DEFINING THE CONCENTRATION GRADIENT OF NERVE GROWTH FACTOR FOR GUIDED NEURITE OUTGROWTH

X. CAO[a] and M. S. SHOICHET[a,b,c]*

[a]Department of Chemical Engineering and Applied Chemistry, University of Toronto, 200 College Street, Toronto, Ontario, Canada M5S 3E5

[b]Department of Chemistry, University of Toronto, 80 St. George Street, Toronto, Ontario, Canada M5S 1A1

[c]Institute of Biomaterials and Biomedical Engineering, University of Toronto, 170 College Street, Toronto, Ontario, Canada M5S 3E3

Abstract—The developing axon is believed to navigate towards its target tissue in response to a concentration gradient of neurotrophic factors, among other diffusible and surface-bound stimuli. However, the minimum concentration gradient required for guidance over the maximum distance is still unknown, largely because well-defined systems have not been utilized to address this question. In this study, a linear concentration gradient of nerve growth factor was achieved across a 5-mm agarose membrane that separated a nerve growth factor source compartment from a sink compartment. The concentrations in both compartments were maintained constant (and different). Both concentration and concentration gradient were well defined across the membrane, allowing us to study the relative importance of concentration gradient vs concentration for neurite guidance. The orientation of PC12 cell neurites was studied in response to a series of nerve growth factor concentration gradients *in vitro*. For effective guidance of PC12 cell neurite outgrowth, a minimum concentration gradient of 133 ng/ml per mm was required, below which guidance was ineffective. Higher gradients were effective for guidance yet were limited by the concentration of nerve growth factor in the source compartment. At a nerve growth factor concentration of 995 ng/ml, the PC12 cells' receptors were saturated, thereby limiting the maximum effective distance for guidance to less than 7.5 mm in response to a diffusible nerve growth factor cue. This distance exceeds the 0.5–2 mm distance observed by others for effective neurite guidance.

Using this model system, we propose that the minimum concentration gradient can be defined for other cells and growth factors. Ultimately, it is anticipated that such concentration gradients could be included in a device to promote regeneration. © 2001 IBRO. Published by Elsevier Science Ltd. All rights reserved.

Key words: neurotrophic factor, growth cone guidance, regeneration, diffusion, PC12 cells.

It is widely accepted that axons are guided to their targets during development by a combination of contact mediated and diffusible cues that are either attractive or repulsive.[7,39] At the nerve fiber terminus, the growth cone is believed to guide the axon by sampling the environment for either positive or negative signals using filopodial and lamellar protrusions.[16,34,49] This sampling, comparing and decision-making procedure is believed to be a concentration gradient-dependent action,[38,49] which evokes a set of intracellular events involving cytoplasmatic second messengers.[43] A gradient of the cytoplasmatic second messenger may signal the preferential incorporation of new plasma membrane material and asymmetric cytoskeleton reorganization at the growth cone that is required for the appropriate orientation of neurites.[24]

Significant research has been devoted to identifying[4,24] and investigating the mechanisms by which guidance cues work, yet surprisingly little research has been devoted to defining the concentration gradient to which growth cones respond. It has been postulated that growth cones will navigate over long distances if: (i) the concentration of the given neurotropic factor does not saturate cell surface receptors, which consequently would be down-regulated,[50] and (ii) the concentration gradient is sufficiently steep for growth cone detection.[14] In the popular micropipette assays,[19,40,44] both concentration and concentration gradient of the neurotrophic factor vary between the cells and the source; thus, it is difficult to determine the minimum concentration gradient for guided neurite outgrowth and whether or not the concentration itself plays any role in this directed growth. The under-agarose assay[26,35,36] and variations thereof (e.g. the linear under-agarose assay[37]) have been successfully used to investigate leukocyte migration in response to various chemotactic stimuli; however, the chemotactic gradient created in this assay varies with time and position.[27] This assay is therefore not applicable to studying the chemotactic effect of a neurotrophic factor gradient on the relatively immobile neuron with a neurite elongation rate of less than 0.5–1 mm per day. Fisher *et al.*[12] prepared a chemotaxis chamber, in which a stable attractant source and sink were provided by a hollow fiber

*Corresponding author. Tel.: +1-416-978-1460; fax: +1-416-978-8605.
E-mail address: molly@ecf.toronto.edu (M. S. Shoichet).

Abbreviations: ELISA, enzyme-linked immunosorbent assay; NGF, nerve growth factor; PC12 cell, pheochromocytoma cell; PLL, poly(L-lysine).
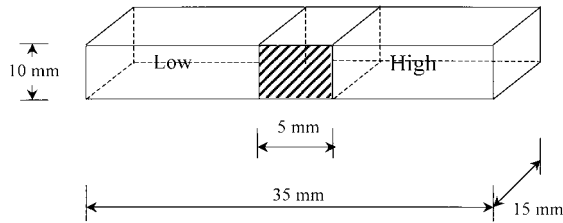
Fig. 1. Compartmentalized diffusion chamber. Shaded area (agarose membrane) separates two equivalent compartments, each of which contains a constant, yet different, concentration of NGF.

perfusion system embedded in thin agarose gels, forming a long-term linear concentration profile. However, this design is complicated and thus has not been widely used. Recently, Knapp et al.[25] created a steep concentration gradient for 24 h by physically constraining the diffusion of fibronectin peptide between a source and a sink. While simple and elegant, this approach is not suitable for nerve guidance studies because the gradient is non-linear and is only stable for 24 h.

We have created a series of stable linear concentration profiles of neurotrophic factors, thereby allowing concentration and concentration gradient to be independently evaluated for axonal guidance. Using this model we can determine both the minimum and the maximum distance for guidance, based on the maximum neurotrophic factor concentration allowed by the cells before saturation and receptor down-regulation. Specifically, we created a series of linear concentration gradients of nerve growth factor (NGF) in a custom-designed diffusion chamber. Using the pheochromocytoma (PC12) cell line in the diffusion chamber, we were able to determine the minimum concentration gradient required to guide PC12 cell neurite outgrowth.

EXPERIMENTAL PROCEDURES

Materials

All chemicals were purchased from Sigma (St. Louis, MO, USA) and used as received unless otherwise indicated. Analytical reagent grade sodium chloride, calcium chloride, sodium carbonate and sodium bicarbonate were purchased from BDH (Toronto, Ontario, Canada) and magnesium chloride was purchased from APC Chemical (Montreal, Quebec, Canada). Low-temperature gelling agarose gel, SeaPlaque®, was obtained from FMC Corp. (Rockland, ME, USA). Mouse NGF-β (2.5S NGF) was purchased from Cedarlane Laboratory (Hornby, Ontario, Canada), and reagents for the NGF enzyme-linked immunosorbent assay (ELISA) were obtained from Boehringer Mannheim (Germany). The adrenal rat PC12 cell line was purchased from American Type Culture Collection (ATCC, Rockville, MD, USA). Deionized distilled water was obtained from Milli-RO 10 Plus and Milli-Q UF Plus system (Bedford, MA, USA) and used at 18 MΩ resistance.

Compartmentalized diffusion study

A custom-built, polycarbonate, rectangular chamber, with three compartments, was autoclaved and then glued to a sterile, 100-mm Petri dish (Falcon, Franklin Lakes, NJ, USA). SeaPlaque® agarose (1%) was dissolved in phosphate-buffered saline (pH 7.4) and sterilized by autoclaving (Yamato SM300 autoclave, Japan). The agarose solution (0.5 ml) was cast in the middle chamber, dividing the chamber into two identical compartments on either side of the gel. One of the compartments was then filled with

400 μl of 66.3 ng/ml (concentration determined by NGF ELISA) of NGF solution (referred to as the high concentration compartment hereafter), and the other was filled with 400 μl of cell culture medium (referred to as the low concentration compartment hereafter), as shown in Fig. 1. The diffusion experiments were carried out at 37°C and 100% humidity (Sanyo incubator, Japan). The solutions in both high and low concentration compartments were withdrawn every 6 h (see Appendix A for the rationale behind the 6-h time frame) and replenished with fresh NGF and cell culture medium, respectively, to maintain constant concentrations in each compartment. After 72 h of diffusion, the agarose gel was frozen and serially sectioned perpendicular to the direction of diffusion using a cryostat at −20°C. This resulted in 20-μm-thick agarose slices. The amount of NGF within each slice was then determined by an NGF ELISA (Boehringer Mannheim, Germany) by simply extracting the NGF from mechanically crushed gels. The NGF concentration profile across the agarose gel was consequently constructed. The diffusion study was carried out in duplicate, and the NGF ELISA was performed in triplicate for every agarose gel slice in both diffusion studies.

PC12 cell culture

PC12 cells were maintained in T-25 cell culture flasks (Falcon) at a plating density of 1.0×10^4 cells/cm². The cell culture medium consisted of 84% RPMI 1460, 10% heat-inactivated horse serum, 5% fetal bovine serum and 1% penicillin/streptomycin. Cells were incubated at 37°C in a 5% CO₂/air atmosphere. Cell culture medium was changed every other day, and cells were subcultured once every week. All cells used in the compartmented culture assay were within three passages.

Directed neurite outgrowth in a compartmentalized culture assay

A 100-mm Petri dish was coated with 10 ml of a poly(L-lysine) (PLL) solution (36,600 g/mol, 50 μg/ml) for 3 h at room temperature, washed thoroughly with deionized distilled water and then air-dried.[17] The three-section, autoclaved, rectangular chamber was then glued to the Petri dish using autoclaved high vacuum grease.[8] PC12 cells were plated within the central compartment on the PLL-coated Petri dish at a cell plating density of 1.0×10^4 cells/cm² and allowed to set for 2 h in the incubator. Agarose gel solution (1%; 0.5 ml) was then cast into the middle compartment, in the same fashion as that in the compartmentalized diffusion study, and on top of the PC12 cells. High concentration NGF and blank cell culture medium were used in the high concentration and the low concentration compartments, respectively. Both high and low concentration compartments were replenished with fresh NGF and cell culture medium every 6 h, respectively. A series of NGF concentration gradients was created by using one of 6.6, 13.2, 66.3, 663 and 995 ng/ml NGF in the high compartment, and either 66.3 ng/ml NGF or cell culture medium in the low compartment. A uniform NGF concentration throughout the agarose was created by inoculating both compartments with the same NGF concentration of 663 ng/ml, which served as a control. After 96 h of culture, neurite outgrowth of PC12 cells was observed by phase contrast microscopy (Axiovert S100, Zeiss) for cells in the low, central and high concentration areas, as illustrated in Fig. 2. Neurites from individual cells were counted in each area and a neurite population of greater than 150 ($n > 150$) was analysed per area (1.2 mm × 15 mm, 1.2 mm in the direction of the concentration gradient), of every concentration gradient studied.

The orientation of neurites extending from PC12 cells was determined by measuring the angle between the neurite and the NGF source. As shown in Fig. 3, an imaginary line was drawn between the geometric center of the cell and the end of the extended neurite, and another imaginary line was drawn between the center of the cell and the NGF source. The angle between these two imaginary lines defined the orientation angle of neurite outgrowth. Neurites that extended from the cell between 0° and 180° were assigned positive orientations, while those extending between 180° and 360° were assigned negative orientations (i.e.
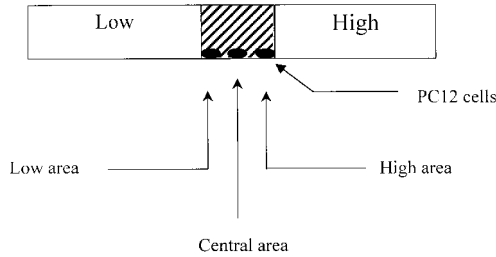
Fig. 2. Compartmentalized cell culture assay is identical to that used in diffusion analysis with the addition of PC12 cells plated beneath the agarose membrane.
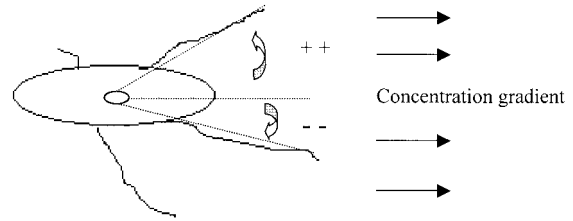

Fig. 3. Neurite orientation is scored relative to the NGF concentration gradient. The angle between an imaginary line from the center of the cell to the high concentration compartment (NGF source) and a line from the center of the cell to the growth cone is utilized to measure orientation.

$0°$ to $-180°$). This symmetrically defined neurite orientation conferred the convenience that highly orientated neurite outgrowth towards the NGF concentration gradient (zero direction using the above-mentioned definition) would cluster around and converge to the proximity of the zero direction. All neurites that were longer than one cell body length were scored, except for those that merged with other cells, to eliminate any artifact associated with cell–cell interactions.

Nerve growth factor dose-dependent neurite outgrowth from PC12 cells

In a separate assay, the effects of NGF concentration alone on PC12 cell neurite outgrowth were studied. PC12 cells were plated on PLL-precoated six-well plates with a cell density of 1.0×10^4 cells/cm^2. Cells were cultured in cell culture media supplemented with NGF to have NGF concentrations of 0.66, 3.3, 6.6, 33.2, 66.3, 332, 663, 995 and 1330 ng/ml. Cells cultured under identical conditions but without NGF served as a blank control. The neurite lengths from 50 random cells were measured after 48 h of culture.

Statistics

The neurite orientation data, which were obtained from the compartmentalized cell culture assay, were analysed first by a uniformity test ($\alpha = 0.01$) and then by a conditional unbiased test. (1) The uniformity test was used to determine whether there was any preferred direction to the neurite outgrowth by a χ^2 test. Only those samples that passed the uniformity test were further tested by the unconditional unbiased test to determine the preferred mean direction of neurite outgrowth relative to the NGF concentration gradient. (2) The preferred mean direction was then compared with the direction of the concentration gradient (i.e. NGF source) with 95% confidence, as described by Mardia.[30] In brief, the preferred mean direction was calculated using Eq. (1), the absolute mean values (with 95% confidence intervals) of which are reported:

$$\phi = \arctan\left(\frac{S}{C}\right), \qquad (1)$$

where C and S are the averaged contributions of all of the scored neurites parallel and perpendicular to the direction of the concentration gradient ($0°$ reference direction), respectively, and were calculated according to:

$$C = \frac{1}{n} \sum_{i=1}^{n} \cos\,\theta_i \qquad (2)$$

and

$$S = \frac{1}{n} \sum_{i=1}^{n} \sin\,\theta_i, \qquad (3)$$

where θ is the angle measured for neurite outgrowth and n is the number of neurites ($n > 150$). The circular variance R was calculated from:

$$R = (C^2 + S^2)^{\frac{1}{2}}, \qquad (4)$$

based on which the 95% confidence interval of the mean direction ϕ is tabulated.[30]

RESULTS

The goals of this study were to create a series of homogeneous concentration gradients and to determine their effect on neurite orientation. Given the constant concentration gradient (i.e. $dc/dx = $ constant), yet the different concentration across the agarose membrane, both the minimum concentration gradient required for guidance and the maximum guidance distance could be determined.

Establishing the time frame for diffusion through agarose gel

Before establishing a linear concentration profile in the agarose gel, we first ran a time course assay to determine the diffusion coefficient and partition coefficient of NGF in agarose gel. Samples from the sink compartment were collected every 6 h and analysed by NGF ELISA. The cumulative amount of NGF that diffused through the agarose gel and was collected in the lower concentration compartment is shown in Fig. 4. (Note that, in this study, unlike the rest of the paper, the lower concentration compartment solution was not changed.) As shown in Fig. 4, a significant amount of NGF was detected in the sink compartment only after 30–36 h, indicating that a steady state of NGF is achieved after 36 h. Thus, after 36 h, a linear relationship was achieved between the amount of diffused NGF and time (cf. graph insert of Fig. 4), which is a characteristic of a steady-state diffusion and from which a diffusion coefficient and a partition coefficient of 7.8×10^{-7} cm^2/s and 0.9 were calculated, respectively[10,14] (see Appendix B for detailed calculations).

Compartmentalized diffusion study of nerve growth factor

To create a linear NGF concentration profile, NGF was allowed to diffuse across an agarose gel membrane that separated two compartments, each with a constant yet different concentration of NGF, thereby achieving steady-state conditions. The agarose gel was frozen and serially sectioned perpendicular to the direction of NGF diffusion to determine the concentration profile across the gel, using an NGF ELISA. The NGF concentration profile was consequently constructed. According to
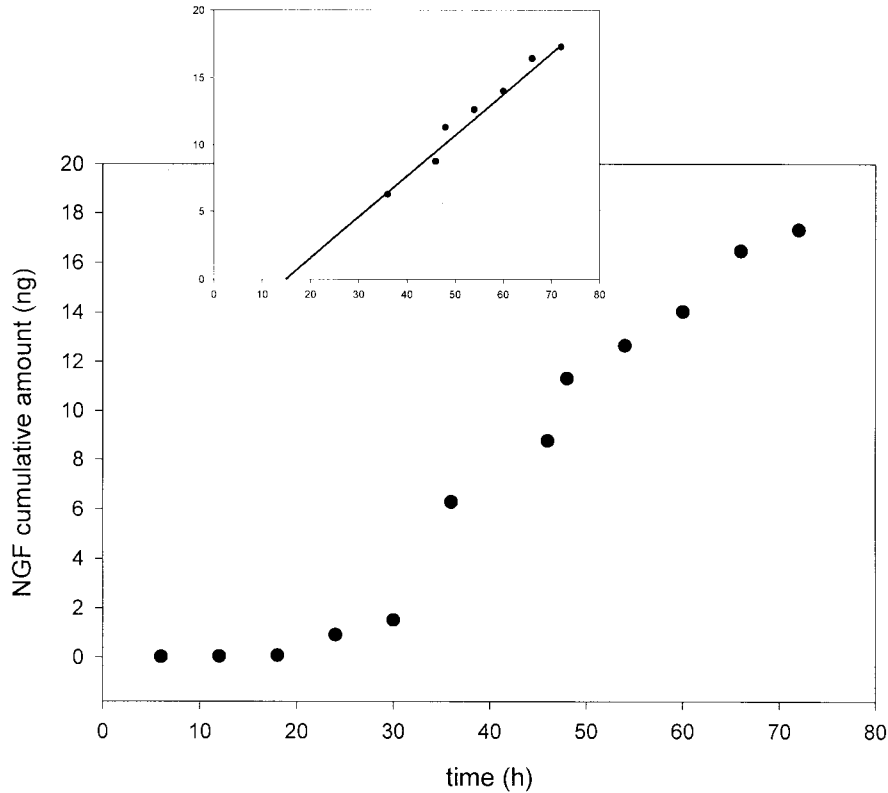
Fig. 4. Cumulative amount of NGF detected in the low concentration compartment in the course of the diffusion study. Insert is a linear regression of the same set of data after 36 h, from which the diffusion coefficient and partition coefficient of NGF in 1% agarose are evaluated to be 7.8×10^{-7} cm^2/s and 0.9, respectively.

Fick's second law, when a steady state is reached in a two-compartment diffusion system, the concentration profile of the solute in the membrane dividing the two compartments is linear.[10,11,33] In this compartmentalized diffusion study, the agarose gel is the membrane that divides the chamber into two compartments, each of which contains high and low concentrations of NGF, and which act as source and sink compartments, respectively. By replenishing solutions in both compartments at

predetermined time intervals, the NGF concentration in each compartment was kept approximately constant; specifically, the NGF concentration in the sink compartment was maintained between 0 and 2.3 ng/ml.

As shown in Fig. 5, the NGF concentration profile within the agarose gel is linear after a 72-h diffusion study. The bulk NGF solution concentrations in both source and sink compartments are nearly identical to those at the gel–solution interfaces. This suggests that resistance to the NGF diffusion at the solution–agarose interface is minimal. While a slight difference in NGF concentration was observed between the bulk and the agarose gel at the interface, this can be accounted for by the methodology employed: the concentration of NGF in agarose was averaged over a 20-μm-thick section. Furthermore, since the NGF bulk concentrations are not consistently higher or lower than those in the agarose at each of the two gel–solution interfaces, the partition coefficient of NGF is likely to be close to unity,[13,45] which is in good agreement with the calculated value of 0.9. The regression line based on experimental data is similar to the theoretical line based on the bulk concentration of NGF in the high and low compartments. This suggests that the linear concentration profile within the agarose gel can be predicted provided that trophic factor concentrations in both high and low compartments are known and constant. Thus, different concentration gradients were designed and accurately prepared by maintaining constant concentrations in the compartments. Five concentration gradients were investigated
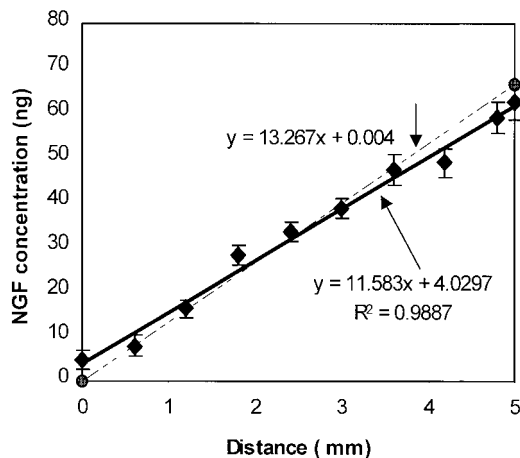

Fig. 5. NGF concentration profile in agarose: (♦) experimental data fitted by least squares (solid line, $n = 2$) are compared to the theoretical prediction (dashed line) connecting low and high concentration data points in bulk (●).

Table 1. Effect of nerve growth factor concentration gradient, across an agarose membrane, on neurite outgrowth of PC12 cells (absolute values shown)

NGF in high concentration compartment (ng/ml)	NGF in low concentration compartment (ng/ml)	Absolute concentration gradient (dc/dx) (ng/ml per mm)	Preferred direction of neurite outgrowth (degrees)* ($n > 150$)		
			Low area	Central area	High area
			(% in parentheses refers to dc/c)		
663	663	0.00 (control)	None (0%)	None (0%)	None (0%)
6.63	0.00	1.32	None (3.3%)	None (0.80%)	None (0.45%)
13.2	0.00	2.64	None (3.3%)	None (0.80%)	None (0.45%)
663	66.3	119	None (1.7%)	None (0.65%)	None (0.40%)
663	0.00	133	0.03 ± 32.0 (3.3%)	15.8 ± 27.0 (0.80%)	4.32 ± 16.0 (0.45%)
995	0.00	199	14.9 ± 18.0 (3.3%)	14.7 ± 22.0 (0.80%)	27.0 ± 18.0† (0.45%)

*Determined by χ^2 test ($\alpha = 0.01$, $n > 150$). Mean ± 95% confidence interval.
†Mean is significantly different from the others of the same concentration gradient ($P < 0.01$), as suggested by one-way ANOVA.

for their effect on neurite guidance: 1.32, 2.64, 119, 133 and 199 ng/ml per mm.

Compartmentalized cell culture assay

In the compartmentalized cell culture assay, PC12 cells were plated underneath the agarose gel, within which there was a defined concentration gradient. The cellular response of the PC12 cells to different NGF concentration gradients was studied in terms of neurite orientation relative to the concentration gradient. Table 1 summarizes preferential PC12 cell neurite outgrowth in response to different NGF concentration gradients after 96 h in culture. As shown in Table 1, below 133 ng/ml per mm, preferred neurite outgrowth was insignificant. It is interesting to note that guided neurite outgrowth from PC12 cells was sensitive to the steepness of the concentration gradient. For example, a concentration gradient of 119 ng/ml per mm did not guide neurite outgrowth significantly, yet provided a concentration gradient that was only 10% less steep than that provided by 133 ng/ml per mm, where significant neurite outgrowth was observed.

A representative light micrograph of PC12 cells cultured in the presence of a 133 ng/ml per mm NGF concentration gradient is shown in Fig. 6A; Fig. 6B shows how the angles are scored with lines for each neurite registered and the angle calculation superimposed.

It is interesting to note that, for the 133 ng/ml per mm NGF concentration gradient, the 95% confidence interval for the mean neurite orientation overlapped with the direction of the concentration gradient, 0° (cf. Table 1) in all three cellular areas, suggesting that the concentration gradient exerts a pronounced guidance effect on neurite outgrowth. Thus, neurite orientation was statistically the same in all three cellular areas (low, central and high). Since the concentration gradient is also the same in all three areas, yet the concentration is different in these three areas, then it is the NGF concentration gradient, and

not the NGF concentration, that guides neurite outgrowth. For the 199 ng/ml per mm concentration gradient, preferred neurite outgrowth was observed in the low and central concentration areas, but was less effective in the high area; the 95% confidence interval for the mean neurite orientation in the high area did not overlap with 0°, the direction of concentration gradient (cf. Table 1). We hypothesized that the PC12 cell receptors were saturated at the high NGF concentration area and tested this hypothesis by measuring neurite length as a function of NGF concentration. As shown in Fig. 7, neurite length increased with NGF concentration to 66.3 ng/ml, remained constant to 663 ng/ml and then decreased dramatically at higher NGF concentrations. Thus, at NGF concentrations >663 ng/ml, the NGF receptors on PC12 cells are probably depleted owing to receptor down-regulation.[50] While a concentration gradient of 199 ng/ml per mm guided neurite outgrowth, cells in the high area experienced an NGF concentration of 995 ng/ml, which was less effective for guidance, probably as a result of NGF receptor down-regulation. The upper NGF concentration limit that can be tolerated by the PC12 cells (without down-regulating receptors) is likely to be between 663 and 995 ng/ml. As shown in Table 1, a uniform concentration (i.e. 663 ng/ml NGF concentration in both compartments) of NGF does not elicit any directional neurite outgrowth from PC12 cells; however, a concentration gradient created by inoculating only one of the compartments (i.e. the high concentration compartment) with 663 ng/ml does. This confirms that an NGF concentration gradient, rather than an NGF concentration, is the driving force for the guided neurite outgrowth from PC12 cells, as outlined above.

It is worthwhile noting that all cells in all areas were exposed to the minimum threshold concentration of NGF for neurite outgrowth.[32] Furthermore, NGF was bioactive throughout the compartmentalized diffusion and cell culture assays. This can be attributed to constant
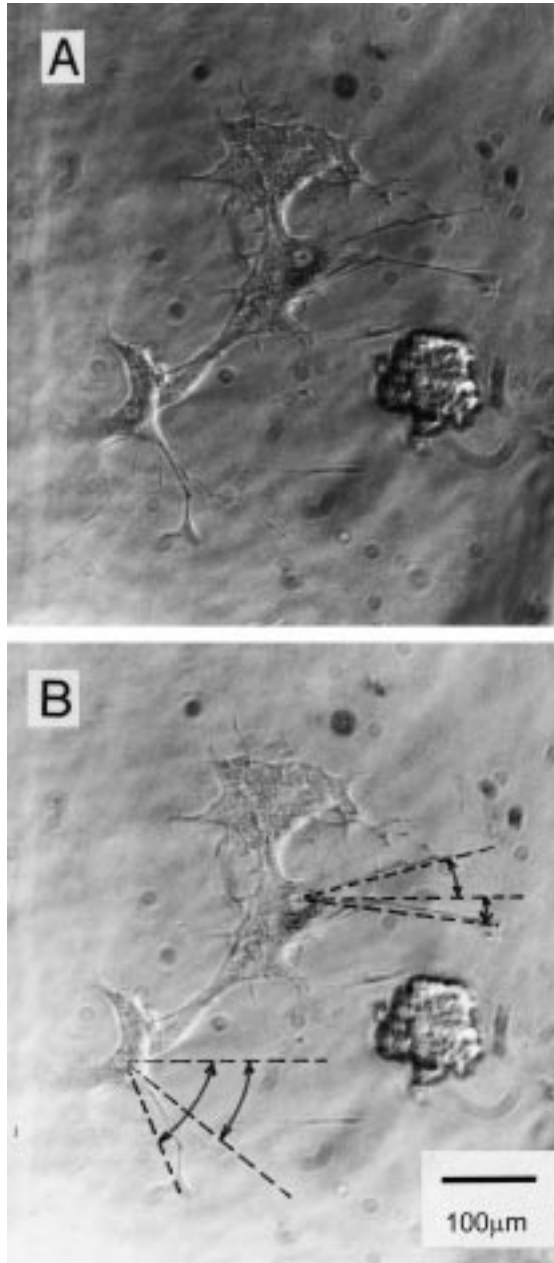
Fig. 6. Neurite outgrowth of PC12 cells towards the NGF source was evident. (A) A representative cell with neurites growing along the gradient after 96 h in culture. (B) A representative angle calculation shown with lines superimposed over neurites extending from PC12 cell body.

replenishing of NGF solutions (every 6 h) and the presence of bovine serum albumin in the cell culture medium.[9,41]

DISCUSSION

The linear and homogeneous NGF concentration gradient throughout the agarose gel provides a unique approach to study the effect of concentration and concentration gradient of neurotrophic factors on directed neurite outgrowth. By controlling concentration and concentration gradient independently, we were able to

demonstrate that concentration gradient (dc/dx) was the major driving force for directed neurite outgrowth and, more importantly, we were able to determine the concentration gradient threshold for effective guidance. While previous studies demonstrated neurite outgrowth towards a neurotrophic factor source, the concentration profile was ill-defined. For example, neurite outgrowth towards a point source was studied, with the source created either by micropipetting factors at a specific location in culture media[18,19,38] or using explants which released the factors of interest.[28] In both examples, the concentration profile was either poorly defined[29] or Gaussian,[22] and depended upon the experimental geometry and other parameters used. The non-linear concentration profile implies that cells may have experienced concentration profiles that differed from one experiment to the next, and that even in one experiment cells may have experienced different concentrations and concentration gradients from one location to the next. This may explain the discrepancy in the literature between cellular response to different concentrations and concentration gradients.[18,28]

To create a linear, stable, homogeneous concentration gradient throughout the agarose gel, a constant concentration of NGF was provided in both source and sink compartments while the steady-state diffusion was established. To achieve constant concentrations of NGF in both source and sink compartments, the NGF solutions in both compartments were replenished every 6 h during the course of the experiment; this ensured a concentration difference fluctuation of less than 1% between each solution replenishment, according to our calculation (cf. Appendix A). To reach a steady-state NGF diffusion throughout the agarose gel membrane, 30–36 h is required. Since PC12 cells did not extend neurites until 36–48 h of cell culture in the current compartmentalized cell culture experiment, and the neurite outgrowth in response to such a linear concentration profile was assessed after 96 h in culture, it is most likely that PC12 cells experienced a steady rather than a transient NGF diffusion state.

The concentration gradient refers to either the fractional concentration gradient dc/c[46] or the absolute concentration gradient dc/dx.[31] While many use dc/c to study chemotaxis of leukocytes, there is no consensus in the neuroscience literature; there are very few quantitative reports for growth cones that distinguish between these two gradient calculations,[15] which sometimes leads to ambiguity in the literature.[3] The linear concentration profile employed in this study is characterized by a homogeneous absolute concentration gradient dc/dx, but a changing fractional concentration gradient dc/c throughout the gel. Therefore, if the absolute concentration gradient is the driving force for guiding neurite outgrowth, then the response of neurites should be similar in the three areas that are under investigation; however, if the fractional concentration gradient is the driving force for guiding neurite outgrowth, then the response of neurites should be different in the three areas. For an absolute concentration gradient of 133 ng/ml per mm, where guided PC12 cell neurite outgrowth is observed, the fractional concentration gradients in the
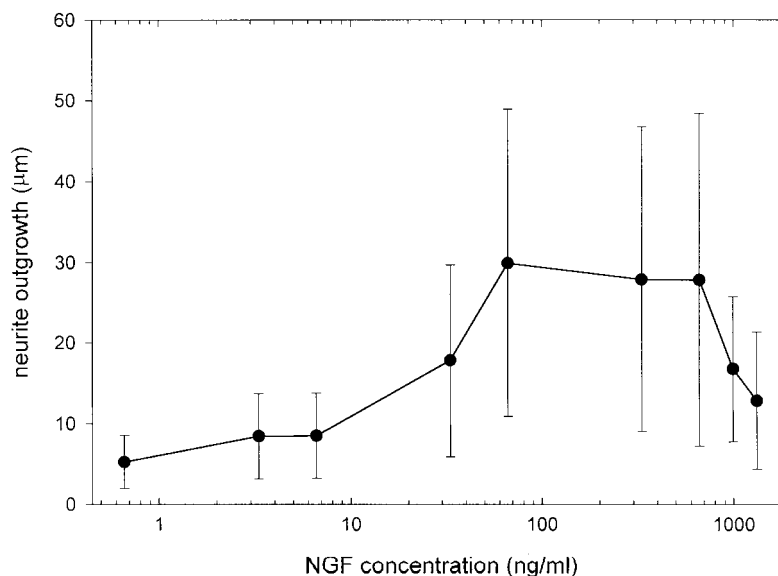
Fig. 7. Neurite outgrowth from PC12 cells (after 48 h of culture) was determined to be dose dependent. Since neurite lengths are not normally distributed, data are presented as median, and error bars indicate a range from the 25th percentile to the 75th percentile.

low, central and high concentration areas are approximated to be 3.3%, 0.80% and 0.45%, respectively, assuming that a growth cone has a diameter of 20 μm;[14] however, the cellular response is not significantly different, as suggested by the one-way ANOVA (cf. Table 1). It is interesting to note that for lower absolute concentration gradients (e.g. 1.32 and 2.64 ng/ml per mm), but the same fractional concentration gradient in all three corresponding locations investigated (cf. rows 1, 2 and 4 in Table 1), no preferred directional growth is observed. This suggests that an absolute concentration is more important than a fractional concentration gradient in guiding PC12 cell neurite outgrowth. Furthermore, the fact that the absolute concentration gradient of 119 ng/ml per mm (a 10% less steep gradient than that of 133 ng/ml per mm) did not elicit any significant neurite outgrowth suggests that the minimum absolute concentration gradient to induce PC12 neurite outgrowth is between 119 and 133 ng/ml per mm, or from approximately 4.6 to 5.1 nM/mm. This result agrees well with that observed by Mato et al.,[31] who demonstrated that an absolute cyclic-AMP concentration gradient of 3.6 nM/mm is the threshold value for a chemotactic response of bacteria.

To guide neurite outgrowth, a minimum absolute concentration gradient (i.e. 133 ng/ml per mm) and a concentration <995 ng/ml is required, as was shown in the 199 ng/ml per mm concentration gradient studies. Thus, the longest distance over which such a diffusible cue could act is <7.5 mm (i.e. <995 ng/ml divided by 133 ng/ml per mm). While others have previously demonstrated guidance over 0.5–2 mm,[14,48] we were able to show guidance over 5 mm (as demonstrated in the experiment), and expect that this could be extended up to ~7.5 mm. We attribute our success to both the linear concentration profile and homogeneous concentration gradient achieved throughout the gel. As we demonstrated, a steep concentration gradient is required to elicit preferential neurite outgrowth. The linear concentration

profile is likely to be the only concentration profile that is capable of maintaining an adequately steep absolute concentration gradient to guide neurite outgrowth over an extended distance.

Axons of developing neurons depend upon both contact-mediated and diffusible cues to navigate to their targets.[16] Contact-mediated cues are provided by extracellular matrix molecules expressed by other cells (or neurons), while diffusible cues are provided by target tissues.[16] These cues act synergistically to precisely navigate growth cones over long distances.[20,34] Recently, Bahr and Schwab[2] showed that regenerating axons regain some of their developing stage characteristics and may also rely on both cues to re-innervate their targets. This view was shared by Woolford,[47] and more recently this concept was echoed by Houwelling et al.[21] and Bregman et al.,[6] who demonstrated experimentally that neurotrophic factors were able to exert a neurotropic influence on injured, mature CNS axons.

Diffusible and contact-mediated cues are probably important to CNS regeneration and a hydrogel matrix loaded with such cues may provide a vital addition to the current device designs[5,23,42] that are intended to augment CNS nerve regeneration. However, there are significant challenges to overcome prior to clinical application. For example, our model demonstrates that the concentration gradient is only stable while concentrations in both source and sink compartments are kept constant. If the source and the sink concentrations are not maintained, the gradient will quickly diminish within 48 h. To translate this model to a device, we are developing an immobilized concentration gradient of neurotrophic factors in a three-dimensional hydrogel which may overcome challenges associated with the unstable concentration gradient discussed above. This well-defined diffusible cue-loaded hydrogel, in conjunction with contact-mediated cues,[5,23,42] may open a new horizon in the quest to enhance axonal regeneration after injury.

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APPENDIX A

To maintain a stable concentration gradient, it is imperative to keep the concentrations in both compartments constant. The stability of the concentration is characterized by:

$$\frac{\Delta C_t}{\Delta C_0} = 0.99 \tag{A1}$$

and

$$\Delta C_t = C_{t,\mathrm{h}} - C_{t,\mathrm{l}},$$
$$\Delta C_0 = C_{0,\mathrm{h}} - C_{0,\mathrm{l}},$$

where C_0 and C_t represent concentrations at times 0 and t, respectively; h and l represent high and low concentration compartments, respectively.

Equation (A1) illustrates that the change in the concentration difference between two solution replenishments would be less than 1%. Thus, for free diffusion across the agarose gel at time t:

$$(C_{t,\mathrm{h}} - C_{t,\mathrm{l}})/(C_{0,\mathrm{h}} - C_{0,\mathrm{l}}) = \mathrm{erf}\ \zeta, \tag{A2}$$

where erf ζ is an error function and is defined by:

$$\mathrm{erf}\ \zeta = \frac{2}{\sqrt{\pi}} \int_0^{\zeta} e^{-t^2} \mathrm{d}t. \tag{A3}$$

Combining Eqs (A1), (A2) and (A3),

$$\mathrm{erf}\ \zeta = \frac{2}{\sqrt{\pi}} \int_0^{\zeta} e^{-t^2} \mathrm{d}t = 0.99;$$

therefore, $\zeta = 1.85$ (cf. Ref. 1).

As

$$\zeta = \frac{L}{\sqrt{4Dt}} \tag{A4}$$

(cf. Ref. 11), where D is the diffusion coefficient of NGF in 1% agarose and L is the thickness of the agarose membrane, solve for time $t = 6.5$ h.

APPENDIX B

The membrane permeation model assumes that if: (i) the donor side of the membrane is held at a constant concentration, (ii) the receiver is held at zero concentration and (iii) the membrane is initially at zero concentration, then the total amount of the diffusing solute "m_i" that has passed through the membrane varies with time according to the following relation:

$$\frac{m_i}{LC_{gi}} = \frac{D_t}{L^2} - \frac{1}{6} - \frac{2}{\pi^2} \sum_{n=1}^{\infty} \frac{(-1)^n}{n^2} \exp\left(\frac{-D_i t}{L^2} n^2 \pi^2\right), \tag{B1}$$

where C_{gi} is the concentration of solute "i" on the gel side of the donor solution–gel interface, L is the thickness of the membrane and D_i is the diffusion coefficient of substance "i". At steady state, the exponential terms of the equation can be ignored and the equation reduces to:

$$m_i = \frac{D_i C_{gi}}{L}\left(t - \frac{L^2}{6D_i}\right). \tag{B2}$$

Since the interfacial concentration C_{gi} cannot be readily measured, it can be replaced with the known donor cell concentration C_{di}, via the definitions of the partition coefficient ($D_i C_{gi} = D_i K_i C_{di} = P_i C_{di}$):

$$m_i = \frac{P_i C_{di}}{L}\left(t - \frac{L^2}{6D_i}\right), \tag{B3}$$

where K_i is the partition coefficient and P_i is the permeability of "i". A plot of "m_i" versus time from this limiting equation [Eq. (B3)] is a straight line (when steady state is established) with a time-axis intercept of $L^2/6D_i$, from which D_i is calculated. The slope of the straight line

is $P_i C_{df}/L$, from which permeability is obtained. The partition coefficient K_i is then calculated from the definition:

$$K_i = P_i/D_i \qquad\qquad (B4)$$

(cf. Ref. 13).

As shown in Fig. 4, the time-axis intercept is 14.8 h (i.e. 5.4×10^4 s); thus, the calculated D_i is 7.8×10^{-7} cm²/s; P_i is calculated to be 6.8×10^{-7} cm²/s, from the slope of the straight line, and K_i is calculated, according to Eq. (B4), to be 0.9.