Regeneration of Neurites in Long-Term Cultures of Sympathetic Neurons Deprived of Nerve Growth Factor

Abstract. *Sympathetic neurons from newborn rats, cultured for 1 month or longer in the virtual absence of nonneuronal cells, were capable of regenerating neurites after neuritotomy. Regeneration occurred even after nerve growth factor was withdrawn from the cultures, although it was much less extensive and appeared limited to a few days following neuritotomy. Even after 29 days of nerve growth factor deprivation, reintroduction of the protein prompted a resumption of neurite growth. Possible roles of both nerve growth factor–independent and –dependent components in adult nerve regeneration are discussed.*

Experiments in vivo and in vitro have shown nerve growth factor (NGF) to be a survival requirement of immature mammalian sympathetic neurons and a promoter of their growth (*1*, *2*). But their dependence on NGF for survival is reduced or lost as they mature in vivo (*3*, *4*) or in virtually pure neuronal cultures (*5*). I report here that sympathetic neurons from newborn rats, cultured in the virtual absence of other cell types for periods exceeding 1 month, were able to regenerate neurites long after NGF withdrawal. However, a strong promotion of neurite growth by NGF, consistent with in vivo observations in adults (*6*), was also observed. More luxuriant and extensive growth resulted when regeneration occurred in the presence of NGF, and neurite growth resumed when NGF was reintroduced after regeneration had occurred in its absence.

Sympathetic neurons from superior cervical ganglia were dissociated and plated into the center compartments of three-compartment culture dishes (*7*) (Fig. 1). Two ganglia were used per dish. The culture medium, Leibovitz-15–CO_2, always contained 1 mg of ascorbate per milliliter and 5 percent rat serum when added to the center compartments; medium given to the side compartments never contained these ingredients. The medium was changed every 3 to 5 days. Initially, 10 μM cytosine arabinoside was included in the center compartments to kill nonneuronal cells and 7*S* NGF (1 μg/ml) was provided in all compartments. Neurites from cell bodies in the center compartments elongated to the left and right along a series of collagen channels, crossed under silicone-grease barriers, and entered the side compartments, where their extension was measured with a stage micrometer (Fig. 1). While penetrable by elongating neurites, such barriers effectively prevent leakage or diffusion of medium components between compartments (*2*, *7*). NGF was withdrawn from the center compartment of several cultures 4 days after plating. The neurons survived, and their neurites continued to elongate in the NGF-containing side compartments (*2*). When the experiments were initiated, the cultures had dense outgrowths—usually extending off the scale of measurement (>5 mm)—in all the channels observed.

After 20 days in culture, the neurites in the left and right compartments of one culture were completely removed by three washings with distilled water squirted from a syringe. This procedure, which will be referred to as bilateral neuritotomy, did not visibly disturb the cell bodies and neurites in the center compartment. Afterward, medium containing 1 μg of NGF per milliliter was added to the left compartment and medium lacking NGF was given to the right compartment. Observations of 18 channels (nine per side compartment) showed that regenerating neurites were present in 16 channels within 24 hours after treatment and that all contained neurites within 3 days. During 4 days of monitoring, the neurites in the left and right compartments advanced at similar rates (about 1 mm/day) (Fig. 2a). However, with one exception, each channel in the left compartment had a higher density of neurites 4 days after treatment than did its counterpart in the right compartment. The exception showed roughly equivalent densities.

A second bilateral neuritotomy was performed 4 days after the first. Again the neurites were allowed to regenerate, with NGF given to the left but not the right compartment. All channels contained regenerating neurites within 24 hours. Results were similar to those for the initial neuritotomy except that neurites in the NGF-deprived compartment ceased advancing after a mean extension of about 2.4 mm (Fig. 2a), while advancement in the NGF-supplied compartment continued off the scale in most channels. The NGF-deprived neurites held their position for the next 15 days, when a third bilateral neuritotomy was performed. The results were about the same. Except for a single channel, neurites regenerated more densely into the NGF-supplied compartment than into the NGF-deprived compartment after the second and third neuritotomies.

A sister culture was subjected to bilateral neuritotomy 33 days after plating and 29 days after NGF withdrawal from the center compartment. In this culture NGF-free medium was given to both side compartments. Within 24 hours, regenerating neurites were present in all nine channels in each side compartment, extended to about 3 mm within 4 days, and held this position for the ensuing 10 days (Fig. 2b). Second and third bilateral neuritotomies, again with no NGF given, were followed by similar regeneration, but only to about 1 mm. The responsiveness of this culture to NGF was tested after neurite advancement had leveled off following the third neuritotomy and 29 days after NGF was withdrawn from the side compartments. Medium containing 1 μg of NGF per milliliter was given to the left compartment, and within 2 days neurites were seen advancing there (Fig. 2b). Thus at least some of the

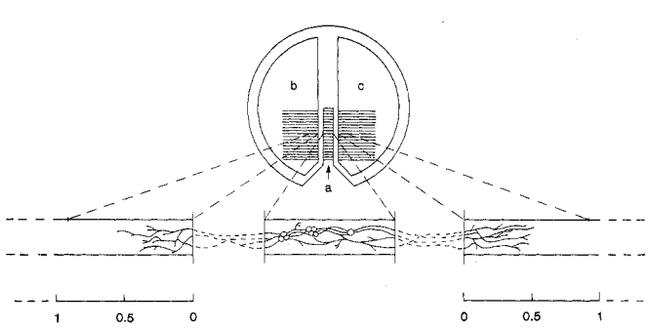

Fig. 1. Schematic diagram of a three-compartment culture. A Teflon divider partitioned the culture dish (not shown) into a narrow (1 by 5 mm) center compartment (*a*), open to the bulk of the culture dish, and left (*b*) and right (*c*) compartments. The collagen-coated floor of the dish was scored with 19 parallel scratches. Eighteen collagen channels, each about 300 μm wide and bordered by the scratches, guided the outgrowth of neurites from neurons in the center compartment to the left and right, as indicated by the enlargement of a single channel. The neurites penetrated silicone-grease barriers beneath the Teflon divider and entered the side compartments, where their extension along the channels was monitored by a digital stage micrometer (Ono Sokki EG-100) affixed to the left-right axis of the mechanical stage of an inverted microscope. The distance from the barrier to the tip of the farthest extending neurite was measured with the aid of an eyepiece cross hair.

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neurons in this culture, which had not received NGF for 29 days, remained responsive to the molecule.

Both cultures showed more extensive regeneration in NGF-deprived compartments after the first neuritotomy than after subsequent ones; residual NGF may have been responsible for this. Bilateral neuritotomy was performed on a third sister culture 55 days after plating, 51 days after NGF withdrawal from the center compartment, and after a 6-day period during which the medium in both side compartments was replaced once each day with NGF-free medium to reduce the level of any residual NGF before the first neuritotomy. The regenerating neurites extended only slightly farther after the first neuritotomy than after a second, when mean extension leveled off at about 1 mm (Fig. 2c). In other respects the results were essentially identical to those obtained with the second culture.

Figure 3 shows photomicrographs of a channel in the right compartment of the third culture. The pictures were taken 9, 14, and 20 days after the second neuritotomy. Neurite elongation had reached a plateau within 4 days, following which many of the regenerated neurites neither degenerated nor elongated further. By 20 days some new neurites had appeared and some had disappeared. But most of the neurite growth in this NGF-deprived culture occurred during the first few days following neuritotomy.

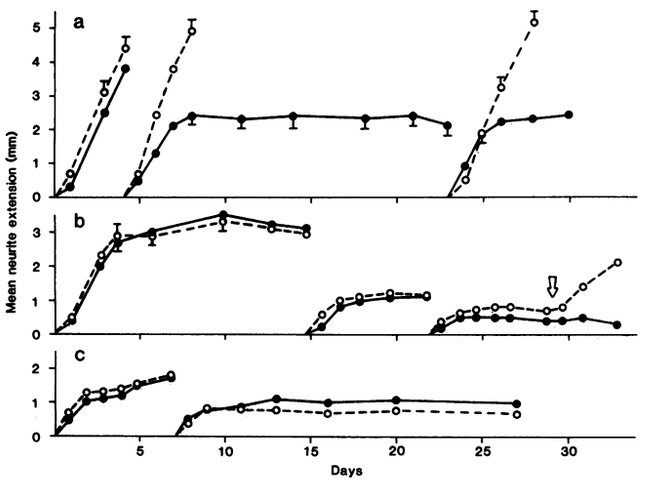

Fig. 2. Plots representing extension of regenerating neurites into the left (○) and right (●) compartments of three cultures. NGF had been withdrawn from the center compartments 16 or more days before testing. (a) Regeneration with NGF (1 μg/ml) provided in the left compartment but withdrawn from the right compartment on day 0 (day 20 of culture). Neuritotomy was performed on days 0, 4, and 23. (b) Regeneration in a second culture, with no NGF provided in any compartment. NGF was withdrawn from the left and right compartments on day 0. The first neuritotomy (day 0) was performed 33 days after plating, and two subsequent neuritotomies were performed on days 15 and 22. NGF (1 μg/ml) was added to the left compartment on day 29 (arrow). (c) Regeneration in a third culture following neuritotomy on day 0 (55 days in culture) and on day 8. No NGF was added to any compartment throughout the experiment, and the side compartments were subjected to one change of NGF-free medium daily for 6 days prior to day 0. The center compartment had not been given NGF for the previous 51 days. Data are means ± standard errors for nine channels in (a) and (b) and 12 channels in (c). Where omitted, the standard error was less than 0.25 mm.

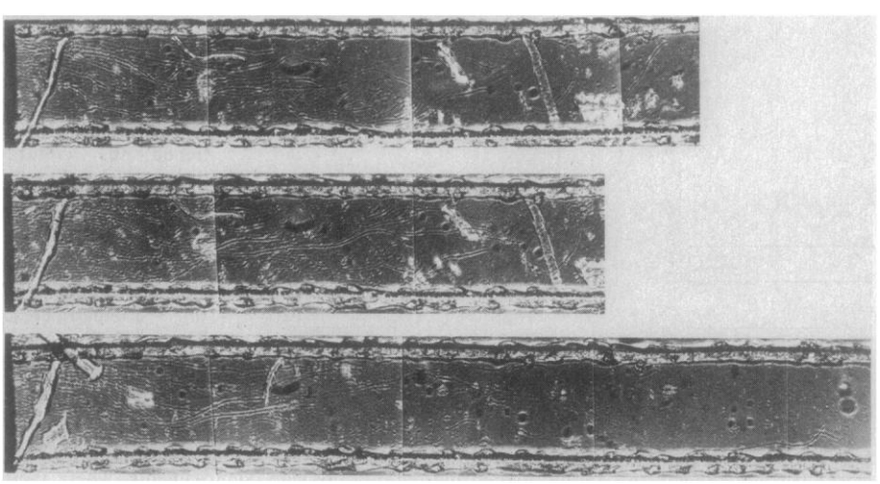

Fig. 3. Phase-contrast photomicrographs of neurites regenerated in a culture in which no NGF was added to any compartment. A single channel in the right compartment is shown at 9, 14, and 20 days (top to bottom) after neuritotomy. Scratches border the channel, and the barrier partitioning the right and center compartments is at the extreme left.

What factors, if any, may support neurite regeneration under conditions of NGF deprivation? Small amounts of residual extracellular NGF, even after 22 days of deprivation and three successive neuritotomies, cannot be ruled out. However, I have found that identical rinsing procedures, performed on cultures up to 12 days old, consistently reduced NGF levels sufficiently to kill sympathetic neurons from newborn rats when NGF was withdrawn from all compartments (*8*). When NGF was withdrawn from the side compartments but retained in the center compartment, neurites in the side compartments ceased to elongate and most degenerated (*8*). Chun *et al.* (*5*) found that nearly all the cells in 10-day-old cultures of sympathetic neurons from newborn rats died within 10 days when NGF was withdrawn; about half the neurons in older cultures survived 10 days after such treatment. Cell death was not increased further in these older cultures by the addition of antiserum to NGF. These findings suggest that residual, extracellular NGF was not responsible for neurite regeneration under conditions of NGF deprivation in the present experiments.

Nerve growth factor–independent outgrowth of neurites was previously reported in explants of superior cervical ganglia from 14-day-old mouse fetuses (*9*). This capability disappeared by fetal day 18. Also, the neurite outgrowth always occurred atop a layer of ganglionic nonneuronal cells. The present study shows NGF-independent neurite regeneration in postnatal sympathetic neurons matured for 1 month or more in the absence of nonneuronal cells. Some nonneuronal cells in the center compartments may have survived cytosine arabinoside treatment and escaped notice, but if so, they were few in number. No nonneuronal cells were observed in the side compartments (where neurite regeneration occurred) and almost certainly were never present there. However, the collagen substrate as well as residual substrate-attached material (*10*), left by neurites grown previously with NGF present, could have contained factors that may have supported neurite growth in the NGF-deprived cultures.

It is not clear whether all or only some of the NGF-deprived neurons were capable of sending regenerated neurites into

side compartments, or whether neurons with this capability retained their responsiveness to NGF. Although reintroduction of NGF into a side compartment after 29 days of deprivation resulted in resumption of neurite advance (Fig. 2b), the neurites of a subpopulation of neurons capable of surviving but not elongating during NGF deprivation could have been responsible for this. Neurites of the hypothetical subpopulation could have been present just out of sight under the barrier in the side compartment, where exposure to reintroduced NGF could have caused their growth to resume. The 1- to 2-day lag between addition of NGF and resumption of neurite advance would have been sufficient for these neurites to overtake and pass the previous growth. Thus, neurons capable of regenerating neurites during NGF deprivation may not, in fact, be responsive to NGF.

Alternatively, neurites of mature sympathetic neurons may be capable of limited regeneration in the absence of NGF, but full regeneration may require reestablishment of contact with an NGF supply. Postganglionic axotomy results in irreversible atrophy of the superior cervical ganglia when performed in rats before the 12th postnatal day (*11*). This atrophy was preventable by a period of intravenous administration of NGF (there was functional recovery of the innervation), but axotomy carried out after 3 weeks of age was followed by reinnervation without the aid of exogenous NGF. The timing of the appearance of the unaided, regenerative capability is well correlated with my observation of regeneration in NGF-deprived neurons maintained 1 month or more in culture. Taken together, the results suggest that NGF-independent neurite regeneration may be a first, crucial stage in the reestablishment of sympathetic innervation after peripheral nerve damage in the adult rat. This capability may develop independent of any direct postnatal influences by nonneuronal cells.

A second stage of regeneration, as well as long-term maintenance of the neurons, may require reestablishment of contact with an NGF source—perhaps the tissues to be reinnervated. Interestingly, NGF production by the iris is stimulated by denervation (*12*), and truly long-term survival of sympathetic neurons may require NGF, even in the intact, adult sympathetic nervous system (*4*).

ROBERT B. CAMPENOT
Section of Neurobiology and Behavior, Cornell University, Ithaca, New York 14850

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Brain Aging Correlates: Retardation by Hormonal-Pharmacological Treatments

Abstract. *Mid-aged rats were either adrenalectomized and chronically maintained, or left intact and treated daily for a 9- to 10-month period with a potent analog of the peptide adrenocorticotropin (residues 4 to 9), which has some stimulant properties, or with the neural stimulant pentylenetetrazole. All three treatments reduced hippocampal morphologic correlates of brain aging (neuronal loss, glial reactivity). The pentylenetetrazole and peptide treatments also improved reversal learning. These results suggest that certain endogenous peptides, with stimulant properties, may also exert long-term, trophic effects on brain structure and function.*

Little is known about the etiological factors that influence the rate of brain aging, although research on possible causes of senile dementia or normal brain aging (or both) is currently focused on cerebrovascular processes, slow viruses, immune reactions, toxic metals, and genetic mechanisms (*1*). Additionally, we have been pursuing the hypothesis that endocrine factors, particularly glucocorticoids, may normally accelerate some aspects of brain aging (*2*). Our prior studies have so far yielded data consistent with predictions of that hypothesis (*2*), and other studies have implicated elevated corticoids in age-like cardiovascular decline (*3*). However, clear evidence of retarded brain aging also seems needed to test a hypothesis of causal factors in normal brain aging. We recently reported, in preliminary form, that long-term adrenalectomy does reduce some morphologic correlates of aging in the rat hippocampus (*4*) [which is rich in corticosterone receptors (*5*)].

Adrenalectomy not only lowers corticoids, however, but also results in widespread changes in endocrine-metabolic measurements. Among the major consequences of adrenalectomy, of course, is an elevation in adrenocorticotropic hormone (ACTH), which exerts direct behavioral and biochemical actions on the brain (*6*); moreover, ACTH and its non-steroidogenic, brain-active fragments (for example, ACTH residues 4 to 10) induce electrophysiological patterns similar to those of neural stimulants (*7*). It therefore seemed feasible that the prolonged neural stimulation resulting from elevated ACTH, rather than the reduction in steroids per se, could be largely responsible for the retardant action of adrenalectomy on brain aging (possibly by maintaining neuronal metabolism). In this study, we examined these possibilities.

We now report that long-term treatment of rats with a neural stimulant, pentylenetetrazole (PTZ), or with a potent and behaviorally active analog of the ACTH[4–9] molecule (ORG 2766), which does not affect glucocorticoid release (*8*), can retard the development of both some neuromorphologic and some behavioral correlates of brain aging in rats. We also report that the long-term absence of the adrenal glands has effects on brain aging correlates different from those of ORG 2766 and PTZ. These studies, then, suggest that peptides, as well as steroids, can influence brain aging and that stimulation may be an important element in these effects.

We used a number of established morphometric correlates to quantify the degree of hippocampal aging. Neuronal loss, lipofuscin, and glial reactivity have

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