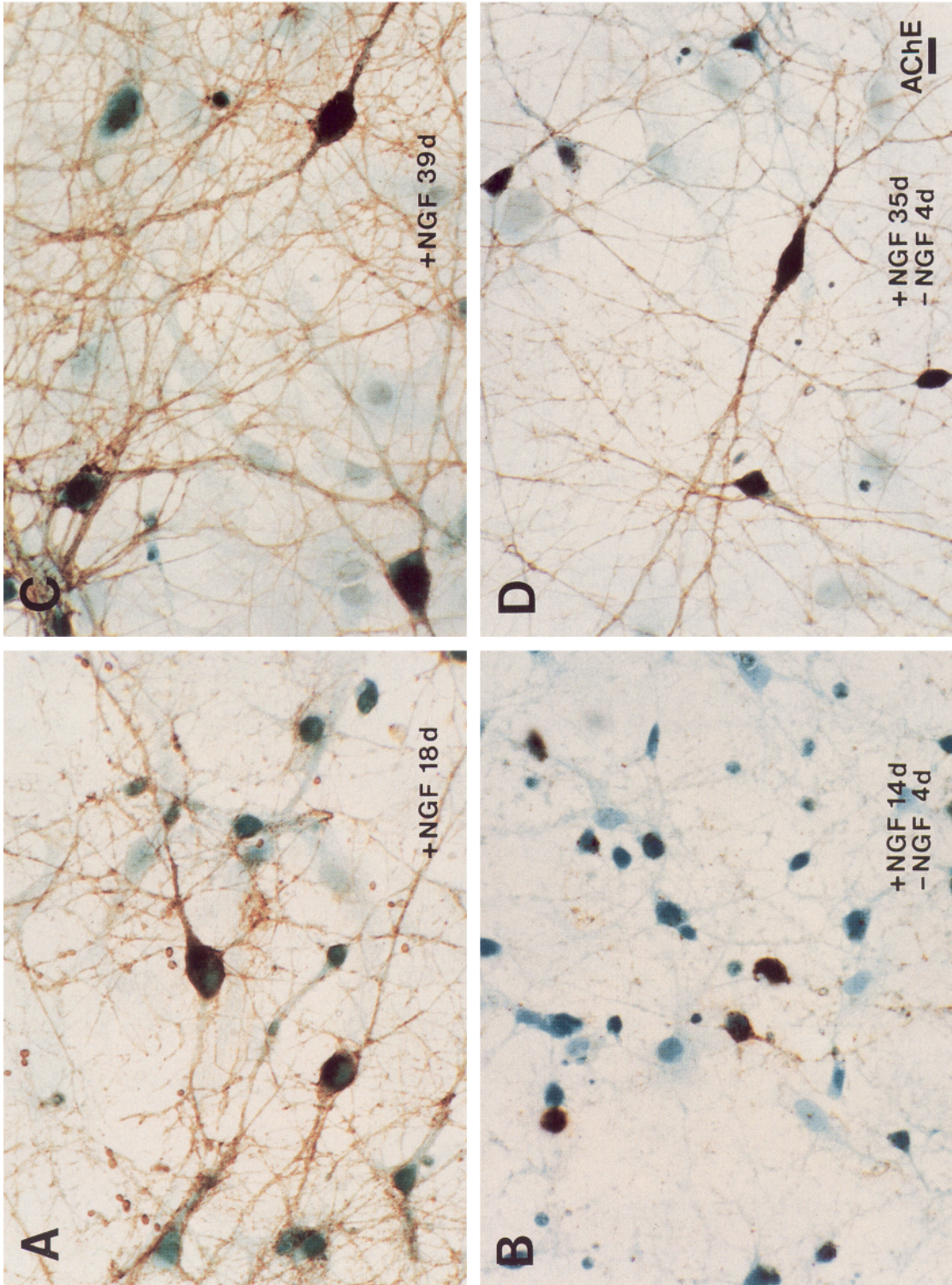
Figure 7. Color photomicrographs comparing the effect of NGF withdrawal at different ages *in vitro* on the appearance of septal cholinergic neurons. NGF was withdrawn and NGF antibodies added to either 14- (*B*) or 35- (*D*) d-old sister sandwich septal cultures grown previously in the continuous presence of exogenous NGF (100 ng/ml). After 4 d, these cultures were harvested along with control sister cultures maintained in NGF respectively for either 18 d (*A*) or 39 d (*C*). All cultures were stained for both AChE and LFB. Cholinergic neurons grown in the continuous presence of NGF for 18 d (*A*) or 39 d (*C*) are large and darkly stained, and exhibit extensive outgrowth and arborization of neurites. After 4 d of NGF withdrawal from 14-d-old cultures, very few intact cholinergic neurons or fibers are present, and there are many darkly stained, round AChE-positive structures that appear to represent the remains of degenerated cholinergic neurons (*B*). After 4 d of NGF withdrawal from 35-d-old cultures, large numbers of cholinergic neurons remain alive but these neurons are markedly shrunken, with a reduction in neurite arborization (*D*). Scale bar, 15 µm.
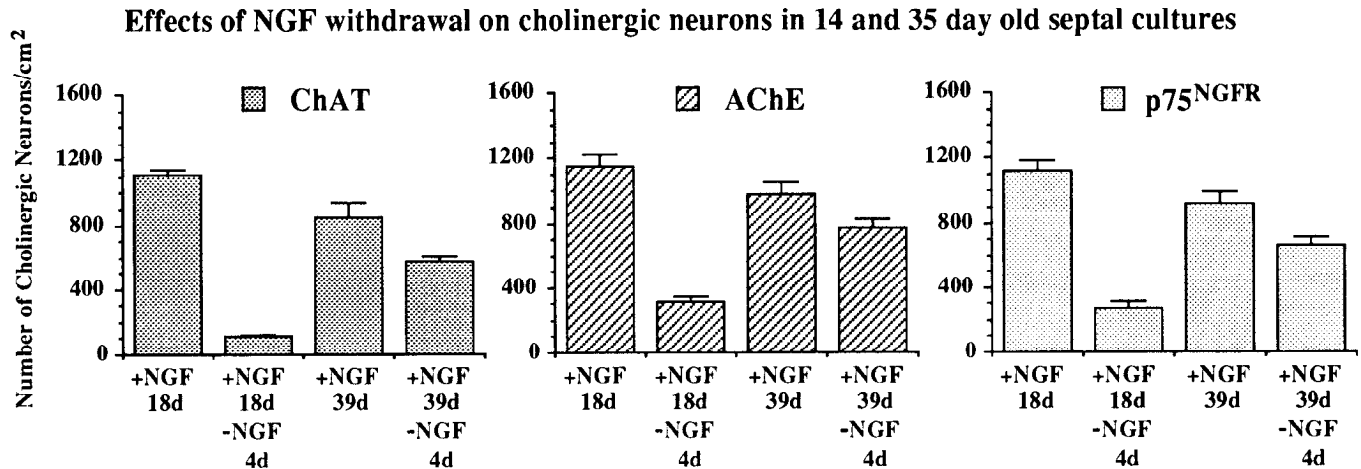
Effects of NGF withdrawal on cholinergic neurons in 14 and 35 day old septal cultures

Figure 8. A, Graph comparing the effect of NGF withdrawal at different ages *in vitro* on cell counts of septal cholinergic neurons. Four groups of sandwich septal cultures were grown in the continuous presence of exogenous NGF (100 ng/ml) for either 14 or 35 d. NGF was then withdrawn and NGF antibodies added to one group at each time point and after a further 4 d, all cultures were harvested. The number of neurons stained for ChAT, p75^{NGFR}, or AChE were counted in five fields per culture well and extrapolated to neurons/cm^2. $N = 6$ culture wells per data point ($\pm$SEM) from a typical experiment. This experiment was conducted three times with similar results. For each marker, values from groups exposed to NGF withdrawal at 14 or 35 d are significantly different from from each other and from values of their respective control groups given NGF for 18 or 39 d (ANOVA, $p < 0.01$).

widespread death of cholinergic neurons, whereas in 35-d-old sister cultures most cholinergic neurons survived NGF withdrawal. In addition, the cholinergic cell death associated with NGF withdrawal from 14-d-old cultures was significantly attenuated by inhibition of protein synthesis. These findings raise a number of questions about the protective effects of the sandwich culture techniques and the role of NGF regarding developing and maturing septal cholinergic neurons.

Methodological considerations

Using standard open-faced culture methods, embryonic neurons from the septal region undergo massive neuronal cell death over the first few days *in vitro* (Hartikka and Hefti, 1988; Alderson et al., 1990; present results). Although this cell death might be reduced by addition of certain chemical supplements to the growth medium, the use of such additives would complicate the interpretation of studies intended to characterize the effects of a neurotrophic factor. We therefore grew septal neurons in unsupplemented medium under glass coverslips using a sandwich technique previously shown to increase neuronal survival and reduce glial proliferation in hippocampal cultures (Brewer and Cotman, 1989).

We found that the sandwich culture technique substantially increased general neuronal survival in septal cultures but did not prevent the eventual death of most cholinergic neurons grown in the absence of exogenous NGF. In addition, in septal cultures maintained for long periods of time in the presence of NGF but not other growth factors there was a gradual loss of non-cholinergic neurons, while cholinergic neurons thrived. The sandwich culture technique also did not prevent the rapid death of DRG neurons grown in the absence of NGF. Embryonic DRG neurons at the age used in this study are completely dependent on NGF for survival *in vivo* (Johnson et al., 1980) and *in vitro* (Levi-Montalcini and Angeletti, 1963). Various lines of evidence suggest that very young neurons are initially not dependent for survival on specific target-derived growth factors, but become dependent during a critical period and may eventually lose this dependence as they mature (Purves and Lichtman, 1985). Thus,

it appears that the sandwich culture technique may be able to improve the survival of neurons in some general way during phases when they do not have specific growth factor requirements, but cannot protect neurons from the lack of specific growth factors during critical periods of development. In addition, the sandwich method substantially improved the long-term maintenance and maturation of neurons provided with their specific growth factor requirements in a serum-free and completely defined medium.

The mechanism underlying the protective effect of the sandwich technique on general neuronal survival is not fully understood. It has been reported in both hippocampal and cortical cultures that lowering the oxygen concentration inside the incubator from 20% to 9% can reduce the postplating cell death to about the same amount seen in sandwich cultures (Kaplan et al., 1986; Brewer and Cotman, 1989; Kushima et al., 1990). Reduced oxygen concentrations may exist within sandwich cultures as a result of the metabolism of cells within a small, slowly circulating diffusion layer.

Between 7 and 14 d *in vitro* our sandwich septal cultures underwent a substantial decline in the total number of neurons. While this decline might reflect the dependence of neurons on specific growth factors during this period, it might also be due to an increased requirement for oxygen in more mature neurons as suggested for telencephalic aggregating cultures and postnatal septal cultures (Honegger, 1985; Kushima et al., 1990). Low oxygen concentration might also be responsible for the variable amount of necrotic cell death at the very center of sandwich cultures noted after long survival times in this study and in hippocampal cultures (Brewer and Cotman, 1989).

Neuronal survival might also be improved in sandwich cultures by the smaller volume of circulating medium into which growth factors and other molecules might be released from neighboring cells. Thermal proteins and poly(dicarboxylic) amino acids have also recently been shown specifically to enhance the survival of septal neurons in culture (Hefti et al., 1991), and similar substances might be produced by neurons or glia. Last, sandwich cultures provide an artificial three-dimensional en-
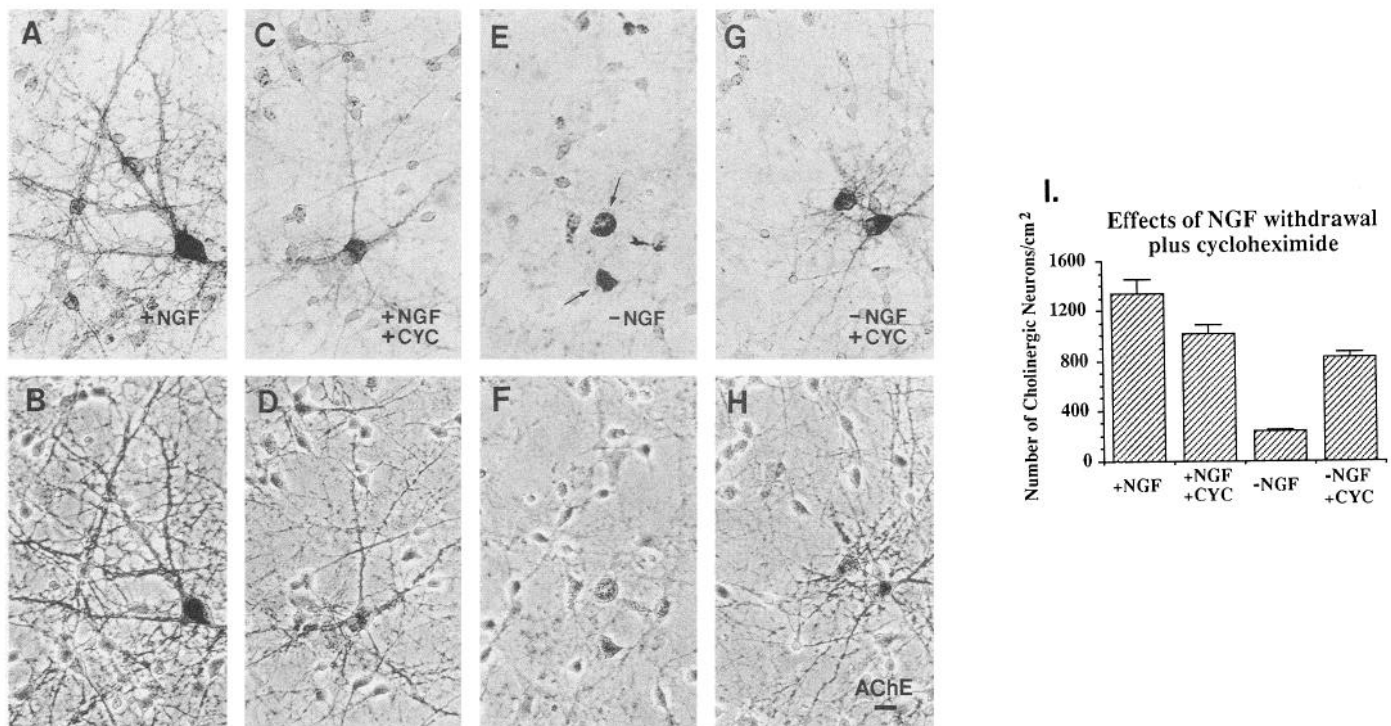
Figure 9. Qualitative (*A–H*) and quantitative (*I*) comparison of the effects of NGF withdrawal in the presence or absence of the protein synthesis inhibitor cycloheximide on the appearance of septal cholinergic neurons. Sister sandwich septal cultures were grown for 14 d in the continuous presence of NGF (100 ng/ml), divided into four groups, and grown a further 4 d in NGF (*+NGF*; *A, B*), NGF plus cycloheximide (0.1 μg/ml) (*+NGF/+CYC*; *C, D*), antibodies to NGF (*−NGF*; *E, F*), and antibodies to NGF plus cycloheximide (0.1 μg/ml) (*−NGF/+CYC*; *G, H*). At 18 d, cultures were harvested and stained for AChE. *A–H,* Representative fields from each experimental condition are shown using both bright-field (*A, C, E, G*) and phase-contrast (*B, D, F, H*) microscopy. Cholinergic neurons grown in the presence of NGF for 18 d are large and darkly stained, and exhibit extensive outgrowth and arborization of neurites (*A, B*). Exposure to cycloheximide for 4 d in the presence of NGF leads to a slight reduction in neuronal number and in the staining intensity of some neurons (*C, D*). After exposure to NGF antibodies for 4 d, few cholinergic neurons or fibers remain, but there are a number of darkly stained, round, AChE-positive structures (*arrows*) that probably represent degenerated neurons (*E, F*). When protein synthesis is inhibited with cycloheximide in combination with exposure to NGF antibodies for 4 d, there is only a slight reduction in the number and appearance of cholinergic neurons and there are no obviously degenerated structures (*G, H*). Treatment with NGF antibodies or cycloheximide has no obvious effect on the number or appearance of non-cholinergic neurons (*B, D, F, H*). Scale bar, 30 μm. *I*, Graph comparing the effects NGF withdrawal in the presence or absence of the protein synthesis inhibitor cycloheximide on cell counts of septal cholinergic neurons. The number of positive cholinergic neurons was counted in five fields per culture well and extrapolated to neurons/cm². $N =$ 6 culture wells per data point (±SEM) from a typical experiment. This experiment was conducted three times with similar results. For AChE-positive neurons, *+NGF/+CYC* and *−NGF/+CYC* groups were not significantly different from each other but were both significantly different from the *−NGF* group, and all three of these groups were significantly different from *+NGF* (ANOVA, $p < 0.01$).

vironment for neurons, which may increase the survival of septal neurons through augmented surface contact.

The sandwich culture technique also markedly decreased glia proliferation and increased glial differentiation. The decreased survival of DRG neurons in sandwich cultures may be related to this effect since glial cells produce various growth factors including NGF (Lindsay, 1979). Reduced glial proliferation may add to the usefulness of sandwich cultures in studying neuronal growth factor requirements under defined conditions.

NGF increases cholinergic but not general neuronal survival and maturation in septal cultures

Exogenous NGF increases the survival of cholinergic neurons in septal cultures at plating densities of under 150,000 cells/cm² (Hartikka and Hefti, 1988), but does not appear to increase the number of detectable cholinergic neurons in open-faced cultures at higher plating densities. In this study, NGF added from plating also substantially increased the number of cholinergic neurons identified by staining for three well-documented cholinergic markers, AChE, p75[NGFR], and ChAT (Eckenstein and Sofroniew, 1983; Dawbarn et al., 1988), in sandwich septal cultures at low plating densities (150,000 cells/cm²). Interestingly, a small number of AChE- and p75[NGFR]- but no ChAT-stained neurons were consistently detectable in sandwich septal cultures grown in the absence of exogenous NGF. The observation that delayed addition of NGF at 14 d *in vitro* failed to increase the number of AChE or p75[NGFR] neurons strongly suggests that the increase in cholinergic cell number seen with the addition of NGF from plating was an effect on cell survival rather than on cell phenotype. Moreover, the small number of AChE and p75[NGFR] neurons seen in the absence of exogenous NGF appear to be cholinergic, since the delayed addition of NGF increased the number and size of ChAT neurons to a level equivalent to those of AChE and p75[NGFR] neurons in similarly treated sister wells. It is not clear whether the small number of cholinergic cells surviving in the absence of exogenous NGF were growth factor independent, or were being maintained by growth factors being produced by cells within the culture. We now have evidence

that growth factors other than NGF can maintain the survival but not the phenotype of cholinergic neurons in these cultures (Kew et al., 1992).

Sandwich cultures also allowed the the long-term maintenance and gradual maturation of cholinergic neurons at low cell density. In open-faced culture systems, long-term survival of septal cholinergic neurons is only possible either when very high initial plating densities are used (300,000 cells/cm^2 or more) or when glial cells are allowed to divide and form a supportive monolayer (Hartikka and Hefti, 1988). In contrast, the sandwich culture technique permitted the gradual maturation and long-term maintenance, for over 40 d, of large numbers of cholinergic neurons in septal cultures at low total neuronal densities (<150,000 cells/cm^2) in serum-free, completely defined NGF-containing medium, with only a sparse glial population. It deserves mention that the maturation of cholinergic neurons in sandwich septal cultures roughly parallels the development of these neurons *in vivo*. In sandwich cultures, the size of NGF-supplemented cholinergic neurons increased substantially between 7 and 21 d *in vitro*. This compares favorably with reports that both ChAT- and p75^{NGFR}-stained basal forebrain neurons undergo pronounced increases in cell size during the third week of postnatal development (Sofroniew et al., 1987; Koh and Loy, 1989) in parallel with a surge in hippocampal production of NGF (Auburger et al., 1987). Thus, the sandwich culture technique provides a means of studying mature septal cholinergic neurons *in vitro* under precisely defined conditions.

NGF has also been reported to increase general neuronal survival at very low plating densities of less than 17,000 neurons/cm^2 (Grothe et al., 1989), but this observation has not been confirmed by others (Knusel et al., 1990). In both our sandwich and open-faced culture conditions NGF had no effect on general neuronal survival in septal cultures at any plating density tested, including those as low 5000 or 10,000 neurons/cm^2, supporting the view that the survival-promoting effects of NGF are limited to cholinergic neurons in septal cultures.

Developing but not mature septal cholinergic neurons are dependent on NGF for survival in vitro

Since the degree to which NGF regulates the survival of developing or mature basal forebrain cholinergic neurons is not well understood, we studied the effects of NGF withdrawal from septal cholinergic neurons that had been allowed to achieve different stages of maturity in the presence of NGF under precisely defined conditions in sandwich cultures. Antibody-mediated withdrawal of NGF at 14 d *in vitro* led to the disappearance and presumed death of about 75% of developing septal cholinergic neurons stained for ChAT, AChE, or p75^{NGFR}. Since axotomized cholinergic neurons can become atrophic and lose their staining for cholinergic markers without dying (Lams et al., 1988), an additional study was conducted. Reapplication of NGF to septal cultures for 4 d after NGF withdrawal failed to increase the number of ChAT, AChE, or p75^{NGFR} neurons, suggesting that the cells that had disappeared were indeed dead rather than merely downregulated in the absence of NGF. The effects of NGF withdrawal on developing septal cholinergic neurons appear to be mediated rapidly. Whereas reapplication of NGF after 3 hr of exposure to NGF antibody rescued cholinergic neurons, our findings suggest that 12 hr of NGF withdrawal is sufficient to commit most developing cholinergic neurons to cell death.

In contrast to our findings *in vitro*, injection of NGF anti-

bodies into the ventricles of neonatal rats at a similar developmental age does not lead to a permanent decline in ChAT activity in the cholinergic basal forebrain (Gnahn et al., 1983; Vantini et al., 1989), suggesting that NGF antibody administration *in vivo* does not kill developing basal forebrain cholinergic neurons. There are several possible explanations for this apparent discrepancy in findings. First, it is possible that antibodies do not penetrate well into brain tissue *in vivo* and are not able to neutralize enough NGF to affect the survival of dependent neurons. In support of this explanation, a study on the sprouting of sympathetic neurons into the injured hippocampus shows that the effects of infused NGF antibody are very restricted around the infusion site (Springer and Loy, 1985). Another possible explanation is that other growth factors produced in the hippocampus, such as brain-derived neurotrophic factor (Alderson et al., 1990), may be able to sustain the survival of septal cholinergic neurons in the absence of NGF. Last, both of the above-mentioned *in vivo* studies measured ChAT activity but did not count the number of histologically detectable cholinergic neurons. It is well documented that levels of ChAT activity will initially decline and then return to near normal levels in the basal forebrain cholinergic system in spite of the experimentally induced loss or degeneration of ChAT-producing neurons (Wenk and Olton, 1984; Stephens et al., 1985). Thus, biochemical determination of ChAT activity in tissue homogenates is not a reliable indicator of the presence or absence of cholinergic cell death in experimental studies.

We also examined whether septal cholinergic neurons remained dependent on NGF for survival as they matured *in vitro*. In contrast to the massive cell death triggered within 12 hr of NGF withdrawal at 14 d *in vitro*, over 75% of septal cholinergic neurons survived 4 d of antibody-mediated withdrawal of NGF at 35 d *in vitro*. Although it might be argued that the mature neurons are simply taking longer to degenerate, these findings are consistent with observations made *in vivo* that developing but not mature septal cholinergic neurons degenerate after lesions that deprive them of neurotophins (including NGF) provided by target neurons (Sofroniew et al., 1990, 1993; Cooper and Sofroniew, 1992). These findings also agree well with previously described changes in the NGF dependence of certain peripheral neurons. Developing DRG neurons are absolutely dependent on NGF for survival but become gradually less dependent on NGF for survival as they mature both *in vivo* and *in vitro* (Greene, 1977b; Barde et al., 1980; Schwarz et al., 1982). Moreover, it appears that the absolute age of the DRG neuron, rather than the time in culture, determines when neurotrophic independence will begin (Eichler and Rich, 1989). This observation suggests that an intrinsic clock regulates the timing of NGF dependence in DRG neurons. It remains to be determined whether the timing of NGF dependence in septal cholinergic neurons is internally regulated in a similar manner.

NGF withdrawal–mediated death of developing septal cholinergic neurons in vitro *requires protein synthesis*

Naturally occurring cell death is an essential feature of neural development and appears to be regulated at least in part by the availability to neurons of specific growth factors (Oppenheim, 1989). Considerable evidence suggests that developmental cell death in certain non-neural tissues occurs via receptor-mediated apoptosis activated by environmental cues that trigger an intrinsic program leading to cell death (Wyllie et al., 1980; Arends et al., 1990). In agreement with this possibility, inhibition of

protein synthesis can block developmental cell death in both non-neural and neural tissue (McConkey et al., 1988; Oppenheim et al., 1990). Moreover, the death of developing sympathetic neurons induced by NGF withdrawal *in vitro* can be prevented by blockade of protein synthesis (Martin et al., 1988; Edwards et al., 1991). In this study we found that blockade of protein synthesis also protects developing septal cholinergic neurons from the cell death induced by NGF withdrawal *in vitro*. This finding suggests that growth factor–regulated developmental cell death may also occur in CNS neurons via activation of programmed cell death requiring protein synthesis. Although the underlying mechanism is not understood, it has been suggested that withdrawal of NGF from sympathetic neurons leads to either the *de novo* expression of lethal proteins (Martin et al., 1988) or the activation of constitutively produced lethal proteins (Batistatou and Greene, 1991).

Neurotrophic regulation of naturally occurring developmental cell death in septal cholinergic neurons

No direct evidence has yet been provided demonstrating that naturally occurring cell death does or does not take place during the development of septal cholinergic neurons, or that such a process might be regulated by a neurotrophic factor like NGF. Addressing this question is difficult because cholinergic neurons comprise only a small proportion of septal cells and can only be identified by chemical phenotype. The problems associated with trying to estimate cell survival based only on chemical markers has been discussed. Nevertheless, naturally occurring developmental cell death has been described in various CNS regions (Purves and Lichtman, 1985; Ferrer et al., 1990). Our findings indicate that septal cholinergic neurons grown in the presence of NGF in tissue culture under defined conditions become dependent on NGF for survival during a critical phase during development and lose this dependence as they mature *in vitro*. These findings are compatible with the observation that ablation of hippocampal neurons and reduction of hippocampal NGF mRNA by over 90% leads to the loss of septal cholinergic neurons in neonatal but not adult or aged rats (Sofroniew et al., 1990, 1991; Cooper et al., 1991; Cooper and Sofroniew, 1992). Moreover, the cell death induced in developing septal cholinergic neurons by NGF withdrawal *in vitro* can be attenuated by inhibition of protein synthesis in a manner similar to growth factor–regulated developmental cell death in other neuronal and non-neuronal cells. Together these findings suggest that NGF might participate in regulating the survival of septal cholinergic neurons during a critical period of development.

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