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# A Single Pulse of Nerve Growth Factor Triggers Long-Term Neuronal Excitability through Sodium Channel Gene Induction

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## Summary

**The continuous presence of nerve growth factor (NGF) is thought to be required for the elaboration of neuronal-like traits in PC12 cells. Surprisingly, we find that a 1 min exposure to NGF is sufficient to engage a longer-term genetic program leading to the acquisition of membrane excitability. Whereas continuous exposure to NGF causes the induction of a family of sodium channels, the effect of a brief exposure is to induce selectively expression of the peripheral nerve-type sodium channel gene PN1, through a distinct signaling pathway requiring immediate-early genes. A 1 min exposure of PC12 cells to interferon-γ also causes PN1 gene induction, suggesting that the "triggered" NGF and interferon-γ signaling pathways share common molecular intermediates.**

## Introduction

Nerve growth factor (NGF) is essential for the development of sympathetic and certain sensory neurons (Levi-Montalcini and Angeletti, 1968; Crowley et al., 1994; Smeyne et al., 1994), and its continual presence is thought to be required for the differentiation of sympathetic neurons in culture (Levi-Montalcini and Angeletti, 1968). Although NGF has been shown to be required for cell survival (Levi-Montalcini and Angeletti, 1968), the precise role it plays in neuronal differentiation has been more difficult to determine. In particular, whether NGF is required for the acquisition of neuronal excitability has remained an open question because the appearance of ion currents precedes the time at which NGF is thought to be present at significant levels in vivo (Davies et al., 1987).

Studies of the role of NGF in sympathetic neuronal development have been facilitated by the use of rat pheochromocytoma (PC12) cells which, unlike their normal neuronal counterparts, require NGF for differentiation but not for survival. These cells also provide the advantage of having had no previous exposure to NGF, allowing for the study of irreversible effects of this growth factor (Greene and Tischler, 1976, 1982; Halegoua et al., 1991). It is well established that PC12 cells acquire the ability to generate action potentials when grown in the continual presence of NGF (Dichter et al., 1977; Greene and Tischler, 1982; O'Lague et al., 1985; Rudy et al., 1987; Mandel et al., 1988). The appearance of excitability can be explained by large increases in the levels of three sodium channel transcripts encoding the brain type II/IIA and peripheral nerve type 1 (PN1) sodium channel α subunits (Mandel et al., 1988; D'Arcangelo et al., 1993). The induction of type II gene expression in PC12 cells by NGF is mediated through activation of the cAMP-dependent protein kinase (Ginty et al., 1992; D'Arcangelo et al., 1993), whereas PN1 gene induction is independent of this activity (D'Arcangelo et al., 1993).

In this paper, we have further examined the mechanism for NGF regulation of membrane excitability in PC12 cells. We find that, contrary to all expectations, a 1 min exposure of PC12 cells to NGF is sufficient to cause long-term excitability. The excitability is due to selective expression of the PN1 gene, in the absence of induction of the brain type II gene, suggesting an important physiological role for this new pathway in peripheral nerve development. The PN1 induction elicited by NGF treatment, in addition to being Ras-independent (D'Arcangelo and Halegoua, 1993), requires prior synthesis of immediate-early genes. A Ras-independent signal transduction pathway also mediates immediate-early gene induction by the cytokine interferon-γ (IFNγ), and this pathway is shared by other growth factors (Bonni et al., 1993; Fu and Zhang, 1993; Ruff-Jaminson et al., 1993; Sadowski et al., 1993; Silvennoinen et al., 1993; Darnell et al., 1994). Indeed, 1 min treatments of PC12 cells with IFNγ, epidermal growth factor (EGF), and basic fibroblast growth factor (bFGF) also trigger PN1 gene induction, suggesting that they share intermediates in the "triggered" pathway.

## Results

### A 1 min Pulse of NGF Causes the Appearance of Functional Sodium Channels

PC12 cells were treated for only 1 min with NGF and then examined for sodium channel expression several hours after removal of the factor. Northern blot analysis (Figure 1A) indicated that, at 5 hr following removal of NGF, levels of PN1 sodium channel mRNA were induced to the same extent in briefly treated (11-fold) and continuously treated (11.5-fold) cultures. In contrast to the effects of continuous NGF treatment, brief treatment did not elicit either the transient decrease or the long-term increase in expression of brain type II/IIA mRNAs (Mandel et al., 1988; D'Arcangelo et al., 1993). Brief treatment with NGF was also not sufficient to induce the growth factor–responsive gene transin (Machida et al., 1989; Figure 1A). This result indicates that the NGF was effectively removed by the 1 min pulse protocol (see Experimental Procedures).

Associated with the triggered PN1 gene induction was the production of functional voltage-gated sodium channels (Figure 1B). The induced voltage-activated sodium current in the briefly treated cells was indistinguishable, in its kinetics and voltage dependence (data not shown), from the sodium currents in PC12 cells treated continuously with NGF (Mandel et al., 1988; D'Arcangelo et al., 1993). In contrast to membrane excitability, a brief exposure to NGF did not result in the elaboration of other major neuronal phenotypic traits such as the cessation of cell

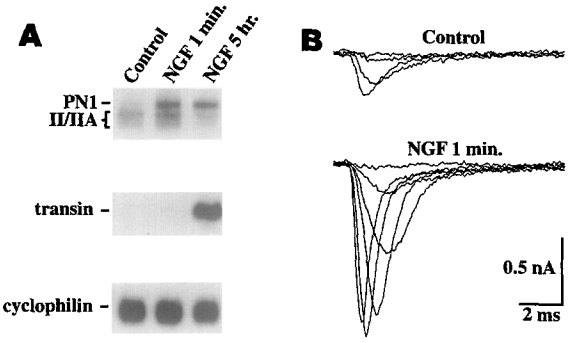


Figure 1. A 1 min Pulse of NGF Triggers the Induction of PN1 Sodium Channel mRNA and Sodium Current in PC12 Cells

(A) Northern blot analysis. Total cellular RNA (10 μg) was prepared from PC12 cells 5 hr after either 1 min or continuous treatment with NGF. The RNA was hybridized with the sodium channel probe pRB211. The PN1 (11 kb) and type II/IIA (9.5–10.5 kb) sodium channel transcripts are indicated. To confirm that the 1 min treatment was effectively terminated, the blot was rehybridized with a probe specific for the growth factor–responsive gene transin (1.9 kb). Rehybridization with a probe for cyclophilin (1 kb) provided an internal standard for the amount of mRNA in each lane. In 11 experiments, the fold inductions correspond to 11 ± 1.8 (mean ± SEM) for briefly treated and 11.5 ± 2 for continuously treated cells.

(B) Electrophysiological analysis. Voltage-activated sodium currents were recorded either from nontreated (control) PC12 cells or from PC12 cells 24 hr after a 1 min treatment with NGF. A control cell with unusually large sodium current is shown to compare more easily the properties of the current with that in the representative NGF-treated cell. The sodium currents were elicited by holding the cell membrane potential at −100 mV and applying depolarizing pulses to −40, −30, −20, and −10 mV (control) or to −55, −50, −45, −35, −25, and −15 mV (NGF-treated).

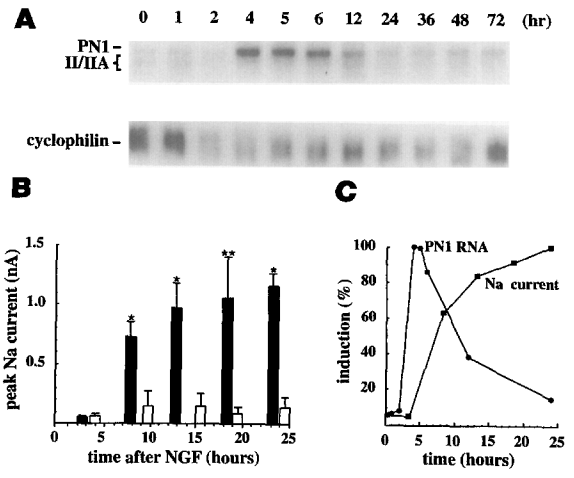


Figure 2. Membrane Excitability Appears Rapidly with Brief NGF Treatment and Follows the Peak of PN1 Sodium Channel mRNA Induction

(A) Time course of PN1 sodium channel mRNA induction. Total RNA (10 μg) was isolated from PC12 cells at the indicated times after 15 min of NGF treatment. The RNA was analyzed by Northern blotting, hybridized to the pRB211 probe, and rehybridized with the cyclophilin probe p1B15. The positions of the PN1 sodium channel (11 kb), type II/IIA sodium channel (9.5–10.5 kb), and cyclophilin (1 kb) mRNAs are indicated.

(B) Histogram of the peak sodium current in PC12 cells measured at the indicated times after a 1 min treatment with NGF. The closed bars indicate NGF-treated cells, and the open bars indicate nontreated PC12 cells. The control cells had the culture media changed at time 0, in lieu of NGF treatment. Electrophysiological measurements were performed using PC12 cells of similar size. The values shown are the mean ± SEM ($n = 5$). The differences in current between control and cells pulsed with NGF are significant (single asterisk, $p < .002$; double asterisk, $p < .02$) according to the two-tailed Student's t test.

(C) Plot of PN1 RNA and sodium current levels at different times after a brief NGF treatment. The data are derived from the experiments shown in (A) and (B) and are normalized to the maximal induction. Levels of PN1 mRNA were determined by densitometric analysis of the autoradiograms and normalized to the levels of cyclophilin mRNA quantitated in the same manner, always using exposures in the linear range of the film.

division or the growth of neurites (Greene and Tischler, 1976).

A time course comparing PN1 mRNA and sodium current induction suggested that the increase in PN1 gene expression was responsible for the acquisition of membrane excitability after brief treatment with NGF. Northern blot analysis indicated that levels of PN1 transcripts began to increase by 3 hr after removal of NGF, peaked between 4 and 5 hr, and declined slowly to near basal levels by 24 hr (Figure 2A). A comparison of PN1 mRNA induction after 1 or 15 min treatments with NGF indicated identical time courses and magnitudes of induction (e.g., compare Figure 1A and Figure 2A). Voltage-activated sodium current was induced approximately 6-fold, as soon as 8 hr after the brief exposure to NGF, and persisted for at least 24 hr (10-fold induction; Figure 2B). The induction of this sodium current following the selective induction of PN1 mRNA (Figure 2C) is consistent with a previous report on PC12 cells treated continuously with NGF (D'Arcangelo et al., 1993).

### PN1 Induction Is Transcriptional and Requires Prior Expression of Immediate-Early Genes

To begin to examine the mechanism of triggered gene induction by NGF, the dependency of induction upon new transcription and translation was determined. The presence of the RNA synthesis inhibitor 5,6-dichlorobenzimidazole riboside (DRB; Sehgal et al., 1976) prevented induction of PN1 mRNA up until the time of maximal mRNA induction (Figure 3A). The presence of the protein synthesis inhibitor cycloheximide (CH; Sisler and Siegel, 1967) also inhibited the induction of PN1 mRNA, but only during the first 1.5 hr of CH treatment (Figure 3B). A comparison of the requirements for RNA and protein synthesis showed that by 2 hr of NGF treatment, PN1 mRNA induction was independent of prior protein synthesis but still dependent upon new RNA synthesis (Figure 3C). These results indicate that the NGF-triggered increase of PN1 mRNA is, at least in part, transcriptional. When CH is added between 2 and 4 hr after NGF treatment, a time at which PN1 induction is still sensitive to DRB, there is no effect on PN1 gene induction. This observation suggests that the sensitivity to CH during the first 1.5 hr is due to the requirement for prior immediate-early gene induction, and not to the presence of a labile transcriptional activator.

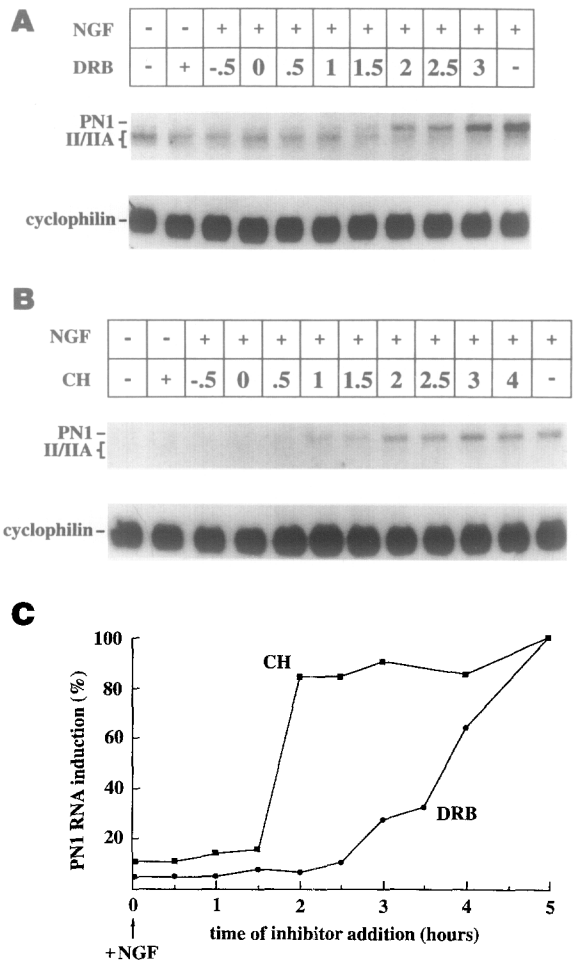


Figure 3. The Induction of PN1 mRNA Requires De Novo Synthesis of Both RNA and Protein

(A) Requirement for RNA synthesis. PC12 cells treated with NGF (20 ng/ml) were cultured in the absence or presence of 200 μM DRB, the inhibitor of RNA synthesis. DRB was applied at the indicated times (in hours), relative to NGF addition. Total RNA was isolated from the cells at 5 hr following addition of NGF to the medium and analyzed by Northern blotting. The positions of the PN1 and type II/IIA sodium channel mRNAs and cyclophilin mRNA are indicated.

(B) Requirement for protein synthesis. The translational inhibitor cycloheximide (CH; 10 μg/ml) was added to NGF-treated (50 ng/ml) PC12 cells, using the same treatment and RNA analysis protocols as in (A).

(C) Sensitivity of PN1 mRNA induction to transcriptional and translational inhibition. Plot of the level of PN1 mRNA induction versus time of addition of the transcriptional (DRB; circles) or translational (CH; squares) inhibitor after NGF treatment (arrow). The PN1 mRNA values were obtained from the experiments shown in (A) and (B) and normalized to the maximal induction.

### Brief (1 min) Exposures of PC12 Cells to IFNγ, as well as to EGF and bFGF, Induce PN1 mRNA

Previous studies have shown that PN1 gene induction, by continuous treatment of PC12 cells with NGF, is independent of Ras proto-oncoprotein activity (D'Arcangelo and Halegoua, 1993). A Ras-independent signal transduction pathway also mediates immediate-early gene induction by the cytokine IFNγ, and this pathway is shared by EGF (Fu and Zhang, 1993; Ruff-Jaminson et al., 1993; Sadowski et al., 1993; Silvennoinen et al., 1993; Darnell et al., 1994).

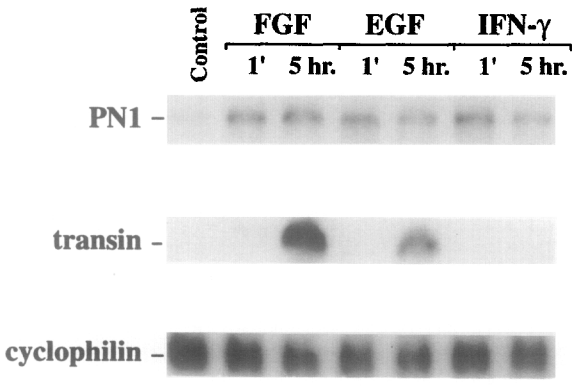


Figure 4. bFGF, EGF, and IFNγ Trigger Induction of PN1 mRNA

PC12 cells were treated for 1 min or continuously with recombinant bovine bFGF (20 ng/ml), mouse EGF (20 ng/ml), or recombinant rat IFNγ (100 U/ml). In all cases, total RNA was extracted 5 hr after exposure to the factor. The positions of the PN1, transin, and cyclophilin mRNAs are indicated.

We therefore tested whether PN1 gene induction could be triggered by a brief exposure to IFNγ, EGF, or bFGF. Indeed, the experiments in Figure 4 demonstrated that a 1 min exposure to each of these factors caused the induction of PN1 mRNA levels. The levels of induction for 1 min and continuous treatment, respectively, with each factor are as follows: bFGF, 5.63 ± 1.1 (n = 5) and 7.8 ± 2 (n = 6); EGF, 4.06 ± 0.82 (n = 7) and 4.92 ± 0.57 (n = 11); IFNγ, 6.73 ± 1.04 (n = 8) and 4.91 ± 0.98 (n = 8). The data are given as the mean of the fold induction ± SEM.

## Discussion

Our results demonstrate that a brief pulse of NGF can trigger a long-term change in neuronal phenotype, thus revealing a new mechanism of NGF action. We refer to the new pathway as being "triggered" because of its ability to engage irreversibly a transcriptional program of gene inductions. In PC12 cells, the program of gene inductions uncouples the regulation of the PN1 sodium channel from other sodium channels responsible for membrane excitability, as well as from the regulation of other phenotypic traits, such as neurite growth, which are dependent upon Ras activity (Hagag et al., 1986; D'Arcangelo and Halegoua, 1993). Although expression of the type II/IIA sodium channel mRNAs is also Ras-independent (D'Arcangelo and Halegoua, 1993; Fanger et al., 1993), only the PN1 sodium channel gene is induced by the brief treatment with NGF. In addition to its independence of Ras, the pathway to PN1 induction is also independent of extracellular calcium, and is not stimulated by depolarization with high potassium or by treatment of PC12 cells with phorbol esters (Toledo-Aral and D'Arcangelo, unpublished data), distinguishing it from all other NGF-stimulated signal transduction pathways.

The signal transducer and activator of transcription (STAT) family of transcription factors mediates cellular re-

sponses to IFNγ and EGF (Fu and Zhang, 1993; Ruff-Jaminson et al., 1993; Sadowski et al., 1993; Silvennoinen et al., 1993; Darnell et al., 1994). Because 1 min exposures of PC12 to IFNγ and EGF induce PN1 gene induction in a manner identical to NGF, it is possible that the STAT family is also involved in the triggered NGF pathway. Alternatively, other Ras-independent pathways may mediate the triggered PN1 induction. For example, another NGF-stimulated, Ras-independent pathway has been identified that involves the phosphorylation of SNT protein (Rabin et al., 1993). However, this pathway is not stimulated by EGF treatment of PC12 cells, reducing the probability that SNT protein is important in PN1 induction. It is not yet known whether the triggered pathway is specific for sodium channel gene induction. However, the recent demonstration that IFNγ induces the expression of choline acetyltransferase in basal forebrain rat neurons (Jonakait et al., 1994), cells known to be responsive to NGF and bFGF, suggests that other genes may also be induced through this pathway.

The triggered pathway provides a mechanism by which neurons can acquire membrane excitability in response to a wide variety of growth factor molecules that may be present during development only transiently. This feature could help explain the paradox posed earlier, that membrane excitability appears in vivo prior to the large increases in NGF normally associated with its other biological activities (Davies et al., 1987), and may account for the apparent functional "redundancy" of neural inducing molecules. Our results support previous suggestions that only short-term exposure to bFGF (2 hr) is required during early embryonic stages for neuronal differentiation (Kengaku and Okamoto, 1993) and could help explain the observed early period of sensitivity to mRNA synthesis inhibitors for the maturation of membrane excitability in developing spinal cord neurons (O'Dowd, 1983; Ribera and Spitzer, 1989). The specific induction of PN1 sodium channels by different growth factors opens the field to the suggestion of an important physiological role for the triggered pathway in the peripheral nervous system.

## Experimental Procedures

### Cell Culture

PC12 cells (Greene and Tischler, 1976) were grown on tissue culture dishes in Dulbecco's modified Eagle's medium (GIBCO BRL, Grand Island, NY) supplemented with 10% donor horse serum (JRH Biosciences, Lenexa, KS), 5% fetal bovine serum (JRH Biosciences), and 1% penicillin/streptomycin (GIBCO BRL) in an atmosphere of 10% $CO_2$ at 37°C. β-NGF was purified from mouse submaxillary glands (Mobley et al., 1976) and used at a final concentration of 100 ng/ml unless otherwise noted. Recombinant bovine bFGF (20 ng/ml; kindly supplied by A. Baird), mouse EGF (20 ng/ml; Collaborative Biomedical Products, Bedford, MA), and recombinant rat IFNγ (100 U/ml; GIBCO BRL) were used at the indicated concentrations.

To ensure the removal of residual NGF after a 1 min treatment, the pulsed PC12 cells were washed twice with culture media and then incubated in media containing a rabbit polyclonal antibody to NGF for the duration of the experiment. This "pulse" protocol was deemed sufficient because the transin gene was no longer induced under these conditions, and because transfer of media containing NGF antibody (from a dish of cells previously treated with NGF) to a new plate of cells did not result in induction of PN1 or transin mRNA levels (data not shown). The brief treatments with bFGF and EGF were performed as described for NGF, using in the wash polyclonal rabbit antibody for bFGF (kindly supplied by P. Maher) or EGF (Collaborative Biomedical Products), respectively. For the brief treatment with IFNγ, the cells were washed several times with new media after the 1 min exposure. In all of the experiments, the control cells were washed with fresh culture media at the time of the experiment, to avoid the possibility of serum-induced responses.

To inhibit the synthesis de novo of RNA or protein, the PC12 cells were cultured in the presence of 200 μM DRB (Sigma, St. Louis, MO; Sehgal et al., 1976) or 10 μg/ml CH (Sigma; Sisler and Siegel, 1967).

### Northern Blot Analysis

Total cellular RNA was isolated from PC12 cells by the method of Cathala et al. (1983). RNA samples (10 μg per lane) were electrophoresed through agarose gels containing 2.2 M formaldehyde, 40 mM MOPS buffer (pH 7), 10 mM sodium acetate, and 1 mM EDTA and then electrophoretically transferred to nylon membranes (Duralon-UV, Stratagene, La Jolla, CA). The blots were cross-linked by using a Stratalinker UV cross-linker (Stratagene) and hybridized to [α-$^{32}$P]UTP-labeled antisense RNA probes in hybridization buffer containing 5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 1× Denhardt's solution, 20 mM sodium phosphate, 50% SDS, and 0.1 mg/ml denatured salmon sperm DNA at 68°C for 16–24 hr. Antisense RNA probes were synthesized using the following linearized cDNAs as templates: pRB211, encoding a fragment of the type II/IIA sodium channel conserved among different sodium channels (Cooperman et al., 1987); pG7TR1, encoding transin (Machida et al., 1989; kindly provided in the vector pGEM7Zf+ by L. M. Matrisian), and p1B15 encoding cyclophilin (Danielson et al., 1988). All riboprobes were generated according to the manufacturer's instructions using SP6 polymerase (Stratagene). The blots were washed once in 2× SSC, 0.1% SDS at 68°C and twice in 0.2× SSC, 0.1% SDS for 20 min at 68°C, then subjected to autoradiography with preflashed Kodak XAR-5 films. Levels of gene expression were determined by densitometric analysis, and all values were normalized to the autoradiographic density corresponding to cyclophilin mRNA.

### Electrophysiology

The whole-cell configuration of the patch-clamp technique (Hamill et al., 1981) was used to measure sodium currents in the PC12 cells. The studies were performed by holding the cell membrane potential at −100 mV and stepping to various potentials for 10 ms durations, at 5 s intervals. The external solution contained 140 mM NaCl, 1 mM KCl, 1 mM $CaCl_2$, and 10 mM Na–HEPES (pH 7.2). The internal solution contained 140 mM CsCl, 10 mM K–EGTA, and 10 mM Na–HEPES (pH 7.2). Data were digitized at 50 μs intervals via an ITC-16 A/D converter and acquired on an ATARI Mega-4 computer. Current measurement and analysis were performed on individual stored current traces using Instrutech software (Acquire and Review). Whole-cell recordings were performed on cells within 1 hr of placement in the external recording solution at room temperature.

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