ceptors are bound by transmitter, a high probability of opening would guarantee large, localized postsynaptic calcium transients and thus facilitate the activation of calcium-dependent processes such as long-term potentiation (*29, 30*).

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15. Experiments were performed on hippocampal neurons from 1- to 3-day-old neonatal rats maintained in primary cell culture for 1 to 3 weeks [as described (*7, 8*)]. Outside-out patch recordings (Axopatch-1C, Axon Instruments, Foster City, CA) from hippocampal neurons were obtained with the use of pipettes containing (in mM): cesium or sodium gluconate or methanesulfonate, 140; NaCl, 10; Hepes, 10; EGTA, 10; and Mg adenosine triphosphate (ATP), 4; adjusted to pH 7.2 with CsOH or NaOH. Control external solutions contained (in mM): NaCl, 160; KCl, 3; Hepes, 5; glycine, 0.02; and CNQX, 0.002; adjusted to pH 7.4 with NaOH. External Ca^{2+} was 0.2 mM to help prevent slow rundown of NMDA receptor channel currents. External solutions were gravity fed into each of the four lumens of four-barreled glass tubing (Vitro Dynamics, Rockaway, NJ), which was pulled to an overall tip diameter of about 200 μm. The patch was positioned within 50 μm of the tip, near the interface formed between the continuously flowing control and drug solutions. The solution exchange was made by rapidly moving the solution interface across the tip of the patch pipette using a piezoelectric translator (Physik Instrumente, Waldbronn, Germany, model P245.30) attached to the flow tube. Outside-out patches were clamped at −60 mV, and the currents were low pass-filtered at 0.5 to 1.0 kHz and digitally sampled at 1 to 2 kHz. Open time histograms were from recordings filtered at 2 kHz and sampled at 20 kHz. High purity salts were obtained from Aldrich Chemical (gold label). Other chemicals were obtained from Sigma except for CNQX (Cambridge Research Biochemicals and Tocris), glycine (Bio-Rad, Richmond, CA), and MK-801 (Merck Sharp and Dohme Research Center). The speed and completeness of solution changes were tested at the end of each recording by "blowing out" the membrane and monitoring the open tip current caused by differences in liquid junction potentials in the control and drug solutions (the drug solution was diluted with water 50:1). Experiments were performed at room temperature (25°C).
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20. The probability that a bound channel opens before it becomes unbound ($P_{o(total)}$) was estimated from $P_{o(total)} = 1 -$ (charge transfer after MK-801)/(charge transfer before MK-801).
21. At 10 μM MK-801, the open time histogram was described better by a double rather than a single exponential. The mean open time used in Fig. 3B for 10 μM was therefore the arithmetic mean of the measured open durations (half-height criteria), which is a slight overestimate of the true mean open time; thus, the slope of the fit (rate constant r) is slightly steeper than it should be ($\alpha = 324$).
22. The probability of channel opening at the peak of the current is $P_{o(peak)} = N_p/N_t$, where N_p is the number of channels open at the peak and N_t is the total number of channels in the patch. $N_p = I_p/i$, where I_p is the amplitude of the peak current and i is the single-channel current amplitude. Because the number of channels that are opened, on average, by a single application of L-glutamate is $N_o = N_t P_{o(total)}$, then $N_t = N_o/P_{o(total)}$. The mean charge transfer evoked by applications of L-glutamate before MK-801 is given by $Q_{(control)} = iTON_o$, where TO is the mean total amount of time that channels that do open spend in the open state before they become unliganded, and thus $N_o = Q_{(control)}/(iTO)$. TO can be estimated as $TO = Q_{(control)}/(Q_{MK}r[MK\text{-}801])$, where Q_{MK} is the charge transfer in the presence of MK-801. Thus, $P_{o(peak)} = (I_p P_{o(total)})/(Q_{MK}r[MK\text{-}801])$. TO was calculated to be 91.1 ± 8.7 ($n = 15$).
23. Using a model that can approximately account for NMDA receptor channel activation and desensitization by a number of agonists (*8*), we fitted control responses to L-glutamate and those in MK-801 by assuming a $P_{o(peak)}$ of 0.3 and a blocking rate of 22 $\mu M^{-1}\ s^{-1}$.
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Axon Guidance by Gradients of a Target-Derived Component

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Spatial gradients of axon guiding molecules have long been suspected to provide positional and directional cues for retinal ganglion cell axons growing within the optic tectum. With the identification of a guiding activity from tectal cell membranes, it has become possible to investigate the potential physiological significance of molecular gradients for retinal growth cone behavior in vitro. A subset of retinal growth cones, those from the temporal half, were highly sensitive to small concentration changes of the guiding component. The degree of response was correlated with the strength of the gradient. These findings demonstrate that the neural growth cone can read gradients of surface-associated information.

DURING NERVOUS SYSTEM DEVELopment elongating axons can be guided to their respective targets by chemical cues (*1*). Concentration gradients of specific molecules have been recognized as a plausible means to generate directional information for axonal pathfinding. Chemotropic factors, for example, could be secreted by the target region to form a diffusion gradient within which the motile tips of the axons, the growth cones, may orient (*2*). Alternatively, local positional cues could be differentially distributed along axonal pathways (*3*). The retinotectal map could also be generated by a similar mechanism (*4*). Retinal ganglion cells project their axons to the optic tectum so that their terminations form a reversed image of the retina. The temporal half of the retina projects to the anterior part of the tectum, the nasal retina to the posterior tectum. Precise mapping also occurs along the dorsoventral axes.

Although it is now widely accepted that retinotectal specificity is based on "chemoaffinity" (*5*) between retinal growth cones and their target cells, the cellular mechanisms governing this process are unknown. Anatomical data suggest that there is a long-range guidance of growth cones within the tectum that could well be achieved by chemical gradients (*6*). Theoretically, a small set of graded cues in the tectum could specify both direction and target position, provided that the retinal growth cones carry differential sets of corresponding receptors (*7*). Growth cone steering may depend on the functional equivalents of attractive (pulling)

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and repulsive (pushing) effects imposed on growth cones by guiding molecules in their environment (7).

A repellent axon guiding activity that is gradedly distributed along the anterior-posterior axis of the embryonic chick tectum has been identified in vitro (*8, 9*) and attributed to a glycoprotein component of tectal cell membranes (*10*). When retinal axons are offered the choice of growing on posterior or anterior tectal cells (*8*) or their membranes (*9*), fibers from the temporal half-retina are repelled by posterior material and grow preferentially on substrate composed of anterior cells or membranes. In the previous "choice" assays, nasal axons did not show any preference for posterior material (that is, their natural target) (*11*).

Negative effects on axon growth (as those observed in the choice assays) can, in principle, be achieved in three ways: (i) by the absence of growth-permissive substrate or by less adhesive substrate (*12*); (ii) by the presence of "antipermissive" (inhibitory) components (*13*); or (iii) by a more complex form of repulsion that allows for a flexible response of a growth cone. In fact, the latter possibility seems to be the case. Nasal and temporal fibers grow almost equally fast on purely anterior or purely posterior membranes (*14*). So the axons' capability to elongate is not impaired by an even distribution of the repellent component. Only concentration differences alter axonal growth and do so by affecting primarily direction rather than speed (*14*).

What happens if growing retinal fibers encounter smooth transitions from one membrane species to the other? To investigate this, we produced membrane carpets that contain spatially defined membrane gradients, spanning from purely anterior to purely posterior material, and observed fiber outgrowth from retinal explants (Fig. 1, A through D). In a first approach, the whole surface of the carpet was made available for outgrowth (Fig. 1E). Under these conditions, temporal growth cones reach the transition zone (the gradient field) and then actively evade increasing concentrations of the repellent component by turning aside or even growing back. Studies in this type of assay, however, are impaired by a severe problem: Axons growing on tectal membranes (as on other two-dimensional surfaces) exhibit an intrinsic tendency to elongate in a large directed ("clockwise") curve, even in the absence of external guiding factors (*15*). Nonetheless, the strong avoidance reaction is to be attributed to the capacity of temporal growth cones to detect graded transitions from anterior to posterior material.

For a closer examination of the gradient's effect a modified assay was developed (Fig. 1F). Here, retinal axons elongate constrainedly in narrow, parallel lanes, separated one from another by membrane-free, nonpermissive stripes. Within each of the "membrane corridors" we have laid down a complete membrane gradient. After growing for a certain time within these lanes from purely anterior membranes to increasing posterior membranes, temporal fibers are significantly shorter than nasal fibers (Fig. 2). Nasal fibers reach control lengths, similar to those observed on homogeneous membrane substrates. This demonstrates that continuous exposure to a repellent gradient impairs the elongation of temporal fibers. A sharp transition from uniformly temporal to uniformly nasal response properties is seen at the midline of the retina (Fig. 2). This finding parallels other reports on position-specific properties along the nasal-temporal axis of the retina (*9, 16*). Because this repulsive effect can be abolished by pretreatment of posterior membranes with phosphatidylinositol-specific phospholipase C (PI-PLC)

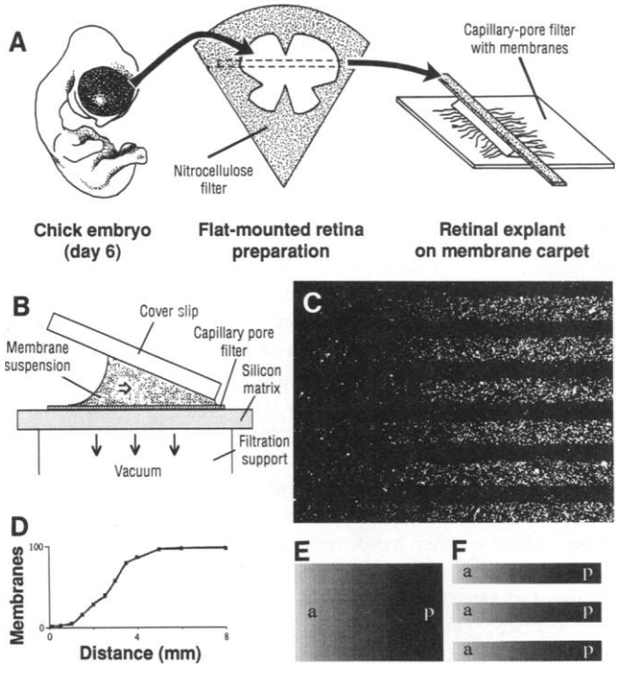


Fig. 1. In vitro experiments on axon guidance. (**A**) Experimental procedure. Retinal explants of nasal and temporal origin are derived from flat-mounted retinas (embryonic day 6) and placed on differently patterned membrane carpets (from embryonic day 9 tecta) (*10*). Experiments are stopped by fixation of cultures with 4% paraformaldehyde, 0.33 M sucrose in phosphate-buffered saline (PBS). (**B**) Gradient production. An inclined glass cover slip holds a small drop of membrane suspension (100 to 500 μg of protein per milliliter) on top of a capillary pore filter. The filter is placed on a silicon matrix that is attached to a filtration support. When suction is applied (three arrows), the drop quickly decreases in volume, and because of its surface tension, contracts in the direction indicated by the open arrow. This continues until the underlying filter is plugged by membrane particles. These particles form a spatial gradient, which for technical reasons is of sigmoid shape (D). The superfluous suspension and the cover slip are removed. The second membrane species is added and sucked onto the filter. By plugging the remaining pores, these membranes form a symmetrical countergradient. (**C**) Photograph of a gradient. A representative detail of a filter containing separate membrane lanes is shown (F). These "corridors" can be formed through a special silicon matrix containing parallel channels (*10*). The distribution of one membrane species is visualized with fluorescent beads (Covaspheres, Duke Scientific) that were added to the respective membrane suspension before filtration. Each lane and each interspace is 90 μm in width. (**D**) Evaluation of the gradient. We determined the spatial distribution of membranes by counting the fluorescent beads (C) with digitized image-processing. Same scale as (B). (**E** and **F**) Two types of gradient assays. The membrane gradients are indicated with gray-scale values (anterior zone: bright; posterior zone: black; no membranes: white). In (E) a coherent area is made available for axon growth, whereas in (F) [as in (C)] fibers are confined to narrow "corridors."

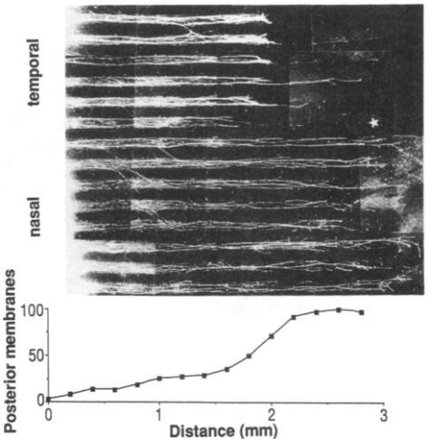
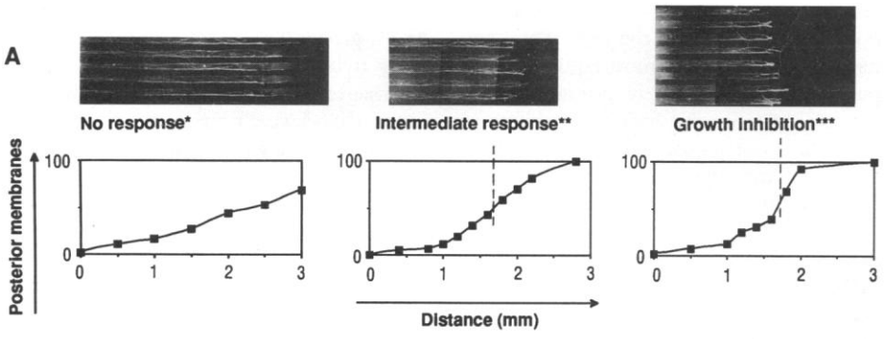


Fig. 2. Temporal and nasal response to a gradient. Nasal and temporal axons [above; stained with the fluorescent dye rhodamine-isothiocyanate (*10*)] grow out on parallel membrane lanes from one and the same explant strip. Each lane contains an "uphill" gradient, which is similar to the one depicted below at the same distance scale. (This special gradient was measured in the lane marked by *.) The transition zone from gradient-sensitive (temporal) to gradient-ignoring (nasal) properties extends over at most one lane (90 μm).



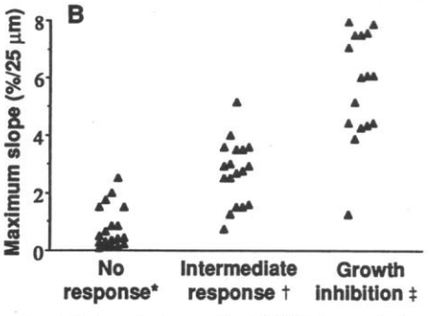


Fig. 3. (**A**) Fiber repulsion by differently shaped gradients. Photographs show representative examples of outgrowth patterns in response to a shallow, an intermediate, and a moderately steep gradient (from left to right). Corresponding gradients are depicted at the same distance scale. Sites of maximum slope within the gradient are indicated if relevant for classification. Fiber outgrowth is more reduced in steeper gradients. (**B**) Distribution of response modes in various gradients. Each dot represents a single gradient situation (N = 53). The graph shows that the degree of response (as classified according to the scheme below) is correlated to the maximum slope of the gradient. Calculation of slopes: Concentrations are set at 0 in anterior and at 100% in posterior membranes; slopes are then given as concentration differences per distance; for convenience, an average growth cone's diameter (25 µm) was taken as the distance unit. *No response, fibers reach distances comparable to that found on homogeneous substrates. Although outgrowth normally extends to about 2.8 mm within 48 hours, 2.5 mm was chosen as the lower limit for this classification. †Intermediate response, fibers are reduced in length (shorter than 2.5 mm), but have passed the steepest part of the gradient by at least 200 µm. ‡Growth inhibition, the fiber front has not traversed the gradient's steepest part (that is, it is found at the site of maximum slope within the gradient).

(*17*), as has been reported for the original choice assay (*14*), we assume that the molecules involved in both assays are identical. Fiber lengths are only changed if the concentration of posterior membranes increases along the axonal tracks, that is, in an "uphill" gradient. Axons growing along a "downhill" gradient from purely posterior to purely anterior substrate are not detectably affected in their growth rates. Thus, temporal growth cone behavior in gradients depends on the "sign of the slope."

Outgrowth of temporal fibers along uphill gradients varies with the shape of the gradient (Fig. 3A). A number of experiments were performed to extract the critical parameters of the gradient that determine how far axons can elongate. The observation time was held constant at 48 hours for all of the experiments to obtain comparable fiber lengths. A set of quantities considered to be likely determinants of axon growth does not correlate to fiber length or position of the fiber front. These include absolute posterior concentrations, up to which fibers extend, average concentrations or average slopes along axonal paths, and position of the retinal explant relative to the gradient. However, degree of fiber repulsion is correlated to the maximum slope (*18*) of the gradient (Fig. 3B). This slope dependence, together with the observation that temporal fibers can in principle grow on any concentration of posterior components, leads to the conclusion that the strength of the gradient (and not absolute concentrations) affect growth cone behavior. Although it is not known if reduced fiber lengths result from a reduced speed of growth or from stoppage of growth (or from both), the observed slope response correlation can best be interpreted as follows: Below the slope of 1% per 25 µm there is no detectable response of axons. On the other hand, temporal axons stall in gradients steeper than about 5% per 25 µm and cannot traverse them (in 10 out of 11 cases) even when exposed to the gradient for about two-thirds of the duration of the experiment (48 hours). Between these two extremes (no response versus growth inhibition), intermediate responses are almost exclusively (in 16 out of 18 cases) observed in gradients from 1 to 5% per 25 µm. This points to a continuum in behavioral repertoire. Although these findings suggest that growth cone repulsion depends primarily on the slope of the gradient, it seems likely that this slope dependence changes with the concentration range investigated.

It is now well established that growth cones can actually be steered by extracellular gradients. For example, a locally applied source of nerve growth factor induces turning of regenerating sensory axons (*19*). Although, in this case, calcium ions and adenosine 3′,5′-monophosphate (cyclic AMP) have been implicated in the intracellular response (*19*), nearly nothing is known of how the gradient reading is accomplished. A plausible model has to incorporate the fact that in a long-distance gradient the relative concentration differences that a growth cone can resolve are extremely small. For diffusible chemotropic factors that can orient axon growth in vitro, the upper limit for the distance between source and growth cone was reported to be 300 to 500 µm (*2*). As demonstrated here, gradients of surface-associated molecules can extend over longer distances. A 1% change over 25 µm corresponds to a linear gradient of 2.5 mm (from 0 to maximum).

Possibly, a growth cone could detect differences in molecule numbers by comparison of receptor occupancy over its extensions. Such a spatial gradient-reading mechanism would either imply contrast enhancement (7), or modulation of sensitivity through an adaptation process (*20*). Both versions demand a reliable intragrowth-cone–signal processing converting minute extracellular concentration differences into a (transiently) stable spatial polarity inside the growth cone. Similar models are under debate in chemotaxis of cellular slime molds, leukocytes, and other eukaryotic cell types (*21*). However, it is possible that growth cone guidance is accomplished by a memory-based cellular strategy comparing outside concentrations with time rather than with space, virtually similar to bacterial chemotaxis (*22*). A decision on these alternatives will be made by the characterization of the intracellular interpreter of outside gradients.

Repellent gradients of the kind produced in this study may well exist in the developing chick tectum. If a natural gradient ranged from 0 at the anterior pole to 100% at the posterior pole, then its slope would be in the same order of magnitude as its artificial counterpart found to be effective in vitro (at least 1% per growth cone diameter). More striking than this coincidence is the finding that the growth cones behave as if they are tuned to detect gradients better than steps. At a sharp boundary between anterior membranes and a mixture of anterior and posterior materials, a response is only seen if the mixing ratio (posterior to anterior) is at least one to four. Thus, in this situation, a concentration difference of at least 25% per growth cone diameter is required to evoke a measurable reaction. Taken together, our

results demonstrate that an immobilized gradient represents adequate conditions for axon guidance. It remains to be seen how the fine-grained mapping of retinotectal connections is achieved, be it by additional gradients or by other mechanisms.

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"As soon as principal Thorndyke comes back, we'll be moving along. By the way, just where did he go?"