ROLE OF NERVE GROWTH FACTOR IN THE DEVELOPMENT OF RAT SYMPATHETIC NEURONS IN VITRO

II. Developmental Studies

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ABSTRACT

Adrenergic sympathetic neurons were grown for 4 wk in submaximal and saturating concentrations of nerve growth factor (NGF) in the virtual absence of non-neuronal cells. In 0.2 or 5 μg/ml 7S NGF, the neurons gradually decreased in number during the first week, although fewer neurons died at the higher level. No significant change in cell number was observed thereafter. Total neuronal protein, a measure of cell growth, increased linearly with age in both concentrations of NGF. At each age, neurons in high NGF exhibited greater growth per cell than those in low NGF. The ability of neurons to produce catecholamine (CA) increased dramatically during the second and third weeks in both concentrations of NGF, and along a similar time-course, although neurons in submaximal NGF developed a lesser capacity for CA production. As neurons developed in culture, they became less dependent on NGF for survival and CA production, but even in older cultures, ~50% of the neurons died when NGF was withdrawn.

KEY WORDS nerve growth factor · sympathetic neurons · cell culture · catecholamine production · neuronal development

In the preceding paper we presented evidence that nerve growth factor (NGF) promoted, in a dose-dependent fashion, cell survival, growth, and differentiation of catecholamine (CA) production in sympathetic neurons grown in the virtual absence of other cell types (6); these effects were studied in cultures 3–4 wk of age. It was important to determine whether the lesser growth and CA production in low NGF concentrations represent a mature state or whether the neurons simply mature along a slower time-course and eventually reach the same state as neurons grown in saturating levels of NGF. Therefore, the time-course of neuronal development in submaximal and saturating concentrations of 7S NGF was studied; sister cultures were assayed for changes in neuronal cell number, total protein, and ability to synthesize CA from radioactive tyrosine as a function of age.

Angeletti et al. (1) observed that adult sympathetic neurons are more resistant to the effects of injected antiserum to NGF than neurons in neonates. This result is consistent with increased NGF reservoirs in the adult animal, with difficulty of access of the antiserum to NGF stores or to the neurons, or with a decreased dependence of the neurons on NGF. We have reexamined this matter in cultures of neurons virtually free of other cell types, where the NGF concentration can be readily manipulated. As has been reported by Lazarus et al. (11) and in preliminary form by us (5), the neurons become less sensitive to NGF withdrawal as they grow older in vitro.

MATERIALS AND METHODS

Many of the methods used in this study were described in the preceding paper (6). Briefly, sympathetic neurons were dissociated from neonatal rat superior cervical ganglia (SCG), plated on collagen-coated dishes into L15-air growth medium containing appropriate concentrations of 7S NGF. At various ages, sister cultures from a plating were assayed for number of neuronal somas, total protein, and [^{3}H]CA production from [2,3-^{3}H]tyrosine.

In the NGF withdrawal experiments, neurons were plated into L15-CO_2 growth medium and treated with 10^{-5} M cytosine arabinoside from days 2-4, 6-8, and 15-17 to kill dividing ganglionic non-neuronal cells, an alternative method to using L15-air medium (15). At various ages, sister cultures were rinsed five times with growth medium lacking Methocel (Dow Corning Corp., Midland, Mich.) and NGF. Then they were provided with L15-CO_2 growth medium containing 5% heat-inactivated adult rat serum and one of the following: (*a*) 5 μg/ml 7S NGF (control), (*b*) no NGF, (*c*) 25 μg/ml anti-NGF rabbit IgG (no added NGF), or (*d*) 25 μg/ml anti-NGF rabbit IgG + 5 μg/ml 7S NGF. After 10 days under these conditions, the cultures were assayed for number of neuronal somas and their ability to synthesize and accumulate [^{3}H]CA and [^{3}H]acetylcholine (ACh) during 4-h incubations with [^{3}H]tyrosine and [^{3}H]choline (15).

The anti-NGF rabbit IgG was a generous gift of Dr. Richard A. Murphy (Department of Anatomy, Harvard Medical School), who determined that 13 μg anti-NGF blocked the fiber outgrowth from chick sensory ganglia elicited by 10 ng 2.5S NGF.

RESULTS

Changes in Neuronal Survival, Protein, and CA Production with Age

As shown in Fig. 1, some cell death occurred during the first week in cultures grown in 0.2 or in 5 μg/ml 7S NGF, although fewer died at the higher level. Other experiments showed that this drop between days 1 and 7 was a gradual one. Subsequently, no significant change in cell number was observed in either concentration up to 28 days in vitro.

Changes in neuronal appearance with time are illustrated in Fig. 2. As the cultures matured, both the soma size and the extent of neurite outgrowth increased in the two NGF concentrations, but at each age soma size and process density were greater in the higher level of NGF. Since the cultures were virtually free of non-neuronal cells (15), neuronal growth could be quantitated by assaying total protein. The results are

FIGURE 1 Cultures were grown in 0.2 (○) or 5 (●) μg/ml 7S NGF, and at each time-point neuronal somas were counted. ⊙ represents the number of cells present in both concentrations of NGF 1 day after plating. In this and all subsequent figures the results are expressed as the mean of at least three sister cultures ± SEM. *N*, neuron.

given in Fig. 3. It can be seen that protein per neuron rose linearly with age in both concentrations of NGF, and at each age, neurons in 5 μg/ml exhibited larger protein values than those in 0.2 μg/ml 7S NGF.

The ability of neurons to produce CA was measured by determining the total amount of radioactive CA and deaminated metabolites synthesized from [^{3}H]tyrosine and accumulated in the cells or released into the medium in an 8-h incubation period (total CA; 6). This synthetic rate is a measure of tyrosine hydroxylase activity in the living cell (6). As shown in Fig. 4, the ability of neurons to synthesize CA increased markedly during the second and third weeks in vitro in both concentrations of NGF. Although the neurons developed a lesser capacity for CA synthesis in submaximal NGF, they did so along a time-course similar to that seen in high NGF. This large increase in CA synthetic capacity represented specific differentiation of CA function, not simply increased neuronal growth. This is illustrated in Fig. 5; total CA per nanogram protein increased to a greater extent in high NGF, but the developmental time-course was similar in both cases.

To study the changing ability of the storage mechanisms to compete with the deaminating mechanisms for newly synthesized CA, the rate at which neurons accumulated [^{3}H]DA and [^{3}H]NE during an 8-h incubation period (accu-

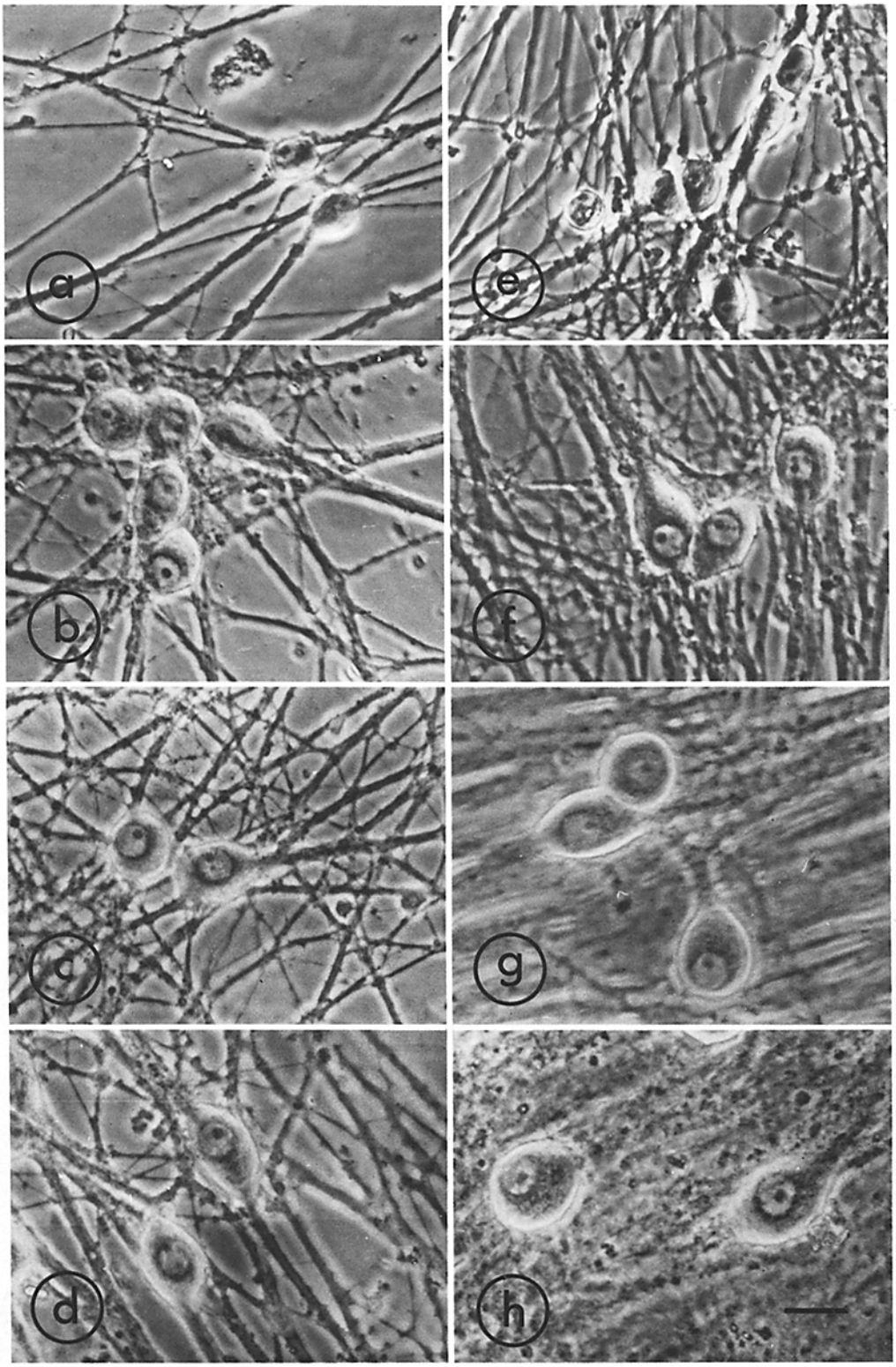

FIGURE 2 Phase-contrast micrographs of neurons grown in 0.2 (*a–d*) and 5 (*e–h*) μg/ml 7S NGF. (*a*) and (*e*) 7 days; (*b*) and (*f*) 17 days; (*c*) and (*g*) 21 days; (*d*) and (*h*) 28 days. Bar, 25 μm. × 400.

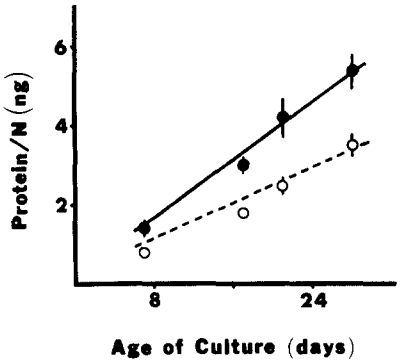

FIGURE 3 Cultures were grown in 0.2 (○) and 5 (●) μg/ml 7S NGF and at each time-point, neuronal protein was assayed (15) and expressed per neuron (*N*).

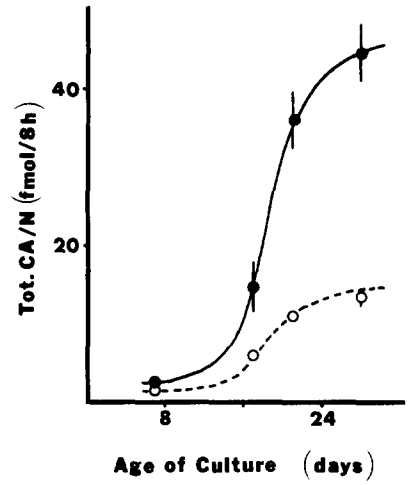

FIGURE 4 Cultures were grown in 0.2 (○) and 5 (●) μg/ml 7S NGF. At each time-point, sister cultures were incubated for 8 h with [^{3}H]tyrosine. Radioactivity in CA and deaminated metabolites was determined (6) and expressed per neuron (*N*).

mulated CA) was examined as a function of age. Fig. 6 illustrates that accumulated CA per neuron increased most rapidly during the second and third weeks in vitro, as did total CA per neuron. Again, the increase occurred with similar time-courses in both submaximal and saturating levels of NGF. Although both total CA per neuron and accumulated CA per neuron increased with similar time-courses in the two levels of NGF, the efficiency of neuronal storage of newly synthesized CA did not increase with age. This is illustrated in Fig. 7. Furthermore, as was seen previously (6), the older cultures in 0.2 μg/ml stored the newly synthesized CA more efficiently than those in 5 μg/ml 7S NGF.

Susceptibility to Change in NGF Levels

Since fewer neurons survived in submaximal levels of NGF and these developed a lesser capacity for CA synthesis, it was of interest to know whether this lesser synthetic capacity was a property of a distinct class of neurons which survived in the lower concentration of NGF, or whether the surviving neurons could synthesize more CA if subsequently exposed to higher levels of exogenous NGF. Neurons were grown in 0.1 or 5 μg/ml 7S NGF for 10 days, by which time neuronal survival appeared stable (Fig. 1). Half the cultures grown in 0.1 μg/ml were then

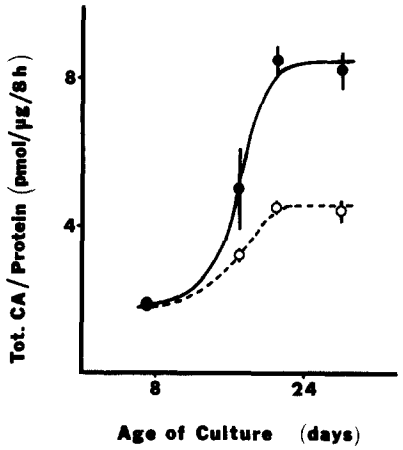

FIGURE 5 Cultures were grown in 0.2 (○) and 5 (●) μg/ml 7S NGF. At each time-point, sister cultures were incubated with [^{3}H]tyrosine. Radioactivity in CA and deaminated metabolites was determined (6) and expressed per protein.

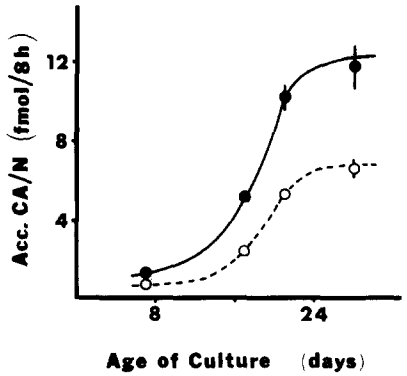

FIGURE 6 Cultures were grown in 0.2 (○) and 5 (●) μg/ml 7S NGF. At each time-point, sister cultures were incubated for 8 h with [^{3}H]tyrosine and the radioactivity accumulated in the cell as DA and NE determined (13) and expressed per neuron (*N*).

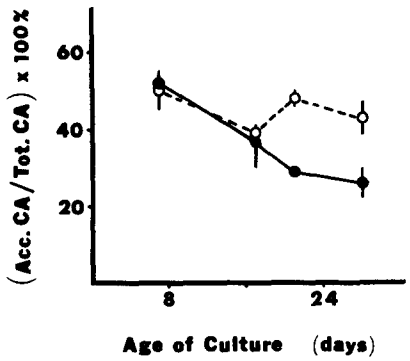

FIGURE 7 Cultures were grown in 0.2 (○) and 5 (●) μg/ml 7S NGF. The fraction of total (*Tot.*) CA derived from [^{3}H]tyrosine in an 8-h incubation which accumulated (*Acc.*) in the neurons is plotted as a function of culture age. The data were derived by dividing the value for each culture in Fig. 6 by the value for the same culture in Fig. 4. The mean was taken for each concentration.

switched to 5 μg/ml and vice versa. After growth for 19 additional days, cell number and accumulated CA were assayed. As shown in Table I, neurons which survived in 0.1 μg/ml and were subsequently switched to 5 μg/ml exhibited the same CA synthetic capacity as those which were exposed to 5 μg/ml from the start. In cultures which were changed from saturating to submaximal NGF, cell death occurred; the surviving neurons displayed the same capacity for CA production as those grown in 0.1 μg/ml from the beginning.

Changes in NGF Dependence with Age

Cultured sympathetic neurons grown in the virtual absence of non-neuronal cells initially exhibited a strong dependence on added NGF, but they became less dependent with time, as illustrated in Fig. 8. After growth for 10 days in medium containing 5 μg/ml NGF, sister cultures were rinsed thoroughly and provided with medium containing no NGF. When assayed 10 days later, no neuronal cell bodies remained (Fig. 8 A); the few remaining neurites had detached from the collagen substrate and synthesized no CA from [^{3}H]tyrosine (Fig. 8 B). On the other hand, when NGF was removed from older cultures, 35–50% of the neurons survived for a further 10 days (Fig. 8 A). Furthermore, these older cultures, whose capacity for CA production had matured (Fig. 6 and see reference 14), were able to synthesize and accumulate 40–50% of control CA 10 days after NGF withdrawal (Fig. 8 B). In anticipation of the results presented in the next paper, it may be noted that these cultures produced no significant ACh (data not shown). The use of NGF antiserum did not further increase cell death or inhibit CA production, provided that the cultures were thoroughly rinsed upon NGF removal. Thus, as the neurons matured, both survival and ability to accumulate newly synthesized CA became less dependent

TABLE I

Susceptibility to Change of NGF Level

NGF			
Day 1–10	Day 11–29	No. neurons/culture	Accumulated CA/neuron
μg/ml			*fmol/8h*
0.1	0.1	810 ± 50	4.57 ± 0.31
5	0.1	790 ± 30	5.86 ± 0.43
0.1	5	920 ± 60	9.97 ± 0.72
5	5	2,390 ± 170	10.80 ± 0.72

Neuronal cultures were grown for 10 days in the indicated levels of NGF, and on day 11 half the cultures were switched to the indicated concentration of NGF. The cultures were assayed for cell number and ability to synthesize and accumulate [^{3}H]tyrosine in an 8-h incubation (13).

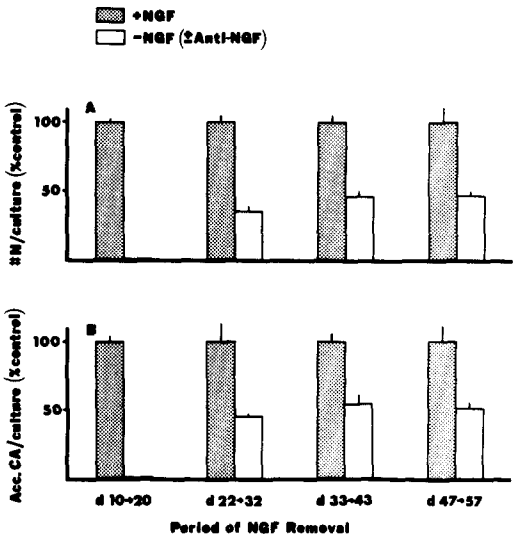

FIGURE 8 The susceptibility of neurons of various ages to NGF removal was determined by withdrawing NGF for 10-day intervals between the ages indicated throughout development. Cell number (A) and ability to synthesize and accumulate CA from [^{3}H]tyrosine during an 8-h incubation (B) were assayed at the end of each period of removal.

on exogenous NGF, but even in older cultures, ~50% of the neurons died when NGF was withdrawn.

DISCUSSION

In contrast to the neuronal hyperplasia reported in the SCG of neonatal mice injected with NGF (12), the number of rat neurons did not increase with time in cultures grown either in submaximal or saturating levels of NGF. This is consistent with the finding that rat sympathetic neurons grown in high levels of NGF do not incorporate significant [^{3}H]thymidine (13). In fact, more neurons were plated than survived even at a level of NGF which was saturating for survival. Hendry and Campbell (9) also found a decrease of neuronal number in developing rat SCG in vivo; in addition, they cited unpublished results which suggested that injected NGF had no effect on neuronal division, assayed by [^{3}H]thymidine autoradiography.

Total protein per neuron increased linearly with age for cultures in both NGF concentrations. At each age, the protein per neuron was greater in high NGF than in low. Thus, the greater process density in high NGF observed morphologically at each age was not due simply to a difference in neuronal survival. Mains and Patterson (14) also observed a linear rise in neuronal lipid and RNA with time for cultures of SCG neurons grown in 1 μg/ml 7S NGF.

The time-course of development of CA functions contrasted markedly with that for neuronal growth. The rate of CA production increased markedly during the second and third weeks in vitro for cultures in both NGF concentrations. Thus, despite a lesser CA synthetic capacity, neurons in low NGF developed along a similar time-course to those grown in high NGF. Furthermore, the changes in total CA per nanogram protein and in accumulated CA per nanogram protein indicate that a specific differentiation of CA mechanisms occurred in both levels of NGF. This time-course of development of CA synthetic ability correlates qualitatively with the increases in tyrosine hydroxylase and dopamine-β-hydroxylase activities in the rat (16) and mouse (3, 4) SCG and in their target organs during the second and third weeks in vivo. In addition, the maturation not only of synthesis but also of accumulation of CA is consistent with the increased CA histofluorescence and dense-cored vesicles in rat SCG neurons during the first 3 wk after birth (8). The efficiency of storage of newly synthesized CA did not increase with age in the present experiments; on the contrary, as the cultures matured, they became less efficient at storage of newly synthesized CA in both levels of NGF, even though both total CA per neuron and accumulated CA per neuron increased with age. Perhaps increased electrical activity, as may be provided by preganglionic innervation and/or innervation of target organs, will be required for full maturation of storage efficiency.

Neurons which survived in low NGF, and were subsequently switched to high NGF after 10 days in vitro, developed the same capacity for accumulation of synthesized CA as those exposed to high NGF from the start. These results indicate that the lesser capacity for CA production is not an irreversible property of the particular subset of neurons which survive in low NGF. It is not known whether this subsequent increase in accumulated CA per neuron is due to growth or to differentiation of CA function, or both. It is, however, not due to an increase in the number of neurons in the culture. These results are consistent with those of Bjerre et al. (2), who showed that injections of NGF into adult mice caused an increase in the fluorescence intensity and the density of innervation of certain sympathetic targets accompanied by increased norepinephrine (NE) levels in the targets.

As first reported by Lazarus et al. (11), cultured sympathetic neurons grown in the virtual absence of ganglionic non-neuronal cells, become less dependent on added NGF with age. Young neurons do not survive NGF withdrawal, whereas some older ones do for at least 10 days. This change is correlated with the maturation of CA production. Two possible causes are: (*a*) a real decrease in requirement for NGF, which might be reflected in disappearance of NGF binding as seen for chick sensory neurons (10), or (*b*) an intracellular accumulation of NGF which could maintain the neurons over the 10-day period of withdrawal. Thus, older cultures might be expected to be more resistant to NGF removal, since they have a greater volume especially in their processes in which to accumulate the NGF. If this were the case, a longer period of NGF withdrawal might result in cell death.

The finding that some neurons survive and others die upon NGF withdrawal suggests some

heterogeneity in the population (see also reference 6). However, the selective survival of cholinergic sympathetic neurons is not the cause, since under these culture conditions the neurons produce very little ACh. Furthermore, as demonstrated in the next paper of this series (7), cholinergic sympathetic neurons display the same developmental dependence on NGF as do adrenergic neurons.

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