

PC12 Cell Mutants That Possess Low- but Not High-Affinity Nerve Growth Factor Receptors Neither Respond to Nor Internalize Nerve Growth Factor

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Abstract. Four mutant PC12 pheochromocytoma cell lines that are nerve growth factor (NGF)-nonresponsive (PC12^{nr}) have been selected from chemically mutagenized cultures by a double selection procedure: failure both to grow neurites in the presence of NGF and to survive in NGF-supplemented serum-free medium. The PC12^{nr} cells were deficient in all additional NGF responses surveyed: abatement of cell proliferation, changes in glycoprotein composition, induction of ornithine decarboxylase, rapid changes in protein phosphorylation, and cell surface ruffling. However, PC12^{nr} cells closely resembled non-NGF-treated PC12 cells in most properties tested: cell size and shape; division rate; protein, phosphoprotein, and glycoprotein composition; and cell surface morphology. All four PC12^{nr} lines differed from PC12 cells in three ways in addition to failure of NGF response: (a)

PC12^{nr} cells failed to internalize bound NGF by the normal, saturable, high-affinity mechanism present in PC12 cells. (b) The PC12^{nr} cells bound NGF but entirely, or nearly entirely, at low-affinity sites only, whereas PC12 cells possess both high- and low-affinity NGF binding sites. (c) The responses to dibutyryl cyclic AMP that were tested appeared to be enhanced or altered in the PC12^{nr} cells compared to PC12 cells. Internalization of, and responses to, epidermal growth factor were normal in the PC12^{nr} cells ruling out a generalized defect in hormonal binding, uptake, or response mechanisms. These findings are consistent with a causal association between the presence of high-affinity NGF receptors and of NGF responsiveness and internalization. A possible relationship is also suggested between regulation of cAMP responses and regulation of NGF responses or NGF receptor affinity.

GENETIC dissection is a powerful tool in the analysis of biological processes. Even complex developmental processes can be so dissected by the use of appropriate model systems. The rat PC12 pheochromocytoma cell line has been developed and used for the study of the mechanism of action of nerve growth factor (NGF)¹ and of neuronal differentiation. PC12 cells respond to NGF by ceasing cell division and acquiring neuronal properties, which include long neurites (Greene and Tischler, 1976, 1982). In the absence of NGF, PC12 cells divide, providing large numbers of homogeneous cells for biochemical or molecular study. This also permits the selection and growth of PC12-derived mutant cell lines for genetic dissection of NGF responses and neuronal differentiation. This approach has already been used in two previous studies (Bothwell et al., 1980; Burstein and Greene, 1982b).

As in certain neurons, NGF responses in PC12 cells are mediated by specific NGF receptors. There appear to be two

affinity classes of binding sites, high- and low-affinity, in neurons (Andres et al., 1977; Sutter et al., 1979; Olender and Stach, 1980) and in PC12 cells (Yankner and Shooter, 1979; Calissano and Shelanski, 1980; Landreth and Shooter, 1980; Bernd and Greene, 1984). Two types of NGF binding sites have also been distinguished by a variety of criteria other than affinity, which include dissociation rate, Triton X-100 extractability, and trypsin sensitivity (Schechter and Bothwell, 1981), and molecular weight of ¹²⁵I-NGF-affinity-labeled protein (Massague et al., 1981; Puma et al., 1983; Ross et al., 1984; Hosang and Shooter, 1985).

There are several issues that have been raised by the apparent existence of two types of NGF receptors. First, are the two types of binding sites different molecules? Observation of two ¹²⁵I-NGF-affinity-labeled proteins of different molecular weights (Massague et al., 1981; Puma et al., 1983; Ross et al., 1984; Hosang and Shooter, 1985) suggests that the two binding sites are separate molecules; Grob et al. (1983), however, reported a single molecular weight protein in their affinity labeled preparations.

Second, which receptor mediates NGF internalization?

¹ Abbreviations used in this paper: AG, 8-azaguanine; dbcAMP, dibutyryl cyclic AMP; EGF, epidermal growth factor; EMS, ethyl methanesulfonate; Int./Ext., internal/external; NGF, nerve growth factor; ODC, ornithine decarboxylase; PC12^{nr}, PC12 pheochromocytoma cell lines that are NGF-nonresponsive.

NGF is internalized by neurons and PC12 cells (Burnham and Varon, 1973; Hendry et al., 1974; Claude et al., 1982; Hogue-Angeletti et al., 1982; Rohrer et al., 1982; Bernd and Greene, 1983) apparently by receptor-mediated endocytosis (Levi et al., 1980). Recent studies (Bernd and Greene, 1984) on the NGF concentration dependence of NGF internalization suggest that internalization is mediated by only the high-affinity site.

Third, which receptor mediates the biological actions of NGF? The concentrations of NGF required to permit survival and neurite outgrowth *in vitro* in sympathetic and dorsal root ganglion neurons (Frazier et al., 1973; Hill and Hendry, 1976) and in PC12 cells (Greene, 1977) are consistent with mediation by the high-affinity site. However, these results could also be consistent with the hypothesis that biological responses to NGF required NGF binding to only a small fraction of the population of low-affinity receptors. Also, the concentration of NGF required for induction of tyrosine hydroxylase in sympathetic neurons (Hill and Hendry, 1976) is much higher than would be expected if this response were mediated by high-affinity receptors. Baribault and Neet (1984) have shown that NGF binding to the "fast" receptor and stimulation of neurite outgrowth have similar sensitivities to tunicamycin treatment and have inferred that the "fast" and not the "slow" receptor mediates this response.

In this study, we have selected and cloned four mutant PC12 cell lines that apparently lack all responses to NGF but are otherwise very similar to the parent PC12 cells in the absence of NGF. These mutant lines also fail to internalize NGF, and they nearly or completely lack high-affinity NGF binding. Our studies of these mutants have provided results that are particularly relevant to the questions raised above: which type of NGF receptor mediates internalization of, and responses to, NGF. As these mutants possess almost exclusively only low-affinity NGF receptors but are otherwise very similar to PC12 cells, their comparison with the parent PC12 cells promises to be of value in answering the questions raised above regarding the molecular identity of the two types of NGF receptors and the mechanism for their possible inter-conversion.

Materials and Methods

Cell Culture

PC12 and PC12^{nm} cells were maintained on rat tail collagen-coated polystyrene tissue culture dishes (Falcon Labware, Oxnard, CA). The medium used was RPMI+5:10: 85% RPMI 1640 (Gibco, Grand Island, NY), 5% fetal calf and 10% heat-inactivated donor horse sera (KC Biological, Lenexa, KS). The cells were subcultured at ~10-d intervals at ratios of 1:5 or 1:6. For NGF-treated cultures, NGF was added at a concentration of 50 ng/ml (1.9 nM) unless otherwise noted.

Mutagenesis and Screen for Mutant Cell Lines

Early passage PC12 cells (10^7 cells in a 100-mm dish) were mutagenized by treatment with 15 mM ethyl methanesulfonate (EMS, Sigma Chemical Co., St. Louis, MO) in RPMI+5:10 for 6 h. This treatment produces a lethality rate of 80–90%.

After 10 d (~3 cell divisions) the cells were separated and replated into 32 dishes at a density of 5×10^4 cells per 100-mm dish. PC12 cells are very adherent to each other and form clumps that must be broken up to obtain suspensions of single cells. The cells were separated by treatment for 8 h with 1 mM EGTA in RPMI+5:10 followed by three washes with RPMI/H₂O, 1:1 and then vigorous trituration with a pasteur pipet. An aliquot of the cell suspension was counted, and the cells were diluted into an appropriate volume of RPMI+5:10 and plated. This procedure generally yields suspensions in

which at least half of the cells are singles and in which there are very few clumps of more than four cells. For the screens performed for neurite-defective mutants, however, there was an additional step: the cell suspension was treated for 10 min with 0.5% trypsin (Difco Laboratories Inc., Detroit, MI) at 37°C before plating. This produced suspensions of single cells only.

The low-density cultures were grown into small, discrete colonies for a month and then treated with NGF. 2 wk after addition of NGF to the cultures they were inspected by low power microscopy and colonies that appeared not to possess neurites were marked. These were then isolated by drawing them up into micropipets pulled from pasteur pipets and replated in 96-well plates. The lines were grown, transferred to 24-well plates, and then, at low density, to individual 35-mm dishes. After further growth, the lines were recloned by picking individual colonies from the 35-mm plates and growing them as for the initial cloning. These clones were then retested for lack of NGF-induced neurite outgrowth and were tested for survival in serum-free RPMI medium in the presence and absence of NGF.

8-Azaguanine (AG) resistant mutants were obtained by plating 10^7 PC12 cells in each of four 100-mm dishes and growing them in the presence of 3 µg/ml AG (Sigma Chemical Co.). 100 surviving colonies in each dish were counted over a known area, and this value was multiplied to obtain the number of surviving colonies per dish.

Phenotypic Analysis of the Mutant Lines

Previously published procedures were used for all of the analyses performed in this study. ³H-Fucose-labeled proteins were identified by methods described in McGuire et al. (1978). ³²P-Orthophosphate-labeled proteins were identified by methods described in Seeley et al. (1984). For these studies SDS polyacrylamide gel electrophoresis was done according to the methods described in Laemmli (1970) but with 32-cm 5–15% gradient gels. Ornithine decarboxylase (ODC) assays were done as described in Greene and McGuire (1978). Scanning electron microscopic studies of cell surface morphology were done as described in Connolly et al. (1979).

Preparation and Binding of ¹²⁵I-NGF

NGF was prepared from adult male mouse submandibular glands according to Mobley et al. (1976) and iodinated using lactoperoxidase according to a modification of the method of Sutter et al. (1979). The ¹²⁵I-NGF was isolated by applying the preparation to a 0.7 × 25-cm Bio-Gel P-100 column (Bio-Rad Laboratories, Richmond, CA) and eluting with phosphate-buffered saline that contained 1 mg/ml bovine serum albumin and 0.5 mg/ml protamine sulfate. The ¹²⁵I-NGF peak was >95% trichloroacetic acid-precipitable and had a specific activity of 3,000–4,000 cpm/fmol. NGF binding and determination of surface-bound and internalized ligand were performed on monolayer cultures by methods described in Bernd and Greene (1984). For these experiments concentrations of 15–600 pM ¹²⁵I-NGF and 0–15 nM NGF were used, the incubations were for 1 h at 37°C with an incubation volume of 1 ml containing 2×10^6 cells.

Results

Mutagenesis and Screen

PC12 cells were mutagenized, dispersed into a single cell suspension, and replated at low density. The cells were grown into small, spatially discrete colonies, and then NGF was added to induce neurite outgrowth. The colonies were inspected visually by low power microscopy to detect those not bearing neurites. This screen produced 24 clones. Three of these were flat, spread, large cell types; the rest were rounded or slightly flat cell types. It is likely that a larger number of neurite-deficient clones could have been obtained. First, because of the low cloning efficiency of PC12 cells at low densities, most of the cells did not survive in the initial growth phase and the period of NGF treatment. Second, some potential mutant colonies may have been overlooked in the visual inspection screen. Therefore, to obtain a better estimate of the efficiency of the mutagenesis, a selection for AG-resistant mutants was done using cells mutagenized by the same protocol as the cells used for the neurite-deficient screen. This screen produced AG-resistant mutants at a frequency of $\sim 10^{-4}$

which is two orders of magnitude greater than the spontaneous frequency we observed and is similar to frequencies reported by others for AG-resistant mutants (Gillin et al., 1972; Sharp et al., 1973). Thus, in EMS mutagenized cultures, induced mutations should outnumber spontaneous mutations by a factor of 100, and therefore the neurite-deficient clonal cell lines we have obtained are all likely to be EMS-induced rather than spontaneous mutants.

The 24 neurite-deficient clones were tested for the ability to show another response to NGF by culturing them in serum-free medium. PC12 cells do not survive in serum-free medium. However, if such medium is supplemented with NGF, they survive and acquire a neuronal phenotype (Greene, 1978). Four of the mutant lines failed to survive in serum-free medium even if supplemented with NGF at concentrations as high as 1 $\mu\text{g}/\text{ml}$ and were designated nnr (not nerve growth factor responsive) lines: PC12^{nnr3}, PC12^{nnr5}, PC12^{nnr6}, and PC12^{nnr14}.

The PC12^{nnr} lines selected by the two-step screening procedure were further characterized to determine their overall similarity to PC12 cells and to determine whether they are completely nonresponsive to NGF or still possess some subset of the normal responses. The following properties were examined in the presence and in the absence of NGF: cell size and shape, cell division rate, glycoproteins, ODC activity, phosphoproteins, and cell surface morphology. These include transcription-dependent and transcription-independent responses as well as responses that require different amounts of time to appear, ranging from seconds to days. Binding and internalization by the PC12^{nnr} cells were also characterized.

Morphology and Neurite Outgrowth

The light microscope morphologies of PC12, PC12^{nnr3}, and PC12^{nnr14} cells in the absence of NGF and after 2 wk in NGF are shown in Fig. 1. In the absence of NGF the PC12^{nnr14} cells appeared to be similar to, but slightly smaller than, PC12 cells. The PC12^{nnr} cells were also more adherent to each other than were PC12 cells and this was especially marked for PC12^{nnr3} cells. Regarding the lines not shown, in cell size, shape, and adherence to each other, PC12^{nnr6} cells most closely resembled PC12^{nnr3} cells, and PC12^{nnr5} cells resembled PC12^{nnr14} cells. In the presence of NGF (50 ng/ml), PC12 cells underwent substantial morphological changes, shown in Fig. 1. These included flattening and enlargement of the soma and the growth of long neurites. The growth of neurites is a transcription-dependent, long-term change that requires days of NGF treatment (Greene and Tischler, 1976; Burstein and Greene, 1978). PC12^{nnr} cells did not undergo short-term flattening, somal enlargement, or neurite outgrowth whether exposed to 50 ng/ml or to 1 $\mu\text{g}/\text{ml}$ NGF. The PC12^{nnr} cultures shown in Fig. 1 after 2 wk in NGF appear denser than the corresponding PC12^{nnr} cultures before NGF treatment because the mutant cells continue to divide in the presence of NGF.

Morphological Changes Induced by Dibutyryl Cyclic AMP (dbcAMP)

PC12 cells treated with 1 mM dbcAMP do not extend neurites but do extend short cytoplasmic processes (Greene et al., 1979; Gunning et al., 1981). A comparison of the effects of dbcAMP and NGF on PC12 and PC12^{nnr} cells is shown in

Fig. 2. Table I summarizes our quantitation of the response to dbcAMP by PC12 cells and by three PC12^{nnr} lines, PC12^{nnr3}, PC12^{nnr5}, and PC12^{nnr14}, to allow for comparison. These results show that the morphological response to dbcAMP was present in the PC12^{nnr} cells and indicate that it was much more pronounced in these cells than in PC12 cells. The processes that extend from dbcAMP-treated PC12^{nnr} cells were much longer than those that extend from similarly treated PC12 cells and were present on a much higher fraction of the cells. However, as shown in Fig. 2, these processes otherwise resembled those induced in PC12 cells by dbcAMP and did not resemble neurites elaborated by NGF-treated PC12 cells: they were broader and much shorter than neurites and they lacked growth cones. Cultures of PC12^{nnr3} or PC12^{nnr5} cells treated with 50 ng/ml NGF + 1 mM dbcAMP were not distinguishable from cultures treated with dbcAMP alone.

Cell Division Rate

Growth curves were established for PC12^{nnr3}, PC12^{nnr6}, and PC12^{nnr14} cell lines. The doubling time for these PC12^{nnr} lines was ~3 d both in the presence and in the absence of NGF. This was the same as for PC12 cells in the absence of NGF. PC12 cells do not increase in number after they have been in NGF-containing medium for more than a few days (Greene and Tischler, 1976).

Glycoproteins

PC12 cell and PC12^{nnr} cell proteins that can be metabolically labeled with ³H-fucose are illustrated in Fig. 3. Three PC12^{nnr} lines were examined: PC12^{nnr3}, PC12^{nnr5}, and PC12^{nnr14}. In the absence of NGF treatment the ³H-fucose-labeled proteins of the PC12^{nnr} cells were very similar to those of PC12 cells. The pattern of Coomassie Blue-stained bands on these gels (not shown) also showed no detectable differences between PC12 and PC12^{nnr} cells.

After long-term treatment with NGF, two major changes could be observed in PC12 cell glycoprotein composition, both of which require transcription: an increase in the 230-kD NGF-inducible large external (NILE) glycoprotein (McGuire et al., 1978) and an increase in a 31-kD glycoprotein (McGuire et al., 1978) that has recently been identified as Thy-1 (Richter-Landsberg et al., 1985). PC12^{nnr} cells showed no marked changes in these or other glycoproteins after NGF treatment.

Ornithine Decarboxylase (ODC) Activity

Treatment with NGF and other agents causes a short-term, transcription-requiring increase in ODC levels in sympathetic neurons and PC12 cells (MacDonnell et al., 1977; Greene and McGuire, 1978; Hatanaka et al., 1978). Exposure to epidermal growth factor (EGF) has also been shown to cause an increase in ODC activity in PC12 cells (Huff and Guroff, 1979). Table II shows ODC activity in PC12 cells and in the four PC12^{nnr} mutant lines. The constitutive ODC specific activities were significantly lower in the PC12^{nnr} cells than in PC12 cells; PC12^{nnr3} and PC12^{nnr6} cells had barely detectable constitutive ODC activity. In contrast to PC12 cells, which exhibited a 50-fold increase in ODC activity after NGF (50 ng/ml) treatment, PC12^{nnr} ODC specific activities were unchanged in response to NGF. Responses to EGF (3 ng/ml),

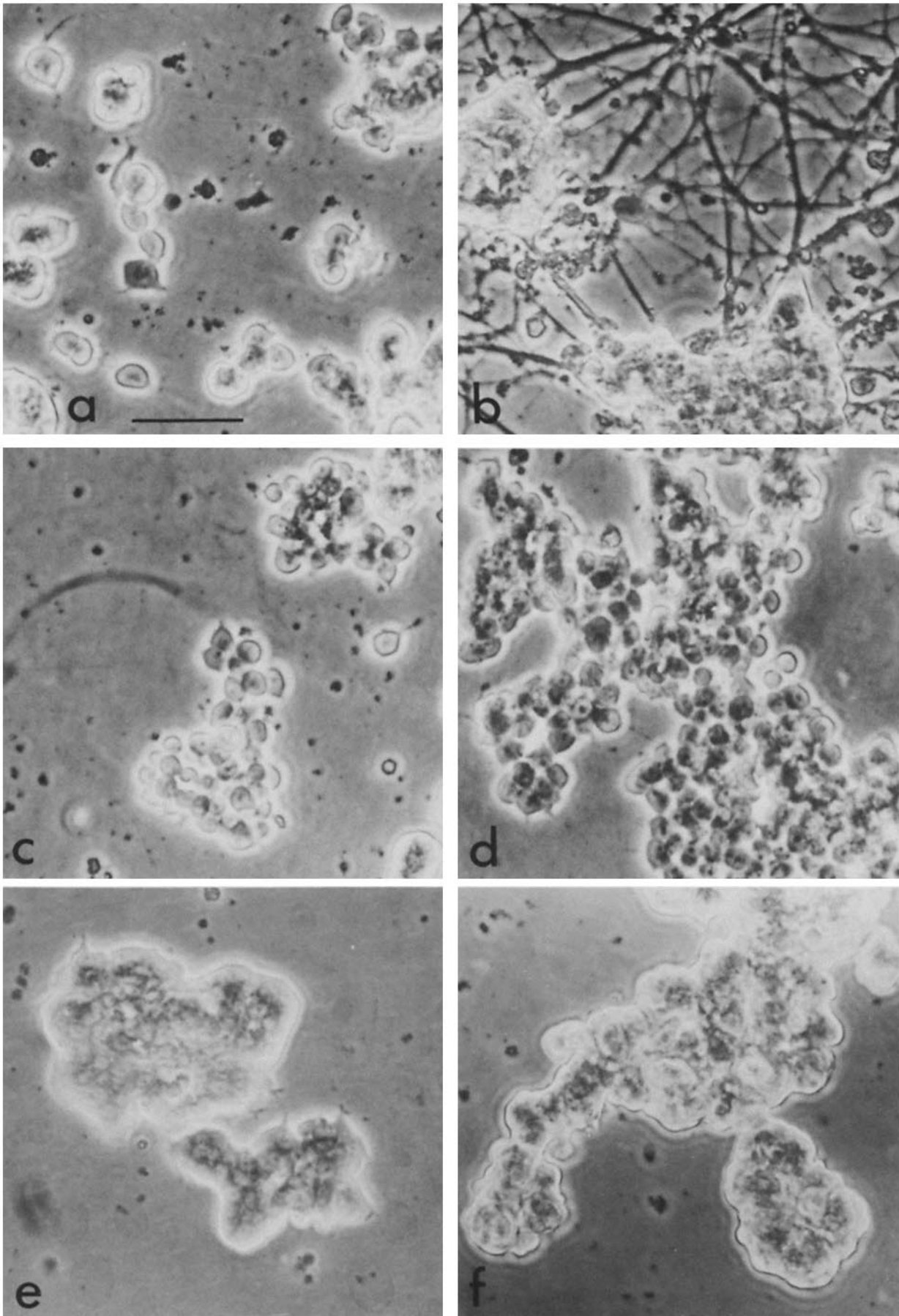


Figure 1. Morphology of, and NGF-induced neurite outgrowth from, PC12 and PC12^{n^{mr}} cells. The cells were plated in the absence of NGF and photographed after 1 d (*a*, *c*, and *e*). NGF (50 ng/ml) was then added and the cells were maintained with NGF for 2 wk and then photographed (*b*, *d*, and *f*). The cell lines shown are PC12 (*a* and *b*), PC12^{n^{mr14}} (*c* and *d*), and PC12^{n^{mr3}} (*e* and *f*). Bar, 50 μ m.

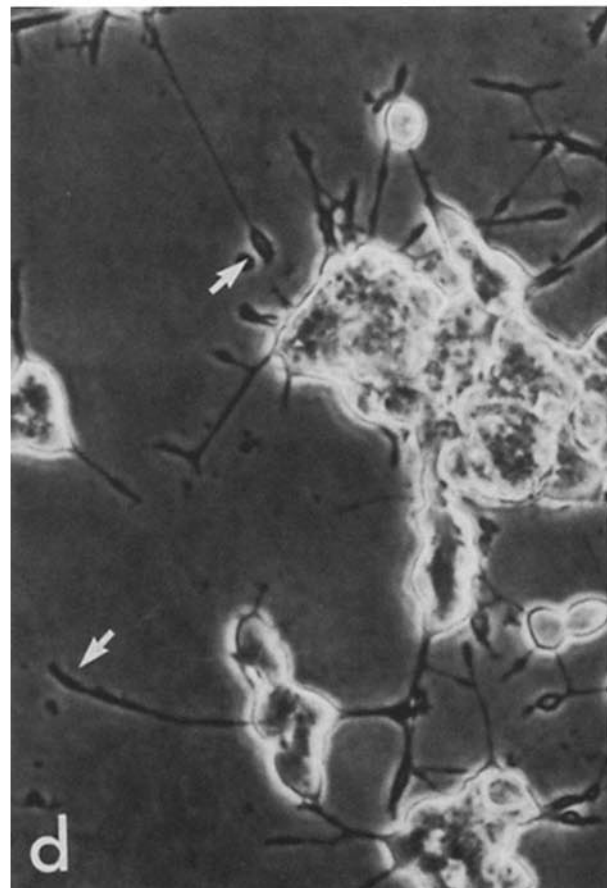
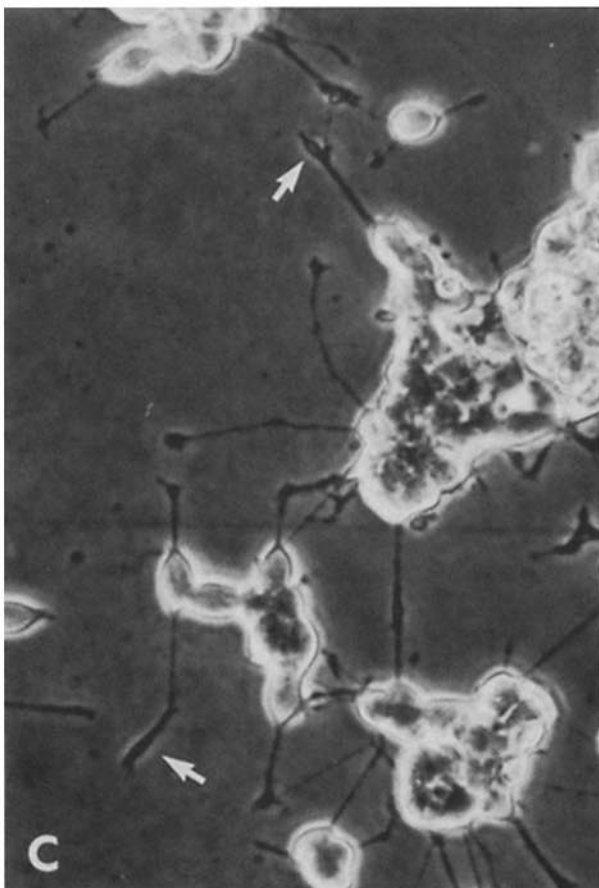
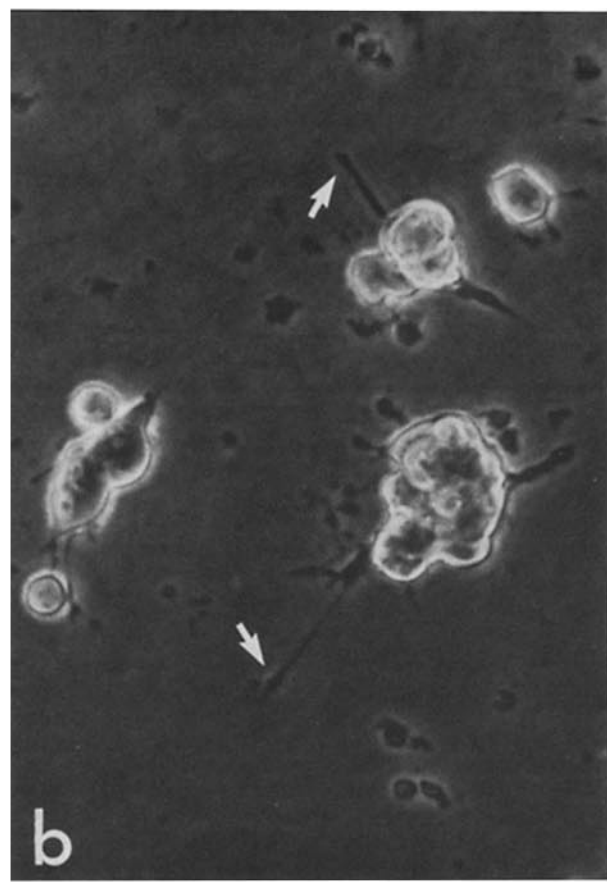
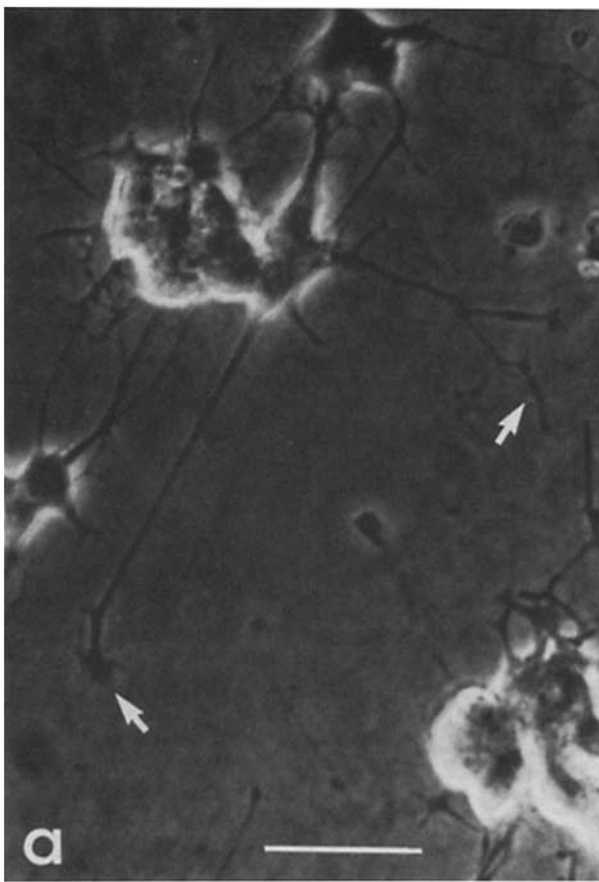


Figure 2. Comparison between NGF- and dbcAMP-induced processes in PC12 and PC12^{nr5} cells. The cells were plated under the conditions described and photographed after 5 d. The arrows indicate examples of the structures at the distal tips of processes induced by NGF or dbcAMP to facilitate comparison. The growth cones at the distal tips of NGF-induced neurites tend to be large, spread, flat, and phase-dark. The distal tips of dbcAMP-induced processes terminate either without obvious specializations or in a bulbous phase-bright structure. (a) PC12 cells in NGF (50 ng/ml); (b) PC12 cells in dbcAMP (1 mM); (c) PC12^{nr5} cells in dbcAMP (1 mM): These processes are longer and more numerous than the processes on PC12 cells in 1 mM dbcAMP but are structurally similar; (d) PC12^{nr5} cells in dbcAMP + NGF. NGF was used at 50 ng/ml and dbcAMP at 1 mM. These processes are not distinguishable from those induced in PC12^{nr5} cells by dbcAMP alone. Bar, 50 μ m.

Table I. Induction of Cell Processes by dbcAMP in Cultures of PC12 and PC12^{nr} Cells

Cell line	Percent cells		
	No processes	Processes <2 cell diam	Processes >2 cell diam
PC12	24	28	48
PC12 ^{nr5}	0	10	90
PC12 ^{nr6}	2	10	88
PC12 ^{nr14}	3	11	86

100 cells or clumps of cells of each of the four lines shown were scored for process outgrowth after 5 d in 1 mM dbcAMP.

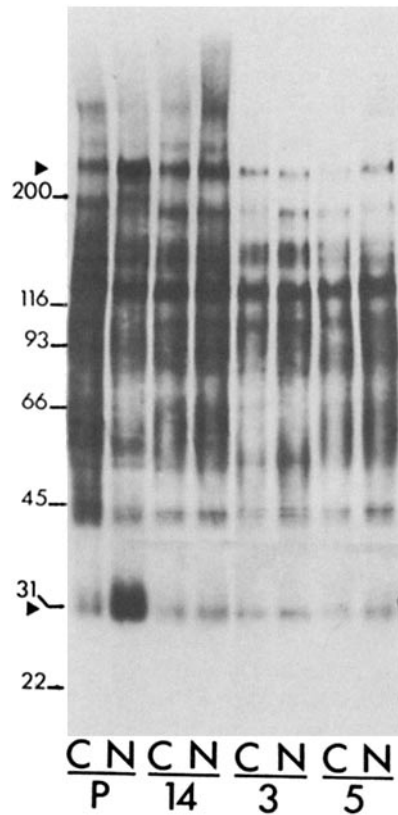


Figure 3. ³H-Fucose-labeled proteins of PC12 and PC12^{nr} cells. PC12 and PC12^{nr} cells were labeled with ³H-fucose as described in McGuire et al. (1978) and subjected to SDS polyacrylamide gel electrophoresis on 32-cm 5–15% acrylamide gradient gels. Gel lanes are of PC12 (P), PC12^{nr14} (14), PC12^{nr3} (3), or PC12^{nr5} (5) cells. Lanes labeled C show control cells in the absence of NGF; lanes labeled N show cells that had been treated with 50 ng/ml NGF for 2 wk. The arrows indicate NGF-induced increases in glycoproteins of apparent molecular weights 230 and 31 kD.

Table II. ODC Activity in PC12 and PC12^{nr} Cells

Cell line	ODC Activity			
	Control	NGF-treated	dbcAMP-treated	EGF-treated
	<i>fmol min⁻¹ μg⁻¹</i>			
PC12	94 ± 4	4,200 ± 180	4,900 ± 270	810 ± 31
PC12 ^{nr5}	32 ± 1	30 ± 2	5,100 ± 160	430 ± 12
PC12 ^{nr14}	32 ± 6	34 ± 6	3,700 ± 380	450 ± 88
PC12 ^{nr6}	2.9 ± 1.0	3.2 ± 0.1	330 ± 7	27 ± 3
PC12 ^{nr3}	<0.6	<0.6	610 ± 98	140 ± 9

ODC activity was assayed in PC12 cells and in the four PC12^{nr} lines as described in Greene and McGuire (1978). Cells were untreated (control) or treated for 4 h with NGF (50 ng/ml), EGF (3 ng/ml), or dbcAMP (1 mM).

however, were normal in the PC12^{nr} cells; as PC12 cells did, they showed about a 10-fold increase in ODC activity after exposure to EGF.

PC12 cells also exhibit a 50-fold increase in ODC activity after treatment with dbcAMP (1 mM) (Hatanaka et al., 1978). Likewise, PC12^{nr} cells responded to dbcAMP, but with a greater than 100-fold increase in ODC specific activity, significantly higher than for PC12 cells. Despite the greater increase in ODC activity after dbcAMP treatment, the level of ODC activity was still not significantly higher in PC12^{nr} than in PC12 cells, and for most PC12^{nr} lines was actually lower, because their constitutive ODC activities were so much lower.

Phosphoproteins

A rapid response to NGF, not requiring transcription, is a change in the level of phosphorylation of specific proteins (Halegoua and Patrick, 1980; Yu et al., 1980; Seeley et al., 1984). Permeant cAMP analogues also cause rapid changes in protein phosphorylation in PC12 cells (Halegoua and Patrick, 1980; Yu et al., 1980). We compared proteins labeled after 2 h of exposure to ³²P-orthophosphate in PC12 cells and the four PC12^{nr} mutant lines. Fig. 4 shows these proteins in PC12 and three PC12^{nr} lines: PC12^{nr3}, PC12^{nr5}, and PC12^{nr14}. In the absence of added NGF or dbcAMP, PC12^{nr} cells had a pattern of phosphoproteins similar to that of PC12 cells, although some phosphoprotein bands that were present in PC12 cells, e.g., 100 kD and 54 kD, were absent in PC12^{nr5} and PC12^{nr14} cells. As shown in Fig. 4, after exposure to NGF (50 ng/ml) for 1 h, there was, in PC12 cells, a marked increase in the incorporation of ³²P into proteins of molecular weights 32, 54, 60, and 64 kD. All four of the PC12^{nr} cell lines (PC12^{nr6} not shown here) showed no increase in phosphorylation of any proteins in response to NGF. The responsive 60-kD phosphoprotein has been identified as tyrosine hydroxylase (Halegoua and Patrick, 1980), and the rapid increase in phosphorylation of this band is correlated with a rapid increase in tyrosine hydroxylase activity (Greene et al., 1984). It is interesting in this regard that the PC12^{nr5} line had tyrosine hydroxylase activity but that there was no rapid increase after treatment with NGF (Müller, T. H., E. Helmer, S. H. Green, and M. Goldstein, unpublished results). The other PC12^{nr} cell lines have not yet been examined in this regard.

In response to dbcAMP there was an increase in the phosphorylation of the 32- and 54-kD proteins in PC12 cells and in PC12^{nr} cells. However, there were two additional bands that showed significant increases in ³²P incorporation after dbcAMP treatment in PC12^{nr} cells but not in PC12 cells: 58 and 66 kD.

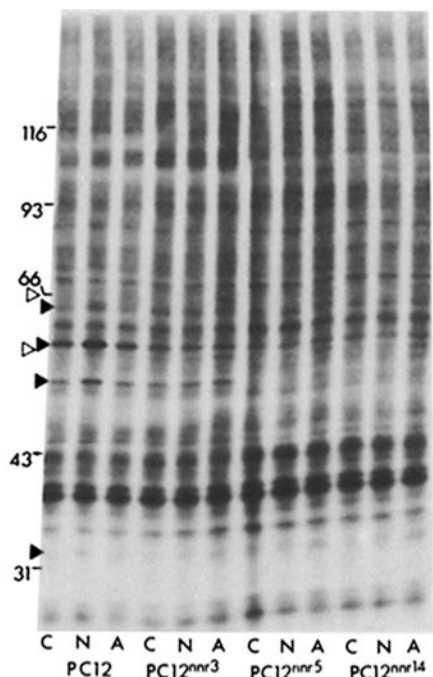


Figure 4. ^{32}P -Orthophosphate-labeled proteins of PC12 and PC12^{nr} cells. PC12 and PC12^{nr} cells were labeled with ^{32}P -orthophosphate as described in Seeley et al. (1984) and subjected to SDS polyacrylamide gel electrophoresis on 32-cm 7.5–15% acrylamide gels. Gel lanes are of PC12, PC12^{nr3}, PC12^{nr5}, or PC12^{nr14} cells as indicated below each set of three lanes. Within each set of three, lane C is from untreated control cells, lane N is from cells that had been treated with 50 ng/ml NGF for 1 h, and lane A is from cells that had been treated with 1 mM dibutyl cAMP for 1 h. The filled arrows indicate NGF induced phosphorylation of PC12 cell proteins of apparent molecular weights 32, 54, 60, and 64 kD. The unfilled arrows indicate dbcAMP-induced phosphorylation of PC12^{nr} cell proteins of apparent molecular weights 58 and 66 kD.

Cell Surface Morphology

In the presence of NGF or EGF, PC12 cells can be seen to rapidly (within 30 s) undergo a series of changes in cell surface morphology, the most striking of which is prominent surface ruffling (Connolly et al., 1979, 1984). This phenomenon was visualized by scanning electron microscopy of PC12, PC12^{nr3}, PC12^{nr6}, and PC12^{nr14} cells. Fig. 5 shows examples of the surface morphology of untreated, NGF-treated, and EGF-treated PC12 and PC12^{nr14} cells. All three PC12^{nr} cells examined behaved similarly. They had a surface morphology very much like that of PC12 cells in the absence of added growth factors. In addition, they responded to EGF (3 ng/ml), as PC12 cells do, by undergoing rapid surface changes. This demonstrates that the PC12^{nr} lines can undergo the same repertoire of surface changes as PC12 cells when appropriately stimulated. However, the PC12^{nr} lines examined differed from PC12 cells in that they showed no surface changes in response to NGF (50 ng/ml).

NGF Binding and Internalization

In view of the finding that the PC12^{nr} cells appear to be completely nonresponsive to NGF, ^{125}I -NGF binding to the cells was characterized to determine whether they might lack NGF receptors. The protocol used allows distinction between surface bound (external) NGF and NGF internalized over the

course of the incubation with ^{125}I -NGF (Bernd and Greene, 1984). Initial experiments, summarized in Table III, were done with an ^{125}I -NGF concentration of 40 pM (1 ng/ml). These showed that while external NGF binding in PC12^{nr} cells was ~35% of PC12 cell levels, internalized NGF was ~7% of PC12 cell levels, measured after 1 h at 37°C. Thus, under these conditions, the internal/external (Int./Ext.) ratio was ~6 in PC12 cells and ~1 in PC12^{nr} cells. However, the mutant lines are not generally defective in receptor-mediated endocytosis; as shown in Table III, the Int./Ext. ratio for ^{125}I -EGF binding was similar in PC12^{nr} and PC12 cells, although the EGF receptor levels were somewhat lower in PC12^{nr} than in PC12 cells.

To examine this apparent loss of NGF internalization in more detail, the Int./Ext. ratio was determined for a number of NGF concentrations over the range of 15–15,000 pM. The results are shown in Fig. 6. For PC12 cells internalization saturated at ~300 pM so that the Int./Ext. ratio dropped above this concentration. The PC12^{nr} cells, however, had no detectable saturable internalization. This suggests that the quantitative difference in the Int./Ext. ratio between the PC12 and PC12^{nr} cells is a result of a qualitative change in the mutants, specifically, a complete or nearly complete lack of the normal saturable mechanism for internalization of bound NGF.

The experimental data used to produce Fig. 6 can also be used to construct Scatchard-type plots, examples of which are shown in Fig. 7. The data were also analyzed by means of the LIGAND (Munson and Rodbard, 1980) and EBDA (McPherson, 1983) computer programs, adapted for use on the IBM PC by G. A. McPherson. The LIGAND program fits binding data to curves by means of a nonlinear regression analysis and provides a measure of goodness of fit which allows statistical comparison of alternative assumptions about receptor parameters. We attempted to fit all of the data for each cell line by assuming either one or two NGF binding sites and then computed the steady-state K_d and number of binding sites on the basis of either assumption. The results of this analysis are summarized in Table IV. The curvilinear shape of the PC12 Scatchard plot in Fig. 7 suggests that a two-site model for NGF binding to PC12 cells is preferable. Testing the fit of both models to the data by use of the F ratio test (Munson and Rodbard, 1980) confirmed that, statistically, the two-site model is a highly significantly better fit; $F = 21.4$, $P < 0.001$. The data for PC12 cells can be best fitted by assuming a low-affinity site with a K_d of 7.7 nM and a high-affinity site with a K_d of 0.15 nM, the high-affinity site comprising ~6% of the total number of binding sites.

The shape of the Scatchard plots for the PC12^{nr} cells suggests the absence of high-affinity binding as shown by the examples in Fig. 7. Analysis of all of the data for each of the PC12^{nr} lines by means of the LIGAND program confirmed that high-affinity binding could not be detected in PC12^{nr14} or PC12^{nr6} cells by assumption of one- or two-site models. NGF binding to PC12^{nr3} and PC12^{nr5} cells could be described by a two-site model having high-affinity sites but in this case these high-affinity sites comprise only 1% or 0.07%, respectively, of the total number of sites. This is a significant reduction from PC12 cells in which high-affinity sites comprise 6% of the total (Table IV). In terms of numbers of receptors per cell these values for PC12^{nr3} and PC12^{nr5}

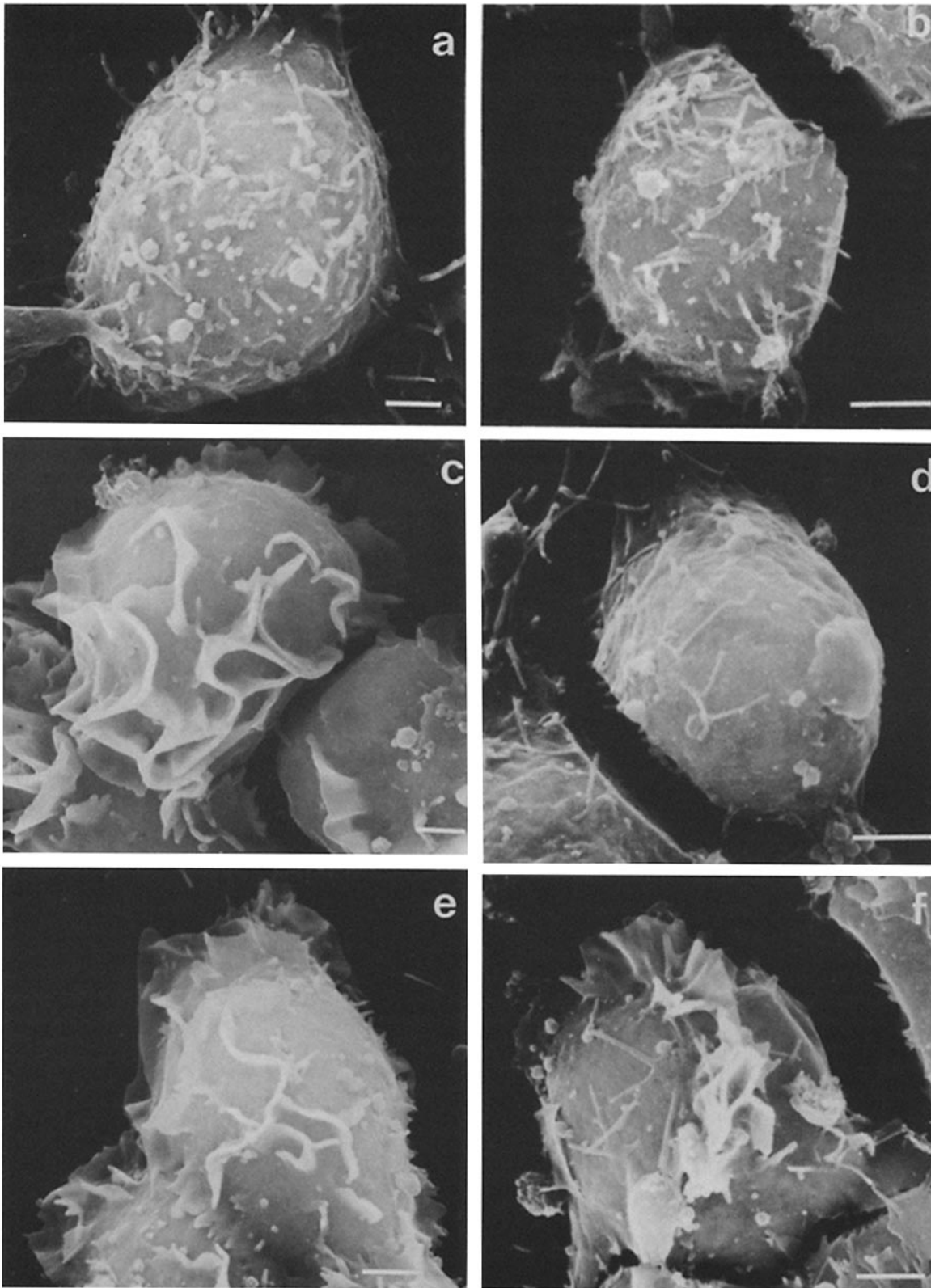


Figure 5. Effects of NGF and EGF on the surface architecture of PC12 and PC12^{nnr14} cells. The cells were fixed and processed for scanning electron microscopy as described in Connolly et al. (1979). (a and b) Control, untreated cells; (c and d) cells treated with 50 ng/ml NGF for 3 min; (e and f) cells treated with 3 ng/ml EGF for 3 min. The cell lines shown are PC12 (a, c, and e) and PC12^{nnr14} (b, d, and f). Bar, 2 μ m.

actually differ very little from no detectable high-affinity binding. The values in Table IV further show that, on the basis of a one-site model, PC12^{nnr} cells have between 12 and

49% of the NGF binding of PC12 cells on a per cell basis and that this binding site has a K_d that resembles the PC12 low-affinity site and not the high-affinity site.

Table III. Binding and Internalization of ^{125}I -NGF (40 pM) and ^{125}I -EGF (1.9 nM) by PC12 and PC12^{nr} Cells

Cell line	^{125}I -NGF			^{125}I -EGF		
	Internal	External	I/E	Internal	External	I/E
	<i>fmol bound/2 × 10⁶ cells</i>			<i>fmol bound/2 × 10⁶ cells</i>		
PC12	2.12 ± 0.05	0.36 ± 0.04	5.9	20 ± 0.4	1.0 ± 0.1	20
PC12 ^{nr3}	0.12 ± 0.03	0.10 ± 0.01	1.2	19 ± 0.8	0.77 ± 0.20	25
PC12 ^{nr5}	0.14 ± 0.03	0.16 ± 0.10	0.9	16 ± 1.0	0.74 ± 0.09	22
PC12 ^{nr6}	0.17 ± 0.01	0.12 ± 0.01	1.4	—	—	—
PC12 ^{nr14}	0.14 ± 0.01	0.12 ± 0.01	1.2	10 ± 0.1	0.43 ± 0.02	24

Surface-bound (external) and internalized ligand were determined for PC12 and PC12^{nr} cells as described in Bernd and Greene (1984). ^{125}I -EGF binding assays were not performed on PC12^{nr6} cells.

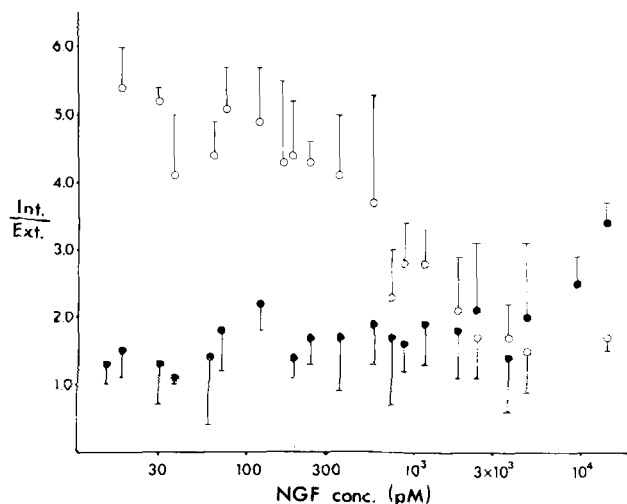


Figure 6. Concentration dependence of the internalization of ^{125}I -NGF by PC12 and PC12^{nr} cells. Surface-bound (external) and internalized ^{125}I -NGF were determined for PC12 (○) and PC12^{nr} cells (●) as described in Bernd and Greene (1984). Int./Ext. is the ratio of internal to external ^{125}I -NGF after an incubation of 1 h at 37°C. The Int./Ext. ratio at any given concentration of NGF is not significantly different among the four different PC12^{nr} lines so these values have been pooled here into a single plot.

Discussion

Isolation of the Mutants

24 clones of PC12 mutants that fail to grow neurites in the presence of NGF were obtained by a visual screen of mutagenized cells. These were further screened for clones of cells completely nonresponsive to NGF by testing them for failure to survive in NGF-supplemented serum-free medium. That this two-step procedure is effective for obtaining PC12 cell mutants completely nonresponsive to NGF is shown by the fact that all four lines that failed to survive in NGF-supplemented serum-free medium also failed to show any other responses to NGF. We observed a mutation frequency of 10^{-4} for AG resistance in EMS-treated PC12 cells so this represents an approximate upper limit for the mutation frequency for any given single gene. It is therefore unlikely that all four PC12^{nr} mutations are allelic; however, complementation analysis is planned to determine the actual number of different genetic loci required for NGF response that we have obtained.

The PC12^{nr} mutant cell lines were generated by treating cultures with EMS, an agent that acts as a mutagen by causing G·C to A·T base pair changes (Coulondre and Miller, 1977).

It is therefore likely that the PC12^{nr} phenotypes result from single-gene changes and not from deletions. Analysis of reversion frequencies will ultimately be needed to determine whether single or multiple mutations gave rise to the PC12^{nr} cell phenotypes. However, in non-NGF-supplemented medium the PC12^{nr} cells are very similar to the parent PC12 cells in most properties tested: cell size and shape; division rate; protein, phosphoprotein, and glycoprotein patterns on SDS polyacrylamide gels; and cell surface morphology. These observations are best explained by the assumption that only a single or a very small number of genetic changes distinguish PC12 from PC12^{nr} cells. Given this assumption, the differences that do exist between PC12 and PC12^{nr} cells and that are consistently seen in all of PC12^{nr} lines, are probably closely and functionally related to each other. These differences, discussed below in detail, are: the PC12^{nr} cells fail to respond to NGF, they lack NGF internalization by the normal, high-affinity, saturable mechanism, they have very little or no detectable high-affinity NGF binding, and they have altered responses to dbcAMP as compared with PC12 cells. The functional relationship between these pleiotropic effects of the PC12^{nr} mutations may be the result of one of two possibilities. The first is that the direct effect of the mutations is to affect some cellular process that in turn independently affects cAMP metabolism, NGF binding, NGF internalization, and NGF responses. By this interpretation there is no causal relationship between these elements of the PC12^{nr} lines' phenotypes. The second possibility is that there indeed is a direct cause and effect relationship between all or some of the elements of the phenotypes. Neither interpretation can be ruled out at this point; however, we consider it less likely that all of the four nonresponsive mutants we could obtain should share the exact same pleiotropic effects without there being a causal relationship between all or some of these effects. Given the consistency of this interpretation with previously published results discussed below, we therefore favor the hypothesis that the lack of high-affinity NGF receptors, NGF internalization, and NGF responses are associated with each other by cause and effect relationships. The data in the existing literature, however, do not presently permit one to conclude that a causal relationship exists between the cAMP-related elements and the NGF-related elements of the mutants' phenotypes.

NGF Responses

Although the list of responses tested is not exhaustive, all general types of NGF responses were represented in the analyses done in this study: survival in NGF-supplemented, serum-free medium; long-term, transcription-dependent

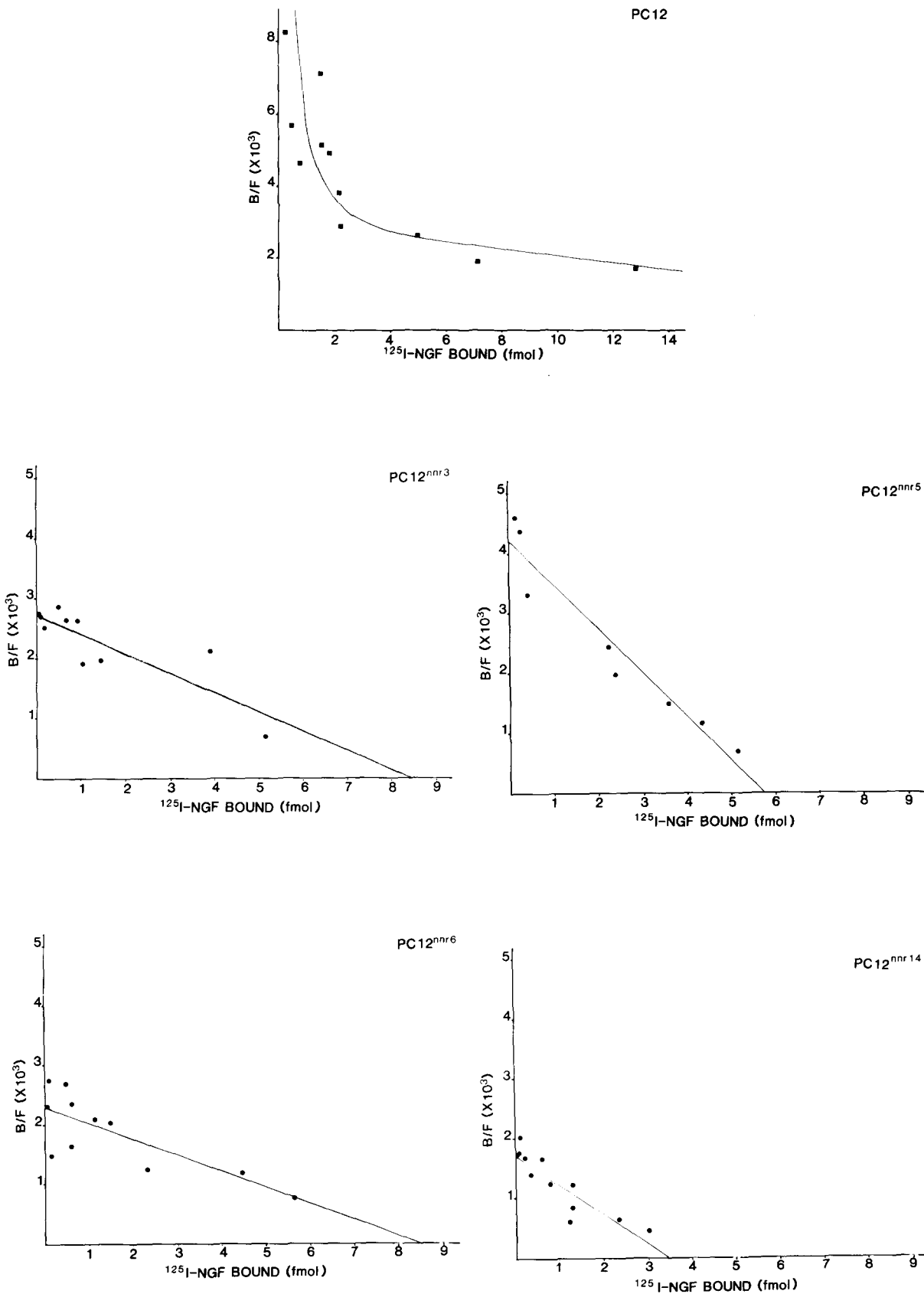


Figure 7. Examples of typical Scatchard-type plots of surface-bound ¹²⁵I-NGF for PC12 and PC12^{nnr} cells. Surface-bound ligand was determined by acid-washing the cells as described in Bernd and Greene (1984). The data from single experiments were plotted as shown. Each point is the mean of three determinations. The lines drawn through the points were drawn assuming a two-site model for PC12 cells (■) and a one-site model for the PC12^{nnr} cells (●). The computed values for receptor number and K_d for both one- and two-site models are given in Table IV and the predictions of each are discussed in the text.

Table IV. Numbers and Dissociation Constants for NGF Binding in PC12 and PC12^{nnr} Cells

Cell line	One-site model		Two-site model			
	R (fmol bound)	K _d (nM)	R ₁ (fmol bound)	K _{d,1} (nM)	R ₂ (fmol bound)	K _{d,2} (nM)
PC12	—	—	1.44 ± 0.96	0.15 ± 0.11	25.9 ± 7.9	7.68 ± 4.79
PC12 ^{nnr3}	5.5 ± 1.4	1.9 ± 0.6	0.073 ± 0.070	0.10 ± 0.08	6.2 ± 2.1	2.4 ± 2.2
PC12 ^{nnr5}	13.3 ± 4.8	4.2 ± 1.8	0.005 ± 0.015	0.014 ± 0.08	7.3 ± 2.2	2.0 ± 1.1
PC12 ^{nnr6}	7.1 ± 1.7	2.9 ± 0.9	—	—	—	—
PC12 ^{nnr14}	3.3 ± 0.4	1.8 ± 0.3	—	—	—	—

Surface-bound ligand was determined by acid-washing the cells as described in Bernd and Greene (1984). The data were analyzed by using the EBDA (McPherson, 1983) and LIGAND (Munson and Rodbard, 1980) computer programs as adapted for the IBM PC by G. A. McPherson to produce the values summarized here. Three separate binding experiments were performed for each of the PC12, PC12^{nnr3}, and PC12^{nnr5} cell lines and two each for the PC12^{nnr6} and PC12^{nnr14} cell lines. R represents the total number of receptors per dish (2 × 10⁶ cells) and is given in fmol NGF bound. The computed values for R and K_d are given for both one-site and two-site models except where no valid model could be assumed.

(growth of neurites, induction of NILE and Thy-1 glycoproteins); short-term, transcription-dependent (ODC induction); short-term, transcription-independent (phosphorylation of 60 kD, 54 kD, and other proteins, increase in tyrosine hydroxylase activity); and extremely rapid responses (cell surface ruffling, internalization of NGF). In a recent study (Greenberg et al., 1985), PC12^{nnr5} cells, in contrast to PC12 cells, failed to show rapid NGF-induced increases in *c-fos* and *c-myc* proto-oncogene transcription. It is therefore extremely unlikely that any NGF responses remain in the PC12^{nnr} cells. In this respect, the PC12^{nnr} cells differ from previously described PC12 subclones with altered NGF responses, obtained by screening for cells that continued to divide in the presence of NGF (Bothwell et al., 1980; Burstein and Greene, 1982a). In the former study, the three lines examined by scanning electron microscopy all showed rapid responses to NGF. Other nonresponsive lines resulting from that screen had no detectable NGF binding or were phenotypically very different from the parent PC12 cells. In the latter study, neurite outgrowth and other responses to NGF were detected in low serum or serum-free media.

Some PC12 cell responses to NGF, e.g., ODC induction (Huff and Guroff, 1979), and rapid changes in cell surface morphology (Connolly et al., 1984) and intracellular pH (Boonstra et al., 1983), can also be triggered by EGF. Since NGF as well as EGF can act as a mitogen for PC12 cells (Burstein and Greene, 1982b; Boonstra et al., 1983), responses elicited, in common, by both of these factors might be related to stimulation of proliferation, whereas responses triggered only by NGF (e.g., neurite outgrowth, induction of NILE glycoprotein) would be part of a specifically NGF-induced program of neuronal differentiation. In the PC12^{nnr} mutant lines EGF responses are present, while NGF responses, both shared and unshared with EGF, are lost. This observation suggests the following: First, genetically altered function(s) that underlie the phenotype of the PC12^{nnr} cells are not ones necessary for biological responses to peptide growth factors in general but are necessary, specifically, for responses to NGF. Second, although NGF and EGF can both trigger some of the same biological responses in PC12 cells, the initial steps in their mechanisms of action must differ. This is consistent with the differential effect of methylation inhibitors on PC12 cell responses to NGF and EGF (Seeley et al., 1984). Third, NGF stimulation of putative mitogenesis-related responses as well as NGF induction of neuronal differentiation are both mediated by common initial steps that are affected by mutation in the PC12^{nnr} lines.

NGF Internalization

The results presented here show that the normal, saturable, high-affinity endocytic pathway for NGF is undetectable in the PC12^{nnr} mutants yet they possess surface receptors for NGF. Mutations that specifically block low density lipoprotein internalization but not surface binding have been described in humans who suffer from familial hypercholesterolemia (Brown and Goldstein, 1976; Lehrman et al., 1985). It is of some interest that mutations exist that block receptor-mediated internalization of specific ligands but that do not block receptor-mediated endocytosis in general. Such mutations, while specific in their effects on particular ligand-receptor systems, may affect analogous functions in these different systems. Similarly, pharmacological distinction between internalization by different receptors has been demonstrated for pancreatic cells by Korc et al. (1984) who have shown strong inhibition by Ca²⁺ of EGF but not of insulin internalization.

NGF Binding

The values reported in this study for numbers and dissociation constants of PC12 cells at 37°C are in close agreement with those previously reported (Yankner and Shooter, 1979; Bernd and Greene, 1984). These measurements have not been done for PC12 cells at low temperature or in the presence of metabolic inhibitors, conditions that would block internalization. However, under these conditions sensory neurons exhibit both high- and low-affinity binding (Sutter et al., 1979), and PC12 cells exhibit both fast and slow dissociating binding (Landreth and Shooter, 1980; Schechter and Bothwell, 1981). Since high-affinity and slow binding can occur in the absence of internalization, it is unlikely that the lack of internalization by PC12^{nnr} cells is the cause of the absence of high-affinity binding.

The numbers and dissociation constants of the NGF receptors described in this study are steady state values and not equilibrium values. Therefore reduced high-affinity receptor number could be due to a reduction in its synthesis or recycling during the 60-min incubation. However, since removal of NGF-receptor complexes from the surface is greatly diminished in the PC12^{nnr} cells, it is unlikely that a reduction in the rate of recycling would make much difference in the steady state surface levels of bound ligand. Rather, we favor the hypothesis that the PC12^{nnr} lines are deficient in the production of the high-affinity NGF receptor. There is evidence suggesting that high and low-affinity NGF receptors are

interconvertible (Landreth and Shooter, 1980; Block and Bothwell, 1983). One possibility, then, is that the PC12^{nmr} mutations affect the conversion of low-affinity receptors to high.

dcAMP Responses

PC12 cells respond to permeant cAMP analogues in a number of ways which include the extension of short cytoplasmic processes (Schubert and Whitlock, 1977; Greene et al., 1979), an increase in ODC activity (Hatanaka et al., 1978), and increases in phosphate incorporation into several proteins (Halegoua and Patrick, 1980). PC12^{nmr} cells share these responses but all four PC12^{nmr} lines differ from PC12 cells in that the cytoplasmic processes are longer and more abundant, the increase in ODC activity is considerably greater, and at least two additional proteins (66 and 58 kD) exhibit increased incorporation of phosphate.

Relationship between High-Affinity Binding and Internalization

PC12^{nmr} cells possess a significant fraction of the total NGF binding levels of PC12 cells. Therefore surface binding alone is not sufficient for NGF responses and internalization. NGF binding to the PC12^{nmr} cells differs from binding to PC12 cells in that there is a reduction of low-affinity binding to between 12 and 49% of PC12 cell levels and a near or total lack of detectable high-affinity binding. It is unlikely that the reduction in low-affinity binding alone is the cause of the lack of detectable saturable NGF internalization by the PC12^{nmr} cells. If such sites were responsible for NGF uptake, then 12–49% of PC12 levels of NGF uptake would have been observed in our studies; this was not seen. Rather, the observation that in PC12^{nmr} cells the lack of NGF internalization is accompanied by a near or complete lack of high-affinity receptors is consistent with recent kinetic experiments (Bernd and Greene, 1984) that strongly suggest that uptake of NGF by PC12 cells is mediated by high-affinity but not by low-affinity NGF receptors.

Relationship between High-Affinity Binding and NGF Responses

It is also unlikely that the reduction in low-affinity binding is the cause of the lack of detectable NGF responses in the PC12^{nmr} cells. This is because no NGF responses were detected in the PC12^{nmr} cells at NGF concentrations (1 µg/ml) at which the number of occupied low-affinity sites would be greater than the number of high and low-affinity sites occupied in PC12 cells treated with concentrations of NGF sufficient to elicit maximal responses (20–30 ng/ml) (Greene, 1978). Our results, rather, support the hypothesis that the high-affinity NGF binding site is associated with NGF responses as well as internalization. This is consistent with the observations that chick embryo dorsal root ganglion neurons with reduced levels of low-affinity NGF receptors but normal levels of high-affinity sites are not affected in their responses to NGF (Stach and Wagner, 1982) and that certain human neuroblastoma cell lines that lack “fast,” but still possess “slow,” NGF receptors retain responses to NGF (Sonnenfeld and Ishii, 1982).

The coincident lack of NGF internalization and responses in PC12^{nmr} cells suggest that they are closely associated. However, we cannot necessarily take our findings to support the

idea that internalization is required for biological responses to NGF. In this regard it should be noted that appreciable amounts of NGF do not enter the cell for ~2 min after its addition (Bernd and Greene, 1984), while cell surface changes occur within 30 s after NGF addition (Connolly et al., 1979). Unless extremely small amounts of internalized NGF are sufficient to induce the cell surface changes it seems likely that internalization is not a necessary prerequisite at least for this response.

Relationship between PC12^{nmr} cAMP- and NGF-related Phenotypes

The consistent association between cAMP-related and NGF-related phenotypes in all four of the PC12^{nmr} cells might indicate a causal relationship between them. This would be consistent with studies that show a synergistic relationship between cAMP and NGF in inducing neurite outgrowth (Gunning et al., 1981; Heidemann et al., 1985). One possibility is that phosphorylation-mediated events could be involved in the regulation of affinity and function of the NGF receptor, and in the mechanisms of NGF internalization and responses. Phosphorylation-mediated changes in ligand binding and function of EGF receptors have been shown by several investigators (Shoyab et al., 1979; Magun et al., 1980; Davis and Czech, 1984; Cochet et al., 1984; Ghosh-Dastidar and Fox, 1984).

However, unlike the proposed causal relationship between the different NGF-related phenotypic elements, such a proposal concerning a causal relationship between cAMP- and NGF-related phenotypes would not be entirely consistent with previously published studies or with our results. Previous studies (Greene et al., 1979; Gunning et al., 1981; Heidemann et al., 1985) have described differences in appearance between NGF-induced neuritic outgrowth and dbcAMP-induced processes in PC12 cells (also shown in Fig. 2), which indicate that cAMP does not function as a second messenger for NGF. Further evidence for this is provided by Hatanaka et al. (1978) and Connolly et al. (1984) who have shown that two early responses to NGF, respectively, ODC induction and changes in cell surface morphology, are independent of cAMP. Treatment of PC12 cells with 1 mM dbcAMP does not result in a phenotype characteristic of PC12^{nmr} cells so the lack of responses in PC12^{nmr} cells would not appear to be caused by an enhanced response to cellular cAMP. Conversely, dbcAMP does not reverse the defect in the PC12^{nmr} cells. The association of altered responses to dbcAMP with the NGF-related phenotypic elements is important as it suggests a relationship between cAMP-dependent and NGF-dependent events, perhaps due to their both being related to the same underlying defect.

Further comparisons between the PC12 and PC12^{nmr} cells should be of great value in determining the nature of the two affinity classes of NGF receptors, the molecular mechanisms of NGF internalization, and induction of cellular responses to NGF, as well as the relationship to cAMP-mediated cellular events.

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