

Effects of Fasciculation on the Outgrowth of Neurites from Spinal Ganglia in Culture

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ABSTRACT This report describes the influence of neurite fasciculation on two aspects of nerve growth from chick spinal ganglia in vitro: the inhibition of outgrowth by high concentrations of nerve growth factor (NGF) and the preferential growth of neurites toward a capillary tube containing NGF. These studies involved a comparison of cultures of single cells, cell aggregates, and intact ganglia and the use of antibodies against the nerve cell adhesion molecule (CAM) to perturb fasciculation under a variety of conditions.

The inhibition of outgrowth, which was observed with ganglia and aggregates but not with single cells, was correlated with a thickening of neurite fascicles. In accord with this observation, anti-CAM, which diminishes fasciculation by inhibiting side-to-side interactions between individual neurites, also partially reversed the inhibition of neurite outgrowth at high NGF concentrations. On the basis of these and other studies, we consider the possibility that neurite bundling causes an increase in the elastic tension of a fascicle without a compensatory increase in its adhesion to substratum. It is proposed that this imbalance could inhibit neurites from growing out from a ganglion and even result in retraction of preexisting outgrowth.

In the analysis of NGF-directed growth, it was found that a capillary source of NGF produced a steep but transient NGF gradient that subsided before most neurites had emerged from the ganglion. Nevertheless, the presence of a single NGF capillary caused a dramatic and persistent asymmetry in the outgrowth of neurites from ganglia or cell aggregates. In contrast, processes of individual cells did not appear to orient themselves toward the capillary. The most revealing finding was that anti-CAM antibodies caused a decrease in the asymmetry of neurite outgrowth. These results suggest that side-to-side interactions among neurites can influence the guidance of nerve bundles by sustaining and amplifying an initial directional signal.

The specification of nerve tracts during embryogenesis is not understood in terms of fundamental mechanisms, particularly at the molecular level. During development, nerve processes often elongate along other nerve fibers or in association with them (9, 15, 27, 28). This fact, together with the results of our previous studies on the mechanism of neural cell adhesion (1, 30, 31, 34), prompted us to reconsider the possible influence of neurite bundling or fasciculation on nerve guidance.

In analyzing neurite fasciculation in cultures of spinal ganglia, we have focused on two previously described phenomena that reveal the effects of local environment on the extent and direction of neurite elongation. The first phenomenon is the halolike outgrowth of neurites from spinal ganglia and its inhibition by high concentrations of nerve growth factor (NGF) (21, 23). In 1968, Levi-Montalcini and Angeletti (24, 25) clearly demonstrated that the inhibitory effect did not reflect an

absence of neurite growth but rather the confining of neurites to the ganglion surface to form a dense capsule. Although these authors drew the important conclusion that NGF stimulates production of neurites at all concentrations, they did not consider the mechanism by which nerve processes are prevented from growing out from the body of a ganglion.

The second phenomenon is pertinent to previous suggestions that NGF may be a chemotactic agent for neurites of certain types of nerve cells (7, 8, 10, 11, 13, 20). Neurites from NGF-sensitive tissues, usually ganglia, have been reported to elongate preferentially in the direction of an NGF source, such as a capillary tube or micropipette (8, 11, 13). Moreover, the injection of NGF into embryos or the inactivation of endogenous NGF by injection of anti-NGF antibodies has been shown to alter or eliminate those cells and neuronal tracts that require this molecule (22, 25, 26).

In the present work, the role of nerve fasciculation in these two systems has been investigated by microscopic and cinematographic studies comparing single cells, cell aggregates, and intact ganglia, by direct inhibition of neurite-neurite interactions using specific antibodies against the neural cell adhesion molecule (CAM) (30, 31, 34) and by observation of cultures containing ganglia from different spinal regions. The response to NGF under these conditions has been recorded in terms of several quantifiable parameters, including the number, length, width, and rate of growth of neuronal processes that emerge from the ganglion surface. Substratum adhesiveness was also included as a variable. Neurite growth toward an NGF source was analyzed by relating the apparent spatiotemporal distribution of NGF to the amount, direction, and morphology of neurite outgrowth. The results suggest that nerve fasciculation is a major determinant in the overall morphology of neurite outgrowth from ganglia.

MATERIALS AND METHODS

Ganglia, Cells, and Cell Aggregates

Dorsal root ganglia were excised from 10-d-old chick embryos. Thoracic ganglia were used in all experiments except where otherwise noted. Suspensions of ganglion cells were obtained by trypsinization (0.5% trypsin; Difco Laboratories, Detroit, Mich.) of 200 ganglia for 20 min at 37°C in calcium-free medium. The ganglia were washed three times and dispersed into cells by trituration with a pipette. Over 90% of the cells were viable as judged by trypan blue exclusion.

To prepare cell aggregates, cells from 100 ganglia were suspended in 1.5 ml of Dulbecco's Modified Eagle's Essential Medium supplemented with one-tenth volume of fetal calf serum (DMEM; Microbiological Associates, Walkersville, Md.). The suspensions were placed in 35-mm plastic petri dishes and incubated under 13% CO₂ at 37°C for 20 h on a gyratory shaker (50 rpm; Fisher Scientific Co., Pittsburgh, Pa.). The aggregates ranged in diameter from 200 to 1,000 μ m. NGF and antibodies were added as indicated in particular experiments.

NGF and Anti-CAM Antibodies

Purified 2.5S NGF was purchased from Collaborative Research, Cambridge, Mass. Hans Thoenen (Max Institute for Psychiatry, Munich, Germany) and Lloyd Greene (New York University Medical School) also kindly provided samples of the purified growth factor. The biological activity (23) of these NGF varied by as much as a factor of two, but otherwise gave identical results in our studies. High molecular weight (7S) NGF gave equivalent results when used at about five times the concentration of 2.5S NGF.

The procedures for purification of CAM from chick embryo retina, production of antibodies to CAM in rabbits, and preparation of monovalent Fab' fragments have been described previously (1, 34). Except where noted, experiments with antibody were carried out with 0.5 mg Fab'/ml of culture medium and included control cultures with Fab' from unimmunized rabbits. At 0.5 mg/ml, anti-CAM Fab' caused an 80–90% decrease in the initial rate of retinal cell aggregation (30). The cultures with nonimmune Fab' were identical to those without antibody.

Cultures

Ganglia, cell aggregates, and single cells were cultured under four separate conditions: in 0.2% agar, on plastic tissue-culture dishes, on plastic dishes coated with collagen, and in agar in which a glass capillary containing NGF was embedded.

The agar cultures were set up by, (a) adding 0.3 ml of DMEM containing ganglia or cells to 0.3 ml DMEM containing NGF and/or Fab' fragments; (b) warming the suspension to 50°C and combining it with 0.2 ml liquified agar (SeaKem agarose, Marine Colloids, Rockland, Maine; 1% wt/vol in H₂O) and 0.2 ml double-strength DMEM, both at 50°C; and (c), pouring the mixture into a 35-mm plastic petri dish containing 0.5 ml of hardened 0.5% agar in DMEM. Ganglia and aggregates were manipulated into appropriate positions before the 0.2% agar solidified.

Cultures with 35-mm plastic tissue-culture dishes (BioQuest, BBL Microbiology Systems and Falcon Products, Cockeysville, Md.) contained 1.5 ml of DMEM to which NGF, Fab', and ganglia or cells had been added. Collagen-coated dishes were prepared by spreading a few drops of rat tail collagen in H₂O on the dish and allowing it to dry.

Cultures with NGF capillaries were made following the same procedures

outlined for agar cultures, except that instead of NGF being added directly to the medium, a 1-cm glass capillary tube filled with 2.5 μ l of DMEM containing 200 ng NGF/ml and 0.5% agar was placed into the dish before solidification of the 0.2% agar. The ganglia or cell aggregates were manipulated to within ~1 mm of the capillary ends. The capillaries were obtained by cutting 10 μ l disposable pipettes (Drummond Scientific Co., Broomall, Pa.).

Observation and Analysis of Neurite Outgrowth

Outgrowth of neurites from ganglia, cell aggregates, and cells was observed at 12-h intervals over a period of 3 d, optimal viewing being between 24 and 36 h. Cinematography with exposure at 15-s intervals was also carried out to observe the dynamic aspects of neurite fasciculation and the filopodial activity of growth cones.

Agar cultures containing 10–20 ganglia were scored with respect to four parameters: the length of neurite outgrowth (linear distance between the ganglion and the neurite or fascicle tips), the surface density of outgrowth (quantity of neurites leaving a ganglion per unit area), an estimate of total outgrowth (the product of outgrowth area and density), and the degree of fasciculation. The degree of fasciculation was represented by the percent of outgrowth contained in thick (15–4 μ m in diameter), medium (4–1.2 μ m), and thin (1.2–0.4 μ m) processes as described in reference 31. Cultures of cells in agar were scored with respect to the number of process-bearing cells and the length of the neurites.

With dense outgrowth, the neurites that emerged from a ganglion could not be enumerated individually. We, therefore, chose to estimate neurite density by light-scattering measurements on agar cultures. Unlike cultures on plastic or collagen, the neurites that grew away from ganglia in agar were not heavily intermixed with fibroblastlike nonneuronal cells (33). It was, therefore, possible to estimate the density of this outgrowth from the amount of light scattered by the neurites. The scattered white light under dark-field illumination was determined with a Zeiss Photometer coupled to a Zeiss Universal Microscope through 1-mm aperture. The average amount of scattering was estimated from four to eight measurements on circular areas of outgrowth having a diameter of 0.1 mm.

The relationship between light scattering and neurite density was established empirically by measurements on ganglia with a pattern of outgrowth favorable for direct counting of processes. These tests indicated that for NGF concentrations between 0.25 and 100 ng/ml, the scattering was proportional to the amount of outgrowth per unit area. Repeated measurements of total neurite outgrowth on the same ganglion indicated a reproducibility of $\pm 5\%$. When a mixture of ganglia from different spinal levels was scored, variation between ganglia cultured under the same conditions was very large ($\pm 20\%$) but narrowed to $\pm 8\%$ when 10–20 thoracic ganglia were used and when those ganglia with no response to NGF (one to two per culture) were not included in the average.

Neurites on plastic or collagen could not be quantitated by light scattering because of the presence of flat nonneuronal cells and the irregular pattern of outgrowth. A visual estimation (with arbitrary units from zero to five to represent the total amount of visible neurites) was, therefore, used to score the outgrowth from each ganglion. Cultures were scored blind by two investigators, with at least 20 ganglia being examined in each culture, and different cultures were extensively cross-compared; the mean scores for parallel cultures were well within one unit of each other.

Analysis of Capillary Cultures

Orientation of neurite outgrowth was estimated by using the light-scattering technique to determine the percent of total growth emerging from the half of a ganglion proximal to the capillary opening. The percent of "attracted" neurites, referred to here as the asymmetry, was determined from the difference in outgrowth between the two ganglion halves divided by the total outgrowth. Repeated measurements of the asymmetry on the same ganglion had a reproducibility of $\pm 7\%$. The standard deviation of the mean for measurements on 10–20 ganglia from the same spinal segment (thoracic, lumbar, or sacral) was also $\pm 7\%$. Direction of outgrowth from single cells was evaluated in terms of growth cone position after 30 h, relative to the positions of the cell body and the capillary. Asymmetry was estimated by averaging these positions for 50–100 cells.

The Distribution of NGF in Capillary Cultures

Given the quantities of NGF used and the dimensions and geometry of the capillary cultures, it was not feasible to determine concentrations of NGF directly. Therefore, the gradients produced by a capillary were estimated by using cytochrome *c* as a tracer after calibration with radiolabeled NGF; under the conditions of this study the two molecules appeared to diffuse at the same rate. Cytochrome *c* is a single polypeptide chain of 28,000 mol wt, whereas 2.5S NGF is composed of two identical subunits each of 14,500 mol wt, which can dissociate at low concentrations. Rather than making assumptions in correcting for shape and

subunit dissociation, the concentrations of cytochrome *c* and ^{125}I -labeled NGF were compared directly at different times and distances from a source of these two molecules. For the purpose of comparison, it was more convenient to follow the diffusion in cultures in which the agar in one half of the dish contained NGF and cytochrome *c*, and the other half did not. The cytochrome *c* concentration was measured by its absorbance at 490 nm with the Zeiss microscope and photometer described above in conjunction with bright-field illumination and a Zeiss monochromator. The NGF concentration was determined by cutting the agar into uniform pieces and measuring their radioactivity. The diffusion behavior of the two proteins did not differ significantly (Fig. 8, *inset*).

RESULTS

Effects of Fasciculation on the Response of Ganglion Cells to NGF

When embryonic spinal ganglia were cultured in media containing a range of NGF concentrations, two distinct phases of response were observed (21, 23–25): at relatively low concentrations (in our experiments 0–10 ng/ml), an increase in neurite outgrowth with increasing NGF (Fig. 1 *a–d*); and at higher concentrations (20–200 ng/ml), a decrease in the size of the neurite halo with increasing NGF (Fig. 1 *e–g*). With >200 ng/ml NGF, the neurites were completely confined to the ganglion, which was increased in size (Fig. 1 *g*).

Initial evidence that cell-cell interactions might affect the response to NGF was obtained in studies comparing whole ganglia, individual ganglion cells, and reaggregated cells. In agreement with previous reports (24, 25), the density of outgrowth from whole ganglia increased over a wide range of NGF concentrations (Fig. 2 *a*). At any given time during the culture, however, the maximum distance of neurite growth away from the ganglion was similar for all concentrations of NGF between 0.4 and 12 ng/ml. At higher concentrations, the radius of outgrowth decreased sharply (Figs. 1 and 2 *a*). The results shown are for cultures in agar, but similar observations were also made with cultures on plastic or collagen. A significant feature of these ganglion cultures was that the radius of outgrowth no longer increased after 24–48 h. Cinematographic analysis indicated that the cessation of outgrowth did not result from a decrease in growth cone activity; instead, the filopodia of the growth cones continued to move but without advancing over the substratum, as if on a treadmill.

In contrast to the complex response observed with ganglia, single neurons isolated by trypsinization of ganglia showed a relatively simple response curve (12, 24) (Figs. 1 *h* and 2 *b*). The number of cells that grew neurites increased with the addition of NGF, reaching a plateau value at ~1 ng/ml NGF and remaining constant for all higher concentrations of NGF. The average length of cellular processes was the same at all growth factor levels (Fig. 2 *b*). These results indicated that cells displayed maximal neurite outgrowth at lower NGF concentrations than ganglia, and that their response did not involve any

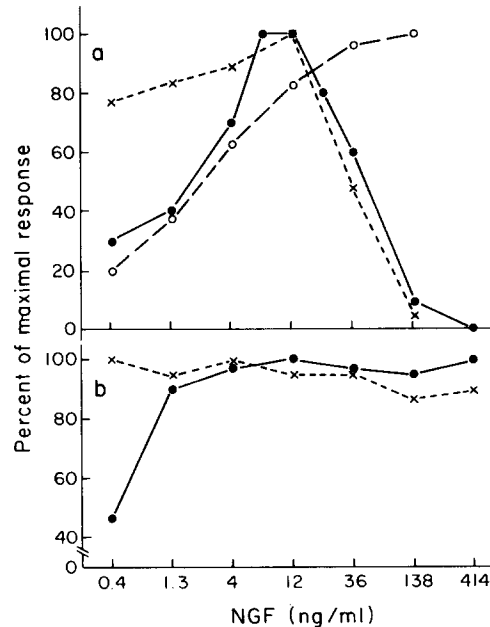


FIGURE 2 Neurite outgrowth after 36 h of culture from thoracic ganglia or reaggregated ganglion cells (*a*), and individual cells prepared from thoracic ganglia (*b*), in agar at various concentrations of NGF. In *a*, the response of ganglia is given in terms of total outgrowth (●), the surface density of neurites in that growth (○), and the greatest distance that neurites had elongated away from the ganglion (×). In *b*, the response of cells is given in terms of the number of cells per culture with neurites (●) and the average length of those neurites (×).

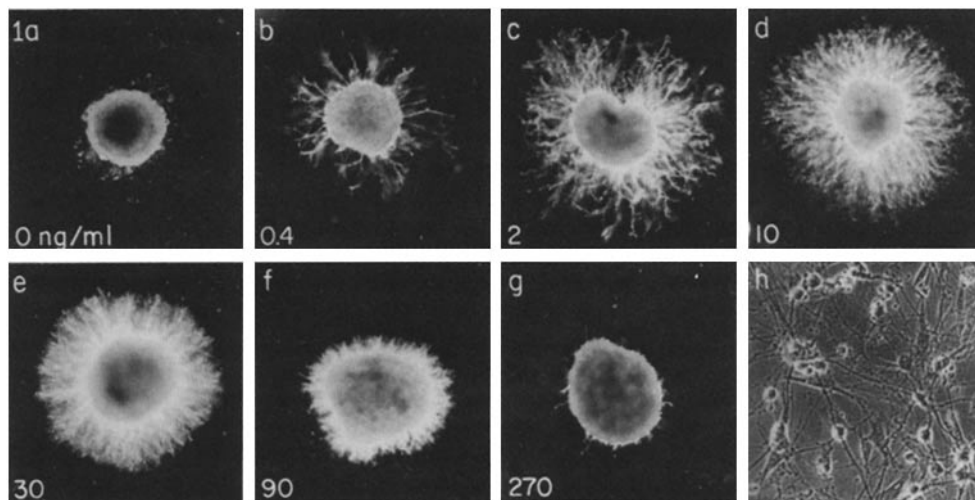


FIGURE 1 (*a–g*) Representative outgrowth in agar of neurites from thoracic spinal ganglia cultured for 36 h in the presence of the indicated concentrations of NGF; dark, field illumination, $\times 40$. (*h*) Outgrowth of neurites from ganglion cells cultured on plastic for 36 h in the presence of 2–300 ng/ml NGF; phase contrast, $\times 600$. Whereas outgrowth from whole ganglia was inhibited by NGF concentrations above 10 ng/ml, the response of individual cells remained constant, above 2 ng/ml.

inhibitory phenomena. It is possible, however, that the requirement for more NGF by ganglia simply reflected a local depletion of the factor.

To test the possibility that the difference in the response of ganglia and single cells was caused by some specific architectural feature of a ganglion as a whole, we also studied aggregates of ganglion cells that were comparable in size to the ganglia. In all the experiments presented here, aggregates and ganglia behaved identically, except that there was less variation in response among a group of cellular aggregates than among ganglia.

The suggestions that membrane-membrane interactions might influence or be influenced by the response to NGF was also supported by the observation that the extent of fasciculation in neurite outgrowth from ganglia increased steadily as higher concentrations NGF were used (Figs. 3 *a* and 4). Although the increase in fascicle diameter was closely correlated with the decrease in diameter of the neurite halo, it was consistently observed that fascicles began thickening at NGF concentrations two to four times lower than that associated with a shortened distance of outgrowth (Fig. 3).

In previous studies on the mechanism of neural adhesion, we prepared antibodies against a cell surface component called CAM, which is a nervous system-specific protein (1, 34). The monovalent Fab' fragments of anti-CAM antibodies inhibit both neural cell aggregation (30) and neurite fasciculation (31) but not cell-substratum adhesion. These antibodies were, therefore, used to probe directly the relationship between the fasciculation and the response of ganglia to NGF. Whereas treatment with anti-CAM decreased the diameter of fascicles at all concentrations of NGF (Fig. 3 *a*), a more gradual thickening of neurite bundles was still observed with increasing NGF concentrations. Of particular importance to this study was the observation that the decrease in fasciculation at each NGF level was accompanied by an increase in the average distance of neurite growth away from the ganglion (Figs. 3 *b* and 4 *c-f*). As might be expected from its known activities, anti-CAM had no detectable effect on the response of single ganglion cells to NGF.

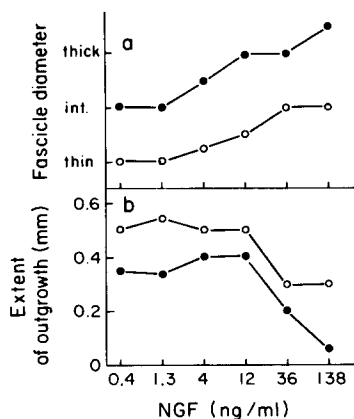


FIGURE 3 Effect of anti-CAM on the response of ganglia to NGF, expressed in terms of the average diameter of neurite fascicles (*a*) and the distance that neurites elongated away from ganglia (*b*). All cultures were of thoracic ganglia in agar containing either 0.5 mg/ml anti-CAM Fab' (O) or 0.5 mg/ml Fab' from unimmunized rabbits (●). Thick, intermediate (int.), and thin fascicles refer to processes with diameters of 15–4, 4–1.2, and 1.2–0.4 μ m, respectively (31). Over 20 ganglia were scored to obtain each value, and the mean variation of these determinations was small compared with the differences shown.

Previous work has suggested that the degree of fasciculation in neurite outgrowth from ganglia also depends on the adhesiveness of the substratum surrounding the ganglion (31). Moreover, it is known that the number and rate of growth of neurites is affected by cell-substratum interactions (18, 19). In the present work, we observed that an increase in adhesiveness to substratum (agar < plastic < collagen) increased the amount of neurite outgrowth at NGF concentrations \sim 12 ng/ml (Fig. 5) and extended the maximum radius of neurite elongation. In the NGF concentration range from 0.4 to 10 ng/ml, however, the relative amount of outgrowth was not significantly different for the three substrata tested.

To interpret the results obtained with NGF in terms of their relationship to fasciculation, it was important to determine whether the growth factor had any direct effect on cell-cell adhesion involving CAM or on cell-substratum interactions. It has been reported that NGF enhances the rate of membrane-membrane and membrane-substratum attachment of the PC 12 pheochromocytoma cell line (32). With cells obtained from ganglia by trypsinization, we found that NGF or anti-NGF had no detectable effect on the rate of cell aggregation (for methods see reference 1) or the rate of cell adhesion to culture dishes. High concentrations of NGF also did not alter the ability of individual growth cones to migrate along the substratum.

Growth of neurites from ganglia requires the continuous presence of NGF. It was, therefore, of interest to determine whether the phenomena observed at one NGF concentration could be reversed by subsequently changing the concentration to lower or higher values. Preincubation of ganglia or cell aggregates with 400 ng/ml NGF for 1 d, followed by culture for 1 d at 10 ng/ml NGF, resulted in a delayed but more vigorous outgrowth of neurites (Fig. 6 *b*) than in equivalent cultures where the level was maintained at 10 ng/ml (Fig. 6 *a*). The enhancement of outgrowth was even more pronounced, and less delayed, when anti-CAM was present in the culture medium (Fig. 6 *c*). Conversely, the shift of NGF concentration from 10 to 400 ng/ml caused a thickening and retraction of the neurites that had initially grown out. Despite this retraction, the growth cones continued to display their filopodial activity. It was also observed that less retraction occurred with substrata having greater adhesiveness, particularly if the culture period at 10 ng/ml NGF was lengthened to 2 d.

Effects of Fasciculation on the Growth of Neurites Toward an NGF Source

To determine the effect of fasciculation on neurite growth toward a capillary tube source of NGF, it was useful first to examine this phenomenon in detail. For this purpose, we used culture medium containing solidified agar (33), which facilitated quantitation both of the amount, rate, and spatial distribution of neurite outgrowth, and of the NGF concentration as a function of time and position.

In preliminary studies, a number of NGF distributions in agar, including transient pulses and preestablished stable gradients of various concentrations, were tested for their ability to orient neurite outgrowth. The small-bore, low-capacity capillary used gave by far the most dramatic and consistent results, with up to 90% of the neurites emerging from the ganglion half proximal to the capillary (Fig. 7 *a* and *b*). The same asymmetry was obtained with reconstituted aggregates of ganglion cells. Fibers emerging from single cells, however, displayed little if

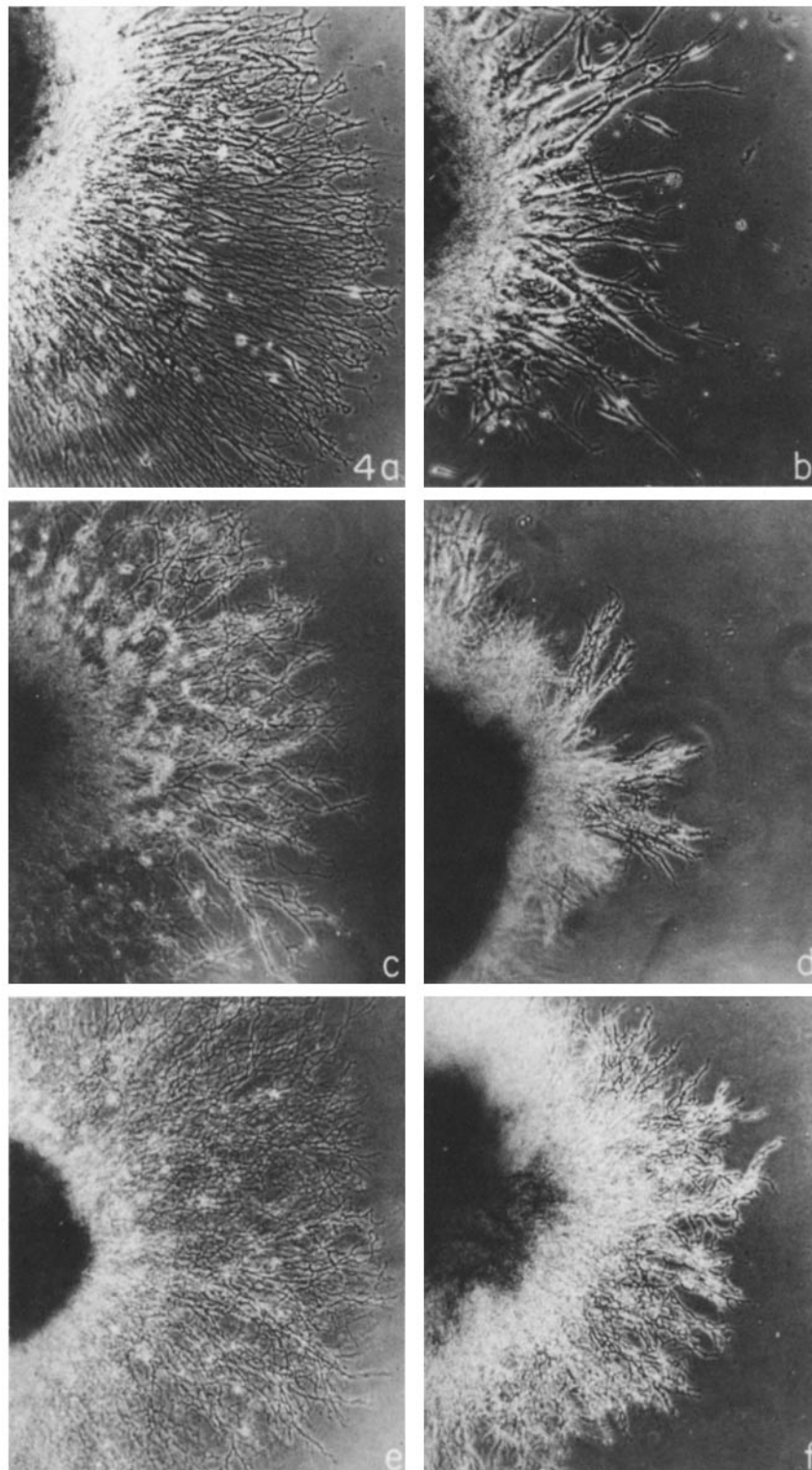


FIGURE 4 Effect of NGF concentration, anti-CAM, and substratum adhesiveness on neurite outgrowth from thoracic ganglia. *a* and *b* represent cultures grown for 36 h on plastic with 12 and 36 ng/ml NGF, respectively. *c* and *d* are the equivalent cultures grown in agar rather than on plastic. *e* and *f* represent the same conditions as *c* and *d*, except that 0.5 mg/ml anti-CAM was added to the medium. Note that increased NGF resulted in thicker fascicles on both substrata and that increased substratum adhesion and addition of anti-CAM increased the extent of outgrowth. Phase contrast, $\times 100$.

any directional preference. In agar, neurites from ganglia did not curve dramatically toward the capillary; instead, the processes, which were heavily fasciculated, extended in a nearly radial direction from the edge of each ganglion, and more of them emerged from the side facing the capillary. Cinemato-

graphic analysis of the outgrowth confirmed that most neurites elongated in a radial fashion, deviations being caused, for the most part, by interactions with other fibers.

Because an NGF capillary so effectively caused asymmetrical neurite outgrowth, an analysis was carried out of the

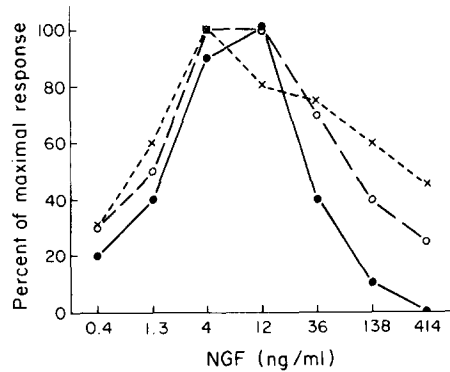


FIGURE 5 Total outgrowth of neurites from thoracic ganglia cultured in agar (●), on plastic (○), or on collagen (×) in the presence of various concentrations of NGF.

spatiotemporal distribution of NGF in the capillary cultures (Fig. 8). A ganglion having a diameter of 0.6 mm and placed 0.6 mm from the capillary experienced a sharp increase in the cytochrome *c* tracer concentration at its proximal edge (in terms of NGF, an increase from 0 ng/ml at the start of the culture to ~8 ng/ml after 1 h.). At the most distal part of the ganglion (1.2 mm from the capillary), the concentration increased more slowly to ~4 ng/ml after 3 h. Therefore, during this period, the NGF concentration gradient across the ganglion was relatively steep. Beyond 10 h of culture, however, diffusion already had decreased the ratio of apparent NGF concentration at these two points to <1.5. Also shown in Fig. 8 are the concentrations measured at distances 0.3 and 1.5 mm from the capillary. These distances represent the approximate positions reached by the proximal and distal edges of the growing neurite halo after 24 h of culture. Neurites are involved

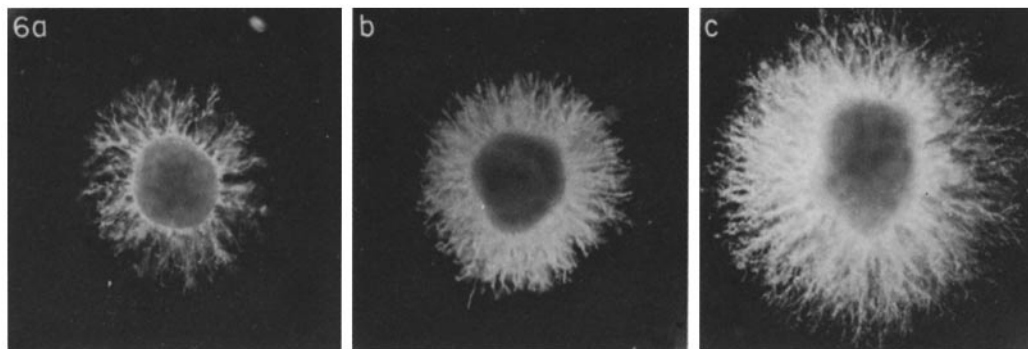


FIGURE 6 Outgrowth of neurites from ganglia that were cultured for 1 d in suspension with NGF at 10 ng/ml (a) or 400 ng/ml (b and c) and then cultured for another day in agar at 10 ng/ml NGF. In c, the cultures also contained 0.5 ng/ml anti-CAM Fab'. Dark-field illumination, $\times 40$.

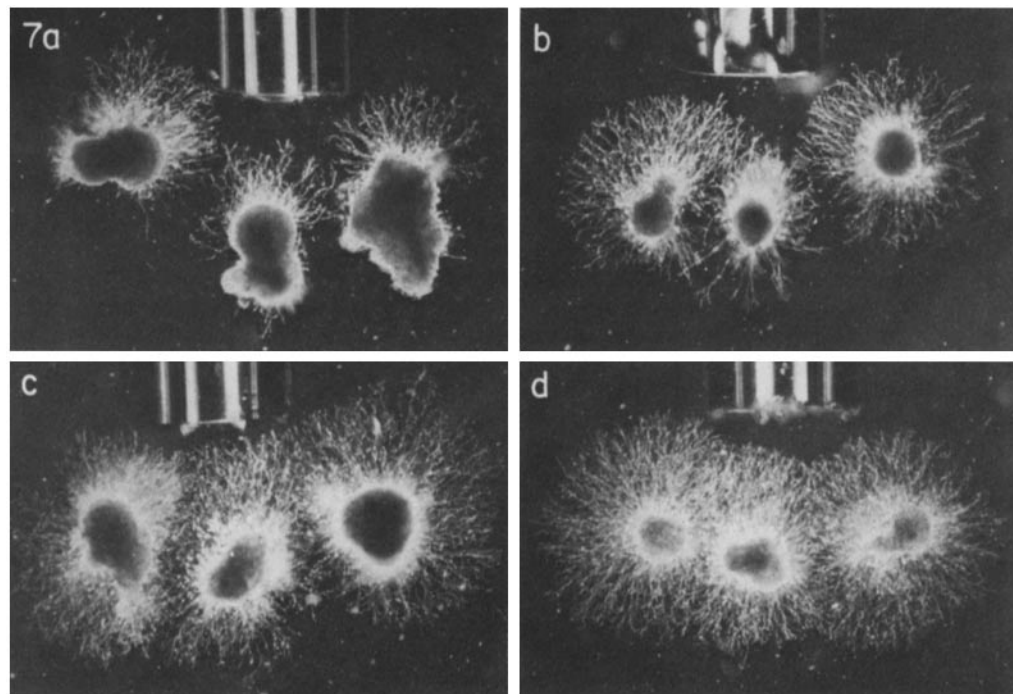


FIGURE 7 Effect of fasciculation on the growth of ganglion neurites toward an NGF capillary: thoracic ganglia cultured without (a) and with (c) anti-CAM Fab' and sacral ganglia cultured without (b) and with (d) anti-CAM Fab'. Note that the asymmetry was more pronounced (91%, see Table II) for the heavily fasciculated outgrowth of thoracic ganglia than for sacral ganglia (49%) and that the anti-CAM decreased the asymmetry in both cases (to 18% and 12%, respectively).

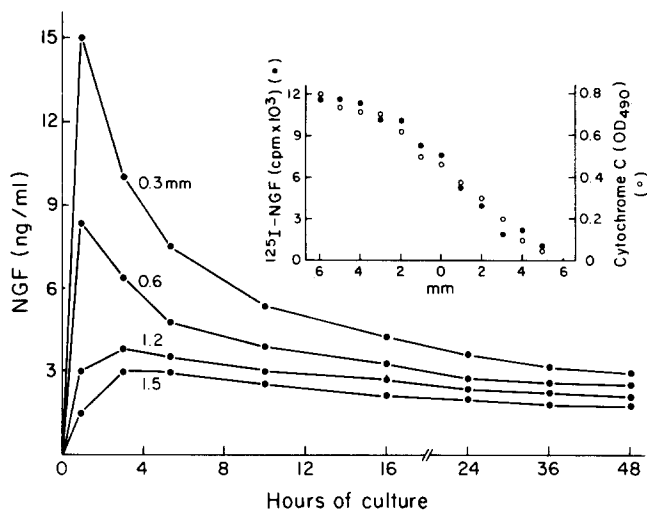


FIGURE 8 Distribution of NGF (ordinate axis) in capillary cultures as a function of time (abscissa) and distance away from the capillary opening. Each curve represents measurements of NGF made at the particular distances indicated (0.3, 0.6, 1.2, and 1.5 mm). In a typical culture, with a ganglion 0.6 mm in diameter, the nearest and furthest edges of the ganglion were ~0.6 and 1.2 mm from the capillary, respectively. Over a period of 24 h, neurites grew to a length of ~0.3 mm, so that nearest and furthest edges of outgrowth were 0.3 and 1.5 mm from the capillary. NGF concentrations were estimated from direct measurements of cytochrome *c* absorbance at 490 nm in the capillary cultures. In the insert, a comparison is shown of the diffusion after 24 h of NGF and cytochrome *c* from one half of an agar culture (without capillary) into the other half.

in NGF uptake (6, 14); this change in position might, therefore, have been expected to enhance the effective gradient across the ganglion. The rate of neurite growth in agar was slow relative to the rate of NGF diffusion, however, and this correction proved to be minor. A potentially more important qualification concerns the fact that the NGF is bound and consumed by a ganglion, whereas the cytochrome *c* tracer is not. Local depletion of NGF might, therefore, have further decreased the amount of growth factor available to the more distal part of the ganglion early in the culture period.

It was surprising that the transient NGF gradient produced by a capillary resulted in highly asymmetric neurite outgrowth. Although this gradient was steep only during the initial hours of culture, the density of processes growing out from a ganglion increased over a period of 24–36 h, with very few neurites appearing during the first 12 h. Furthermore, the percent of the neurites that grew toward the capillary remained nearly constant during the entire period of increasing outgrowth (Table I). To help in interpreting these results, the effect on neurite outgrowth of shallow NGF gradients, similar to those existing in the capillary cultures between 12 and 48 h, was also determined. To produce shallow gradients, NGF was added to the agar in one half of the culture dish and allowed to diffuse into the other half for 24 h. The ganglia were then added in a thin layer of agar. Although many neurites appeared, only a slight asymmetry (<10% of the neurites growing preferentially toward the NGF) was observed.

The lack of asymmetry obtained with single cells, and the asynchrony between the NGF gradient and the growth of most neurites toward the capillary, suggested the possibility that interactions between processes (in particular among those neurites appearing earlier and later in the culture) might be necessary to achieve the observed distribution of outgrowth.

The effect of fasciculation was, therefore, examined directly with anti-CAM antibodies and by comparing results obtained with ganglia from different regions of the spine. In addition to their known ability to decrease the diameter of fascicles (31), Fab' fragments of anti-CAM antibodies also decreased the asymmetry of neurite growth in NGF-capillary cultures (Table II). The magnitude of this effect depended on the amount of the Fab' present, with maximal reduction at concentrations of 0.5 mg/ml or higher. The antibody did not, however, completely eliminate the orienting effect of the NGF capillary.

The neurite outgrowth of ganglia from different segments of the spine have been shown to differ in their degree of fasciculation (thoracic > sacral > lumbar) (31). The same hierarchy was found in the degree of asymmetry obtained in cultures containing an NGF capillary (Table III and Fig. 7*a* and *b*). The ganglia with thinner neurite bundles also gave the most uniform outgrowth in the presence of anti-CAM Fab' (Fig. 7*c* and *d*).

TABLE I
Effect of Culture Time and the Amount of Outgrowth on Growth Toward NGF Capillaries

Hours of culture	Percent of maximum outgrowth*	Outgrowth toward capillary‡	Asymmetry§
			%
10	5		
18	23	85 ± 8	70
30	78	90 ± 4	80
42	100	87 ± 5	74

* Relative to the amount of outgrowth obtained after 36–48 h of culture.

‡ The standard error of the mean is given for measurements on 10–20 ganglia.
§ Expressed as $(P - D/P + D) \times 100$ where *P* and *D* are the total outgrowth from the ganglion halves proximal and distal to the capillary, respectively.

TABLE II
Effect of Anti-CAM on Growth of Neurites toward an NGF Capillary

Anti-CAM Fab'	Outgrowth toward capillary*	Asymmetry*
mg/ml	%	%
0	93 ± 4	86
0.125	75 ± 5	50
0.25	66 ± 4	32
0.5	58 ± 6	16
1.0	62 ± 4	24

* See footnotes to Table I. Outgrowth was measured after 36 h of culture in the presence of the Fab'.

TABLE III
Response of Ganglia from Different Spinal Segments to NGF Capillaries

Ganglion	Fascicles*			Outgrowth toward capillary‡	Asymmetry‡
	15–4 μm	4–1.2 μm	1.2–0.4 μm		
	%	%	%	%	%
Lumbar	30	50	20	67 ± 7	34
Sacral	35	50	15	75 ± 5	50
Thoracic	50	35	15	95 ± 4	90

* Data from reference 31; fasciculation was scored in terms of average percent of outgrowth contained in nerve bundles of the indicated diameter.

‡ See footnotes to Table I. Outgrowth was measured after 36 h of culture.

DISCUSSION

In this study we provide a detailed analysis of two phenomena associated with the response of spinal ganglia to NGF: the inhibition of neurite outgrowth by high concentrations of the growth factor and the preferential growth of processes toward a source of NGF. On first consideration, these subjects might not appear closely related, and in the past they have been investigated separately; however, the focus and major conclusion of this report is that both phenomena involve or are influenced by membrane-membrane interactions that occur via CAM. These interactions have been shown previously to be a primary basis for the formation of neurite bundles or fascicles (31).

The observation of inhibitory effects by NGF in cultures of whole ganglia, but not of isolated cells, indicated that some aspect of ganglion structure is required for the inhibition. The behavior of reconstituted cell aggregates was identical, however, to that of ganglia. These findings suggest that the decrease in neurite outgrowth is not associated with a subtle tissue pattern, but rather reflects the fact that the cells (and their neurites) are in very close proximity. In connection with this observation, it is particularly pertinent that this study suggests a relationship between changes in the extent of outgrowth and the fasciculation of neurites, a phenomenon that occurs extensively in ganglion cultures but not in cell cultures.

This correlation could reflect independent influences of NGF on fasciculation and neurite elongation. We have previously suggested that patterns of neurite bundling can reflect a competition between side-to-side adhesions involving CAM and the movement of growth cones in different directions along the substratum (31). When neurite adhesions dominate, thick and stable fascicles are observed; if these interactions are overcome by the pull of growth cones migrating along the substratum, the bundles are thinner, have many branches, and have a transient existence. To relate the present work to our previous studies (31), we first searched for a direct influence of NGF on either membrane-membrane or membrane-substratum interactions. No evidence was obtained in support of such effects of NGF on individual ganglion cells, although the possibility remains that they do occur with intact ganglia. In any case, neurite-neurite interactions in cultures having different levels of NGF are very likely to be affected by another parameter, namely, differences in the density of processes surrounding the ganglion. We believe that the simplest explanation of the increase in fascicle diameter with NGF concentration is that with denser outgrowth the frequency of neurite-neurite contact increases relative to interactions of growth cones with substratum.

If NGF only indirectly causes an increase in fasciculation, it remains to be explained how neurite bundling could cause the observed inhibition of outgrowth. The ability of neurites to elongate is known to depend on three growth cone functions: adhesion to the substratum (3, 18, 19), migration in a direction opposite to the vector sum of the tension exerted on the growth cone by the neurite shaft (4), and the addition of new membrane at or near the neurite tip (3, 5). In general, neurite shafts do not adhere to substratum, so that nerve processes in culture often are attached only at their ends (3, 29). When this attachment is broken either spontaneously or by perturbation, the nerve process quickly retracts, causing an increase in thickness and distortion of its shape (2, 17, 18, 19). These observations indicate that a neurite is elastic and is under tension when attached to substratum (4). These properties suggest a mechanism to account for the diminished ability of neurites to grow

out from ganglia at high NGF concentrations. It is necessary only to assume that the increased number of neurites causes them to grow out as thicker fascicles, and that the tension created in a fascicle during elongation can exceed the force that attaches it to the substratum. Such conditions would seem likely, in that the tension of individual neurite shafts should sum in a bundle, whereas many of the growth cones would not be in contact with the substratum because they had not reached the fascicle tip or were located within or on top of the bundle. This mechanism is consistent with the observation that when the NGF concentration is increased, thickening of fascicles is observed before the processes become shorter. The fact that, at any concentration of NGF, the neurites eventually ceased to elongate without an apparent loss of their growth cone activity, is also consistent with a dynamic balancing of neurite tension with the pull of growth cones along the substratum. Neurite growth in or on a ganglion would, of course, also be supported by lateral adhesions and, therefore, would not be expected to be inhibited at high NGF concentrations.

Direct evidence in favor of this hypothesis was provided by the perturbation experiments with anti-CAM and various substrata. Anti-CAM not only reduces the diameter of fascicles, but also increases the number of actively moving growth cones on the surface of the remaining bundles (31). As would be expected from the model, the antibody caused a shift of the inhibitory effects to higher NGF concentrations and increased the maximum extent of outgrowth. The same effects were produced by increasing the adhesiveness of the substratum. The hypothesized balance of forces would account for this result in that an increase in growth cone-substratum adhesion would be expected to counteract the tension of the neurite shafts.

The experiments in which the NGF concentration was changed during the culture period provided additional insight into the mechanics of neurite outgrowth. When ganglia grown at 200 ng NGF/ml were shifted to 10 ng NGF/ml, there was a delayed but enhanced outgrowth that was more immediate and further enhanced by the presence of anti-CAM. These observations confirm that what is inhibited by NGF is growth away from the ganglion but not neurite growth itself (24, 25); they also suggest that escape from the inhibited state is facilitated by a reduction in membrane-membrane adhesion. Conversely, when the NGF was shifted from low to high concentrations, more and more neurites elongated along existing fascicles, increasing their diameter and eventually causing retraction. If, however, the substratum was very adhesive, or if the fascicle had reached another ganglion, retraction did not always occur. In other words, if the tip of a process found a suitable "anchor," its previous history of migration was not erased. This behavior is of particular interest in that it may be relevant to the development of nerve tracts. For example, a number of pilot neurites could grow out independently in search of an adhesive pathway. With the subsequent outgrowth of nerves along these initial fibers, a pilot that had failed to find such a pathway would be retracted, leaving only those fibers that were successfully anchored.

The results of our experiments with NGF capillary cultures suggest that growth of ganglion neurites toward an NGF source represents a complex phenomenon, influenced not only by the amount and spatiotemporal distribution of NGF, but also by interactions among the neurites themselves. Whereas uncertainties in the measurement of NGF concentrations prevented a very precise description of these cultures, the results nevertheless suggest that there was a steep gradient of NGF across

the ganglion during the first few hours of culture. By this time, however, only a few short processes had appeared. The number and length of neurites then increased steadily over the next 24 h when the concentration gradient had become too shallow to cause a significant asymmetry of outgrowth. Despite this asynchrony between the NGF gradient and the appearance of neurites, the percent of ganglion processes growing toward the capillary did not change significantly during the entire culture period. Therefore, if the initial difference in NGF concentration across the ganglion was responsible for producing the asymmetry, the results imply that its effect was maintained throughout the entire time of neurite growth and in some way was communicated to fibers that appeared later in the culture.

An important feature of this phenomenon has been revealed by the experiments involving single cells as well as anti-CAM antibodies. In contrast to process outgrowth from intact ganglia or reaggregated cells, the neurites produced by individual ganglion cells failed to adopt a perceptible and lasting orientation with respect to the capillary. It is known, however, that isolated growth cones will reorient themselves according to movements of a micropipette containing NGF (13) and that a small but significant outgrowth asymmetry can be produced by exposure of ganglion cells to a stable gradient of NGF (20). The absence of such effects in this study was probably caused by the transient nature of the NGF source. In any case, our observations suggest that the dramatic asymmetry of neurite outgrowth produced by the NGF capillary used in our experiments requires cell-cell contacts as found in ganglia or cell aggregates. This possibility was strongly and directly supported by the demonstration that the asymmetry obtained with ganglia and NGF capillaries was sharply decreased by anti-CAM Fab' fragments.

Two observations suggest that the relevant interactions occurred among neurites rather than among cell bodies within a ganglion. First, preexisting contacts between cells in an aggregate were not disturbed by anti-CAM nearly as effectively as were the side-to-side interactions that occurred among nerve processes during the cultures. Second, the degree of outgrowth asymmetry obtained with intact ganglia from different spinal segments also was correlated with the amount of fasciculation observed among their neurites.

Neurite-neurite interactions involving CAM could provide a mechanism to account for the phenomena obtained with intact ganglia and NGF capillaries. We assume that a small number of cells in a ganglion initially respond to the NGF gradient in a directed manner, either by differential growth or chemotaxis. The bulk of outgrowth, which occurs subsequently and without a substantial gradient, uses fasciculation to follow these initial pioneering paths. Fascicles, being relatively rigid, tend to grow in straight lines and, therefore, would also help maintain the orientation of the initial fibers throughout the culture period. In the presence of anti-CAM, however, interactions among individual neurites decreases and the outgrowth would assume a more random orientation.

An important issue remains concerning the relevance of our observations to the development of the nervous system. A number of tissues appear to contain sufficient NGF to produce either of the two phenomena discussed here (16). It is not known, however, whether the growth factor is distributed appropriately in time and space. In any case, growth of a nerve in vivo often takes place in the presence of other neurites that could influence its path. In this respect, the results of our studies on fasciculation may be of particular significance in

deciphering a possible role for NGF (or any other directing influence) in the guidance of nerves during embryogenesis.

We are grateful to Ms. Helvi Hjelt for excellent technical assistance.

This work was supported by U.S. Public Health Service grants HD-09635, AI-11378, and AM-04256, and by a Scholars' Award to Urs Rutishauser from the McKnight Foundation.

Received for publication 25 March 1980, and in revised form 7 July 1980.

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