MYC AND E1A ONCOGENES ALTER THE RESPONSE OF PC12 CELLS TO NERVE GROWTH FACTOR AND BLOCK DIFFERENTIATION

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ABSTRACT

PC12 rat pheochromocytoma cells respond to nerve growth factor (NGF) by neuronal differentiation and partial growth arrest. Mouse c-myc and adenovirus E1A genes were introduced into PC12 cells to study the influence of these nuclear oncogenes on neuronal differentiation. Expression of myc and E1A blocked morphological differentiation and caused NGF to stimulate rather than inhibit cell proliferation. NGF binding to cell surface receptors, activation of ribosomal S6 kinase, and ornithine decarboxylase induction were similar in myc and E1A expressing clones compared with wild-type PC12 cells, suggesting that changes in the cellular response to NGF were at a post-receptor level. The ability of myc and E1A expression to block the transcription-dependent induction of microtubule associated proteins by NGF further suggested that these genes may inhibit differentiation by interfering with NGF's ability to regulate transcription. These results illustrate that NGF can promote either growth or differentiation of PC12 cells, and that myc or E1A alter the phenotypic responses to growth factors

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ABREVIATIONS

NGF	Nerve Growth Factor
EGF	Epidermal Growth Factor
CT	Cholera Toxin
DME	Dulbecco's Modified Eagle's Medium

CHAPTER I
INTRODUCTION

HISTORICAL PERSPECTIVE

Since its discovery in the early 1950's, nerve growth factor (NGF) has become the prototype of neurotrophic factors (reviewed: Thoenen and Barde, 1980; Yanker and Shooter, 1982; Thoenen and Edgar, 1985; Levi-Montalcini, 1987). The identification of NGF was a consequence of early experiments performed by Bueker (1948) who demonstrated the invasion of sensory neurons from the dorsal root ganglia of a chick embryo into a mouse sarcoma transplanted into the body wall of the embryo adjacent to the ganglia. Levi-Montalcini and Hamburger extended this work to show that sympathetic ganglia also participated in the nerve ingrowth to the tumor and most significantly, that ganglia not in direct connection with the tumor also underwent extreme hyperplasia (Levi-Montalcini and Hamburger, 1951 and 1953). These observations led to the suggestion that the sarcoma tissue released a diffusible factor into the circulation which had nerve growth promoting effects. Direct evidence supporting this hypothesis came from in vitro tissue culture experiments in which co-cultivation of sensory or sympathetic ganglia with sarcoma tissue resulted in marked nerve fiber outgrowth from the ganglia explants (Levi-Montalcini et al., 1954).

Stanley Cohen was instrumental in the continued progress of NGF research. Through the identification of potent sources of NGF, Cohen was able to partially purify NGF and subsequently produce NGF antibodies (Cohen, 1960). The resulting destruction of the peripheral sympathetic

system after injection of these NGF antibodies into neonatal mice provided strong evidence for a physiological role of NGF (Cohen, 1960). These pioneering studies laid the foundation for further research which demonstrated participation of NGF in at least three processes of the developing nervous system: cell survival, axonal orientation, and neuronal differentiation.

One of the most dramatic processes in neural morphogenesis is the regression of neurons by cell death. Transplantation and ablation experiments indicate that survival of developing neurons is determined by innervation of their target tissues. It has been suggested that release of neurotropic factors from these target tissues determines neuronal survival. In support of this proposal, NGF has been shown to be required for the survival of developing sympathetic and sensory neurons both in vivo and in vitro (Thoenen and Barde, 1980; Yankner and Shooter, 1982).

The accurate formation of synaptic connections requires that growing axons be oriented in the proper direction. A chemotactic effect of NGF has been demonstrated both in vivo and in vitro by the rapid reorientation of the axonal growth cone to a gradient of NGF (Levi-Montalcini and Hamburger, 1951 and 1953). Release of NGF from specific tissues may be directly responsible for the growth of neurons to that tissue.

Finally, NGF appears to be directly involved in the neuronal differentiation of several neural crest-derived tissues including sympathetic and certain sensory neurons, as well as cells of the adrenal medulla (Thoenen and Barde, 1980; Yankner and Shooter, 1982). NGF's capacity to modulate phenotypic expression has been most thoroughly characterized in the

pheochromocytoma cell line, PC12 (Greene and Tischler, 1976; Tischler and Greene, 1978). Unlike primary neuronal cultures, PC12 cells do not require NGF when grown in serum containing medium. Under these conditions they lack neurites, replicate, and possess the phenotypic properties of their non-neoplastic counterpart, adrenal chromaffin cells. These properties include the presence of chromaffin granules and the capacity to synthesize, store and release catecholamines (Greene and Rein, 1977). Upon exposure to NGF, PC12 cells partially growth arrest and acquire several properties of sympathetic neurons, including the outgrowth of long branching neurites, the appearance of evocable sodium action potentials, the presence of synaptic-like vesicles; and the enhanced synthesis of several neuronal proteins.

Because PC12 cells can be grown in the absence of NGF but undergo a well characterized differentiation program upon its exposure to NGF, these cells provide an excellent model system for studying the initial responses to NGF. Thus, PC12 cells are valuable for studying early signalling mechanisms activated by the binding of NGF to its receptor and for identifying rapid events committing the cell to differentiation. In addition, since these cells replicate they can be used to genetically dissect NGF's mechanisms either by somatic mutation or by introducing a specific gene into the cells through transfection.

BIOLOGICAL ACTIONS OF NGF IN PC12 CELLS

An outline of the temporal events that are known to occur during the differentiation program of PC12 is given in Table 1. The initial step in the differentiation of PC12 cells is the binding of NGF to specific cell surface

Table 1: Summary of biological events known to occur in PC12 cells in response to NGF

TIME	BIOCHEMICAL EVENT	REF
0	Binding of NGF to its receptor	Buxser et al., 1985a
	Conversion from a low affinity monomer to a high affinity dimer	
5-15 m	Plasma membrane ruffling	Schubert et al., 1978
	Increased activity Na+/K+ and Na+/H+ pumps	Varon and Skaper, 1983
	Increase in cytosolic Ca2+	Pandiella-Alonso et al., 1986
	Increased metabolic transport	Greene and Tischler, 1982
	Stimulation phosphoinositol turnover	Traynor et al., 1983
	Activation Protein Kinase C	Hama et al., 1986
	Activation S6 protein kinase	Halegoua and Patirck, 1980
	Phosphorylation of specific proteins	
15m-2h	Induction of transcription: c-fos, c-myc, actin and ODC	Greenberg et al., 1985
24 h	2-fold increase mRNA, tRNA, rRNA	Gunning et al., 1981
	Increase in somatic size	Greene and Tischler, 1982
	Increase in cell adhesion	Schubert and Whitlock, 1977
	Down-regulation of EGF receptor	Lazarovica et al., 1987
	Short neurite processes	Greene and Tischler, 1976
3d	Induction of neurotransmitters & related enzymes	Greene and Tischler, 1982
	Induction of HMW MAPs, tau proteins	Drubin et al., 1986
	Increase in microtubule polymers	Drubin et al., 1986
	Stable neurites	Greene and Tischler, 1976
7d	Induction of tubulin	Drubin et al., 1986
	Long branching neurites	Greene and Tischler, 1976
	Cessation of DNA synthesis	Greene and Tischler, 1976

receptors on the plasma membrane (reviewed, Buxser et. al , 1985a). Kinetic and equilibrium binding studies have revealed two apparent affinity states of the receptor (Yanker and Shooter, 1979; Landreth and Shooter, 1980; Bothwell and Schecter, 1981; Buxser et al., 1983). The high affinity NGF-receptor complex is trypsin resistant and has an apparent K_d of 0.3 nM. The second NGF-receptor state has a lower affinity for ^{125}I-NGF (K_d 5.2 nM) and is trypsin sensitive. Furthermore, photoaffinity crosslinking experiments with ^{125}I-NGF (Buxser et al., 1983), and analysis of highly purified NGF-receptor by SDS-PAGE electrophoresis (Puma et al., 1983) reveal two proteins with molecular weights of 80 and 200 kDa. Partial proteolytic peptide mapping indicates that the 200 and 80 kDa NGF binding proteins are indistinguishable (Puma et al., 1983).

A model to explain the molecular interactions between NGF and its receptor based on the structure and binding properties of the NGF receptor and the dimeric structure of β-NGF, has been recently proposed (Buxser et al., 1985b). β-NGF is composed of two identical 13 kDa peptides which are non-covalently associated to form a 26 kDa molecule (Angelletti and Bradshaw, 1971; Greene et al., 1971). The dimeric structure appears to be important functionally, since attempts to produce biologically active monomeric β-NGF have not been successful. The dimeric structure of β-NGF may allow each 13 kDa monomer of the molecule to bind one 80 kDa receptor. The hypothesis further proposes that low affinity rapidly dissociating binding results when one of the subunits of β-NGF associates with a single 80 kDa receptor molecule. Binding of the second subunit of β-NGF to a separate receptor molecule produces a dimeric receptor structure

bridged by the dimeric β-NGF (200 kDa complex). Since dissociation of NGF from two receptors simultaneously is significantly less probable than dissociation from a single receptor, the resulting 200 kDa complex has a slower dissociation rate and a higher apparent affinity. Consistent with this model, Green and co-workers (1986), have isolated chemically mutagenized PC12 cells whose failure to respond to NGF is correlated with the absence of high affinity binding sites. These results suggest that the high affinity NGF receptor mediates the biological actions of NGF.

In spite of the rapid progress in defining the kinetic and structural features of the NGF receptor, it is still not known how the receptor "signals" the cell to undergo neuronal differentiation. Recent cloning of the receptor (Johnson et al., 1986) has provided no further clues to a possible signalling mechanism. While the NGF receptor shares general structural features with other growth factor receptors, there is no homology with growth factor receptors containing intrinsic tyrosine kinase activity or with other known tyrosine or serine/threonine specific protein kinases. This is consistent with the failure of several laboratories to demonstrate an intrinsic tyrosine kinase activity of the purified NGF receptor (Boonstra et al., 1985). In addition, the predicted amino acid sequence does not contain structural features common to receptors which serve as ion channels. Finally, the receptor lacks amino acid homology with proteins which are presently known to interact with GTP-binding proteins (G-proteins), as well as the conserved amino acid residues involved in GTP binding to G-proteins. This indicates that the receptor is not a G protein but does not exclude the possibility that the receptor interacts with an unknown G-protein.

Recent experiments predict that the NGF signalling pathway may indeed include the interaction of the receptor with a GTP-binding protein. Microinjection of the v-ras protein (p21) into PC12 cells (Bar-Sagi and Feramisco, 1985) or infection of these cells with ras-containing retrovirus (Noda et al., 1985) leads to apparent growth arrest and morphological differentiation. Moreover, microinjection of the monoclonal antibodies to Ha v-ras p21 inhibits NGF-induced neurite outgrowth (Hagag et al., 1986). While the simplest interpretation of these results is that the ras proto-oncogene mediates NGF's actions, one must be cautious about such a conclusion. First, microinjection of c-ras into PC12 does not produce the same effects on cellular differentiation as does injection of v-ras (Bar-Sagi and Feramisco, 1985). It is perhaps not surprising that the expression of exogenous v-ras produces different effects in PC12 cells since v-ras p21 may escape the normal regulatory controls that c-ras p21 is subjected to. Second, the ability of the anti-ras p21 antibody to inhibit NGF function in PC12 cells may be explained by cross-reactivity of this antibody in vivo with a yet unidentified G-protein. The antibody used in this study does in fact bind weakly to G_s and G_i proteins (Beckner et al., 1985). Third, there may be multiple pathways for induction of neuronal differentiation in PC12. Clearly many agents are capable of inducing cell proliferation within a given cell type, therefore it is not unreasonable to speculate that a variety of agents may also induce a specific differentiation state. This is illustrated by the fact that fibroblast growth factors (FGFs) mimic many of NGF's effects in PC12 cells (Rydel and Greene, 1987; Togari et al., 1983) even though FGFs apparently utilize a different receptor system (Neufeld and Gaspadarowica, 1986).

Furthermore, infection of PC12 cells with Rous sarcoma virus containing the v-src oncogene also induces morphological alterations associated with neuronal differentiation (Alemo et al., 1985). Thus, while these studies support a possible role of G-proteins in the NGF-induced pathway of neuronal differentiation they do not provide conclusive evidence that c-ras is the G-protein involved.

It has also been postulated that cAMP mediates NGF's effects in PC12 cells. This hypothesis is based on the observation that cAMP and NGF elicit similar protein phosphorylation patterns (Halegoua and Patrick, 1980; Cremins et al., 1986) and on the ability of cAMP to mimic NGF's induction of neurites (Schubert et al., 1977; Schubert et al., 1978). However, cAMP-induced neurites are clearly distinct from those induced by NGF. The transient neurites produced in response to cAMP are short and unbranched. In contrast, NGF produces long, branched neurites which are maintained in the continued presence of NGF. Furthermore, several lines of evidence indicate that NGF and cAMP elicit their effects on neurite outgrowth by dissimilar mechanisms. First, RNA synthesis inhibitors block NGF's induction of neurites but do not interfere with the ability of cAMP to direct neurite extension (Burstein and Greene, 1978; Greene et al., 1982). Second, NGF and cAMP act synergistically to produce neurites that are longer and more branched than those produced by either agent alone (Gunning et al., 1981; Heidemann et al., 1985). In addition, NGF does not appear to require cAMP as a second messenger since cAMP antagonists do not block NGF-induced morphological differentiation (Richter-Landsberg and Jastroff, 1986). Finally, NGF does not activate adenylate cyclase (Race and Wagner,

1985) or cAMP-dependent protein kinase (L. Heasley, unpublished observations) indicating that NGF's actions are not mediated through the classic adenylate cyclase system.

Internalization of NGF and retrograde transport of the molecule along the axons to the cell bodies of differentiated neurons is well documented (Levi et al., 1980; Stockel et al.,1974; Stockel and Thoenen, 1975). While several lines of evidence suggest that these mechanisms may be involved in some of NGF's actions, NGF does not act as its own second messenger to promote neuronal differentiation. This is illustrated by the failure of microinjected NGF to promote neurite outgrowth as well as by the inability of microinjected anti-NGF antibodies to block NGF-induced morphological differentiation in PC12 cells (Heuman et al., 1981; Seeley et al., 1983). Furthermore, the time course for internalization is too slow to account for early cellular responses to NGF (Buxser et al., 1983). In PC12 cells, the major pool of internalized NGF appears to be targeted to the lysosome for degradation (Kasaian and Neet, 1988).

Perhaps the most significant reason for the difficulty in defining NGF's signalling mechanism is the inability to identify early events which contribute directly to the neuronal phenotype. As shown in Table 1, all the events known to occur rapidly in response to NGF are those typically elicited by mitogenic factors rather than differentiation-promoting agents. Early events occuring within minutes of NGF binding include membrane ruffling (Schubert, et al., 1978; Connolly et al., 1979 and 1981), increased levels of cytosolic calcium (Pandiella-Alonso et al., 1986), and activation of the Na^+/K^+ and Na^+/H^+ pumps (Boonstra et al., 1981 and 1983; Varon and

Skaper, 1983). NGF also enhances the turnover of phosphoinositols resulting in an increased production of diacyglycerol and increased activity of protein kinase C (Contreras and Guroff, 1987; Traynor et al., 1983; Hama et al., 1986). Several proteins are phosphorylated rapidly in response to NGF (Halegoua and Patrick, 1980; Landreth and Rieser, 1985; McTigue et al., 1985), and at least one of these, tyrosine hydroxylase is phosphorylated in vitro by protein kinase C (Cremins et al., 1986). Nerve growth factor also stimulates within minutes of its addition the S6 protein kinase and correspondingly, an increased phosphorylation of its known substrate, the ribosomal S6 protein (Blenis and Erikson, 1986; Matsuda and Guroff, 1987).

In addition to these non-transcriptional events, NGF also induces rapid and transient increases in the transcription of several genes including c-fos , c-myc, actin and ornithine decarboxylase genes (Greenberg et al., 1985; Feinstein et al., 1985; Curran and Morgan, 1985). c-fos transcription is increased greater than 100-fold within 5 minutes of NGF addition, peaks between 15-30 min and is rapidly shut-off by 60 minutes. Similarily, actin transcription is also induced by 5 minutes, but transcription peaks within 30-60 min and returns to its basal level by 2 hours. In contrast to c-fos and actin transcription, the level of c-myc transcription peaks within 1-2 h after NGF treatment and remains high for several hours. Transcription of the gene for ornithine decarboxylase, the rate limiting enzyme in polyamine synthesis, is induced by 2 hours and reaches its greatest level by 4 hours after NGF addition. The corresponding mRNA's for each of these genes are induced with time courses similar to their rates of transcription.