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COLLEGE ON NEUROPHYSICS

"Neural correlates of behaviour, development, plasticity and memory"

1-19 October 1990

Strategies of perturbation of the function and the development of neural networks

A. Cattaneo
Institute of Neurobiology, CNR
Rome, Italy

Major contributions to the understanding of the (development an function of the) mammalian brain have come in recent years from

- global recording techniques
  - PET scanning
  - NMR, magnetic activity etc.
  - optical parallel recording

  ("functional anatomy")
- computational neuroscience
  (insight into the nature of the problems dealt with by the nervous system)
- spin glass models of neural networks
  [help in - defining concepts
  - posing new questions
  - role (or independence from) randomness
  - inverse statistical mechanics]
- "Simple" nervous systems
  - Aplysia, Drosophila

  (molecular and cellular explanations for memory and learning processes)

  but the logic of invertebrate and vertebrate nervous system may be totally different!

What is lacking?

- General methods to perturb in a selective and specific manner the development and/or function of subsets of neurons.

  Working out such a general strategy should allow to address important questions

- utilize the tools of molecular biology

enetics at the level of the phenotype

pproaches used so far in mammalian cells

- GENETIC ABLATION
- ANTISENSE RNA
- GENE DISRUPTION BY HOMOLOGOUS RECOMBINATION
- RIBOZYMES

GENETIC ABLATION

- Regulatory elements from highly tissue-specific genes linked to toxic genes, such as A subunit of diphteria toxin* or ricin.
  As a consequence, cells expressing the toxin are killed.
- Killing can be made conditional, if the thymidine kinase gene from herpes virus is used, instead of DT-A or ricin. Expression of the tK gene from HSV confers sensitivity to the drug acyclovir (but only dividing cells can be killed)

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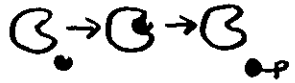
* DT-A catalyzes the NAD-dependent ADP-ribosylation of elongation factor 2, resulting in the inhibition of protein synthesis.

R-A acts as a ribonuclease specific for the 28S ribosomal RNA.

A



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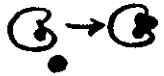

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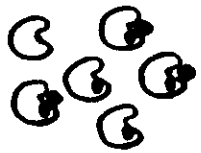
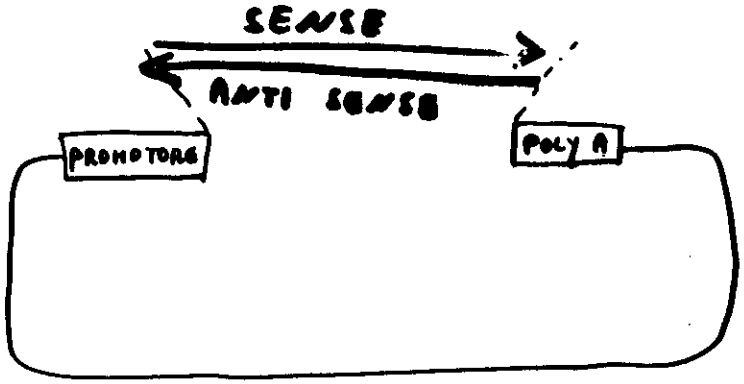
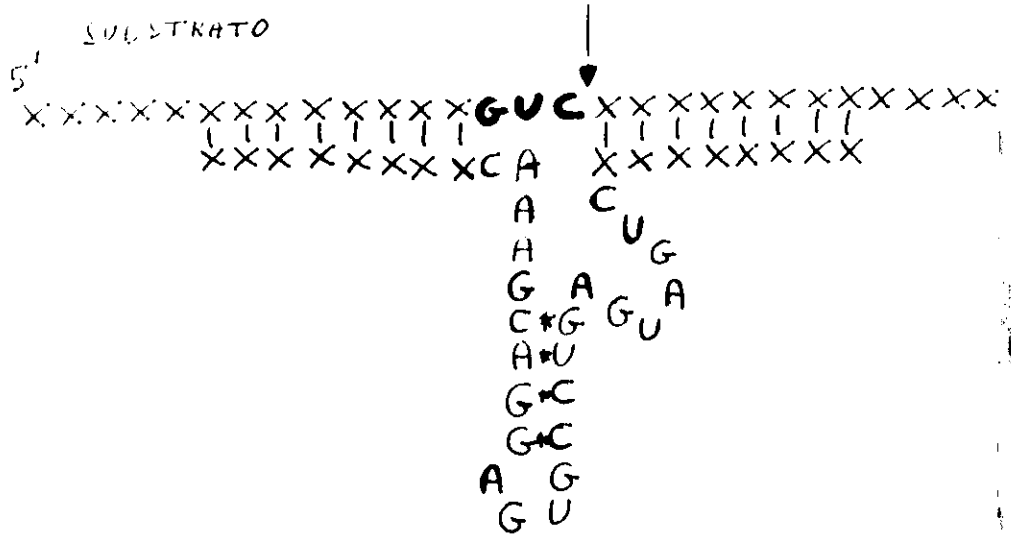




RIBOZIMI



GENE DISRUPTION BY HOMOLOGOUS RECOMBINATION

- Target homologous recombination events to genes whose inactivation is selectable
  e.g. Hprt → hypoxanthine phosphoribosyl transferase

Recombination frequency ~ 10^{-3}

- Technique has been refined to achieve disruption of non selectable genes

⇒ POSITIVE NEGATIVE SELECTION

- Positive selection for cells that have integrated the vector (either by legitimate or illegitimate recombination
- Negative selection against cells that have randomly integrated the vector.

⇒ results in enrichment for cells in which bona fide homologous recombination events have occured.

'lection procedure that enriches 2,000-fold for those cells that contain a targeted the isolation of hprt⁻ *and* int-2⁻ *mutants, but it should be applicable to any gene.*

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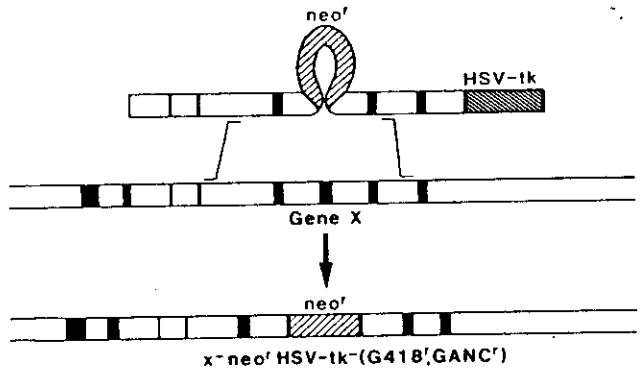
a **Gene Targeting**



b **Random Integration**

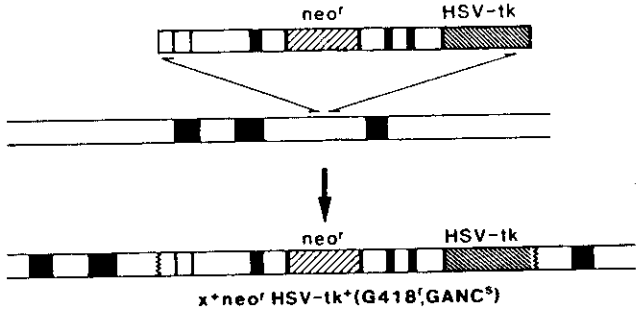


Fig. 1 The PNS procedure used to enrich for ES cells containing a targeted disruption of gene *X*. *a*, A gene *X*-replacement vector, that contains an insertion of the *neo*^{r} gene in an exon of gene *X* and a linked HSV-*tk* gene, is shown pairing with a chromosomal copy of gene *X*. Homologous recombination between the targeting vector and genomic *X* DNA results in the disruption of one copy of gene *X* and the loss of HSV-*tk* sequences. Such cells will be X^-, *neo*^{r} and HSV-*tk*$^-$ and will be resistant to both G418 and GANC. *b*, Because non-homologous insertion of exogenous DNA into the genome occurs through the ends of the linearized DNA[9-11], the HSV-*tk* gene remains linked to the *neo*^{r} gene. Such cells will be X^+, *neo*^{r} and HSV-*tk*$^+$ and therefore resistant to G418 but sensitive to GANC. Open boxes denote introns or flanking DNA sequences, closed boxes denote exons and cross-hatch boxes denote the *neo*^{r} or HSV-*tk* genes.

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THE NEUROANTIBODIES APPROACH

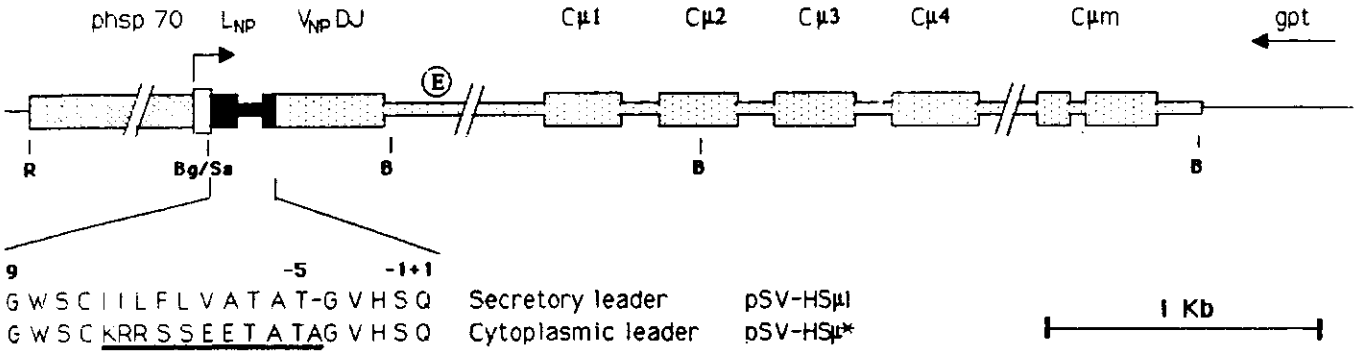
Two fields of molecular biology provide the prerequisite for this approach

- Availability of GENE TRANSFER techniques
- Monoclonal antibodies

Is it not possible to get cells of the nervous system to express antibodies against specific molecules, to block their function?

USES OF NEUROANTIBODIES

- Blocking of a selected class of synapses with antibodies against neurotransmitters, neuropeptides or their receptors
- Facilitation of synaptic transmission (e.g. with antibodies to acetylcholinesterase or to inhibitory synaptic transmitters)
- Destruction of selected neuronal subsets with antibodies against growth factors or their receptors, or with introcellular toxic antibodies.
- Preventing neurons to reach their final differentiated state (e.g. intracellular antibodies to signal transducing molecules


phsp 70
L_{NP}
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gpt
E
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Cytoplasmic leader
pSV-HSμI
pSV-HSμ*
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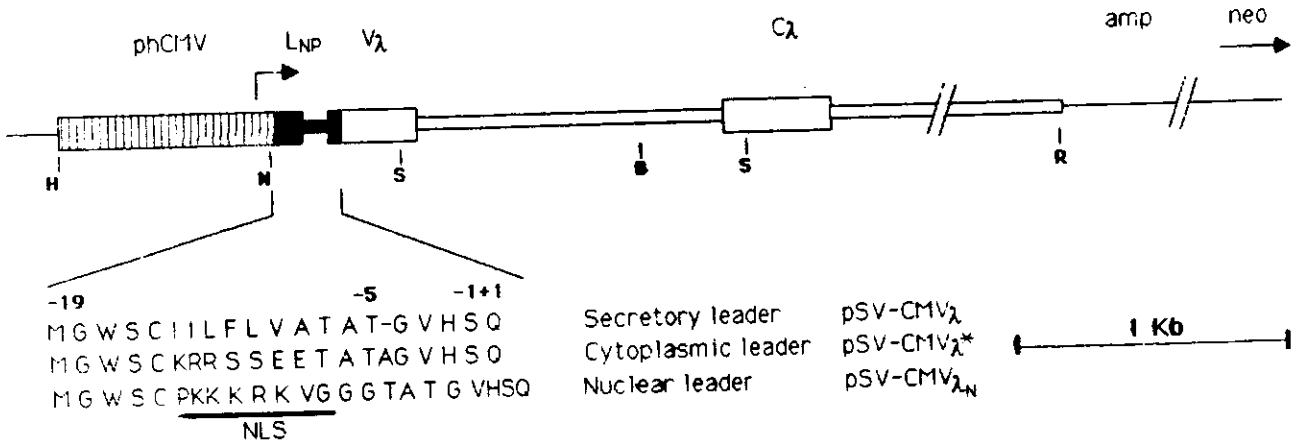
phCMV
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Nuclear leader
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pSV-CMV$_{\lambda}$*
pSV-CMV$_{\lambda_N}$
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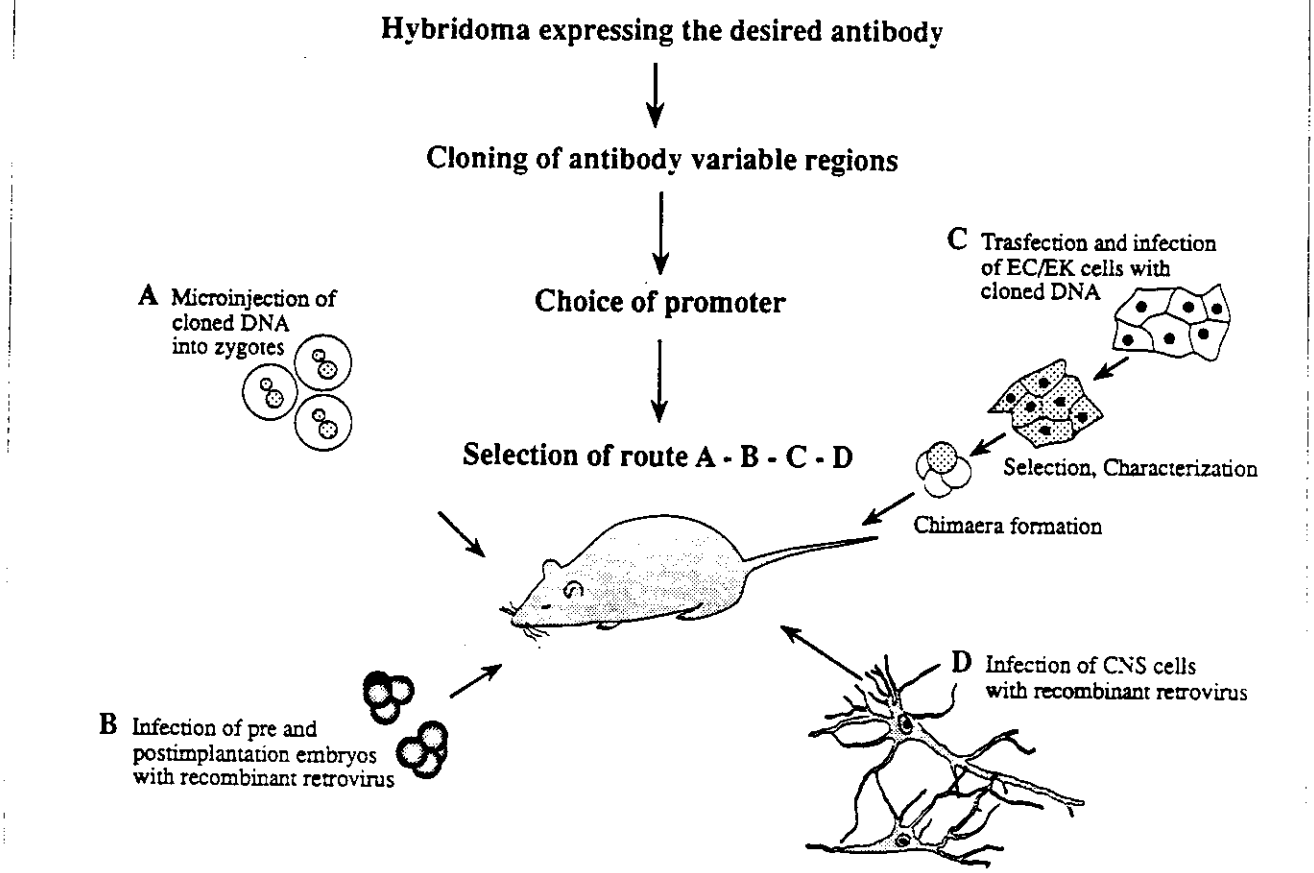
Routes for introducing immunoglobulin genes into mice



The Mode of Action of Nerve Growth Factor in PC12 Cells

A. Levi, S. Biocca, A. Cattaneo, and P. Calissano*

Institute of Neurobiology, CNR,
Viale Marx 15, 00156 Rome, Italy

Contents

*Author to whom all correspondence and reprint requests should be addressed.

Abstract

This review deals with the mechanism of nerve growth factor action. In view of the many and diversified effects of this growth factor, and since it could utilize different mechanism(s) in distinct types of cells, we have confined our analysis to the best characterized and more extensively studied target, the clonal cell line PC12.

When exposed to NGF in vitro, these neoplastic cells recapitulate the last major steps of neuronal differentiation, i.e., the commitment to become a neuron and the acquisition of the neuronal phenotype. This is characterized by electrically excitable neurites, a display of a highly organized cytoskeleton, and the specific chemical and molecular neuronal properties. These effects are elicited upon the interaction of NGF with a receptor whose gene has been cloned and whose kinetic properties are now relatively well charaterized. It is not yet clear, on the contrary, if and which of the several potential second messengers (cAMP, Ca, or phosphoinositides) that undergo marked fluctuations following NGF binding, transduce and amplify the NGF message. Among both the early and late effects of NGF is the modulation of expression of several genes. Some of the products of these genes are mainly restricted to nerve cells and others appear to play a crucial role in regulating the proper assembly of cytoskeletal elements.

It is hypothesized that this complex array of chemical, molecular, and ultrastructural changes is triggered by NGF, not through activation of a single pathway, but more likely via combinatorial processes whereby several intracellular signals interplay before the irreversible commitment of becoming a neuron is undertaken.

Index Entries: NGF, action of; PC12; neuronal differentiation; second messengers; intracellular signals.

Introduction

Several reviews have been published in recent years on NGF (Levi-Montalcini, 1987; Levi-Montalcini and Calissano, 1986; Calissano et al., 1984; Thoenen and Barde, 1980; Greene and Shooter, 1980; Bradshaw, 1978). The reader is referred to them for a comprehensive description of the historical background of the discovery of this protein, its molecular properties, and biological functions. This review is confined to the mechanism of NGF action.

The biological effects exerted by this growth factor are so numerous and qualitatively different that there is *a priori* no reason to postulate that they are all elicited through the same molecular mechanism. Each distinct type of target cell may be genetically programmed to respond to NGF in a different fashion. Thus it is possible, for instance, that distinct molecular pathways are activated by NGF 1) in neurite growth 2) during the chemotactic action on the growth cone (Levi-Montalcini 1976) (Gundersen and Barret, 1979); 3) in the mitogenic effect on chromaffin cells (Lillien and Claude, 1985); or 4) in the numerical increase of mast cells (Aloe and Levi-Montalcini, 1977; Bohm and Aloe, 1986). The present experimental evidence indicates that NGF acts during the late developmental stages, when cells derived from the neural crest are engaging, under the influence of epigenetic factors, in their final differentiative commitments. Among these cells are precursors that may give rise either to chromaffin or to sympathetic cells. Glucocorticoids impose the former choice whereas NGF, both in vivo (Aloe and Levi-Montalcini, 1979) and in vitro (Unsicker et al., 1978), channels these cells towards the sympathetic type. Although the studies on the biological properties of NGF were steadily progressing, those on its mode of action were proceeding much more slowly, and still would be, had not clonal cell line derived from a rat pheochromocytoma named PC12

come to the aid of the investigator. At variance with other NGF targets, PC12 cells (Tischler and Greene, 1975; Greene and Tischler, 1976) provide a homogeneous cell population that is available in large quantities and does not depend on the presence of NGF for its survival. Therefore, the vast majority of studies on the molecular aspects of the mechanism of NGF action has been performed with these cells and we will limit the present review to this particular experimental model. The reader is also referred to previously published reviews on the effects of NGF on PC12 cells (Greene and Tischler, 1982; Guroff, 1985).

PC12 cells, in response to NGF, acquire and maintain a neuronal phenotype by a process that has been operationally divided into two distinct phases. First, transcription dependent phase (priming), and second, a transcription independent one (regeneration) that takes place when neurite-bearing cells are dislodged from the culture dishes and subsequently replated in the presence of NGF (Burnstein and Greene, 1978; Greene et al., 1982). Both processes are simultaneously needed for the outgrowth of neurites to take place, in keeping with the observation that NGF must be continuously present in the culture medium in order to induce its effects (Greene, 1984). Some remarks are in order before analyzing the effects of NGF on PC12 cells:

1. The neoplastic nature of these cells raises the question as to whether it is justified to extrapolate all the findings on these cells to normal, untransformed NGF target cells and;
2. Not all the measurable effects elicited by NGF in PC12 cells are necessarily relevant to the acquisition and maintenance of the neuronal phenotype. Some may be related to the initial mitogenic action of NGF (Boonstra et al., 1983) or may be epiphenomena, i.e., side effects of the cascade of events set in motion by this growth factor.

Finally, we would like to stress that there is presently a growing recognition that the action of many growth factors may be responsible for the activation of several distinct and independent pathways in target cells in order to achieve the final biological response. This is particularly true for cells that undergo a process such as neuronal differentiation: the induction of several "second messengers" (possibly interacting between themselves) will activate distinct pathways necessary to accomplish such a complex task, and at the same time will guarantee, by a combinatorial mechanism, that the cell will not respond to false stimuli. Accordingly, NGF-receptor complexes formed at the level of the plasma membrane may interact with more than one transducing molecule and modulate the production of several second messengers.

This hypothesis, of course, does not rule out the possibility that in some NGF triggered events only a few of the combinatorial phenomena are set in motion. For instance, control of the motility of a growth cone (Gundersen and Barrett, 1979) or induction of secretion in mast cells (Bruni et al., 1982) may be achieved by a simple modulation of intracellular Ca^{2+}, concentration; mitotic stimulation of adrenal chromaffin cells (Lillien and Claude, 1985) may require a second step after, or in addition to, Ca^{2+} mobilization whereas an even more complex repertoire of responses may have to be triggered in order to induce neuronal differentiation.

This review has been divided in three distinct sections. The first one deals with the molecular properties of the NGF receptor, its kinetic behavior and the intracellular fate of NGF-receptor complexes. The second section is devoted to the analysis of the early effects elicited by NGF in PC12 cells. The third and last section is centered on the late effects that result in the acquisition of the neuronal phenotype by PC12 and include the modulation of the expression of neuronal specific genes and the supramolecular reorganization of cellular structures.

PC12 cells are the object of an extraordinary number of studies some of which are not directly related to the mode of action of NGF. We

apologize for not being able to mention all of these studies and for any inadvertent failure to cite other relevant contributions.

The NGF Receptor

Cloning of the Gene and Primary Structure

Monoclonal antibodies have been produced which recognize the NGF receptor on human melanoma cells (Ross et al., 1984), on rat PC12 cells (Chandler et al., 1984), and on rabbit sympathetic neurons (Morgan and Bradshaw, 1985). The availability of these antibodies has recently allowed the isolation of genomic and cDNA clones containing the entire coding sequence of the human NGF receptor (Chao et al., 1986; Johnson et al., 1986) and of the rat NGF receptor (Radeke et al., 1987). The human gene is localized in the chromosome region 17q12q22 distal to the 17 breakpoint present in acute leukemias (Heubner et al., 1986; Rettig et al., 1986). Analysis of the mRNA sequence suggests that in PC12 cells, the receptor is synthesized as a precursor with a molecular weight of 45432 dalton containing a 29 amino acid *N*-terminal signal peptide that is removed, leaving the mature form of 396 amino acids with a molecular weight of 42478 dalton. The *N*-terminal extracellular domain, very rich in cysteine residues, is characterized by four repeating elements with the cysteines at conserved positions. A similar structure with numerous disulfide bonds has also been observed in the extracellular domain of a number of membrane proteins including receptors for low density lipoprotein (LDL) (Yamamoto et al., 1984), epidermal growth factor (EGF) (Ullrich et al., 1984), insulin (Ebina et al., 1985: Ullrich et al., 1985), the *Drosophila* "notch" protein (Wharton et al., 1985), and the *neu* oncogene (Bargmann et al., 1985). However, computer analysis indicates very little homology between the NGF receptor and these proteins or any other polypeptide. The extracellular region of the protein appears to be very acidic and contains two putative sites for *N*-linked glycosilation. The remaining portion of the molecule consists of a single plasma membrane spanning domain and a small intracellular one that is essentially neutral in charge.

The cytoplasmic domain lacks the consensus sequence for an ATP binding site suggesting that the NGF receptor has no kinase activity. It is one of the smallest receptors so far cloned and is similar, in this respect, to the interleukin-2 (IL2) receptor which has 251 amino acid residues, half of its mass is carbohydrate and displays two different affinities for its ligand (Nikaido et al., 1984; Leonard et al., 1984).

The identification and cloning of the gene has only partially settled a long lasting controversy about the existence of one or more types of NGF receptors. Only a single mRNA species has been found even in those cells (PC12 cells or sensory neurons) that express two kinetically distinguishable NGF receptors (*see*, Kinetic studies).

Molecular Properties and Posttranslational Modifications

Several attempts have been made to purify the NGF receptor, to assess its molecular weight, and to identify potentially associated proteins. The first insight into the molecular properties of this polypeptide came from studies on the detergent extracted fraction from mature rabbit cervical ganglia (Costrini et al., 1979). However, the characterization of the molecule has relied essentially upon photoaffinity cross-linking procedures. When NGF is cross-linked to its receptor, depending on the reagent used as well as on the experimental conditions, two different molecular weights of the complexes are detected. Grob et al. (1983) and Green and Greene (1985) describe a 100–105 kD band, whereas Massague et al. (1982) and Hosang and Shooter (1985) find a species of 148–158 kD. A

minor component of approximately 200 kD, probably a result of dimerization, is also detected (Grob et al., 1983). Hosang and Shooter (1985) postulate that the 158 kD band is derived from the cross-linking of NGF, the monomeric receptor and a 60 kD protein possibly involved in the transduction of the NGF message.

Purification to near homogeneity of the NGF receptor has been obtained either by affinity chromatography on WGA columns (Puma et al., 1983) or by immunoprecipitation with a monoclonal antibody directed against this protein (Grob et al., 1985) from extracts of A875 melanoma cells. These cells, in fact, possess 10 times more receptors than PC12 cells (Fabricant et al., 1977).

Kinetic labeling studies have shown that the receptor is synthesized as a precursor of an apparent mol wt of 59 kD, slightly higher than the one deduced from the cDNA sequence. The molecule is subsequently glycoslylated via *N*-linked and probably *O*-linked sugar moieties to produce the mature form (Grob et al., 1985).

Phosphorylation of NGF receptors has been reported to occur only in serine and threonine residues and is apparently not affected by pretreatment of the cells with NGF (Taniuchi et al., 1986).

Kinetic Studies

A variety of studies have shown that PC12 cells possess NGF receptors that are comparable, in terms of kinetic and molecular properties, with those present on the sympathetic and sensory neurons (for review, *see* Greene and Tischler, 1982; Stach and Perez-Polo, 1987). At least two classes of ^{125}I-NGF binding sites have been described on the surface of PC12 cells on the basis of different criteria. By equilibrium binding analysis, high affinity NGF receptors with apparent Kd of 0.2nM and low affinity receptors with a Kd of 5 nM have been reported (Bernd and Greene, 1984; Green et al., 1986). When the dissociation rates of ^{125}I-NGF bound to PC12 cells are measured in the presence of an excess of unlabeled NGF, a percentage of the bound ^{125}I-NGF dissociates within seconds, whereas the remaining molecules dissociate with a slower kinetic (t 1/2 = 30 min). The fast and slow dissociating populations have been tentatively identified with, respectively, the low and the high affinity components reported above (Schechter and Bothwell, 1981; Landreth and Shooter, 1980). When the NGF binding to PC12 cells is measured at low temperatures, only the low affinity, fast dissociating component is detectable (Herrup and Thoenen, 1979). Moreover, if PC12 cells are fixed with a mild paraformaldehyde treatment and permeabilized with methanol only the fast dissociating, low affinity species is found (Cattaneo et al., 1983). The high affinity, slow dissociating component is not extractable by low concentrations of Triton-X 100 and is resistant to inactivation by diluted trypsin. NGF-binding to this class of receptors is inhibited by phenyl-methyl-sulfonyl-fluoride (PMSF) suggesting the presence of a reactive serine (Stach et al., 1986). This pool of receptors is postulated to be associated with some constituent(s) of the cytoskeletal matrix (Vale et al., 1985). The high affinity receptors are apparently the only ones responsible for the process of internalization of NGF (*see below*) (Bernd and Greene, 1984; Hosang and Shooter, 1987; Gunning et al., 1981) as well as for the induction of the biological effects (Green et al., 1986; Stach and Wagner, 1982).

Though it is possible that the heterogeneity in the properties of the receptors exists prior to NGF exposure, it is well established that the binding of this factor induces a conversion of low affinity to high affinity receptors in metabolically active cells (Landreth and Shooter, 1980; Block and Bothwell, 1983) and is characterized by positive cooperativity (Woodruff and Neet, 1986). A kinetically similar conversion can also be achieved by wheat germ agglutinin (WGA) (Vale and Shooter, 1982; Buxser et al., 1983; Vale and Shooter, 1985) or by cluster-

ing NGF-receptor complexes with anti-NGF antibodies (Vale and Shooter, 1983), but this does not necessarily imply that the same kind of modification has occurred. In fact, after WGA treatment, PC12 cells lose the capacity to respond to NGF with rapid ruffling of the membrane, *in situ* phosphorylation of a 250 kD cytoskeletal protein, and regeneration of neurites (Landreth et al., 1985). These findings indicate that, although WGA binding results in a quantitative conversion of the receptors to the slow, high affinity state, accompanied by a corresponding decrease of fast, low affinity species, this event is not sufficient *per se* to trigger the cellular response (Landreth et al., 1985). However, conversion from one state to another appears to represent a necessary condition for transduction of the NGF message. In fact, monoclonal antibodies against the receptor that prevent the NGF-induced conversion from fast to slow receptors also inhibit neurite regeneration (Chandler et al., 1984) and the induction of *c*-fos (Milbrandt, 1986) by low concentrations of NGF. In summary, the most relevant findings that emerge from the above mentioned studies are the following: 1) a single mRNA species coding for the NGF receptor has been found; 2) only the high affinity receptors appear to be involved in triggering the biological effects; and 3) the molecular basis of the observed kinetic heterogeneity of the NGF receptors remains to be established, but clearly the NGF binding modulates the dynamic properties of the NGF-receptor complexes.

Internalization

NGF is retrogradely transported from peripheral target tissues to the perikarya of sympathetic neurons (Hendry et al., 1974). This process requires a specific interaction with membrane receptors (Dumas et al., 1979; Schwab et al., 1982) and the subsequent transport of the endocytosed NGF-receptor containing vesicles along microtubules (Schnapp et al., 1985). Since discontinuation of this process by chemical (Levi-Montalcini and Booker, 1960; Angeletti and Levi-Montalcini, 1970) or surgical (Hendry et al., 1974) procedures leads to death of the target cells, it has been suggested that it exerts a vital role.

In PC12 cells internalization of NGF has the characteristics of receptor-mediated endocytosis (Levi et al., 1980) and is accompanied by down regulation of receptors (Calissano and Shelanski, 1980; Biocca et al., 1980; Layer and Shooter, 1983). More detailed studies on the process of internalization have shown that only the high affinity receptors are involved in this event (Shooter et al., 1981; Bernd and Greene, 1984; Green et al., 1986; Hosang and Shooter, 1987). It should be pointed out that the amount of NGF internalized is greater than that expected from the number of high affinity receptors present on the cell membrane, indicating that this population should be replaced continuously by its recycling, by exposure of hidden receptors (Cattaneo et al., 1983), by new synthesis (Calissano and Shelanski, 1980), or by conversion of fast low affinity to slow high affinity receptors (Roher et al., 1982; Bernd and Greene, 1984).

The intracellular distribution of NGF in target cells has been determined by cell fractionation techniques and by quantitative ultrastructural autoradiography. Yankner and Shooter (1979) detected Triton X100-insoluble binding of ^{125}I-NGF in PC12 cells. A considerable amount of ^{125}I-NGF was found associated with the nuclear fraction. This finding has been, however, questioned on the basis of cytoplasmic contamination of the nuclear fraction used in these studies (Rohrer et al., 1982). More recently, the presence of NGF receptors in the chromatin of melanoma cells has been reported (Rakowicz-Szulczynska and Koprowski, 1986). Morphological studies employing gautoradiography and immunofluorescence have suggested a perinuclear and nucleolar accumulation of NGF within a few hours of initial binding

in PC12 cells (Marchisio et al., 1980). Electron microscopic autoradiography of PC12 cells incubated with ^{125}I-NGF has added more information on this issue (Hogue-Angeletti et al., 1982; Roher et al., 1982; Bernd and Greene, 1984). After 15 min, 70–90% of the grains resulted to be on the internal side of the cytoplasmic membrane. Most of ^{125}I-NGF was found in the plasma membrane and lysosomes but an appreciable amount was also visible in the cytoplasm and on the nuclear membrane (Bernd and Greene, 1984).

No morphological effect has been observed when NGF is microinjected in PC12 by different procedures, and injected antibodies do not block the effects of externally added NGF (Heuman et al., 1981; Seeley et al., 1983; Heuman et al., 1984). These findings would rule out a direct role of NGF in the cell cytoplasm. If the internalization of the NGF-receptor complexes is instrumental to the biological activity of NGF, it is conceivable that the internalized NGF-receptor complexes, or metabolic products derived from them, have an intracellular target. Thus, a local action of the internalized complexes may add up to the effects elicited by the production of second messenger(s) at the level of the plasma membrane.

A schematic view of the events possibly occurring following NFG-receptor interaction is shown in Fig. 1.

Early Effects of NGF

As mentioned previously, the NGF receptor does not appear to possess, at variance with other growth factor receptors, a kinase, nor any other intrinsic enzymatic activity, suggesting that transduction of the NGF message involves the interaction of the receptor with an as yet unidentified untracellular system(s). Whatever the mechanism of the signal transduction, it is now largely documented that several potential second messengers are activated in PC12 cells within seconds or minutes after NGF binding. Among these messengers, a role has been attributed by various investigators to cyclic nucleotides, cations, and phosphoinositides.

cAMP

Conflicting reports have been presented on activation of adenylate cyclase by NGF. The increase of cAMP inside the cells may be a local and transient phenomenon and for this reason difficult to detect. Schubert and Whitlock (1977) described a transient increase of cAMP levels in PC12 cells caused by NGF but this finding has been subsequently challenged by others (Hatanaka et al., 1978). More recently, Race and Wagner (1985) showed that although NGF alone is unable to activate adenylate cyclase, it potentiates the stimulation induced by adenosine agonists or cholera toxin. The suggestion that cAMP lays a role in mediating the NGF action came from observations that some of the effects of NGF can be mimicked by conditions that increase the intracellular concentrations of cAMP. Moreover, in several instances, NGF and cAMP are not additive when present in saturating amounts, suggesting that they act through a common pathway. In particular, phosphorylation of a number of proteins induced by NGF can be accounted for by activation of a cAMP-dependent protein kinase (Halegoua and Patrick, 1980; Cremins et al., 1986) (*see below*). Conversely, the argument of additivity of some effects of NGF and cAMP has been used by some authors to conclude that NGF acts through a cAMP-independent pathway. Guroff et al. (1981) demonstrated that a rise of cAMP or cAMP analogs acts additively with NGF in inducing an increase of ornithine decarboxylase. Moreover, in PC12 mutants with an impaired cAMP-dependent protein kinase, ornithine decarboxylase is still induced by NGF (Van Buskirk et al., 1985).

As far as neurite extension is concerned, NGF and conditions that raise the endogenous level of cAMP show similar but distinct features and are additive (Schubert et al., 1978; Guroff et al.,

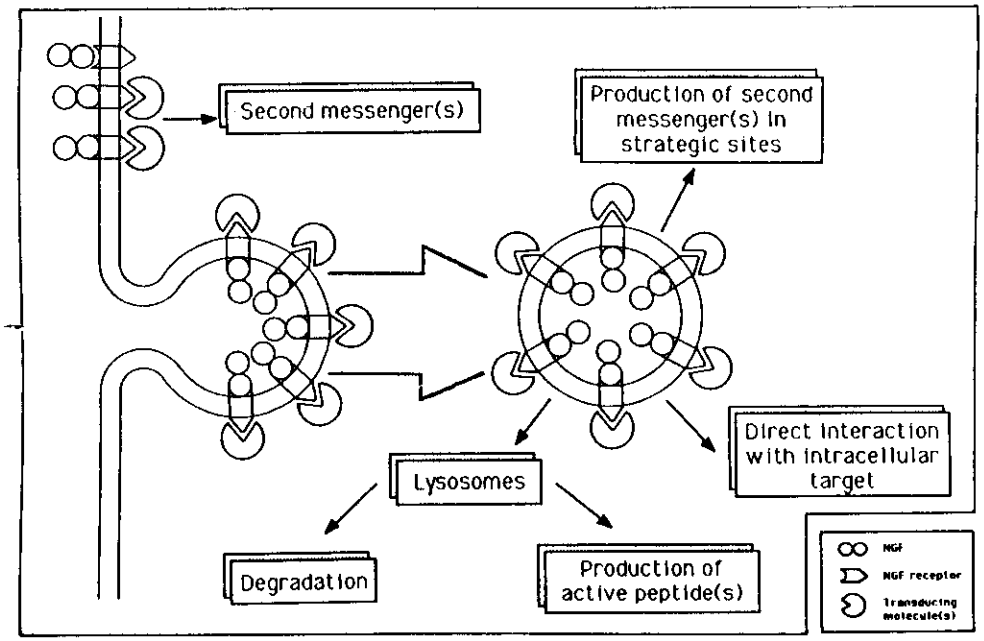


Fig. 1. Schematic representation of the fate of NGF-receptor complexes. This drawing depicts the internalization of the receptor-bound NGF and the hypothetical mechanism(s) through which the biological signal is transduced. Transduction may occur at the level of the plasma membrane via production of a second messenger(s) or by the internalized NGF-receptor complexes. More than one of the depicted pathways may be operative at the same time.

1981; Huffaker et al., 1984; Richter-Landsberg and Jastorff, 1986). In particular, the cAMP-mediated and the NGF-induced neurite extension show different sensitivity to inhibitors of RNA synthesis (Gunning et al., 1981) and NGF and cAMP act synergistically on the speed of neurite outgrowth. Microtubules present in neurites grown in response to elevation of cAMP, to NGF treatment, or to both these agents together, display different stability in the presence of depolimerizing agents (Heidemann et al., 1985). Moreover, NGF-induced, but not cAMP-induced neurite extension can be blocked by anti-*ras* antibodies (Hagag et al., 1986). Finally, it is noteworthy that increased levels of cAMP have an antagonistic role on the NGF-induced regeneration of PC12 neurites, phosphorylation of microtubule associated proteins, and expression of neurofilament protein or Thy-1 glycoprotein (Greene et al., 1986; Doherty et al., 1987; Doherty and Walsh, 1987). It has been suggested that this inhibitory effect may be mediated by cAMP counteracting activation of protein kinase C (*see below*).

In conclusion, there is still room for discussion on the role of cAMP in mediating the response of PC12 cells to NGF; it is clear, however, that conditions that elevate the cAMP content of the cells are not sufficient to substitute for NGF and that the pathways elicited by these two agents are distinct, even if possibly partially overlapping.

Ionic Fluxes, Intracellular Ca^{2+}, and Phospholipid Turnover

Boonstra et al. (1981,1983) demonstrated that NGF stimulates, within minutes from its binding to PC12 cells, the Na^+/K^+ pump by a mechanism mediated by an amiloride sensitive Na^+ influx (Na^+/H^+ exchange). This effect is similar to the activation of Na^+/K^+ATPase, induced by NGF in primary cultures (Boonstra et al., 1982). The relevance of the stimulation of amiloride-sensitive Na^+/H^+exchange to the differentiative effect of NGF is questionable since amiloride fails to prevent neurite outgrowth. The authors concluded that activation of the Na^+/K^+ATPase in PC12 cells is more likely related to the mitogenic effects of NGF in these cells.

Modulation of Ca^{2+} levels in PC12 cells by NGF has been investigated by different groups in view of the widespread role of this cation in signal transduction. Schubert et al. (1978) reported a NGF stimulated Ca^{2+} efflux from PC12 cells, a finding not confirmed by others (Landreth et al. 1980). More recently, Pandiella-Alonso et al. (1986) and Vicentini et al. (1986) measured a very rapid increase (in a few seconds) in free intracellular Ca^{2+} concentration in NGF treated PC12 cells as well as in chromaffin cells. This rise is the result of both an increased influx and a release from internal stores. Though it is very difficult to assess the relevance of such a Ca^{2+} increase in the mechanism of action of NGF, it is interesting to note that this effect is not induced by EGF or by an increased level of cAMP. Ca^{2+} influx has been implicated also in mediating the decrease in phosphorylation of a soluble protein named Nsp100 induced by NGF (Hashimoto et al., 1986).

In many different systems, there is a clear interplay between modulation of Ca^{2+} levels and phospholipid metabolism (for a recent review on this topic, *see* Berridge, 1987). It has been shown that NGF increases incorporation of phosphate in phosphatidylinositol within minutes of binding to PC12 cells (Traynor et al., 1982). Moreover, NGF also stimulates the turnover of phosphatidic acid which can play a role in the Ca^{2+} permeability of plasma membranes (Traynor, 1984). On the other hand, Ca^{2+} fluxes may influence phosphatidylinositol turnover as exemplified by recent data from Contreras and Guroff (1987) who demonstrated a very rapid (15 sec) formation of inositol mono, bis, or trisphosphate in cells treated with NGF by a mechanism strictly dependent on extracellular calcium. Moreover, NGF potentiates the bradykinin-stimulated accumulation of inositol phosphates in PC12 cells (Van Calker and Heumann, 1987; Volonté et al.). Increased Ca^{2+} mobilization and phospholipid turnover are probably connected with activation of protein kinase C as discussed below.

Protein Kinases

Hama et al. (1986) demonstrated that NGF increases (within 15 to 30 min) the activity of cytosolic protein kinase C in PC12 cells assessed after fractionation on diethylamio ethyl (DEAE) cellulose columns. Moreover, some of the effects of NGF could be mimicked by the activation of kinase C, and the two were not additive.

The NGF-induced phosphorylation of tyrosine hydroxylase in one of its tryptic fragments (T3) is blocked by inhibition of protein kinase C and is induced by its activation (Cremins et al., 1986).

Halegoua and Patrick (1980) demonstrated rapid phosphorylation of cytoplasmic (S6 ribosomal protein, tyrosine hydroxylase) and nuclear proteins (histones H3, H1A, and one protein of the high mobility group, chromosomal protein HMG17) in PC12 cells following NGF treatment. Phosphorylation of these proteins was induced also by increasing the intracellular concentration of cAMP. Since increased cAMP levels and NGF were not additive, the authors concluded that they were acting through the same mechanism(s). (For a detailed study

on tyrosine hydroxylase phosphorylation, *see* McTigue et al., 1985). In addition, phosphorylation of the T1 fragment of tyrosine hydroxylase as well as phosphorylation of the ribosomal protein S6 is absent in PC12 cell mutants defective in protein kinase A.

Three groups independently demonstrated activation via NGF of kinases distinct from kinase A and C and other known kinases (Blenis and Erikson, 1986; Matsuda et al., 1986; Rowland et al., 1987). The activation is measurable within minutes of cell exposure to NGF, being one of the fastest responses of PC12 cells to this factor. Substrates for these kinases are S6 ribosomal protein and tyrosine hydroxylase. Further characterization of these activities in terms of enzymatic properties and substrate specificity is needed to ascertain whether the same or distinct polypeptides are responsible for the reported phosphorylation. Recently it has been reported (Romano et al., 1987) that synapsin I, a neuron-specific phosphoprotein, is phosphorylated following NGF treatment of PC12 cells and that this phosphorylation cannot be accounted for by previously described kinases. The NGF-induced phosphorylation of synapsin I is detectable within 15 min.

The fact that NGF induces phosphorylation of a set of proteins which are also targets for protein kinase C, A, and other kinases, suggests that second messengers such as cAMP, calcium ions, and phosphatidylinositol breakdown products mediate at least some of the responses of the cells to NGF. However, this does not clarify the specific role played by the phosphorylated substrates in the subsequent cellular differentiation. The phosphorylation of cytoskeletal associated proteins that may regulate the assembly of microtubules and, in general, the cell architecture, will be reported later in this study. The hypothesis that NGF may induce phosphorylation of transcriptional factors which, in turn, regulate gene expression, has been presented to explain the finding that NGF induces within minutes the translation-independent, increased transcription of a number of genes (Greenberg et al. 1985).

Early Activated Genes

Particular attention has been devoted to rapid activation in gene transcription following NGF addition to PC12 cells. Several authors (Greenberg et al., 1985; Curran and Morgan, 1985; Kruijer et al., 1985; Mildbrandt, 1986; Kujubu, et al., 1987) showed that in a very short time (5–60 min) NGF transiently stimulates transcription of a number of genes, such as the proto-oncogenes *c-fos* and *c-myc*, actin, ornithine decarboxylase, and others. Moreover, the induction of some of them does not require ongoing protein synthesis (Greenberg et al., 1986a). It is important to note that transcription of some of these sequences is also induced in other cells as well as in PC12 by agents such as tumor-promoting agent tetradecanoyl phorbol acetate (TPA), cAMP, Ca^{2+}, and EGF postulated to act through protein kinase activation. It is worth noting that, in the case of *c-fos*, multiple regulatory sequences of the gene allow its regulation in response to distinct stimuli in various cell types (for a review, *see* Verma and Sassone-Corsi, 1987). In the case of PC12 cells, for instance, Greenberg et al. (1986b) showed that nicotine activation of *c-fos* proceeds by a different pathway than activation by NGF, whose stimulation of *c-fos* is independent of external Ca^{2+}.

It has not yet been possible to assign a clear functional role to each of these early NGF-activated genes in the process of PC12 differentiation. It seems very likely in any case that some of them are responsible for the subsequent translation-dependent activation of transcription of other sequences. In this respect, it is noticeable that one of the genes induced early by NGF in the presence of cycloheximide, is structurally related to the *Xenopus laevis* transcription factor TF-IIIA and other DNA binding proteins (Milbrandt, 1987).

The Acquisition of the Differentiated Neuronal Phenotype

The reader is referred to recent reviews and references (Greene and Tischler, 1982; Guroff, 1985) for a detailed description of what can bedefined as "the differentiated phenotype" of NGF treated PC12 cells, i.e., the collection of the NGF-induced phenotypic changes such as induction of electrical excitability, increase in sodium channels, muscarinic acetylcholin (Ach) receptors, opiate receptors, and induction of the 16S form of acetylcholinesterase (AchE) and of membrane glycoproteins such as the NGF inducible large external protein NILE and Thyl. In the following we shall concentrate only on some aspects dealing with the acquisition of theneuronal phenotype.

Identification of Gene Products Potentially Involved in NGF-Induced Differentiation

As mentioned in the introduction in experiments designed to test the role of regulation of gene transcription in the NGF mode of action, it was found that, although initiation of neurite growth by NGF in native PC12 cells was inhibited by low concentrations of RNA synthesis inhibitors, NGF-dependent neurite regeneration from cells pretreated with the factor failed to show such a sensitivity (Burstein and Greene, 1978). This observation led to the so-called "priming" model (*see*, also Greene et al., 1982), whereby promotion of neurite outgrowth by NGF has a component dependent on RNA transcription and another that is not. It is hypothesized that during the priming period the syn thesis and accumulation of proteins required fo neurite growth takes place and that these pro teins are available in cells treated with NGF i suspension or mechanically divested of the neurites. This transcription-dependent phas however, is not sufficient to bring about neurit outgrowth. For this to happen, a fast, transcri tion independent response to NGF must als occur.

Only a small number of minor changes wer found in early experiments aimed at detectin proteins differentially expressed in the nativ and NGF-treated PC12 cells by two-dime sional electrophoresis (Garrels and Schube 1979: McGuire and Greene, 1980). Thus, it wa initially surmised that the differentiation pro ess triggered by NGF did not involve majo changes in the pattern of gene expression bu mostly a reorganization of the preexisting po of proteins. However, by restricting the pool proteins analyzed, for instance by selectiv labeling of membrane glycoproteins (McGui et al., 1978; Richter-Landsberg et al., 1985), b cell fractionation (Biocca et al., 1983; Tiercy an Shooter, 1986), or by affinity purification pr cedures (Biocca et al., 1984), it became evide that, following NGF action, several chang occur in the levels and/or synthetic rate of number of proteins. Thus, at least part of th mechanism of action of NGF appears to involv the regulation of the expression of specif genes.

These observations have prompted th search for mRNAs that are regulated by NGF PC12 cells, an approach that, because of th great sensitivity of the technique, would allo the detection of subtle modifications in ger expression. One obvious advantage of this ap proach over that of identifying changes at th protein level is provided by the possibility of d rectly studying the regulation of transcription control by NGF in PC12 cells, i.e., the search f the so-called "NGF box(es)." NGF-induce changes in specific mRNAs have been observe

at different times following addition of NGF (Feinstein et al., 1985; Greenberg et al., 1985; Levi et al., 1985; Leonard et al., 1987) and have been identified with the use of available probes (Anderson and Axel, 1985; Dickson et al., 1986; Prentice et al., 1987) or through a process of cDNA cloning and differential hybridization (Levi et al., 1985; Leonard et al., 1987). These studies led to the identification of gene products present not only in PC12 cells but also in some regions of the nervous system and upregulated by NGF. Among these genes, one has been the object of further investigations that have led to the intracellular localization of the protein in PC12 cells (Possenti et al., 1987) and its localization in the central nervous system by immunohistochemical techniques (Levi et al., sub mitted).

Though it has not been possible to attribute a functional role to all the NGF-induced genes in PC12 cells, the relevance of proteins involved in the reorganization of cell shape and in the outgrowth of neurites is self evident. NGF has been shown to increase the level of tubulin, microtubule associated proteins MAP-1, MAP-2 and tau, vimentin, neurofilament proteins of 68 and 200 kD and other nonidentified microtubule associated proteins (Black et al., 1986; Drubin et al., 1985; Greene et al., 1983; Lee and Page, 1984). It is conceivable that neurite outgrowth may indirectly influence the biosynthesis of cytoskeletal protein since it is well documented that tubulin synthesis is under an autoregulatory control dependent upon the state of microtubule assembly (Ben-Ze'ev et al., 1979; Cleveland et al., 1981).

The Role of the Cytoskeleton in Neurite Outgrowth

One of the intracellular events that appears to be essential for neurite outgrowth is the formation of microtubule bundles that extend from the cell body to the tips of the growing neurites. (Luckenbill-Edds et al., 1979; Spiegelman et al., 1979; Jacobs and Stevens, 1986). Microtubule polymerization *per se*, however, is not sufficient to induce neurite outgrowth in PC12 cells, as shown by the failure of the polymerizing drug, taxol, to induce neurite extension in the absence of NGF (Corvaja et al., 1982).

Microtubules (MT) in long term NGF treated cells are more stable than the ones in native PC12 cells, as revealed by their resistance to the depolymerizing action of colchicine and low temperature (Black and Greene, 1982). The proportion of polymerized tubulin increases after NGF treatment (Drubin et al., 1985; Black et al. 1986). Moreover, fully differentiated PC12 cells contain a macromolecular structure favoring microtubule assembly, which allowed the identification of a set of *bona fide* microtubule associated proteins whose synthesis is positively or negatively regulated by NGF (Biocca et al., 1983).

Microtubule stability can be greatly influenced by microtubule associated proteins (MAPs). As stated before, several changes in microtubule associated proteins of PC12 cells occur after exposure to NGF. The composition of a group of MAPs originally observed in PC12 cells grown without NGF, designated as chartins (64, 67, and 80kD) (Zieve and Solomon, 1984; Pallas and Solomon, 1982; Magendantz and Solomon, 1985) is also altered by NGF treatment, in that the abundance of the more acidic variant of each chartin MAP class is markedly enhanced relative to the more basic members. This occurs without substantial changes in the abundance of each chartin MAP class and is a result of differential phosphorylation of some chartin MAP isoforms. Moreover, Aletta and Greene (1987) have demonstrated that the state of phosphorylation of these chartin MAPs is dependent on the presence of polymerized microtubules, since drug-induced depolymerization of MTs leads to an overall decrease in incorporation of phosphate into chartins. The MT-dependent phosphorylation of chartins is reminiscent of the MT-dependent phosphorylation of beta-tubulin that accompanies differentiation of the N115 neuroblastoma cells (Gard and

Kirschner, 1985).

The relevance of this posttranslational modification of chartins in the process of neurite outgrowth is suggested by the finding that millimolar concentrations of LiCl, which block this morphological event, also specifically inhibit phosphorylation of several chartins (Burstein et al., 1985).

Under MT depolymerizing conditions, neurite elongation is arrested but, despite depolymerization of microtubules, neurites remain extended for at least 24 h. This is in agreement with the finding of Joshi et al. (1985) who observed that neurites in long term NGF-treated PC12 cultures remain extended even in the presence of concentrations of MT depolymerizing drugs 10-fold higher than those sufficient to cause rapid retraction and disappearance of neurites in PC12 cell cultures that have been exposed to NGF for a few days. One possible source of this change in dependence of the neurites on microtubule integrity is the appearance of neurofilaments (*see below*).

It has been recently realized that dynamic and stable populations of microtubules can coexist within the same cell (see, Schulze and Kirschner, 1987). Selective stabilization of microtubule subclasses can be generated by microtubule-associated proteins or by posttranslational modifications of the tubulin molecules such as detyrosination and acetylation (detyrosinated and acetylated MTs being less dynamic). It is noteworthy, therefore, that after 2 w with NGF the alpha-tubulin in PC12 cell neurites becomes acetylated (Black and Keyser, 1987). The reported two-fold increase in specific activity of tyrosyltubulin ligase (Levi et al., 1978) that accompanies PC12 differentiation may reflect local regulation of MT stability.

The overall picture emerging from these studies is that of a progressive shift towards an increase and stabilization of microtubules, achieved through different concurrent means. How does such a shift of the assembly equilibrium induce initiation and growth of neurites in PC12 cells? In keeping with the suggestion by Bray (1979), it has recently been found (Joshi et al., 1985) that PC12 cell bodies occupy an equilibrium position in a network of tensioned neurites. Microtubules within PC12 cell neurites are under compression, supporting an opposite tension exerted by the actin network. Hill and Kirschner (1982) have shown that compression of MTs destabilizes them, favoring disassembly. An increase in microtubule stability would therefore be required to enable the MT to bear compression without depolymerization. Release of compression, which in a growing neurite would occur by the pulling force exerted by the advancing growth cone, should provide a local signal for further MT elongation by decreasing the critical concentration of tubulin for assembly.

In order for the growing neurite to exert tension, adhesion to the substrate, and possibly among neurites themselves, must be strong. Early reports demonstrated that NGF increases cell to cell and cell to substratum adhesion in PC12 cells (Schubert and Whitlock, 1977; Schubert et al., 1978). Proteins or protein complexes such as vinculin and integrin provide molecular links between the extracellular matrix and the intracellular actin filaments. Halegoua (1987) demonstrated that growth cone formation and elongation in PC12 cells correlate with changes in cell substrate adhesion and in the phosphorylation and distribution of vinculin in response to NGF., Within 4 h of NGF treatment both vinculin and focal adhesion sites become restricted to discrete protruding portions of the cell periphery and eventually remain within the tips of the growth cone extensions. Vinculin phosphorylation was found to be increased within 1 h of NGF treatment. Vinculin has been identified in other systems as a good substrate for protein kinase C and for the virally encoded *src* kinase (Sefton et al., 1981). Finally, it is noteworthy that one of the first identified NGF-induced protein, NILE, (McGuire et al., 1978) belongs to the category of the so called cell adhesion molecules (Cam) and that *N*-Cam is also induced by NGF

(Prentice et al., 1987).

The intermediate filaments undergo in PC12 cells major rearrangements during NGF-induced differentiation, both in terms of spatial organization (Tischler and Greene, 1978) and of molecular composition (Lee and Page, 1984; Lee, 1985). Treatment with NGF results in an intensely fluorescent ball-like staining by antibodies recognizing the 68 kD neurofilament subunit, together with staining of neurites. The 150 and 200 kD neurofilament subunits show a weak and diffuse staining pattern both in native and NGF treated PC12.

Recently (Parysek and Goldman, 1987) it has been shown that the putative vimentin containing filaments in PC12 cells are in fact made not of vimentin but of an immunologically related 57 kD protein. By indirect immunofluorescence it was shown that this vimentin-like polypeptide costains with the 68 kD neurofilament subunit.

In view of the fact that the state of assembly of intermediate filament proteins can be modulated by site-specific phosphorylation events, the rearrangement of these filaments in PC12 cells, following NGF action, might be also controled by a hitherto undescribed modulation of the phosphorylation state of the vimentin or neurofilament proteins.

Mitotic Arrest in PC12 Differentiation

One of the effects caused by NGF in PC12 cells is their withdrawal from the cell cycle and the induction of mitotic arrest as measured by growth curves and thymidine incorporation (Greene, 1978; Greene and Tischler, 1976). This effect is rather slow and, as already discussed, within the first 24 h NGF is in fact mitogenic for PC12 cells, which undergo one or two rounds of accelerated cell division before being removed from the cell cycle (Boonstra et al., 1983). Arrest of cell division with a variety of agents acting at different levels of the replicative cycle leads to PC12 cell death unless NGF is also present (Improta et al., 1988; Corvaja et al., 1982). As opposed to these agents, gamma-interferon (IFN) causes a rapid arrest of cell division without leading to cell death, and at the same time increases the speed of neurite outgrowth. NGF, in turn, potentiates gamma-IFN-activation of 2'–5'A synthetase, prospecting a peculiar interplay between these two factors (Improta at al., 1988). Similar effects on the arrest of cell division and potentiation of NGF-induced neurite outgrowth has been reported for adenosine (Huffaker et al., 1984).

The antimitotic activity of NGF for PC12 cells allows the selection of NGF unresponsive mutants by prolonged incubation of PC12 cells in NGF (Bothwell et al., 1980; Burstein and Greene, 1982). Some mutant PC12 clones respond morphologically to NGF by forming neurites notwithstanding the lack of inhibition of their proliferation (Bothwell et al., 1980). This finding demonstrates that the cessation of division is not necessarily coupled to morphological differentiation and neurite outgrowth, and that the two processes are not strictly linked.

Another group of variant PC12 cells (Burstein and Greene, 1982) that also continues to proliferate in the presence of NGF under normal culture conditions, when exposed to NGF in growth-restrictive media responds to the factor with an increase in cell proliferation. Although these variant cells do not cease cell division in response to NGF, they retain the cellular machinery to undergo NGF-mediated neurite outgrowth, which can occur when division is arrested by exposure to antimitotic drugs (Burstein and Greene, 1982). A mitogenic effect of NGF has also been shown on immature chromaffin cells (Lillien and Claude, 1985) showing that the cellular response to a growth factor is governed more by the transduction mechanisms present within the cell than by any inherent activity associated with the factor itself.

Work by Gunning et al. (1981) and by Goodman et al. (1979) has challenged the notion that NGF completely blocks DNA replication in

PC12 cells. These studies demonstrate that thymidine incorporation continues in NGF-treated neurites bearing cells even after 10 d. This issue has been further analyzed by Ignatius et al. (1985), who demonstrate that NGF treatment induces polyploidism in at least one subpopulation of the cells, resulting in a threefold increase in the number of G2+M/4N cells along with the appearance of 8N and 16N cells. Polyploid neurite-bearing PC12 cells were shown not to be multinucleate. The persistent DNA synthesis in these extensively differentiated PC12 cells may merely reflect a partial retention of their transformed phenotype, but it is tempting to relate it to reveal other scattered examples reported in the literature describing continued DNA synthesis or extensive polyploidy in cells of the invertebrate nervous system or of both the peripheral and central vertebrate nervous systems (*see* references quoted in Ignatius et al., 1985).

The overall picture that emerges from these results is that induction of neurite outgrowth by NGF is not necessarily associated with a cessation of DNA synthesis, although blockage of cell division facilitates the NGF-induced neurite outgrowth. Therefore we surmise that the mitotic arrest in PC12 cells is a secondary event with respect to neurite formation induced by NGF. The selective stabilization of a microtubule and/or a microfilament pool involved in neurite formation, as opposed to that necessary for the mitotic apparatus, could prevent the PC12 cell from undergoing cytokinesis but would not necessarily prevent the duplication of its genome, which might require a further signal of arrest to be blocked. In this context it is worth recalling that a differential sensitivity of cell cytokinesis and karyokinesis to cell shape and to cell–surface contact with the substrate has been described in fibroblasts (Ben-Zé ev and Raz, 1981).

A metabolic correlate of mitotic arrest in NGF-treated PC12 cells is the shift from anaerobic glycolisis, typical of neoplastic cells, to a progressively higher utilization of glucose in the Krebs cycle (Morelli et al., 1986). Such change that is presumably induced to supply more energy for the onset of the differentiative program has an enzymatic counterpart in the inhibition of the synthesis and decrease in total content of lactic dehydrogenase (Biocca et al., 1984; Calissano et al., 1985). NGF control of energy metabolism via an action on this enzyme, whose activity is particularly elevated in neoplastic cells, together with modulation of some other glycolitic enzymes (Salvatore and Calissano unpublished observation) has been postulated to represent a prerequisite for the action of NG leading to neuronal differentiation (Morelli et al., 1986).

Interplay Between NGF, Oncogenes, and Other Growth Factors

In recent years, new perspectives have been opened in the field of growth factors with growing awareness that an interplay exists between genes defined as able to confer a transformed phenotype to cells in culture (oncogenes) and genes that control cell division and differentiation. In the case of PC12 cells, studies dating from the past three years have demonstrated that the activity of viral *src* (*v-src*) (Alema et al., 1985; Casalbore et al., 1986), viral Kirsten and Harvey *ras* (Noda et al., 1985), and activated Ha- and *N-ras* proteins (Bar-Sagi and Feramisco 1985; Guerrero et al., 1986) induce in these same cells neuronal differentiation indistinguishable both from a morphological and a biochemical point of view, from that induced by NGF. These findings indicate that *ras* and *src* proteins may have the capacity to affect diverse cellular pathways controling proliferation or differentiation according to intrinsic properties of a given cell type rather than to their inherent properties. It is not surprising that these two viral oncogenes share a similar effect on PC12 cells, since their action in conferring a transformed phenotype to fibroblasts has also been shown to be intercon

nected (Smith et al., 1986; Noda et al., 1983). It has yet to be ascertained whether they activate in PC12 cells the same molecular pathways as NGF, or whether they are acting through different mechanisms, converging on the same endpoint. The fact that microinjection of anti-p21 (*c-ras*) antibodies in PC12 cells blocks NGF induced, but not cAMP induced, neurite formation speaks in favor of the first possibility (Hagag et al., 1986). Anti-p21 antibodies inhibit neurite formation even if microinjected up to 36 h after initiation of NGF treatment.

Recent experiments (Satoh et al., 1987) have demonstrated that also the proto-oncogene Ha-*ras* induces a differentiated phenotype in PC12 cells if microinjected together with a nonhydrolyzable GTP analog. This suggests that the activated *ras* protein eliminates the dependence on NGF for the induction of the differentiated phenotype, and that NGF receptor complexes may, directly or indirectly, activate the cellular proto-oncogene p21 in a way similar to other hormone–receptor complexes activating members of the G protein family. Hanley and Jackson (1987) suggest that in mammalian cells the *ras* family may constitute relays between receptor activation and structural, possibly local, changes in the cytoskeleton. This hypothesis is based on a structural analysis of the two yeast *ras*-like gene products, YPT1 and SEC4, which seem to function as mechanotransducers. It is tempting to speculate that a similar role might be played by NGF-activated in PC12 p21 protein.

The differentiative effect of *v-src* would suggest that its cellular homolog *c-src* involved in this process. The products of these two genes, $pp60^{v\text{-}src}$ and $pp60^{c\text{-}src}$, however, are structurally different and may activate distinct pathways. On the other hand, it is possible that $pp60^{v\text{-}src}$ acts in PC12 cells as a constitutively activated $pp60^{c\text{-}src}$. Interestingly, treatment of PC12 cells with orthovanadate, which is a phosphotyrosine phosphatose inhibitor, mimicks some of the NGF or *v-src*-induced differentiating actions (Alema, personal comunication).

A study of the interaction between NGF and other growth factors in the induction of neurite outgrowth in PC12 is also likely to shed some light on the mechanistic aspects of this process. PC12 cells can be triggered to differentiate, in the absence of NGF, by acidic and basic fibroblast growth factor (FGF) (Wagner and D'Amore 1986; Togari et al., 1983, 1985; Rydel and Greene, 1987) but not in response to (EGF) (Huff et al., 1981) or phorbol esters. The case of acidic and basic fibroblast growth factor (aFGF and bFGF) is particularly illuminating, since they are well characterized peptide hormones with potent angiogenic activity and with mitogenic activity for a variety of cell types (for a review, *see* Gospodarowicz et al., 1986). FGFs can reproduce most (Togari et al., 1983, 1985) or all (Rydel and Greene, 1987) PC12 cell responses to NGF, including the rapid ones and the more delayed, transcription dependent ones. Interestingly, the regeneration of neurites in response to both factors is blocked in a similar way by the addition of inhibitors of methylation, previously shown (Seeley et al., 1983, 1984) to block neurite regeneration from NGF primed cells.

It should be noted that the mitogenic growth factor EGF acts on PC12 cells through specific receptors and induces specific responses, many of which are in common with those elicited by NGF itself. These are: induction of ODC, stimulation of ionic fluxes through activation of the Na^+/K^+/ATPase, and early induction of the transcription of the nuclear proto oncogenes *c-fos* and *c-myc*. EGF, however, is not able to induce PC12 cells to differentiate and its receptors are downregulated by previous exposure of these cells to NGF. It was suggested that this heterologous receptor downregulation is part of the mechanism by which differentating cells become insensitive to mitogens (Lazarovici et al., 1987; Boonstra, et al., 1987). It is noteworthy that simultaneous addition of EGF and the tumor promoting phorbol ester 12-*O*-tetradecanoylphorbol-13-acetate (TPA) elicits a

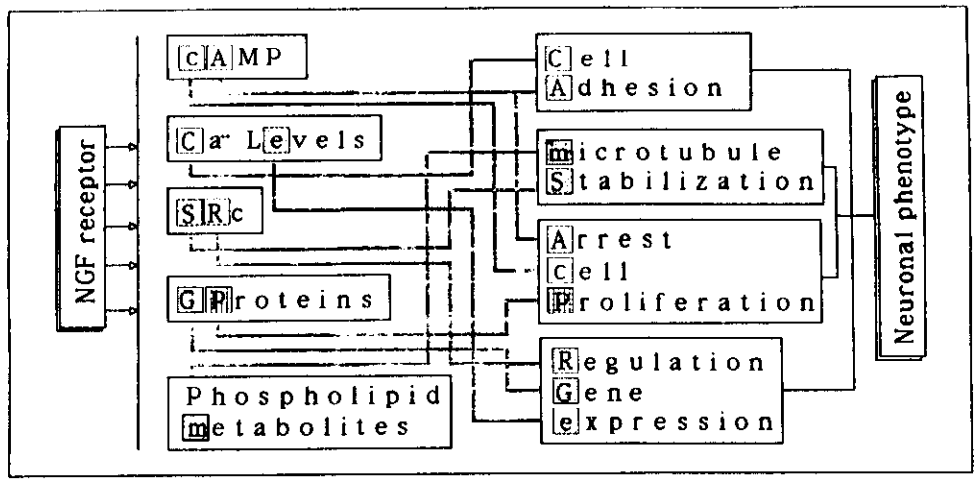


Fig. 2. Combinatorial mechanism in the transduction of the NGF signal. The left part of the figure lists several second messengers and proteins potentially involved in the transduction of the NGF signal. A proper combination of some of them (dashed letters on the right part of the panel) will cause distinct cellular responses that together result in the acquisition of the neuronal phenotype. Notice that the particular combinations of second messengers leading to the different cellular responses illustrated in the figure are purely arbitrary.

rapid extension of slender cellular processes (End et al., 1982).

A further example of interaction between NGF and other growth factors is provided by the fact that whereas PC12 cells are not viable in the absence of serum in the culture medium, supplementation of serum-free medium with NGF promotes both PC12 cell survival and neuronal differentiation (Greene, 1978). Thus, under these experimental conditions, NGF displays a trophic activity, in addition to its differentiative one, which is reminiscent of that exerted by the factor in vivo, or in primary neurons in culture, and which is normally masked in PC12 cells by the presence of other factors present in the serum of the culture medium.

Conclusion

PC12 cells, notwithstanding their neoplastic nature, provide an excellent experimental model for studying the mechanism of action of NGF. The increasing number of papers published yearly on these cells represent the best quantitative estimate of this conclusion.

When exposed to NGF, PC12 cells undergo the two final, major steps of neuronal differentiation: the commitment of a precursor to become a neuron and the definitive acquisition of the neuronal phenotype. In this respect NGF is acting on evolving target cells and it is therefore conceivable that not the same molecular mechanisms are operative during onset of these two differentiative stages. The possibility of experimentally dissecting these two NGF actions by the use of substances such as LiCl or colchicine, or by growing the cells in suspension has already provided valuable information.

The structure of the NGF receptor and its kinetic properties are presently well characterized. Moreover the translational and posttranslational regulation of gene products involved in the cytoskeletal architecture and adhesion properties of the cells has been thoroughly investigated and their relevance for the acquisition of the morphological differentiated state is self explanatory. In contrast, the bulk of phenomena observed as early responses of the cells to NGF are more difficult to visualize as a clear cut chain of cause-and-effect events. We have sur-

mised that the activation, in parallel, or several second messengers may, by a combinatorial mechanism, mediate the transduction of the NGF signal (Fig. 2). Since it is conceivable that some of these pathways are also operative in the transduction of the message of other agents, it is not surpising that they are activated not only by NGF but also by factors such as EGF. Therefore, the specificity of the differentiating response must also rely on some other signal elicited by NGF or on a specific combination of such signaling pathways. It is inherent to the combinatorial hypothesis that different factors activate multiple overlapping cascades of events and each response is mediated only by a subset of them. The other events may be devoid of a functional role. To determine which are the necessary and sufficient steps for the action of NGF and which are epiphenomena is a major goal of future investigations.

Acknowledgments

We wish to thank Rita Levi-Montalcini for her encouragement and suggestions during the preparation of this manuscript and S. Alemá and A. Grasso for their critical comments. We also thank Linda Delucchi for her help in typing this paper. This work was supported by Progetto Finalizzato Oncologia to P. Calissano.

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