Proc. Natl. Acad. Sci. USA
Vol. 75, No. 12, pp. 6059–6063, December 1978
Cell Biology

Evidence for RNA synthesis-dependent and -independent pathways in stimulation of neurite outgrowth by nerve growth factor

(pheochromocytoma cells/differentiation/PC12 cell line)

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Communicated by Rita Levi-Montalcini, September 11, 1978

ABSTRACT Studies on the mechanism of action of nerve growth factor (NGF) were carried out with PC12 rat pheochromocytoma cells. PC12 cells are uniquely useful for such studies because they respond to, but (unlike normal neurons) do not require, NGF and may undergo either generation or regeneration of neurites in response to NGF. Regeneration is defined here as NGF-dependent regrowth of neurites within 24 hr after subculture of NGF-treated PC12 cells. As in cultures of normal NGF-responsive neurons, neurite regeneration by PC12 cells occurs even in the presence of high concentrations of RNA synthesis inhibitors. Generation of neurites is defined as the *de novo* initiation of outgrowth when PC12 cells are exposed to NGF for the first time. In contrast to regeneration, neurite generation takes place with a lag of at least 24 hr and is blocked by low concentrations of RNA synthesis inhibitors. Such findings suggest that there are both RNA synthesis-dependent and -independent pathways in the mechanism whereby NGF stimulates neurite outgrowth. In addition, NGF-treated PC12 cells undergo a time-dependent loss of the capacity for neurite regeneration after pretreatment with RNA synthesis inhibitors or withdrawal of NGF. Such findings suggest that (*i*) initiation of neurite outgrowth requires NGF-stimulated, RNA synthesis-dependent accumulation of intracellular material(s), (*ii*) once such accumulation occurs, RNA synthesis-independent regeneration can occur (but only in the presence of NGF), and (*iii*) the turnover of such material(s) in the absence of their replacement leads to loss of the capacity for regeneration. A tentative sequence is presented for the events whereby NGF may stimulate neurite outgrowth.

Among the biological actions of nerve growth factor protein (NGF) (for review, see ref. 1) is stimulation of neurite outgrowth from responsive sympathetic and dorsal root ganglionic neurons both *in vivo* (1) and *in vitro* (1–3). Early studies on RNA and protein synthesis by ganglia treated with NGF *in vitro* led to the suggestion that the effects of the factor on neurite outgrowth are mediated via transcription of DNA into new RNA (1, 4). More recent experiments, however, indicate that explanted sympathetic (3) or dorsal root (5) ganglia undergo at least 24 hr of NGF-stimulated neurite outgrowth in the presence of actinomycin D at levels that block nearly all cellular RNA synthesis. Such findings have suggested that NGF-stimulated neurite outgrowth does not require new RNA synthesis (3). However, several aspects of such *in vitro* experiments must be considered. The ganglia used in all probability have already responded to endogenous NGF *in vivo*. This means that the *in vitro* experiments may involve continuation rather than initiation of metabolic responses to NGF (3, 5) and that such experiments may test NGF-stimulated *regeneration* rather than *generation* (or initiation) of neurite outgrowth.

In addition to cultures of normal neurons, pheochromocytoma cells represent another class of cells that respond to NGF *in vitro* (6). A clonal line (designated PC12) of rat pheochromocytoma cells has been established (7) and characterized (7–10), and this line promises to be a useful model system for study of the mechanism of action of NGF. Such cells do not require NGF for survival and replication in serum-containing medium, but after several days in the presence of NGF they cease replication and extend long, microtubule-containing neurites (7). One particularly unique advantage of the line is that, because PC12 cells do not require NGF for survival under normal culture conditions, non-NGF-treated cultures may be used as experimental controls. This contrasts with normal NGF-responsive neurons which require NGF for survival *in vitro* and which thus die in its absence (2). A second experimental advantage of PC12 cells is that, unlike normal cells, they may be used to study the initial steps whereby NGF stimulates initiation of neurite outgrowth from cells that have never had previous exposure to detectable levels of the factor. Furthermore, PC12 cultures may also be used to study NGF-stimulated regeneration of neurites. That is, if NGF-treated PC12 cells are divested of their neurites by mechanical means, such cells begin NGF-dependent regrowth of neurites within 24 hr (11).

We report here that NGF-stimulated *regeneration* of neurites can occur in the presence of RNA synthesis inhibitors but *generation* of neurites cannot. We suggest a tentative model for the mode of action of NGF based on such findings.

MATERIALS AND METHODS

Cell Culture. PC12 cells or their noncholinergic subclone PC12-A1 (10) were maintained as described (7, 9) on collagen-coated 35-mm tissue culture dishes in the presence (50 ng/ml) or absence of 2.5S mouse salivary gland NGF (12). Cells were plated at 0.2–1 × 10^6 per dish, and culture medium was changed three times weekly. Proportions of process-bearing cells were determined by strip counts as described (7, 11). At least 300 cells were scored per culture. Neurites were considered to be processes greater than 20 μm in length. The procedures used for subculturing process-bearing cells for neurite regeneration experiments are given elsewhere (11). Actinomycin D was purchased from Calbiochem; cordycepin and 1-β-arabinofuranosylcytosine (cytosine arabinoside) from Sigma. Camptothecin was obtained through Harry B. Wood (Drug Synthesis and Chemistry Branch, Division of Cancer Treatment, National Cancer Institute) and was converted to its sodium salt before use by means of a protocol prepared by the Research Triangle Institute (Research Triangle Park, NC).

Incorporation of [^{3}H]Leucine and [^{3}H]Uridine. Sister cultures were treated for 24 hr with NGF followed by an additional 19–21 hr with NGF together with the indicated level of drug. The cultures were then labeled in complete medium

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Abbreviations: NGF, nerve growth factor; hnRNA, heterogeneous nuclear RNA.

Table 1. Effect of RNA synthesis inhibitors on neurite regeneration by PC12 cells

Inhibitor	% cells regenerating processes	
	With NGF	No NGF
None	77	6
Camptothecin (78 μM)	81	3
Actinomycin D (8 μM)	63	4
Cordycepin (40 μM)	79	1

PC12 cells were maintained in presence of NGF for 8–12 days and then were subcultured in the presence or absence of NGF and of inhibitors. Approximately 1 day after subculture, the cultures were scored for proportion of neurite-bearing cells.

with NGF and drug and either [^{3}H]leucine (2 μCi/ml) or [^{3}H]uridine (0.5 μCi/ml) (New England Nuclear; specific activity, 54.6 and 48.0 Ci/mmol, respectively) for 2–4 hr at 37°C. Uptake was linear for this entire period. After labeling, the medium was removed, the cultures were rapidly washed three times with saline, and the cells were scraped off the dishes in ice-cold water. After removal of an aliquot for total counts, macromolecules were precipitated in ice-cold 5% trichloroacetic acid and collected on glass filters (GF/A; Whatman). Radioactivity was measured by liquid scintillation counting using Packard Instagel. Incorporation was calculated on the basis of total non-acid-precipitable intracellular radioactivity and was thus corrected for drug effects on uptake. At drug concentrations that suppressed neurite generation, uptake was affected by less than 15%. Three to six sister cultures were assayed for each point, and data are expressed as mean ± SEM.

RESULTS

Neurite Regeneration. To assess the dependence of neurite regeneration on RNA synthesis, clonal PC12 pheochromocytoma cells were treated for 1–2 weeks with NGF and then dislodged from the culture dishes by repeated aspiration of medium with a pasteur pipette. As a result of dislodgment, neurites were lost from the cells both by mechanical shearing and by resorption into the cell body. After several washes with NGF-free medium, the cells were replated in the presence or absence of NGF and of RNA synthesis inhibitors. These inhibitors were: actinomycin D, an intercalating agent which blocks synthesis of rRNA and heterogeneous nuclear (hnRNA) (13); camptothecin, an alkaloid which inhibits synthesis of rRNA, hnRNA, and mRNA by an as yet unclear mechanism (14); and cordycepin (3-deoxyadenosine), a base analogue which interferes with synthesis of rRNA and formation of mRNA (15). In the presence of NGF (see also ref. 11), 50–80% of the cells regenerated neurites by 24 hr, whereas only 2–10% did so when NGF was not added to the cultures (Table 1). Significantly, a comparable or only slightly reduced level of NGF-dependent regeneration occurred in the presence of each of the above drugs at concentrations that produced substantial inhibition of [^{3}H]uridine incorporation (Table 1; Fig. 1).

Neurite Initiation. In contrast to regeneration, *de novo* initiation of neurite outgrowth by PC12 cells after treatment with NGF was preceded by a lag of at least 24 hr. This lag period was also present in cells whose proliferation had been blocked by treatment with arabinofuranosylcytosine or serum-free medium (16) and thus did not appear to be merely a consequence of cell cycle. After the initial lag, outgrowth of identifiable neurites (i.e., processes at least 20 μm in length) commenced so that the proportion of fiber-bearing cells in NGF-treated cultures increased progressively to about 90% within 1 week (Fig. 2). Fig. 2 also shows the effects of various concentrations of the RNA synthesis inhibitors on NGF-induced initiation of neurite outgrowth in PC12 cultures. Initiation of outgrowth was significantly blocked in the presence of each of these drugs at concentrations 0.1–10% of those at which neurite regeneration took place. Moreover, neurite generation could also be inhibited in cultures that had already been treated with NGF for short periods of time. For example, when cultures were exposed to NGF alone for 24 hr and then to NGF and inhibitor for the next 24 hr, initiation of outgrowth was also blocked (Fig. 1). Fig. 3 shows the effect of adding camptothecin to PC12 cultures after various times of pretreatment with NGF. Addition of camptothecin within the first few days resulted in loss of existing neurites as well as blockade of further neurite generation. However, at times after which most of the cells had already generated lengthy neurites, addition of camptothecin had little observable effect on neurite maintenance over the next several days.

The efficacy of the drugs used in blocking generation of neurites was not due to effects on cell viability. In each case, the

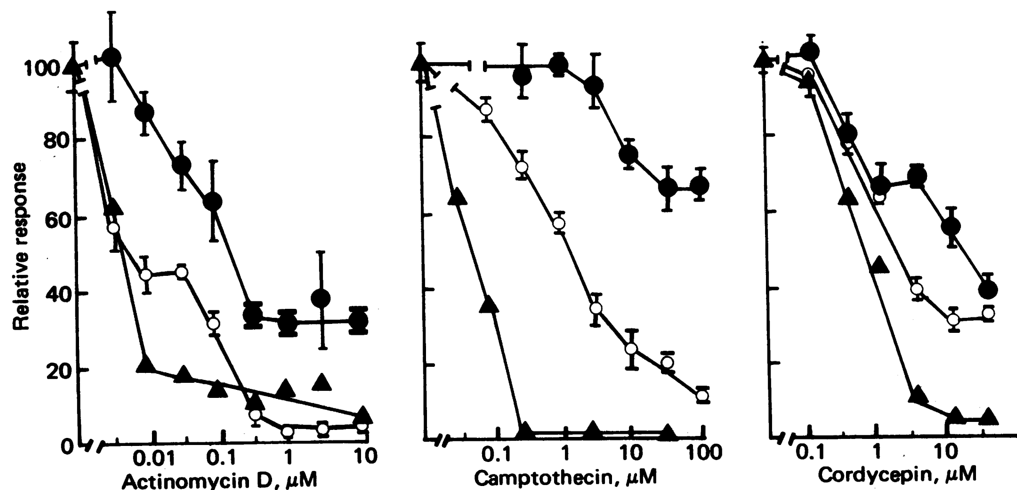

FIG. 1. Effect of RNA synthesis inhibitors on incorporation of [^{3}H]leucine (●) and [^{3}H]uridine (○) and on neurite generation (▲) in NGF-treated PC12 cultures. For each experiment, sister cultures were incubated according to the following sequence: 24 hr with NGF alone; 19–21 hr with NGF plus inhibitor; and then 2–4 hr with NGF and inhibitor plus radiolabeled precursor. The proportion of process-bearing cells was determined after the second incubation, and the incorporation of precursor into trichloroacetic acid-insoluble material was measured after the third incubation. Responses at each concentration are expressed relative to values in control cultures not treated with inhibitors. Values for incorporation of [^{3}H]leucine and [^{3}H]uridine into control cultures were 0.63 and 9.1 pmol/3 hr per mg of protein, respectively.

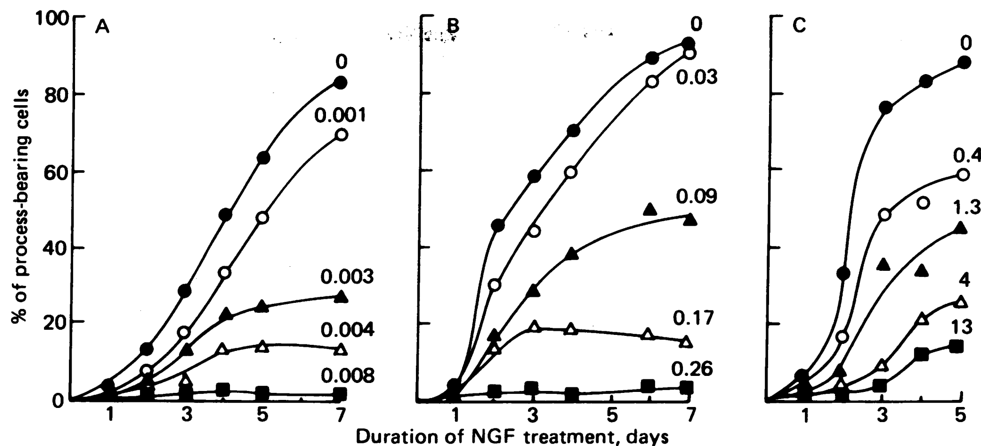

FIG. 2. Effect of RNA synthesis inhibitors on NGF-stimulated initiation of neurite outgrowth by PC12 cells. In each experiment, sister cultures were plated in the presence of NGF and the indicated concentration of drug (μM as shown on figure) and were scored at various times thereafter for proportion of process-bearing cells. (*A*) Actinomycin D; (*B*) camptothecin; (*C*) cordycepin.

cells excluded trypan blue and morphologically were similar to NGF-untreated controls. Further evidence for cell viability and vitality was gained in the case of camptothecin. One advantage of this drug is that its effects on RNA synthesis are completely and rapidly reversible (14). The experiment shown in Fig. 4 illustrates the effects of withdrawing camptothecin from PC12 cultures after various times of pretreatment with both the drug and NGF. At each time, removal of camptothecin was followed by commencement of neurite outgrowth. Significantly, the lag period normally observed prior to initiation of neurite outgrowth was not present, and 20–40% of the cells had generated neurites within 24 hr after drug withdrawal. Comparison of the curves for rate of initiation of neurite outgrowth after addition of NGF alone or removal of camptothecin shows that the latter is about 18 hr more rapid than the former. These results suggest that at least part of the sequence of events whereby NGF initiates neurite outgrowth may be carried out in presence of concentrations of camptothecin that suppress neurite outgrowth.

Fig. 1 compares the relative levels of neurite generation and incorporation of [^{3}H]leucine and [^{3}H]uridine by PC12 cultures after 24 hr of exposure to various levels of camptothecin, actinomycin D, or cordycepin between the first and second days of NGF treatment. In each case, neurite generation was blocked by doses of drug that spared a substantial proportion of leucine incorporation. With camptothecin, leucine incorporation was not affected at concentrations that completely inhibited initiation of neurite outgrowth. Such findings suggest that the observed effects of drugs on neurite outgrowth were not due to a general insufficiency of protein synthesis. Furthermore, neurite generation was suppressed at drug concentrations that only partially blocked uridine incorporation. Camptothecin in particular affected neurite outgrowth at concentrations that inhibited uridine incorporation by less than 30%. In preliminary experiments, sedimentation of cytoplasmic poly(A)$^+$ RNA and rRNA from PC12 cultures labeled with [^{3}H]uridine for 24 hr in the presence of 0.26 μM (0.1 μg/ml) camptothecin and NGF revealed normal profiles on 15–30% sucrose gradients (17).

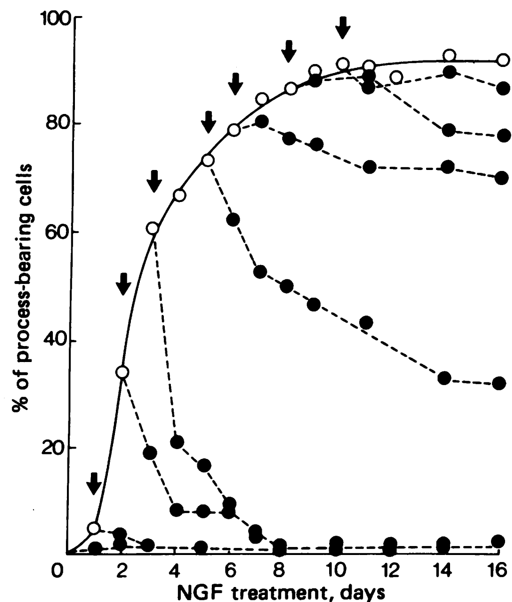

FIG. 3. Effect of camptothecin on neurite outgrowth and maintenance in PC12 cultures after various times of pretreatment with NGF. Sister cultures were plated in the presence of NGF and, at various times thereafter (indicated by arrows), camptothecin (final concentration, 0.36 μM) was included in the medium. O, Cultures with NGF alone; ●, cultures with both NGF and camptothecin.

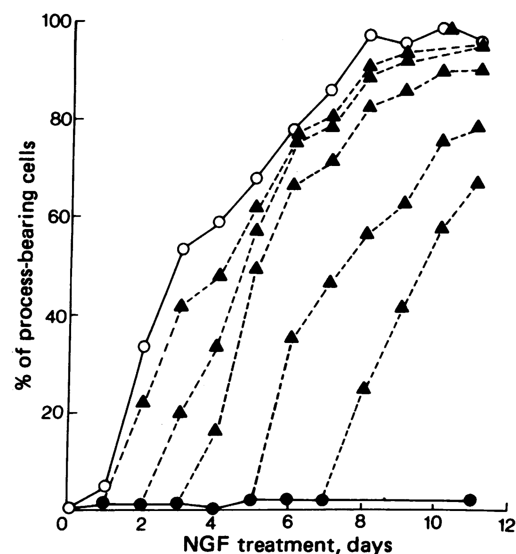

FIG. 4. Reversibility of the effect of camptothecin on initiation of neurite outgrowth in NGF-treated PC12 cultures. Sister cultures were treated with both NGF and camptothecin (final concentration, 0.65 μM) on day 0. After various times as indicated, the camptothecin was withdrawn. O, Cultures with NGF alone; ●, with NGF and camptothecin; ▲, with NGF alone after removal of camptothecin.

Table 2. Effect of pretreatment with camptothecin on neurite regeneration by PC12 cells

Pretreatment	Regeneration conditions*	% cells regenerating neurites
0	0	72
0	Camptothecin	55
Camptothecin (0.26 μM)	Camptothecin	10
Camptothecin (26 μM)	Camptothecin	3

PC12 cultures were maintained for 12 days with NGF and then pretreated for 1 day with NGF and the indicated level of camptothecin. The cells were then subcultured under the indicated conditions and were scored 1 day later for regeneration of neurites in presence of NGF.

*When present, camptothecin was at 26 μM.

Loss of "NGF-Priming." One interpretation of the presently observed differences in time course and drug sensitivities of neurite generation and regeneration is that NGF treatment causes a time- and RNA synthesis-dependent alteration in cell composition that is required for neurite outgrowth. In NGF-treated (or "NGF-primed") cells, such changes would already be present and, upon blockade of RNA synthesis, might persist for a sufficient length of time to sustain neurite regeneration. Results of several experiments are consistent with this model. Table 2 shows the results of an experiment in which fiber-bearing NGF-primed PC12 cultures were treated with a low dose of camptothecin for 1 day before being subcultured for regeneration. Although the camptothecin-treated cells did not show altered morphology, in contrast to untreated controls they no longer showed the capacity for RNA synthesis-independent neurite regeneration.

In a second type of experiment, fiber-bearing NGF-primed cells were subcultured for regeneration but in the absence of NGF. After various times, NGF was added to the cultures and the cells were scored 1 day later for neurite regeneration. In the absence of NGF, the cells showed a time-dependent loss of the capacity for neurite regeneration (Fig. 5). In six separate experiments, the cells lost their NGF priming with a mean (± SEM) half-life of 32 ± 4 hr. Furthermore, the longer the cells were maintained without NGF, the less was their capacity for neurite outgrowth in the presence of a RNA synthesis-inhibitor. This was lost with a half-life of 16 ± 4 hr (six experiments). Loss of priming by withdrawal of NGF did not appear to be due to recommencement of cell division because similar results were obtained with cultures in which DNA synthesis was blocked by continuous exposure to 10 μM arabinofuranosylcytosine.

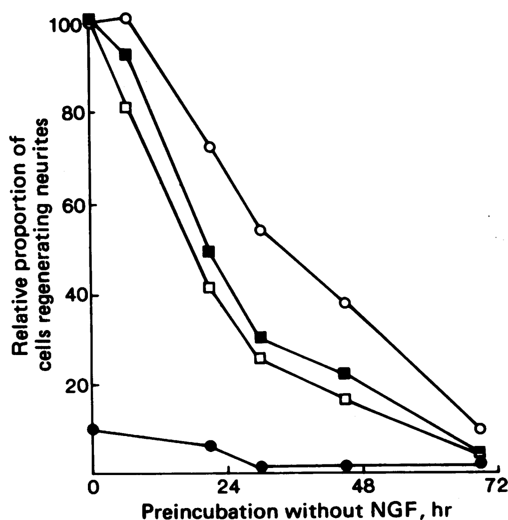

FIG. 5. Loss of capacity of PC12 cells to regenerate neurites caused by withdrawal of NGF. Cells were treated with NGF for 10 days and then subcultured without NGF for the times indicated. After this, NGF, with or without actinomycin D (8 μM) or camptothecin (26 μM) was added to the cultures which were then scored 24 hr later for proportion of process-bearing cells. Results are expressed relative to the proportion of cells that regenerated processes without preincubation in the absence of NGF (74%). ○, With NGF alone; □, NGF plus camptothecin; ■, NGF plus actinomycin; ●, without NGF.

DISCUSSION

The present results demonstrate that NGF-stimulated generation (or initiation) and regeneration of neurite outgrowth may be distinguished from one another both by their rates and by their sensitivities to inhibitors of RNA synthesis. Such findings imply at least two required pathways by which NGF stimulates neurite outgrowth; one of these is, and the other is not, inhibited by drugs that are known to interfere with RNA synthesis.

The point of stimulation of neurite outgrowth at which the multiple pathways of NGF's action are mechanistically independent from one another is not clear. There is evidence for NGF receptors of several different affinities on responsive ganglion cells (18, 19) as well as on PC12 cells (G. Landreth, K. Simon, and E. M. Shooter, personal communication) and for the uptake and retrograde transport of NGF from ganglion cell nerve endings (20). It is possible that the multiple pathways of action are related to such different means by which NGF interacts with responsive cells. Alternatively, it is conceivable that the multiple pathways may share a number of initial steps.

The nature of the RNA synthesis-independent role of NGF in neurite outgrowth is not presently known. Among possible mechanisms that have been suggested are the direct interaction of NGF with and influence on assemblable macromolecules such as tubulin and actin (21) and a rapid effect of NGF on uptake of small molecules (22). It is also relevant to note that experiments with sympathetic neurons in dissociated cell culture have provided evidence for local effects of NGF on growth of neurites (23).

Consideration of the sensitivity of NGF-induced initiation of neurite outgrowth to actinomycin D, camptothecin, and cordycepin raises the question of the molecular level at which these drugs affect the cells. The lack of effect of such drugs (even at very high concentrations) on NGF-dependent regeneration apparently rules out their direct actions on neurite outgrowth mechanisms. Also, because the levels of drugs that blocked initiation of outgrowth caused only partial or (in the case of camptothecin) no inhibition of leucine incorporation and because regeneration, in contrast, occurred even at drug levels that inhibited a significant proportion of leucine incorporation, it appears that the efficacy of the drugs was not due to suppression of overall protein synthesis. In addition, although actinomycin D and camptothecin can inhibit DNA synthesis (13, 14), such a mode of action is unlikely to be involved. For example, cultures in which DNA synthesis was blocked by treatment with arabinofuranasylcytosine displayed NGF-stimulated initiation of neurite outgrowth (8, 10), and such outgrowth was inhibited by exposure to actinomycin D and camptothecin. The above considerations thus appear to favor the interpretation that the drugs block NGF-stimulated initiation of neurite outgrowth by inhibiting RNA synthesis. This is further supported by the attainment of similar results with three different drugs, each of which affects RNA synthesis by a different mechanism of action and by the efficacy of these drugs at concentrations much lower than those at which they are known to have other nonspecific actions.

The present results are thus consistent with the previous

hypothesis (1, 4) that one role of NGF in promoting neurite outgrowth is selective stimulation of RNA synthesis. If this interpretation is correct, it is intriguing to consider the observation that neurite generation was almost totally suppressed at drug concentrations that inhibit uridine incorporation by as little as 25–30%. This suggests that initiation of neurite outgrowth by NGF requires species of RNA whose synthesis may be particularly sensitive to the drugs used. Although these drugs are known to have differential effects on the synthesis of various types of RNA (13–15), speculation at present as to the species involved in the action of NGF appears to be premature.

Another question raised by the present findings is the means whereby the transition takes place between the capacities for generation and regeneration of neurites. One appealing mechanism for this, which is supported by the present data, is that the initial effects of NGF include commencement of RNA synthesis-dependent accumulation of intracellular material(s) required for initiation of neurite outgrowth. Once a sufficient store of such material(s) accumulates, NGF-stimulated regeneration could occur even when RNA synthesis (and further accumulation) is inhibited. The gradual loss of the capacity for RNA synthesis-independent regeneration caused by withdrawal of NGF or by pretreatment (Table 2) with RNA synthesis inhibitors would thus be due to the turnover and nonreplacement of such material(s).

If the action of NGF requires newly synthesized species of RNA, then the question arises as to the role of such RNA in stimulating neurite outgrowth. One possibility is that the RNA itself plays a structural role in organization and assembly of neurites. There is recent evidence, for example, that RNA in centrioles may play a functional role in nucleating assembly of microtubules (24). A second possibility is that such RNA plays a role in the synthesis of differentiation-associated proteins. In this regard, experiments involving electrophoretic resolution of peptides from NGF-treated and untreated PC12 cells indicate that NGF does not bring about major changes in the overall pattern of PC12 cell proteins but that it does result in a relatively small number of quantitative alterations in protein synthesis (25). Two of these NGF-dependent changes in particular are selectively blocked by low levels of camptothecin (ref. 25; J. C. McGuire and L. A. Greene, unpublished data).

On this basis, and in consideration of the above discussion and experimental evidence, one tentative model for the types of events that comprise the mode of action of NGF in initiating neurite outgrowth is as follows. (*i*) NGF interacts with one or more types of surface receptors. This leads to subsequent events: (*ii*) As suggested by the rapid initiation of neurite outgrowth that occurs when camptothecin is withdrawn from NGF-treated cultures, there occurs one or more required events that are not blocked by levels of this drug that suppress neurite outgrowth. (*iii*) NGF treatment leads to stimulated synthesis of selective species of RNA. (*iv*) At least one consequence of the change(s) in RNA synthesis is a minor but required change in the pattern of protein synthesis. (*v*) As indicated by the findings that neurite regeneration requires NGF but is not blocked by high concentrations of RNA synthesis inhibitors, NGF also triggers required events that are independent of new RNA synthesis. This could include the events described in *ii* above. (*vi*) Neurite outgrowth occurs for the most part via assembly or rearrangement of types of structural proteins already present prior to NGF treatment and whose relative rates of synthesis are little, if at all, affected by NGF. Further experiments with normal responsive neurons and with the PC12 line should provide means for more extensive testing and refining of this and other models for the mechanism of NGF-stimulated neurite outgrowth.

We thank Mrs. M.-N. Chou and Mrs. J. Graham for expert technical assistance, Dr. H. Abelson for suggesting the use of camptothecin and for his very helpful comments and suggestions, and Drs. J. McGuire and E. Trenkner for helpful criticisms. This work was supported in part by grants from the National Foundation–March of Dimes and the U.S. Public Health Service (NS11557 and NS12200).

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