

## Chapter 4

# TROPHIC FACTORS DIRECTED TO NERVE CELLS\*

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**O**UR LONG-TERM RESEARCH interest has been the investigation of humoral and cellular influences that may regulate development, maintenance, functional performances, and (possibly) repair mechanisms in neuronal and glial cells. Our main approach has been the use of *in vitro neural systems*, which we pioneered several years ago and which have since been adopted by many others as a fundamental tool for neurobiological research.

This presentation will be focused mainly on *Neurotrophic Factors* (NTF), i.e. soluble macromolecular agents exerting trophic support on selected nerve cells. The concept of NTF derives from two phenomena, developmental neuronal death and Nerve Growth Factor (NGF).<sup>1, 2</sup>

*During development*, all subsets of neurons studied thus far become dependent for their survival on the target tissues they innervate. Within each subset, a large number of neurons die at a specified time coinciding with the period of synaptic connection. If the target tissue has been ablated before arrival of the nerve terminals, all neurons of that subset will die. Conversely, if additional target tissue has been provided (e.g., implantation of an extra limb), neuronal death is much reduced. The general hypothesis has been that (a) target tissues supply the surviving neurons with a NTF, (b) the NTF is picked up by the axonal terminals and retrogradely transported to the neuronal somata, (c) the supplied NTF supports survival, further growth, and functional maturation of the neurons, and (d) the same NTF may continue its supportive role for the mature neuron and be critical for its regenerative capabilities.

Until recently, the only known macromolecule with NTF characteristics has been *Nerve Growth Factor* (NGF). NGF elicits the expanded trophic consequences from its specific neuronal targets in sympathetic and dorsal root ganglia and protects mature sympathetic neurons from the consequences of anatomical or functional axotomy. It has been shown to bind to specific "receptors" on the neuronal surface, and also to be picked up by the nerve terminals for internalization and retrograde transport. The

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NGF protein has been purified, sequenced, and crystalized. However, progress has been much slower with regard to the mechanisms by which NGF exercises its trophic role on its target neurons.

We shall first describe the second NTF, which we have recently found to be directed to certain cholinergic neurons, and then present our current views on the mode of action of NGF (and possibly other NTF).

### Search for Cholinergic NTFs

The strategy for such a search involves (a) selection of a neuronal system, and identification of promising sources of NTF for those neurons, with a putative physiological significance, (b) use of the chosen neuronal system for an NTF bioassay, and titration of NTF activity in the chosen source and materials derived therefrom, (c) purification and characterization of the new NTF molecule. With the purified factor, and antiserum raised against it, one can then investigate the responses of the target neurons *in vitro* and *in vivo*, and the neuronal spectrum on which the new factor is active.

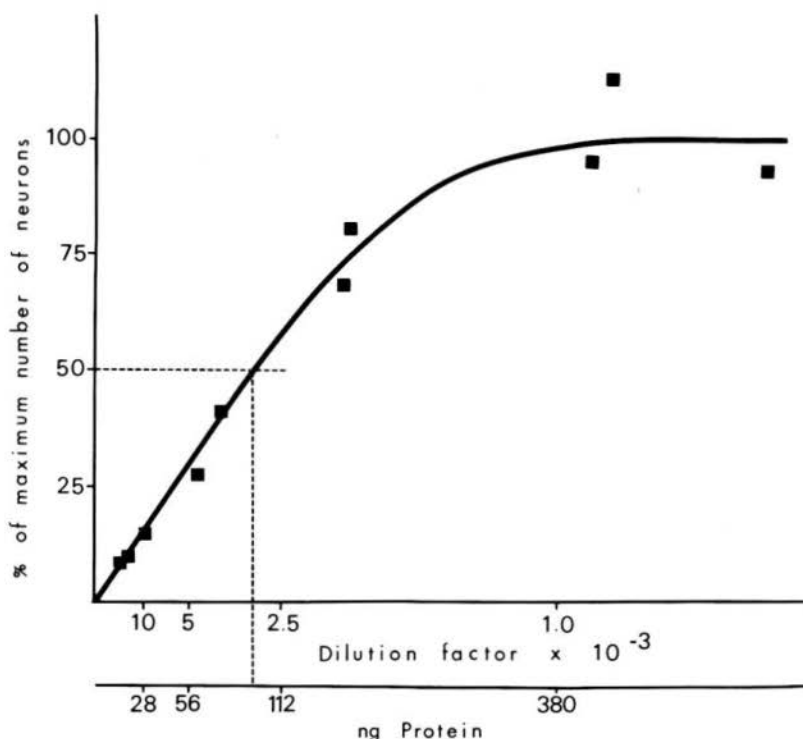


Figure 4-1. *Bioassay for ciliary neurotrophic factors (CNTFs)*. Dissociates from eight day chick embryo ciliary ganglia are seeded in serum-supplemented medium containing serially diluted active material. After twenty-four hours, surviving neurons are counted under phase contrast microscopy. The dilution factor required to achieve 50 percent survival represents the number of Trophic Units per ml of original material.

We have chosen the 8 day chick embryo ciliary ganglion (CG) as our neuronal system. The CG neurons comprise only two subpopulations, both of which are cholinergic and innervate intrinsic muscles of the eye. Between eight and fourteen embryonic days, when synapses are established on the intraocular target muscles, half the CG neurons die in the normal embryo, and death is classically enhanced by prior removal of the eye primordium or reduced by prior implantation of an extra eye. Thus, the intraocular target tissues should be a "physiological" source of ciliary neuronotrophic factor, or *CNTF*.

When CG are dissociated and seeded in monolayer cultures with serum-containing medium, neurons attach but fail to survive for even twenty-four hours unless special supplements are also supplied. This provides the basis for a *CNTF* bioassay, where surviving neurons are counted in twenty-four hour cultures that contain increasing amounts of *CNTF* materials (Fig. 4-1). We have defined 1 trophic unit (TU) as the activity present in 1 ml of medium capable of supporting 50 percent survival.<sup>3, 4, 5</sup> A similar assay can be used to count differentially neurite-bearing neurons and, thus, titrate neurite-promoting activities separately from trophic activities.<sup>6</sup>

We have directed our efforts mainly to chick eye extracts,<sup>3, 4</sup> and the results are summarized in Table 4-I. In the twelve day embryo, as much as one-third of the *CNTF* activity of a whole embryo is concentrated in the eye, where specific *CNTF* activity exceeds by five to six times that of whole embryo extract. Subdissection of the eye tissue shows that most of the eye activity resides in the intraocular targets for CG innervation (choroid,

TABLE 4-I  
CILIARY NEURONOTROPHIC FACTOR (CNTF) ACTIVITY IN EXTRACTS AND FRACTIONS DERIVED FROM CHICK EMBRYO EYE TISSUES

| <i>Extracts and Fractions</i>                                | <i>TU/Embryo</i> | <i>mg Protein/Embryo</i> | <i>TU/mg Protein</i> |
|--|------------------|--------------------------|----------------------|
| <i>12 day chick embryo</i>                                   |                  |                          |                      |
| whole embryo   | 8,000            | 55.0                     | 145                  |
| whole eye  | 2,500            | 2.9                      | 862                  |
| choroid + ciliary body +<br>pigment epithelium + sclera      | 1,985            | 0.82                     | 2,421                |
| iris   | 33               | 0.06                     | 550                  |
| <i>15 day chick embryo</i>                                   |                  |                          |                      |
| whole eye  | 7,100            | 4.70                     | 1,511                |
| iris + choroid + ciliary body +<br>pigment epithelium = CIPE | 6,000            | 0.40                     | 15,000               |
| <i>Batch purification (1 work day)</i>                       |                  |                          |                      |
| 15 d. CIPE (36 embryos)                                      | 200,000          | 13.0                     | 15,000               |
| DE-52 pool   | 100,000          | 3.2                      | 31,200               |
| CTNF 1   | 96,000           | 1.5                      | 64,300               |

ciliary body, iris) with another threefold increase in specific activity. Total and specific CNTF activities in the eye increase markedly between twelve and fifteen embryonic days — the period concerned with developmental CG neuronal death and survival. An eye fraction, containing all the CG-innervated tissue and only the non-target pigment epithelium, is responsible for most of the eye CNTF. This fraction (CIPE) contains as much activity as the entire twelve day embryo, but its specific CNTF activity is 100 times higher. Thus, strong evidence for a physiological role of this CNTF is provided by its coincidence in both *space and time* with the developmental CG neuron death phenomenon.

Furthermore, the CIPE fraction of fifteen day chick embryo eye constitutes a sufficiently abundant source for *purification* of the CNTF molecule. We have now developed a procedure for producing, within a single work-day, about 1.5 mg of partially purified factor, with a final specific activity of 60-70,000 TU/mg protein.<sup>5</sup> Despite the incomplete purification, this preparation has a potency of 15 ng/TU, similar to that of the most pure NGF preparations (10 ng/TU).

### Neurite-Promoting Factors

Over the past several years, we have proposed that extrinsic macromolecular influences over neural (or, for that matter, other) cells be categorized into two distinctive classes, i.e. *trophic* and *specifying* influences.<sup>1, 2</sup> Trophic influences would control quantitative anabolic activities of the cell and, thus, be equally responsible for death (insufficient trophic support), maintenance (adequate trophic support to match anabolic and catabolic activities), or overproduction (trophic support above maintenance levels). Specifying influences would be additionally required to direct selective uses of this overproduction by the cell, such as proliferation, hypertrophy, elongation of a process, or secretory activities. The distinction between trophic and specifying influences has been of great help to us to conceptualize the role of NGF and other neuron- or glia-related factors, and similar distinctions are now being adopted by other research groups. An illustration of this distinction is provided by the study of neurite-promoting agents for CG neurons.

Eye-CNTF supports survival of CG neurons cultured on either collagen- or polyornithine-coated dishes but promotes neurites only on the collagen substratum.<sup>7</sup> Neurite outgrowth on polyornithine requires the additional presence of *neurite-promoting materials*, adsorbed to the polyornithine itself (Fig. 4-2). Two such materials<sup>6</sup> are under study. One is a microexudate released into the culture medium by chick embryo heart cells<sup>6, 7, 8</sup> as well as by glial and other cell cultures.<sup>9</sup> The other is a microexudate deposited by CG explants in a zone concentric to the explant itself.<sup>6</sup> Neither material allows for survival of CG neurons in the absence of

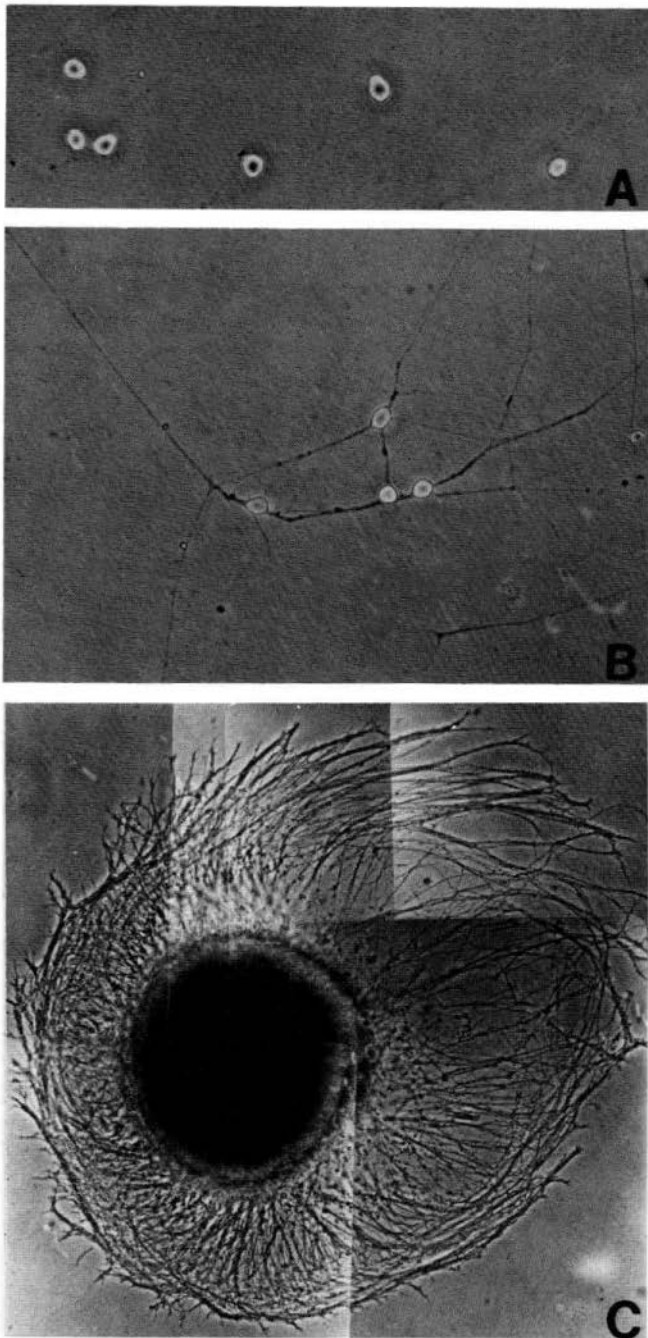


Figure 4-2. *Neurite promotion in ciliary ganglionic cultures.* Dissociated or explant cultures were set up on polyornithine-coated tissue culture plastic (PORN), provided with CNTF-containing medium to ensure neuronal survival, and examined by phase contrast twenty-four hours later. *A.* dissociates on untreated PORN display neuronal survival but no neurite growth. *B.* the same cells show profuse neuritic growth on PORN that was preexposed to heart cell conditioned medium. *C.* explants on untreated PORN deposit their own microexudate on the surrounding PORN, and their neuritic outgrowth remains confined to the modified substratum.

CNTF supplements and, thus, has the basic property of a neuronotrophic factor. The question under investigation is whether such materials only confer to polycationic surfaces better neurite-supportive properties, or whether they actually represent true neurite-specifying factors, such as those that may occur on cell surfaces to direct axonal growth in developing or regenerating systems *in vivo*.

### Mechanisms of Neuronotrophic Action

NGF has a thirty-year longer history than the newcomer CNTF and should, therefore, be the ideal agent for an investigation of the mechanisms of neuronotrophic action. One feature inherent to the neuronotrophic function, however, is that without their NTF neurons fail to survive and, thus, offer inadequate controls to their NTF-treated counterparts. This has been a particularly serious difficulty in the study of NGF mode of action, as long as the NGF consequences to be examined (survival, neurite elongation, new synthesis of transmitter enzymes) develop over a similar time span as does the death of NGF-deprived neurons. We therefore sought to establish an *experimental system* in which (a) quantitatively measurable "deficits" would develop over a period of hours in NGF-deprived neurons, (b) delayed administration of NGF would fully reverse such deficits, thereby verifying the still viable status of the cells, and (c) the NGF-induced recovery would take place within minutes of the factor presentation, while untreated replicate samples undergo negligible additional changes. Such an experimental system was achieved by use of freshly prepared cell suspensions from eight day chick embryo dorsal root ganglia (DRG), one of the traditional NGF target tissues. With this system, we have been able to backtrack "short-latency" responses to NGF from changes in RNA labeling, to changes in uridine uptake and hexose transport and, eventually, changes in intracellular  $\text{Na}^+$  levels to which the action of NGF appears primarily directed. Only some salient features of these studies<sup>10</sup> will be reviewed here.

The main features of the uptake of [<sup>3</sup>H]2-deoxyglucose (2DG)<sup>11</sup> are summarized in Figure 4-3. In freshly prepared DRG cells, 2DG uptake occurs to the same extent regardless of the presence or absence of NGF in the medium. The ability to take up 2DG declines by about one-third over six hours of incubation without NGF, but not in the presence of the factor. Presentation of NGF to six hour deprived cells restores the lost ability in a transcription- and translation-independent manner. The concentration at which NGF is provided controls both magnitude and speed of the recovery, suggesting that the characteristics of this "2DG response" reflect the extent and the rapidity of a binding reaction between NGF and its receptors on the neuronal surface. A similar regulation by NGF had been



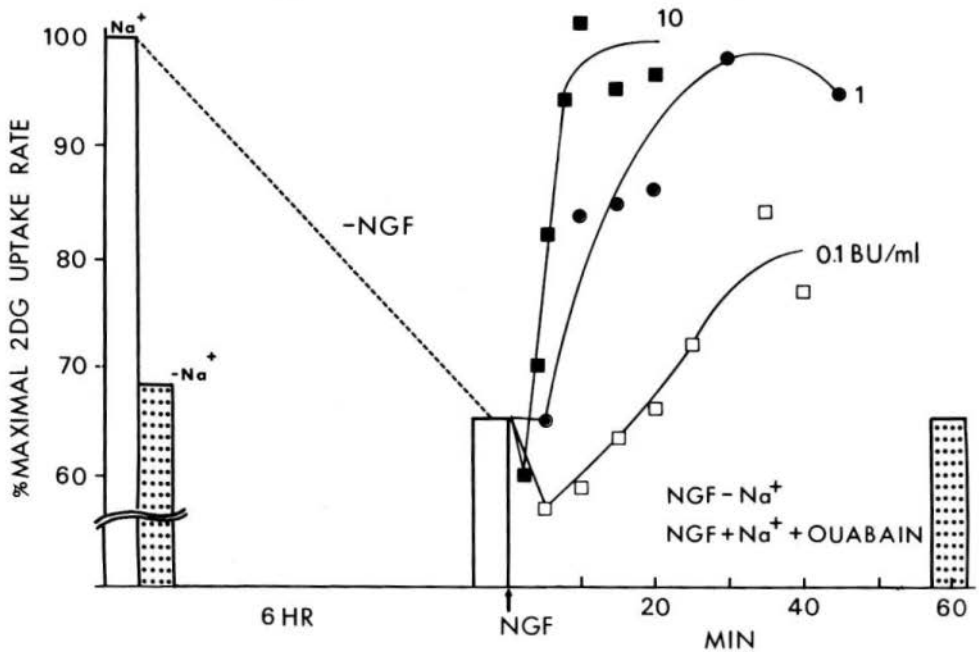


Figure 4-3. *NGF and Na<sup>+</sup> effects on 2-deoxyglucose uptake by dorsal root ganglion neurons.* Cell suspensions from eight day chick embryo ganglia are incubated with or without NGF and pulsed for six minutes with [<sup>3</sup>H]2-deoxyglucose at different times. *Open bars* = uptake before and after six hours of NGF = deprivation. *Right-side curves* = of uptake capabilities by six hour deprived cells upon delayed presentation of NGF at concentrations indicated. *Stippled bars* = omission of Na<sup>+</sup> (replaced by choline) in the medium reduces uptake to the same extent as does a six hour deprivation of NGF, and prevents recovery on delayed NGF administration. The same behavior is achieved by treatment with ouabain (10<sup>-3</sup>M).

measured for the transport of uridine and  $\alpha$ -aminoisobutyric acid but not, for example, leucine.<sup>12</sup>

The ability of NGF to control selective transport systems suggested that they share a common feature to which NGF would be directed. Such a feature was found to be the coupling of the NGF-regulated transports to Na<sup>+</sup> gradient.<sup>13</sup> In the absence of extracellular Na<sup>+</sup>, hence of a Na<sup>+</sup> gradient (Fig. 4-3, stippled bars), even freshly prepared cells reduce their 2DG uptake, with or without NGF, to the same extent to which they would reduce it over six hours of NGF deprivation. Furthermore, NGF no longer succeeds in restoring transport losses in NGF-deprived cells when it is presented in a Na<sup>+</sup>-free medium or together with ouabain — both situations being characterized by a diminished Na<sup>+</sup> gradient across the cell membrane. These and other data indicated that transport of NGF-sensitive substrates is driven by Na<sup>+</sup> gradient and suggested that NGF might control such transports by controlling the Na<sup>+</sup> gradient itself.

We know now that NGF does indeed *control the intracellular  $\text{Na}^+$  levels* of its target neurons.<sup>14</sup> As shown in Figure 4-4, NGF-deprived cells accumulate  $^{22}\text{Na}^+$  to a six to eight times greater level than their NGF-supported counterpart. This develops over the same six hours during which transport abilities are lost reversibly. If NGF is now provided, the cells lose their  $\text{Na}^+$  content to a degree and at a speed that are again controlled by the NGF concentration. Additional studies<sup>15, 16</sup> on this " $\text{Na}^+$  response" have indicated the following:

- It is a general NGF effect, since it occurs with either beta or 7S NGF (but not with other proteins, including insulin) and with both sympathetic and spinal sensory ganglionic targets;
- It does not reflect particularly restrictive conditions, since it is elicited in intact as well as dissociated ganglia, and in nutrient- and hormone-rich media as well as in buffered saline;
- It involves the control by NGF of an outward  $\text{Na}^+$  pump, or of corresponding mechanisms involving other ionic species.

The action of NGF on neuronal ionic features is likely to have *pervasive consequences* (Fig. 4-5). Both the intracellular  $\text{Na}^+$  level and the transmembrane  $\text{Na}^+$  gradient are bound to have considerable impact on several cell behaviors. In addition, intracellular  $\text{K}^+$  levels and consequently mem-

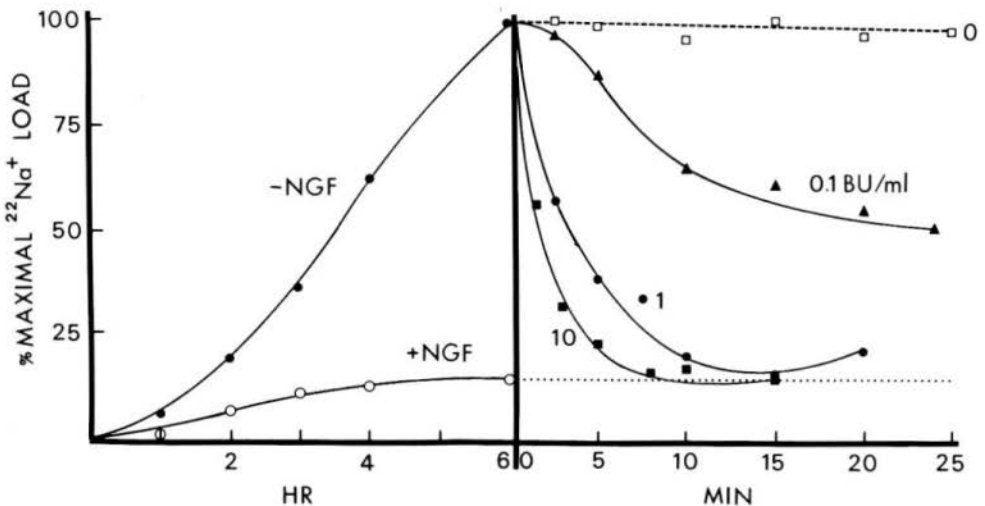


Figure 4-4. NGF control of intracellular  $\text{Na}^+$  in dorsal root ganglion neurons. Left side = Ganglionic cell suspensions, incubated for six hours in the presence of  $^{22}\text{Na}^+$ , accumulate radioactivity to eightfold higher levels when deprived of NGF than in its presence. Right side = NGF-deprived,  $^{22}\text{Na}^+$  preloaded cells release their sodium (on further incubation in  $^{22}\text{Na}^+$ -medium) within minutes of NGF administration. Both magnitude and speed of the release are regulated by the concentration of NGF.



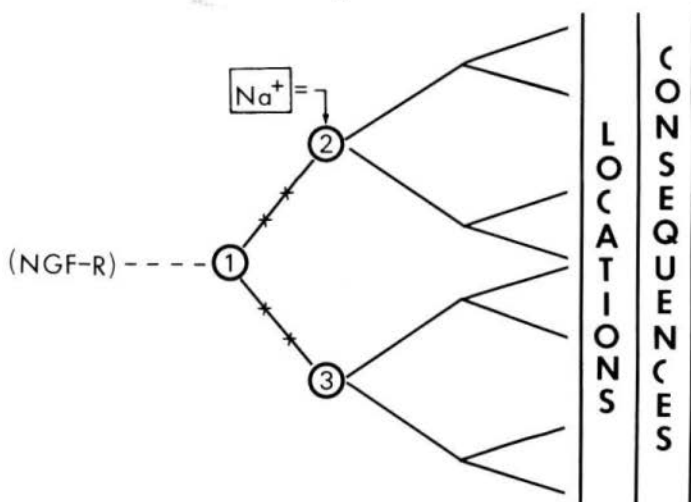


Figure 4-5. Schematic diagram of the mode of action of NGF. Association of NGF with its surface receptors (NGF-R complex) triggers a yet unknown sequence of fast events (broken line) leading to the regulation of a critical neuronal function (1). This function in turn controls other important neuronal functions (e.g. 2, 3), which will affect several cell machineries in various cell locations and eventually elicit the overt consequences traditionally perceived as "NGF effects." Control by NGF of  $\text{Na}^+$  regulation warrants a prominent position in this scheme (arrow). (For more details, see Varon and Skaper.<sup>10</sup>)

brane potentials are also likely to be affected by the action of NGF (S. D. Skaper and S. Varon, unpublished data), and they too must regulate a variety of cell behaviors. Finally, one should note that different consequences may result from an ionic control by NGF in different cellular locations (soma, nucleus, growth cone). This confers to the  $\text{Na}^+$  response a high rank in the sequences of events that must link the formation of an NGF-receptor complex on the cell surface with the ultimate manifestation of the NGF action.<sup>2, 10</sup>

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