Characterization of the Turning Response of Dorsal Root Neurites toward Nerve Growth Factor

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ABSTRACT This study reports that chick dorsal root ganglion neurites undergo a rapid (20 min) reorientation of their direction of growth in response to nerve growth factor (NGF) concentration gradients in vitro. Dorsal root ganglia from chick embryos were explanted onto a collagen-poly-L-lysine substrate. After 24–48 h in culture, NGF gradients were applied to individual growth cones via a micropipette containing 50 biological units NGF/ml. The growth cones turned and grew toward these NGF sources. This turning response was not caused by the trophic effects of NGF on neurite initiation, survival, or growth rate.

Dorsal root neurites also grew toward sources of mono- and dibutyryl cyclic adenosine monophosphate (dB cAMP), cyclic guanosine monophosphate (cGMP), and elevated calcium in the presence of the calcium ionophore A23187. These results are consistent with the hypothesis that intracellular levels of cAMP and/or cGMP and calcium may play a role in the turning response of dorsal root neurites toward NGF, but do not establish a causal relationship between the mechanisms of action of NGF, cyclic nucleotides and calcium.

Total growth cone adherence to the substrate was measured using a timed microjet of perfusion medium. NGF increased the adherence of growth cones to the substrate, but caffeine and dB cAMP which also elicit the positive turning response, decreased growth cone adherence. Calcium, which did not elicit the positive turning response, produced a greater growth cone adherence to the substrate than that observed with NGF. Although these results do not rule out a role of adhesion changes in axonal turning to NGF, they show that a general increase in adherence does not correlate well with the rapid turning response observed in this study.

When growth cones of chick dorsal root neurites are exposed to a local concentration gradient of nerve growth factor (NGF), they are observed to rapidly turn and grow toward the NGF source within 9–21 min (14). Even though this response appears to be chemotactic in nature, the possibility remains that the turning response may involve increased growth rate produced by a trophic influence of NGF (21). It is also possible that this oriented growth may have resulted from increased adhesion of the growth cone to the substrate because neurites have been observed to preferentially grow down tracts of highly adhesive substrate (17, 18).

The purpose of this investigation is to study the possible roles of changes in adhesion and growth rate in the rapid turning response of chick dorsal root neurites toward NGF. The possible roles of internal messengers such as cyclic adenosine monophosphate (cAMP) and calcium are also studied, as NGF may increase the intracellular levels of cAMP and calcium in NGF-sensitive cells (25), but these observations are not well established.

The evidence presented here indicates that the fast turning response of chick dorsal root neurites toward NGF is probably not produced by gross changes in growth rate or adhesion, and is consistent with the hypothesis that chemotaxis toward NGF involves increases in the intracellular levels of cAMP and calcium.

MATERIALS AND METHODS

Tissue Culture and Preparation Observation

Lumbosacral dorsal root ganglia or spinal cords from Leghorn chick embryos 7 and 12 d of age were explanted onto glass coverslips coated with a collagen-poly-L-lysine mixture (25/1 wt/wt). The explants were incubated in air at 34°C

THE JOURNAL OF CELL BIOLOGY · VOLUME 87 DECEMBER 1980 546-554

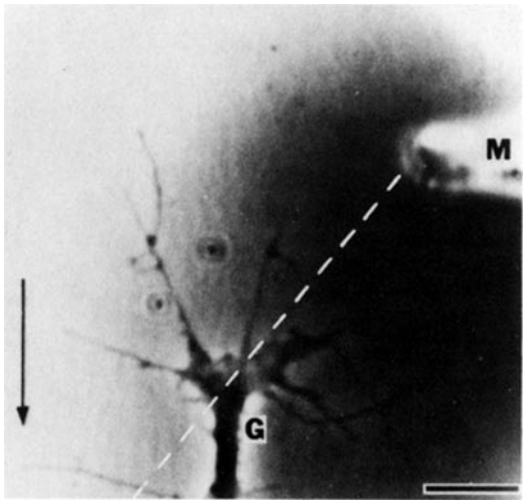

FIGURE 1 Spatial relationship of growth cone (*G*) and NGF-containing micropipette (*M*) during a typical experiment. The micropipette is positioned approximately 25 μm from the growth cone on a line 45° to the axon's longitudinal axis. In this picture the dye methylene blue replaced NGF in the micropipette to illustrate the shape of the concentration gradient along the growth cone. Material coming from the pipette has a higher concentration on the near side of the growth cone. Arrow indicates direction of flow of the background perfusion medium. Dashed line represents the line along which the gradients depicted in Fig. 2 were measured. Bar, 10 μm.

in 35-mm plastic petri dishes with nutrient medium (similar to L 15 except buffered with PIPES[1]) supplemented with 5–10 biological units (BU) NGF/ml (20, 22).

After 24 h in culture, the coverslips with attached explants were placed in a petri dish with a glass coverslip bottom. The chamber containing the explant was perfused with a medium identical to the nutrient medium, except containing only 1 BU NGF/ml. The temperature of the perfusion medium was maintained at 34°C with a small heating coil attached to the bottom of the dish. The preparation was viewed through an inverted microscope with phase contrast optics at × 750.

Establishing the Drug Gradient

A micropipette (2–4 μm tip diameter) filled with 1 or 50 BU NGF/ml or drugs dissolved in perfusion medium was used to produce a local gradient. The tip of this micropipette was initially placed ~25 μm from the tip of a growth cone, at an angle of ~45° to the axon's longitudinal axis (Fig. 1), and slightly above the surface of the substrate. A separate background perfusion system was placed so as to direct 25 ml/h of perfusion medium past the neurite in a direction opposite to the initial direction of neurite growth (Fig. 1). This perfusion system added medium to one edge of the dish while removing it from the opposite side with a vacuum line. Drugs flowing from the micropipette source (gravity flow pressure head of 4 cm) at 1–2 μliter/h, were carried along with the background flow, so that the concentration of the applied drugs was always higher on the side of the growth cone nearest the micropipette. The concentration gradient across the growth cone produced using this perfusion arrangement is illustrated in Fig. 1, using methylene blue in place of NGF in the micropipette.

As the neurites grew, the micropipette was moved to prevent contact between the growth cone and the pipette, and also to maintain approximately the same geometrical relationship to the axis of the neurite tip. The direction of flow of the background medium was also adjusted so that the flow remained parallel to the axis of the neurite tip.

[1] The major differences between the standard L15 medium and the medium used in this study are the following deletions and additions. Deletions: ascorbic acid, glutathione, and glycine. Additions (mg/liter): 100 fructose, 4,500 glucose, 50 mannose, 100 sorbitol, 1 *p*-aminobenzoic acid, 0.2 biotin, 1 carnitine, 1 cyanocobalamine, 8 niacinamide, 40 proline, and 0.5 pyridoxamine (L. Dribin, R. Gundersen, R. Kadingo, and J. Barrett, Department of Physiology and Biophysics, University of Miami School of Medicine, manuscript in preparation).

Criteria for Turning Response

Micropipettes were placed near isolated growth cones as described above, and observed for up to 30 min. The criterion utilized for distinguishing turning responses is lateral displacement, measured as the shortest distance between a line drawn through the original axis of the neurite and the position of the growth cone. A lateral displacement of the growth cone of 20 μm or more towards the micropipette during the 30-min test observation period was scored as a "positive response."

Precautions must be taken to insure the viability of the growth cones after transfer from the nutrient medium to the perfusion medium in the observation chamber. The pH (7.3) and osmolarity (306 mosmol) of the nutrient medium must be duplicated in the perfusion medium. The simplest way to accomplish this is to make sufficient medium (without NGF) for both culturing and perfusion, and to store the perfusion aliquot along with the cultures in the incubator. Other variables that reduce the viability of growth cones are sudden changes in temperature and observation with intense light. If the proper growth conditions are not provided, the viability of the growth cone and its ability to respond to NGF gradients are decreased.

Measuring Growth Cone Adherence to Substrate

The adherence of growth cones to the collagen-poly-L-lysine substrate was measured by detaching growth cones from the substrate with small graded microjets of perfusion medium. To accomplish this, a micropipette with a 3-μm bore at its tip was filled with perfusion medium and attached to a regulated pressure line. Air pressure delivered to the micropipette was increased in increments of 2.5 lb/in^2. Perfusion medium was delivered by microjet to the side of a single growth cone from a distance of 20 μm, allowing 1 S for each increment of pressure. The pressure increment at which the growth cone detached from the substrate was taken to be an indirect measure of total growth cone adherence to the substrate.

Determining the Shape of Drug Gradients Produced by the Microperfusion System

The concentration gradients produced by the microperfusion system were very difficult to measure as they were localized to a very small area and were established using low source concentrations. Thus we determined the approximate shape of gradients produced during microperfusion by *in situ* local resistance measurements, which were very sensitive to local ion concentrations. Electrolytes with molecular weights similar to the drugs used for growth cone tests were used to estimate the microperfusion gradients.

These local resistance measurements were made by using microelectrodes for which the total electrode resistance was very sensitive to the conductivity of the bath solution surrounding the electrode tip. Such electrodes were made by filling fine tipped (0.1 μm) micropipettes with a low concentration of electrolyte solution (25 mM KCl). The resistance of the microelectrodes was measured by passing current pulses of constant amplitude (10^{-8} to 10^{-9} A) through the electrode and recording the voltage response using a high input resistance (10^{13} ohm) preamplifier. The pipette resistance was calculated as the amplitude of the voltage response to the current pulse, divided by the magnitude of the current pulse. Because these electrodes tapered rapidly to a fine tip size, a large portion of the total electrode resistance was actually located outside the electrode tip. More than 99% of the external electrode resistance was located within 1 μm from the tip, because the electrode tips were very small and the geometry of current spread from the electrode tip was approximately spherical. Thus, these electrodes provided a means for measuring electrolyte concentration in a very localized area. The electrode resistance decreased monotonically with increasing bath electrolyte concentrations, and the electrodes could be calibrated empirically to give the local bath electrolyte concentration corresponding to any given electrode resistance measurement. Separate calibration curves had to be obtained for each electrolyte system and microelectrode used, because the relationship of voltage resistance and current was different for each electrolyte and electrode.

The sensitivity of the measuring technique was increased using rapidly tapering electrodes to minimize their internal resistance and a wheatstone bridge circuit to subtract most of the remaining internal resistance. The sensitivity was also increased by using background perfusion solutions of relatively low conductivity (e.g., 1 mM KCl) and microperfusion solutions of high conductivity (e.g., 100 mM KCl). The bridge circuit was balanced so that no voltage response was obtained in response to the applied current pulse in a solution equal in concentration to that within the microperfusion pipette. The measuring microelectrode was then calibrated by measuring its response to the applied current pulse of constant amplitude in various dilutions of the source electrolyte solution. The voltage response to the applied current pulse decreased in an approximately

hyperbolic manner with increasing electrolyte concentrations. The same microelectrode was then used to measure the gradient of electrolyte concentration produced by the microperfusion pipette.

The concentration gradients produced by micropipette sources containing 100 mM KCl (background concentration 1 mM) or the buffer combination of 100 mM Bis-Tris ([bis(2-hydroxymethyl)imino-tris(hydroxymethyl)methane]) and 100 mM EPPS (4-[2-hydroxymethyl]-1-piperazine propane sulfonic acid; HEPPS), background concentrations 1 mM, were measured.

Because the rate of diffusion of a molecule decreases with increasing molecular weight, the results obtained with the KCl and buffer combination might not give a good indication of the gradient formed by NGF. Thus we also measured the gradient produced by a protein electrolyte source, specifically a mixture of a basic protein, cytochrome *c* (1 mM) and an acidic protein, ovalbumin (1 mM). The proteins were dissolved in a 10-mM glycerol solution and the resulting mixture ultrafiltered (nominal 10^4 mol wt cut-off) to remove any small ionic comtaminants from the final solution (dilution factor of 1.6×10^5). The final pH of the solution was 6.8 and the proteins remained in solution, apparently acting as counter ions for one another. Using this protein solution, a calibration curve of voltage response to the applied current pulse as a function of protein concentration was constructed and the concentration gradient produced by the micropipette source was measured using the same techniques as for the smaller electrolyte solutions. The background perfusion solution contained 10 mM glycerol.

The results of the gradient measurements are shown in Fig. 2 as the distribution of KCl, buffers (Bis-Tris and EPPS), and ovalbumin-cytochrome *c* along a line extending from the micropipette tip through the center of the growth cone as shown in Fig. 1. Micropipette sources of KCl, buffers, or ovalbumin-cytochrome *c* produce gradients across the region that the growth cone normally occupies. Because these gradients are similar, despite great differences in molecular weights of the compounds, these gradients are probably not produced by diffusion alone, but rather some combination of solution mixing and diffusion. The similarity in the profiles of these gradients also indicates that they should serve as an example of the probable profile of the gradients produced by the micropipette sources of drugs used in this study, and that the approximate drug concentration at the center of the growth cone is 10% of the pipette concentration.

Because the growth cones used in this study have an average width of 25 μm, the concentration of NGF (50 BU source, 1 BU background) at the side of the growth cone proximal to the micropipette is ~12 BU and the side distal to the micropipette is 3 BU.

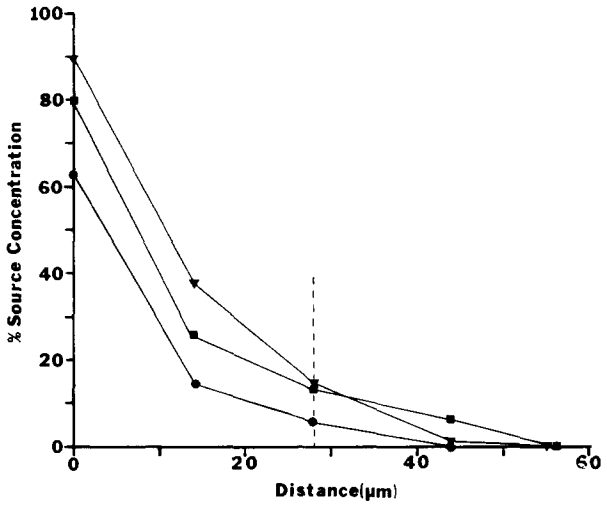

FIGURE 2 Concentration gradient formed by the microperfusion system utilized in the NGF turning experiments. Gradient profiles are expressed as a percent of the source concentration vs. distance (μm) from the tip of the micropipette source (Fig. 1). Micropipette sources contained 100 mM KCl (filled triangles), 100 mM Bis-Tris and EPPS (filled squares), or 1 mM ovalbumin-cytochrome *c* (filled circles). The profiles of the three electrolyte gradients are similar despite varying molecular weights, indicating that the gradients are probably formed by some combination of diffusion and solution mixing. The concentration of electrolyte in the region of the growth cone (vertical dashed line) is ~10% of the source concentration.

RESULTS

Characterization of the Turning Response toward NGF

Fig. 3 shows a rapid positive turning response produced by a local gradient of NGF. A dorsal root neurite bathed in a background level of 1 BU NGF/ml was exposed to a 50 BU NGF/ml micropipette source (Fig. 3*a*). The axon grew toward the NGF source, such that after 16 min (Fig. 3*b*) the growth cone was displaced 20 μm laterally away from its original position and toward the micropipette. All 40 neurites tested under these conditions exhibited similar positive turning responses. The growth cones of these neurites were displaced 20 μm laterally toward the NGF source within 9–21 min. The rate of turn, measured as the final angular deviation divided by the time required to produce that deviation, was 3.3° ± 0.2°/min towards the micropipette. The total angular deviation produced by this rate of turn averages 46° after 14 min.

Fig. 4 illustrates the results of a control experiment in which the micropipette contained only the background level of NGF, 1 BU/ml. After 18 min of observations, the growth cone has advanced 23 μm from its original position without any significant displacement towards or away from the micropipette. In similar experiments, no significant turning or lateral displacement was observed even if the mciropipette was moved in the same pattern as if a positive response had occurred. Thus the positive turning response shown in Fig. 3 is not merely an artifact produced by the presence of pipette or by fluid movement from the pipette. The average rate of turn for control neurites was near zero, 0.01° ± 0.14°/min. The maximum angular deviation for control axons after 14 min was only ±5°, compared to the average 46° observed for axons undergoing the positive turning response.

Fig. 5 is a plot of average lateral displacement vs. time for growth cones exposed to micropipettes containing 50 BU NGF/ml or 1 BU NGF/ml (control). The background level of NGF was 1 BU/ml. Control neurites showed small (up to 5 μm) random displacements toward or away from the micropipette, while the average lateral displacement of the control population remained close to zero (0.2 μm) at all times. In contrast, the lateral displacement of neurites exposed to the 50 BU NGF/ml pipette was always toward the micropipette and increased with time. As indicated by the standard deviation bars on Fig. 5, the responses of axons to 1 BU or 50 BU NGF/ml in the micropipette were significantly different. Thus it is clear that an NGF gradient can produce a dramatic effect on the direction of axonal growth.

To check the possibility that this result might somehow be caused by a subjective bias on the part of the experimenter, we performed a series of double blind experiments, in which the observer did not know whether the micropipette contained 1 BU (background) or 50 BU NGF/ml. The results of these double blind experiments confirm the previous results in that 100% of the neurites tested turned toward the 50 BU NGF/ml source, whereas none of the axons tested turned toward a 1 BU NGF/ml source. This result, together with the use of the objective criteria (20 μm lateral displacement for a positive response) provides evidence that the results reported here are probably not because of subjective bias on the part of the observer. Further evidence is seen in Fig. 5 which shows that the responses of control and experimental axons are readily separable from one another.

The observed turning of neurites towards an NGF source appears to be true directed turning, rather than just an NGF-induced increase in the rate of neurite outgrowth. The growth rate of 10 neurites under our standard experimental conditions

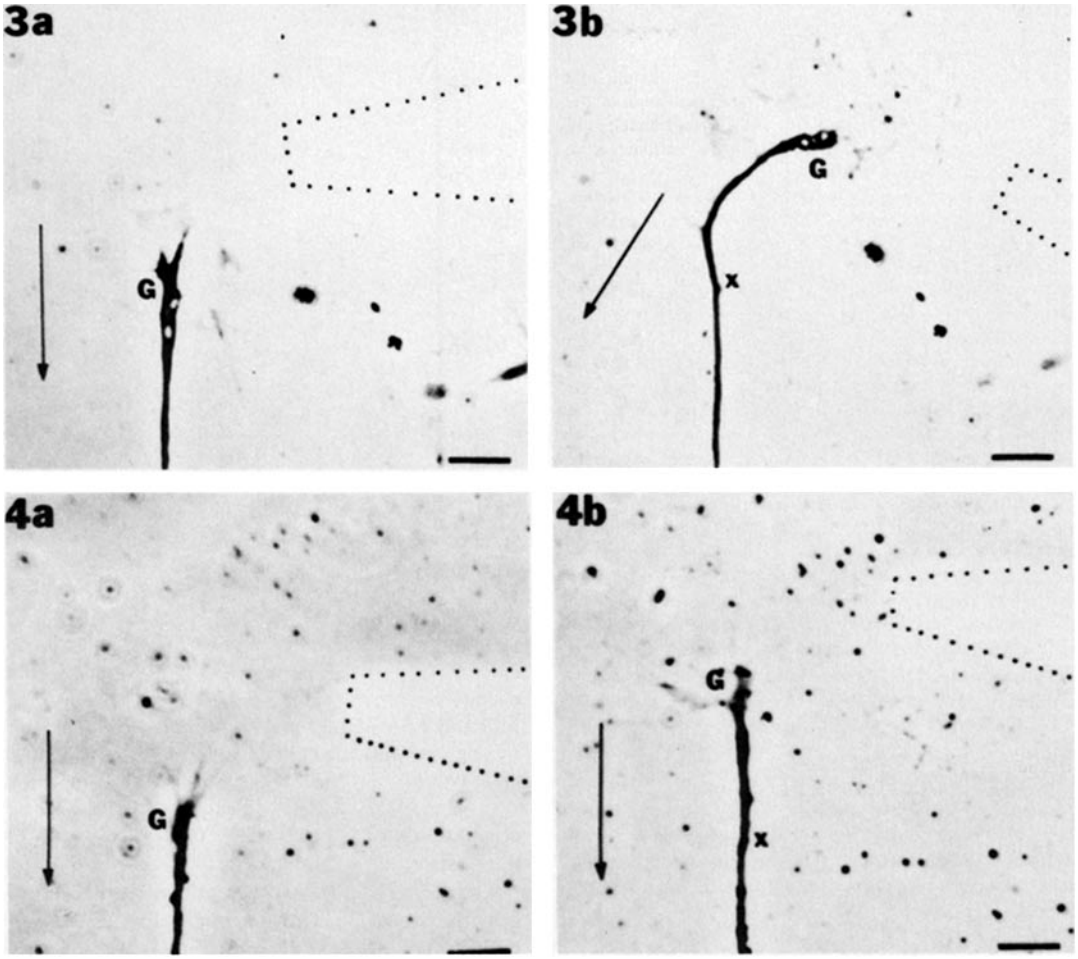

FIGURE 3 Chemotactic response of a dorsal root growth cone to NGF. (*a*) Growth cone (*G*) and micropipette (dotted area) at the beginning of localized perfusion with 50 BU NGF/ml. Arrow indicates direction of flow of background medium. (*b*) 16 min after *a*. The growth cone has turned and the axon is growing toward the micropipette NGF source. This turn and subsequent growth has displaced the growth cone 20 μm laterally from its original position (*X*). The growth rate was 83 μm/h, the angle of turn 80°. Bar, 10 μm. The position of the micropipette and direction of background flow are changed during experiments to maintain the same approximate geometrical relationship to the tip of the neurite.

FIGURE 4 Lack of turning in a dorsal root growth cone perfused from a micropipette containing only background levels of NGF (1 BU/ml). (*a*) Growth cone (*G*) and micropipette (dotted area) at the onset of perfusion. Arrow indicates direction of background flow. (*b*) 18 min after *a*. The growth cone has advanced 23 μm from its original position (*X*) without any significant displacement towards or away from the micropipette. Growth rate 75 μm/h. Bar, 10 μm.

(50 BU NGF/ml in micropipette), 85 ± 39 μm/h, was indistinguishable from the control rate of 84 ± 33 μm/h measured in 10 other neurites exposed to only background (1 BU/ml) levels of NGF in the pipette (Fig. 6). Also, if the turning response were simply a growth rate phenomenon, we would have expected to see at least a few growth cones that momentarily turned in the wrong direction or grew past the NGF source, but these growth patterns were never observed.

Two of the neurites (4%) observed in this study branched during the application of the NGF gradient. In each case, only the branch nearest the NGF source exhibited the turning response. The distal branch grew away from the NGF source. The percentage of neurites undergoing branching in response to an NGF gradient is not appreciably different from the 5% observed spontaneous branching. Thus the application of an NGF gradient does not appear to promote branching of neurites.

Not all kinds of neurites turn rapidly toward an NGF source. Spinal cord neurons, which do not require NGF in vivo or in vitro for growth of survival, did not exhibit a turning response toward or away from a micropipette containing 50 BU NGF/ml during the 30-min observation period. Thus spinal cord neurites do not respond to NGF at the concentrations and over the time period studied here.

To determine whether the effective NGF was free in the medium or bound to the collagen-poly-L-lysine substrate, growth cones were perfused with a 50 BU NGF/ml micropipette source for 5 min, and then the pipette was removed. Growth cones were observed for 30 min after pipette removal. None of these neurites continued to turn toward the original location of the micropipette NGF source, but rather grew in straight lines which did not cross through the original location of the NGF source. This result suggests that substrate-bound NGF is not responsible for the turning response, although the possibility remains that any substrate-bound NGF dissociated from the substrate rapidly after pipette removal.

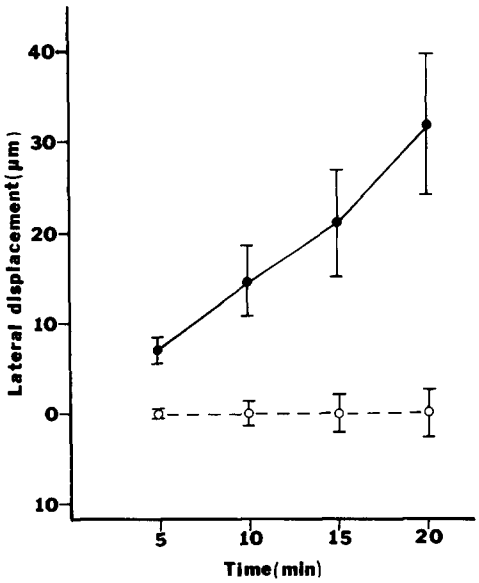

FIGURE 5 Plot of average lateral displacement vs. time for growth cones exposed to micropipettes containing either 50 BU NGF/ml (filled circles, solid line) or 1 BU NGF/ml (controls, open circles, dashed lines). The background (NGF) was 1 BU/ml. Lateral displacement was measured as the shortest distance between a line drawn through the original axis of the axon and the position of the growth cone at the indicated time after the onset of perfusion. Each point represents the average for 10 growth cones (±SD). The lateral displacement of control axons was either towards (+) or away (−) from the micropipette source, but averaged close to zero at all times. The lateral displacement of growth cones exposed to the NGF gradient was always towards the micropipette NGF source, and increased steadily with time.

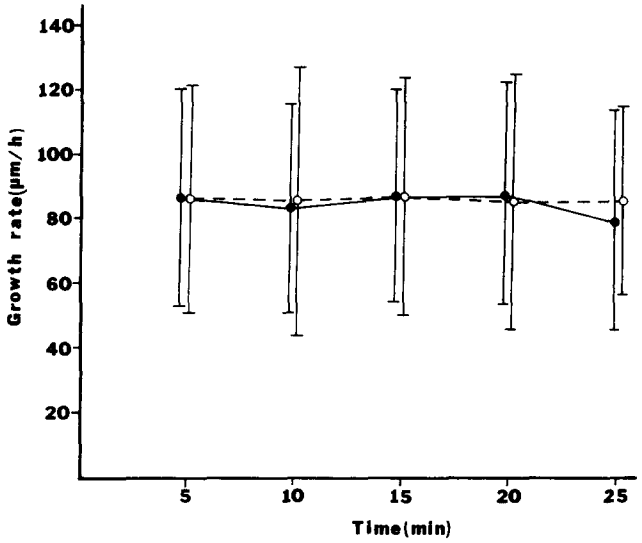

FIGURE 6 Plot of growth rate vs. time for growth cones exposed to 1 BU NGF/ml (open circles) or 50 BU NGF/ml (filled circles). The growth rates of growth cones not undergoing the turning response (1 BU NGF/ml) are not significantly different from those undergoing the turning response (50 BU NGF/ml).

Microspike Distribution during the Turning Response

During the first 6 min of applying an NGF gradient to a growth cone, the length and number of microspikes and a lamellapodia on the side of the growth cone proximal to the NGF source appear to increase. Table I presents the number and mean length of the microprocesses (microspikes and lamellapodia) on 26 growth cones, 13 growth cones before perfusion, and on the same 13 growth cones 6 min after perfusion with 1 BU or 50 BU NGF/ml. An analysis of variance was performed as described by Steel and Torrie (26), and the means were compared using Duncan's Multiple Range test at the 1 ($P < 0.01$) and 5% ($P < 0.05$) confidence levels. This statistical test indicated that there is a significant increase in the relative number of microspikes on the proximal side of the growth cone after 6 min perfusion with 50 BU NGF/ml ($P < 0.01$; Duncan test). A comparison of the number of microspikes on the distal sides and proximal sides of the growth cones before and after perfusion indicates that there is an increase in the absolute number of microspikes on the proximal side ($P < 0.01$; Duncan test). The number of microspikes on the distal sides of the growth cones decreases after perfusion ($P < 0.01$; Duncan test). In control experiments, both the analysis of variance and Duncan Multiple Range tests indicated that there was no significant difference between the proximal and distal sides of growth cones before or after perfusion with 1 BU NGF/ml (1 BU NGF/ml background). Thus, the increase in number of microspikes on the proximal sides of growth cones perfused with 50 BU NGF/ml is not caused by mechanical artifacts induced by the pipette. This analysis indicates that in response to an NGF gradient the growth cone may extend more microspikes on the side proximal to the NGF source while retracting some on the distal side. No significant increase in the length of microspikes was observed.

Possible Role of cAMP and Calcium in the Turning Response

Previous work on NGF-sensitive cells suggests that NGF causes an increase in the cellular levels of cAMP, and that cAMP can also elicit limited neuritic outgrowth in the absence of NGF (15, 23–25). These experiments suggest that cAMP may mediate NGF's effects on neuritic growth. Experiments conducted on NGF-sensitive PC 12 cells have shown that both NGF and cAMP cause a rise in intracellular-free calcium (24, 25). Because dorsal root neurites turn toward sources of NGF and cAMP, it is possible that they might also turn in response to an increase in intracellular-free calcium levels. However, there is a conflict as to whether or not NGF increases cAMP and calcium (15, 16, 23, 24).

To determine whether cAMP is involved in the turning response of dorsal root neurites toward NGF, growth cones were presented with sources of various nucleotides. Growth cones did not turn toward a micropipette containing 1 mM cAMP (Table II). However, when 1×10^{-7} M of the phosphodiesterase inhibitor Roche 20-1724 was included in the perfusion medium and micropipette, 30% of the axons tested turned toward a micropipette source containing 1 mM cAMP. All of the axons tested turned toward the more lipid soluble cAMP analogues mono- and dibutyryl cAMP (mB cAMP, 1 mM; dB cAMP, 1 mM). Turning responses were also observed when the micropipette source contained 1 mM cyclic guanosine monophosphate (cGMP). 1 mM ATP did not elicit a turning response during the 30-min test period.

Growth cones also turned toward micropipette sources of the phosphodiesterase inhibitors caffeine (1 mM), theophylline (0.01 mM), and Roche 20-1724 (1 μM) (Table II).

The results of these experiments with cAMP and its ana-

TABLE I

Number and Mean Length of Microspikes and Lamellapods on the Sides of 13 Growth Cones Proximal and Distal to the NGF Micropipette before the Onset of Perfusion and after 6-min Perfusion with 1 BU NGF/ml or 50 BU NGF/ml

	Before perfusion (1 BU NGF)				After perfusion (1 BU NGF)			
	Distal		Proximal		Distal		Proximal	
	No.	Length	No.	Length	No.	Length	No.	Length
1	2	16.3	0	0	4	9.1	6	10.3
2	2	10.6	4	18.9	2	6.1	4	3.7
3	4	4.7	3	3.3	6	6.8	4	2.5
4	5	18.0	5	9.1	3	9.3	2	19.6
5	6	9.3	7	10.3	5	10.6	4	11.1
6	1	8.4	2	15.1	5	8.9	6	14.4
7	0	0	3	18.2	5	19.1	4	26.1
8	6	2.5	7	7.4	8	15.0	8	8.9
9	5	19.4	2	10.6	2	9.6	3	15.1
10	4	6.5	6	3.7	5	18.0	3	5.3
11	6	10.2	7	13.9	4	10.3	4	19.4
12	6	8.5	8	17.3	0	0	1	8.4
13	4	6.4	4	12.9	5	18.8	4	16.6
Mean	3.0 ± 0.6 (SE)	9.3 ± 1.6	4.5 ± 0.7	10.8 ± 1.7	4.2 ± 0.6	10.9 ± 1.5	4.1 ± 0.5	12.4 ± 1.9
	Before perfusion (1 BU NGF)				After perfusion (50 BU NGF)			
1	8	17.9	7	18.5	3	9.3	7	19.6
2	4	19.4	4	18.0	3	13.1	4	14.8
3	7	15.1	6	23.4	6	14.5	7	16.3
4	1	3.3	1	8.4	1	16.8	3	5.3
5	2	9.3	2	3.3	1	10.3	3	8.4
6	1	18.9	2	25.7	0	0	2	6.1
7	7	0	0	10.6	0	0	0	0
8	8	14.6	6	7.9	5	26.1	8	24.8
9	5	4.7	4	4.7	3	10.3	5	11.8
10	1	0	0	6.5	1	3.7	1	3.7
11	1	4.7	1	13.8	1	14.0	3	11.5
12	1	9.1	1	3.7	0	0	2	7.0
13	5	28.9	4	16.8	6	11.9	4	18.2
Mean	3.9 ± 0.8 (SE)	11.2 ± 2.4	2.9 ± 0.7	12.4 ± 2.1	2.3 ± 0.6	10.0 ± 2.1	3.8 ± 0.7	11.3 ± 2.0

logues, cGMP and phosphodiesterase inhibitors suggest that cAMP and/or cGMP may be involved in the turning response to NGF.

To test the possibility that calcium is involved in the chemotactic response, we presented growth cones with a 20-mM calcium micropipette source. Growth cones did not turn toward the elevated calcium concentration calcium, nor did they turn toward the calcium ionophore A23187 (10^{-7} M). However, when A23187 was placed in the background perfusion solution, the growth cones did turn toward a 20-mM calcium source (Table III), suggesting that turning can be produced by a local increase in internal calcium caused by a calcium influx. To determine if calcium influx is necessary for the chemotactic response to NGF, the level of calcium in the micropipette and background perfusion solution was reduced to $<5 \times 10^{-9}$ M with 4 mM EGTA (total calcium 10^{-5} M). Low external calcium can make cells detach from a collagen substrate, but this difficulty was not observed with the collagen-poly-L-lysine substrate used here. Growth cones continued to turn toward 50 BU NGF/ml in this very low calcium concentration, indicating that extracellular calcium is not necessary for the turning response toward NGF.

To investigate whether the response to NGF might involve release of intracellular calcium stores, we added 40 μM dantrolene to the 10^{-9} M-calcium perfusion solution and micropipette source. This concentration of dantrolene has been shown to reduce the release of calcium from intracellular stores in skeletal muscle (7, 10, 28). This combination of low calcium and dantrolene blocked the turning response toward micropipette sources of 50 BU NGF/ml and/or 1 mM dB cAMP in all 30 growth cones tested (Table III). This inhibition of the turning response was not caused by a decrease in growth rate, as the growth rates of all 30 neurites remained within the normal range observed during chemotaxis to NGF. In addition, dantrolene is not nonspecifically inhibiting the turning response because turning toward a 20-mM calcium source was observed in the presence of dantrolene and A23187.

The results of these experiments are consistent with the hypothesis that calcium influx is not necessary for turning of dorsal root neurites toward NGF to occur, but release of calcium from intracellular stores may be important.

Total Growth Cone Adherence to the Substrate

The drugs that elicited a positive turning response (Tables II and III) were tested to determine if they produced increases or

TABLE II

Response of Neurites to Cyclic Nucleotides and Phosphodiesterase Inhibitors

Reagent in micropipette	Positive response	No response
1 mM cAMP	0	10
1 mM cAMP in the presence of 1×10^{-7} M Roche 20-1724*	6	14
1 mM mB cAMP	10	0
1 mM dB cAMP	20	0
1 mM cGMP	10	0
1 mM ATP	0	10
0.01 mM Theophylline	10	0
1 mM Caffeine	10	0
1 μM Roche 20-1724	10	0

* Roche 20-1724 was at identical concentrations in both the micropipette and background perfusion medium.

TABLE III

Response of Neurites to Reagents That Modulate Intracellular Calcium

Reagent in micropipette	Positive response	No response
20 mM Calcium chloride	0	10
10^{-7} M A23187	0	10
20 mM Calcium chloride in the presence of 1×10^{-7} M A23187*	10	0
50 BU NGF/ml in the presence of 10^{-9} M calcium*	10	0
50 BU NGF/ml in the presence of 40 μM dantrolene and 10^{-9} M calcium*	0	10
1 mM dB cAMP in the presence of 40 μM dantrolene and 10^{-9} M calcium*	0	10
50 BU NGF/ml and 1 mM dB cAMP in the presence of 40 μM dantrolene and 10^{-9} M calcium*	0	10
20 mM Calcium chloride in the presence of 40 μM dantrolene and 10^{-7} M A23187*	10	0

* "In the presence of" indicates that the specified compound(s) were at identical concentrations in both the micropipette and background perfusion medium.

decreases in the total adherence of the growth cone to the collagen-poly-L-lysine substrate. The experiments were performed by adding the drugs directly to the background medium, and then after a period of 30 min, a microjet of medium was used to measure adherence as described in Materials and Methods. Adherence measurements were performed over a range of concentrations for each drug tested. The concentrations used were adjusted to 100, 10, and 1% of the drug concentration in the micropipette source during the chemotaxis experiments. The measurements of electrolyte gradients described in Materials and Methods indicate that the drug concentration in the region of the growth cone during the turning response experiments is ~10% of the micropipette source concentration. The control medium contained 2 mM calcium and 1 BU NGF/ml. The concentrations of calcium and NGF were held constant when measuring growth cone adherence in response to the drugs tested, except when the effects of different levels of calcium and NGF were tested. Unpaired *t* tests were conducted to determine if the changes in adherence observed with the different durgs tested were significantly different from control measurements.

An increase in NGF concentration from 1 to 5 BU/ml or from 5 to 10 BU/ml increases growth cone adherence to the

TABLE IV

Effects of Drugs on Growth Cone Adhesion *

Drug	Maximal background concentration‡	Adherance measured at % maximal drug concentration 100%	10%	1%
Control	—	25.6	—	—
Calcium	20×10^{-3} M	30.3§	28.5‖	22.6‖
Caffeine	1×10^{-3} M	14.3§	20.9§	26.2
Roche 20-1724	1×10^{-6} M	27.3‖	26.0	25.6
cGMP	1×10^{-3} M	27.8‖	26.9	25.5
dB cAMP	1×10^{-3} M	20.9‖	23.3‖	25.6
A23187	1×10^{-9} M	25.8	—	—
Dantrolene	1×10^{-2} M	24.7	—	—
Cytochrome *c*	500 ng/ml	25.5	—	—
NGF¶	10 BU/ml	28.8§	27.0‖	25.6

* All adhesion measurements are in lbs/in^2. Standard errors of all adhesion measurements ranged from 0.2 to 1.4 with a mean value of 0.9. $n = 60$ (20 growth cones in each of three cultures).

‡ Unless different concentrations of calcium and NGF are indicated, the background solution also contained 2×10^{-3} M calcium and 1 BU NGF/ml.

§ $P < 0.01$, unpaired *t* test.

‖ $P < 0.05$, unpaired *t* test.

¶ NGF was tested instead at 100, 50, and 10% dilutions.

substrate ($P < 0.05$) (Table IV). Cytochrome *c* added to the background medium in equal weight amounts (1 BU NGF = 10 ng) did not increase growth cone adherence. Because cytochrome *c* and NGF have similar molecular weights and isoelectric points, the increase in adherence observed with NGF is not simply because of the fact that NGF is a basic protein.

Both caffeine and dB cAMP decrease growth cone adherence ($P < 0.01$, $P < 0.05$) at 1×10^{-4} M, when present at the same concentration as that in the region of the growth cone during the turning response experiments. Roche 20-1724 (1×10^{-6} M) and cGMP (1×10^{-3} M) increased adherence ($P < 0.05$), but only at concentrations 10 times greater than those at which they elicit the positive turning response. Calcium, which did not elicit the positive turning response, increases the adherence of the growth cone at 4 mM ($P < 0.05$), the approximate concentration applied to the growth cone in the turning response experiments.

These results suggest that NGF may guide the direction of axonal growth by increasing adherence, but that a simple increase in adherence, such as that observed with calcium is not sufficient by itself to guide the direction of axonal growth.

DISCUSSION

The results of these experiments demonstrate that chick dorsal root neurites undergo a rapid re-orientation of growth and turn toward micropipettes containing 50 BU NGF/ml, even in the presence of sufficient background NGF to promote vigorous axonal growth. Ebendal (8) has demonstrated that the direction of neurite growth can be guided by contact with an aligned collagen matrix. Therefore, the turning response toward elevated concentrations of NGF might possibly be induced by contact with the pipette. Because all of the growth cones studied had equal opportunities to contact the micropipette, then at least some neurites should have turned toward pipettes containing background levels or NGF, if contact with the pipette produced the turning response. However, neurites were never observed to turn outward pipettes containing background levels of NGF, even if the pipette was moved as if a turning response was occurring. Therefore the turning response is not caused by the presence of the pipette itself or contact with the pipette.

The trophic effects of NGF on neurite initiation (12, 13) and survival do not play a role in the turning response of chick dorsal root neurites toward NGF, because the neurites used in this study were actively growing and none were observed to die or retract. Growth rate of neonatal rat sympathetic fibers is stimulated in a dose-dependent manner by NGF (6), but Letourneau (19) did not observe any difference in the length of chick dorsal root neurites after growing 22 h in 0.1 or 1,000 ng NGF/ml. This lack of an increased growth rate in response to increasing concentrations of NGF is supported by our observation that growth rates were not significantly different whether the micropipette contained 50 BU NGF/ml or 1 BU NGF/ml. Thus the observed turning response was not caused by trophic effects on neurite initiation, survival, or growth rate, but rather appears to be a chemotactic re-orientation of the direction of neurite growth in response to the NGF gradient.

Letourneau (19) has observed a chemotactic response of chick dorsal root neurites to NGF. He found that after 22–72 h, 60% of the neurite tips of dissociated dorsal root neurons were oriented up an NGF concentration gradient, compared to the 50% expected if growth were random (a net increase of 10%). In agreement with our results, this response was not caused by trophic effects of NGF on neurite growth and initiation. However, the quantitative difference between ours and Letourneau's results is marked: he saw oriented growth in only a small percentage of the neurites exposed to an NGF gradient, whereas under our standard conditions (50 BU NGF/ml in pipette, 1 BU NGF/ml background), all tested dorsal root neurites turned rapidly toward the NGF source. There are several possible reasons for this discrepancy. Our NGF gradients were steeper, and were always (initially) applied with the same orientation to the growth cone, whereas in Letourneau's study the growth cones were initially distributed in many different orientations with respect to the NGF gradient. In preliminary experiments we found that the geometry of NGF application is important; the geometry used here (Fig. 1) was empirically determined to be optimal for eliciting rapid axonal turning. Another possibly important difference between our and Letourneau's experiments is the nature of the substrate. His neurites moved through a three-dimensional, low-adhesivity agar matrix, whereas ours moved across a two-dimensional, higher adhesivity collagen-poly-L-lysine substrate. More extensive experiments will be required to determine the contribution of our rapid turning response to the long-term orientation of neurites demonstrated in Letourneau's (19) and other in vitro and in vivo experiments that may involve chemotrophic as well as chemotactic responses (4, 5, 9).

The observed 100% turning response toward a source of NGF is not consistent with the hypothesis that only a subpopulation of neurites are directionally responsive to NGF (19). Because the neurites used in this study were well separated from neighboring axons and growing in relatively straight lines, the possibility remains that not all neurites from chick dorsal root ganglia are directionally responsive to NGF. In addition no distinction can be made between peripheral and central neurites branches of the dorsal root neurites or developing and regenerating neurites.

Campenot (3) has found that the tips of growing sympathetic ganglion neurites in tissue culture withdraw from or die in regions lacking NGF, even when the somata are exposed to NGF. Thus neurite tips may survive only in areas that contain a sufficient level of NGF. Perhaps during embryonic development, selective survival operates in regions low in NGF and chemotaxis directs neurite growth in the vicinity of target tissues where the level of NGF is higher. The effect of NGF on neurite survival may be mediated by high affinity NGF receptors that have a dissociation constant of approximately 10^{-11} M (about 1 BU NGF/ml), whereas the chemotactic response may involve lower affinity receptors with a dissociation constant of $\sim 10^{-4}$ M (~250 BU NGF/ml) (27). These two mechanisms involving high and low affinity NGF receptors would greatly extend the range of NGF concentration which would be effective in shaping the pattern of neurite growth.

How might the turning response toward elevated concentrations of NGF be produced? The number of microspikes increases significantly on the side of the growth cone proximal to the NGF source. This increase in the number of microspikes on the proximal side of the growth cone might indicate an increase in membrane addition. Increased membrane addition to the side of the growth cone proximal to the NGF source would shift the direction of growth toward the NGF source.

Letourneau (18) observed that large relative increases in the adhesion of growth cones to the substratum, such as that produced by poly-L-ornithine, can guide the direction of neurite growth. Increasing the concentration of NGF from 1 to 10 BU/ml does slightly increase growth cone adherence to the substrate. Therefore the chemotactic response of chick dorsal root neurites toward NGF may be mediated by increased adherence. However, directed growth toward sources of caffeine and dB cAMP was also observed even though these drugs decrease growth cone adherence. A general increase in adherence such as that observed with calcium which is greater than that produced by NGF, is not sufficient to guide the direction of neurite growth.

The microjet technique used here gives a measure of the total adherence of the growth cone to the substrate, but this total adherence is dependent on both the total area of the growth cone membrane contacting the substrate and on the specific adhesion per unit area of contact. Thus the drugs studied might affect the measured total adherence by affecting the number of growth cone attachment sites (areas of contact between the substrate and the growth cone membrane) and/or the specific adhesion at each site. Our measurements could also be affected by changes in the shape of the growth cone that would influence the force applied to the growth cone by the microjet, although no obvious correlation was found between the width of the growth cone or degree of flattening of the growth cone and the measured adherence.

Letourneau (18) observed that growth cone microspike extension precedes the direction of axonal growth down a highly adhesive tract on the substratum. Because the turning response does not correlate well with increases in total growth cone adhesion, the observation that the growth cone extends more microspikes on its side closest to the NGF source may indicate that there is a differential increase in adhesion on the side of the growth cone nearest the NGF source. Therefore, differential increases in growth cone adhesion, rather than changes in total adhesion, may guide the direction of neurite growth.

Bray (2) has presented evidence that growth cones can exert tension and that the direction of axonal growth is determined by this tension. The microspikes (filipods) of the growth cone may be responsible for generating part or all of this tension since the filipod extensions of fibroblasts and Physarum (1, 11) are able to contract and exert tension. The observed increase in the number of microspikes on the side of the growth cone nearest the NGF source, if they do contract and exert tension,

could align the cytoplasm of the growth cone (11) and direct the flow and addition of membrane precursors to the side of the growth cone nearest the NGF source. This could effectively guide the direction of axonal growth up the NGF gradient.

Other mechanisms that do not rely directly on differential adhesion or tension could also produce the turning response. For example, if the main body of the growth cone advances by following one of the microspikes, then it would be more likely to turn toward the side having the greatest number of microspikes. Because our observations indicate that there is an increase in the relative number of microspikes on the side of the growth cone nearest the NGF source, this mechanism would be effective in turning the direction of axon growth toward the NGF source. Alternatively, the probability that the main growth cone body will follow a given microspike may depend on the local NGF concentrations around the microspike.

NGF has been reported to increase the intracellular cAMP concentration in dorsal root neurones (23). Our results indicate that mB cAMP, dB cAMP, and phosphodiesterase inhibitors can guide the direction of growth of dorsal root neurites. Thus it is possible that an increase in the intracellular concentration of cAMP may play a role in the chemotactic response to NGF. However, because cGMP also elicited the rapid turning response, it is also possible that cyclic nucleotides and phosphodiesterase inhibitors nonspecifically activate the chemotactic mechanism. Alternatively, cGMP might activate the chemotactic response simply by saturating the intracellular phosphodiesterases that normally hydrolyze cAMP, thus effectively elevating endogenous levels of cAMP. The converse argument could be used to suggest that cGMP, not cAMP, is the primary nucleotide involved. The phosphodiesterase inhibitors used could elevate endogenous levels of both cyclic nucleotides.

Schubert et al. (25) have shown that both NGF and cAMP increase calcium influx from PC 12 cells, thus suggesting a rise in intracellular-free calcium. The combination of the calcium ionophore A23187 and a calcium source did elicit the turning response. However, chemotaxis to NGF still occurred in the virtual absence of external calcium. These observations indicate that an elevation of internal free calcium may play a role in chemotaxis to NGF, but this elevation of internal calcium is not dependent upon calcium influx. The combination of low extracellular calcium and dantrolene, which reduces the release of intracellular calcium in skeletal muscle (27), blocked the chemotactic response of dorsal root axons toward NGF and/or dB cAMP. Therefore the response to NGF and cyclic nucleotides may produce a release of calcium from intracellular stores.

The results of these experiments concerning the possible roles of cyclic nucleotides and calcium in the turning response of dorsal root neurites toward NGF are consistent with the hypothesis that the turning response toward NGF involves cyclic nucleotides and calcium, but does not establish a causal relationship between the two.

There is a conflict as to whether or not NGF increases the intracellular levels of cAMP and calcium (15, 16, 24, 25). Because the growth cone is such a small portion of the cell, it will be difficult to determine if NGF is actually increasing the levels of cAMP, cGMP, and calcium in the growth cone. Thus it may be difficult to determine whether or not these cyclic nucleotides and calcium play a direct role in the turning response of chick dorsal root neurites to NGF or whether they exert their effects via independent mechanisms.

Received for publication 8 April 1980, and in revised form 31 July 1980.

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