# Rapid Retraction of Neurites by Sensory Neurons in Response to Increased Concentrations of Nerve Growth Factor

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ABSTRACT The phenomenon of growth cone (GC) and neurite retraction resulting from a rapid increase in concentration of the trophic molecule NGF was studied. Neurite outgrowth from explants of 8-d chick embyro dorsal root ganglia was achieved at very low NGF concentrations with heart conditioned medium during overnight culture. Quickly increasing the NGF concentration in the growth medium dramatically affected GC and neurite morphology: the majority of GCs and neurites collapsed and retracted towards the cell body over a course of ~2-5 min. Retraction was elicited by increasing NGF levels from 0 or 0.05 ng/ml to as little as 0.5 ng/ml but did not occur if the NGF concentration during the initial overnight culture period exceeded 0.8 ng/ml, regardless of how much the concentration was elevated. Similar concentration changes of cytochrome *c* or insulin did not result in retraction. Neurites that had been separated from their cell bodies by cutting close to their exit from the explant still retracted when NGF levels were raised. Cytochalasin B reversibly inhibits retraction, whereas colchicine allows retraction to occur. Observation of cell-substratum adhesion during retraction revealed that some adhesion points remain during retraction and that they correspond to the ends of retraction fibers. We conclude that retraction is a sensitive, dramatic response to increased NGF levels and that it may involve microfilaments in the neurite cytoskeleton. The NGF concentration changes that elicit neurite retraction suggest that a primary event in retraction may be increased occupancy of a high-affinity NGF receptor on neurites.

Chemotactic responses to gradients of specific molecules have been postulated to play a part in the directionality of cell migrations during embryogenesis (31, 32). Several in vitro studies have indicated that neurite growth from embryonic peripheral neurons to target tissues may involve chemotaxis (4, 5, 11, 16). A dramatic in vivo finding is that the injection of nerve growth factor (NGF) into the brains of young rats induces abnormal growth of axons from peripheral sympathetic neurons into the spinal cord and up to the site of NGF injection (19).

The studies reported here began as an attempt to determine whether elongating neurites respond to an NGF gradient with chemotactic growth. We hoped to examine how an NGF gradient influences the behavior of the growth cone, the portion of a nerve tip responsible for neurite extension and growth (17, 34). In initial studies we were unable to reliably generate and maintain gradients of NGF in a situation that allowed highly magnified observation of growth cones. Therefore, we adopted the technique of rapidly changing the NGF concentration of the culture medium and then observing the response of growth cones extended from sensory neurons. This approach has been used to study the mechanisms of bacterial and leukocyte chemotaxis (23, 35). Leukocytes, for example, round up, form many surface ruffles, and transiently stop moving when exposed to chemotactic peptides (35).

A startling finding of our experiments was that an abrupt increase from 0 or 0.05 ng/ml to 40 ng/ml NGF resulted in a rapid and drastic change in growth cone and neurite morphology, best described as retraction. Further study revealed that the retraction response is limited to NGF among several molecules tested, that it is triggered by local events within the neurite, and that it occurs at NGF concentrations that suggest



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the involvement of a high-affinity NGF receptor. The relationship of NGF-induced retraction to the postulated chemotaxis is unclear; however, neurite retraction resembles other rapid responses to increased levels of surface ligands and suggests that NGF binding to surface receptors can modulate cytoskeletal function in the neurite (9, 22, 35).

## MATERIALS AND METHODS

### Media and Solutions

Tissue culture medium was Ham's F12 (Grand Island Biological Co., Grand Island, N. Y.) buffered in two ways: (*a*) at pH 7.4 with 5 mM *N*-tris(hydroxymethyl)methyl-2-minoethanesulfonic acid (TES) and 5 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanol sulfonic acid (HEPES) and supplemented with 10% fetal calf serum (F12THS10), and (*b*) at pH 7.4 with sodium bicarbonate and supplemented with 10% fetal calf serum (F12BS10). Heart conditioned medium (HCM) was prepared from dissociated hearts of 7–8-d chicken embryos using the method described by Helfand et al. (15). Purified $\beta$-NGF was a generous gift from Dr. Eric Shooter. NGF was kept frozen in a stock solution of F12THS10 in concentrations varying from 10 ng/ml to 1,000 $\mu$g/ml.

### Culture Dishes

Culture dishes were made by drilling a 1-cm hole in a petri dish (Falcon 1006, Falcon Labware, Div. of Becton, Dickinson & Co., Oxnard, Calif.) and gluing over the hole a coverslip tht had been cleaned with nitric acid and flamed to deposit a thin layer of carbon. The dishes were then treated with a 0.1 mg/ml solution of polyornithine in a borate buffer, pH 8.4, for 24 h, washed, and sterilized by ultraviolet irradiation (17).

### Culture of Dorsal Root Ganglia

Dorsal root ganglia were dissected from 8-d chick embryos and cut into 2–4 explants per ganglion. These pieces were then placed in a culture dish with 1 ml of F12BS10 and 0.5 ml of HCM, plus NGF. The dishes were incubated at 37°C in a 5% $CO_2$–humidified atmosphere for 20 h. Before experimentation, the medium was changed to F12THS10 with the same NGF concentration and allowed to equilibrate for 1 h at 37°C. The organic buffers maintained pH 7.4 at atmospheric $CO_2$ levels while the dishes were on the microscope.

### Neurite Cutting

Neurites were cut with a micro blade consisting of glass shard glued to a microelectrode tip. The neurites were cut as close as possible to their origin from the ganglionic explant with a Leitz micromanipulator under direct visualization.

### Cell Observation

Explants and neurites were observed with phase and interference reflection optics with a Zeiss IM inverted microscope (17). The dishes were maintained at 38°C on the microscope stage with a Zeiss Air Stream Incubator. A field of neurites of intermediate length (~250 $\mu$m) projecting in an even radial distribution from the explant was chosen for observation. At least four neurites were present in each field, and the field was observed for at least 10 min before NGF levels were changed to be sure that there was no spontaneous retraction. The NGF levels were increased by carefully adding a volume of warm F12THS10 equal to that present (1 ml) and that contained twice the desired final NGF concentration. Convection currents produced by the airstream incubator helped to mix the liquid media. Micrographs were taken with Kodak Plus X film.

Cytochalasin B (Sigma Chemical Co., St. Louis, Mo.) was dissolved at 1 mg/ml in dimethyl sulfoxide (DMSO) and put into F12THS10 before being added to the culture dishes, and colchicine (Sigma Chemical Co.), dissolved at 1 mg/ml in Hanks' salts solution, was added to the dishes in a similar manner.

## RESULTS

### Characterization of the Retraction Response

Retraction is the word we have used to denote the rapid change in growth cone and neurite morphology that occurs after an increase in NGF concentration in the culture medium. Retraction becomes apparent within 1–5 min after NGF is added: the majority of neurites begin to shorten towards their cell body without any obvious foreshadowing change in ruffling activity of the growth cone margin. Small neurites often coalesce into bundles as they shorten, and retraction fibers of ~0.2 $\mu$m in diameter are drawn out from many retreating neurites (Fig. 1). We do not know how fast the NGF levels reequilibrate, but retraction begins simultaneously at the perimeter of explants placed several millimeters apart. This retraction of neurite tips need not result in total withdrawal of a neurite into the perikaryon, and we have observed growth cone motility and reextension of retracted neurites within 2 h of NGF-induced retraction.

A series of experiments was undertaken to characterize the NGF levels at which neurite retraction can be elicited. In the first group of studies, the NGF concentration of the culture medium for the initial 20–24 h of culture was 0.05 ng/ml. Neurite outgrowth was obtained from the explants at this low NGF level because HCM was included in the medium and because the glass substratum was treated with polyornithine (7, 14). HCM contains an active component that binds to the substratum and stimulates neurite outgrowth from chick parasympathetic, sensory, and sympathetic neurons (7, 15, 20). NGF has not been shown to be present in HCM from chick hearts; in fact, antiserum to NGF does not affect the stimulation of sympathetic neurite outgrowth by HCM (15). Table I shows that rapid neurite retraction occurs when the NGF concentration is elevated from 0.05 ng/ml to 0.5 ng/ml or higher. The data should be viewed as indicating a threshold concentration for eliciting retraction, rather than as reflecting a dose-response analysis. When the NGF level was raised to 0.25 ng/ml, some neurites retracted, but not the majority, as they did when higher NGF amounts were added. Cultures raised to 0.25 ng/ml were observed for 30 min to ensure that the absence of retraction was not the result of slow mixing of the added NGF.

### Characterization of the Initial NGF Concentration Associated with the Retraction Response

These experiments established the range of initial NGF concentrations from which elevation of the NGF levels results in neurite retraction (Table II). We found that the NGF concentration during the initial culture period must be <0.8 ng/ml for a retraction response to rapidly increased NGF levels (Figs. 1 and 2) to occur. Even a 500-fold increase in NGF concentration from 2 ng/ml to 1,000 ng/ml does not produce neurite retraction. These results indicate that neurite retraction occurs only if the explants are cultured with a low initial NGF concentration, and that it is not merely a response to all large increases in NGF levels.

### Specificity of the Retraction Response

We assessed whether increased concentrations of insulin, a peptide hormone similar to NGF (12), and of cytochrome *c*, a protein with a charge similar to that of NGF also cause neurite retraction. Ganglionic explants were cultured for 24 h in normal medium containing 0.5 ng/ml NGF and no insulin or cytochrome *c* and then subjected to an increase to 10 ng/ml or 1,000 ng/ml of cytochrome *c* or insulin. There was no change in ruffling or microspike extension at the growth cone margin and no retraction of the neurites. After these treatments, the explants were still able to respond to an NGF concentration change to 40 ng/ml with neurite retraction. Thus, retraction is

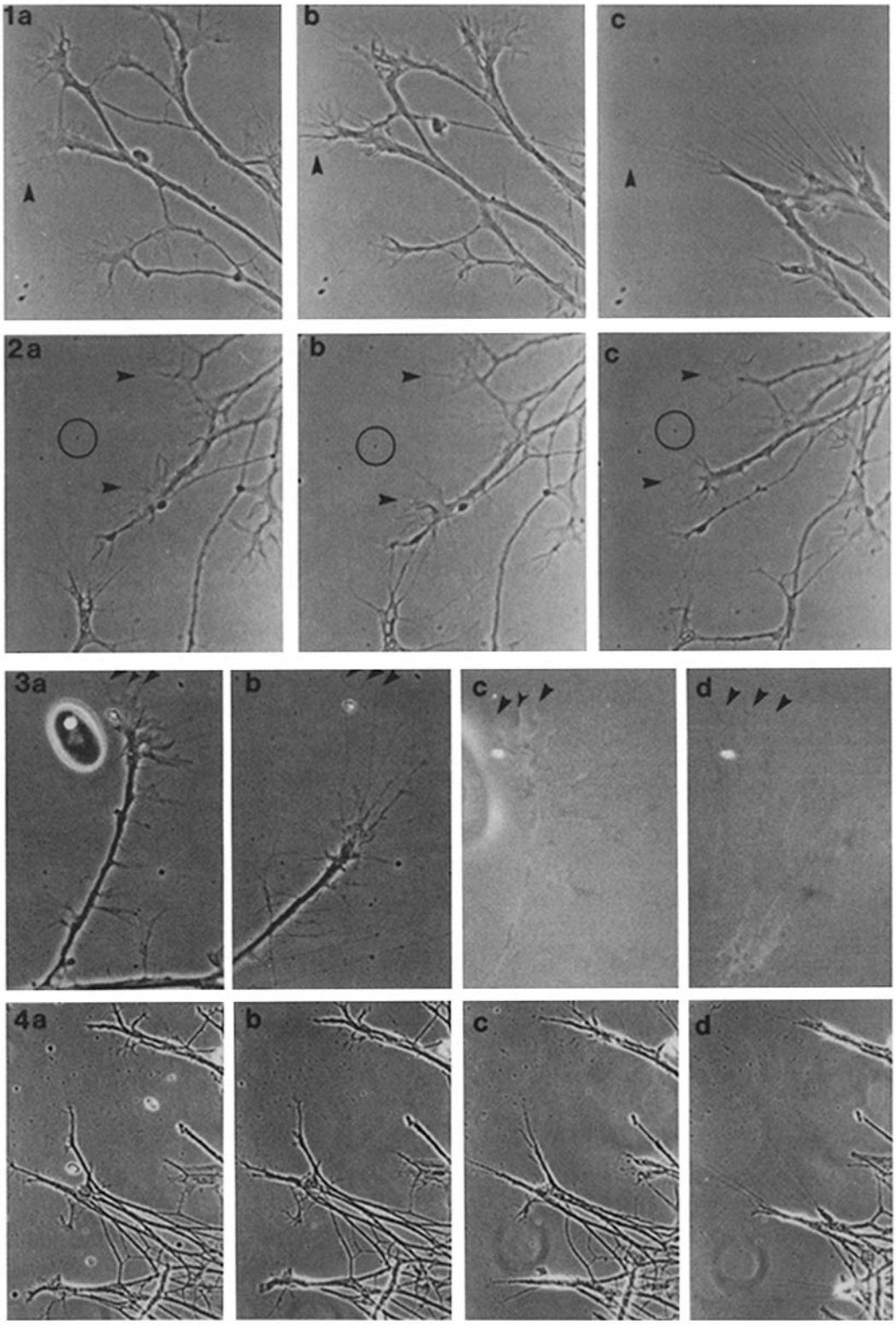


FIGURE 1 Retraction of neurites after NGF levels were increased from 0.25 ng/ml to 40 ng/ml. (*a*) Neurite morphology immediately before NGF was added; (*b*) 2 min after NGF levels were increased, retraction was already apparent; (*c*) 4.5 min after NGF levels were increased. Arrowhead at the same spot in each picture indicates the extent of retraction. × 630.

FIGURE 2 Absence of retraction when NGF level was increased from 2 ng/ml to 40 ng/ml. (*a*) Just before NGF was added; (*b*) 10 min after NGF was added; (*c*) 38 min after NGF was added. Circle indicates a marker on the substratum that shows that growth cones (arrowheads) have advanced since NGF levels were increased. × 630.

FIGURE 3. Phase-contrast and interference reflection micrographs of a growth cone before (*a* and *c*) and 30 min after (*b* and *d*) retraction began, after an increase in NGF from 0.05 ng/ml to 40 ng/ml. Arrowheads indicate points of correspondence of phase-contrast and interference reflection images in *a* and *c* and *b* and *d*, respectively. Adhesive contacts are the dark areas of the reflection images, as indicated by the arrowheads and as seen at other points. × 630.

FIGURE 4. Reversible inhibition of NGF-induced neurite retraction by cytochalasin B. (*a*) Neurites cultured for 24 h in 0.05 ng/ml NGF, photographed just before CB was added; (*b*) 4.5 min after the addition of 2.5 μg/ml CB, 20 ng/ml NGF was added; motility has ceased; (*c*) 13 min after CB was added, neurites are still present in spite of elevated NGF; (*d*) 12 min after CB was washed out (20 ng/ml NGF remains), neurites have retracted. × 250.

not simply a response to increased protein concentration. Specificity is also indicated by the fact that retraction occurs in response to subnanogram changes in NGF concentration in the presence of culture medium containing 10% fetal calf serum.

### Retraction of Neurites Isolated from their Cell Body

Neurites can be separated from their perikarya with glass needles and still remain motile and continue neurite elongation for at least 5 h (3, 28, 33). We used this separation technique to examine whether retraction results from local events within the neurite or whether it is triggered from the cell body. Groups of neurites extended from a ganglionic explant in the presence of 0.05 ng/ml NGF were cut near their exit from the explant. Medium containing sufficient NGF to increase the levels to 20 ng/ml was then added, and the cut neurites, as well as other neurites around the explant, were observed to retract. Some cut neurites contacted uncut neurites during retraction, but isolated cut neurites, free of cell contacts, also retracted in response to elevated NGF levels. Cut neurites subjected to a similar addition of medium with no change in NGF levels did not retract during a 30 min observation. This demonstrates that NGF-induced retraction can result solely from events within a neurite.

### Observation of Neurite-Substratum Adhesions during Neurite Retraction

Retraction might result from the loss of adhesion to the culture substratum by the growth cone, although, when a growth cone is detached from the substratum with a microneedle, the neurite remains transiently extended in the medium and does not rapidly retract, as it does when NGF levels are raised. Using interference reflection optics to observe neurite retraction, we found close contacts beneath the tips of retraction fibers and beneath the collapsed, retracted neurites (Fig. 3). Some of the contacts were at the same sites as adhesive contacts of the growth cone before NGF-induced retraction, suggesting that retraction is not the result of a complete loss of adhesion to the substratum, although changes in neurite-substratum adhesion certainly do occur during retraction.

### Effects of Cytochalasin B and Colchicine on NGF-induced Neurite Retraction

The involvement of microfilaments and microtubules in neurite retraction was assessed by using the drugs cytochalasin B (CB) and colchicine (Table III). When explants grown with 0.05 ng/ml NGF were exposed to 2.5 μg/ml CB, microspike extension and all growth cone motility stopped immediately, but the neurites did not retract (confirming previous reports; 34). After 3 min, 20 ng/ml NGF was added, but neurite retraction did not occur in the presence of CB. At 13 min, the medium containing CB and NGF was replaced with medium containing 20 ng/ml NGF only, and normal retraction occurred (Fig. 4). Control neurites exposed to CB, but not to increased NGF levels, resumed growth cone activity after CB was washed out. In another control experiment, DMSO alone did not inhibit NGF-induced retraction. These data showing retraction to be reversibly inhibited by CB suggest that microfilament activity is necessary for the retraction response.

The effects of colchicine on neurite retraction were assessed by incubating explants with 1 μg/ml colchicine for 1 h after an initial 24-h period of culture with 0.05 ng/ml NGF. Many neurites and growth cones were present after 1 h with colchicine, and, when the NGF concentration was raised to 20 ng/ml, neurite retraction did occur in the presence of colchicine. Although we did not demonstrate the disruption of microtubules by this colchicine treatment, it is a level that has previously been shown to be effective on dorsal root neurons (3, 10, 34). These data suggest that intact microtubules are not necessary for retraction to occur.

TABLE I

*Minimum Concentration Change that Induces Neurite Retraction*

| Initial [NGF] | Final [NGF] | Retraction |
|---|---|---|
| *ng/ml* | *ng/ml* | |
| 0.05 | 0.05 | − |
| 0.05 | 0.25 | ± |
| 0.05 | 0.5 | + |
| 0.05 | 1.0 | + |
| 0.05 | 10 | + |
| 0.05 | 40 | + |
| 0.05 | 1,000 | + |

Explants cultured for 20–24 h at the initial NGF concentration were subjected to the increased NGF levels noted above and observed for neurite retraction.
+, Most of the neurites in the field of observation retracted.
±, A few but not the majority of neurites retracted.
The data are based on observation of at least two microscope fields from different dishes for each reported concentration change.

TABLE II

*Initial Concentration from Which Elevation of NGF Level Induces Retraction*

| Initial [NGF] | Final [NGF] | Retraction |
|---|---|---|
| *ng/ml* | *ng/ml* | |
| 0 | 40 | + |
| 0.05 | 40 | + |
| 0.5 | 40 | + |
| 0.8 | 40 | ± |
| 1.0 | 40 | − |
| 2.0 | 40 | − |
| 2.0 | 1,000 | − |

Ganglionic explants were treated as described for Table I.

## DISCUSSION

The elevation of NGF levels in the culture medium of explanted chick embryo dorsal root ganglia induces a rapid retraction of the neurites extended from the ganglia. Although we did not expect retraction as a response to NGF, we believe that a reasonable explanation can be presented for the involvement of NGF receptors and the neurite cytoskeleton in causing retraction. These conclusions also suggest how chemotactic growth of neurites toward NGF might occur.

### NGF Receptors

Two distinct NGF receptors on chick embryo sensory neurons with dissociation constants of $2 \times 10^{-11}$ M (0.52 ng/ml) and $1.7 \times 10^{-9}$ M (43 ng/ml) have been described (29). Retraction occurs when the NGF levels are raised to as little as 0.5 ng/ml from initial concentrations of 0 or 0.05 ng/ml NGF. This increase would elevate occupancy of the high-affinity receptor to ~50% (at 0.5 ng/ml NGF) from <5% initial occupancy, with no measurable binding to the low-affinity receptor

TABLE III

*Effects of Cytochalasin B and Colchicine on Neurite Retraction*

| Time, *min* | 0 | 3 | 13 |
|---|---|---|---|
| Treatment | 0.05 ng/ml NGF 2.5 μg/ml CB | 20 ng/ml NGF 2.5 μg/ml CB | 20 ng/ml NGF 0 CB |
| Effect | cessation of ruffling activity | no retraction, ruffling still absent | retraction within 5 min |
| Time, *min* | 0 | 75 | |
| Treatment | 0.05 ng/ml NGF 1.0 μg/ml colchicine | 20 ng/ml NGF 1.0 μg/ml colchicine | |
| Effect | no retraction, ruffling continues | retraction | |

Ganglionic explants were cultured for 20–24 h in 0.05 ng/ml NGF. The explants were then exposed to NGF changes and drug treatments at the time points indicated. The effects of these treatments are reported in the column below the time and type of treatment.

in this concentration range (29). Retraction does not occur, even when NGF levels are raised to 1,000 ng/ml, if the initial concentration exceeds 0.8 ng/ml, a level that corresponds to ~67% occupancy of the high-affinity receptor but <5% occupancy of the low-affinity receptor. Thus, retraction of neurites occurs only after concentration changes that substantially increase occupancy of the high-affinity receptor and does not occur if the high-affinity receptors are roughly half occupied at first, regardless of what large increases may occur in occupancy of the low-affinity receptor. Therefore, neurite retraction is not a response to all increases in NGF concentration but, rather, may result from rapid, large increase in occupancy of the high-affinity NGF receptor. Initiation of retraction may require a threshold number of receptor-linked events, in which case the threshold would not be reached when the majority of NGF receptors are occupied initially.

Several explanations can be offered for the different involvement of these two NGF receptors in the retraction response. Our experiments with cut neurites show that local neurite components are sufficient to induce and carry out retraction. High-affinity NGF receptors must, therefore, be present on neurite and/or growth cone membranes, but low-affinity receptors may be absent from the neurite and may exist only on the perikaryon. If so, NGF binding to the low-affinity receptors may not affect the neurite. Alternatively, low-affinity NGF receptors are present on neurites but elevation in their occupancy does not induce neurite retraction. Another possibility is that the neurites extended when initial NGF levels exceed 0.8 ng/ml represent a different neuronal population than when 0.5 ng/ml or less is present. However, examination of the dose-response curve to NGF for neurite extension by sensory neurons suggests a single responsive population, requiring only binding to the high-affinity receptor (13, 29).

## *What is Neurite Retraction?*

Retraction of neurites in response to NGF presents the paradox that neurites appear to shun the trophic hormone that promotes neurite growth. However, neurite retraction is similar to other rapid changes in cell morphology after the addition of ligands that bind to surface receptors. PC12 cells, carcinoma cells, and leukocytes all respond to specific ligands with extensive ruffling and filopodial extension of the whole cell surface (9, 22, 35). It is not surprising that similar responses within a neurite <1 μm wide might prompt retraction of the neurite. Our observation that retracted neurites can recover and reinitiate elongation further suggests that neurite retraction is a transient response, like other morphological responses to added ligands.

These morphological changes suggest that cytoskeletal activity in these cells is sensitive to ligand-receptor interactions (9, 22, 35), which in the case of neurite retraction must involve the cytoskeleton of the neurite since retraction can be triggered in neurites isolated from their cell body. The principal cytoskeletal components of the neurite are a network of microfilaments in the growth cone and in the subplasmalemmal cortex of neurites and a linear array of microtubules and neurofilaments extending the length of neurites (34). Our cytochalasin B experiments suggest that microfilament activity is necessary for retraction, and our colchicine data suggest that intact microtubules are not necessary for retraction.

How, then, might a large increase in NGF-receptor binding alter the neurite cytoskeleton to produce retraction? One possibility involves the common observation that binding of a ligand to surface receptors induces large-scale redistribution and concentration (e.g., capping) of both the external receptor-ligand complexes and the internal cytoskeletal proteins (8, 30). It has been assumed that the force that rearranges the receptors is provided by the cytoskeletal elements through a transmembrane link that may be formed in response to the receptor-ligand interaction (1, 2). By this scheme, an abrupt increase in occupancy of high-affinity NGF receptors on the neurite and growth cone may induce a massive redistribution and concentration of NGF receptors and membrane-associated microfilaments that would withdraw the growth cone margin and induce retraction of the neurite. One of us has previously shown that concanavalin A–receptor complexes on cultured retinal neurons undergo a cytochalasin B–sensitive withdrawal from the growth cone margins and sides of the neurites to collect in centralized aggregates on the neurite membrane (18). Concanavalin A has also been reported to induce retraction of neurites extended from embryonic dorsal root ganglia, although details of this effect were not reported (26). It is not known whether NGF receptors undergo ligand-induced surface rearrangements; however, two hormones similar to NGF, insulin and EGF, induce clustering of their receptors on other cells (21).

NGF binding could affect the neurite cytoskeleton in other ways than by direct interactions of receptors with cytoskeletal proteins. Small ions and molecules, such as $Ca^{++}$ and cyclic AMP, may modulate the functions and organization of microfilaments and microtubules (6, 27). A large increase in NGF binding may alter plasmalemmal $Ca^{++}$ channels or adenyl cyclase, triggering widespread contractile events involving microfilaments or depolymerization of microtubules and eventual neurite retraction (27).

Disruption of growth cone and neurite adhesion to the substratum following the cell surface rearrangements associated with NGF binding might induce neurite retraction. Long retraction fibers adhering to the substratum at their tips are drawn out from the retreating neurite to mark the former position of the growth cone. Very similar retraction fibers have been noted when fibroblasts round up before mitosis or when they are treated with low concentrations of proteases, and other investigators have concluded, as we might here, that withdrawal of the cell margin and formation of these retraction

fibers result from a change in the association between cytoskeletal elements and the plasma membrane (24, 25).

It is worth considering whether neurite retraction can be related to chemotactic responses to NGF. We have concluded that the abrupt, large increase in occupancy of high-affinity NGF receptors on the neurite induces widespread, immediate changes in the neurite and growth cone cytoskeleton to produce retraction. In an NGF gradient, however, the changes in receptor occupancy experienced by a growing neurite would be much smaller. On a small scale, receptor-mediated effects may not include major redistribution of the cytoskeleton but, rather, merely modulate the action of microfilaments, microtubules, etc., in filopodial extension from the growth cone margin, in the formation of adhesive contacts, and in the transport and positioning of the structural components of the neurite (17, 34).

A model of sensory adaptation, originally created for bacteria, has been offered to explain the spatial detection of chemotactic gradients by leukocytes (23, 35). This is applicable to neurite growth as well inasmuch as neurite retraction depends on the concentration of NGF. An important feature of this model is that the postulated effects of ligand-receptor interactions on the motility system are not widespread, as they may be during retraction but, rather, are restricted to motile components in the cellular region exposed to the highest ligand concentration (35). Thus, we propose that small increases in receptor occupancy on portions of a growth cone situated along an NGF gradient produce not only subtle, but, importantly, local effects on neurite growth. Like adhesive differences in a neurite's microenvironment, NGF gradients may influence the cellular apparatus for neurite elongation to direct growing axons to the proper target tissues (17).

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