Annu. Rev. Pharmacol. Toxicol. 1991. 31:205–28

THE MECHANISM OF ACTION OF NERVE GROWTH FACTOR

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KEY WORDS: neuronal differentiation, growth factors, PC12 cells, tyrosine kinases, *ras* gene

INTRODUCTION

Nerve growth factor (NGF) was the first discovered and the best-characterized of the polypeptides that exert neurotrophic effects (1). The earliest studies identified peripheral sympathetic and sensory neurons of the chick embryo and newborn rodents as target cells for NGF. The major biological function of NGF, its trophic action, was operationally defined on these cells by the effects elicited by deprivation of the growth factor by various means. Beside its trophic role, other important properties of NGF are (*a*) its neurotropic action both in vitro and in vivo (*b*) its ability to modulate the differentiative program (fate) of neuronal precursor cells and (*c*) its contribution to the maintenance of the acquired differentiated phenotype (1, 2). Recently, two advances, made possible by the acquisition of molecular probes for the hormone and its receptor, have brought new impetus to the field of NGF: the identification of molecules structurally related to NGF (3–6) and the discovery that the spectrum of its potential target cells is larger than previously anticipated. In particular, it is now well established that NGF also acts on defined populations of neurones within the central nervous system (7–9) and probably exerts some biological influence on some nonneuronal cells (10–12). The

0362-1642/91/0415-0205$02.00

biochemical properties and physiological functions of NGF have been the subject of a number of recent reviews (1, 7, 8, 13).

The biochemical correlates of NGF trophic action can be best inferred from primary cultures of NGF-dependent neurons (14–16). However, the NGF requirement for survival as well as the limited availability of the cells have prevented extensive use of this experimental model. Among NGF-responsive cells, the clonal cell line PC12 has become the model of choice for understanding the mode of action of NGF in the modulation of differentiation. These cells, which do not require NGF for survival and growth, acquire and maintain a sympathetic-like neuronal phenotype in response to the factor (17–19). Here we have chosen to concentrate on some recent developments in the mechanism of action of NGF in PC12 cells and, when possible, in other cell types. In view of the previously mentioned wide spectrum of potential targets such an approach may appear too restrictive, although we expect that the effects exerted by NGF on other target cells are mediated by activation of similar biochemical pathways. This assumption is as yet only tentative but is amenable to direct experimentation.

DIFFERENTIATION OF PC12 CELLS IN RESPONSE TO NGF AND OTHER FACTORS

The differentiation response of PC12 cells to NGF is a complex phenomenon that occurs with a time scale of several days. After an initial mitogenic phase (20), which appears dispensable for the ultimate acquisition of the differentiated phenotype (21), PC12 cells stop dividing (17). DNA replication in some differentiated PC12 cells, however, is not completely extinguished and a proportion of the cells become polyploid (22). The acquisition of a neuronal-like morphology, i.e. outgrowth of long, branching neurites, is accompanied by the de novo expression of a number of nerve cell-specific antigens and neurospecific functions such as synthesis and storage of neurotransmitters, electrical excitability, and the ability to form functional synapses with cocultured muscle cells (reviewed in ref. 17, 18). In this respect PC12 cells, which are derived from rat pheochromocytoma, behave like their untransformed counterparts, the immature chromaffin cells of the adrenal medulla, which respond to NGF both in vivo and in vitro by acquiring a sympathetic-like neuronal phenotype (23–25). The NGF-induced phenotype is reversible: removal of NGF leads to loss of differentiated traits and resumption of the cell cycle (17). Both transcription-dependent and transcription-independent pathways are needed to achieve complete differentiation, a finding on which the priming model has been based (26). This model suggests that a transcription-dependent slow accumulation of a set of (specific?) proteins is a prerequisite for morphological differentiation. The accumulation of this material *primes*

the cells for rapid, transcription inhibitor-resistant, NGF-dependent neurite outgrowth (26). Even though cells exposed to NGF undergo the transcription-dependent and transcription-independent processes over similar time scales, the experimental dissection of these two events has validated the priming model (27).

The acquisition of the mature differentiated phenotype is well defined by the above-mentioned ensemble of neuronal properties as well as by a number of molecular markers that represent their biochemical correlates. However, it has been very difficult to subsume the terminal differentiation in terms of a number of early—seconds to hours—measurable biochemical changes induced by NGF. In the ensuing review, we focus on these fast responses in the hope of gaining some clues about their possible relevance to the acquisition of the terminal phenotype. Since genetic dissection of the relevant cellular functions is not feasible in PC12 cells at this time, the second best approach, we believe, is to compare the biochemical pathways activated by NGF with those set in motion by other growth factors or activated oncogenes that partly or almost totally mimic its effects. Beside NGF receptors, PC12 cells express receptors for epidermal growth factor (EGF), fibroblast growth factor (FGF) and interleukin 6 (17, 18, 28, 29). EGF is a well-characterized mitogenic factor in several cell lines and in PC12 cells and its pathway is initiated by activation of the tyrosine kinase intrinsic to the receptor (30). In PC12 cells EGF induces a number of early biochemical responses similar to those induced by NGF, but fails to induce terminal differentiation. It is surmised that most of the events elicited by both EGF and NGF are relevant to the early mitogenic effect of NGF, even though this conclusion does not rule out their involvement in the triggering of differentiation as well. FGF (acidic and basic FGFs) is also a mitogen in several cell lines as well as a differentiative factor for a subpopulation of CNS neurons (31). Its mitogenic actions and supposedly also its differentiative functions are mediated by activation of the tyrosine kinase activity intrinsic to its receptor (32). FGF induces both early and delayed responses in PC12 almost indistinguishable from those elicited by NGF (28, 33). Interleukin-6 also induces differentiation of PC12 cells (29). The mechanism of action of interleukin-6 has yet to be defined. Finally, expression of two viral oncogenes, namely v-*src* and v-*ras*, fully activate the differentiative program of PC12 cells (34, 35). Understanding the extent to which the biochemical pathways activated by these growth factors and oncogenes are relevant to the mechanism of action of NGF is a potentially fruitful approach.

PROPERTIES OF THE RECEPTOR FOR NGF (NGF-R)

The ability of NGF to exert its trophic, differentiative, and mitogenic actions on target cells is mediated by high-affinity binding to specific cell surface

receptors. Although this dominant role of receptors in initiating NGF effects is well established, the question of whether NGF can also act from within the cell is not fully settled.

NGF-R Identification and Structure

In recent years, cloning and sequencing of human (36), rat (37), and chicken (38) cDNAs coding for the receptor protein have allowed the determination of its primary structure. Moreover, analysis of genomic clones of the promoter has begun to define the upstream regions responsible for the restricted expression of this protein in vivo (39). The cDNAs predict a protein with an apparent molecular mass of 45 kd that increases to about 75 kd after glycosylation. The receptor polypeptide can be divided into several distinct domains, each with differences in the degree of intraspecies conservation. The signal peptide is followed by a stretch of about 160 amino acids rich in cysteine residues that presents a high degree of identity. Similarly to the EGF receptor and the genes belonging to its family, this region appears to be composed of four repeats of about 40 amino acids probably caused by repeated gene duplication events. Sequence comparison has shown that the cysteine-rich region shows a significant degree of homology with the OX 40 T lymphocyte-specific antigen (40), the 50 Kd CD40 antigen expressed on B lymphocytes and carcinoma cells (41), and the tumor necrosis factor receptor (42). The most conserved region with 95% intraspecies homology is composed of 21 aa spanning the membrane flanked by 19 aa in the extracellular portion of the molecule and by the first 46 residues of the cytoplasmic region. A number of observations (see below), beside the degree of evolutionary conservation, strengthen the notion that this domain is essential for transduction of the NGF signal. The cytoplasmic domain of the receptor neither reveals the presence of an ATP binding site, nor bears any other resemblance to other receptors for growth factors with an intrinsic protein kinase activity. The NGF receptor moiety recognized by available antibodies is phosphorylated on serine residues only and the state of phosphorylation does not change with binding of NGF, again behaving differently from receptors for growth factors with associated tyrosine kinase activity. It has been suggested that phosphorylation of NGF-R might be correlated with constitutive internalization (43).

High-Affinity Receptor Functions

Most biochemical studies on NGF receptors have been carried out using PC12 cells, even though the kinetic parameters of binding of labeled-NGF have also been extensively characterized in chick embryo sympathetic and sensory neurons (44, 45), melanoma (46), neuroblastoma (47), and bovine and rat adrenal chromaffin cells (48). While most NGF-responsive cells display two

classes of NGF receptors, type I or slow and type II or fast, differing in affinity by more than one order of magnitude (from 10 pM to nM), transfection of cDNA clones to express the NGF receptor in a number of different cell backgrounds resulted in the appearance of the low-affinity species (type II) only. Other evidence strongly suggests that the cloned receptor is present in both high- and low-affinity receptor species: (*a*) crosslinking with water-soluble agents of labeled NGF with its receptor generated only a single molecular species that accounted for both forms of the receptor (49); (*b*) the same mRNA is detected in cells expressing predominantly type II receptors and in cells that express only the type I form (50). Under appropriate conditions type II can be converted to the type I receptor, but conversion occurs only in cells capable of responding to the factor (51). The general consensus is that the high-affinity receptor is the result of the association of the low-affinity moiety with a different polypeptide, likely to participate in the signal-transducing machinery (and perhaps expressed only in NGF-responsive cells), or to be involved in the internalization of the NGF-receptor complexes (52, 53). This conclusion is based on two observations: (*a*) crosslinking experiments with lipid-soluble agents have suggested the presence of a second protein of about 50 to 60 kd bound to the NGF-receptor complex (54); (*b*) the human type II receptor introduced into mutant PC12 cells that are defective for the expression of the endogenous gene causes both forms of the receptor to appear (55). In this case it has been possible to demonstrate that the expressed receptor is at least partially functional (55). Where the interaction between the two putative subunits of the NGF-R occurs is not known, but the observation that only hydrophobic reagents are capable of crosslinking the type II receptor to the putative transducing protein, suggests the conserved membrane-spanning domain of the receptor as a candidate for the interaction. Furthermore, other regions of the receptor are also involved in the association since deletions in the cytoplasmic domain of the receptor abolish the formation of the high-affinity form as shown by transfecting the human cDNA in receptor-deficient PC12 cells (M. Chao, personal communication).

The association of a membrane-spanning receptor with a protein deputed to signal transduction and bound to the cytoplasmic side of the plasma membrane is reminiscent of the recently described association of the T lymphocyte CD4 and CD8 antigens with the intracellular, membrane-bound tyrosine kinase $p56^{lck}$ (56). A slightly different situation holds for the interleukin 2 receptor: in this case two distinct polypeptide chains are both able to bind the ligand and to cooperate in creating a range of different affinities (57). As to the nature of the accessory protein, it has been speculated that the products of the *src* or *ras* proto-oncogenes might participate in the interconversion from low- to high-affinity NGF receptors (37, 53) (see below).

Since the NGF-receptor exists in a dynamic state in the cell membrane and is known to be quickly internalized following binding of the ligand, the endocytic process itself may easily mimic a slowly dissociating complex (58,

51). If the "accessory subunit" of the NGF-R participates in the internalization of the NGF-receptor complex, dissociates from it and recycles to the membrane via a different pathway, it might represent a mechanism for the observed interconversion between low- and high-affinity receptors. Whatever the molecular basis of type I receptors, they might help the cell to discriminate between NGF and related molecules. In particular, the high-affinity receptors have about 1,000-fold higher affinity for NGF than for the brain-derived neurotrophic factor (BDNF), whereas type II receptors do not appear to discriminate between the two growth factors (59).

Is There a Function for Low-Affinity Receptors?

What is the functional relevance of the low-affinity receptor? In trying to answer this question two extreme points of view may be considered: the low-affinity receptor is not involved in signal transduction (but may be in other functions) or it cooperates with high-affinity receptors in mediating the full response to NGF. A complicating factor arises from the present lack of information on the local concentrations of NGF available for binding in vivo. Current thinking is that NGF is produced in small amounts by peripheral tissues or cells that stimulate NGF-responsive neurons. The local concentration of the growth factor is possibly suboptimal for saturation of the receptors, since exogenously added NGF has profound effects on survival and differentiation of presumptive target cells (1, 7). The local level of NGF, where axotomy has occurred, may be increased either by neosynthesis (reviewed in ref. 7) or by the induced expression of low-affinity NGF receptors on the surface of Schwann cells, these receptors being predicated to act by concentrating circulating NGF (60). In these conditions the local concentration of NGF may reach levels compatible with occupancy of type II receptors. PC12 cell mutants that possess low- but not high-affinity receptors neither respond to nor internalize NGF (52). Moreover, low-affinity receptors ectopically expressed in a number of cell types do not seem to be capable of activating second messengers or early genes (M. Chao, personal communication). However, the expression of NGF-R in *Xenopus* oocytes potentiates the ability of progesterone to induce maturation, a property in common with the v-*src* and v-*ras* oncogenes (61). A few examples are known of NGF-induced responses that may be attributed solely to the occupancy of the low-affinity receptors. The concentrations of NGF required for induction of tyrosine hydroxylase in sympathetic neurons are consistent with this effect being mediated by occupancy of the low-affinity receptors (62). It has also been reported that induction of VGF8a mRNA in PC12 cells reaches half maximum at 50 ng/ml NGF, i.e. at concentrations of NGF close to the Km of the low-affinity receptor (63). More definitive is the finding that melanoma cells that express only the low-affinity receptor species, but at high density, respond to NGF by surviving in the absence of serum (46).

As postulated for the two known types of glucocorticoid receptors (64), two receptor species with high and low affinity could provide a continuum of control and thus expand the range of the physiological response. Incidentally, note that most studies aimed at tracing NGF-responsive target cells in the central nervous system rely on specific antibodies or nucleic acid probes that recognize only the low-affinity receptor. If the notion that only high-affinity receptors are of biological relevance holds true, then identification of NGF-responsive cells should await the characterization of new components of the receptor complex. Moreover, the similar affinities of NGF and BDGF for type II NGF-R noted above advise against equalizing type II NGF-R-bearing cells with NGF-responsive cells.

Receptor Physiology

In PC12 cells the NGF bound to the cell surface is internalized by receptor-mediated endocytosis (65, 66) and the internalized NGF has a relatively long half-life (67). More strikingly, specific retrograde transport of NGF occurs in vivo in endocytotic vesicles from the tips of the axons to the cell body (68). On the one hand, if the biological effects of NGF are presumably mediated at the level of the plasma membrane with the production of one (or more) second messengers responsible for generating an intracellular cascade of biochemical events, then the internalization of NGF-receptor complexes may be instrumental only to the down-regulation of the signal. On the other hand, the existence of a specific mechanism for retrograde transport suggests that the function of the internalization process is to deliver NGF to strategic intracellular compartments. Although the role of internalized NGF-receptor complexes is still subject to experimental investigation, intracellular binding of growth factors to their receptors is sufficient in other cellular contexts to produce a functional autocrine response (69, 70). One possible role of internalized NGF is suggested from the finding that it has a perinuclear localization (67) and that chromatin binding of internalized NGF has been reported (71; but see ref. 72). A reconciling hypothesis could be that multiple signals are generated, some at the level of the plasma membrane, others following internalization and transport to the cell body, and that these signals cooperate synergistically to elicit the full biological response. If such a "multiple hit" mechanism exists, it would provide the target cell with a means of double checking for the presence of NGF.

SECOND MESSENGERS IN THE MECHANISM OF ACTION OF NGF

Like a mystery novel with too many suspects, many classical second messengers have been shown to be modulated by NGF, and are thus possible mediators of NGF action. However, no single second messenger has clearly

been demonstrated to be responsible for expressing the full range of changes induced by NGF, and virtually all second messengers and other early events downstream identified to date are also activated with similar kinetics and quantitative variations by other growth factors (e.g. EGF) and a multitude of pharmacological agents, which, however, do not lead to differentiation. The simplest, but minimally heuristic, conclusion would be that a large number of the early biochemical responses may not be directly relevant to the final outcome, i.e. increased transcription of differentiation-specific genes. For instance, if the target cell needs to double-check for the presence of NGF, some of the first responses may be aimed at putting the cell on a stand-by position. Finally, to understand the role of second messengers as mediators of NGF-induced differentiation two points should be considered: (*a*) most second messengers are only transiently modulated, yet the requirement for NGF to be continuously present suggests that some long-lasting modification of cellular pathways is needed; (*b*) NGF could conceivably utilize different pathways in the initiation of the differentiative program of PC12 and in the maintenance of the acquired phenotype.

Calcium Ions

Activation of the Na^+/K^+ ATPase with consequent increase in the influx of monovalent ions is one of the earliest events in the response to NGF (20). By analogy with EGF action, this effect may be linked to the initial mitogenic stimulation exerted by NGF on PC12 cells.

Modulation of intracellular concentration of calcium by NGF has been a controversial hypothesis but it now seems to be established by direct measurements (73; G. Guroff, personal communication) as well as by indirect evidence (74). It is the consequence of both calcium influx and release from internal stores. Downstream events, possibly regulated by an elevated calcium level, are Ca/calmodulin-dependent protein kinase III, phosphorylation of elongation factor 2 (75), catecholamine release (76), and phosphatidylinositol turnover (77).

cAMP

The existence of a cAMP connection in the action of NGF was mainly postulated because (*a*) cell-permeable cAMP-analogues induce phenotypic changes similar to early NGF-induced events and (*b*) NGF activates protein kinase A (PK-A). However, it has been difficult to demonstrate a direct activation of adenylate cyclase by NGF and conflicting reports exist on the existence of positive variations in cAMP (78, 79). In addition, no changes in the activity and level of expression of G_s and G_i proteins after NGF treatment have been observed in PC12 cells (A. M. Salvatore, P. Calissano, personal communication). Furthermore, the effects of cAMP analogues on PC12 cells

and primary sympathetic and sensory neurons are clearly distinct from those induced by NGF as far as activation of gene expression (80) and growth of neurites are concerned (14, 81). On the other hand, some of the phosphorylation events caused by NGF can be accounted for by activation of PK-A (see below). That PK-A activity may be involved in the modulation of the expression of the differentiated phenotype is suggested by the observation that the induction of sodium channels in differentiated PC12 cells is specifically blocked by injection of an expression plasmid coding for an inhibitor of the catalytic subunit of PK-A (82).

Phospholipids

Very rapid modulation by NGF of the synthesis of inositol mono, bis, and trisphosphate were reported by Contreras & Guroff (77), who also showed that the NGF-stimulated hydrolysis of polyphosphoinositides was dependent on extracellular calcium. The production of IP_3 was observable within seconds after exposure to NGF and may be responsible for the reported rapid increase in intracellular calcium (73).

A recent report links the binding of NGF to its functional receptors in PC12 cells to a rapid (less than one minute) production of myristate-containing diacylglycerol and an inositol phosphate glycan, generated by the digestion of a glycosyl-phosphatidylinositol (83). The release of diacylglycerol by this previously unidentified pathway represents an alternative way of stimulating protein kinase C without breakdown of phosphatidylinositols. A different kind of phospholipid modification, phospholipid methylation, has been invoked as a possible mediator of NGF action mainly on the evidence that exposure of primary target cells to NGF is rapidly followed by an increase of methylated phospholipids (84, 85). However, no significant enhancement of phospholipid methylation of PC12 cells exposed to NGF has been measured and inhibitors of phospholipid methylation fail to prevent neurite outgrowth (86).

PROTEIN METHYLATION

Inhibition of protein methylation has profound effects on the response of PC12 cells to NGF. Both early and late effects of NGF are inhibited without any appreciable effect on NGF binding. Interestingly, prevention of protein methylation discriminated between NGF and EGF in the sense that the same early responses elicited by the two factors were unaffected in the case of EGF (87). A subsequent report also links protein methylation to the trophic effects of NGF on primary cultures (88). Although very promising, the results reported above are not definitive because they rely on the use of methyltrans-

ferase inhibitors that may affect nucleic acid methylation and it has not been possible to identify specific protein substrates for methylation.

PROTEIN PHOSPHORYLATION

As stated above, a number of serine- and threonine-specific protein kinases are activated by NGF binding to its receptor; the list includes PK-A (89), protein kinase C (PK-C)(89–91), S6 protein kinase (92), MAP-2 protein kinase (93), and the newly described protein kinase N (94). This impressive amount of data has been recently reviewed (see ref. 95). The activation of each of these different kinases is probably not an independent event in the cells but results from "crosstalk", in the sense that NGF is probably initiating a cascade of interrelated activations. Since it is difficult to arrange a genealogical tree based only on temporal criteria—these activations all occur within minutes—the pharmacological approach with specific kinase inhibitors has proved more profitable. Some of these inhibitors, moreover, offer the advantage of being able to discriminate between NGF and EGF, which, as far as activation of the kinases and phosphorylation of protein substrates is concerned, behave with an almost superimposable pattern.

The kinase inhibitor K-252a blocks the action of NGF while leaving unaltered most of the effects triggered by EGF (96). A note of caution is however necessary since conflicting observations on the effects of K-252a on the trophic effect of NGF in primary cultures have been reported (see 97 and ref. therein). An example of the power of kinase inhibitors in dissecting the pathways activated by NGF is provided by the inhibition exerted by the purine analog 6-thioguanine (6-TG). 6-TG specifically blocks the activity of protein kinase N (98) and consequently some but not all transcription-dependent and transcription-independent effects of NGF (99; L. A. Greene, personal communication). Similarly, the inhibition of protein kinase C exerted by sphingosine defines a set of responses in PC12 cells that are probably mediated through this kinase (91), a finding reinforced by the use of PC12 cells in which protein kinase C is down-regulated (100). It is worth mentioning, however, that down-regulation of PKC fails to prevent neurite outgrowth in PC12 cells both in response to NGF (100, 101) and to an activated *ras* gene (102).

The main difficulty in the use of a pharmacological approach to dissect the cause-effect relationship in the activation of several kinases is the lack of absolute specificity of some of the inhibitors used. For instance, the kinase inhibitor K-252a blocks most biological effects of NGF in PC12 cells. While this finding might imply that it interferes with a very early, essential step in the NGF-induced pathway, it is also consistent with K-252a's blocking several independent kinases. This reasoning is further supported by the use of purine analogs, described above: the activation of kinase N is itself a consequence of a phosphorylation event and can be blocked by the inhibitor

2-aminopurine. The latter substance, however, blocks pathways both upstream and downstream from those operationally defined by interference with kinase N activity (L. A. Greene, personal communication).

One final issue related to the phosphorylation of proteins is worth mentioning as more relevant to the maintenance of the differentiated phenotype in PC12 cells than to the induction of the differentiative program. There is a good temporal correlation between outgrowth of neurites, enhanced stability of microtubules and phosphorylation of a class of microtubule-associated proteins designated as chartins (103). Moreover, inhibition of morphological differentiation of NGF-treated cells by lithium ions also results in decreased phosphorylation of chartins (27).

The raf *Proto-oncogene*

The cytoplasmic serine/threonine kinase *raf*-1 has been identified as a potential down-stream effector of a variety of receptors in fibroblasts (104). Infection of PC12 cells with a truncated, transforming version of *raf*-1 causes the emission of neurites, thus suggesting that *raf*-1 may be a proto-oncogene component of the NGF signaling pathway for differentiation (K. W. Wood, T. M. Roberts, personal communication). In parallel experiments, both NGF and aFGF treatment of PC12 cells resulted in the phosphorylation of endogenous *raf*-1. With the exception of c-AMP, other treatments such as EGF, insulin, and high K^+ also caused phosphorylation of *raf*-1. In fibroblasts the transforming ability of *raf* is not blocked by anti-*ras* antibodies, implying that it functions downstream of both *src* and *ras* (104). It would be interesting to learn by microinjection of specific antibodies in PC12 cells where *raf*-1 is positioned in a hypothetical hierarchy of NGF-generated signals (See Figure 1).

The Involvement of Tyrosine Kinases

The involvement of tyrosine-phosphorylation was already suggested by the finding that FGF reproduces NGF effects in PC12 as well as in primary cells. The FGF receptor on PC12 cells is a single protein of 145 kd; it has an

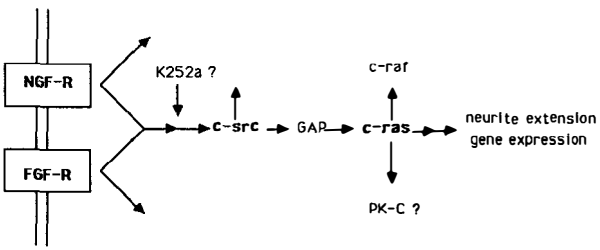

Figure 1 Model for proto-oncogene interactions in NGF-activated signaling pathway in PC12 cells.

apparent single affinity class and is not known to associate with other proteins (105). Although the cloned NGF-R is not a tyrosine kinase, two lines of evidence are consistent with a role of phosphorylation of tyrosine residues in mediating the differentiative response to NGF:

1. When PC12 cells are exposed to NGF there is a rapid (within 5 min) and transient phosphorylation of tyrosine residues in a limited number of cellular proteins (106, 107). This is in contrast to the more general phosphorylation of proteins on serine/threonine residues that is maintained as long as NGF is present. The molecular masses of these proteins are different from those found to be phosphorylated on tyrosine residues, showing that NGF activates several classes of protein kinases that phosphorylate distinct subsets of proteins. Recently, it has been shown that treatment of PC12 cells with NGF induces the phosphorylation of phospholipase C-γ on both serine and tyrosine (G. Guroff, personal communication). EGF and dibutyryl-cAMP also stimulate extensive protein tyrosine phosphorylation. However, NGF-induced tyrosine phosphorylation, but not that elicited by EGF, is inhibited by K252a and MeSAdo (107), suggesting that (*a*) NGF and EGF induce tyrosine phosphorylation by different mechanisms, (*b*) the inhibition of NGF action by K252a, already shown to be independent of PK-A and PK-C (96), is perhaps exerted directly on an NGF-stimulated tyrosine kinase, and (*c*) the NGF-induced tyrosine phosphorylation may be a very early step in the pathway. Is the activation of a tyrosine-protein kinase the first event elicited by the activated NGF receptor? Preliminary data do not seem to support this view; for instance, although K-252a blocks the induction of protein tyrosine phosphorylation (107), it does not prevent NGF-stimulated activation of MAP2 kinase (93).

2. Infection of PC12 cells with retroviruses expressing v-*src*, a viral oncogene homologous to the proto-oncogene *c-src* and which encodes the protein tyrosine-specific kinase $pp60^{v\text{-}src}$, efficiently induces these cells to progress further along their differentiative pathway, as demonstrated by the emission of long neurites, survival in serum-free conditions, and progressive withdrawal from the cell cycle (34, 108). The constant expression of $pp60^{v\text{-}src}$ is required for maintaining the differentiated phenotype, as shown by the use of temperature-sensitive conditional mutants (34, 109; S. Thomas, J. Brugge, personal communication). Although at the biochemical level v-*src*-differentiated cells express markers such as neurofilament proteins, AChase, NILE, and VGF8a proteins (108; E. Agostini, S. Alemà, unpublished data), the v-*src*-induced phenotype differs from that elicited by NGF in being refractory to inhibition by K252a (109). v-*src* effects are not confined to the PC12 cell line since v-*src* expression also allows differentiation of primary quail NGF-sensitive sympathetic neurons (110) and rescues differentiation in nnr5 cells, an NGF-unresponsive PC12 variant (105).

$pp60^{v\text{-}src}$ is located in the plasma membrane (and cytoskeleton) and the NGF pathway also originates peripherally, yet one level of action of both NGF and v-*src* that leads to phenotypic consequences (i.e. differentiation and transformation) is selective modulation of gene expression. Can we discern events both early or late in v-*src*-transformed cells that could help identify critical steps in NGF-signaling pathways? Recent reports indicate that neither activation of the canonical PI cycle nor direct activation of PK-C appears necessary for transformation by $pp60^{v\text{-}src}$ (reviewed in ref. 111). Identification of protein substrates crucial for transformation has proven difficult, (yet phosphorylation by $pp60^{src}$ of proteins such as vinculin, talin, or connexin clearly seems pertinent to some aspects of differentiation into neuronlike cells). The ability of v-*src* to phosphorylate the GAP protein appears more significant, establishing a link with *c-ras* (112) (see below). Recent preliminary results suggest that some of the substrates phosphorylated in PC12 cells by $pp60^{v\text{-}src}$ and the NGF-activated tyrosine kinase may overlap, although unique targets for each kinase distinguish the pathways activated by $pp60^{v\text{-}src}$ and NGF (S. M. Thomas, J. S. Brugge, personal communication).

The question arises whether the product of *c-src* or other known intracellular tyrosine kinases are involved in NGF-signal transduction. The *c-src* proto-oncogene has been implicated in neuronal differentiation and maintenance of specific functions because it is expressed in early differentiating and postmitotic neurons (113, 114). Furthermore, neurons also express $c\text{-}src^{+}$, a specific isoform with higher specific activity in vitro (115, 116). *c-src* differs from v-*src* in many structural and functional traits, including the finding that overexpression of *c-src* does not induce the growth effects of v-*src* in fibroblasts or neuroretinal cells (117). Recent experiments have shown that some transforming variants of both the receptor and nonreceptor classes of tyrosine kinases such as activated *c-fyn*, v-*fms* and polyoma middle T are capable of transducing signals that lead to neurite extension in PC12 cells, whereas partially transforming and nontransforming tyrosine kinases such as *c-src*, $c\text{-}src^{+}$ or *c-fyn* are unable to direct the complete response necessary for morphological differentiation (S. M. Thomas, J. S. Brugge, personal communication). Moreover, treatment of PC12 cells with sodium orthovanadate, a relatively specific inhibitor of phosphotyrosine phosphatases, can "prime" the cells to the action of NGF but by itself leads only to an abortive morphological and biochemical differentiation that is not as complete as that triggered by v-*src* or NGF (E. Agostini, S. Alemà, unpublished data). Perhaps the most convincing evidence for the involvement of $pp60^{c\text{-}src}$ in NGF-induced differentiation comes from microinjection experiments of a $pp60^{c\text{-}src}$ specific monoclonal antibody, whereby morphological differentiation induced by NGF and FGF was blocked, suggesting that $pp60^{c\text{-}src}$ may function downstream from early receptor-mediated events (S. M. Thomas, S.

Halegoua, J. S. Brugge, personal communication; Figure 1). In these experiments, however, the transient lifetime of intracellular antibodies made it impossible to rule out the notion that priming or specific gene expression were also affected, in addition to neurite extension. Other treatments such as lithium ions affect neurite extension without interfering with the priming process by NGF (27). Given this caveat, a simple explanation to reconcile the finding that elevated levels of *c-src* do not fully mimic the effects of v-*src* and NGF with the outcome of the microinjection experiments, might be that the activity of $pp60^{c\text{-}src}$ is necessary but not sufficient and that other pathways cooperate. $pp60^{v\text{-}src}$ may be endowed with properties that allow the kinase to serve as a substitute for these pathways.

ROLE OF *ras* GENES

A family of closely related oncogenes (Ha-, Ki-, and N-*ras*) encode proteins that, because of their structural and biochemical homology to G-proteins, are thought to act as coupling factors between receptors/primary transducers and a number of effector systems (111, 118). Expression of all viral counterparts of *ras* p21 protein by either microinjection or infection with retroviruses induces in PC12 cells a differentiated phenotype similar to that induced by NGF or v-*src* (35, 102, 119). In other experiments the oncogenic T24-*ras* protein, when introduced into the cytoplasm of primary chick embryonic neurons, promotes the in vitro survival and neurite outgrowth of NGF-responsive DRG neurons as well as of BDNF- and ciliary neurotrophic factor-responsive neurons (120). The proto-oncogene c-Ha-*ras* also promotes neuronal survival, albeit much less efficiently. These results suggest a generalized involvement of *ras* proteins in the signal transduction pathways for neurotrophic factors. An elegant series of experiments using a variety of mutants of the *ras* gene established that the protein domains responsible for induction of the differentiated phenotype in PC12 cells overlap with those required for transformation of fibroblasts (121). Transforming *ras* proteins expressed in PC12 cells activate a pathway that leads to the induction of *c-fos* and increased transcription from TPA-responsive elements, independently of the cAMP-dependent pathway of signal transduction (121; see below and Table I). Both oncogenic *ras* and TPA are commonly supposed to activate the PK-C pathway (111). Using an inducible cell system the functional relationship between *ras* p21 and PK-C during v-*ras*-induced differentiation of PC12 cell has been investigated (102). No formal proof of a requirement of functional PK-C as a downstream effector of $p21^{ras}$ was obtained. Contrary to results obtained previously in 3T3 cells, removal of endogenous PK-C did not interfere with the *ras*-p21-induced differentiation process, rather it enhanced *ras* function (102). This conforms with earlier speculation that PK-C is not required for NGF-induced differentiation (100, 101; see above).

Irrespective of the putative pathways involved in v-*ras*-induced differentiation, other lines of evidence are consistent with a role of c-*ras* in the NGF-activated pathway that leads to morphological (and biochemical) differentiation. First, microinjection of specific antibodies to $p21^{ras}$ block NGF-induced neurite extension and leave unaffected morphological changes elicited by elevated cAMP (122). Interestingly, recent experiments have shown that although antibodies to $p21^{ras}$ inhibit v-*src*- and v-*ras*-induced neurite extension, antibodies to $pp60^{src}$ do not inhibit v-*ras*-induced differentiation (S. M. Thomas, J. S. Brugge, S. Halegoua, personal communication). These results imply a hierarchy in which $pp60^{src}$ functions reside upstream of $p21^{ras}$ in the NGF-induced signal transduction pathway (Figure 1) and conform to previous conclusions reached in fibroblasts as far as the mitogenic signaling pathway is concerned (104). A possible link between the activity of *ras* proteins and that of some growth factor receptors and tyrosine kinase oncogenes is provided by the recent finding that the GTPase activator protein (GAP) is phosphorylated in tyrosine in cells expressing v-*src* or treated with PDGF (reviewed in ref. 112). The GAP protein enhances the GTPase activity of p21*ras,* thus down-regulating the *ras*-GTP active form, which is postulated to interact with downstream effectors of *ras*. It has been shown that the proto-oncogene Ha-*ras* induces a differentiated phenotype in PC12 cells when microinjected together with a nonhydrolyzable GTP analogue (123). Second, a dominant inhibitory *ras*-H mutant strongly inhibits differentiation by NGF or FGF, but not effects induced by TPA or elevated cAMP (J. Szeberenyi, G. M. Cooper, personal communication). Since clones expressing low levels of the mutated *ras* were blocked in differentiation, but normal in terms of c-*fos* activation, induction of c-*fos* and c-*jun* appears insufficient for secondary gene activation. This suggests that *ras* proteins are involved in at least two distinct signal transduction pathways in PC12 cells.

BINDING OF NGF IS ACCOMPANIED BY INCREASED EXPRESSION OF EARLY RESPONSE GENES

One consequence of the binding of NGF to type I receptor-bearing cells is the transient transcription of a specific set of genes, named immediate early response genes. These genes, which are classically expressed at augmented levels following mitogenic stimulation of resting cells, are considered to be required for progression through the G1 phase of the cell cycle and, in the capacity of "third" messengers, to convert extracellular stimuli into changes in the genetic program (111, 124). The activation of the immediate early genes is a mirror of the mobilization of the second messengers and the more we learn at the molecular level about the control elements of these genes, the more evident it becomes that they behave like sensors for a large number of environmental changes (124, 125). The proto-oncogene c-*fos* is a paradigm; it

displays both a serum-regulated element (target for the serum responsive factor, in turn activated by protein kinase C) and a cAMP-responsive element (target for the DNA binding protein CREB, in turn activated by at least PK-A and Ca/calmodulin binding protein) in the 300 bp preceding the start of transcription (126). In PC12 cells this class of genes is transcriptionally activated by a protein synthesis-independent process in response not only to NGF but also to EGF, serum, cAMP, phorbol esters, Ca ionophores, and depolarization (124, 127; Table I). NGF and depolarization may use distinct pathways to activate c-*fos* (127, 128). The activation of the immediate early genes in PC12 has been excellently reviewed in the larger context of early genes activated in the nervous system (125) and we refer the reader to it for an extensive bibliography.

At least some immediate early genes may be induced only as a stereotyped response to the varied levels of second messengers. Thus, the relevance of the induction of each of these genes needs to be validated experimentally to state that their function, if not sufficient, is at least necessary for the subsequent acquisition of the differentiated phenotype. One further aspect of the activation of immediate early genes deserves comment: in most cases the activation is very prominent, almost two order of magnitude above the resting level, and is also transient so that within minutes to a few hours the transcription and the level of mRNA return to unstimulated levels. One simple explanation is that the cell "overshoots" because the molecular machinery is ill-suited for a more graduated response and the excess produced is simply not utilized. Alternatively, considering that homeostasis of transcriptional factors is a crucial cellular function (and feed-back mechanisms exist (129)), overexpression of early genes may be the only way to bring their steady-state levels to a modest increase that could still be of functional relevance for late responses.

Most products of immediate early genes induced by NGF in PC12 cells are transcription factor themselves (Table I). Some, like c-*fos,* may be pleiotropic proteins acting as both a transrepressor and a transactivator, and interact to form multimeric complexes with sequence-specific DNA binding activity (126). A major problem is to understand the mechanisms whereby the specificity of the response to NGF is realized—in other words, how the immediate early genes are responsible for modulating the expression of genes induced later (Table I). Two possibilities are: (*a*) the activity of the known early genes is largely dispensable for NGF differentiation and the difference between EGF and NGF is then explained by activation of genes not yet identified; (*b*) different stimuli may differentially affect the function of early gene products through specific post-translational modifications. These would in turn affect their turnover or localization and the formation of multiprotein complexes. It is conceivable that these mechanisms cooperate in generating divergent pathways of activation of gene expression. Although it is generally

Table 1 Immediate early and late genes induced by nerve growth factor and other stimuli in PC12 cells

Gene	Specific induction by NGF/FGF	Function	Reference
Immediate early			
c-*fos*	−	transcription modulator/binds c-*jun*	133
c-*jun*	−	transcription factor	134
c-*myc*	−	transcription modulator	133
egr-1(NGF1A/D2/TIS8)	−	transcription factor/zinc finger containing protein	124, 135
nur 77/NGF1B	−	transcription factor of the steroid and thyroid hormone receptor superfamily	124, 136
D5	−	not known	80
PC4 (TIS7)	−	putative cytokine	137
PC3	−	putatively secreted protein	*
TIS11	−	not known	124
pip92	−	not known/cytoplasmic/short-lived	
Late			
VGF8a	+	secreted protein	63, 138
N-CAM	+	nerve cell adhesion molecule	139
41A/41C	+	members of the S100 family	140
Peripherin	+	neuronal-specific intermediate filament protein	141
SCG10	+	vesicle-associated protein	131
Transin	+	metalloprotease	130
Thymosin	+	not known	141

*F. Tirone, personal communication.

surmised that a subset of the activated immediate early genes cooperates to regulate the expression of the specific later genes, this hypothesis has not yet been fully demonstrated by studying the regulative sequences of the late genes directly. Which genomic sequences confer tissue specificity and NGF inducibility to these late genes is a question that is beginning to be answered (130–132; R. Possenti, A. Levi, unpublished data).

CONCLUSIONS

Studies on the biochemical changes induced by NGF in the experimental model provided by PC12 cells have begun to give clues to the pathways responsible for transducing its signal. Furthermore, the comparison with the intracellular events produced by other growth factors and activated oncogenes, as well as the use of pharmacological agents and PC12 mutants, should help in discriminating which events are relevant to the acquisition of the differentiated phenotype and which are redundant or dispensable. The

main unanswered question, for which only hypotheses can presently be advanced, is the means by which the specificity of the response to NGF is achieved. It is possible that activation of the NGF receptor leads to the production of a single, as yet unidentified, second messenger (specific for the NGF/NGF-R system) that, in turn, is responsible for generating all the subsequent events. All the available evidence, however, points to the fact that NGF is utilizing the same set of second messengers and effectors modulated by other factors in other cellular contexts to elicit other biological responses. Two, not mutually exclusive, viewpoints can explain how the specificity of the response to NGF is obtained: (*a*) the specificity relies on the PC12 cells themselves; (*b*) a peculiar topological or temporal combination of second messengers allows the cells to discriminate between NGF and, for instance, EGF. Hence, chromatine structure, DNA methylation, the presence of positive or negative transcription factors and similar mechanisms related to the previous history of restriction of potentiality of PC12 cells, may all contribute in providing PC12 cells with a limited choice of responses to epigenetic factors. The ability of NGF—as well as of other growth factors—to activate several second messengers in parallel has been proposed by many authors. The existence of multiproteic complexes associated with at least one growth factor receptor (112) may provide the biochemical basis for such a multisignal activation. Understanding how the distinct signals are integrated for generating the differentiative response in PC12 cells is probably the key to elucidating the mechanism of action of NGF.

ACKNOWLEDGMENT

We thank J. Brugge, M. Chao, L. Greene, G. Guroff, P. Mahrer, J. Meldolesi, and J. Wagner for sending preprints and for communicating unpublished results. We are grateful to our many colleagues from our laboratories for thoughtful discussions and comments on the manuscript. This review was written with the support of grants from CNR-"Progetto Finalizzato Biotecnologie e Biostrumentazione" and Associazione Italiana Ricercha sul Cancro.

Literature Cited

1. Levi-Montalcini, R. 1987. The nerve growth factor 35 years later. *Science* 237:1154–62
2. Calissano, P., Cattaneo, A., Aloe, L., Levi-Montalcini, R. 1984. The nerve growth factor (NGF). *Horm. Proteins Pept.* 12:1–56
3. Hohn, A., Leibrock, J., Bailey, K., Barde, A. 1990. Identification and characterization of a novel member of the nerve growth factor/brain derived neurotrophic factor family. *Nature* 344:339–41
4. Maisonpiere, P. C., Belluscio, L., Squnto, S., Ip, N., Furth, M. E., Lindsay, R. M. 1990. Neurotrophin-3: a neurotrophic factor related to NGF and BDNF. *Science* 247:1446–51
5. Stockel, K. A., Lottspeich, F., Sendtner, M., Masiakowski, P., Carroll, P., et al. 1989. Molecular cloning, expression and regional distribution of rat

ciliary neurotrophic factor. *Nature* 342: 920–23
6. Rosenthal, A., Goeddel, D. V., Nguyen, T., Lewis, M., Shih, A., et al. 1990. Primary structure and biological activity of a novel human neurotrophic factor. *Neuron* 4:767–73
7. Thoenen, H., Bandtlow, C., Heumann, R. 1987. The physiological function of nerve growth factor in the central nervous system: comparison with the periphery. *Rev. Physiol. Biochem. Pharmacol.* 109:145–78
8. Whittemore, S. R., Steiger, A. 1987. The expression, localization and functional significance of β-nerve growth factor in the central nervous system. *Brain Res. Rev.* 12:439–64
9. Vantini, G., Schiavo, N., Di Martino, A., Polato, P., Triban, C., et al. 1989. Evidence for a physiological role of nerve growth factor in the central nervous system of neonatal rats. *Neuron* 3:267–73
10. Levi-Montalcini, R., Aloe, L, Alleva, E. 1990. A role for nerve growth factor in nervous, endocrine and immune systems. *Progr. Neuroendocriimmunol.* 3:1–10
11. Cattaneo, A. 1986. Thymocytes as potential target cells for nerve growth factor. In *Molecular Aspects of Neurobiology,* ed. R. Levi-Montalcini, P. Calissano, E. R. Kandel, A. Maggi, pp. 31–36. Berlin: Springer-Verlag
12. Otten, U., Ehrhard, P., Peck, R. 1989. Nerve growth factor induces growth and differentiation of human B lymphocytes. *Proc. Natl. Acad. Sci. USA* 86:10059–63
13. Levi-Montalcini, R., Calissano, P. 1986. Nerve growth factor as a paradigm for other polypeptide growth factors. *Trends Neurosci.* 8:473–77
14. Rydel, R. E., Greene, L. A. 1988. cAMP analogs promote survival and neurite outgrowth in cultures of rat sympathetic and sensory neurons independently of nerve growth factor. *Proc. Natl. Acad. Sci. USA* 85:1257–61
15. Martin, D. P., Wallace, T. L., Johnson, E. M. 1990. Cytosine arabinoside kills postmitotic neurons in a fashion resembling trophic factor deprivation: evidence that a deoxycytidine-dependent process may be required for nerve growth factor signal transduction. *J. Neurosci.* 10:184–93
16. Koike, T., Martin, D. P., Johnson, E. M. 1989. Role of Ca^{++} channels in the ability of membrane depolarization to prevent neuronal death induced by trophic factor deprivation; evidence that levels of internal Ca^{++} determine nerve growth factor dependence of sympathetic ganglionic cells. *Proc. Natl. Acad. Sci. USA* 86:6421–25
17. Greene, L. A., Tischler, A. S. 1982. PC12 pheochromocytoma cultures in neurobiological research. *Adv. Cell. Neurobiol.* 3:373–414
18. Guroff, G. 1985. PC12 cells as a model of neuronal differentiation. In *Cell Culture in Neuroscience,* ed. J. E. Bottenstein, J. Sato, pp. 245–66. New York: Plenum
19. Levi, A., Biocca, S., Cattaneo, A., Calissano, P. 1988. The mode of action of nerve growth factor in PC12 cells. *Mol. Neurobiol.* 2:201–26
20. Boonstra, J., Moolenar, W. H., Harrison, P. H., Moed, P., van der Saag, P. T., de Laat, S. W. 1983. Ionic response and growth stimulation induced by nerve growth factor and epidermal growth factor in rat pheochromocytoma PC12 cells. *J. Cell Biol.* 97:92–98
21. Rudkin, B. B., Lazarovici, P., Levi, B.-Z., Abe, Y., Fujita, K., Guroff, G. 1989. Cell cycle-specific action of nerve growth factor in PC12 cells: differentiation without proliferation. *EMBO J.* 8:3319–25
22. Ignatius, M. J., Chandler, C. R., Shooter, E. M. 1985. NGF-treated neurite-bearing PC12 cells continue to synthesize DNA. *J. Neurosci.* 5:343–51
23. Aloe, L., Levi-Montalcini, R. 1979. Nerve growth factor-induced transformation of immature chromaffin cells in vivo into sympathetic neurons: effects of antiserum to nerve growth factor. *Proc. Natl. Acad. Sci. USA* 76:1246–50
24. Unsicker, K., Krisch, B., Otten, J., Thoenen, H. 1978. Nerve growth factor-induced fiber outgrowth from isolated rat adrenal chromaffin cells: impairment by glucocorticoids. *Proc. Natl. Acad. Sci. USA* 75:3498–502
25. Lillien, L. E., Claude, P. 1985. Nerve growth factor as a mitogen for cultured chromaffin cells. *Nature* 317:632–34
26. Burstein, D. E., Greene, L. A. 1978. Evidence for RNA synthesis-dependent and -independent pathways in stimulation of neurite outgrowth by nerve growth factor. *Proc. Natl. Acad. Sci. USA* 75:6059–63
27. Burstein, D. E., Seeley, P. J., Greene, L. A. 1985. Lithium ions inhibit nerve growth factor-induced neurite outgrowth and phosphorylation of nerve-growth factor-modulated microtubule-associ-

ated proteins. *J. Cell Biol.* 101:862–70
28. Togari, A., Dickens, G., Kuzuya, H. Guroff, G. 1985. The effect of fibroblast growth factor on PC12 cells. *J. Neurosci.* 5:307–16
29. Satoh, T., Nakamura, S., Taga, T., Matsuda, T., Hirano, T., et al. 1988. Induction of neuronal differentiation in PC12 cells by B-cell stimulatory factor 2/interleukin 6. *Mol. Cell. Biol.* 8:3546–49
30. Ullrich, A., Schlessinger, J. 1990. Signal transduction by receptors with tyrosine kinase activity. *Cell* 61:203–12
31. Knusel, B., Michel, P. P., Schwaber, J. S., Hefti, F. 1990. Selective and nonselective stimulation of central cholinergic and dopaminergic development in vitro by nerve growth factor, basic fibroblast growth factor, epidermal growth factor, insulin and the insulin-like growth factors I and II. *J. Neurosci.* 10:558–70
32. Ruta, M., Burgess, W., Givol, D., Neiger, N., Kaplow, J., et al. 1989. Receptor for acidic fibroblast growth factor is related to the tyrosine kinase encoded by the fms-like gene (FLG). *Proc. Natl. Acad. Sci. USA* 86:8722–726
33. Rydel, R. E., Greene, L. A. 1987. Acidic and basic fibroblast growth factors promote stable neurite outgrowth and neuronal differentiation in cultures of PC12 cells. *J. Neurosci.* 7:3639–53
34. Alemà, S., Casalbore, P., Agostini, E., Tatò, F. 1985. Differentiation of PC12 phaeochromocytoma cells induced by v-*src* oncogene. *Nature* 316:557–59
35. Noda, M., Ko, M., Ogura, A., Lin, D., Amano, T., et al. 1985. Sarcoma viruses carrying *ras* oncogenes induce differentiation-associated properties in a neuronal cell line. *Nature* 318:73–75
36. Johnson, D., Lanahan, A., Buck, C. R., Sehgal, A., Morgan, C., et al. 1986. Expression and structure of the human NGF receptor. *Cell* 47:545–54
37. Radeke, M. J., Misko, T. P., Hsu, C., Herzenberg, L. A., Shooter, E. M. 1987. Gene transfer and molecular cloning of the rat nerve growth factor receptor. *Nature* 325:593–97
38. Large, T. H., Weskamp, G., Helder, J. C., Radeke, M. J., Misko, T. P., et al. 1989. Structure and developmental expression of the nerve growth factor receptor in the chicken central nervous system. *Neuron* 2:1123–34
39. Patil, N., Lacy, E., Chao, M. V. 1990. Specific neuronal expression of human NGF receptors in the basal forebrain and cerebellum of transgenic mice. *Neuron* 2:437–47
40. Mallet, S., Fossum, S., Barclay, A. N. 1990. Characterization of the MRC OX40 antigen of activated CD4 positive T lymphocytes—a molecule related to nerve growth factor receptor. *EMBO J.* 9:1063–68
41. Stamenkoovic, I., Clark, E. A., Seed, B. 1989. A B-lymphocyte activation molecule related to the nerve growth factor receptor and induced by cytokines in carcinomas. *EMBO J.* 8:1403–10
42. Loetscher, H., Pan, Y. C. E., Lahm, H.-W., Gentz, R., Brockhaus, M., et al. 1990. Molecular cloning and expression of the human 55 kD tumor necrosis factor receptor. *Cell* 61:351–59
43. Taniuchi, M., Johnson, E. M., Roach, P. J., Lawrence, J. C. 1986. Phosphorylation of nerve growth factor receptor proteins in sympathetic neurons and PC12 cells. *J. Biol. Chem.* 261:13342–49
44. Frazier, W. A., Boyd, L. F., Bradshaw, R. A. 1974. Properties of the specific binding of ^{125}I nerve growth factor to responsive peripheral neurons. *J. Biol. Chem.* 249:5513–19
45. Sutter, A., Riopelle, R. J., Harris-Warrick, R. M., Shooter, E. M. 1979. Nerve growth factor receptors. *J. Biol. Chem.* 254:5972–82
46. Fabricant, R. N., De Larco, J. E., Todaro, G. J. 1977. Nerve growth factor receptors on human melanoma cells in culture. *Proc. Natl. Acad. Sci. USA* 74:565–69
47. Sonnenfeld, K. H., Ishii, D. N. 1985. Fast and slow nerve growth factor binding site in human neuroblastoma and rat pheochromocytoma cell lines: Relationship of sites to each other and to neurite formation. *J. Neurosci.* 5:1717–28
48. Hofmann, H. D., Ebener, C., Unsicker, K. 1987. Age-dependent differences in ^{125}I nerve growth factor binding properties of rat adrenal chromaffin cells. *J. Neurosci. Res.* 18:574–77
49. Green, S. H., Greene, L. A. 1986. A single Mr 103,000 ^{125}I-β nerve growth factor-affinity-labeled species represents both the low and high affinity forms of the nerve growth factor receptor. *J. Biol. Chem.* 261:15316–26
50. Hemptstead, B., Patil, N., Olson, K., Chao, M. 1988. Molecular analysis of the nerve growth factor receptor. *Cold Spring Harbour Symposia Quantitative Biol.* 53:477–85
51. Eveleth, D. D., Bradshaw, R. A. 1988. Internalization and cycling of nerve

growth factor in PC12 cells: interconversion of type II (fast) and type I (slow) nerve growth factor receptors. *Neuron* 1:929–36
52. Green, S. H., Rydel, R. E., Connolly, J. L., Greene, L. A. 1986. PC12 cell mutants that possess low- but not high-affinity nerve growth factor receptors neither respond to nor internalize nerve growth factor. *J. Cell. Biol.* 102:830–43
53. Hosang, M., Shooter, E. M. 1987. The internalization of nerve growth factor by high-affinity receptors on pheocromocytoma PC12 cells. *EMBO J.* 6:1197–12
54. Hosang, M., Shooter, E. M. 1985. Molecular characteristic of the nerve growth factor receptor in PC12 cells. *J. Biol. Chem.* 260:655–62
55. Hempstead, B. L., Schleifer, L. S., Chao, M. 1989. Expression of functional nerve growth factor receptors after gene transfer. *Science* 243:373–75
56. Robey, E., Axel, R. 1990. CD4: collaborator in immune recognition and HIV infection. *Cell* 60:697–700
57. Hatakeyama, M., Tsudo, M., Minamoto, S., Kono, T., Doi, T., et al. 1989. Interleukin-2 receptor β chain gene: generation of three receptor forms by cloned human α and β chain cDNA's. *Science* 244:551–56
58. Cattaneo, A., Biocca, S., Nasi, S., Calissano, P. 1983. Hidden receptors for nerve growth factor in PC12 cells. *Eur. J. Biochem.* 135:285–90
59. Rodriguez-Tebar, A., Dechant, G., Barde, Y-A. 1990. Binding of brain-derived neurotrophic factor to the nerve growth factor receptor. *Neuron* 4:487–92
60. Taniuchi, M., Clark, H. B., Schweitzer, J. B., Johnson, E. M. 1988. Expression of nerve growth factor receptors in Schwann cells of axotomized peripheral nerves: ultrastructural location, suppression by axonal contact and binding. *J. Neurosci.* 8:664–81
61. Seghal, A., Wall, D. A., Chao, M. 1988. Efficient processing and expression of the human nerve growth factor receptors in *Xenopus laevis* oocytes: effect on maturation. *Mol. Cell. Biol.* 8:2242–46
62. Hill, C. E., Hendry, I. A. 1976. Differences in sensitivity to nerve growth factor of axon formation and tyrosine hydroxylase induction in cultured sympathetic neurons. *Neuroscience* 1: 489–96
63. Levi, A., Eldridge, J. D., Paterson, B. M. 1985. Molecular cloning of a gene sequence regulated by nerve growth factor. *Science* 229:393–95
64. Evans, R. M., Arriza, J. L. 1989. A molecular framework for the action of glucocorticoid hormones in the nervous system. *Neuron* 2:1105–12
65. Calissano, P., Shelanski, M. L. 1980. Interaction of NGF with pheochromocytoma cells: evidence for tight binding and sequestration. *Neuroscience* 5: 1033–39
66. Levi, A., Schechter, Y., Neufeld, E. J., Schlessinger, J. 1980. Mobility, clustering and transport of nerve growth factor in embryonal sensory cells and in a sympathetic neuronal cell line. *Proc. Natl. Acad. Sci. USA* 77:3469–73
67. Marchisio, P. C., Naldini, L., Calissano, P. 1980. Intracellular distribution of nerve growth factor in rat pheochromocytoma PC12 cells: evidence for a perinuclear and intranuclear location. *Proc. Natl. Acad. Sci. USA* 77:1656–60
68. Hendry, I. A. 1980. Retrograde axonal transport. In *Proteins of the Nervous System*, ed. R. A. Bradshaw, D. M. Schneider, pp. 183–211. New York: Raven
69. Bejcek, B. E., Li, D. Y., Deuel, T. F. 1989. Transformation by v-*sis* occurs by internal autoactivation mechanism. *Science* 245:1496–99
70. Dunbar, C. E., Browder, T. M., Abrams, J. S., Nienhuis, A. W. 1989. COOH-terminal-modified interleukin-3 is retained intracellularly and stimulates autocrine growth. *Science* 245:1493–96
71. Rakowicz-Szulczynska, E. M., Rodeck, L., Merlyn, M., Koprowski, H. 1986. Chromatin binding of epidermal growth factor, nerve growth factor, and platelet-derived growth factor in cells bearing the appropriate surface receptors. *Proc. Natl. Acad. Sci. USA* 83:3728–32
72. Hogue-Angeletti, R., Stieber, A., Gonatas, N. K. 1982. Endocytosis of nerve growth factor by PC12 cells studied by quantitative ultrastructural autoradiography. *Brain Res.* 241:145–56
73. Pandiella-Alonso, A., Malgaroli, A., Vicentini, L. M., Meldolesi, J. 1986. Early rise of cytosolic Ca^{2+} induced by NGF in PC12 and chromaffin cells. *FEBS Lett.* 208:48–51
74. Lazarovici, P., Levi, B.-Z., Lelkes, P. I., Koizumi, S., Fujita, K., et al. 1989. K252a inhibits the increase in *c-fos* transcription and the increase in intracellular calcium produced by nerve growth factor in PC12 cells. *J. Neurosci. Res.* 23:1–8
75. Koizumi, S., Ryazanov, A., Hama, T.,

Chen, H-C., Guroff, G. 1989. Identification of Nsp 100 as elongation factor 2 (EF-2). *FEBS Lett.* 253:55–58
76. Nicodijevic, B., Creveling, C. R., Koizumi, S., Guroff, G. 1990. Nerve growth factor and K252a increase catecholamine release from PC12 cells. *J. Neurosci. Res.* 26:288–95
77. Contreras, M. L., Guroff, G. 1987. Calcium-dependent nerve growth factor-stimulated hydrolysis of phosphoinositides in PC12 cells. *J. Neurochem.* 48:1466–1472
78. Schubert, D., Witlock, C. 1977. Alterations of cellular adhesion by nerve growth factor. *Proc. Natl. Acad. Sci. USA* 74:4055–58
79. Hatanaka, H., Otten, U., Thoenen, H. 1978. Nerve growth factor-mediated selective induction of ornithine decarboxylase in rat pheochromocytoma: a cyclic AMP-independent process. *FEBS Lett.* 92:313–16
80. Cho, K., Skarnes, W. C., Minsk, B., Palmieri, S., Jackson-Grusby, L., Wagner, J. A. 1989. Nerve growth factor regulates gene expression by several distinct mechanisms. *Mol. Cell. Biol.* 9:135–43
81. Heidemann, R. S., Joshi, H. C., Schechter, A., Fletcher, J. R., Bothwell, M. 1985. Synergistic effects of cyclic AMP and NGF on neurite outgrowth and MT stability of PC12 cells. *J. Cell. Biol.* 100:916–27
82. Kalman, D., Wong, B., Horvai, A. E., Cline, M. J., O'Lague, P. H. 1990. Nerve growth factor acts through cAMP-dependent protein kinase to increase the number of sodium channels in PC12 cells. *Neuron* 2:355–66
83. Liwah Chan, B., Chao, M. V., Saltiel, A. L. 1989. Nerve growth factor stimulates the hydrolysis of glycosyl-phosphatidylinositol in PC12 cells: a mechanism of protein kinase C regulation. *Proc. Natl. Acad. Sci. USA* 86:1756–50
84. Pfenninger, K. H., Johnson, M. P. 1981. Nerve growth factor stimulates phospholipid methylation in growing neurites. *Proc. Natl. Acad. Sci USA* 78:7797–800
85. Skaper, S. D., Varon, S. 1984. Nerve growth factor stimulates phospholipid methylation in target ganglionic neurons independently of the cAMP and sodium pump responses. *J. Neurochem.* 42: 116–22
86. Ferrari, G., Greene, L. A. 1895. Does phospholipid methylation play a role in the primary mechanism of action of nerve growth factor? *J. Neurochem.* 45:853–59
87. Seeley, P. J., Rukenstein, A., Connolly, J. L., Greene, L. A. 1984. Differential inhibition of nerve growth factor and epidermal growth factor effects on the PC12 pheocromocytoma line. *J. Cell Biol.* 98:417–26
88. Acheson, A., Vogl, W., Huttner, W. B., Thoenen, H. 1986. Methyltransferase inhibitors block NGF-regulated survival and protein phosphorylation in sympathetic neurons. *EMBO J.* 5:2799–803
89. Cremins, J., Wagner, J. A., Halegoua, S. 1986. Nerve growth factor action is mediated by cyclic AMP and Ca^{2+}/phospholipid-dependent protein kinases. *J. Cell Biol.* 103:887–93
90. Hama, T., Huang, K. P., Guroff, G. 1986. Protein kinase C as a component of nerve growth factor-sensitive phosphorylation system in PC12 cells. *Proc. Natl. Acad. Sci. USA* 83:2353–2357
91. Hall, F. L., Fernyhough, P., Ishii, D. N., Vulliet, P. R. 1988. Suppression of nerve-growth factor-directed neurite outgrowth in PC12 cells by sphingosine, an inhibitor of protein kinase C. *J. Biol. Chem.* 263:4460–66
92. Blenis, J., Erikson, R. L. 1986. Regulation of protein kinase activities in PC12 pheochromocytoma cells. *EMBO J.* 5:3441–47
93. Miyasaka, T., Chao, M. V., Sherline, P., Saltiel, A. R. 1990. Nerve growth factor stimulates a protein kinase in PC12 cells that phosphorylates microtubule-associated protein-2. *J. Biol. Chem.* 265:4730–35
94. Rowland, E. A., Muller, T. H., Goldstein, M., Greene, L. A. 1987. Cell-free detection and characterization of a novel NGF-activated protein kinase in PC12 cells. *J. Biol. Chem.* 262:7504–13
95. Mutoh, T., Guroff, G. 1989. The role of phosphorylation in the action of nerve growth factor. *Bio-Factors* 2:71–76
96. Koizumi, S., Contreras, M. L., Matsuda, Y., Hama, T., Lazarovici, P., Guroff, G. 1988. K252a: a specific inhibitor of the action of nerve growth factor on PC12 cells. *J. Neurosci.* 8:715–21
97. Borasio, G. D. 1990. Differential effects of the protein kinase inhibitor K252a on the in vitro survival of chick embryonic neurons. *Neurosci. Lett.* 108:207–12
98. Volontè, C., Rukenstein, A., Loeb, D. M., Greene, L. A. 1989. Differential inhibition of nerve growth factor re-

sponse by purine analogues: correlation with inhibition of a nerve growth factor-activated protein kinase. *J. Cell Biol.* 109:2395–403

99. Greene, L. A., Volontè, C., Chalazonitis, A. 1990. Purine analogs inhibit nerve growth factor-promoted neurite outgrowth by sympathetic and sensory neurons. *J. Neurosci.* 10:1479–85
100. Damon, D. H., D'Amore, P. A., Wagner, J. A. 1990. Nerve growth factor and fibroblast growth factor regulate neurite outgrowth and gene expression in PC12 cells via both protein kinase C- and cAMP-independent mechanisms. *J. Cell Biol.* 110:1334–39
101. Reinhold, D. S., Neet, K. E. 1989. The lack of a role for protein kinase C in neurite extension and in induction of ornithine decarboxylase by nerve growth factor in PC12 cells. *J. Biol. Chem.* 264:3538–44
102. Lacal, J. C., Cuadrado, A., Jones, J. E., Trotta, R., Burstein, D. E., et al. 1990. Regulation of protein kinase C activity in neuronal differentiation induced by the N-*ras* oncogene in PC12 cells. *Mol. Cell. Biol.* 10:2983–90
103. Black, M. M., Aletta, J. M., Greene, L. A. 1986. Regulation of MT composition and stability during NGF-promoted neurite outgrowth. *J. Cell Biol.* 103:545–57
104. Smith, M. R., DeGudicibus, S. J., Stacey, D. W. 1986. Requirement for c-*ras* proteins during viral oncogene transformation. *Nature* 320:540–43
105. Eveleth, D. D., Hanecak, R., Fox, G. M., Fan, H., Bradshaw, R. A. 1989. v-*src* genes stimulate neurite outgrowth in pheochromocytoma (PC12) variants unresponsive to neurotrophic factors. *J. Neurosci. Res.* 24:67–71
106. Maher, P. 1988. Nerve growth factor induces protein-tyrosine phosphorylation. *Proc. Natl. Acad. Sci. USA* 85: 6788–91
107. Maher, P. 1989. Role of protein tyrosine phosphorylation in the NGF response. *J. Neurosci. Res.* 24:29–37
108. Casalbore, P., Agostini, E., Tatò, F., Alemà, S. 1986. Expression of the v-*src* oncogene induces differentiation in PC12 cells. See Ref. 11, pp. 37–39
109. Rausch, D. M., Dickens, G., Doll, S., Fujita, K., Koizumi, S., et al. 1989. Differentiation of PC12 cells with v-*src*: comparison with nerve growth factor. *J. Neurosci. Res.* 24:49–58
110. Haltmeier, H., Rohrer, H. 1990. Distinct and different effects of the oncogene v-*myc* and v-*src* on avian sympathetic neurons: retroviral transfer of v-*myc* stimulates neuronal proliferation whereas v-*src* transfer enhances neuronal differentiation. *J. Cell Biol.* 110:2087–98
111. Macara, I. G. 1989. Oncogenes and cellular signal transduction. *Physiol. Rev.* 69:797–820
112. Hall, A. 1990. *ras* and GAP—Who's controlling whom? *Cell* 61:921–23
113. Sorge, L. K., Levy, B. T., Maness, P. F. 1984. pp60$^{c\text{-}src}$ is developmentally regulated in the neural retina. *Cell* 36:249–57
114. Shartl, M., Barnekow, A. 1984. Differential expression of the cellular *src* gene during vertebrate development. *Dev. Biol.* 105:415–22
115. Lynch, S. A., Brugge, J. S., Levine, J. M. 1986. Induction of altered c-*src* product during neural differentiation of embryonal carcinoma cells. *Science* 234:873–76
116. Martinez, R., Mathey-Prevot, B., Bernards, A., Baltimore, D. 1987. Neuronal pp60$^{c\text{-}src}$ contains a six-amino acid insertion relative to its non-neuronal counterpart. *Science* 237:411–15
117. Iba, H., Jove, R., Hanafusa, H. 1985. Lack of induction of neuroretinal cell proliferation by Rous sarcoma virus variants that carry the c-*src* gene. *Mol. Cell. Biol.* 5:2856–59
118. Barbacid, M. 1987. *Ras* genes. *Annu. Rev. Biochem.* 56:779–827
119. Bar-Sagi, D., Feramisco, J. 1985. Microinjection of the *ras* oncogene protein into PC12 cells induces morphological differentiation. *Cell* 42:841–48
120. Borasio, G. D., John, J., Wittinghofer, A., Barde, Y.-A., Sendtner, M., Heumann, R. 1989. *ras* p21 protein promotes survival and fiber outgrowth of cultured embryonic neurons. *Neuron* 2:1087–96
121. Sassone-Corsi, P., Der, C. J., Verma, I. M. 1989. *ras*-induced neuronal differentiation of PC12 cells: possible involvement of *fos* nd *jun*. *Mol. Cell. Biol.* 9:3174–83
122. Hagag, N., Halegoua, S., Viola, M. 1986. Inhibition of nerve growth factor induced differentiation of PC12 cells by microinjection of antibodies to *ras* p21. *Nature* 319:680–82
123. Satoh, T., Nakamura, S., Kaziro, Y. 1987. Induction of neurite formation in PC12 cells by microinjection of proto-oncogene Ha-*ras* protein preincubated with guanosine 5'-O-(3-thiotriphosphate). *Mol. Cell. Biol.* 7:4553–56
124. Herschman, H. R. 1989. Extracellular

signals, transcriptional responses and cellular specificity. *Trends Biol. Sci.* 14:455–58
125. Sheng, M., Greenberg, M. E. 1990. The regulation and function of c-*fos* and other immediate genes in the nervous system. *Neuron* 4:477–85
126. Verma, I. M. 1990. *fos-jun* conspiracy: implications for the cell. In *Proto-oncogenes in Cell Development*. pp. 128–146. Chichester: Wiley, (Ciba Found. Symp. 150)
127. Morgan, J. E., Curran, T. 1986. Role of ion flux in the control of c-*fos* expression. *Nature* 322:552–55
128. Sheng, M., McFadden, G., Greenberg, M. E. 1990. Membrane depolarization and calcium induce c-*fos* transcription via phosphorylation of transcription factor CREB. *Neuron* 4:571–82
129. Sassone-Corsi, P., Sisson, J. C., Verma, I. M. 1988. Transcriptional autoregulation of the proto-oncogene *fos*. *Nature* 334:314–19
130. Machida, M. C., Rodland, K. D., Matrisian, L., Magun, B. E., Ciment, G. 1989. NGF induction of the gene encoding the protease Transin accompanies neuronal differentiation in PC12 cells. *Neuron* 2:1587–96
131. Mori, N., Stein, R., Sigmund, O., Anderson, D. J. 1990. A cell type-preferred silencer element that controls the neural-specific expression of the SCG10 gene. *Neuron* 4:583–94
132. Gizang-Ginsberg, E., Ziff, E. B. 1990. Nerve growth factor regulates tyrosine hydroxylase gene transcription through a nucleoprotein complex that contains c-*fos*. *Genes Dev.* 4:477–91
133. Greenberg, M. E., Greene, L. A., Ziff, E. B. 1985. Nerve growth factor and epidermal growth factor induce rapid transient changes in proto-oncogene transcription in PC12 cells. *J. Biol. Chem.* 260:14101–10
134. Wu, B., Fodor, E. J. B., Edwards, R. H., Rutter, W. J. 1989. Nerve growth factor induces the proto-oncogene c-*jun* in PC12 cells. *J. Biol. Chem.* 264:9000–3
135. Milbrandt, J. 1987. A nerve growth factor-induced gene encodes a possible transcriptional regulator. *Science* 238: 797–99
136. Milbrandt, J. 1988. Nerve growth factor induces a gene homologous to the glucocorticoid receptor gene. *Neuron* 1:183–88
137. Tirone, F., Shooter, E. 1989. Early gene regulation by NGF in PC12 cells: induction of an interferon-related gene. *Proc. Natl. Acad. Sci. USA* 86:2088–92
138. Possenti, R., Eldridge, J. D., Paterson, B. M., Grasso, A., Levi, A. 1989. A protein induced by NGF in PC12 cells is stored in secretory vesicles and released through the regulated pathway. *EMBO J.* 8:2217–23
139. Prentice, H. M., Moore, S. E., Dickson, J. G., Doherty, P., Walsh, F. S. 1987. Nerve growth factor induced changes in nerve cell adhesion molecule NCAM in PC12 cells. *EMBO J.* 6:1859–63
140. Masiakowski, P., Shooter, E. M. 1988. Nerve growth factor induces the genes for two proteins related to a family of calcium-binding proteins in PC12 cells. *Proc. Natl. Acad. Sci. USA* 85:1277–81
141. Leonard, D. G. B., Ziff, E. B., Greene, L. A. 1987. Identification and characterization of mRNA regulated by nerve growth factor in PC12 cells. *Mol. Cell. Biol.* 7:3156–67