Ciliary Neurotrophic Factor Induces Cholinergic Differentiation of Rat Sympathetic Neurons in Culture

S. Saadat, M. Sendtner, and H. Rohrer

Max-Planck-Institute for Psychiatry, Department of Neurochemistry, D-8033 Martinsried-Munich, Federal Republic of Germany

Abstract. Ciliary neurotrophic factor (CNTF) influences the levels of choline acetyltransferase (ChAT) and tyrosine hydroxylase (TH) in cultures of dissociated sympathetic neurons from newborn rats. In the presence of CNTF both the total and specific activity of ChAT was increased 7 d after culture by 15- and 18-fold, respectively, as compared to cultures kept in the absence of CNTF. Between 3 and 21 d in culture in the presence of CNTF the total ChAT activity increased by a factor of >100. Immunotitration demonstrated that the elevated ChAT levels were due to an increased number of enzyme molecules. In contrast to the increase in ChAT levels, the total and specific activity levels of TH were decreased by 42 and 36%, respectively, after 7 d in culture. Half-maximal effects for both ChAT increase and TH decrease were obtained at CNTF concentrations of ~0.6 ng and maximal levels were reached at 1 ng of CNTF per milliliter of medium. The effect of CNTF on TH and ChAT levels were seen in serum-containing medium as well as in serum-free medium. CNTF was shown to have only a small effect on the long-term survival of rat sympathetic neurons. We therefore concluded that the effects of CNTF on ChAT and TH are not due to selective survival of cells that acquire cholinergic traits in vitro, but are rather due to the induction of cholinergic differentiation of noradrenergic sympathetic neurons.

Sympathetic ganglia contain several neuronal subpopulations that differ in their transmitter phenotype. Whereas most peripheral sympathetic neurons are noradrenergic, a small population of neurons are functionally cholinergic. The developmental expression of cholinergic properties by sympathetic neurons of the rat superior cervical ganglia has been analyzed in great detail in vitro and in vivo. These neurons have noradrenergic properties in vivo (Cochard et al., 1979; Teitelman et al., 1979) but when grown in vitro in the presence of certain factors, these cells show plasticity with respect to their expression of transmitter phenotype. Under the influence of factors present in rat heart muscle-conditioned medium (Patterson and Chun, 1974; Furshpan et al., 1976; Patterson and Chun, 1977; Weber 1981) and in rat skeletal muscle–conditioned medium (Swerts et al., 1983; Raynaud et al., 1987*b*), catecholaminergic properties, including tyrosine hydroxylase (TH)[1], are reduced and cholinergic characteristics, such as choline acetyltransferase (ChAT), are induced. The induction of cholinergic properties by the heart muscle–conditioned medium was demonstrated to be due to the presence of a 45-kD glycoprotein, named cholinergic neuronal differentiation factor (Fukada, 1985). Cholinergic differentiation of cultured rat sympathetic neurons was also demonstrated under the influence of factors present in human placental serum and/or embryo extract (Johnson et al., 1976; Ross et al., 1977; Iacovitti et al., 1981). Interestingly a similar transition in the transmitter phenotype of sympathetic neurons has also been observed in vivo. The sympathetic neurons innervating the sweat glands of the rat foot pad are functionally cholinergic in adult rats (Langley, 1922; Hayashi and Nakagawa, 1963; Sato and Sato, 1978; Landis and Keefe, 1983; Landis et al., 1988). During early development, however, these neurons express first noradrenergic properties. The noradrenergic properties are lost during later development and the neurons become cholinergic which, among other changes, is reflected by the appearance of vasoactive intestinal peptide and ChAT immunoreactivity (Landis and Fredien, 1986; Yodlowski et al., 1984).

During our analysis of the development and differentiation of chick sympathetic neurons we have observed that ciliary neurotrophic factor (CNTF) induces the expression of vasoactive intestinal peptide immunoreactivity and reduced TH immunoreactivity in cultured chick sympathetic neurons (Ernsberger et al., 1989). Thus, it seemed of great interest to investigate whether CNTF has the properties of a factor that can promote the switch from the noradrenergic to the cholinergic phenotype. Since this transition has been studied extensively in rat sympathetic neurons of the superior cervical ganglia, we decided to study the effect of CNTF on ChAT activity levels in these cells.

1. *Abbreviations used in this paper*: ChAT, acetyl cholinetransferase; CNTF, ciliary neurotrophic factor; NGF, nerve growth factor; TH, tyrosine hydroxylase.

The present study demonstrates that CNTF increases ChAT and at the same time decreases TH activity levels. Together with the previously obtained evidence on the induction of vasoactive intestinal peptide immunoreactivity in chick sympathetic neurons (Ernsberger et al., 1989), these data suggest that CNTF may be considered a factor that promotes the transition from a noradrenergic to a cholinergic phenotype in sympathetic neurons.

Materials and Methods

Materials

Collagen from calf skin (type III), poly-D-L-ornithine (type I-B), penicillin G, streptomycin sulfate, cytosine β-D-arabinofuranoside, protein A crude cell suspension (formalin-fixed *Staphylococcus aureus*, Cowan strain), rabbit anti-mouse IgG (whole molecule) antiserum, IgG-free albumin, ovalbumin, and molecular mass standards for one-dimensional gel electrophoresis were purchased from Sigma Chemical Co. (St. Louis, MO); six-well 35-mm dishes were purchased from Costar (Cambridge, MA); mouse sarcoma laminin, Leibovitz's L-15 medium, and nutrient mixture Ham's F12 were purchased from Gibco Laboratories (Grand Island, NY); ^{3}H-acetyl-CoA (1-6 Ci/mmol) was purchased from New England Nuclear (Boston, MA). Molecular mass standards for two-dimensional gel electrophoresis were obtained from Bio-Rad Laboratories (Richmond, CA).

Neurotrophic Factors

CNTF was purified from adult rat sciatic nerve by a modification of the method of Manthorpe et al. (1986) using DEAE-ion-exchange chromatography and preparative SDS-PAGE. The protein eluted from the gel was then subjected to a further chromatographic step to remove SDS. The purified CNTF migrated as a single band on a 10-20% SDS polyacrylamide gradient gel under reducing conditions. The molecular mass was estimated to be ~22.5 kD, if related to lysozyme, 14.3 kD; PMSF-treated trypsinogen, 24 kD; and ovalbumin ~45 kD. The isoelectric point was determined to ~4.8. Two-dimensional gel electrophoresis was performed as describe O'Farrell (1975). The first dimension was isoelectric focussing in a gradi of pH 3.5 to 10. The second dimension was done on a 12% SDS polyac amide gel. The CNTF migrated as a single spot (Fig. 1). In some cas faint second spot with the same molecular mass and a slightly more ac isoelectric point was observed on the gel: we attribute this spot to a degra form of CNTF, probably due to oxidation. The purified protein displa the same biological properties as described previously by Manthorpe e (1986), i.e., the protein was able to maintain neurons from E8 ciliary g glia, E8 and E10 sympathetic ganglia, E10 dorsal root ganglia, but had effect on the survival of E8 dorsal root ganglia neurons. 2.5 S NGF was p pared from male mouse submaxillary glands as described by Bocchini Angeletti (1969) using modifications described by Suda et al. (1978).

Cell Culture

Adrenergic sympathetic neurons were obtained from superior cervical g glia of newborn rats by a procedure of Mains and Patterson (1973; C and Patterson, 1977*a*,*b*) using modifications described by Schwab Thoenen (1985). The dissociated sympathetic neurons were plated on mm dishes which were coated with collagen (1 μg/ml of PBS per dish sequentially with polyornithine (0.5 mg/ml 0.15 M borate, pH 8.3 per di and laminin (10 μg/ml of PBS per dish). Routinely 17,000 cells were pla on one 35-mm dish and the neurons were kept in 2-3 ml of Leibovitz's L medium, 5% (vol/vol) rat serum obtained from adult rats, 100 U of peni lin, and 100 μg of streptomycin per milliliter and, if not otherwise sta 50 ng of nerve growth factor (NGF) per milliliter. Medium was changed ery 2-3 d and 10 μM of cytosine arabinofuranoside was added 2 d after p ing and then once every week to suppress the growth of nonneuronal ce

Serum-free medium consisted of Ham's F12 medium supplemented w transferrin, putrescine, insulin, selenium, and progesterone as described Bottenstein and Sato (1979). The number of neurons was determined counting the large, phase bright cells (with or without processes) in 10 r domly chosen visuals fields at 125-fold magnification. The area coun corresponded to 2.6% of the total area. Neuron cultures were washed tw before harvesting with PBS to remove serum proteins. Then the cells w scraped off the dish in PBS with a rubber policeman, pelleted by centrifu tion, and stored frozen at −20°C until further use.

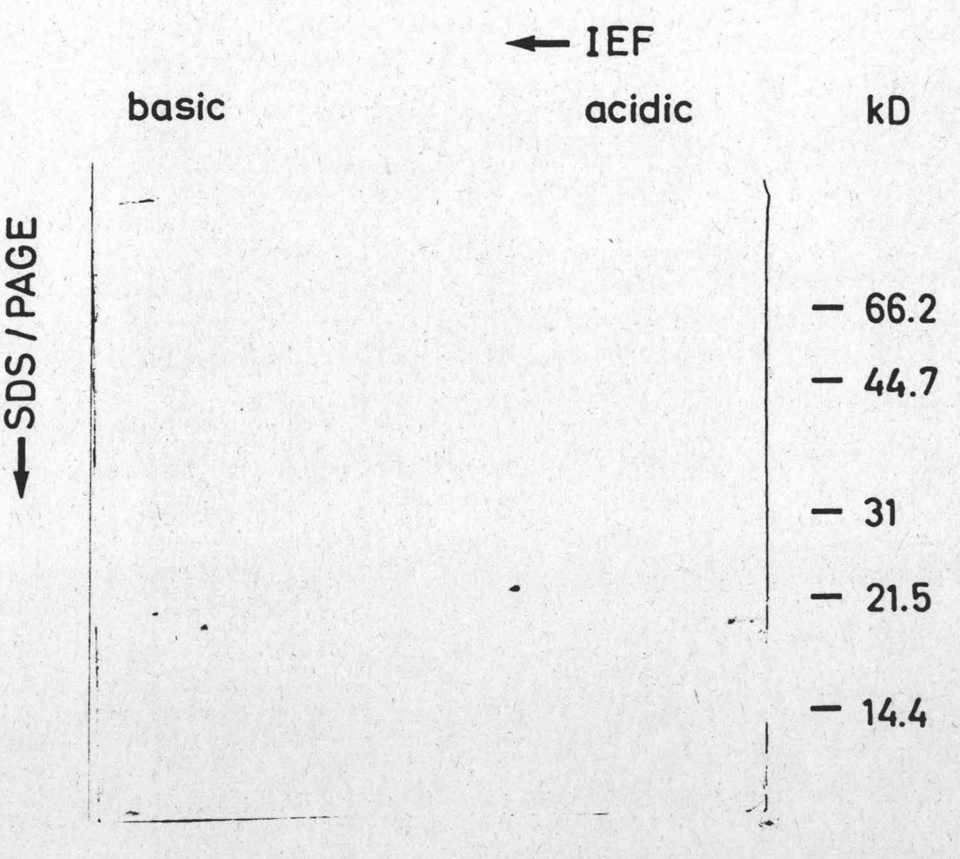


Figure 1. Two-dimensional ele trophoresis of CNTF. 4 μg purified CNTF (5 × 10^4 T were subjected to isoelectric cusing (*IEF*) on a gradient of p 3.5 -10.0. The second dimensi was a 12% SDS polyacrylami gel. Proteins were visualized Coomassie blue staining. Molec lar mass standards were run on separate gel: lysozyme (14.4 kD soybean trypsin inhibitor (21 kD), carbonic anhydrase (31 kD ovalbumin (44.7 kD), BSA (66 kD).

Assays

Frozen neurons (~10,000–15,000 surviving cells from one culture dish) were suspended in 130 μl of homogenization buffer (0.1% Triton X-100 and 5 mM Tris-HCl, pH 7.4) and homogenized by repetitive pipetting. Undissolved material was pelleted by centrifugation for 2 min at 10,000 *g*. 20 μl were assayed for TH (Acheson et al., 1984), 15 μl for ChAT (Fonnum, 1969; Raynaud et al., 1987*a*) enzyme activity measurements, and 40 μl for protein determination by the Bradford procedure (Bradford, 1976) with ovalbumin as standard. All determinations were done in duplicate. Specific TH and ChAT activity levels were expressed as nanomoles of dihydroxyphenylalanine (dopa) formed/min per mg of total cellular protein and as picomoles of acetylcholine formed/min per mg of protein, respectively. Due to the small amounts of ChAT, the sensitivity of the enzyme assay was increased using subsaturating concentrations of acetyl-CoA (2 μM with a specific activity of ~1 Ci/mmol) described by Raynaud et al. (1987*a*). The activity was blocked completely in the presence of 0.5 mM *N*-hydroxyethyl-4[1-naphthylvinyl]pyridinium bromide. Both assays were linear with time up to 15 min and with the amount of enzyme present in the assay mixture until ~40% of the substrate was used.

Immunotitration

Neurons were harvested and solubilized in homogenization buffer as described above. Homogenates from cells cultured in the presence of CNTF contained high levels of ChAT and were therefore diluted 20-fold with homogenization buffer to obtain ChAT activity levels that were similar to those found in cells cultured in the absence of CNTF. To 20 μl of homogenate 3 μl of the mouse monoclonal anti-ChAT antibody 1E6 (Crawford et al., 1982; a generous gift of Dr. P. Salvaterra, Beckman Research Institute, Duarte, CA) was added and the mixture was incubated for 1 h at 4°C. The lyophilized monoclonal antibody was dissolved in PBS supplemented with 0.1% albumin at a concentration of 200 ng/ml and diluted up to 100-fold. The antibody–ChAT complexes were then mixed with 7 μl of a 10% (wt/vol) protein A–cell suspension (formalin-fixed *S. aureus* cells) and incubated for 1 h at 4°C. After the incubation the cells were pelleted and the amount of ChAT activity that remained in the supernatant solutions was determined. The protein A–cell suspension was prepared as follows: The cells were first washed four times with homogenization buffer, then incubated for 1 h at 4°C with rabbit anti-mouse IgG antiserum, and finally washed four times with homogenization buffer.

Results

Cell Culture

The conditions for culturing adrenergic sympathetic neurons of superior cervical ganglia from newborn rats have been analyzed in detail previously (Mains and Patterson, 1973; Hefti et al., 1982). In these studies collagen has been used as culture substrate. Since laminin has been shown to be a more preferable substrate than collagen for the culture of different types of peripheral neurons (Baron-Van Evercooren et al., 1982; Rogers et al., 1983), the effect of these two substrates on morphology and survival was investigated. The neurons cultured on laminin were more evenly distributed on the dish and produced mainly thin neurite bundles, whereas the neurons cultured on collagen did aggregate and their neurites formed thick bundles. Neurite outgrowth was detectable after 1 d on laminin-coated dishes whereas on collagen-coated dishes neurite outgrowth was observed only after 2 d in culture (data not shown). The maximal survival of neurons cultured on laminin was reached with 10 ng of NGF per milliliter of medium (Fig. 2), the concentration also reported to result in maximal neuronal survival on collagen (Hefti et al., 1982).

NGF affects not only the survival of rat sympathetic neurons but also the activity levels of TH and ChAT (Hefti et al., 1982; Raynaud et al., 1988). To analyze the effect of CNTF on the expression of ChAT and TH, conditions were

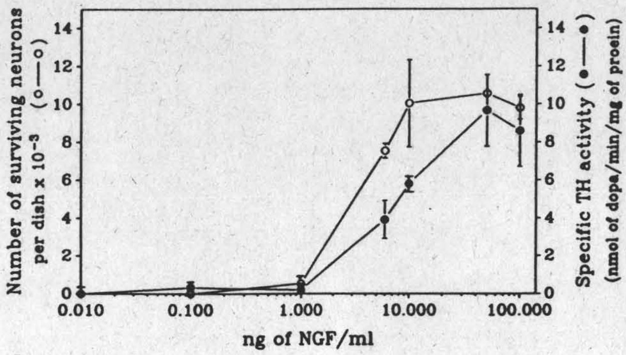


Figure 2. NGF-dependent survival of rat sympathetic neurons cultured on laminin. The neurons were plated on laminin and cultured for 7 d. The number of cells surviving at different NGF concentrations was determined by cell counting (○). For the determination of the specific TH activity (●), the neuron cultures were washed with PBS and then scraped off the dish with a rubber policeman.

chosen where survival, TH, and ChAT activities were maximally stimulated by NGF. Whereas the maximal survival of neurons cultured on laminin was reached with 10 ng of NGF per milliliter of medium, TH activity levels reached maximal values at 50 ng/ml (Fig. 2). Previous studies indicated that both TH and ChAT activity reach maximal levels at the same NGF concentration (Raynaud et al., 1988). Thus all experiments were performed at a NGF concentration of 50 ng/ml. The specific TH activity of cells cultured on laminin was not significantly different from the enzyme levels of cells kept on collagen as shown in Table I.

Effect of CNTF on the Activity Levels of ChAT and TH

Dose Response Curve. Neurons were cultured either in the presence of NGF alone or in the presence of NGF and increasing amounts of CNTF. The levels of ChAT and TH were analyzed 7 d after the addition of CNTF. CNTF addition led to a strong increase in ChAT activity and to a decrease in TH activity (Fig. 3). ChAT activity levels began to increase significantly at a CNTF concentration of 0.1 ng/ml and reached maximal values at 1 ng/ml. Half-maximal ChAT increase was observed in this experiment at 0.5 ng/ml and on the average at 0.6 ± 0.2 ng/ml (three independent experiments with SEM). Addition of up to 8 ng/ml did not further

Table I. TH Activity Levels in Rat Sympathetic Neurons Cultured on Either Laminin or Collagen

Substratum*	Units of TH per well	μg of protein per well	Specific TH activity
	%	%	%
Collagen	100 ± 13	100 ± 12	100 ± 5
Laminin	135 ± 4.6	113 ± 17	108 ± 13

* The sympathetic neurons were cultured on either collagen or laminin as substratum. The neurons were harvested 7 d after plating and TH and protein content were determined. Results obtained with neurons cultured on collagen were set as 100% and ranged between 2.2 and 12.4 nmol of dihydroxyphenylalanine (dopa)/min per mg of protein. Numbers represent the average of three independent experiments with SEM.

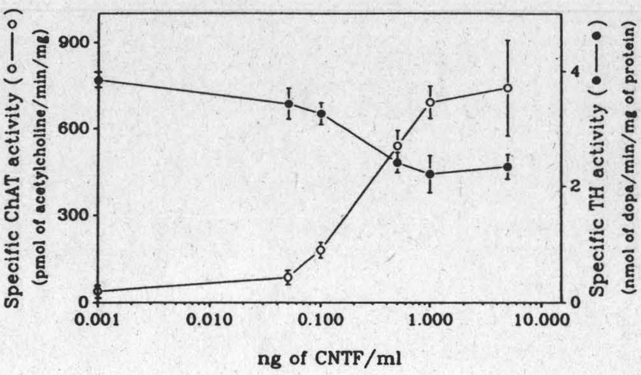


Figure 3. Dose-response curve of CNTF on ChAT and TH activity levels. The neurons were plated in CNTF-containing medium and cultured for 7 d. The specific activities of TH (●) and ChAT (O) were determined and numbers represent the average of three dishes with SEM of one experiment.

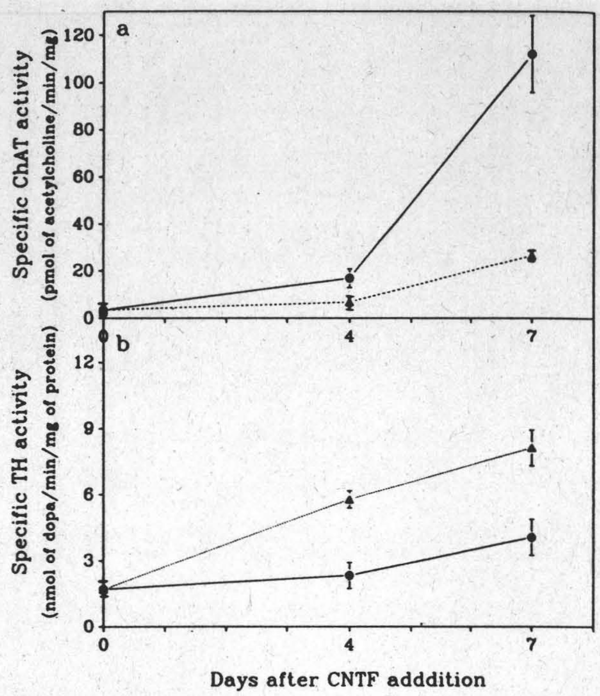


Figure 4. Short-term time course of the effect of CNTF on Ch/ and TH. The neurons were first plated in NGF-containing mediu and CNTF (1 ng/ml) was added 3 d after plating. The neurons we harvested at the indicated time points and the specific ChAT (*a*) a TH (*b*) activities were determined in the presence (●) or absen (▲) of CNTF. The 0 time-point represents the time of CNTF add tion which was 3 d after plating. Data points represent the avera of three dishes with SEM from one experiment.

increase ChAT levels (data not shown). After 7 d in culture in the presence of CNTF, the specific ChAT levels were increased on the average by 18-fold (Fig. 3 and Table II).

The total and specific TH activity were determined in parallel and were found to be decreased by 42 and 36%, respectively, as compared to control cultures in the absence of CNTF (Table II and Fig. 3). Both ChAT and TH activities were expressed as specific activities, that is, normalized to the total cellular protein content. Protein content per dish decreased in the presence of 1 ng/ml CNTF by 27 ± 4.1% in 10 independent experiments. Since CNTF decreased protein and TH levels, it is concluded that the ChAT increase is due to a selective induction by CNTF in a dose-dependent manner.

Time Course. After culturing the neurons for 3 d with NGF alone, CNTF was added and the levels of ChAT and TH were determined 0, 4, and 7 d after CNTF addition (Fig. 4). Control cultures were kept with NGF alone. In the absence of CNTF the specific activity levels of TH increased 4.8-fold between day 3 and 10 in culture (*0* and *7* in Fig. 4 *b*). During the same culture period in the presence of CNTF, however, TH levels were only increased by 2.3-fold.

Table II. Effect of CNTF on ChAT and TH Activity Levels of Rat Sympathetic Neurons Cultured in Serum-containing and Serum-free Medium

Factor added*	Specific activity of			
	ChAT	TH	ChAT	TH
	Serum-containing		Serum-free	
NGF	40.8 ± 7.1	6.8 ± 0.7	24.8 ± 5.3	12.3 ± 4.4
NGF and CNTF	723.5 ± 173 (17.7×)	4.2 ± 0.5 (0.62×)	193.3 ± 13.3 (7.8×)	3.7 ± 1.8 (0.3×)

* The neurons were cultured for 7 d on laminin-coated dishes in serum-containing or serum-free medium with NGF (50 ng/ml) and CNTF (1 ng/ml) as indicated. ChAT is expressed as picomoles of acetylcholine/min per mg and TH as nanomoles of dopa/min per mg of protein. Numbers represent the average of four to eight independent experiments (±SEM). The magnitude of the CNTF effect on ChAT and TH levels as compared to cultures with NGF are given in brackets.

In contrast to TH, the specific activity levels of ChAT i creased 23-fold during the first 7 d after CNTF addition (Fi 4 *a*). The effect of CNTF showed a slow time course: A though a small increase in the specific activity levels ChAT was observed as early as 2 d after CNTF additic (data not shown), the maximal increase was observed 7 d a ter CNTF addition (Fig. 4 *a*). The specific ChAT activity CNTF-treated cultures was increased fivefold, as compare to untreated cultures (Fig. 4 *a*), and on the average was ir creased 17.7-fold (Table II).

These results demonstrated that CNTF has significa (and opposite) effects on the activity levels of ChAT and T in rat sympathetic neurons that were cultured for 7 d in th presence of CNTF. However, most of the previous work o the effects of cholinergic factor was carried out with sym pathetic neurons that were cultured for several weeks. There fore, we also investigated the effect of CNTF on ChAT level of neurons cultured for 21 d (Fig. 5). The specific activit of ChAT continued to increase up to 14 d but was found t be constant between 14 and 21 d. The total ChAT activity pe dish, in contrast, continued to increase up to 21 d, as de scribed previously (Swerts et al., 1983), for the effects c cholinergic factor from rat skeletal muscle-conditioned me dium. Total ChAT activity increased between 3 and 24 d i the presence of CNTF by 177-fold in this experiment and o the average by 154 ± 22-fold. TH levels were decreased i the presence of CNTF after 21 d in culture by 56 ± 13 an 50 ± 2% in total and specific activity, respectively.

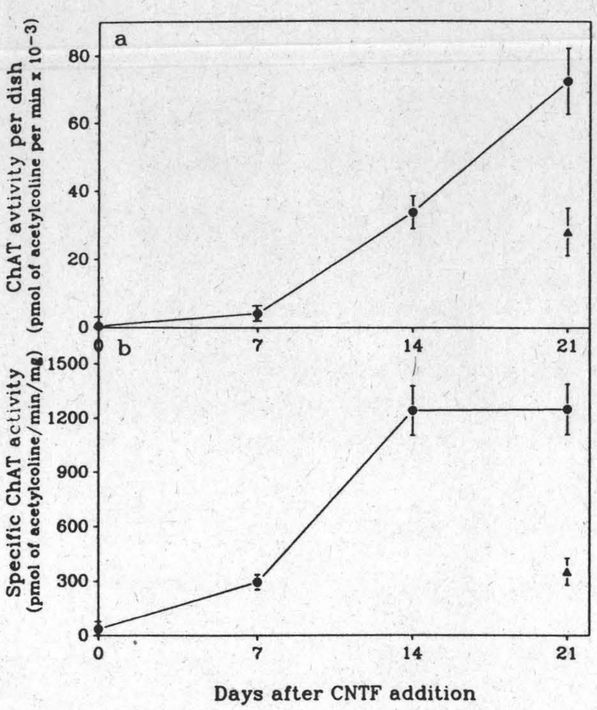


Figure 5. Long-term time course of the increase of ChAT by CNTF. The experimental conditions were the same as described in the legend of Fig. 4 except that the cells were cultured for up to 21 d.

CNTF Effects in Serum-free Medium. Rat serum has been demonstrated to increase the levels of ChAT and the ability to synthesize acetylcholine in cultured rat sympathetic neurons as compared to cells cultivated in serum-free medium (Iacovitti et al., 1982; Wolinsky and Patterson, 1985*a*). In the absence of serum, acetylcholine production is very low. It can, however, be induced by the addition of the cholinergic inducing factor from heart cell–conditioned medium (Wolinsky and Patterson, 1985*b*). To exclude the possibility that the effect of CNTF on ChAT levels is due to a potentiation of the serum effect rather than due to a direct effect, ChAT levels of rat sympathetic neurons were also analyzed in serum-free medium (Table II). In the absence of serum the total ChAT activity per dish was 2.5 $\pm$ 0.83 pmol of acetylcholine per min (three independent experiments with SEM) and thus, lower by a factor of 20 as compared to cultures kept in serum-containing medium. CNTF addition resulted in a sevenfold increase in the specific ChAT activity. The effect of CNTF on the decrease of TH was more extensive than in serum-containing medium and resulted in a threefold reduction of TH levels (Table II).

Effect of CNTF on the Survival of Rat Sympathetic Neurons

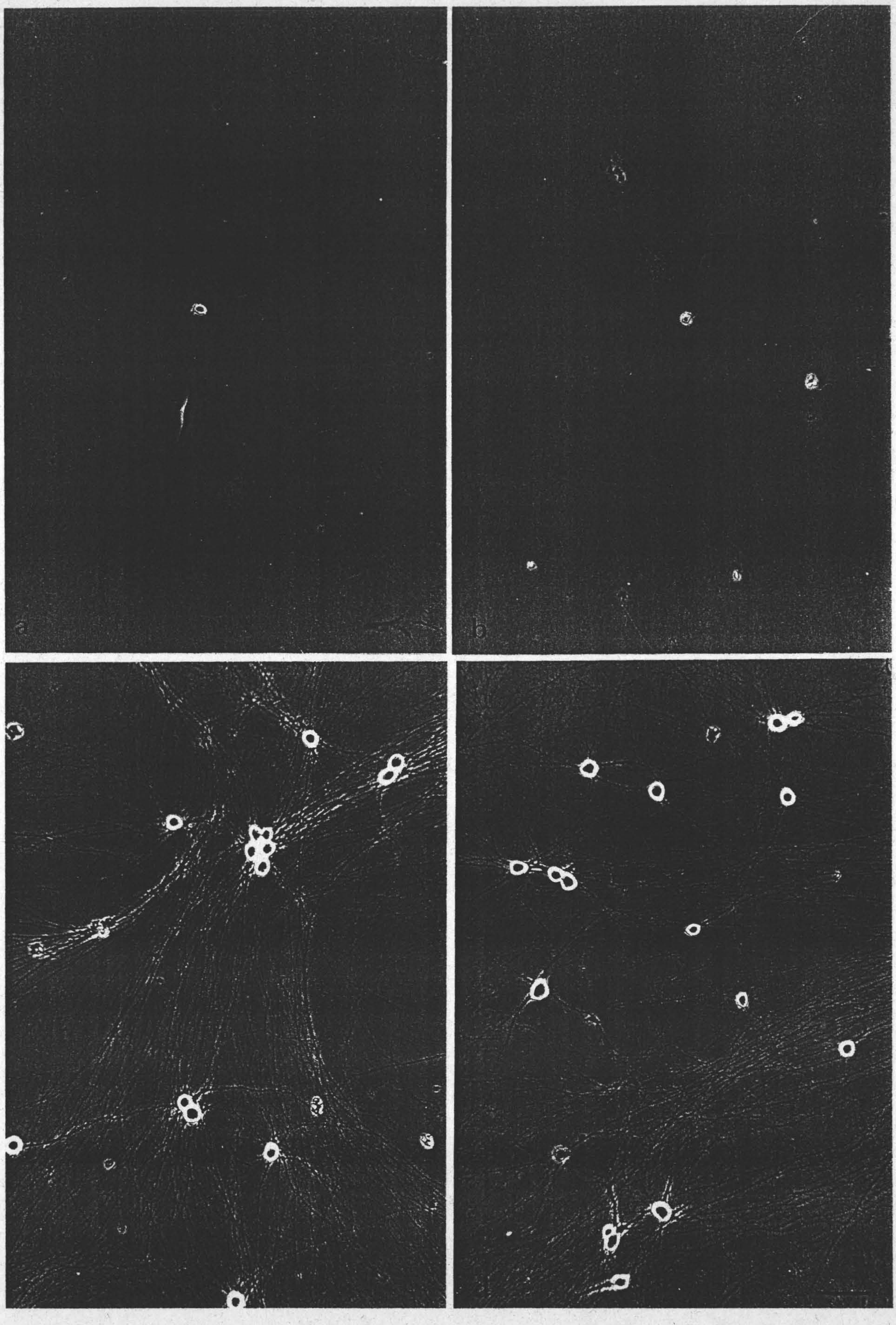
The effect of CNTF on ChAT and TH could either be due to selective survival of a cholinergic (or presumptive cholinergic) subpopulation of sympathetic neurons or due to a general induction of ChAT and depression of TH in all sympathetic neurons. To decide between these two possibilities, the survival effect of CNTF on sympathetic neurons from newborn rats was analyzed and compared to the effect of NGF. The neurons were plated either in the absence of any factor, with NGF alone, CNTF alone, or with both factors, and the number of surviving cells was determined (Table III). 18 h after plating, similar survival was observed under all four culture conditions, indicating that these cells can survive for that period of time in the absence of added survival factors. Similar observation have been made previously (Hefti et al., 1982). However after 2 d in the absence of any survival factor almost all cells were dead (Table III). In the presence of CNTF alone, 41% of the cells were still alive after 2 d in culture, and after 7 d ~9% of neurons were observed which, however, showed signs of degeneration (Table III and Fig. 6 *b*). These data are in agreement with previous observations that CNTF has an effect on the survival of rat sympathetic neurons in 24-h cultures (Barbin et al., 1984). In contrast, in the presence of NGF alone 80% of the cells survived after 2 d and 72% after 7 d of culture (Table III and Fig. 6 *c*). In the presence of both NGF and CNTF, 63% of the cells survived 7 d after plating (Table III and Fig. 6 *d*). The simultaneous addition of NGF and CNTF did not increase the number of neurons as compared to cultures kept with NGF alone. In four independent experiments the number of neurons was slightly decreased by 9.2 $\pm$ 3.4% (SEM). Also in serum-free medium CNTF had only a small survival effect after 7 d in culture. In the presence of both CNTF and NGF again no additional survival was observed in serum-free medium as compared to NGF cultures (data not shown). These data exclude a selective survival effect of CNTF on a subpopulation of sympathetic neurons as the cause for the effects of CNTF on ChAT levels in culture.

CNTF Increases the Number of ChAT Enzyme Molecules

To investigate whether the effect of CNTF on ChAT activity levels was due to an increased number of enzyme molecules or due to an activation of the enzyme, immunotitration experiments were carried out. Increasing amounts of the monoclonal anti-ChAT antibody 1E6 (Crawford et al., 1982) were added to neuron homogenates and the enzyme–antibody complexes were precipitated by a protein A–cell suspension. Immunoprecipitation was performed with homogenates obtained from neurons that were cultured for 7 d in 0 and 1 ng of CNTF/ml. The homogenates of the CNTF-treated cultures which contained ~17-fold more ChAT activity were diluted 1:20. The ChAT activity that was still present in the supernatant was determined and plotted against the amount of antibody (Fig. 7). The slopes of the curves were very similar indicating that the increased ChAT activity was due to an increased number of enzyme molecules.

Discussion

This study shows that CNTF increases the levels of ChAT activity in cultured rat sympathetic neurons and that this increase is parallelled by a reduction in the levels of TH activity. Evidence is presented that the CNTF effect on ChAT activity is due to an increase in the number of enzyme molecules. Since CNTF did not increase the number of surviving neurons as compared to NGF alone, a selective effect of CNTF on the survival of precholinergic neurons was excluded. It is concluded that the selective effect of CNTF on



a
b

Table III. NGF- and CNTF-dependent Survival of Rat Sympathetic Neurons Cultured in Serum-containing Medium

Factor added*	Time after plating 2 d	7 d
	%	%
NGF	80 ± 11	72 ± 10
CNTF	41 ± 18	9 ± 9‡
NGF and CNTF	79 ± 5	63 ± 8
None	2.3 ± 1.2	0

* The neurons were plated on laminin-coated dishes in the presence or absence of NGF (50 ng/ml) and CNTF (1 ng/ml) and the surviving neurons were counted 18 h and 2 and 7 d after plating. Cell numbers obtained after 18 h were set as 100% and were similar for all four culture conditions. Numbers represent the average cell counts with SEM of three independent experiments.
‡ Neurons kept in the presence of CNTF alone had a small cell diameter and often displayed signs of degeneration, i.e., beaded structures of neurites. This value is the mean of three experiments where in two experiments virtually no neuron was detectable.

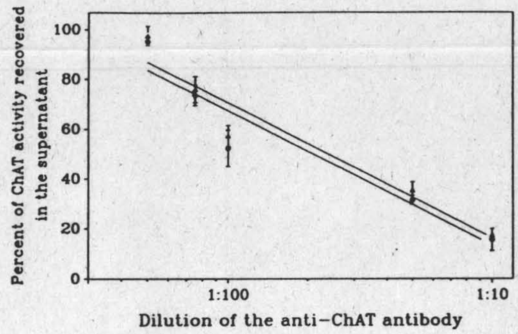


Figure 7. Immunotitration of ChAT activity. The neurons were cultured in the presence (1 ng/ml) (●) or absence (▲) of CNTF and harvested 7 d after plating. Homogenates of CNTF-treated cultures were diluted 1:20. The ChAT activity in the cell homogenates was immunoprecipitated with the mouse monoclonal anti–ChAT antibody 1E6 and the ChAT activity remaining in the supernatant was determined as described in Materials and Methods. The amount of ChAT activity present before immunoprecipitation was set as 100%. Data points represent the average of three experiments with SEM.

ChAT and TH is due to effects on the expression of these properties rather than to selective survival. The observed effects of CNTF classify this factor as a molecule involved in the transition of sympathetic neurons from adrenergic to cholinergic phenotype.

The sympathetic neurons kept on laminin in the presence of NGF were found to respond to CNTF in a dose-dependent manner with a simultaneous increase in ChAT and a reduction in TH activity. The half-maximal effect of CNTF was in the range of 0.6 ng/ml and maximal effects were obtained at 1 ng/ml. Survival effects on chick ciliary neurons reached half-maximal values at 0.1 ng/ml and maximal effects at ~0.4 ng/ml (Ernsberger et al., 1989). Thus, ~2–5 times more CNTF was needed for ChAT induction in cultured rat sympathetic neurons than for survival of chick ciliary neurons. This could be due to inactivation and/or degradation of CNTF in the rat cultures compared to chick neuronal cultures. CNTF has been observed indeed to lose biological activity under a variety of conditions, for instance upon freezing and thawing (Hughes et al., 1988). On the other hand the difference could also be due to different receptor properties or due to different receptor occupancies required for these two effects. In the case of NGF it has been shown that survival and noradrenergic differentiation; i.e., catecholamine production and induction of TH in rat sympathetic neurons are affected by different concentrations of NGF (Chun and Patterson, 1977*a*; Hefti et al., 1982; Raynaud et al., 1988; and the present study).

The effect of CNTF on ChAT activity levels is a slow process that leads to a small increase during the first 4 d after CNTF addition and to a large effect 14–21 d after CNTF addition. Similar results have been obtained when the effect of rat heart or skeletal muscle–conditioned medium on the development of cholinergic properties has been analyzed (Patterson and Chun, 1977; Swerts et al., 1983; Raynaud et al., 1987*a*,*b*).

In the absence of CNTF, ChAT levels did also increase with time in culture. This development of ChAT activity has been attributed to the presence of cholinergic factor(s) in rat serum (Wolinsky and Patterson, 1985*b*). In accordance with this interpretation ChAT levels were dramatically reduced in cells cultured under serum-free conditions. CNTF induced ChAT activity also under these conditions which excludes the possibility that CNTF may act indirectly by interacting with the factor(s) present in serum.

The levels of TH were decreased in the presence of CNTF. CNTF suppressed the increase of TH activity in culture and in some experiments TH levels were reduced by CNTF below the levels observed at the beginning of the culture period. In the absence of serum, TH activity was reduced by CNTF addition to even lower levels. This finding is in agreement with the results on the effects of cholinergic factor from heart muscle–conditioned medium in the absence of serum (Wolinsky and Patterson, 1985*a*). The extent of development of adrenergic properties in the presence of the cholinergic differentiation factor(s) present in serum and/or embryo extract (Johnson et al., 1980; Iacovitti et al., 1981), rat heart muscle–conditioned medium (Patterson and Chun, 1977; Wolinsky and Patterson, 1983), and rat skeletal muscle–conditioned medium (Swerts et al., 1983) has been found to be very variable and it has been suggested to depend on the strength of the cholinergic stimulus (Wolinsky and Patterson, 1983; Raynaud et al., 1987*b*). It should be noted that the levels of both TH and ChAT in sympathetic neurons are influenced by several culture variables; i.e., cell contact (Acheson and Thoenen, 1983; Adler and Black, 1985; Kess-

Figure 6. Morphology of rat sympathetic neurons cultured in the presence of NGF and/or CNTF. The neurons were plated either without any factors (*a*), with CNTF (1 ng/ml) alone (*b*), with NGF (50 ng/ml) alone (*c*), or with both NGF and CNTF (*d*). Photomicrographs were taken 7 d after plating. Bar, 100 μm.

ler et al., 1986; Saadat and Thoenen, 1986; Acheson and Rutishauser, 1988), substrate (Acheson et al., 1986), and serum (Wolinsky and Patterson, 1983). Differences in the time course and the extent of both the increase in ChAT and the decrease in TH by CNTF as compared to the cholinergic factors may be due to small differences in culture conditions.

The effects of CNTF on ChAT levels could be explained by a selective effect of CNTF on the survival of a subpopulation of sympathetic neurons with the ability to acquire cholinergic properties. This possibility had to be considered since CNTF has been discovered as a survival factor for the cholinergic neurons of the chick ciliary ganglion (Varon et al., 1979) and since a short-term survival effect of CNTF on rat sympathetic neurons has been described (Barbin et al., 1984; Manthorpe and Varon, 1985). It was now demonstrated that the survival effect of CNTF on rat sympathetic neurons is only transient. In contrast to NGF, CNTF was not able to support the long-term survival of rat sympathetic neurons. When CNTF was present in addition to NGF, the number of surviving neurons was not increased. These findings exclude a selective survival effect of CNTF in the presence of NGF as the cause for the ChAT increase. Also, it was found that the addition of CNTF after several days in culture results in the induction of ChAT. This finding and also the observed decrease in TH activity cannot be explained by the survival of an additional cell population. There have been several reports on the effect of CNTF on ChAT activity in cultured neurons (Varon et al., 1979; Unsicker et al., 1987; Hoffman, 1988). In these cases it has however not been clear whether the effect of CNTF is due to an increase of ChAT or due to a selective survival effect or both. In the present culture system the neurons are cultured in the presence of saturating doses of NGF and thus, the increase in ChAT reflects increased enzyme activity per cell. Immunotitration experiments demonstrated that the increase in ChAT activity is due to an increased number of enzyme molecules. It is thus concluded that CNTF causes the induction of ChAT.

The present data, together with the previously observed effect of CNTF on the expression of vasoactive intestinal peptide in chick sympathetic neurons (Ernsberger et al., 1989) indicate that CNTF is a cholinergic differentiation factor. What is the relationship between CNTF and the cholinergic differentiation factor present in rat heart and rat skeletal muscle–conditioned medium? With respect to the in vitro effects, there were no significant differences observed between CNTF and the cholinergic factor. Both factors lead to an increase in ChAT and a decrease in TH. Both factors have no long-term survival activity for rat sympathetic neurons. Cholinergic factor is present in rat heart cell–conditioned medium (Patterson and Chun, 1977; Weber, 1981; Fukada, 1985), and CNTF or CNTF-like factors, i.e., factors with survival properties for chick ciliary neurons, are present in chick heart cell–conditioned media (Helfand et al., 1976).

There is, however, a major difference in the molecular structure. The cholinergic factor is a glycoprotein with an apparent molecular mass of 45 kD as judged by polyacrylamide gel electrophoresis. CNTF has a molecular mass of 22 kD and we have so far no evidence for glycosylation of CNTF. CNTF has an isoelectric point of 4.8–5.0 (Barbin et al., 1984) whereas the cholinergic factor from muscle-conditioned medium is considered to be a basic protein (Fukada, 1985; Weber, 1981; Weber et al., 1985). The cholinergic differentiation factor loses its activity when subjected to mercaptoethanol in the presence of SDS (Fukada, 1985) whereas CNTF does not. Thus, the most likely possibility is that CNTF and the cholinergic factor from muscle-conditioned medium are different proteins with similar functions. There is, however, the finding that the cholinergic factor can be deglycosylated resulting in a protein with an apparent molecular mass of 21 kD which retains its biological activity. A molecular mass of 21 kD was determined for the cholinergic differentiation factor from skeletal muscle–conditioned medium using molecular sieving and sucrose gradient centrifugation (Weber et al., 1985). Thus the apparent molecular mass of 45 kD on SDS-PAGE may be an overestimate due to the large number of carbohydrate chains. Although CNTF may be the unglycosylated form of the cholinergic factor as it has a similar molecular mass, this possibility is unlikely since CNTF carries a net negative charge; glycosylation would presumably result in more negative charges whereas glycosylated cholinergic factor is a basic protein. Amino acid sequence analysis will eventually answer the question if there is any structural similarity between these two molecules which have similar effects on the cholinergic differentiation of rat sympathetic neurons and will also clarify the relation of CNTF to other factors with cholinergic function (Nishi and Berg, 1981; Henderson et al., 1984; McManaman et al., 1988).

Although the physiological function of CNTF is not clear at present, the observed in vitro effects of CNTF suggest a possible involvement of CNTF in the transition of sympathetic neurons from noradrenergic to cholinergic phenotype. It should be noted however that CNTF-like activity has been demonstrated in tissues like heart, iris (Adler et al., 1979; Hill et al., 1981; Watters and Hendry, 1988), which are innervated by noradrenergic rather than by cholinergic sympathetic neurons. In addition the high concentrations of CNTF in sciatic nerve (Manthorpe et al., 1986) seem not to affect the differentiation of noradrenergic neurons projecting in the nerve. A specific biological effect of CNTF would thus require a strict local control of availability of CNTF or a control of the ability of the neurons to respond to CNTF.

We thank H. Thoenen for continuous support and critical evaluation of this work. We thank Dr. Maria Kehl and Kerstin Andersson for performing the two-dimensional gels; Christine Müller, Cornelia Krieger, and Eva Braun for technical assistance; and Elfi Grossmann for secretarial assistance.

This work was supported in part by a grant from the Deutsche Forschungsgemeinschaft (SFB 220).

Received for publication 24 August 1988 and in revised form 27 December 1988.

References

Acheson, A. L., and H. Thoenen, 1983. Cell contact-mediated regulation of tyrosine hydroxylase synthesis in cultured bovine adrenal chromaffin cells. *J. Cell Biol.* 97:925–928.

Acheson, A. L., and U. Rutishauser. 1988. Neural cell adhesion molecule regulates cell contact-mediated changes in choline acetyltransferase activity of embryonic chick sympathetic neurons. *J. Cell Biol.* 106:479–486.

Acheson, A. L., K. Naujoks, and H. Thoenen. 1984. Nerve growth factor-mediated enzyme induction in primary cultures of bovine adrenal chromaffin cells: specificity and level of regulation. *J. Neurosci.* 7:1771–1780.

Acheson, A. L., D. Edgar, R. Timpl, and H. Thoenen. 1986. Laminin increases both levels and activity of tyrosine hydroxylase in calf adrenal chromaffin cells. *J. Cell Biol.* 102:151–159.

Adler, R., K. Landa, M. Manthorpe, and S. Varon. 1979. Cholinergic neuronotrophic factors. II. Intraocular distribution of trophic activity for ciliary neu-

rons. *Science (Wash. DC)*. 204:1434–1436.
Adler, J. E., and I. B. Black. 1985. Sympathetic neuron density differentially regulates transmitter phenotypic expression in culture. *Proc. Natl. Acad. Sci. USA*. 82:4296–4300.
Barbin, G., M. Manthorpe, and S. Varon. 1984. Purification of the chick eye ciliary neuronotrophic factor. *J. Neurochem.* 43:1468–1478.
Baron-Van Evercooren, A., H. K. Kleinmann, S. Ohno, P. Marangos, J. P. Schwartz, and M. E. Dubois-Dalcq. 1982. Nerve growth factor, laminin and fibronectin promote neurite outgrowth in human fetal sensory ganglia cultures. *J. Neurosci. Res.* 8:179–193.
Bocchini, V., and P. N. Angeletti. 1969. The nerve growth factor: purification as a 30,000-molecular weight protein. *Proc. Natl. Acad. Sci. USA*. 64:787–794.
Bottenstein, J., and G. H. Sato. 1979. Growth of a rat neuroblastoma cell-line in serum-free supplemented medium. *Proc. Natl. Acad. Sci. USA*. 76:514–517.
Bradford, M. M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* 72:248–254.
Chun, L. L. Y., and P. H. Patterson. 1977*a*. Role of nerve growth factor in the development of rat sympathetic neurons in vitro. I. Survival, growth, and differentiation of catecholamine production. *J. Cell Biol.* 75:694–704.
Chun, L. L. Y., and P. H. Patterson. 1977*b*. Role of nerve growth factor in the development of rat sympathetic neurons in vitro. II. Developmental studies. *J. Cell Biol.* 75:705–711.
Cochard, P., M. Goldstein, and I. B. Black. 1979. Initial development of the noradrenergic phenotype in autonomic neuroblasts of the rat embryo in vivo. *Dev. Biol.* 71:100–114.
Crawford, G. D., L. Correa, and P. M. Salvaterra. 1982. Interaction of monoclonal antibodies with mammalian choline acetyltransferase. *Proc. Natl. Acad. Sci. USA*. 79:7031–7035.
Ernsberger, U., M. Sendtner, and H. Rohrer. 1989. Proliferation and differentiation of embryonic chick sympathetic neurons: effects of ciliary neurotrophic factor. *Neuron*. In press.
Fonnum, F. 1969. Radiochemical micro-assays for the determination of choline acetyltransferase and acetylcholinesterase activities. *Biochem. J.* 115:465–472.
Fukada, K. 1985. Purification and partial characterization of a cholinergic neuronal differentiation factor. *Proc. Natl. Acad. Sci. USA*. 82:8795–8799.
Furshpan, E. J., P. R. MacLeish, P. H. O'Lague, and D. D. Potter. 1976. Chemical transmission between rat sympathetic neurons and cardiac myotubes developing in microcultures. Evidence for cholinergic, adrenergic and dual function neurons. *Proc. Natl. Acad. Sci. USA*. 73:4225–4229.
Hayashi, H., and T. Nakagawa. 1963. Functional activity of the sweat glands of the albino rat. *J. Invest. Dermatol.* 41:365–367.
Hefti, F., H. Gnahn, M. E. Schwab, and H. Thoenen. 1982. Induction of tyrosine hydroxylase by nerve growth factor and by elevated K^+ concentrations in cultures of dissociated sympathetic neurons. *J. Neurosci.* 2:1554–1566.
Helfand, S. L., G. A. Smith, and N. K. Wessells. 1976. Survival and development in culture of dissociated parasympathetic neurons from ciliary ganglia. *Dev. Biol.* 50:541–547.
Henderson, C. E., M. Huchet, and J. P. Changeux. 1984. Neurite-promoting activities for embryonic spinal neurons and their developmental changes in the chick. *Dev. Biol.* 104:336–347.
Hill, C. E., F. A. Hendry, and R. E. Bonyhady. 1981. Avian parasympathetic neurotrophic factors: age related increases and lack of regional specificity. *Dev. Biol.* 85:258–261.
Hoffmann, H.-D. 1988. Ciliary neuronotrophic factor stimulates choline acetyltransferase activity in cultured chicken retina neurons. *J. Neurochem.* 51:109–113.
Hughes, S. M., L. Lillien, M. C. Raff, H. Rohrer, and M. Sendtner. 1988. Ciliary neurotrophic factor (CNTF) as an inducer of type-2 astrocyte differentiation in rat optic nerve. *Nature (Lond.)*. 355:70–73.
Iacovitti, L., T. H. Joh, D. H. Park, and R. P. Bunge. 1981. Dual expression of neurotransmitter synthesis in cultured autonomic neurons. *J. Neurosci.* 1:685–690.
Iacovitti, L., M. I. Johnson, T. H. Joh, and R. P. Bunge. 1982. Biochemical and morphological characterization of sympathetic neurons grown in a chemically-defined medium. *Neuroscience*. 7:2225–2239.
Johnson, M. I., D. Ross, M. Meyers, R. Rees, R. Bunge, E. Wahshull, and H. Burton. 1976. Synaptic vesicle cytochemistry changes when cultured sympathetic neurones develop cholinergic interactions. *Nature (Lond.)*. 262:308–310.
Johnson, M., C. D. Ross, M. Meyers, E. L. Spitznagel, and R. P. Bunge. 1980. Morphological and biochemical studies on the development of cholinergic properties in cultured sympathetic neurons. I. Correlative changes in choline acetyltransferase and synaptic cytochemistry. *J. Cell Biol.* 84:680–691.
Kessler, J. A., G. Conn, and V. B. Hatcher. 1986. Isolated plasma membranes regulate neurotransmitter expression and facilitate effects of a soluble brain cholinergic factor. *Proc. Natl. Acad. Sci. USA*. 83:3528–3532.
Landis, S. C., and D. Keefe. 1983. Evidence for neurotransmitter plasticity in vivo: developmental changes in the properties of cholinergic sympathetic neurons. *Dev. Biol.* 98:349–372.
Landis, S. C., and J. R. Fredieu. 1986. Coexistence of calcitonin gene-related peptide and vasoactive intestinal peptide in cholinergic sympathetic innervation of rat sweat glands. *Brain Res.* 377:177–181.
Landis, S. C., R. E. Siegel, and M. E. Schwab. 1988. Evidence for neurotransmitter plasticity in vivo. II. Immunocytochemical studies of rat sweat gland innervation during development. *Dev. Biol.* 126:129–140.
Langley, J. N. 1922. The secretion of sweat. Part I. Supposed inhibitory nerve fibres on the posterior nerve roots. Secretion after denervation. *J. Physiol.* 56:110–129.
McManaman, J. L., F. G. Crawford, S. S. Stewart, and S. H. Appel. 1988. Purification of a skeletal muscle polypeptide which stimulates choline acetyltransferase activity in cultured spinal cord neurons. *J. Biol. Chem.* 263:5890–5897.
Mains, R. E., and P. H. Patterson. 1973. Primary cultures of dissociated sympathetic neurons. I. Establishment of long-term growth in culture and studies of differentiated properties. *J. Cell Biol.* 59:329–345.
Manthorpe, M., and S. Varon. 1985. Regulation of neuronal survival and neuritic growth in the avian ciliary ganglion by trophic factors. *In* Growth and Maturation Factors. Vol. 3. G. Guroff, editor. John Wiley & Sons, New York. 77–117.
Manthorpe, M., S. Skaper, L. R. Williams, and S. Varon. 1986. Purification of adult rat sciatic nerve ciliary neuronotrophic factor. *Brain Res.* 367:282–286.
Nishi, R., and D. K. Berg. 1981. Two components from eye tissue that differentially stimulate the growth and development of ciliary ganglion neurons in cell culture. *J. Neurosci.* 1:505–513.
O'Farrell, P. H. 1975. High resolution two-dimensional electrophoresis of proteins. *J. Biol. Chem.* 250:4007–4021.
Patterson, P. H., and L. L. Y. Chun. 1974. The influence of nonneuronal cells on catecholamine and acetylcholine synthesis and accumulation in cultures of dissociated sympathetic neurons. *Proc. Natl. Acad. Sci. USA*. 71:3607–3610.
Patterson, P. H., and L. L. Y. Chun. 1977. The induction of acetylcholine synthesis in primary cultures of dissociated rat sympathetic neurons. I. Effects of conditioned medium. *Dev. Biol.* 56:263–280.
Raynaud, B., N. Faucon-Biguet, S. Vidal, J. Mallet, and M. J. Weber. 1987*a*. The use of a tyrosine-hydroxylase cDNA probe to study the neurotransmitter plasticity of rat sympathetic neurons in culture. *Dev. Biol.* 119:305–312.
Raynaud, B., D. Clarons, S. Vidal, C. Ferrand, and M. J. Weber. 1987*b*. Comparison of the effects of elevated K^+ ions and muscle-conditioned medium on the neurotransmitter phenotype of cultured sympathetic neurons. *Dev. Biol.* 121:548–558.
Raynaud, B., N. Faucon-Biguet, S. Vidal, J. Mallet, and M. J. Weber. 1988. Regulation of neurotransmitter metabolic enzymes and tyrosine hydroxylase mRNA level by nerve growth factor in cultured sympathetic neurons. *Development (Camb.)*. 102:361–368.
Rogers, S. L., P. C. Letourneau, S. L. Palm, J. McCarthy, and L. T. Furcht. 1983. Neurite extension by peripheral and central nervous system neurons in response to substratum-band fibronectin and laminin. *Dev. Biol.* 98:212–220.
Ross, D., M. I. Johnson, and R. P. Bunge. 1977. Development of cholinergic characteristics in adrenergic neurons is age dependent. *Nature (Lond.)*. 267:536–539.
Saadat, S., and H. Thoenen. 1986. Selective induction of tyrosine hydroxylase by cell-cell contact in bovine adrenal chromaffin cells is mimicked by plasma membranes. *J. Cell Biol.* 103:1991–1997.
Sato, F., and K. Sato. 1978. Secretion of a potassium-rich fluid by the secretory coil of the rat paw eccrine sweat gland. *J. Physiol.* 274:37–50.
Schwab, M. E., and H. Thoenen. 1985. Dissociated neurons regenerate into sciatic but not optic nerve explants in culture irrespective of neurotrophic factors. *J. Neurosci.* 5:2415–2423.
Suda, K., Y.-A. Barde, and H. Thoenen. 1978. Nerve growth factor in mouse and rat serum: correlation between bioassay and radioimmunoassay determinations. *Proc. Natl. Acad. Sci. USA*. 75:4042–4046.
Swerts, J.-P. A. Le Van Thai, A. Vigny, and M. J. Weber. 1983. Regulation of enzymes responsible for neurotransmitter synthesis and degradation in cultured rat sympathetic neurons. I. Effects of muscle-conditioned medium. *Dev. Biol.* 100:1–11.
Teitelman, G., H. Baker, T. H. Joh, and D. J. Reis. 1979. Appearance of catecholamine-synthesizing enzymes during development of the rat sympathetic nervous system: possible role of tissue environment. *Proc. Natl. Acad. Sci. USA*. 76:509–513.
Unsicker, K., H. Reichert-Preibsch, R. Schmidt, B. Pettmann, G. Labourdette, and M. Sensenbrenner. 1987. Astroglial and fibroblast growth factors have neurotrophic functions for cultured peripheral and central system neurons. *Proc. Natl. Acad. Sci. USA*. 84:5459–5463.
Varon, S., M. Manthorpe, and R. Adler. 1979. Cholinergic neurotrophic factors. I. Survival, neurite outgrowth and choline acetyltransferase activity in monolayer cultures from chick embryo ciliary ganglia. *Brain Res.* 173:29–45.
Watters, D. J., and I. A. Hendry. 1987. Purification of a ciliary neurotrophic factor from bovine heart. *J. Neurochem.* 49:705–713.
Weber, M. J. 1981. A diffusible factor responsible for the determination of cholinergic functions in cultured sympathetic neurons. *J. Biol. Chem.* 256:3447–3453.
Weber, M. J., B. Raynaud, and C. Delteil. 1985. Molecular properties of a cholinergic differentiation factor from muscle-conditioned medium. *J. Neu-*

rochem. 45:1541–1547.
Wolinsky, E., and P. H. Patterson. 1983. Tyrosine hydroxylase activity decreases with induction of cholinergic properties in cultured sympathetic neurons. *J. Neurosci.* 3:1495–1500.
Wolinsky, E. J., and P. H. Patterson. 1985*a*. Expression of noradrenergic and cholinergic traits by sympathetic neurons cultured without serum. *J. Neurosci.* 5:1497–1508.
Wolinsky, E. J., and P. H. Patterson. 1985*b*. Rat serum contains a developmentally regulated cholinergic inducing activity. *J. Neurosci.* 5:1509–1512.
Yodlowski, M., J. R. Fredieu, and S. C. Landis. 1984. Neonatal 6-hydroxydopamine treatment eleminates cholinergic sympathetic innervation and induces sensory sprouting in rat sweat glands. *J. Neurosci.* 4:1535–1548.