−0.574, $P = 0.00655$). The point can be expressed in less abstract terms: where occupied cells at a coarse scale occur in dense clusters, each cell is likely to include a relatively large number of populations or occurrences at finer scales, but where coarse-scale cells are sparsely scattered, each is likely to contain only one or a very few finer scale records (Fig. 3).

Scale-area curves may provide a useful descriptive and predictive tool in the study and management of species abundance. Current conservation prioritization schemes often rely on arbitrary scales of analysis; British red data lists, for example, are based on abundance measured at a 100-km^2 scale, but the ranking of species could be quite different if they were analyzed at a different scale (*10*). The use of scale-area curves (or parameters fit to them) would allow more robust prioritization and would permit explicit consideration of different forms of rarity (*2*) in conservation decision-making. Furthermore, if the patterns documented here hold across a wider range of species and scales, it may be possible to extrapolate these curves to estimate abundance at scales that would otherwise be difficult or impossible to study.

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8. If the log area occupied at "coarse" (2500 km^2), "moderate" (100 km^2), and "fine" (4 km^2) scales are represented as A_c, A_m, and A_f, respectively, and if both scale transitions are equal in magnitude (in this case, 25-fold), the predicted fine-scale value with a linear scale-area curve can be expressed as: $A_F = 2A_M - A_C$.
9. The computation of probabilities is complicated by inbuilt positive relationships across scales: a species cannot occupy fewer cells or more area at a fine scale than it does at a coarse scale of analysis. Where possible, supplementary analyses have been added to permit probability estimates. The analysis of scale-area curve slopes, for example, has an in-built negative correlation (due to a shared 100-km^2 scale term that affects one slope positively and the other negatively), making the observed positive relationship and probability value conservative estimates. The accuracy of model predictions is measured throughout using a technique analogous to an R^2, but comparing observed values to predictions, rather than to lines of best fit. The fraction of variance explained is expressed as $(SSY - SSE)/SSY$, where $SSY = \Sigma(Y_i - \bar{Y})^2$, $SSE = \Sigma(Y_i - \hat{Y})^2$, and $\hat{Y}$ is the model prediction.
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12. *Gladiolus* field work was supported by the Centre for Population Biology, Imperial College, and benefited from the co-operation of the Forestry Commission and English Nature. I thank A. Shmida and J. Antonovics for raising the question, J. H. Lawton for raising the challenge, and G. H. Orians for inspiration. P. D. Sasieni, the Joint Nature Conservation Committee, and two anonymous referees provided invaluable assistance and advice.

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Conversion of Neuronal Growth Cone Responses from Repulsion to Attraction by Cyclic Nucleotides

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Nerve growth is regulated by attractive and repulsive factors in the nervous system. Microscopic gradients of Collapsin-1/Semaphorin III/D (Sema III) and myelin-associated glycoprotein trigger repulsive turning responses by growth cones of cultured *Xenopus* spinal neurons; the repulsion can be converted to attraction by pharmacological activation of the guanosine 3′,5′-monophosphate (cGMP) and adenosine 3′,5′-monophosphate signaling pathways, respectively. Sema III also causes the collapse of cultured rat sensory growth cones, which can be inhibited by activation of the cGMP pathway. Thus cyclic nucleotides can regulate growth cone behaviors and may be targets for designing treatments to alleviate the inhibition of nerve regeneration by repulsive factors.

The development of specific connections between neurons and their targets is determined in part by selective pathway choices made by growing axons, which are directed by guidance factors present in the embryo (*1*). These factors may exert either attractive or repulsive action on the extension of axonal growth cones (*1, 2*). There is evidence that attractive and repulsive responses might be mechanistically related. Attractive responses to netrins, mediated by the DCC/UNC-40 family of proteins, can be converted to repulsion by coexpression of proteins of the UNC-5 family (*3*). In addition, attractive effects of brain-derived neurotrophic factor (BDNF) and netrin-1 on *Xenopus* spinal neurites in culture can be converted to repulsion by inhibition of protein kinase A activity (*4, 5*). That a conversion (rather than an inhibition) of the response can occur suggests that some of the same cytoplasmic components may be used for both attractive and repulsive responses. This also raised the question of whether the action of repulsive factors can be converted to attraction.

Collapsin-1/semaphorin III/D (Sema III), a diffusible member of the semaphorin family, can repel or cause collapse of growth cones in culture (*6*). Defects in Sema III knockout mice suggest that Sema III creates exclusion zones for axons and drives axonal fasciculation through surround repulsion (*7*). We analyzed the effect of a microscopic gradient of Sema III on growth cones of cultured *Xenopus* spinal neurons. Sema III–containing saline was applied in pulses from a micropipette positioned 100 μm from the center of the growth cone and at a 45° angle with

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respect to the original direction of neurite extension (Fig. 1) (*8*). Most growth cones grew away from the pipette (Fig. 1, A to C). The repulsive response was dose-dependent with a minimal response occurring at an effective concentration of about 10 ng/ml at the growth cone (*8*). Heat-inactivated Sema III was ineffective (Fig. 1G). The repulsive turning was initiated by active protrusion of filopodia in the direction away from the pipette, with no obvious growth cone collapse during the turning process (*9*). The rate of neurite extension was unaffected by the presence of the Sema III gradient.

When 8-bromo-cGMP (8-Br-cGMP) (*10*) or Sp-cGMPS (*11*), a membrane-permeable agonist of endogenous cGMP signaling pathways, was present in the culture medium, nearly all growth cones turned toward rather than away from the pipette in the same Sema III gradient (Figs. 1, D to F, and 2A). Protoporphyrin-9 (PP-9), a guanylate cyclase activator (*12*), had a similar effect (Fig. 2A). Application of a nitric oxide (NO) donor, *S*-nitroso-*N*-acetylpenicillamine (SNAP), which may activate soluble guanylate cyclase by releasing NO (*13*), abolished the repulsive turning response without causing a significant attractive response. On the other hand, bath application of Rp-cGMPS (*11*), a cGMP antagonist and a specific inhibitor of protein kinase G, did not affect the growth cone response. Thus, cGMP regulates the direction of growth cone turning induced by Sema III. NO and cGMP may regulate establishment of the central connections of developing retinal axons and stimulate synapse formation of developing and regenerating olfactory neurons (*14*). In contrast to the effect of cGMP analogs, we found that adenosine 3′,5′-monophosphate (cAMP) analogs had no significant effect on the repulsion induced by Sema III gradients (Fig. 2B). However, the cAMP antagonist Rp-cAMPS, but not agonist Sp-cAMPS (*15*), blocked conversion of the turning response in

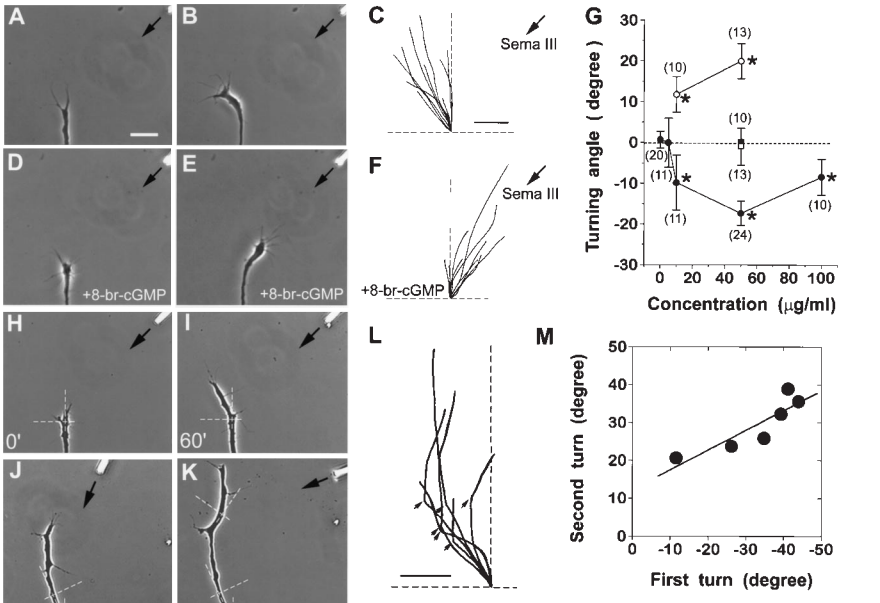

Fig. 1. Repulsive and attractive turning responses of *Xenopus* growth cones induced by Sema III. A gradient of Sema III (50 μg/ml in the pipette) was applied in normal culture medium (**A** to **C**) or in medium containing 100 μM 8-Br-cGMP (**D** to **F**). Images of the growth cone are shown at the onset (A and D) and at the end of 1 hour (B and E). Bar in (A) = 20 μm. Superimposed traces (C and F) depict the trajectory of neurite extension during the 1-hour period for a random sample of 10 neurons. Origin represents the center of the growth cone at the onset, with the original direction of neurite extension shown as vertical. Arrows indicate direction of the gradient. Bar in (C) = 10 μm. (**G**) Dependence of turning responses on the Sema III concentration in the pipette. Turning angles (mean + SEM) (*8*) in normal medium (filled symbols), in medium containing 8-Br-cGMP (100 μM; open symbols), and for heat-inactivated Sema III (squares). Total number of neurons examined is shown in parentheses. *, Significant difference from the data set at zero concentration ($P < 0.05$; Kruskal-Wallis test). (**H** to **K**) Conversion of Sema III–induced turning responses in the same neuron. A neuron was exposed to the same Sema III gradient (50 μg/ml in the pipette) for 1 hour (H and I). The pipette was then repositioned to 100 μm away from the center of the growth cone and at 45° with respect to the new direction of neurite extension, and the same gradient was applied for another hour in the presence of 100 μM 8-Br-cGMP (J). After the second hour, 8-Br-cGMP was washed away, the pipette was repositioned, and the same gradient was applied for the third hour (K). Number refers to time (minutes) after the onset of the experiment. Dashed lines depict reference frame for pipette positioning. (**L**) Superimposed traces of the trajectories of neurite extension from six neurons for which sequential repulsive and attractive turning responses have been examined (same as H to J). Arrow marks end of the first hour. Bar = 10 μm. (**M**) Correlation of turning angles induced by a Sema III gradient in normal medium (first turn) and in medium containing 8-Br-cGMP (second turn) for the same neuron [same data set as in (L)]. Line represents best linear fit of the data ($r = -0.88$; $P = 0.02$).

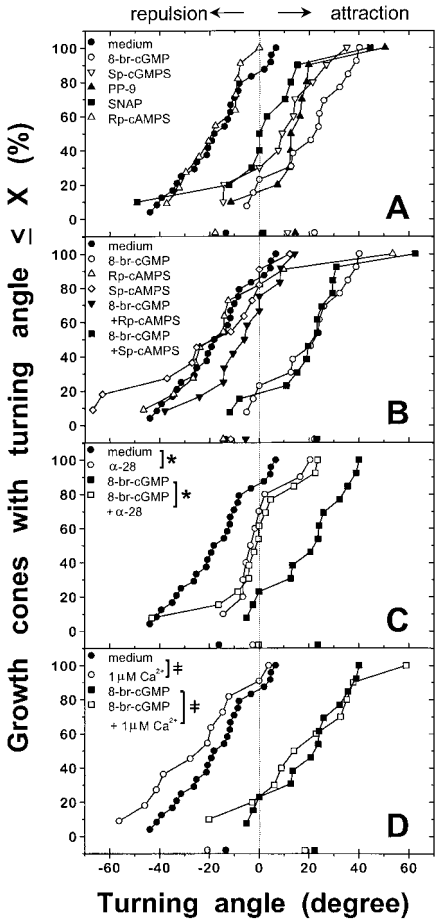

Fig. 2. Growth cone turning in a gradient of Sema III. (**A**) Effects of manipulating cGMP-dependent activities. Angular positions of all growth cones at the end of the 1-hour exposure to a Sema III gradient (50 μg/ml in the pipette) are shown in a cumulative distribution plot for the following conditions: normal culture medium, medium containing 8-Br-cGMP (100 μM), Sp-cGMPS (10 μM), PP-9 (10 μM), SNAP (300 μM), and Rp-cGMPS (10 μM). Isolated symbols along the abscissa are median values for corresponding data shown above. (**B**) Effects of manipulating cAMP-dependent activities. Shown are distributions of turning angles for the following conditions: normal culture medium, medium containing 8-Br-cGMP (100 μM), Rp-cAMPS (20 μM), Sp-cAMPS (20 μM), both 8-Br-cGMP (100 μM) and Rp-cAMPS (20 μM), or both 8-Br-cGMP (100 μM) and Sp-cAMPS (20 μM). (**C**) Distribution of turning angles in the absence or presence of α-28 (20 μg/ml) in normal medium and in medium containing 8-Br-cGMP (100 μM). *, Significant difference ($P < 0.01$; Kolmogorov-Smirnov test). (**D**) Effects of reducing $[Ca^{2+}]_o$ on turning responses in a Sema III gradient. Because of increased growth rate, the turning in 1 μM $[Ca^{2+}]_o$ was assayed 30 min after the onset of the gradient. Distribution of turning angles in normal (1 mM) or low (1 μM) Ca^{2+} medium in the absence or presence of 8-Br-cGMP (100 μM). ‡, Not significantly different ($P > 0.2$; Kolmogorov-Smirnov test).

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the presence of 8-Br-cGMP (Fig. 2B), which suggests that there is some interaction between cAMP- and cGMP-dependent pathways in these neurons.

The opposite turning responses are not due to behaviors of different types of neurons in these *Xenopus* cultures (*16*) as they can be displayed by the same neuron (Fig. 1, H to L). After a repulsive turning response was first elicited by a Sema III gradient, we tested the same neuron with the same gradient in the presence of 8-Br-cGMP. We found that the growth cone exhibited an attractive response. The repulsive response was restored after 8-Br-cGMP was washed away. Thus, the response of the growth cone to a Sema III gradient does not desensitize with time and it can be switched between repulsion and attraction in a cGMP-dependent manner. The extent of repulsion versus attraction for each individual neuron appears to be correlated (Fig. 1M), which suggests that cGMP affects only the directionality of the response and not the extent to which the growth cone turns.

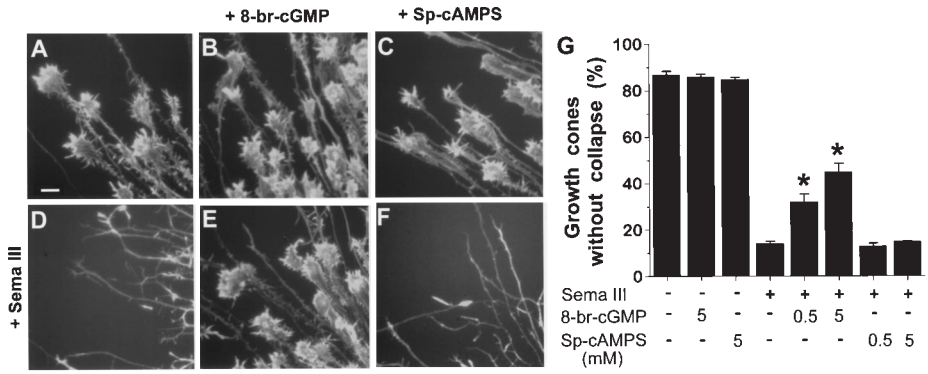

Fig. 3. Inhibition of the collapse-inducing activity of Sema III by 8-Br-cGMP. Fluorescence images of DRG cultures incubated in normal culture medium (**A** and **D**) or in medium containing 5 mM 8-Br-cGMP (**B** and **E**) or 5 mM Sp-cAMP (**C** and **F**) for 1 hour before addition of Sema III (100 ng/ml) (D to F). Cultures were maintained for another 30 min after addition of Sema III before fixation and visualization with rhodamine-phalloidin. Bar = 10 μm. (**G**) Percentage of intact growth cones from the explants treated with cyclic nucleotides or Sema III or both. Error bars refer to SEM. *, Significant difference from the set without pretreatment with 8-Br-cGMP and Sp-cAMPS ($P < 0.001$; *t* test).

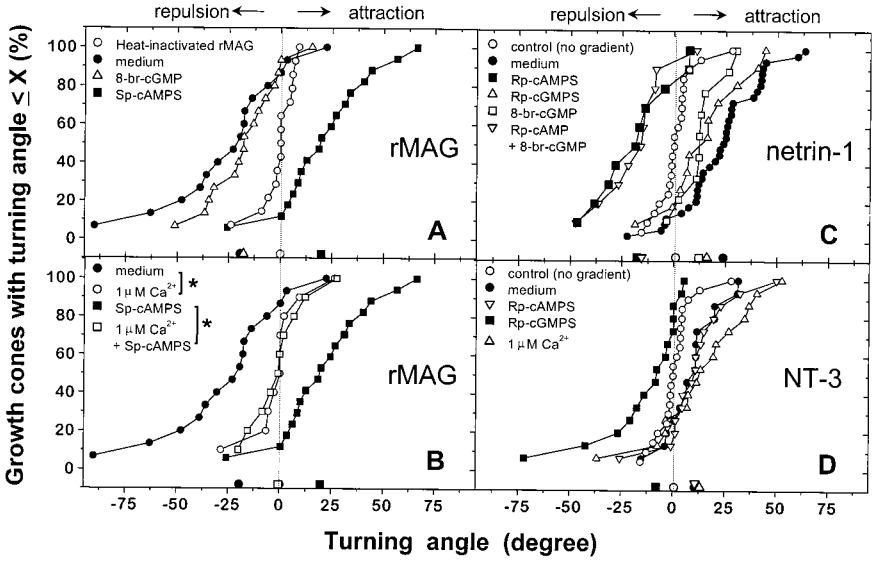

Fig. 4. (**A**) Effects of manipulating cyclic nucleotide levels on turning responses induced by a rMAG gradient (150 μg/ml in the pipette) in normal medium, in medium containing 8-Br-cGMP (100 μM) or Sp-cAMPS (20 μM), and by heat-inactivated rMAG in normal medium. Results from the latter were significantly different from the three other groups [$P < 0.01$; Kolmogorov–Smirnov test; same test for (B) to (D)]. (**B**) Effects of reducing $[Ca^{2+}]_o$ on turning responses induced by rMAG. Distribution of turning angles in normal (1 mM) or low (1 μM) Ca^{2+} medium in the absence or presence of Sp-cAMPS (20 μM). *, Significant difference ($P < 0.05$). (**C**) Turning responses induced by a gradient of netrin-1 (5 μg/ml in the pipette) (*5*) in normal medium and in medium containing Rp-cGMPS (10 μM), 8-Br-cGMP (100 μM), Rp-cAMPS (20 μM), or both Rp-cAMPS (20 μM) and 8-Br-cGMP (100 μM). Also shown is control distribution of turning angles observed when the pipette contained only culture medium (no gradient), which is significantly different from all other data sets ($P < 0.05$). (**D**) Turning induced by a NT-3 gradient (50 μg/ml in the pipette) in normal medium and in medium containing Rp-cAMPS (20 μM) or Rp-cGMPS (10 μM) or in medium containing 1 μM Ca^{2+}. All data sets were significantly different from the no-gradient control ($P < 0.05$).

Neuropilins are receptors for several members of the semaphorin family (*17–19*), and antibodies against the extracellular domain of neuropilin-1 block the effects of Sema III in vitro (*17, 18*). A function-blocking antiserum to rat neuropilin-1 (α-28) cross reacts with the *Xenopus* protein (*20*). We found that bath application of the α-28 antiserum abolished both the repulsion induced by Sema III under normal conditions and the attraction toward Sema III in the presence of 8-Br-cGMP (Fig. 2C). Thus, neuropilin-1 function is required for both repulsion and attraction of these growth cones induced by Sema III.

Cyclic nucleotides also change the responsiveness of developing rat dorsal root ganglion (DRG) axons to Sema III. When added to cultures of DRG explants (*21*), 8-Br-cGMP, but not Sp-cAMPS, inhibited the collapsing activity of Sema III in a dose-dependent manner (Fig. 3), but 8-Br-cGMP or Sp-cAMPS alone had no detectable effect on growth cones in these cultures. In these experiments, higher concentrations of 8-Br-cGMP (0.5 to 5 mM) were needed to inhibit the collapsing activity of Sema III, which suggests that the turning response of *Xenopus* spinal neurons might be more sensitive to modulation by cGMP than is the collapse of growth cones of rat DRG neurons (*9*).

To examine whether the conversion from repulsive to attractive behavior occurs for other repulsive factors, we studied myelin-associated glycoprotein (MAG), a component of myelin and an inhibitor of axonal regeneration (*22*). A soluble proteolytic fragment of MAG consisting of its extracellular domain is released in abundance from myelin in vivo and can potently inhibit axon regeneration (*23*). We found that a gradient of recombinant protein consisting of the extracellular domain of MAG (rMAG) (*24*) repelled growth cones of *Xenopus* spinal neurons (Fig. 4A). However, the repulsion by rMAG was not affected by addition of 8-Br-cGMP. On the other hand, when Sp-cAMPS was added to the medium, the growth cone responses were converted to attraction in the same rMAG gradient (Fig. 4A). Thus, the turning response induced by rMAG can be modulated by cAMP-dependent activities. This is reminiscent of the turning responses induced by gradients of BDNF and netrin-1 (*4, 5*), although the latter factors are normally attractive and attraction is converted to repulsion by inhibition of cAMP-dependent activities.

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Cytosolic Ca^{2+} regulates growth cone motility (*25*). An increase in cytosolic Ca^{2+} concentration correlates with growth cone collapse induced by some myelin-associated proteins but not other factors (*26*). To examine the involvement of Ca^{2+} in Sema III– and rMAG-induced turning, we reduced the extracellular Ca^{2+} concentration ($[Ca^{2+}]_o$) from the normal amount of 1 mM to 1 μM (*4, 5*). This resulted in a two- to threefold increase in the rate of neurite extension but no change in guidance responses in a Sema III gradient (Fig. 2D). In contrast, the repulsion and attraction induced by gradients of rMAG were both abolished by the reduction of $[Ca^{2+}]_o$ (Fig. 4B). Thus, growth cone turning induced by rMAG, but not Sema III, requires normal $[Ca^{2+}]_o$.

The above studies on growth cone turning induced by Sema III and MAG point to the existence of at least two distinct pathways involving cAMP and cGMP, with different Ca^{2+} dependences. Growth cone turning induced by BDNF, acetylcholine, and netrin-1, but not NT-3, depends on both $[Ca^{2+}]_o$ and cAMP (*4, 5*). Here we found that inhibition or activation of cGMP-dependent pathways by Rp-cGMPS or 8-Br-cGMP, respectively, did not affect the attractive turning toward netrin-1 ($P > 0.1$; Kolmogorov-Smirnov test) (Fig. 4C). Moreover, repulsive turning induced by the same netrin-1 gradient in the presence of Rp-cAMPS also was not affected by 8-Br-cGMP. On the other hand, the attractive response in a NT-3 gradient was converted to a repulsive response by inhibiting cGMP-dependence pathways with Rp-cGMPS, whereas depletion of $[Ca^{2+}]_o$ or addition of Rp-cAMPS had no effect on the attractive turning (Fig. 4D). Thus, the dependence of turning behavior on $[Ca^{2+}]_o$ for the four factors examined here (rMAG, netrin-1, Sema III, and NT-3) correlates with a dependence on cAMP, not cGMP.

Studies of the turning response of *Xenopus* neuronal growth cones induced by a number of diffusible factors (*4, 5*), including those examined here, have implicated cAMP and cGMP in setting the neuronal response to different guidance cues. The guidance cues examined can all be either attractive or repulsive, depending on the status of cytosolic cyclic nucleotides. Manipulations to increase the level of cyclic nucleotide activity favor attraction and manipulations to decrease the level of cyclic nucleotide activity favor repulsion. Because cyclic nucleotides are known to serve as second messengers for a large number of cell surface receptors (*27*), the response of a growth cone to a particular guidance cue may thus depend critically on other coincident signals received by the neuron. The susceptibility to conversion between attraction and repulsion may enable a growing axon to respond differentially to the same guidance cue at different points along the journey to its final target (*28*). Reversal of the action of repulsive factors by elevated cyclic nucleotides has potential implications for promoting nerve regeneration in the central nervous system, as effective regeneration in the central nervous system is blocked by inhibitory factors (*22, 29*), and modulation of cyclic nucleotide concentrations may help relieve this inhibition and therefore help stimulate regeneration.

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8. Dissociated *Xenopus* spinal neurons were prepared as described in (*4, 5*). Isolated spinal neurons were used for experiments at room temperature (22° to 24°C) 14 to 22 hours after plating. Sema III was purified from conditioned medium of stable 293-EBNA (Epstein-Barr virus–encoded nuclear antigen) cell lines, secreting Sema III–AP as described in (*17*). To inactivate Sema III activity, the supernatant containing Sema III–AP was heated at 85°C for 45 min [J. Fan and J. A. Raper, *Neuron* **14**, 263 (1995)]. Microscopic gradients of diffusible factors were produced as described in (*4, 5*). The average concentration of factors at the growth cone was about 10^3-fold lower than that in the pipette [A. M. Lohof, M. Quilian, Y. Dan, M-m. Poo, *J. Neurosci.* **12**, 1253 (1992)], and a concentration gradient of 5 to 10% was created across a growth cone 10 μm wide and 100 μm away from the tip of the ejecting pipette. The original direction of neurite growth was defined by the last 10-μm segment of the neurite. The turning angle was defined by the angle between the original direction of neurite extension and a straight line connecting the positions of the growth cone at the onset and the end of the 1-hour period. Only growth cones with a net extension > 5 μm over the 1-hour period were scored.
9. No obvious collapse of *Xenopus* growth cones was observed even when a higher concentration of Sema III (up to 100 μg/ml in the pipette) was applied to cultured *Xenopus* spinal neurons. We have not examined whether a uniform concentration of Sema III causes collapse as it does for chick and rat growth cones.
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16. These *Xenopus* cultures contain spinal neurons sensitive to various neurotransmitters [J. L. Bixby and N. C. Spitzer, *J. Physiol. London* **353**, 143 (1984)] and exhibiting a wide range of capacity in secreting acetylcholine [J. Evers, M. Laser, Y. A. Sun, Z. P. Xie, M-m. Poo, *J. Neurosci.* **9**, 1523 (1989)]. The variability of turning responses observed within a population of these neurons (see Fig. 2) could be attributed to either the heterogeneity of neuronal types or variability in the cytoplasmic concentration of cyclic nucleotides or other signal transduction components.
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20. The antibody to neuropilin-1(α-28) is directed against the ectodomain of rat neuropilin-1, purified on a protein A–agarose column as described in (*17*), and does not cross react with neuropilin-2 (*19*). Using α-28, we detected a putative *Xenopus* neuropilin by protein blotting. Interaction of α-28 with putative *Xenopus* neuropilin in cultured *Xenopus* spinal neurons was confirmed by immunostaining. For antibody blocking experiments, α-28 (20 μg/ml) was added 30 min before the onset of the gradient. During the turning assay, the concentration of α-28 was 5 μg/ml.
21. DRG explants derived from embryonic day 14 rat embryos were cultured with NGF (25 ng/ml) on plates precoated with poly-D-lysine and laminin for 20 hours before experiments as described in (*17*). Pharmacological agents were added an hour before the collapse assay. The collapse assay was performed on sensory axons from these explants with Sema III–AP–containing medium essentially as described in (*17*). For visualization, growth cones were stained with rhodamine-phalloidin and then washed and mounted.
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24. To generate rMAG, SF9 cells were infected with baculovirus expressing the extracellular domain of MAG and conditioned medium was collected 5 or 6 days after infection and purified as described [M. Li *et al., J. Neurosci. Res.* **46**, 404 (1996)]. For experiments with inactivated rMAG, rMAG was heated at 80°C for 35 min.
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