To our knowledge, this is the first demonstration of an anti-tumour property of βhCG, and offers a new strategy for treating patients with Kaposi's sarcoma. □

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Nitric oxide triggers a switch to growth arrest during differentiation of neuronal cells

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ARREST of cell division is a prerequisite for cells to enter a program of terminal differentiation. Mitogenesis and cytostasis of neuronal cell precursors can be induced by the same or by different growth or trophic factors[1–9]. Response of PC12 cells to nerve growth factor (NGF) involves a proliferative phase that is followed by growth arrest and differentiation. Here we present evidence that the cytostatic effect of NGF is mediated by nitric oxide (NO), a second messenger molecule with both para- and autocrine properties that can diffuse freely and act within a restricted volume[10–14]. We show that NGF induces different forms of nitric oxide synthase (NOS) in neuronal cells, that nitric oxide (NO) acts as a cytostatic agent in these cells, that inhibition of NOS leads to reversal of NGF-induced cytostasis and thereby prevents full differentiation, and that capacity of a mutant cell line to differentiate can be rescued by exogenous NO. We suggest that induction of NOS is an important step in the commitment of neuronal precursors and that *NOS* serves as a growth arrest gene, initiating the switch to cytostasis during differentiation.

When PC12 cells were tested for the diaphorase cytochemical reaction, no staining was observed. But after treatment with NGF, the cells gradually acquired an intense NADPH-dependent blue colour after diaphorase staining, indicating that NOS accumulates in PC12 cells in response to NGF treatment (Fig. 1*A*). This increase in staining was specific for NGF and did not appear after addition of fetal calf serum or epidermal growth factor (Fig. 1*A*). The NGF-treated cells that were first to undergo initial morphological changes characteristic of the differentiated phenotype were also the first to show bright blue

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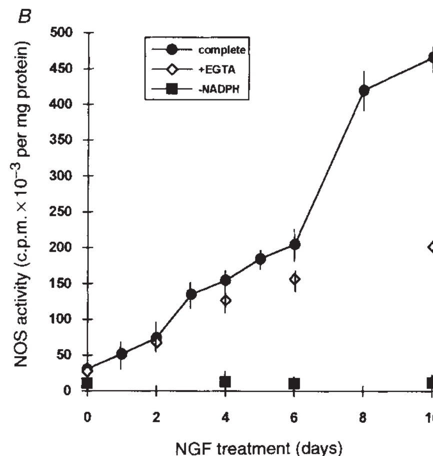
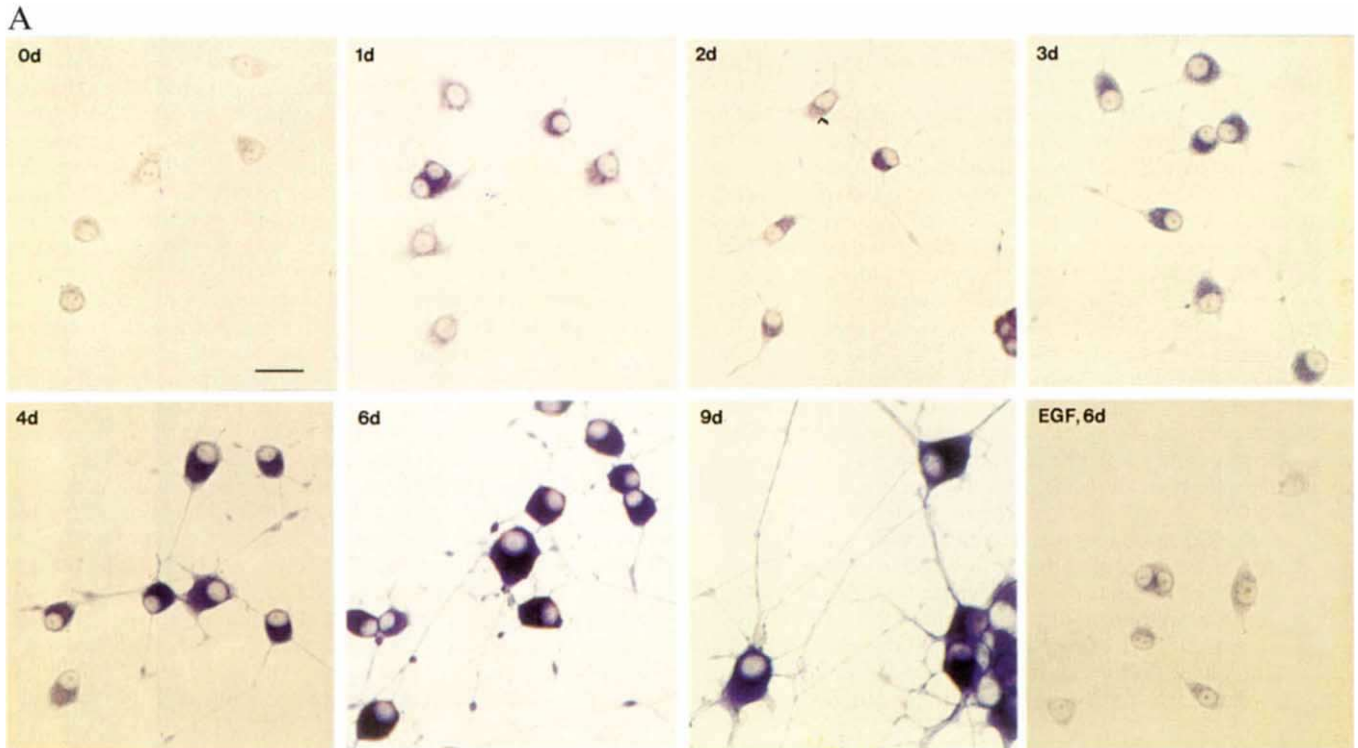


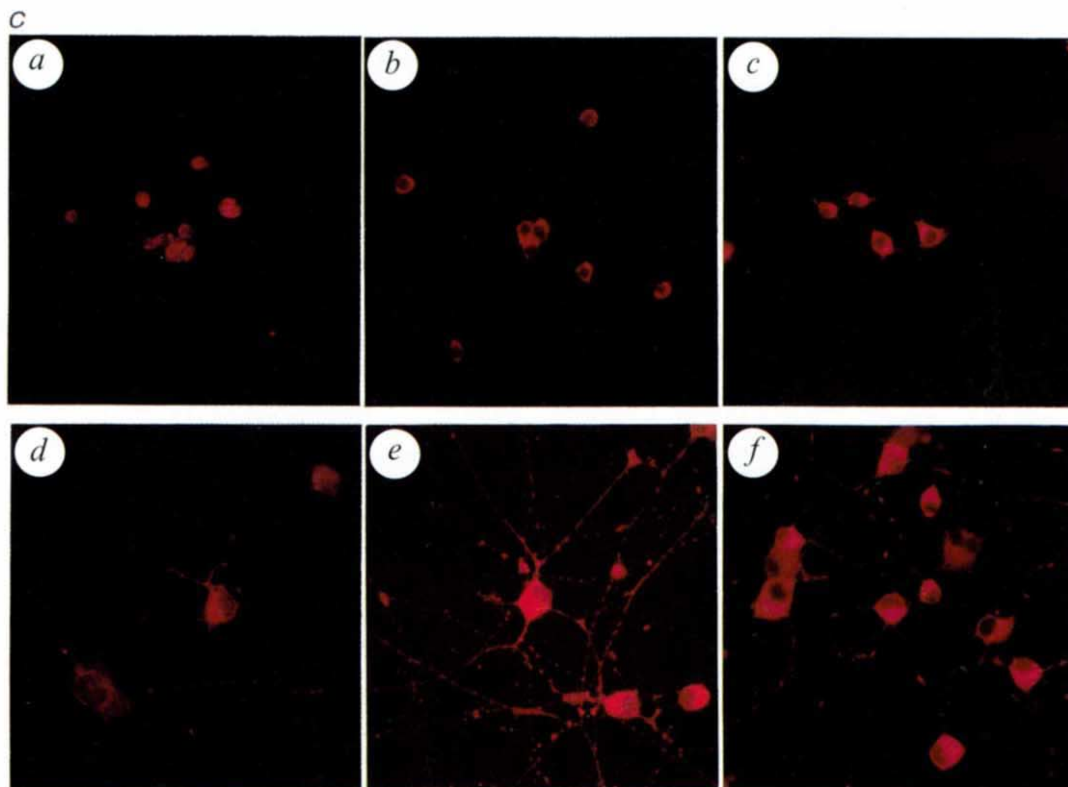
FIG. 1 NGF treatment induces NOS activity in PC12 cells. *A*, Diaphorase staining is induced by NGF treatment. PC12 cells were plated at low density (5×10^4 cells per ml) on collagen and poly-L-lysine-treated plates and NGF (50 ng ml^{-1}) or EGF (25 ng ml^{-1}) was added. At the indicated times cells were fixed with glutaraldehyde and tested for diaphorase staining. Scale bar, 25 μm. *B*, Arginine-citrulline converting activity is induced by NGF treatment. Cell extracts were prepared after NGF treatment and NOS activity was determined by conversion of ^{3}H-arginine to citrulline and NO in the presence or absence of EGTA and NADPH[23]. NOS activity is expressed as c.p.m. of ^{3}H-citrulline produced per mg of protein in 30 min. Error bars represent the standard error of the mean (s.e.m.). *C*, Immunostaining with anti-NOS antibodies. *a*, *d*, Anti-neuronal NOS monoclonal antibodies; *b*, *e*, anti-macrophage NOS monoclonal antibodies; *c*, *f*, anti-endothelial NOS monoclonal antibodies. Cells were fixed before (*a–c*) or after (*d–f*) 9 days of NGF treatment and processed for immunofluorescence using monoclonal antibodies. The exposure time for the photomicrographs of untreated cells was ~10 times longer than for NGF-treated cells; this length of exposure time was necessary to visualize the low level of immunofluorescent signal from the untreated cells. Note the differences in subcellular distribution of different NOS isoforms. Scale bar, 25 μm.

METHODS. PC12 cells were an early passage stock obtained from E. Ziff's laboratory (where they were provided by L. Greene). Cells were grown on collagen-coated plates in DMEM supplemented with 5% calf serum and 10% horse serum (HyClone). For diaphorase staining, cells were fixed with 0.2% glutaraldehyde, washed in PBS and tested for diaphorase staining with 2 mM NADPH, 0.02% tetrazolium blue and 0.3% Triton X-100 as described[25,26]. Control experiments demonstrated that this reaction was NADPH-dependent. Diaphorase staining was not induced when cells received calf, horse, or fetal calf serum. For the ^{3}H-arginine–citrulline conversion assay, 0.5 μCi of ^{3}H-arginine was incubated with 100 μg of cell extract together with 0.45 mM $CaCl_2$, 2 mM NADPH, 500 μM arginine, 10 μg ml^{-1} calmodulin and 50 mM HEPES, pH 7.5, in a total volume of 50 μl for 30 min at 37 °C as described[27]. Citrulline was separated from arginine on Dowex AG50 columns and the radioactivity in the flow-through (citrulline-containing fraction) was determined. For determination of Ca^{2+}-independent activity, the reaction was done in the presence of 3 mM EGTA. Control determinations of the enzyme activity were done in the presence of 500 μM L-NAME, or 20 μl of heat-treated extract, or in the absence of NADPH. For immunochemical analysis, cells were fixed with 0.5% paraformaldehyde for 5 min at room temperature followed by acetone for 15 min at −20 °C. The expression of various forms of NOS was visualized using anti-NOS monoclonal antibodies (Transduction Laboratories), biotinylated sheep anti-mouse antibodies (Amersham), and streptavidin-Texas red complex (Amersham). Control experiments with non-NON-specific antibodies and with omitted first antibodies gave negative results (not shown).

staining. An *in vitro* assay showed gradual elevation of NOS activity starting at about 1 day after NGF addition, agreeing with our data on diaphorase staining (Fig. 1*B*).

Various forms of NOS can be discriminated by their dependence on calcium. Our data show that a large proportion of the NGF-induced NOS activity in PC12 cells (especially at the early stages of NGF action) is independent of Ca^{2+}, suggesting that different isoforms of NOS are present in NGF-treated PC12 cells. This was confirmed using immunocytochemical analysis with specific antibodies for three different types of NOS, which revealed that differentiated cells express all three enzymes, including the calcium-dependent and independent forms (Fig. 1*C*). Taken together, these data indicate that elevation of NOS activity precedes further development of the differentiated phenotype, and that a substantial part of NO is produced by an inducible form of the enzyme, similar to the isoform that is induced in macrophages, hepatocytes, smooth muscle cells and other cell types after appropriate stimulation[15–18].





NO can inhibit DNA synthesis in several systems[19-22]. To test if NO can act as an antimitogenic agent in PC12 cells, we treated cells with increasing concentrations of three different chemical NO donors and tested the ability of the cells to incorporate ^{3}H-thymidine. These compounds suppressed DNA synthesis in a concentration-dependent manner (albeit with different potencies), implying that NO is an active suppressor of DNA replication in PC12 cells (Fig. 2*a*). Both ^{3}H-thymidine incorporation and cell proliferation were restored once NO sources were washed away, indicating that the decrease in ^{3}H-thymidine incorporation that is seen with NO donors is caused by a decrease in DNA synthesis and cell proliferation (cytostasis) and is not due to cytotoxicity.

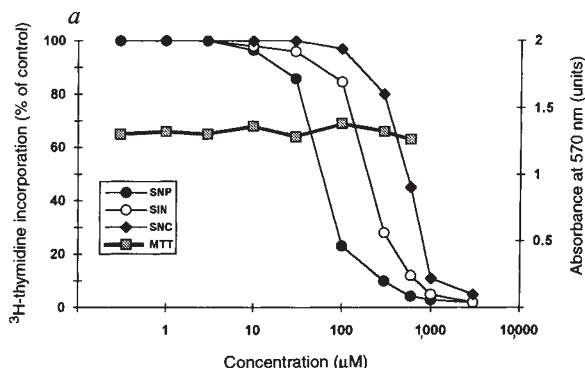
The changes in cell-cycle phase distribution induced by NO in PC12 cells were determined by fluorescence-activated cell sorting (FACS) analysis (Fig. 2*b*). At concentrations of sodium nitroprusside (SNP) up to 150 μM, NO causes the cells to accumulate specifically in the G2 phase, whereas the proportion of cells in S phase decreases. Remarkably, within the range of SNP concentration of 30–150 μM, the proportions of PC12 cells in G2 and S phases are similar to the levels reached after prolonged treatment with NGF (Fig. 2*b* and ref. 23).

After several days of NGF treatment, PC12 cell proliferation ceases and differentiation occurs[5,6]. If NO produced by the NGF-induced NOS in PC12 cells can indeed act as an antiproliferative factor, then inhibition of the enzyme should uncouple the proliferative and cytostatic components of NGF action and prolong the proliferative phase while suppressing the cytostatic effect of NGF. To test this hypothesis, we treated PC12 cells with specific NOS inhibitors in the presence or absence of NGF and monitored both proliferation arrest and neurite outgrowth as principal manifestations of the differentiated phenotype.

NOS inhibitor *N*-nitro-L-arginine methyl ester (L-NAME) indeed reversed the cytostatic action of NGF and forced the cells to continue to proliferate instead of ceasing to divide after 6–8 days of NGF treatment (Fig. 3*a*, *b*). In the absence of NGF, similar concentrations of L-NAME did not have a detectable effect on PC12 cell growth; in particular, L-NAME did not accelerate cell proliferation (Fig. 3*a*). Other NOS inhibitors behaved in a similar way, also permitting the cells to overcome the cytostatic activity of NGF. The inactive D-isomers of these compounds did not interfere with the cytostatic action of NGF, the anti-cytostatic action of NOS inhibitors was concentration-dependent, and finally, the action of L-NAME on PC12 cell division can be partially overcome by the addition of an excess of L- (but not D-) arginine (Fig. 3*b*).

The most visible consequence of NGF action on PC12 cells is neurite outgrowth[5] and this was also affected by NOS inhibition. Under normal conditions, almost every cell gradually extended neurites in response to NGF but, when PC12 cells were treated with a combination of NGF and NOS inhibitor, the number of cells with neurites decreased dramatically; this effect was concentration dependent and was L-isomer specific (Fig. 3*c*, *d*). Thus, inhibition of NOS blocked the later stages of the terminal differentiation process; the cells did not stop dividing and they did not extend neurites.

A reciprocal test of the role of NOS induction in the differentiation cascade is to examine whether treatment with exogenous NO can influence differentiation. For these experiments we used a mutant PC12-U2 cell line[6]. These cells retain the early steps of the response to NGF, but have lost the capacity to execute the later steps. They do not stop dividing after NGF treatment and, as a consequence, they do not develop the fully differentiated phenotype; in particular, they do not send out processes. To ask if the deficit is connected to NO production, we tested if this phenotype can be overcome by the addition of NO. Figure 4*a* shows that although neither NGF nor NO alone had any effect on the phenotype of U2 cells, in combination these treatments restored the differentiated neuronal phenotype. The cells stopped dividing (not shown) and grew extensive branched neurites. To show the involvement of NOS in the differentiation of U2 mutant cells, the induction of NOS by NGF in these cells



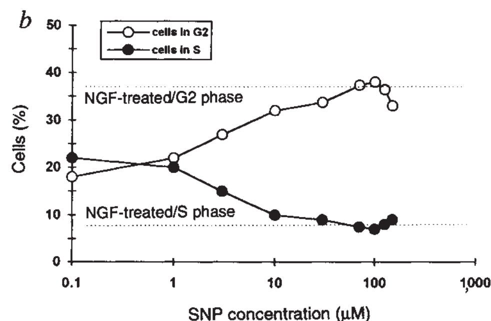


FIG. 2 Nitric oxide act as an antimitogenic cytostatic agent. *a*, Effect of NO donors on ^{3}H-thymidine incorporation. PC12 cells were incubated with increasing concentrations of NO donors and thymidine incorporation was measured after labelling for 2 h. SNP, Sodium nitroprusside; SIN-1, 3-morpholinosydnonimine; SNC, S-nitrosocysteine; MTT, MTT tetrazolium salt conversion assay. The cells remained attached throughout the experiment and excluded trypan blue. NO-mediated inhibition of DNA synthesis overcomes induction of ^{3}H-thymidine incorporation by serum, NGF, EGF, bFGF, serum + *N*-nitro-L-arginine methyl ester (L-NAME), and NGF + L-NAME (data not shown). *b*, Effect of SNP on cell-cycle phase distribution measured by FACS analysis. Changes in cell-cycle phase distribution after exposure to increasing concentrations of the NO donor sodium nitroprusside (SNP) are presented as the proportion of cells in G2 and S phases. For comparison, the fractions of cells in G2 and S phases after 9 days of NGF treatment are shown by the dotted lines.

METHODS. For thymidine incorporation experiments, cells were grown on medium with 1% serum for three days, transferred to medium with 15% serum, incubated with various concentrations of NO donors for 16 h as indicated (duplicates for each concentration) and labelled with tritiated thymidine for 2 h. Cells were collected and the incorporated radioactivity was determined in duplicate for each sample. Control experiments have shown that NO donors affect the incorporation of radioactive thymidine into DNA rather than interfere with the thymidine uptake. For the cytotoxicity analysis the cellular conversion of the MTT tetrazolium salt into formazan after incubation with increasing concentrations of SNP was determined using a reagent kit from 'Promega'. The assay was done as recommended by the manufacturer and data are presented as absorbance of formazan at 570 nm. For FACS analysis, cells were incubated with increasing concentrations of SNP for 16 h; nuclei were prepared in the hypotonic buffer with 0.2% NP40, fixed with ethanol, treated with RNAse, incubated with 50 μg ml^{-1} propidium diiodide, and analysed for DNA content distribution using Coulter EPICS Elite flow cytometer. Similar data were obtained when cells were analysed by combining propidium diiodide staining with an immunofluorescent antibody to BrdU after BrdU incorporation (data not shown).

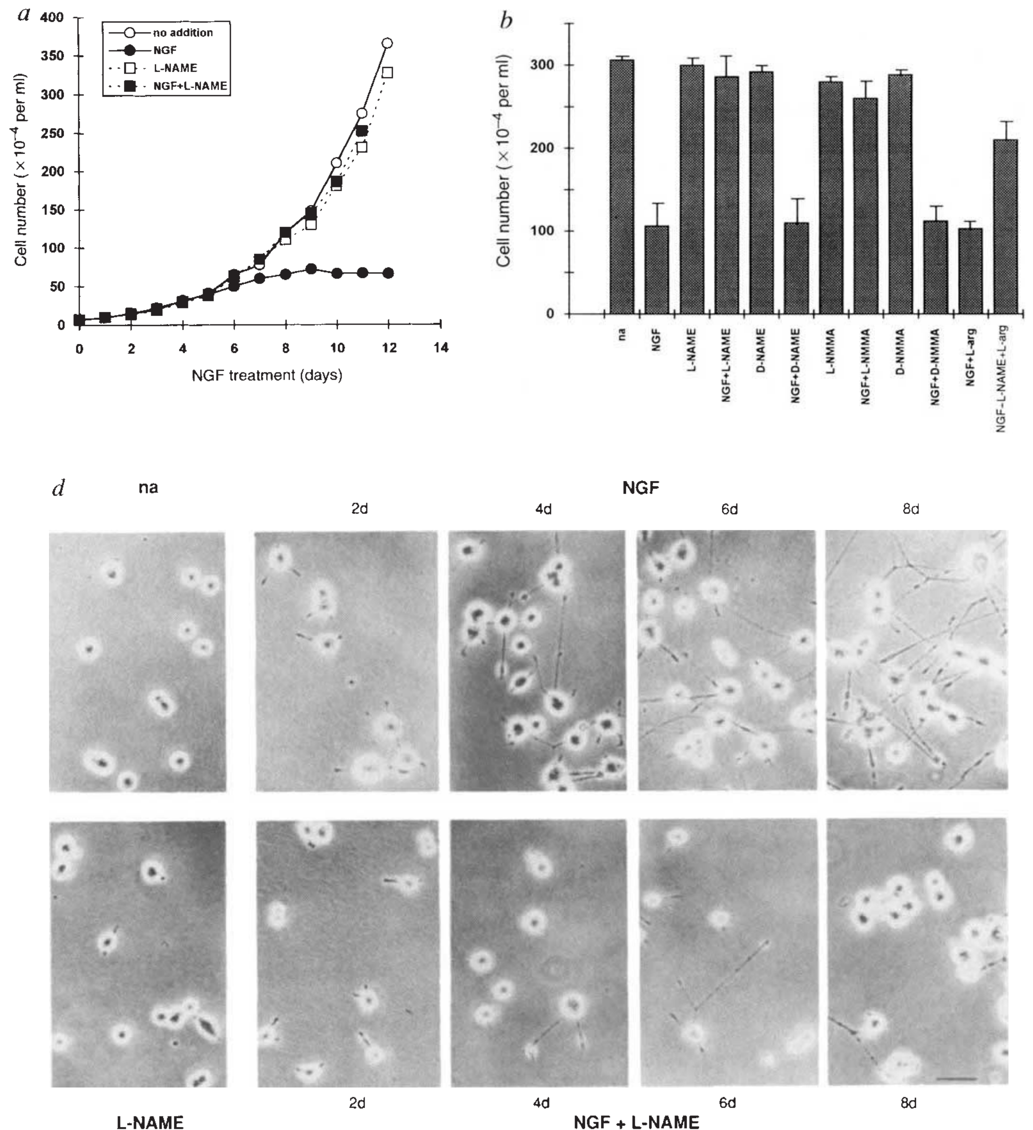
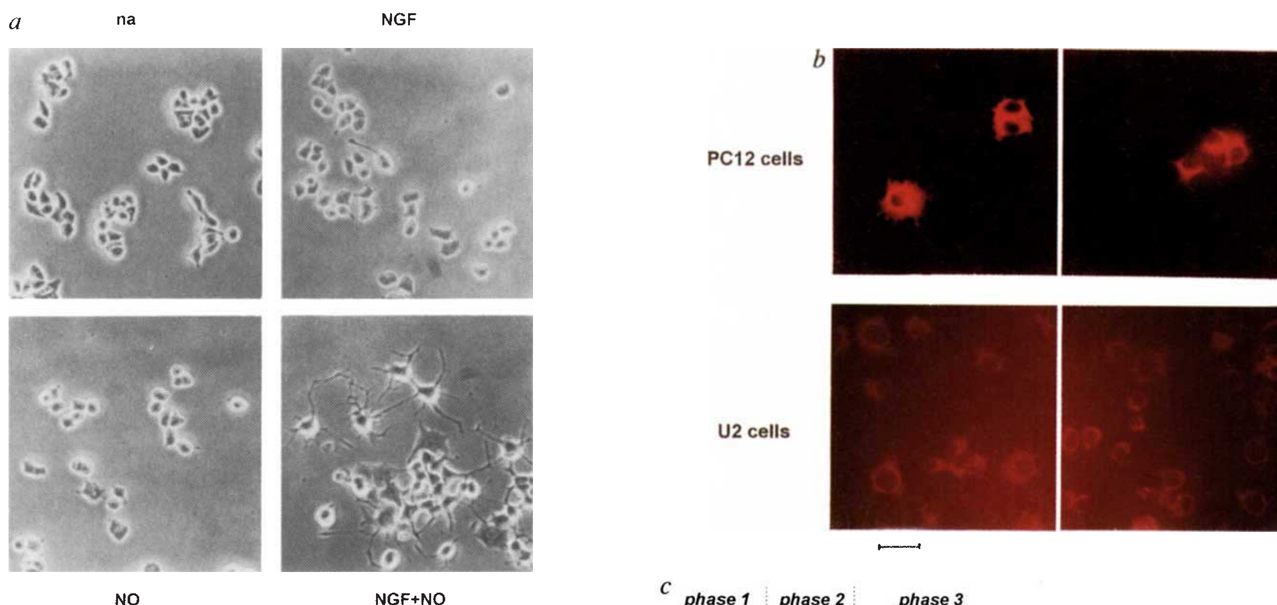


FIG. 3 Inhibition of nitric oxide synthase prevents cytostatic action of NGF and neurite outgrowth. *a*, Growth of NGF-treated PC12 cells with and without L-NAME. PC12 cells were plated at low density and were treated with NGF (50 ng ml^{-1}), L-NAME (1 mM), a combination of both, or received no additions. *b*, Effect of NOS inhibitors on cell proliferation. PC12 cells were treated with NGF alone or in combination with L-NAME (1 mM), D-NAME(5 mM), *N*-monomethyl-L-arginine (L-NMMA) (3 mM) or D-NMMA (3 mM), and L-arginine (8 mM). At day 9, the cells were counted in triplicate as described. Error bars represent s.e.m. *c*. Neurite outgrowth in NGF-treated PC12 cells with and without L-NAME. Cells received NGF (50 ng ml^{-1}) with or without 2 mM or 20 mM L-NAME. Cells with neurites were counted as a percentage of total cells in five independent areas of the plate. Only the cells with neurites longer than two cell diameters were scored as positive. Error bars represent s.e.m. At low concentrations of inhibitor (0.5–5 mM), about 25% of cells still extended neurites, although these neurites were fewer in number (per cell), shorter and less branched than those observed in cells that were treated with NGF without L-NAME. At high L-NAME concentrations (8–20 mM), NGF-induced neurite outgrowth was almost completely suppressed. *d*, Phenotype of PC12 cells treated with NGF, L-NAME and combinations. PC12 cells were plated at low density on collagen-treated plates and NGF (50 ng ml^{-1}) and L-NAME (5 mM) were added. Photomicrographs were taken at the days indicated. Scale bar, 50 μm.

METHODS. Cells (after proteolytic dispersion with collagenase and papain) and nuclei (after hypotonic NP40 lysis) were quantified in triplicate (three plates per point). Values for s.e.m. are not shown in *a* for clarity, but they were similar to those in *b*. The experiments were done with a PC12 cells isolate provided by E. Ziff (originally provided by L. Greene), but similar results were obtained with PC12 cells from several independent sources, including an original isolate provided by L. Greene. The anti-cytostatic action of L-NAME was concentration-dependent, starting at ~100 μM; these experimental numbers probably reflect the fact that L-NAME is a relatively weak competitive inhibitor of NOS and that the concentration of free arginine in the media is ~1 mM. The effect of L-NAME on general cell metabolism and on NGF action is rather specific, in accordance with the data[11] on the high selectivity of NOS inhibitors. L-NAME did not affect major metabolic functions of the cell, so that growth rates and the appearance of the cells were unchanged in comparison with untreated cells. It also did not interfere with the rapid induction of a set of immediate early genes (IEGs) by NGF. We tested a panel of IEGs and found no changes in their basal levels of expression or in their induction by NGF when L-NAME was added (data not shown).

was examined by immunofluorescence. Figure 4*b* shows that inducible NOS is expressed at much lower levels in U2 cells than in the wild-type PC12 cells after 4 days of NGF treatment (note the difference in exposures). At this same time, the levels of the neuronal and endothelial forms of NOS in U2 cells are indistinguishable from those in PC12 cells (data not shown). Taken together, these data demonstrate that the NGF-treated U2 cells are deficient in inducible NOS and that this defect can be rescued by complementing NGF action with NO.

Experiments in which exogenous NO was added to the mutant U2 cells complement the experiments with wild-type PC12 cells in which NO production was blocked. Together they indicate a



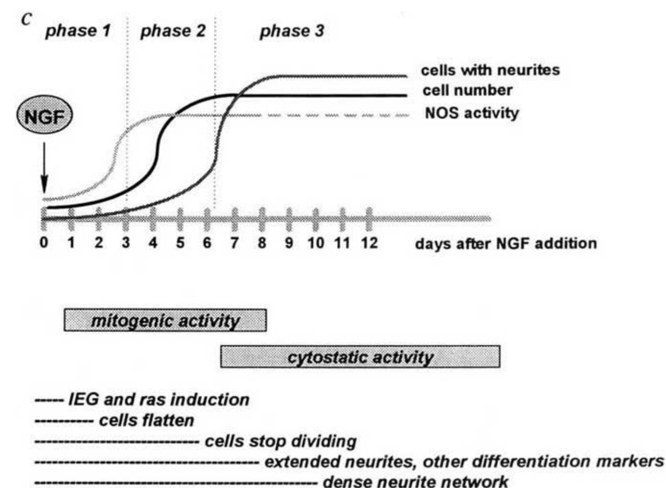


FIG. 4 *a*, Capacity of mutant PC12-U2 cells to differentiate is rescued by NO. Phenotype of PC12-U2 cells treated with NGF, NO and their combination. Mutant U2 cells (a gift from L. Greene) were plated at low density on collagen-treated plates and NGF (50 ng ml^{-1}), SIN-1 (300 μM) or their combination were added. Photomicrographs were taken at day 9 after NGF addition. Scale bar, 50 μm. Similar results were obtained with another NO donor, sodium nitroprusside (not shown). *b*, Immunostaining of PC12 cells and U2 cells with anti-iNOS antibodies. Cells were fixed after 4 days of NGF treatment and iNOS expression was visualized using anti-iNOS monoclonal antibodies as described for Fig. 1. The exposure time for the photomicrographs of U2 cells was ~10 times longer than for PC12 cells in order to visualize the low level of immunofluorescent signal from U2 cells. Scale bar, 25 μm. c, Schematic summary of NGF action, NOS activity and differentiation of PC12 cells. The model relates NGF-induced NOS activity to PC12 cell proliferation and differentiation. NGF activates a cascade of genes, eventually inducing NOS activity. NOS produces enough NO to inhibit DNA synthesis and, probably, to activate further checkpoints, thereby blocking the cell cycle progression, completing the proliferative phase and switching the cells to the cytostatic phase. Finally, after growth arrest, the cell starts to implement those differentiation traits that can only occur after cell division has ceased (such as neurite outgrowth). Various forms of NOS, induced with different kinetics, might perform different physiological functions during PC12 cell differentiation. Inducible NOS might be primarily responsible for a slow accumulation of cytostatic activity (independent of temporary calcium stimulation), whereas the neuronal (and, perhaps, endothelial) form of NOS might be primarily important for the later stages of development in fully differentiated cells, where transient modulations of calcium level activate NOS to induce secretion of neurotransmitters[28].

causative role for NO action in NGF-induced growth arrest and differentiation in PC12 cells.

The results of this study suggest a model for NGF action in PC12 cells in which at least three stages can be outlined (Fig. 4*c*). In the first, proliferative stage NGF activates a cascade of genes, eventually leading to the induction of the *NOS* gene. In the second stage, the accumulated NOS enzyme produces enough NO to inhibit DNA synthesis and, probably, to 'alert' further checkpoints, thereby blocking further progression of the cell cycle, completing the proliferative phase of NGF action, and switching the cells to the cytostatic phase; probably, NO also directly induces some of the later differentiation markers by promoting gene activity[24]. Finally, as soon as the cell perceives and processes the cytostatic signal, it starts to implement the remaining programme of differentiation traits (such as neurite outgrowth), which can only occur after cell division has ceased.

Is a similar NOS-mediated mechanism used by other systems (including, but not limited to, the nervous system) to maintain mitogenic quiescence? NOS-dependent cytostasis could explain the action of other growth factors during terminal differentiation in which the initial mitogenic response is replaced by a cytostatic phase. It might also help to explain the regulated mitogenesis in tissues expressing NOS (endothelial cell regeneration, liver injury, atherosclerotic smooth muscle cell lesions, wound healing and so on) where initial rapid proliferation has to be followed by controlled cessation in order not to provoke excessive growth. Given the unconventional properties of NO it is possible that NO diffuses from the cell that produces it to promote cessation of growth in adjacent cells. If NO molecules, produced by a group of adjacent cells, act additively within a limited volume, this could contribute to the synchronization of development of a domain of precursor cells. Finally, because NO is also involved in neuronal plasticity, induction of NOS could provide a link between the activity of a cell and the developmental fate of it and its nearest neighbours. □

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A calcium-channel homologue required for adaptation to dopamine and serotonin in *Caenorhabditis elegans*

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PROCESSING and storage of information by the nervous system requires the ability to modulate the response of excitable cells to neurotransmitter. A simple process of this type, known as adaptation or desensitization, occurs when prolonged stimulation triggers processes that attenuate the response to neurotransmitter. Here we report that the *Caenorhabditis elegans* gene *unc-2* is required for adaptation to two neurotransmitters, dopamine and serotonin. A loss-of-function mutation in *unc-2* resulted in failure to adapt either to paralysis by dopamine or to stimulation of egg laying by serotonin. In addition, *unc-2* mutants displayed behaviours similar to those induced by serotonin treatment. We found that *unc-2* encodes a homologue of a voltage-sensitive calcium-channel α-1 subunit. Expression of *unc-2* occurs in two types of neurons implicated in the control of egg laying, a behaviour regulated by serotonin. Unc-2 appears to be required in modulatory neurons to downregulate the response of the egg-laying muscles to serotonin. We propose that adaptation to serotonin occurs through activation of an Unc-2-dependent calcium influx, which modulates the postsynaptic response to serotonin, perhaps by inhibiting the release of a potentiating neuropeptide.

To identify molecules involved in adaptation in the *C. elegans* nervous system, we searched for mutants that failed to adapt to neuroactive substances. We focused primarily on two biogenic amines, dopamine and serotonin. Both compounds are found in *C. elegans* neurons[1,2], and both have striking effects on nematode behaviour. Exogenous serotonin has several behavioural effects: stimulation of egg laying, inhibition of locomotion, stimulation of feeding, and activation of a specific step in the male mating program[2–4]. We found that dopamine treatment had at least two effects on *C. elegans* behaviour: inhibition of movement, and inhibition of egg laying (Table 1). Thus dopamine and serotonin had opposing effects on egg laying and similar effects on movement, although serotonin, unlike dopamine, also caused animals to move in a twisting, 'kinking' manner. Sets of neurons expressing either serotonin or dopamine have been identified[1], including the serotonergic hermaphrodite-specific neurons (HSNs)[5], required for egg laying, and the CP neurons, which also contain serotonin and are involved in male mating[4].

To determine whether *C. elegans* could adapt to these neurotransmitters, we tested the response to dopamine and serotonin after prolonged exposure. Treatment with 3 mg ml^{-1} (16 mM) dopamine initially inhibited egg laying and locomotion in wild-type animals. However, animals treated with dopamine in this manner for 4 hours or more recovered the ability to move and lay eggs normally. These pretreated animals became resistant to inhibition of both egg laying and locomotion by up to 6 mg ml^{-1} dopamine, indicating that they had adapted to dopamine (Table 1; Fig. 1*a*, *c*). Moreover, when these adapted animals were transferred to a solution that did not contain dopamine, they laid eggs at an abnormally high rate, suggesting that they had become dependent on exogenous dopamine for the control of egg laying (Table 1). Adapted animals regained sensitivity to dopamine over the course of approximately 4 hours (Fig. 1*b*). Long-term exposure to serotonin also led to adaptation. Serotonin (3 mg ml^{-1}, 7.7 mM) initially stimulated egg laying; however, animals exposed to serotonin overnight accumulated unlaid eggs, and were unable to lay eggs in response to a fresh dose of serotonin (Table 1, Fig. 2*d*).

TABLE 1 Adaptation to dopamine and serotonin

Experiment	Strain genotype	Pretreatment	Test conditions	Eggs laid (eggs per worm per hour)	Percentage active
1	wild-type	no drug	no drug	2.5 [±1.1]	100
	wild-type	no drug	6 mg ml^{-1} dopamine	0.5 [±0.1]	0
	wild-type	3 mg ml^{-1} dopamine	6 mg ml^{-1} dopamine	2.5 [±1.7]	100
2	*egl-1*(*n987*)	no drug	no drug	0.6 [±0.2]	
	egl-1(*n987*)	no drug	3 mg ml^{-1} serotonin	45.2 [±6.8]	
	egl-1(*n987*)	3 mg ml^{-1} serotonin	3 mg ml^{-1} serotonin	0.8 [±0.8]	
3	wild-type	no drug	no drug	3.6 [±2.8]	
	wild-type	3 mg ml^{-1} dopamine	no drug	9.8 [±2.8]	

Effects of adaptation on egg laying and locomotion. Wild-type or *egl-1* adult hermaphrodites were grown overnight on 1.5% agar plates spread with *Escherichia coli* strain OP50, and with dopamine hydrochloride or serotonin creatinine sulphate added at the indicated concentrations (2 mM acetic acid was added to dopamine plates to stabilize the dopamine). To eliminate effects of endogenous serotonin, *egl-1* mutants, which lack HSN neurons, were used for the serotonin experiment. After overnight incubation in the presence or absence of drug, 10 animals were transferred to freshly poured test plates containing dopamine or serotonin as indicated, and were allowed to lay eggs at room temperature. Eggs were counted after 45 minutes for experiment 1, 1 hour for experiment 2, and 30 minutes for experiment 3. These data represent the mean rate of egg laying in 4 or more independent experiments (experiment 3 involved 11 independent trials). The sample standard deviation of these data is indicated in brackets.