

Wheat Germ Agglutinin Blocks the Biological Effects of Nerve Growth Factor

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ABSTRACT The binding of nerve growth factor (NGF) to specific cell surface receptors initiates a variety of effects that lead to the morphological and biochemical differentiation of clonal pheochromocytoma, PC12, cells. The lectin wheat germ agglutinin (WGA) alters the characteristics of NGF-receptor interaction. We have found that treatment of PC12 cells with WGA dramatically and reversibly inhibits the ability of NGF to elicit three distinct biological effects characteristic of NGF action. Two of these events, the rapid ruffling of cell-surface membranes and the stimulation of the phosphorylation of a 250-kD cytoskeletal protein *in situ*, occur rapidly and are an immediate consequence of receptor occupancy. Both of these effects are blocked by pretreatment of the cells with WGA. WGA was also found to inhibit the NGF-stimulated regeneration of neurites that occurs over 1–2 d. Both the WGA inhibition of neurite outgrowth and the phosphorylation of the 250-kD cytoskeletal protein were reversed upon addition of the specific sugar *N*-acetylglucosamine. These data demonstrate that the WGA-induced changes in the NGF-receptor interaction reflect important alterations in the ability of the receptor to transmit biological signals, resulting in the abrogation of the biological effects of NGF on these cells.

Nerve growth factor (NGF)¹ directs the morphological and biochemical differentiation of sympathetic and certain sensory neurons (1). The cellular mechanisms that subserve NGF action on its target cells are unknown. The clonal rat pheochromocytoma line, PC12, responds to NGF by ceasing division and acquiring various phenotypic characters similar to those of sympathetic neurons (2, 3). This cell line has proven to be a valuable model for investigating the mechanism of NGF action.

PC12 cells have two interconvertible classes of cell-surface receptors for NGF (4–7). The two receptor populations can be distinguished based on their different kinetic properties, sensitivity to trypsin digestion, and solubility in nonionic detergents. One class of receptors demonstrates rapid dissociation kinetics and has been termed the “fast” receptors; this population of receptors is trypsin-labile and is solubilized upon extraction of PC12 cells in Triton X-100 (5). A second class of receptors, or “slow” receptors, from which NGF dissociates slowly, is trypsin-stable and remains with the Triton-insoluble cytoskeleton after detergent extraction of the cells. The latter class of NGF receptors appears to be respon-

sible for the internalization of the NGF-receptor complex (8), and based on the NGF dose-dependence of various biological effects is likely to mediate many of the actions of NGF on these cells (3). Some controversy exists as to whether the two populations of receptors preexist on the cell surface or are generated as a consequence of ligand-dependent conversion of “fast” to “slow” receptors (4, 5); it is also unresolved whether the two classes of receptors demonstrate different equilibrium dissociation constants (5, 7, 8).

The NGF receptor is a membrane glycoprotein of at least 85 kD; other species of ~150 and 200 kD have also been identified (9–13). The NGF receptor has *N*-acetylglucosamine residues that bind the lectin wheat germ agglutinin (WGA) (10). It is interesting that binding of the lectin has dramatic effects on the interaction of NGF with its receptor (10, 14–16). WGA treatment of PC12 cells induces the conversion of kinetically “fast” receptors to “slow” receptors, confers trypsin-resistance to the hormone-receptor complex, and results in the association of the NGF-receptor complexes with the Triton-insoluble cytoskeleton. These effects are reversible upon dissociation of WGA from the receptor by the addition of the specific sugar, *N*-acetylglucosamine.

Although the ability of WGA to alter the binding characteristics of the NGF receptor has been well characterized, the

¹ *Abbreviations used in this paper:* NGF, nerve growth factor; WGA, wheat germ agglutinin.

significance of these changes with respect to the ability of NGF to produce its specific effects on these cells has not been examined. We report here that the interaction of WGA with the NGF receptor abrogates the ability of NGF to elicit three distinct biological effects characteristic of NGF action on these cells. The inhibition of NGF action is reversible and specific for WGA.

MATERIALS AND METHODS

Materials: WGA was purchased from Calbiochem-Behring Corp. (San Diego, CA). NGF was prepared by the method of Smith et al. (17). Horse serum was obtained from KC Biologicals Inc. (Lenexa, KS), fetal calf serum from Gibco Laboratories Inc. (Grand Island, NY). The lectins *Dolichos biflorus* agglutinin, peanut agglutinin, and concanavalin A were from Sigma Chemical Co. (St. Louis, MO).

Cell Culture: PC12 cells were grown in an atmosphere of 10% CO₂ in Dulbecco's modified Eagle's medium containing 10% horse serum, 5% fetal calf serum.

Measurement of Neurite Outgrowth: Neurite outgrowth was measured using the bioassay of Greene (18). PC12 cells were treated for 11 d with 50 ng/ml NGF, with the medium being changed every 2 d. The cells were removed from the tissue culture dish by trituration, washed, and replated in polylysine-treated 16-mm tissue culture wells in serum-free medium containing 1 mg/ml bovine serum albumin. NGF was added at a final concentration of 50 ng/ml along with lectins or *N*-acetylglucosamine as indicated. The cellular aggregates were examined 24 h later for neurite outgrowth, and the fraction of neurite-bearing cellular aggregates was determined. After the cultures were scored, some wells received 10 mM *N*-acetylglucosamine and were incubated for 24 h more, and the number of neurite-bearing aggregates was again determined. The assay was performed using triplicate wells with at least 80 aggregates counted per well.

Phosphorylation of PC12 Cytoskeletal Proteins In Situ: The phosphorylation of PC12 cytoskeletal protein was performed as described by Landreth and Rieser (19). PC12 cells were removed from the tissue culture dishes by trituration in calcium- and magnesium-free phosphate-buffered saline (PBS), washed, and then resuspended at a final concentration of 10⁶ cells/ml in PBS containing 1 mg/ml bovine serum albumin and 1 mg/ml glucose. The cells were incubated at 37°C in the presence of 50 ng/ml NGF or lectin, as indicated. The cells were collected by centrifugation and extracted in 0.15% Triton X-100, 25 mM HEPES, pH 7.4, 2 mM MnCl₂, and 1 mM phenylmethylsulfonyl fluoride for 2 min at 4°C. The resulting cytoskeletons were pelleted by centrifugation for 30 s in a Beckman Microfuge B (Beckman Instruments Inc., Palo Alto, CA) and resuspended in the same buffer but without Triton. The phosphorylation reaction was initiated by the addition of [³²P]ATP (10 μM, 50 dpm/fmol ATP) and carried out for 10 min at 4°C. The reaction was stopped by the addition of electrophoresis sample buffer and 5 min of boiling. The labeled cytoskeletal proteins were separated by electrophoresis on 4–13% polyacrylamide gels as described by Laemmli (20). The gels were stained with 0.1% Coomassie Blue, dried, and exposed to Kodak XAR film.

Scanning Electron Microscopy: PC12 cells were grown on polylysine-treated glass coverslips. The cells were then incubated in serum-free medium in the presence of 50 ng/ml NGF or 50 μg/ml WGA. WGA-treated samples were incubated for 30 min at 37°C; one group of cells was preincubated with WGA but then received NGF and was incubated for 3 min more at 37°C. The NGF-treated samples were incubated for 3 min at 37°C. The treatment was terminated by a rapid wash with cold PBS followed by fixation in 2% glutaraldehyde for 5 min at room temperature. The cells were washed with PBS, postfixated in 1% osmium tetroxide for 3 min, washed extensively, and dehydrated through graded alcohols. The samples were critical point dried, coated with gold-palladium, and examined using a JEOL 35C scanning electron microscope. The figures are representative of triplicate samples with ~200 cells examined per sample.

RESULTS

Effect of WGA on NGF-stimulated Neurite Outgrowth

NGF treatment of PC12 cells produces transcriptionally based events, termed "priming," which lead to morphological differentiation with the growth of extensive neuritic processes

over a period of several days (2, 3). Once the PC12 cells have been primed, if they are divested of their neurites by removal from the tissue culture dish and replated, they will regenerate their neurites in the presence of NGF. The fraction of PC12 cells that exhibits neurites is NGF dose-dependent and is the basis of a sensitive bioassay for NGF (18).

The ability of WGA to modify the effects of NGF on neurite regeneration was tested by incubating primed PC12 cells with 50 ng/ml NGF for 24 h in the presence or absence of WGA (Table I). WGA inhibited the NGF-induced neurite outgrowth in a dose-dependent manner, with half-maximal inhibition occurring at a WGA concentration of between 2.5 and 5 μg/ml. This effect was abolished by inclusion of the specific sugar, *N*-acetylglucosamine, in the incubation. The effects of WGA were fully reversible, since the addition of 10 mM *N*-acetylglucosamine to the cultures after a 24-h exposure to WGA restored the ability of the cells to regenerate neurites. The lectin-mediated inhibition of neurite outgrowth was a specific effect of WGA, as concanavalin A and *Dolichos* agglutinin were without effect (Table I). We have also been unable to inhibit neurite outgrowth with peanut agglutinin at doses as high as 50 μg/ml (data not shown).

WGA Inhibits NGF-mediated Phosphorylation of a 250-kD Cytoskeletal Protein

NGF treatment of PC12 cells produces stable changes in the cells that persist upon extraction of the cells in nonionic detergent and that can be detected by phosphorylation of a 250-kD cytoskeletal protein in situ (19). In this system, protein phosphorylation occurs between immobilized protein kinases and their protein substrates, both of which are associated with the detergent-insoluble cytoskeleton. The changes that lead to the phosphorylation of this protein occur rapidly after NGF

TABLE I. Effect of Lectins on NGF-mediated Neurite Outgrowth

	% with neurites	
	24 h	48 h (+10 mM GlcNac)
	mean (±SD)	
Experiment 1		
NGF	95.7 (1.5)	97.1 (0.8)
NGF + 2.5 μg/ml WGA	67.7 (5.0)	95.5 (0.3)
NGF + 5 μg/ml WGA	34.2 (1.7)	84.9 (1.8)
NGF + 10 μg/ml WGA	9.5 (4.0)	64.8 (0.3)
NGF + 10 μg/ml WGA + 10 mM GlcNac	83.2 (5.8)	—
No additions	11.6	9.8 (0.2)
Experiment 2		
NGF	92.7 (2.2)	
NGF + 5 μg/ml WGA	44.1 (7.0)	
NGF + 10 μg/ml WGA	8.9 (1.8)	
NGF + 25 μg/ml ConA	97.4 (1.9)	
NGF + 25 μg/ml <i>Dolichos biflorus</i> agglutinin	95.3 (0.5)	
No additions	4.2 (1.3)	

PC12 cells were treated with 50 ng/ml NGF for 11 d and then replated in polylysine-treated wells in serum-free medium containing 1 mg/ml bovine serum albumin. NGF was added as indicated at a final concentration of 50 ng/ml alone or with lectin. The fraction of cellular aggregates exhibiting neurite outgrowth was determined 24 h later. At this time 10 mM *N*-acetylglucosamine (GlcNac) was added to the indicated cultures, and neurite outgrowth was measured 24 h later. The data are the mean ±SD of triplicate determinations. ConA, concanavalin A.

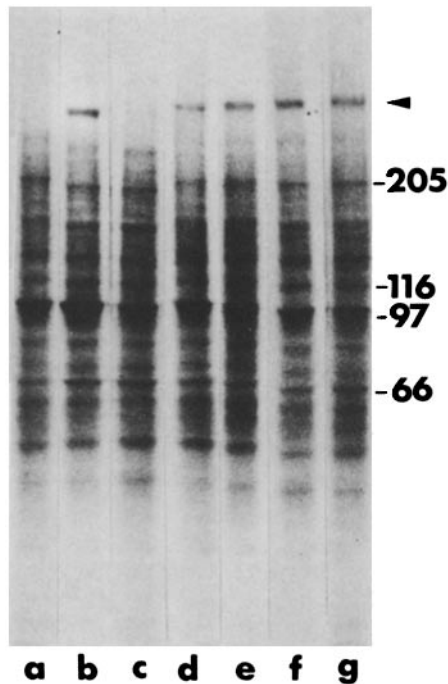


FIGURE 1 Effect of lectins on the phosphorylation of a 250-kD cytoskeletal protein. PC12 cells were preincubated for 30 min at 37°C alone or with 50 $\mu\text{g}/\text{ml}$ of the indicated lectin. NGF was then added at a concentration of 50 ng/ml and incubated for 5 min more at 37°C. The cells were extracted in detergent-containing buffer and the resulting cytoskeletons were incubated for 10 min at 4°C with [^{32}P]ATP. An autoradiogram of the labeled phosphoproteins is shown, and the position of the 250-kD protein is indicated. Lane a, untreated control. Other lanes were treated with: (lane b) NGF; (lane c) WGA then NGF; (lane d) concanavalin A then NGF; (lane e) peanut agglutinin then NGF; (lane f) *Dolichos* agglutinin then NGF; (lane g) WGA then 10 mM *N*-acetylglucosamine and NGF.

binding to its receptors; however, the phosphorylation is a transient consequence of hormone action, occurring maximally within 5 min of hormone exposure and diminishing thereafter.

Incubation of PC12 cells for 5 min at 37°C with 50 ng/ml NGF leads to the phosphorylation of the 250-kD protein *in situ* (Fig. 1). If the cells were pretreated for 30 min at 37°C with 50 $\mu\text{g}/\text{ml}$ WGA, 50 ng/ml NGF was added, and cells were incubated for 5 min more at 37°C, the 250-kD protein did not become phosphorylated. The inhibitory effect of WGA was reversible; if at the end of the 30-min incubation with WGA, 10 mM *N*-acetylglucosamine was added with NGF, the effect of WGA on the phosphorylation of the 250-kD cytoskeletal protein was abolished. Treating PC12 cells with *Dolichos* or peanut agglutinin or concanavalin A before NGF had no effect on the NGF-stimulated phosphorylation of the 250-kD cytoskeletal protein.

The inhibition of 250-kD cytoskeletal protein phosphorylation by WGA was dose-dependent; half-maximal inhibition occurred at a WGA concentration of $\sim 5 \mu\text{g}/\text{ml}$ (Fig. 2).

Similar experiments were performed to test if WGA would alter the ability of epidermal growth factor to stimulate the phosphorylation of the 250-kD protein (19). WGA inhibited the epidermal growth factor-stimulated phosphorylation of the 250-kD protein, although not as much as NGF did (data not shown). This result is probably due to binding of WGA

to the epidermal growth factor receptor, inhibiting the binding of the hormone (21).

WGA Inhibits NGF Effects on Cell-Surface Morphology

The most immediate effect of NGF on PC12 cells is the dramatic alteration of cell-surface architecture (22). The cell surface of PC12 cells has numerous small blebs and microvilli; however, within 1 min of the addition of NGF large ruffles appear over the dorsal surface. The development of these ruffles occurs maximally within 2–4 min after NGF exposure.

Treatment of the cells with 50 ng/ml NGF for 3 min resulted in the appearance of large ruffles over the dorsal surface of the cell (Fig. 3); these observations are similar to those previously reported by Connolly et al. (22). If PC12 cells were pretreated for 30 min with WGA alone, the lectin had no significant effect on the morphology of the cells, although very small blebs were frequently observed on the cell surface. Pretreatment of the cells with WGA for 30 min, followed by the addition of NGF and a further incubation for 3 min, blocked the NGF-mediated changes in cell-surface architecture and development of ruffles.

DISCUSSION

The ability of the lectin WGA to modify the behavior of the NGF receptor has been extensively studied (10, 14–16), yet the impact of these changes on the ability of the receptor to elicit biological responses has, until now, not been explored. We report here that whereas the binding of WGA to the NGF receptor results in the quantitative conversion of NGF receptors to the “slow” state, there is a dramatic inhibition of the ability of the cells to respond to the hormone. This observation

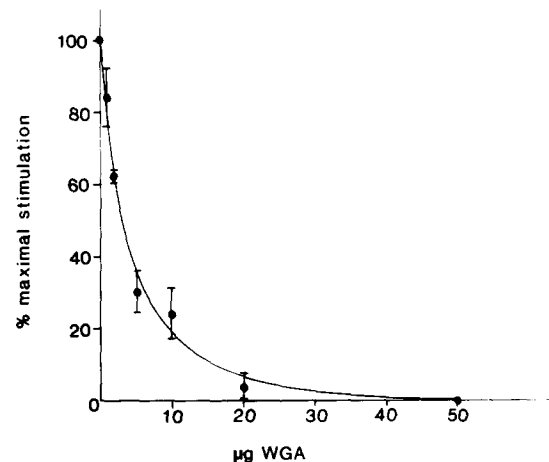


FIGURE 2 Dose-dependence of WGA inhibition of the phosphorylation of a 250-kD cytoskeletal protein. PC12 cells were incubated for 30 min at 37°C with the indicated concentrations of WGA, then 50 ng/ml of NGF was added and incubated for 5 min more at 37°C. Cytoskeletons prepared from these cells were incubated with [^{32}P]ATP for 10 min at 4°C, and the labeled phosphoproteins were separated by gel electrophoresis. The radioactivity in the 250-kD protein was determined by excision of the band and measurement by scintillation counting. The data are the mean of duplicate determinations and are representative of two independent experiments. NGF stimulated the incorporation of 320 cpm ^{32}P into the 250-kD protein in the absence of WGA.

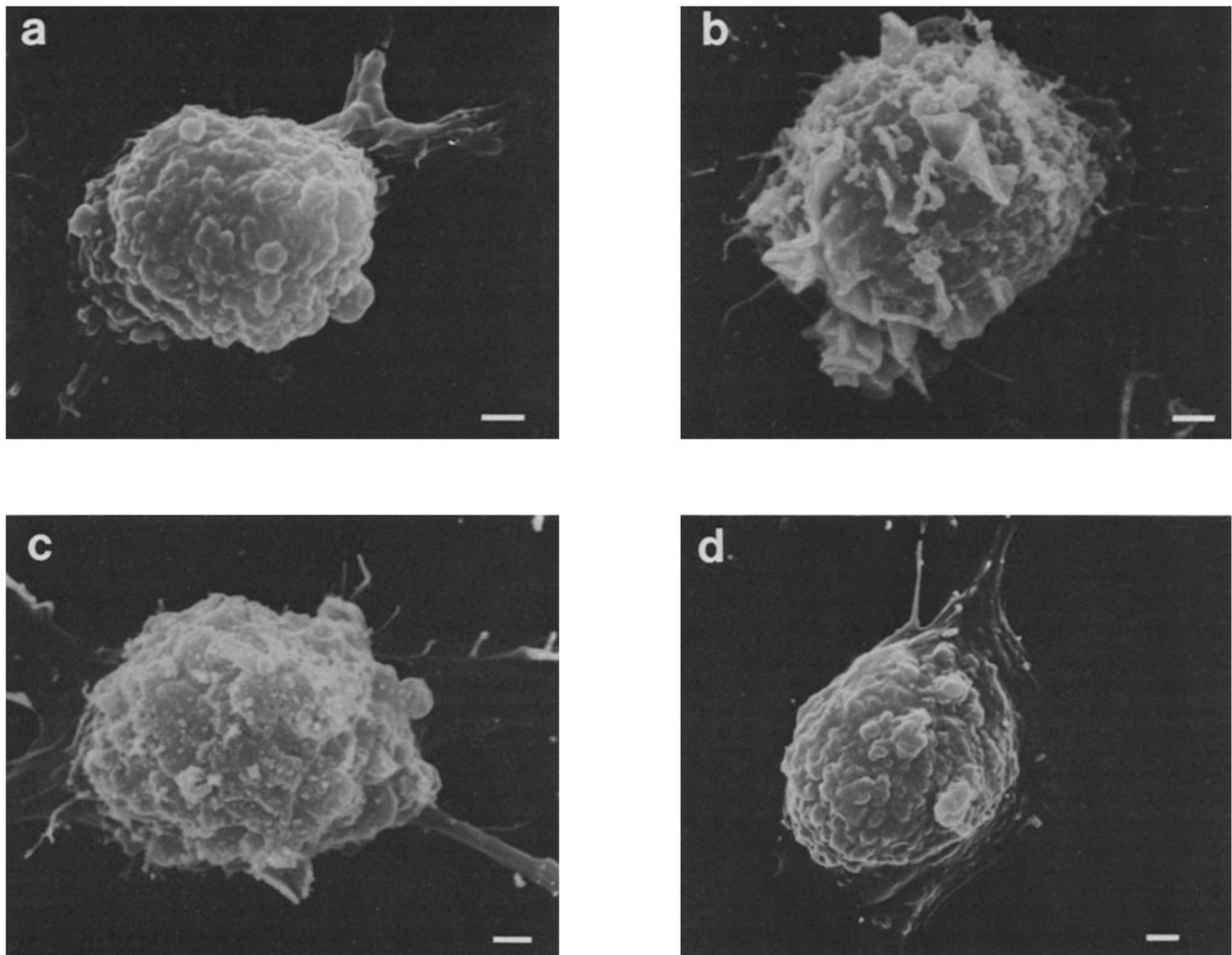


FIGURE 3 Effect of WGA on NGF-induced changes in cell surface morphology of PC12 cells. PC12 cells were incubated (a) alone; (b) in the presence of 50 ng/ml NGF for 3 min; (c) with 50 µg/ml WGA for 30 min at 37°C; (d) with 50 µg/ml WGA for 30 min at 37°C and then incubated for 3 min with 50 ng/ml NGF. Bar, 1 µm.

is paradoxical since the “slow” form of the NGF receptor is likely to be responsible for internalization of the hormone (8) and initiation of many of its biological effects. The present data indicate that the association of the NGF receptor with the cytoskeleton, the acquisition of trypsin resistance, and the “slow” rate of ligand dissociation from the receptor are not of themselves directly involved in the signal transduction that leads to initiation of the biological responses examined here. These alterations may involve the association of the receptor with other receptors or with membrane glycoproteins; indeed, the “slow” form of the receptor was reported to have a greater molecular mass than did the “fast” form (13, 23). It is apparent that the binding of ligand to the receptor induces a conformational change, resulting in a diminished rate of NGF dissociation and in resistance to trypsin (4, 14–16). Whereas NGF can produce these changes through its specific association with the receptor-binding domain, WGA binding at different sites produces similar effects. Furthermore, Vale and Shooter have reported that cross-linking the NGF receptors with antibodies can elicit identical changes (14).

We interpret these data to indicate that the NGF receptor, upon binding of the hormone, undergoes a conformational change that alters its affinity for NGF. This alteration is

accompanied by sequestration of trypsin-sensitive sites on the receptor and the binding of the hormone-receptor complex to the cytoskeleton. Similarly, WGA can produce an analogous conformational change, leading to trypsin resistance and altered NGF affinity; however, it is plausible that the tetra-valent lectin may immobilize the receptor by cross-linking to other membrane constituents. Alternatively, the WGA-induced conformational change may be distinct from that produced from NGF, as Grob and Bothwell suggested (15). In either case, the NGF receptor cannot undergo physiologically relevant interactions, blocking the ability of NGF to produce its biological effects on the cells.

One effect of preincubation of the cells with WGA is to diminish the binding of NGF to PC12 cells by ~50% (14, 15). The experiments were performed at NGF concentrations of 50 ng/ml, a condition under which the level of NGF receptor occupancy on the PC12 cells, even if reduced by 50%, would be more than sufficient to stimulate maximally each of the biological effects examined here. Both the NGF-stimulated neurite outgrowth and the phosphorylation of the 250-kD cytoskeletal protein occur maximally at NGF concentrations below 1 ng/ml (18, 19). The NGF-mediated alteration of cell surface morphology is also observed at low NGF

concentrations (24). Thus, the inhibition of the biological response of the cells to NGF was not a consequence of insufficient receptor occupancy.

The dose of WGA required for half-maximal inhibition of NGF action was $<5 \mu\text{g/ml}$. The effect of WGA on NGF binding was half-maximal at WGA concentrations of $\sim 30\text{--}50 \mu\text{g/ml}$, which suggests that the inhibition of the effect of NGF on these cells may occur at doses of WGA below that required to maximally inhibit NGF binding (14, 15).

We have chosen to examine three different effects of NGF on PC12 cells, two of which occur as an immediate consequence of receptor occupancy. The extension of neurites from these cells requires longer periods (1–2 d). Thus, WGA acts both acutely and chronically to block the receptor-mediated events that lead to the biological effects of NGF on these cells. The molecular mechanism by which WGA inhibits NGF action is still unclear, yet these observations provide insight into receptor mediation of the biological effects of NGF.

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