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Proliferation and Differentiation of Embryonic Chick Sympathetic Neurons: Effects of Ciliary Neurotrophic Factor

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Summary

At early developmental stages (embryonic day 7, E7), chick paravertebral sympathetic ganglia contain a cell population that divides in culture while expressing various neuronal properties. In an attempt to identify factors that control neuronal proliferation, we found that ciliary neurotrophic factor (CNTF) specifically inhibits the proliferation of those cells expressing neuronal markers. In addition, CNTF affects the differentiation of sympathetic ganglion cells by inducing the expression of vasoactive intestinal peptide immunoreactivity (VIP-IR). After 1 day in culture, tyrosine hydroxylase immunoreactivity (TH-IR) was expressed by about 86% of the cells whereas VIP-IR was virtually absent. In the presence of CNTF, 50%–60% of the cells expressed VIP-IR after 4 days in culture; however, none of the cells expressed VIP-IR in the absence of CNTF. These results, and the demonstration of cells that express both VIP and TH-IR, indicate that VIP is induced in cells that initially express tyrosine hydroxylase. The findings suggest a potential role for CNTF as a factor affecting the proliferation and differentiation of developing sympathetic neurons.

Introduction

The neurons and glial cells of peripheral ganglia are derived from precursor cells of the neural crest and ectodermal placodes. After a phase of migration, the cells aggregate to form primitive ganglia. During migration and aggregation, the precursor cells proliferate to give rise to a fixed number of postmitotic neurons. The final number of ganglionic neurons is then adjusted by selective cell death to match the size of the target field innervated by the neurons of the ganglion. Whereas the control of neuronal death by neurotrophic factors like nerve growth factor is well understood and documented (Levi-Montalcini and Angeletti, 1968; Hamburger et al., 1981; Oppenheim et al., 1982), very little is known about the control of neuronal proliferation in the peripheral nervous system. This lack of knowledge is at least partly due to the lack of tissue culture systems available to investigate this question; it is necessary to identify cells of the neuronal lineage and to provide conditions that allow the proliferation and differentiation of these cells. We have previously described a system that fulfills these criteria, using sympathetic ganglion cells from seven-day-old chick embryos. These cells divide in vivo (Cohen, 1974; Rothman et al., 1978) and in culture (Rohrer and Thoenen, 1987), and they can be identified as cells of the neuronal cell lineage as they express neuron specific properties. We have used this system in an attempt to define factors involved in the control of neuronal proliferation.

Chick sympathetic neurons differentiate during normal development into two distinct neuronal subpopulations which can be identified in vivo by staining for either tyrosine hydroxylase or vasoactive intestinal peptide (Hayashi et al., 1985; New and Mudge, 1986). In the mammalian sympathetic nervous system, VIP is localized in a subpopulation of cholinergic sympathetic neurons (Hökfelt et al., 1977; Lundberg et al., 1979, 1982; Yodlowski et al., 1984). Whereas in mammals it has been demonstrated clearly that cholinergic sympathetic neurons are derived from adrenergic neurons both in vitro (for review, see Patterson, 1978) and during development in vivo (Landis and Keefe, 1983; Yodlowski et al., 1984), the generation of different cellular phenotypes in the chick sympathetic ganglia is unclear. Thus it was of interest to analyze not only the proliferation but also the differentiation of E7 chick sympathetic ganglion cells.

Evidence is presented that the proliferation of chick sympathetic neurons is subject to extrinsic control and that adrenergic cells can acquire a cholinergic property, i.e., VIP immunoreactivity. The factor affecting proliferation and cholinergic differentiation in vitro is CNTF.

Results

Characterization of Cells Present in E7 Sympathetic Ganglia

The cellular composition of sympathetic lumbosacral ganglia has been analyzed previously using specific markers for neurons, glial cells, and fibroblasts (Rohrer and Thoenen, 1987). At embryonic day 7 (E7), the neuron-specific cell surface antigen Q211 is expressed by 95 ± 2% of the cells. Glial cells and fibroblasts represent only 3 ± 2% and 2 ± 1% of the cell population, respectively. These data have been obtained using short-term (3 hr) cultures. The morphology of the Q211-positive cells after culture periods of 1–3 days was heterogeneous (Figure 1). Some cells had a typical neuronal morphology, i.e., phase-bright cell body, long processes; others had short processes and appeared phase-dark. However, the morphology of Q211-positive cells could easily be distinguished from that of nonneuronal, Q211-negative cells (Figure 1). All cells expressing the neuron-specific Q211 antigen (Roesner et al., 1985; Rohrer et al., 1985; Rohrer and Thoenen, 1987) are referred to as neurons in the present study. This terminology seemed appropriate despite their ability to proliferate, since these cells express various neuron-specific properties (Rothman et al., 1978; Rohrer and Thoenen, 1987).

The neuronal, Q211-positive cell population was further analyzed for the presence of markers specific for

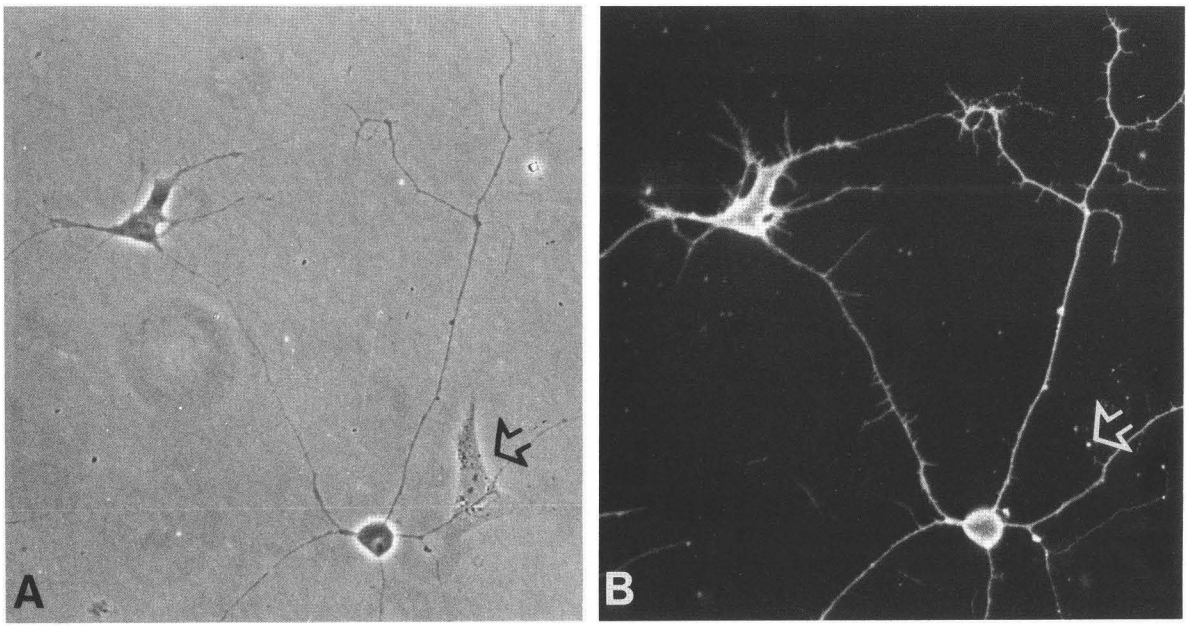

Figure 1. E7 Sympathetic Ganglion Cells Stained for the Presence of Q211 Antigen after 1 Day in Culture

(A) Phase-contrast. (B) Immunofluorescence. Staining was carried out as described in Experimental Procedures. Note the absence of Q211 antigen on a nonneuronal cell (arrow). Magnification, 320×.

adrenergic cells. The adrenergic marker enzyme tyrosine-hydroxylase (TH) was detected in cultures by immunohistochemical techniques (Figures 2A and 2B) in about 86% (see Table 3) of the cells. A comparable proportion (73 ± 12%) expressed imipramine-sensitive norepinephrine uptake, demonstrated by autoradiography (Figures 2C and 2D). Somatostatin-immunoreactive cells, which mostly had short processes after 1 day in culture, were only present in low numbers (10 ± 4%; Figures 2E and 2F). Also, in vivo somatostatin-immunoreactive cells only represent a small subpopulation of sympathetic chain ganglion cells at this stage of development (Maxwell et al., 1984; Garcia-Arraras et al., 1984), suggesting that the cultures are representative of the in vivo situation. Only a small proportion of the cells (2 ± 1%) demonstrated aldehyde-induced catecholamine fluorescence (Figures 2G and 2H). Since the method used detects cells with high levels of catecholamines, i.e., chromaffin cells from E12 chick adrenals (see Experimental Procedures), these data suggest that the majority of E7 sympathetic ganglion cells differ from SIF-like cells.

Since the sympathetic neurons in the rat may be derived from a bipotential precursor that can differentiate to chromaffin cells rather than to sympathetic neurons in the presence of glucocorticoids (Doupe et al., 1985a, 1985b; Anderson and Axel, 1986), we analyzed the effect of dexamethasone (10 μg/ml) on the E7 sympathetic neurons. After 4 days in culture (with or without NGF), neither the proportion of cells with long processes nor the proportion of CA-fluorescent cells or TH-positive cells changed (data not shown).

Characterization of Sympathetic Ganglion Cells Dividing In Vitro

It has been shown previously that cells from E7 sympathetic chain ganglia are able to divide in vitro. The dividing cells were identified by [^{3}H]thymidine-labeling and by the demonstration of mitotic figures after DNA staining (Rohrer and Thoenen, 1987). During the first day in culture, 58 ± 9% of the Q211-positive cell population incorporated [^{3}H]thymidine (24 hr pulse). This proportion decreased to 37 ± 9% and 22 ± 6% during the second and third days in culture, respectively. The dividing cell population was analyzed by staining the cells for specific antigens after a 3 hr pulse with [^{3}H]thymidine, followed by autoradiography. The population of dividing cells was found to contain (during the first day of culture) a proportion of TH-positive cells (84 ± 4%) similar to the total population of E7 sympathetic neurons.

CNTF Affects the Proliferation of Cells

The proliferation of sympathetic neurons in culture was quantified by determining the increase in cell number during the first four days in culture and by determining the proportion of cells incorporating [^{3}H]thymidine during the second day in culture.

Whereas the cell number increased during the first 3 days in the absence of CNTF, no significant increase was observed from 1 day onward in the presence of CNTF (Figure 3). As demonstrated in Table 1, CNTF also strongly inhibited the incorporation of [^{3}H]thymidine in sympathetic neurons. The low numbers of nonneuronal

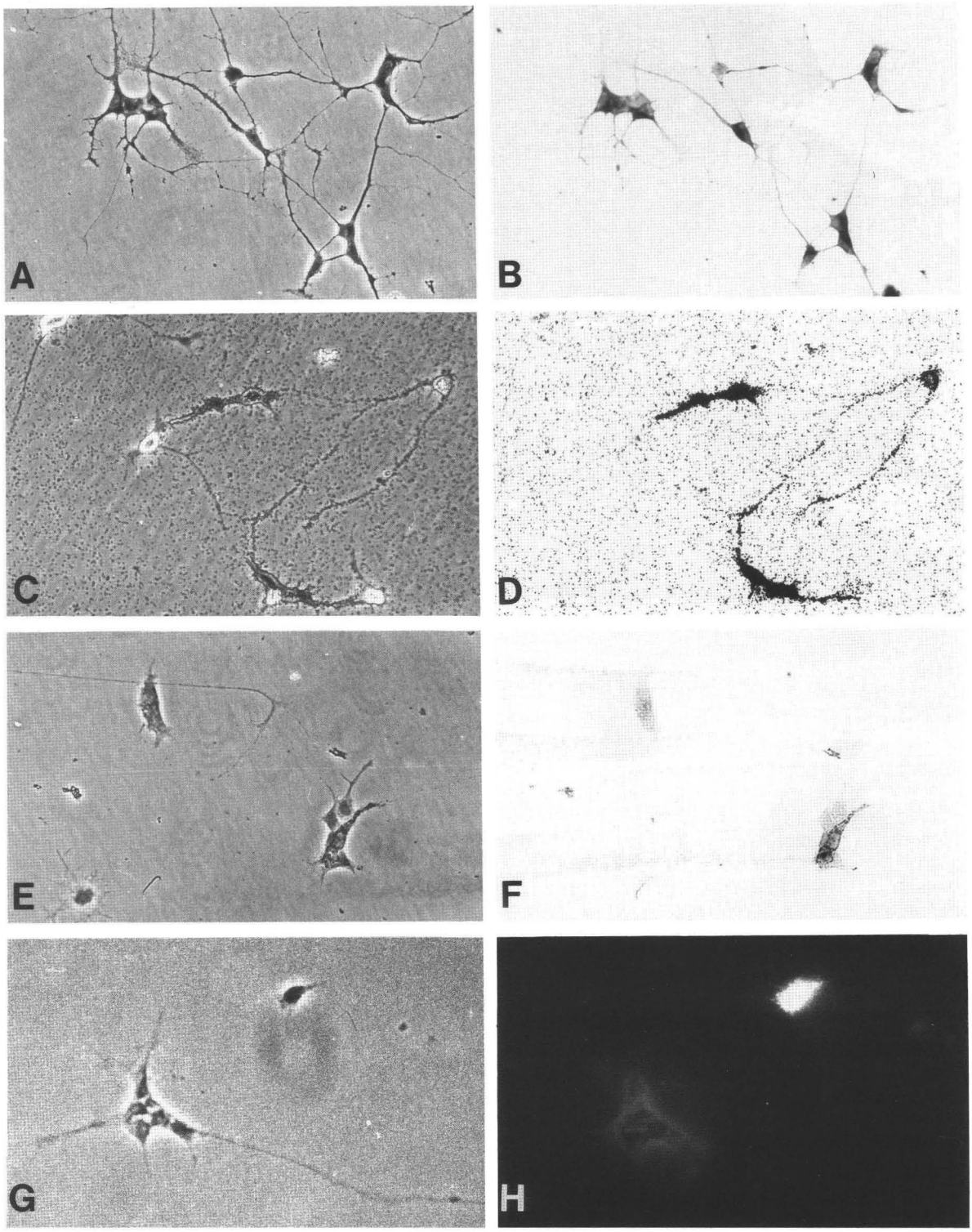

Figure 2. Adrenergic Properties of E7 Sympathetic Ganglion Cells after 1 Day in Culture

Cells were stained for tyrosine hydroxylase (A, B) and somatostatin (E, F) using the PAP method. Norepinephrine uptake is demonstrated by autoradiography. Labeled cells are identified by silver grains in the photographic emulsion (C, D). Cells with high levels of catecholamines were identified by glyoxylic acid–induced fluorescence (G, H). (A, C, E, G) Phase-contrast optics. (B, D, F) Bright-field optics. (H) Fluorescence optics. Magnification A–F, 280×; G, H, 400×.

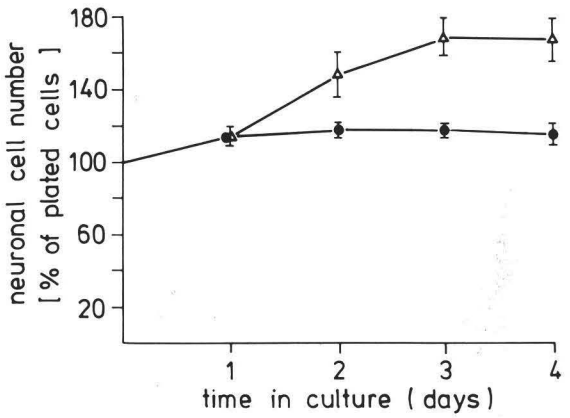

Figure 3. The Increase in the Number of E7 Sympathetic Ganglion Cells in Culture Is Blocked by CNTF

The number of sympathetic neurons was determined after 1, 2, 3, and 4 days in culture. Three hour counts were obtained by counting all cells attached and subtracting the proportion of nonneuronal cells (5%, Rohrer and Thoenen, 1987). Cells were cultivated either in the presence of NGF alone (△) or in the presence of both NGF and CNTF (●). All data points represent the mean of triplicate determinations ± SEM.

cells present in the same cultures were not affected by this treatment, indicating the specificity of the CNTF effect (Table 1). The CNTF preparation used produced half-maximal survival of E8 chick ciliary ganglion neurons at a concentration of about 0.04 ng/ml (Figure 4). The concentration necessary for half-maximal inhibition of neuronal proliferation was in the same order of magnitude (0.1 ng/ml). Not only the pure factor, but also fractions along the purification pathway, had the same biological activity in both systems. The neurotrophic factors NGF (15 ng/ml) or BDNF (1 ng/ml) (Barde et al., 1982) had no influence on the proliferation. In this context it should be mentioned that E7 sympathetic neurons under the present culture conditions do not depend on NGF for survival, which will be described in detail elsewhere (Ernsberger et al., submitted). The growth factors FGF_a, FGF_b, GGF, IL-1β, IL-2, and IL-3 had also no effect on the proliferation of neurons (Table 2). The growth factors were tested both in the presence of serum (Table 2) (10% horse serum, 5% fetal calf serum) and under low serum conditions (N2 medium with 0.1% horse serum and 0.05% fetal calf serum). Under low serum conditions neither basal proliferation rate nor effects of growth factors were different from those in high serum containing medium (data not shown).

Table 1. Effect of CNTF on the Proliferation of Neurons and Nonneuronal Cells ([^{3}H]Thymidine Incorporation)

Factor Present	% of Neurons Labeled	% of Nonneuronal Cells Labeled
NGF	37 ± 9	44 ± 8
NGF + CNTF	7 ± 2	44 ± 7
CNTF	5 ± 2	47 ± 5

Cultures of E7 sympathetic ganglion cells were kept in the presence of NGF or CNTF or both as indicated. [^{3}H]thymidine was present during the last 24 hr of a 48 hr culture period. The cultures were then washed, fixed, and processed for autoradiography.

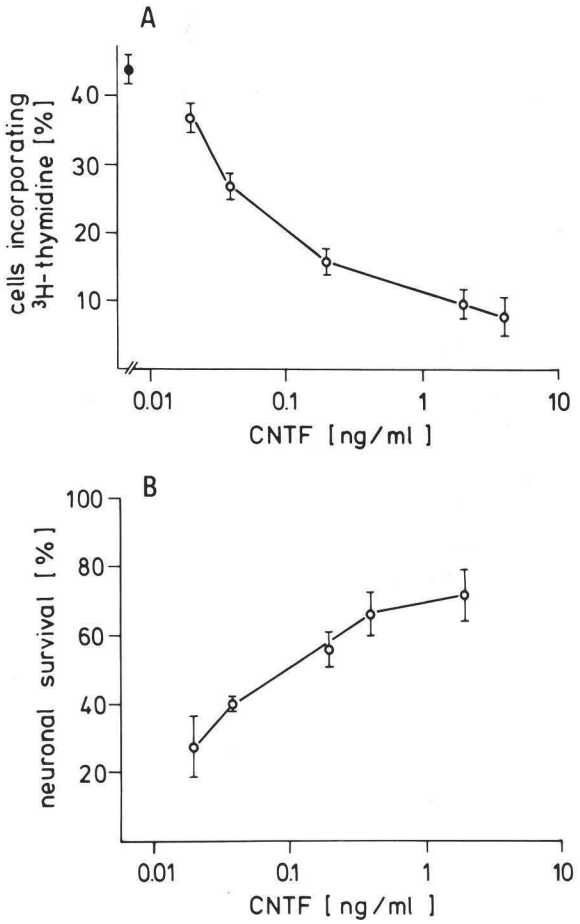

Figure 4. CNTF Interferes with the Proliferation of E7 Sympathetic Neurons at the Same Range of Concentrations as It Produces E8 Ciliary Neuron Survival

(A) Sympathetic neurons were grown for 2 days in the presence of a constant amount of NGF (15 ng/ml) and in addition CNTF was present in the concentrations indicated. [^{3}H]thymidine was present during the last 24 hr, and the proportion of cells that incorporated [^{3}H]thymidine during that period was determined by autoradiography.

(B) Ciliary neurons were grown for 1 day in the presence of CNTF at the concentrations indicated. The number of neurons initially plated was determined by counting the neurons 2 hr after plating. After 24 hr the number of surviving neurons was counted. Values represent the mean ± SD of triplicate determinations.

CNTF Induces the Expression of VIP in Cultured Sympathetic Neurons

Since CNTF caused the sympathetic neurons to leave the proliferation phase, it was of interest to analyze in vitro the effects of CNTF on the further differentiation of these cells. Sympathetic ganglion cells differentiate during normal development in vivo to neurons expressing either TH or VIP immunoreactivity (Hayashi et al., 1985; New and Mudge, 1986), and therefore the cultured cells were analyzed for these phenotypes.

Table 2. Effect of Growth Factors on Neuronal Proliferation ([^{3}H]Thymidine Incorporation)

Factor Present	Number of Neurons Labeled (% of Control)
None	100
FGF_a	109 ± 1
FGF_b	97 ± 9
PDGF	86 ± 13
GGF	102 ± 6
IL-1β	105 ± 17
IL-2	101 ± 8
IL-3	104 ± 22
CNTF	19 ± 5

Cultures of E7 sympathetic ganglion cells were kept either in the presence of NGF alone or with NGF and the factors at concentrations indicated in Experimental Procedures. [^{3}H]thymidine was present during the last 24 hr of a 48 hr culture period. The cultures were then washed, fixed, and processed for autoradiography.

Sympathetic ganglion cells from E7 chick embryos were cultured for 4 days either with NGF alone or with both NGF and CNTF and then analyzed for TH and VIP immunoreactivity (Figure 5). In cultures grown in the presence of NGF alone, the proportion of TH-immunoreactive cells decreased from about 85% at 1 day in culture to 47% after 4 days in culture (Table 3). The expression of VIP was never observed under these culture conditions.

However, when the cells were grown in the presence of both CNTF and NGF or with CNTF alone, about 50% of the neuronal cells showed strong VIP immunoreactivity after 4 days in culture (Table 3, Figure 5). The presence of a small number (<10%) of cells with very faint VIP-IR in the presence of CNTF after 1 day in culture may indicate the onset of VIP expression. Since about 86% of the cells after 1 day in culture expressed TH immunoreactivity, and since neuronal proliferation is inhibited (Table 1) and cell number remained constant in the presence of CNTF over a period of 4 days (Figure 3), it must be assumed that a major part of the VIP-positive cell population is derived from TH-positive cells. However, double-label experiments after 4 days in culture demonstrated only a small proportion of VIP-positive cells (2 ± 1%) that express also TH immunoreactivity (Figure 6). This is due to the fact that TH-IR is lost with time both in cultures kept with NGF alone and in the presence of NGF and CNTF (Table 3). Elevated potassium concentrations prevent the rapid loss of TH immunoreactivity in culture (Ernsberger and Rohrer, unpublished data), and therefore double-labeled cells were found in greater numbers in cultures kept both in the presence of elevated potassium concentrations and in the presence of CNTF (60 ± 3% of VIP-positive cells were also positive for TH-IR).

Discussion

Proliferating neurons from chick sympathetic ganglia (Rohrer and Thoenen, 1987) have been used as a model in identifying in vitro factors affecting the control of proliferation and differentiation of these immature cells. Proliferation was demonstrated to be subject to extrinsic control by CNTF, and in addition it was shown that VIP immunoreactivity is acquired in vitro by adrenergic TH-positive sympathetic ganglion cells under the influence of CNTF.

The sympathetic ganglia are generated by migrating neural crest cells during early development, the neurons being characterized by the early expression of adrenergic properties (Enemar et al., 1965; Cohen, 1974; Kirby and Gilmore, 1976; Rothman et al., 1978). In vivo studies have demonstrated that a large proportion of the cells contain catecholamines and tyrosine hydroxylase immunoreactivity (TH-IR) (Rothman et al., 1978; Garcia-Arraras et al., 1986). In addition, it has been shown that a large proportion of catecholamine-containing cells incorporate [^{3}H]thymidine in vivo (Cohen, 1974; Rothman et al., 1978). Minor populations of ganglion cells can be distinguished from the majority of cells by very high levels of tyrosine hydroxylase immunoreactivity and catecholamines and by the expression of somatostatin immunoreactivity (~7% somatostatin-IR positive cells in E6 quail embryos, which corresponds to the developmental stage of E7 chick embryos) (Luckenbill-Edds and van Horn, 1980; Garcia-Arraras et al., 1984, 1986; Maxwell et al., 1984; Hayashi et al., 1985; New and Mudge, 1986). We have shown previously that up to 95% of the ganglion cells at E7 express the neuron-specific surface marker Q211 and that these cells are able to divide in vitro (Rohrer and Thoenen, 1987). In other ganglia of the chick peripheral nervous system, the Q211 antigen is specific for postmitotic neurons and expression of the antigen starts several hours after the terminal S phase (Rohrer et al., 1985; Rohrer and Thoenen, 1987, see also Roesner et al., 1985). In accordance with our previous definition of neuronal differentiation, the cells from E7 sympathetic ganglia expressing the Q211 antigen may be termed neurons in the present study despite their ability to proliferate. This terminology was chosen to distinguish the cells of the neuronal cell lineage from glial cells and fibroblasts, being aware of the fact that the Q211-positive cells in E7 sympathetic ganglia, although expressing several neuron-specific properties (Rothman et al., 1978, and the present study), differ from mature, postmitotic sympathetic neurons. Here we show that the cultured sympathetic ganglion cells (and the cells incorporating [^{3}H]thymidine) have properties qualitatively and quantitatively similar to those of the cells in the ganglion, i.e., a high proportion of TH-positive cells, low numbers of somatostatin-positive cells. These results indicate that the cell population analyzed in vitro is representative of the in vivo situation, and is thus a useful model for investigating the neuronal proliferation.

Proliferation was demonstrated both by an increase in the number of neurons in culture and by the incorporation of [^{3}H]thymidine. During the first day in culture, about half of the cells incorporated [^{3}H]thymidine. The ability of the cells to proliferate gradually declined, as evidenced from determinations of cell numbers (Figure

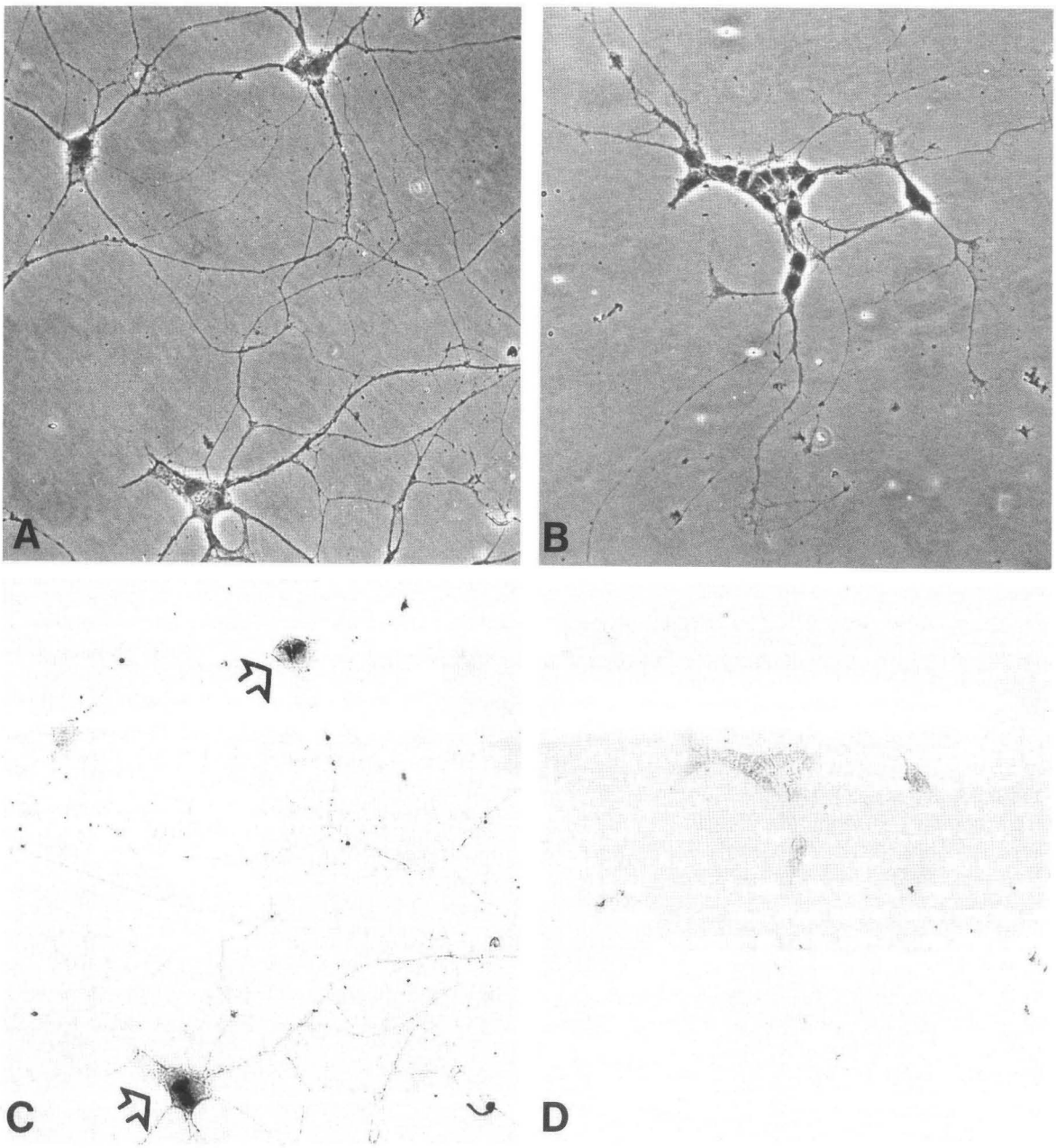

Figure 5. CNTF Induces VIP-IR in Cultures of E7 Sympathetic Ganglion Cells

Sympathetic ganglion cells were grown for 4 days in the presence of NGF alone (B, D) or in the presence of both NGF and CNTF (A, C) and were then stained for VIP-IR using the PAP method. (A, B) Phase-contrast. (C, D) Bright-field. Arrows indicate VIP-positive cells. Magnification, 320×.

3) and the proportion of cells incorporating thymidine. Although the number of proliferating cells also declines during development in vivo, at E9 a considerable proportion of sympathetic ganglion cells has been shown to incorporate [^{3}H]thymidine, and the majority of the cells are able to divide at least twice (Rothman et al., 1978). Thus it seemed that a factor might be absent in vitro which is responsible for sustained proliferation in vivo. Positive control of neuronal proliferation has indeed been demonstrated recently in vivo by Rothman et al. (1987), where a stimulatory influence of endodermal tissue on cells of the neuroepithelium was shown, and in vitro by DiCocco-Bloom and Black (1988), who demonstrated a stimulatory effect of insulin growth factors on the mitotic cycle of embryonic rat sympathetic neurons.

A variety of factors were analyzed in an attempt to determine what regulates neuronal proliferation in vitro. Surprisingly, a factor was detected which inhibited proliferation rather than stimulating it. CNTF, a 24 kd protein that was identified as a factor affecting survival of chick ciliary neurons (Varon et al., 1979; Adler et al., 1979; Barbin et al., 1984; Carnow et al., 1985; Man-

Table 3. Effect of CNTF on the Expression of TH-IR and VIP-IR in Cultures of E7 Sympathetic Neurons

	1 Day in Culture		4 Days in Culture	
	% of Neurons Labeled for		% of Neurons Labeled for	
Factor Present	TH-IR	VIP-IR	TH-IR	VIP-IR
NGF	85 ± 6 (3)	0 (4)	47 ± 9 (8)	0.2 ± 0.4 (6)
CNTF	86 ± 1 (3)	0+ (2)	39 ± 9 (4)	56 ± 16 (4)
NGF + CNTF	87 ± 1 (3)	0+ (2)	ND	47 ± 9 (4)

E7 sympathetic ganglion cells were cultured for 1 or 4 days in the presence of the factors indicated. The cultures were then fixed and stained for tyrosine hydroxylase and vasoactive intestinal peptide as described in Experimental Procedures. Data are given as the mean ± SD. +: After 1 day in culture in the presence of CNTF, extremely faint VIP immunoreactivity could already be detected in some sympathetic neurons (<10%). Whereas after 4 days in culture the somata of VIP-positive cells were filled by granular DAB reaction product (see Figure 5), faintly stained cells after 1 day in culture displayed only a few spots of reaction product in the vicinity of the nucleus. For this reason, the cells were not included in this table. ND: not determined. The numbers in parentheses indicate the number of independent experiments.

thorpe and Varon, 1985; Manthorpe et al., 1986), inhibited the proliferation of sympathetic neurons. Other neurotrophic factors like NGF and BDNF did not affect proliferation, and also the growth (mitotic) factors tested did not influence the incorporation of [^{3}H]thymidine into sympathetic neurons. CNTF specifically blocked the proliferation of neurons, but did not affect the proliferation of nonneuronal cells. The antiproliferative effect of CNTF on sympathetic neurons was observed in the same low range of concentrations as that exerting a survival effect on ciliary neurons, indicating that the same molecule is responsible for both activities, presumably by acting via a receptor-mediated mechanism. This notion is further supported by the fact that the biological activities copurify during the purification procedure and that effects on ciliary neuronal survival, sympathetic neuronal proliferation, and induction of VIP were obtained by CNTF migrating as a single spot on two-dimensional SDS gel electrophoresis.

The differentiation of chick sympathetic ganglion cells during normal development in vivo results in two distinct neuronal subpopulations which can be defined by immunostaining for tyrosine hydroxylase or vasoactive intestinal peptide (Hayashi et al., 1985; New and Mudge, 1986). VIP-positive cells are first detectable at E10, and double-labeling experiments indicate that VIP-positive and TH-positive cell populations do not overlap (Hayashi et al., 1985; H. Rohrer, unpublished data). The VIP-

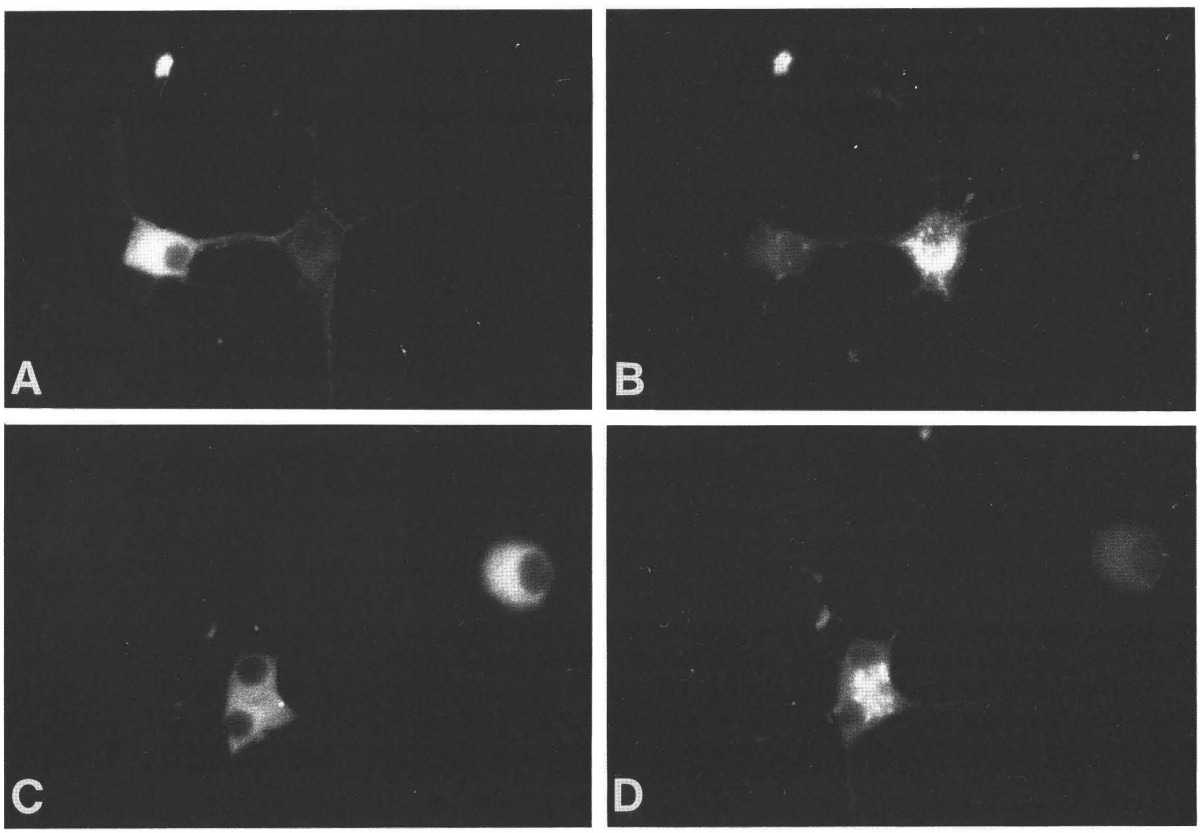

Figure 6. Double-Staining for VIP-IR and TH-IR in Cultures of E7 Sympathetic Ganglion Cells

Cells were grown for 4 days in the presence of both CNTF and NGF and were then stained for both TH-IR (A, C) and VIP-IR (B, D) (see Experimental Procedures). Cells that are either positive for TH-IR or VIP-IR (A, B) and cells that express both TH-IR and VIP-IR (C, D) are demonstrated. Magnification, 500×.

positive cell population accounts for a considerable proportion of the total cell population. At embryonic day 14, about 10% of the neuronal cell population was VIP-positive after 1 day in culture (U. Ernsberger and H. Rohrer, unpublished data). In addition, evidence has previously been presented that chick sympathetic neurons are heterogeneous with respect to their survival factor requirements and the expression of imipramine-sensitive norepinephrine uptake (Edgar et al., 1981; Rohrer et al., 1983). Although there is considerable evidence for neuronal heterogeneity, the lineage relationship and the development of these different phenotypes has been unclear. We now demonstrate that VIP-IR is induced by CNTF in cultured sympathetic ganglion cells from E7 chick embryos. More than half of the neurons expressed VIP-IR after 4 days in culture in the presence of CNTF, whereas in the absence of CNTF no VIP-positive cells could be detected. Since after 1 day in culture about 86% of the neuronal cell population expressed TH-IR, and since in the presence of CNTF the proliferation was inhibited and the number of cells per dish did not vary significantly during that time, VIP-positive cells must be derived from TH-positive cells. This conclusion was supported by the demonstration of cells that express both TH-IR and VIP-IR. These double-labeled cells were present in low numbers under normal culture conditions since TH-IR is rapidly lost. When TH levels were maintained in culture by elevating the potassium concentration, a large proportion of the VIP-positive cells also contained TH-IR.

The present in vitro findings suggest that the precursors of the VIP-positive subpopulation in vivo may be generated during development from TH-positive adrenergic cells. The absence of cells that express both TH and VIP in vivo could be due to a loss of TH in those cells that later express VIP-IR, similar to the observations in vitro.

A switch of rat sympathetic neurons from adrenergic to cholinergic phenotype has been described previously both in vivo and in vitro for sympathetic neurons of the superior cervical ganglion (Johnson et al., 1976, 1980; Furshpan et al., 1976; Patterson and Chun, 1974; Patterson, 1978; Landis and Keefe, 1983; Yodlowski et al., 1984), and a 45 kd glycoprotein that induces cholinergic differentiation in vitro has been purified from heart-conditioned medium (Fukada, 1985). The present finding that VIP-positive sympathetic neurons are derived in vitro from TH-positive sympathetic neurons suggests that these cells may display a similar type of plasticity as demonstrated for postmitotic rat sympathetic neurons. The physiological role of CNTF during neural development in vivo still has to be established, but in view of the present findings, CNTF may be considered as a factor with a potential role in regulating not only neuronal survival and glial differentiation (Hughes et al., 1988; Lillien et al., 1988), but also neuronal proliferation and differentiation.

Experimental Procedures

Cell Preparation and Culture

Lumbosacral paravertebral sympathetic chains were dissected from 7-day-old chick embryos (E7). The ganglia were rinsed in PBS and then treated for 25 min with 0.1% trypsin. Trypsinization was stopped by adding soybean-trypsin inhibitor (Sigma). After washing twice with serum-containing tissue culture medium, the ganglia were triturated by pipetting through a siliconized Pasteur pipet. The resulting cell suspension was plated onto polyornithine/laminin-coated dishes. For all immunocytochemical experiments, cells were plated at a density of 3000 cells/cm^2 in 35 mm dishes (Greiner, 4-well). The dishes were treated before plating overnight with 0.5 mg/ml polyornithine (Sigma) in borate buffer (0.15 M, pH 8.3). After washing with water, laminin (BRL) was applied for 2 hr (35 mm dishes, 10 μg/ml) or overnight (60 mm dishes, 4 μg/ml).

The culture medium used was Hams F14, supplemented with 10% horse serum and 5% fetal calf serum. NGF (prepared according to Bocchini and Angeletti [1969] using modifications as described by Suda et al. [1978] and CNTF (prepared using a modification of the procedure of Manthorpe et al. [1986]; see below) were added at saturating concentrations (NGF 15 ng/ml, CNTF 25 trophic units/ml which corresponds to 1–2 ng/ml). The medium was changed every 2–3 days.

Cell counts to determine survival of neurons were obtained by examining cells in randomly selected fields of the culture dish. The number of neurons initially plated per well was determined by counting the cells 3 hr after plating. Triplicates were counted for each data point. Ciliary ganglion neurons were prepared from 8-day chick embryos and cultured at a density of 1–2 × 10^3 cells/well in multiwell dishes (Costar, 16 mm) in F14 medium with 10% horse serum, as previously described (Harper et al., 1983), except that the culture dishes were coated with laminin (BRL, 2 μg/500 μl/well) instead of heart-conditioned medium.

Purification of CNTF

CNTF was prepared from adult rat sciatic nerve by the following modifications of the method of Manthorpe et al. (1986): the sciatic nerves were homogenized in phosphate buffer (5 mM NaP_i [pH 7.0] with 30 mM NaCl) instead of water. After ultracentrifugation the supernatant was applied to a DEAE-cellulose column as described by Manthorpe et al. (1986). The column was eluted with 5 mM sodium phosphate-buffer (pH 7.0) containing 180 mM NaCl. The eluted protein was loaded on a preparative SDS-polyacrylamide gradient (12%–18%) gel. After KCl staining of the gel, the CNTF was eluted (using the electroelution procedure described by Braun et al. [1987]) from a single band visible at about 22.5 kd (MW standards were lysozyme, chymotrypsinogen [PMSF-treated], and ovalbumin, all obtained from Sigma). The purified CNTF migrated as a single silver-stained band when analyzed by SDS–PAGE (Hughes et al., 1988). The apparent MW on SDS–PAGE of CNTF is slightly higher under nonreducing conditions (Hughes et al., 1988).

In addition to the biological activity of this preparation of CNTF described in the present paper, we also found that CNTF that was prepared for sequencing (Sendtner et al., unpublished data) and which migrated as a single spot on 2D gel electrophoresis (Saadat et al., 1989) displayed identical activity (data not shown).

Immunocytochemistry

Neuron-specific cell surface antigens were detected by immunofluorescence on cultured cells stained as described in detail previously (Rohrer et al., 1985; Ernsberger and Rohrer, 1988), using the monoclonal antibody Q211 (a generous gift from S. Henke-Fahle, MPI für Entwicklungsbiologie, Tübingen). Intracellular antigens (single staining) were detected using the peroxidase-anti-peroxidase (PAP) method. After washing the cultures, fixation was performed with 4% paraformaldehyde in PBS for 15 min. The cells were then washed twice with PBS and permeabilized with PBS containing 0.1% Triton X-100, 10% goat serum, and 10% fetal calf serum (P buffer) at room temperature for 15–20 min. Then primary antibodies were applied for either 1 hr at room temperature or overnight at 4°C. All of the following steps were done at room temperature. Rabbit antisera against tyrosine hydroxylase (1:100–1:400, a gift from Dr. J. Thibault, College de France, Paris), VIP (1:200, INC), and Somatostatin (1:100, INC) were used as first antibodies.

Goat-antisera against the Fc part of rabbit IgG were used as second antibodies at a 1:10 dilution for 30 min (Nordic). Complexes of rabbit PAP (1:100; Miles-Yeda) were then added for 30 min. Be-

tween all antibody steps the cultures were washed three times for at least 5 min each with P buffer. Staining reactions were performed with 0.05% diaminobenzidine (Sigma) in Tris-phosphate buffer (pH 7.8). The reaction was stopped by diluting the incubation medium after 30 min. The cultures were then mounted in PBS/glycerol (1:1; v/v) viewed, and photographed with Leitz Orthoplan phase-contrast and bright-field optics. Control incubations were routinely included using rabbit polyclonal antibodies raised against snake venom. The staining for TH and VIP was completely abolished by preincubating the antibodies with purified rat tyrosine hydroxylase (a gift from Dr. J. Thibault) or VIP (Sigma) at a concentration of 40 μg/ml for 1 hr at room temperature.

Double-staining for tyrosine hydroxylase and VIP was carried out using the biotin-streptavidin method. Cells were fixed for 15 min with 4% paraformaldehyde in PBS, washed, permeabilized for 15 min with PBT1 (PBS supplemented with 1% BSA and 0.1% Triton X-100), and then incubated for 30 min with both rabbit anti-VIP (1:200; Seralab) and mouse anti-TH (1:200; clone 40) in the same buffer. After washing (four times), FITC-labeled goat anti-rabbit antiserum (1:100) and biotinylated goat anti-mouse antibodies (1:100, Amersham) dissolved in PBT1 were added for 30 min. After washing (two times with PBT1 and PBT2 each), Texas Red streptavidin (1:100, Amersham) dissolved in PBT2 (PBS supplemented with 0.1% BSA and 0.1% Triton X-100) was added for 20 min. Then the cultures were washed and mounted in PBS/glycerol.

Norepinephrine Uptake

Norepinephrine uptake was carried out as described in detail by Rohrer and Sommer (1983). In brief, cultures were washed and incubated with 0.5 μM [^{3}H]norepinephrine (NEN) for 1 hr at 37°C. Control incubations contained in addition the inhibitor desipramine (DMI) at a concentration of 0.5 μM. After fixation, norepinephrine uptake was visualized by autoradiography.

Catecholamine Fluorescence

Cell cultures were kept on glass cover slips coated with polyornithine/laminin as described for the cultures on plastic dishes. Cultures were washed with buffer PR (PBS/Ringer, 1:1) for 30 min at 4°C. Glyoxylic acid dissolved in PR buffer (13.6 mg/ml; pH 7.0) was added for 5 min. Then the cover slips were removed from the glyoxylic acid solution and allowed to dry. After heating for 5 min at 100°C, the cells were mounted immediately in immersion oil. Fluorescence was viewed with a Leitz Orthoplan microscope using appropriate filter combinations. Cultures of E12 chick adrenal cells after 1 day in culture under the same conditions as used for E7 sympathetic neurons were used as a positive control. About 30 ± 5% of these cells were strongly fluorescent. A similar proportion of the cells displayed TH immunoreactivity (38 ± 4%).

[^{3}H]Thymidine Labeling

Methyl-[^{3}H]thymidine (Amersham; 40–60 Ci/mmol) was added at a concentration of 1 μCi/ml to the culture medium. After an incubation period of 18–24 hr, the cultures were washed several times with KRH/A and then fixed for 20 min with glutaraldehyde (2%–5%) or formaldehyde (4%). To determine the proportion of cells incorporating thymidine, cells were dehydrated with ethanol and covered with photographic emulsion (Kodak NTB2). After an exposure period of 4–5 days, the emulsion was developed using Kodak D19 developer. Labeled cells were identified by the presence of silver grains, using bright-field optics and phase-contrast. To investigate the effect of various growth factors on the incorporation of [^{3}H]-thymidine, the cells were grown either in F14 medium supplemented with 10% horse serum and 5% fetal calf serum, or in N2 medium (Bottenstein and Sato, 1979) supplemented with 0.1% horse serum and 0.05% fetal calf serum. The growth factors used were acidic and basic FGF (gifts from P. Böhlen, M. Sensenbrenner, and W. Risau), used at concentrations of 50 ng/ml and 5 ng/ml, respectively; glial growth factor (GGF) (heparin-sepharose fraction) was a gift from J. Brockes and was used at concentrations stimulating glial cell proliferation maximally (50 ng/ml). PDGF (Collaborative Research) was used at concentrations of 3 U/ml. Interleukin-1β (a gift from Biogen) and IL-2 (Polysciences) were used at concentrations of 30 U/ml. IL-3 (a gift from P. Vassalli) was used at a concentration of 28 ng/ml.

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