A new chemotaxis assay shows the extreme sensitivity of axons to molecular gradients

William J Rosoff^{1,4}, Jeffrey S Urbach^{2,4}, Mark A Esrick², Ryan G McAllister², Linda J Richards³ & Geoffrey J Goodhill¹

Axonal chemotaxis is believed to be important in wiring up the developing and regenerating nervous system, but little is known about how axons actually respond to molecular gradients. We report a new quantitative assay that allows the long-term response of axons to gradients of known and controllable shape to be examined in a three-dimensional gel. Using this assay, we show that axons may be nature's most-sensitive gradient detectors, but this sensitivity exists only within a narrow range of ligand concentrations. This assay should also be applicable to other biological processes that are controlled by molecular gradients, such as cell migration and morphogenesis.

A key hypothesis in developmental neuroscience is that axons are often guided to their targets by sensing molecular gradients¹⁻⁴. Precise measurements of axonal sensitivity to gradients in physiologically relevant environments are essential for understanding how gradients direct axons along particular trajectories in vivo, and for designing effective therapeutic interventions using guidance factors. Several quantitative gradient assays have greatly aided the study of chemotaxis in fibroblasts, leukocytes and neutrophils⁵⁻⁸. These assays, however, are generally unsuitable for studying axonal guidance, and previous approaches to quantifying axonal sensitivity to gradients have so far provided only limited information^{9–14}. Here we present a new technology that is compatible with the demands of long-term neuronal cell culturing and that allows for the efficient generation of precise, reproducible and arbitrarily shaped gradients of diffusible molecules. Using this technology, we show that growth cones, the motile sensing structures at the tips of developing axons, are capable of detecting a concentration difference as small as about one molecule across their spatial extent. Furthermore, we show that this sensitivity exists across only a relatively small range of ligand concentrations, indicating that adaptation in these growth cones is limited.

RESULTS

Gradient generation

We established gradients by 'printing' drops of solution in a a series of ten lines 1 mm apart with increasing amounts of chemotropic molecules onto the surface of a thin collagen gel (Fig. 1a). After a relatively short time, molecules diffuse to fill in the gaps between the printed lines and to create a profile that is independent of the depth. The resulting smooth gradient can be quite stable, as in general the time τ required for significant diffusion over a distance *L* scales as the square of distance, $\tau \approx L^2/D$, where D is the diffusion coefficient¹⁵. For nerve growth factor (NGF), the guidance factor used in these experiments, we have measured $D = 8 - 10^{-7} \text{ cm}^2/\text{s}$ in collagen (see Methods), which gives $\tau \approx 52$ min for L = 0.5 mm, the distance over which molecules must diffuse to fill in the space in between the lines or to reach the midplane of the gel (where the axons are growing), but $\tau \approx 3.6$ d for L = 5 mm, the horizontal distance from the center to the end of the printed pattern. The time-dependent concentration profile can be calculated using finite element modeling of the diffusion equation. Figure 1b,c shows the result of a two-dimensional simulation of an exponential gradient applied to a gel, appropriate to the situation depicted in Figure 1a, assuming no significant variation in the direction parallel to the printed lines. The printed molecules diffused quickly into the thin gel, and the concentration rapidly became independent of depth (Fig. 1b). The initial oscillations along the length of the block quickly died away, followed by a long period of a relatively stable gradient, particularly at the lowconcentration end (Fig. 1c).

The actual concentration gradients produced by this method can be measured with quantitative fluorescence imaging. Concentration profiles of fluorescently labeled casein for exponential and linear patterns are shown (Fig. 2a,b; casein is of similar molecular weight to NGF but is relatively inexpensive and easy to label). The actual timedependent concentration profiles of casein that were extracted from the fluorescence imaging show a good match with the results of the finite element modeling using an independently determined value of D = 6 10⁻⁷ cm²/s (Fig. 2c). This gradient generation method has several important advantages over previous approaches: large numbers of identical gradients can be generated quickly, the often precious chemotropic molecules are required in only very limited quantities, the gradients are established in a short time but can remain stable for a day or more, nonlinear gradient shapes can be used and gradients of multiple factors with different shapes and arbitrary spatial relationships can be generated. This technique could also be used to provide a source of factor while axons are growing, as no direct contact is made with the gel.

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Application to axon guidance

We have applied this technology to a well-characterized model system, the response of the rat dorsal root ganglion (DRG) to NGF^{9,16-18}. The sensitivity of DRG axons was measured by culturing explants in a three-dimensional collagen gel in the presence of an exponential NGF concentration gradient. This gradient shape produced a percentage change in concentration across the growth cone that was independent of its position in the gradient. Several explants were placed in a row that was parallel to the printed lines, between the third and fourth line of the pattern. This region of the gradient (between 1.2 and 1.3 cm) is expected to be the most stable (Fig. 1c). The overall NGF concentration was adjusted to be 1 nM at the location of the explants, independent of the steepness of the gradient (see Methods). As a simple way of quantifying the guidance response, we defined the guidance ratio as the number of bright pixels representing axons on the high side of the gradient minus the number on the low side, normalized by the total number of bright pixels representing axons (ref. 19; see Methods). If all neurites emerged from the explant on the high-concentration side, the guidance ratio would be 1.

Figure 1 Gradient generation technique. (a) Lines of chemotropic factor were printed onto the surface of a gel using a computer-controlled pump. By increasing the density of droplets from one line to the next and allowing the factor to diffuse into the gel, a smooth gradient was created. (b,c) Concentration profile at various times in a block of gel 3.5 cm long by 1 mm deep generated by finite element modeling. The initial pattern consisted of ten equally spaced lines 1 mm apart. The quantity of factor in each subsequent line was increased by a constant factor of 1.22 in this case, producing an exponential concentration gradient with a percentage change of 0.2% every 10 m. (b) Profile through the vertical thickness of the collagen. (c) Profile along the length of the collagen (least-concentrated line at 1.5 cm). After the initial transients have died away, a smooth profile remains. Although the concentration at the high end decays, at the low end both the concentration and gradient steepness remain relatively stable. The time for stabilization and eventual decay of the gradient is inversely proportional to the diffusion coefficient. For gradients steeper than 0.2%, the concentration at the low end of the gradient increases slowly over time as a result of diffusion from the region of higher concentration, and the gradient flattens somewhat.

We have measured the width of rat DRG growth cones in threedimensional collagen to be no larger than 10 m including filopodia (data not shown; see also ref. 20). We therefore defined the gradient steepness *s* as the percentage change in concentration across 10 m. The value of the guidance ratio after 36–40 h in culture for s = 0, 0.1, 0.2 and 0.4% is shown (Fig. 3a). There was an increase in the guidance ratio as the steepness of the gradient increased, and significant guidance occurred even for s = 0.1%. This was despite the fact that DRG explants are comprised of a heterogeneous population of neurons, not all of which are responsive to NGF²¹. Representative explants with guidance ratios close to the mean for each steepness are shown (Figs. 3d–f). As the gradient steepness increased, the asymmetry increased markedly.

These explants did not show obvious turning of neurites up the gradient (Figs. 3d-f), and a statistical analysis of turning for the data set reported in Figure 3a found no significant neurite turning overall. To determine whether the asymmetry in outgrowth as measured by the guidance ratio was simply the result of a trophic effect, whereby the higher concentration of NGF on the up-gradient side of the explant caused more undirected outgrowth than on the down-gradient side, we measured total neurite outgrowth as a function of absolute concentration of NGF (Fig. 3b). The curve is bell shaped with a peak at about 1 nM, consistent with earlier studies²². There was little change in outgrowth between 0.3 nM and 1 nM, but there was a significant decrease in outgrowth between 1 nM and 3 nM ($P < 10^{-5}$). In our gradient experiments, the difference in concentration between the two sides of each explant varied between about 10% (s = 0.1%) and 50% (s = 0.4%), which is small compared with that required for significant variations in outgrowth. In any case, the decrease in outgrowth between 1 nM and 3 nM indicates that the trophic effect of NGF by itself would actually produce a negative guidance ratio for concentrations around 1 nM.

To determine the range of concentrations for which growth cones are sensitive to shallow gradients, we established exponential NGF gradients of steepness s = 0.2% but with NGF concentrations at the explants in the range of 0.0001–100 nM (Fig. 3c). The guidance ratio curve is bell shaped with a peak at 1–10 nM, where the guidance is highly significant ($P < 10^{-7}$). The guidance ratio is more weakly significant for 0.1 nM (P < 0.005) and is not significant for 100 nM and for 0.01 nM and below. Unlike the 1 nM case described above, in the 10 nM case we saw clear neurite turning (turning strength = 0.023; P < 0.0001; Fig. 3g). Weaker turning was also observed in the 100 nM condition (turning strength = 0.015; P < 0.02) and the 0.1 nM condition (turning

Figure 2 Fluorescence imaging of casein concentration gradients. (a) Concentration profile determined from fluorescence intensity 1 h after printing a pattern of lines of exponentially increasing strength (s = 0.4%; ten lines 2 cm wide and 1 mm apart). (b) Concentration profile for a pattern of lines of linearly increasing strength 1 h after printing (ten lines 2 cm wide and 1 mm apart). (c) Comparison of the gradient shown in **b** with a numerical simulation. Points, measured concentration along the center of the linear gradients; lines, simulated values; 3 h, red; 24 h, blue.

strength = 0.010; P < 0.09, not significant). No significant turning was seen in other conditions. We conclude that these growth cones are unable to adapt their sensitivity to shallow gradients over a concentration range of more than about two to three orders of magnitude.

DISCUSSION

The minimum concentration difference across a motile cell that is required for directional sensing is typically quoted as 1-2% (refs. 23,24), although statistically significant responses for values as low as 0.5% have been found for neutrophils^{6,7}. Our results show that axons can be guided by gradients that are substantially shallower than this. This sensitivity is particularly remarkable considering the noisy environment in which the growth cone must operate. Statistical fluctuations in receptor binding limit the accuracy of any instantaneous measurement of concentration^{25,26}. For a growth cone with a diameter of 10 m in a 0.1% gradient at 1 nM, the number of molecules in the vicinity of half of the growth cone is on the order of 1,000, so the average difference in the number of molecules between the high- and low-concentration sides of the growth cone is about one molecule. We also found a guidance ratio significantly different from zero for s = 0.2% at 0.1 nM, an average difference of about one-fifth of a molecule across the growth cone. By contrast, the effects of Brownian motion will produce instantaneous fluctuations in the number of molecules of approximately the square root of 1,000 or about 30 at 1 nM. Overcoming this noise requires averaging over many independent measurements. A simple calculation indicates that growth cones would need to average concentration measurements for at least several minutes to achieve the sensitivity we have measured^{25,26}, but specific mechanisms by which this might be accomplished are unknown.

Turning of neurites in the gradient at a concentration of 1 nM was not seen. We believe that we have, however, observed a guidance effect in this case because the degree of asymmetry in outgrowth from the explants is much larger than one would expect given the small difference in NGF concentration between the two sides of the explant. One possibility is that turning predominately occurs before the neurites leave the explant. Neurite turning outside the explant was, however, strongly significant at 10 nM and more weakly significant at 100 nM. Thus, surprisingly, measureable turning and biased outgrowth are not always correlated and seem to be related in a nontrivial way. Dissociated neurons can also be used in our assay, which will allow the dependence of individual axon trajectories on gradient steepness to be probed more directly.

A recent study examining adaptation of gradient sensitivity of *Xenopus laevis* spinal axons to external ligand concentrations²⁷ measured the response of axons growing on a two-dimensional substrate to very steep gradients (5–10% over 10 m) over a relatively short period of time (1–2 h). Adaptation was probed by introducing relatively small step changes in ligand concentration in the fluid above the growth cone. In contrast, we have examined mammalian axons guided by very shallow gradients for long periods of time in a three-dimensional assay, more akin to the *in vivo* situation, and have varied ligand concentration at the growth cone over seven orders of magni-



tude. We find a similar bell-shaped dependence on concentration to that previously measured for leukocytes⁵, which can be explained theoretically by the effect of stochastic concentration fluctuations²⁸. As the peak is expected to be roughly centered at the dissociation constant, K_d , this provides indirect evidence that the effective K_d mediating guidance responses for the binding of NGF to its receptors trkA and p75 is about 1 nM (refs. 29,30). The relatively narrow window of concentration for effective guidance of axons indicates that there must be tight *in vivo* regulation of the absolute concentration of guidance factors present. Based on the less-quantitative experimental measurements of gradient sensitivity and the absolute concentration range that was previously available, we estimated the maximum distance an axon could be guided by a single gradient of exponential shape to be about 1 cm (ref. 31). Results from our more quantitative assay now extend this estimate to about 2 cm.

Our results show the power of the technology we have developed to quickly and reliably produce molecular gradients that can robustly guide axons. The exquisite sensitivity of axonal gradient detection



Figure 3 Guidance of DRG axons by NGF gradients. (a) Guidance ratio of DRG explants as a function of gradient steepness. Error bars are s.e.m. For s = 0.1%, the guidance ratios were significantly different from zero ($P < 10^{-8}$), and 80% of explants (43 of 54) had positive guidance ratios. Responses for s = 0.2% and 0.4% were also significantly different (P < 0.005). For s = 0.4%, 53 of 54 explants had positive guidance ratios (54 explants per condition pooled over three separate experiments; similar results were seen in each experiment). (b) Outgrowth in response to NGF after 36–40 h in culture, as measured by the number of bright pixels representing neurites, divided by the area of the explant (36 explants per condition pooled over three separate experiments). (c) Guidance as a function of absolute NGF concentration at the explants for s = 0.2%. The probability that the mean guidance ratio is zero in each case is given above each data point (54–108 explants per condition pooled over six separate experiments). Although the 1 nM condition represents equivalent conditions to the 0.2% condition in **a**, the guidance ratios in these two cases are not exactly the same as they are derived from a different series of experiments. (**d**-f) Typical explants from experiment in **a** for s = 0.1%, 0.2% and 0.4%, respectively (guidance ratios within 0.01, that is 5–10%, of the mean). The NGF gradient points upwards. (**g**) Complete row of explants for one dish from experiment in **c**, s = 0.2%, 10 nM. Clear turning of the neurites is apparent. Scale bars, 500 m.

demonstrates that axons have developed a perhaps uniquely effective mechanism for integrating guidance cues. This sensitivity, and its variation with absolute concentration, provide important constraints for the *in vitro* control of axonal trajectories and therapies for axonal regeneration after injury, and for hypotheses about how ligand gradients are transduced into signals for directed motion in growth cones.

METHODS

Tissue preparation. DRGs were removed from the lumbar region of P2 rat pups, trimmed and washed in DMEM and enzymatically digested for 12 min in 0.25% trypsin/10 g/ml DNAse1/Ca²⁺- and Mg²⁺-free Hanks Balanced Salt Solution to loosen the capsule. The reaction was stopped by addition of FBS, and the explants were centrifuged and resuspended in DMEM three times. Explants were then equilibrated in 0.2% liquid collagen at 4 °C before being plated.

Dry collagen gels. A 0.2% collagen gel solution was prepared under sterile conditions by mixing on ice type I rat tail collagen stock (Collaborative Biomedical Products) diluted with water to contain 0.2 mg/ml collagen, 27 l of a 7.5% sodium bicarbonate solution per ml of original collagen stock, 10 Optimem (Invitrogen) to a final concentration of 1, 50 U penicillin, 50 g/ml streptomycin. We uniformly spread 750 l of the solution over the

bottom of a 35-mm dish and allowed it to set, forming the bottom layer of collagen (~0.5 mm thick). We added a fresh layer of 750 l of collagen and then arranged six explants in a line in the dish before the collagen set (Fig. 3g). The final collagen culture was approximately 1 mm thick. The dishes were returned to the incubator for 15 min for the top layer of collagen to set and were then ready for molecular printing. No fluid culture medium was added on top of the collagen, as this would provide a short circuit for the gradient; we therefore refer to these as dry gels. No serum was added to the collagen. Dishes were returned for 36–40 h to a 37 °C incubator with 5% CO₂. We chose this time interval because it is typical of more standard collagen gel coculture experiments; it is unlikely that axons are exposed to a single gradient for this length of time *in vivo*.

Fixation and immunohistochemistry. Explants were fixed by covering the collagen with 1.5 ml of 10% formaldehyde and 0.1% Triton X-100 (J.T. Baker) in PBS for several hours. Explants were washed five times with PBS for 1 h each and then incubated overnight in an antibody directed against neuronal β -tubulin (TUJ1; Babco; 1:1,000), followed by a further five washes in PBS for 1 h each. The explants were then incubated overnight in the secondary antibody Alexafluor 488–conjugated goat anti-mouse IgG (Molecular Probes; 1:1,000), washed five times in PBS for 1 h each and photographed with a CCD (charge-coupled device) camera on a Nikon TE300 inverted fluorescence microscope.

Molecular printing. For printing, we used a commercially available pump (Gesim) that can deliver precisely repeatable nanoliter droplets at rates of up to 1,000 drops/s. A dish containing the gel was mounted on a computer-controlled commercially available high-precision x-y translation stage. The spacing between drops can be controlled by varying the rate of droplet ejection and the rate of translation of the x-y stage. Gradients were established within the middle 10 mm 20 mm of a 35-mm culture dish in the manner suggested in Figure 1a. The gradient was positioned so that the line of explants was between the third and fourth line of the gradient. To produce an exponential gradient with steepness s per 10 m, the number of drops in each successive line increased by a factor of e^{100s} . This factor is 1, 1.11, 1.22 and 1.49 for steepnesses s = 0, 0.1, 0.2 and 0.4%, respectively. The droplet volume was measured to be 1.4 nl. For Figure 3a, the solution in the pump was 117 nM NGF (Roche Diagnostics) in PBS. The number of drops in the first line of the gradient was adjusted so that the concentration at the explant position was 1 nM after the initial concentration variations smoothed out. The total volume of fluid deposited on each line for every condition was equalized by the application of the appropriate amount of the vehicle solution (PBS) after the deposition of the gradient of NGF solution. In addition, a 5-mm plateau of the vehicle solution was added adjacent to the low-concentration side of the gradient. For Figure 3c, procedures were the same, except that the concentration of NGF in the pump was varied to achieve the desired concentration at the explant position.

Statistical analysis. Fluorescent images of TUJ1-stained neurites were processed using Scion Image (Scion Corporation) to outline the explant. The image containing neurites outside the explant was then thresholded to give a binary image. The total number of nonzero pixels, divided by the number of pixels contained in the explant, was used to calculate outgrowth (Fig. 3b). The guidance ratio R (Fig. 3a,c) was determined from the number of nonzero pixels on the high-concentration side of the explant, H, and the number on the low-concentration side, L, according to R = (H - L)/(H + L). Because this is normalized by total neurite outgrowth, it is expected to be relatively insensitive to total outgrowth. We quantified turning by detecting neurite segments in the explant images using a ridge-tracking algorithm that is similar to one presented previously³², although we developed ours independently. The turning vector associated with each neurite segment was calculated as the difference vector between a unit vector that is parallel to the segment and the radial unit vector measured from the explant center. The deflection of the neurite up the gradient was measured as the component of the turning vector along the gradient, and the average of these components across all explants was defined as the turning strength for that condition. Significance values quoted for the guidance ratio and turning strength give the probability that the mean of the distribution is zero, as determined by a *t*-test.

Measurement of diffusion coefficients. Using the micropump, a single line of the diffusant was delivered onto a 1-mm layer of collagen in a 35-mm petri dish. The concentration profile along the length of the gel perpendicular to the line was measured by fluorescence imaging of either directly labeled protein (casein, Sigma; labeled with Oregon Green488 FluoReporter kit, Molecular Probes) or immunofluorescence labeling of fixed protein (NGF). The narrow line spreads out into a Gaussian concentration profile and the width of the Gaussian evolves linearly in time, with a slope determined by *D*. The profile measured at each time was fit to a Gaussian curve, and *D* was determined from a linear fit to the width versus time.

Finite element modeling. The simulations shown in Figures 1b,c and 2c were produced by two-dimensional finite element modeling (PDEase2D, Macsyma Inc.) of the diffusion equation in a domain of 3.5 cm - 0.1 cm, with no flux boundary conditions. The printing of the lines was modeled by a constant flux for a brief period (10 s) in narrow regions (0.01 cm) along one of the long edges of the domain.

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COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

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The production process introduced several errors into Fig. 3c. A minus sign was omitted from the negative value of "0.05" on the y-axis, and the label for the x-axis incorrectly stated the concentration as " $(nM \times 10^{-1})$ "; it should read "(nM)". Furthermore, the image quality of the micrographs in Fig. 3d–g was poor due to color conversion problems. The corrected Fig. 3 is shown below.



Figure 3 Guidance of DRG axons by NGF gradients. (a) Guidance ratio of DRG explants as a function of gradient steepness. Error bars are s.e.m. For s = 0.1%, the guidance ratios were significantly different from zero ($P < 10^{-8}$), and 80% of explants (43 of 54) had positive guidance ratios. Responses for s = 0.2% and 0.4% were also significantly different (P < 0.005). For s = 0.4%, 53 of 54 explants had positive guidance ratios (54 explants per condition pooled over three separate experiments; similar results were seen in each experiment). (b) Outgrowth in response to NGF after 36–40 h in culture, as measured by the number of bright pixels representing neurites, divided by the area of the explant (36 explants per condition pooled over three separate experiments). (c) Guidance as a function of absolute NGF concentration at the explants for s = 0.2%. The probability that the mean guidance ratio is zero in each case is given above each data point (54–108 explants per condition pooled over six separate experiments). Although the 1 nM condition represents equivalent conditions to the 0.2% condition in a, the guidance ratios in these two cases are not exactly the same as they are derived from a different series of experiments. (d–f) Typical explants from experiment in a for s = 0.1%, 0.2% and 0.4%, respectively (guidance ratios within 0.01, that is 5–10%, of the mean). The NGF gradient points upwards. (g) Complete row of explants for one dish from experiment in c, s = 0.2%, 10 nM. Clear turning of the neurites is apparent. Scale bars, 500 µm.