Neuron, Vol. 19, 1225–1235, December, 1997, Copyright ©1997 by Cell Press

cAMP-Dependent Growth Cone Guidance by Netrin-1

Guo-li Ming,* Hong-jun Song,* Benedikt Berninger,* Christine E. Holt,*§ Marc Tessier-Lavigne,† and Mu-ming Poo*‡
*Department of Biology
University of California at San Diego
La Jolla, California 92093-0357
†Howard Hughes Medical Institute
Department of Anatomy
Department of Biochemistry and Biophysics
University of California at San Francisco
San Francisco, California 94143-0452

Summary

Netrin-1 is known to function as a chemoattractant for several classes of developing axons and as a chemorepellent for other classes of axons, apparently dependent on the receptor type expressed by responsive cells. In culture, growth cones of embryonic Xenopus spinal neurons exhibited chemoattractive turning toward the source of netrin-1 but showed chemorepulsive responses in the presence of a competitive analog of cAMP or an inhibitor of protein kinase A. Both attractive and repulsive responses were abolished by depleting extracellular calcium and by adding a blocking antibody against the netrin-1 receptor Deleted in Colorectal Cancer. Thus, nerve growth cones may respond to the same guidance cue with opposite turning behavior, dependent on other coincident signals that set the level of cytosolic cAMP.

Introduction

The pathway choice of nerve growth in developing nervous systems depends on guidance cues in the form of diffusible or substrate-bound molecules (Tessier-Lavigne and Goodman, 1996). These molecules serve as either attractants or repellents, thereby influencing the direction of growth cone extension. Members of the netrin family of guidance cues function as attractants for several classes of axons extending toward the ventral midline of the nervous system and as repellents for other axons growing away from it. In vertebrates, commissural axons originating from the dorsal spinal cord appear to be attracted toward the ventral side by netrin-1 released from floor plate cells (Kennedy et al., 1994; Serafini et al., 1994, 1996). Conversely, in tissue culture, netrin-1 can repel the axons of trochlear motor neurons and branchiomotoneurons, two classes of axons that normally grow away from the floor plate (Colamarino and Tessier-Lavigne, 1995; Varela-Echavarria et al., 1997). A similar bifunctionality has been documented in nematodes, where the netrin homolog UNC-6 has been implicated in attracting sensory axons to the ventral body wall, while motor axons are thought to avoid UNC-6, growing dorsally away from it (Hedgecock et al., 1990; Wadsworth et al., 1996). In Drosophila melanogaster, an attractive function of the netrins Netrin-A and Netrin-B has been documented in the guidance of commissural axons to the midline and of motor axons to subsets of their target muscles (Harris et al., 1996; Mitchell et al., 1996). Whether netrins are also involved in repulsion in Drosophila is not at present known.

The receptor and signal transduction mechanisms through which netrins produce their attractive and repulsive actions are still poorly understood, though available evidence indicates that distinct receptors are responsible for the attractive and repulsive effects documented to date. In the case of attraction, genetic loss-of-function and antibody perturbation studies have demonstrated a requirement for the receptor Deleted in Colorectal Cancer (DCC) or its homologs UNC-40 and Frazzled for chemoattraction mediated by netrins in rodents, Caenorhabditis elegans, and Drosophila, respectively (Hedgecock et al., 1990; Chan et al., 1996; Keino-Masu et al., 1996; Kolodziej et al., 1996; Fazeli et al., 1997). It is not known whether each of these DCC-related proteins constitutes an entire receptor involved in attraction or simply a necessary binding component of an attractive receptor complex. In the case of repulsion, studies in the nematode have shown that migrations away from an UNC-6 source (presumed repulsions) depend on the normal presence of the UNC-5 protein (Hedgecock et al., 1990), while misexpression of UNC-5 in touch neurons of C. elegans steers their axons dorsally away from an UNC-6 source (Hamelin et al., 1993). Thus, UNC-5 appears to be a receptor or a component of a receptor complex involved in netrin-mediated repulsion, a possibility further supported by the finding that vertebrate homologs of UNC-5 are netrin-binding proteins (Leonardo et al., 1997).

Little is known about the signal transduction mechanisms underlying the differential responses of different classes of axons to netrins. At one extreme, the mechanisms involved in attraction and repulsion by netrins could be completely distinct, mediated by unrelated receptor complexes and unrelated intracellular signaling pathways in the growth cones. However, the finding in nematodes that migrations away from the source of the netrin UNC-6 are also slightly dependent on the function of the DCC homolog UNC-40 (Hedgecock et al., 1990) has raised the possibility that UNC-40 might also be part of a repulsive receptor complex involving UNC-5 and that the signaling mechanisms involved in attraction and repulsion might be closely related. Further support for the idea that mechanisms involved in attraction and repulsion are related has come from recent studies on the chemoattractant effect of brain-derived neurotrophic factor (BDNF) on the growth cones of embryonic Xenopus laevis spinal neurons in culture. Remarkably, the attractive effect of BDNF on growth cones in this culture situation was found to be converted to a repulsive effect when these neurons were exposed to agents that inhibit cAMP-dependent protein kinase A (PKA)

‡To whom correspondence should be addressed.
§Present address: Department of Anatomy, Cambridge University, Downing Street, Cambridge, CB2 3DY, United Kingdom.

(Song et al., 1997). This has suggested a close relationship between signaling mechanisms involved in attraction and repulsion within the growth cone and raised the possibility that neurons may interpret the same extracellular guidance cue as either attractive or repulsive, dependent upon the status of cytosolic PKA activity.

In this paper, we explore whether the conversion of an attractant to a repulsive growth cone response can also be demonstrated in the case of netrin-1. It has recently been shown that Xenopus retinal growth cones turn toward a microscopic gradient of netrin-1 (de la Torre et al., 1997 [this issue of *Neuron*]). In the present study, we found similarly that netrin-1 induces attractive responses of a large majority of growth cones of embryonic Xenopus spinal neurons in culture. However, in the presence of a competitive cAMP analog, Rp-cAMPS, or a specific inhibitor of PKA, KT5720, we observed a repulsive response for most growth cones in the same netrin-1 gradient. The response of a given axon can be rapidly switched from attraction to repulsion, consistent with the suggestion from in vivo experiments in nematodes that receptor mechanisms involved in mediating attractive and repulsive effects of the netrins tap into interconnected signal transduction pathways. Our results also suggest that different guidance behaviors of neurons toward netrin sources in vivo are not necessarily predetermined characteristics of the neuronal phenotype. Rather, cytosolic cAMP-dependent activity, which may depend on coincident signals received by the neuron, may be a central determinant of the growth cone's response to netrin-1.

Results

Attractive and Repulsive Turning in Netrin-1 Gradients

Microscopic gradients of recombinant chick netrin-1 were created near the growth cones of isolated Xenopus spinal neurons in 14–20 hr cultures by repetitive pulsatile application of picoliters of netrin-1-containing saline (Lohof et al., 1992; Zheng et al., 1994; Song et al., 1997). The tip of the micropipette was positioned at a distance of 100 μm from the growth cone and at a 45° angle with respect to the direction of neurite extension. The direction and total length of neurite extension were measured 1 hr after the onset of gradient. When the pipette concentration of netrin-1 was 5 μg/ml, we consistently observed a marked chemotropic turning response of the growth cone toward the pipette, as illustrated in Figures 1A and 1B. The growth cone behavior of a population of neurons is shown by superimposing traces of individual trajectories of neurite extension over the 1 hr period for a random sample of ten neurons. As depicted in Figure 1C, growth cones exhibited a clear bias toward the source of netrin-1 at the end of a 1 hr period in the netrin-1 gradient. As controls, the effect of a gradient of heat-inactivated netrin-1 (75°C for 45 min, 5 μg/ml) was tested. We found that growth cones exhibited no apparent bias in their direction of extension, with the final growth cone positions at the end of the 1 hr period being scattered symmetrically around the original direction of growth (Figures 1D–1F).

To examine whether the netrin-1-induced turning response was affected by changes in the activity of cAMP-dependent pathways, we applied a specific inhibitor of PKA, KT5720 (200 nM; Kase et al., 1987), to the culture prior to the turning assay. Using the same netrin-1 gradient as that described above (5 μg/ml in the pipette), we observed marked repulsive turning of growth cones of Xenopus spinal neurons away from the pipette. An example of the repulsive turning together with a composite tracing of a population of neurons is shown in Figures 1G–1I. In addition, we have also examined the effect of a nonhydrolyzable competitive analog of cAMP, Rp-cAMPS (Rothermel and Parker Botelho, 1988), which is also known to inhibit PKA. Bath addition of Rp-cAMPS (20 μM) resulted in similar repulsive responses in the presence of the netrin-1 gradient as that found for KT5720. It has recently been shown that such Rp-cAMPS treatment abolishes the attractive turning response of these growth cones induced by a gradient of forskolin (Song et al., 1997), a drug known to activate endogenous production of cAMP. Thus, the Rp-cAMPS treatment is indeed effective in competing with endogenous cAMP in these neurons.

The turning responses of these growth cones were normally assayed after 1 hr in the netrin-1 gradient. However, the action of netrin-1 was apparent within minutes after the onset of the gradient. As shown by the images obtained during the first 20 min in the gradient (Figures 1J and 1K), for both attractive and repulsive responses, the growth cone exhibited a persistent asymmetry in the orientation and the number of filopodia at ~5 min after the onset of the netrin-1 gradient. At the present resolution of growth cone morphology, the repulsive response appeared to be a mirror image of the attractive response. The behavior during the netrin-1-induced attractive response was similar to that found for the response induced by a gradient of glutamate (Zheng et al., 1996). In the latter study, the presence of filopodia was shown to be essential for the growth cone turning response, since elimination of the filopodia by a low concentration of cytochalasin B treatment abolished the turning response without significantly affecting neurite extension.

Dependence on Netrin-1 Concentration

Previous studies using similar microscopic gradients have shown that for the pipette tip positioned at a distance of 100 μm from the center of the growth cone, the average concentration of the chemical at the growth cone is approximately 10^3-fold lower than that in the pipette (Lohof et al., 1992). To determine the concentration range in which netrin-1 effectively induces turning, we have varied the concentration of netrin-1 in the pipette. The turning response was quantitatively measured by determining the average turning angle of the growth cone at the end of the 1 hr period in the netrin-1 gradient. While clear turning was observed at a concentration of 5 μg/ml in the pipette, we found no significant turning at concentrations of 0.5 μg/ml, 1.5 μg/ml, or when heat-inactivated netrin-1 (at 5 μg/ml) was used. Increasing the netrin-1 concentration from 5 to 10 μg/ml did not further increase the turning response (Figure

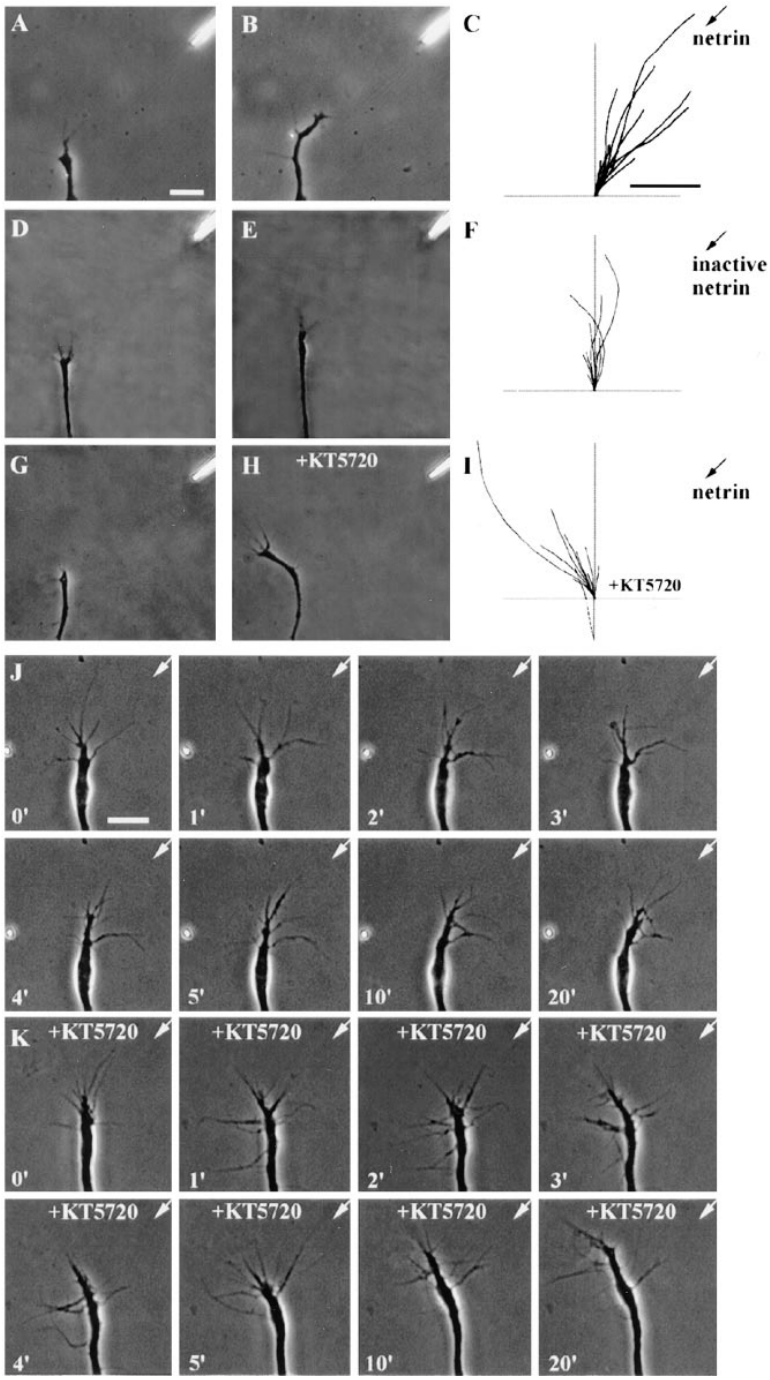

Figure 1. Attractive and Repulsive Turning of Nerve Growth Cones Induced by Netrin-1

(A–C) A gradient of netrin-1 was applied to cultured Xenopus spinal neurons by pulsatile application of picoliters of netrin-1 solution (5 μg/ml) from a micropipette (shown at the right upper corner). Microscope images were recorded at the onset (A) and the end (B) of 1 hr exposure to the netrin-1 gradient. In (C), superimposed traces depict the trajectory of neurite extension during the 1 hr period for a random sample population of ten neurons. The origin is the center of the growth cone at the onset of the experiment, and the original direction of growth was vertical. The arrow indicates the direction of the gradient. Bar: 20 μm.

(D–F) Control experiments carried out in the same manner as in (A–C), except that the netrin-1 solution in the pipette was heated at 75°C for 45 min.

(G–I) The same as in (A–C), except that an inhibitor of PKA, KT5720 (200 nM), was added to the medium.

(J and K) Changes in growth cone morphology during the first 20 min in a netrin-1 gradient in the absence (J) and the presence (K) of 200 nM of KT5720. The number depicts the time in minutes after the onset of the gradient. Arrow indicates the direction of the gradient. Bar = 5 μm.

2). This suggests that the effective average concentration of netrin-1 at the growth cone is about 5 ng/ml, which is close to that found to promote neurite growth in cultures of Xenopus retinal neurons (de la Torre et al., 1997). This result is consistent with the notion that a threshold number of netrin-1 receptors must be activated in order for the netrin-1 gradient to exert its guidance effect.

In addition to the turning angle, we have also measured the total length of neurite extension during the 1 hr period. Compared to the growth rate found in the presence of heat-treated netrin-1, there was no statistically significant change in the rate of extension in various netrin-1 gradients ($p = 0.07$ at 5 and 10 μg/ml, Kruskal-Wallis test, data not shown).

Dependence on DCC

Recent studies have identified a receptor for netrin-1, the DCC protein, which is a member of the immunoglobulin superfamily. DCC is a netrin-binding protein, and a monoclonal antibody against the extracellular domain of DCC was shown to block the netrin-1-induced neurite

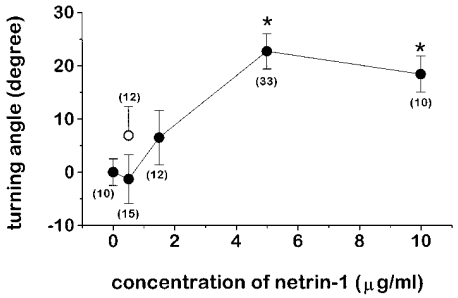

Figure 2. Attractive Turning Responses in Gradients of Different Netrin-1 Concentrations

The average turning angle for growth cone responses to netrin-1 gradients created by pipettes containing different concentrations of netrin-1. Closed circles are data obtained in normal culture medium. Data at 0 μg/ml were from experiments using heat-treated netrin-1 (5 μg/ml). Open circles represent data from experiments in which Sp-cAMPS (20 μM) was added in the medium. The error bar represents SEM, and the number associated with each bar refers to the total number of growth cones examined. Values marked with an asterisk were significantly different from the control (heat-treated netrin-1), using the Kruskal-Wallis test ($p < 0.001$).

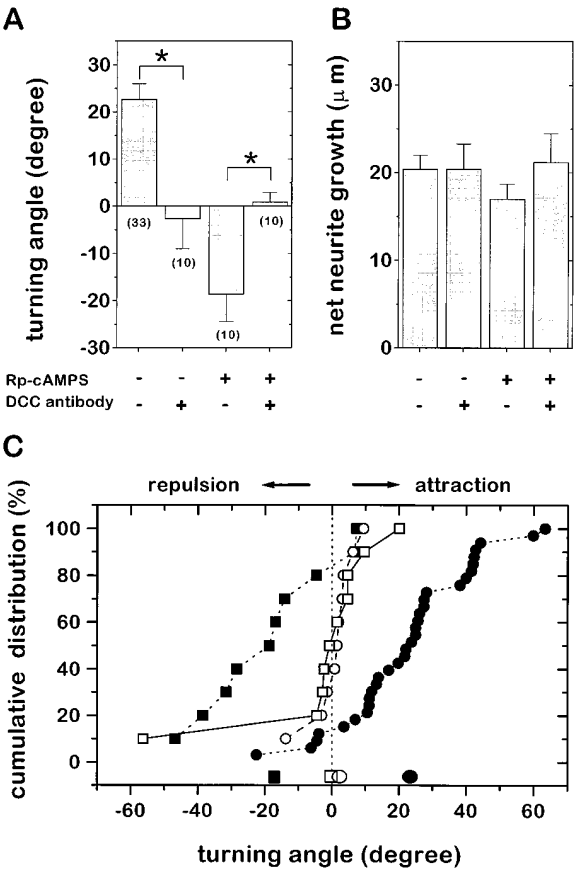

Figure 3. Effect of a Blocking DCC Antibody on Netrin-1-Induced Attractive and Repulsive Responses

(A and B) The average turning angle and net neurite extension in a netrin-1 gradient in the absence or presence of a DCC antibody (1 μg/ml) and Rp-cAMPS (20 μM), respectively. The error bar represents SEM, and the number associated with each bar is the total number of growth cones examined. An asterisk indicates significant difference (Kruskal-Wallis test, $p < 0.001$).

(C) Distribution of turning angles. For each experimental condition, angular positions of all growth cones at the end of 1 hr of exposure to a netrin-1 gradient are shown in a cumulative distribution plot. The percent value refers to the percentage of growth cones with angular position less than or equal to a given angular value on abscissa. Data shown are turning in normal culture medium (closed circles), in medium containing a DCC antibody (open circles), in medium containing Rp-cAMPS (closed squares), and in medium containing both Rp-cAMPS and a DCC antibody (open squares). Isolated data points along the abscissa are median values for corresponding data shown above.

outgrowth of commissural axons from rat spinal cord explants and of retinal ganglion axons from rat retinal explants, as well as the turning of Xenopus retinal axons to a source of netrin-1 (Keino-Masu et al., 1996; Deiner et al., 1997; de la Torre et al., 1997). In the present study, we have examined whether DCC is involved in either the attractive or the repulsive response of Xenopus neurons induced by a netrin-1 gradient. As shown in Figure 3A, attractive turning induced by netrin-1 was totally abolished in the presence of the DCC antibody (1 μg/ml), suggesting that the netrin-1 effect requires DCC. Furthermore, when the DCC antibody (1 μg/ml) was present together with Rp-cAMPS (20 μM) in the culture medium, the repulsive turning of the growth cone was likewise abolished (Figure 3A). No change of the net rate of neurite extension was found (Figure 3B). To depict turning angles for all growth cones examined in these experiments, the distribution of turning angles was shown in a cumulative distribution plot in Figure 3C. The median values of the attractive and repulsive turning angles were 23.8° and −17.8°, respectively, and they were reduced to 0.4° and 1.7°, respectively, in the presence of the DDC antibody. These results implicate DCC in mediating both repulsive and attractive turning of these growth cones induced by a netrin-1 gradient in this in vitro assay.

Transition between Attractive and Repulsive Responses

Changes in the growth cone turning behavior may depend on the cytosolic cAMP concentration in an "all-or-none" or a graded manner, in analogy to either a "switch" or a "dial". In the switch mode, a narrow range of concentration of cAMP may be responsible for switching between attractive and repulsive responses of similar magnitude. In the dial mode, the neuron may respond to the netrin-1 gradient by graded degrees of attraction and repulsion and, in the simplest case, with a monotonic dependence on the level of cAMP. We addressed this issue by examining the turning response in netrin-1 gradient (5 μg/ml in the pipette) in the presence of increasing concentrations of the competitive cAMP analog Rp-cAMPS. As shown in Figures 4A and 4C, there was a clear transition from attractive to repulsive response when the Rp-cAMPS concentration was increased from 1 to 5 μM. The repulsive response quickly reached a plateau level with a Rp-cAMPS level of 5 μM, which is similar to the effect of KT5720 (200 nM). The net neurite extension, however, was not significantly affected by the drug treatment (Figure 4B). Given the 100-fold higher affinity of cAMP for PKA as compared

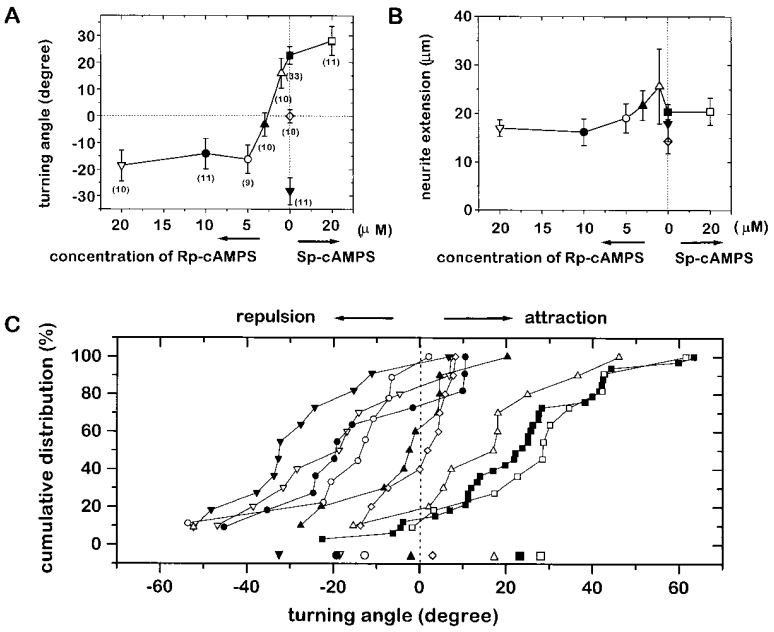

Figure 4. Transition between Attractive and Repulsive Turning Responses

(A and B) The average turning angles and the net neurite extension for growth cones after 1 hr of exposure to a netrin-1 gradient (same as that described in Figure 1) in the presence of different concentrations of Rp-cAMPS (1, 3, 5, 10, and 20 μM), 200 nM KT5720 (closed upside-down triangle), and 20 μM of Sp-cAMPS (open circle). Data for 0 μM of the drug were the same set as that shown in Figure 3. For comparison, data from experiments using heat-inactivated netrin-1 (5 μg/ml) were also shown (open diamond). The error bar represents SEM, and the number associated with each bar refers to the total number of growth cones examined.

(C) The distribution of turning angles was plotted as the cumulative distribution at various angles for the same set of data as that shown above. Different experimental conditions are represented by data points with different symbols, corresponding to those shown in (A) and (B). Isolated data points along the abscissa are median values for corresponding data shown above.

to Rp-cAMPS (Rothermel and Parker Botelho, 1988), this result suggests that switching of the turning response from attraction to repulsion can occur with a reduction of intracellular cAMP levels in a narrow range of only 10–50 nM.

In separate experiments, we examined whether bath application of Sp-cAMPS (20 μM), a cAMP analog that activates PKA (Rothermel and Parker Botelho, 1988), can enhance netrin-1-induced attractive responses. As shown in Figure 4, there was a small increase in the mean and median turning angle, although the increase was not statistically significant. Under the standard gradient generated by a pipette containing 5 μg/ml of netrin-1, the attractive response observed in the normal culture medium may have already reached saturation. We thus examined whether Sp-cAMPS can enhance the attractive response at a lower netrin concentration (0.5 μg/ml in the pipette) that produced no significant attractive turning in normal medium. As shown in Figure 2A, the attractive response was not significantly affected when Sp-cAMPS (20 μM) was applied. Taken together, the results from the studies using cAMP analogs suggest that the action of cAMP more closely resembles that of a switch, without significant effects on the sensitivity or the magnitude of the turning response.

Dual Turning Experiments

Neurons in these Xenopus spinal cord cultures are heterogeneous in phenotypes (Bixby and Spitzer, 1984a), and differences in the extent of turning response by various neurons might in principle reflect this heterogeneity. However, the marked cAMP-dependent switching of the turning response for essentially all neurons examined argues in favor of a general mechanism operating in all neurons in these cultures. To ensure that diametrically different turning behaviors can indeed occur in the same neuron, we have carried out "dual" turning experiments. First, a standard netrin-1 gradient (5 μg/ml in the pipette) was applied to the growth cone of an isolated neuron for 1 hr in normal culture medium, which resulted in an attractive turning response. The tip of the netrin-containing pipette was then repositioned to a distance of 100 μm and at a 45° angle with respect to the new direction of neurite extension for another hour, during which KT5720 (200 nM) was added to the culture medium. As shown by the example depicted in Figures 5A–5D, the attractive response switched to a repulsive response within 60 min after adding KT5720 (200 nM) to the medium. The transition of the growth cone response from attractive to repulsive turning was not accompanied by any significant change in the growth rate. In a separate set of experiments, a netrin-1 gradient was first applied in the presence of Rp-cAMPS (10 μM), which resulted in a repulsive response of the growth cone. At the end of the first hour, Rp-cAMPS was washed out by extensive perfusion with fresh culture medium. After repositioning the pipette, the same gradient was then applied for another hour. Extensive washing had apparently resulted in a slight inhibition of neurite growth in the second hour. However, a consistent attractive turning was observed (Figures 5F–5I). The results from all dual turning experiments (n = 6) are summarized in the composite graphs shown in Figures 5E and 5J. From these experiments, we conclude that the same growth cone can exhibit both attractive and repulsive responses toward a netrin-1 gradient. Furthermore, the growth cone response to netrin-1 does not become desensitized after 1 hr of exposure to the gradient.

Long-Term Behavior in Gradients

In a gradient created by pulsatile application of netrin-1 from a pipette, does the growth cone grow directly toward the tip of the pipette after an initial attractive turning response? In some experiments, we have followed the course of neurite growth in netrin-1 gradients for up to 4 hr. Interestingly, we consistently observed that the

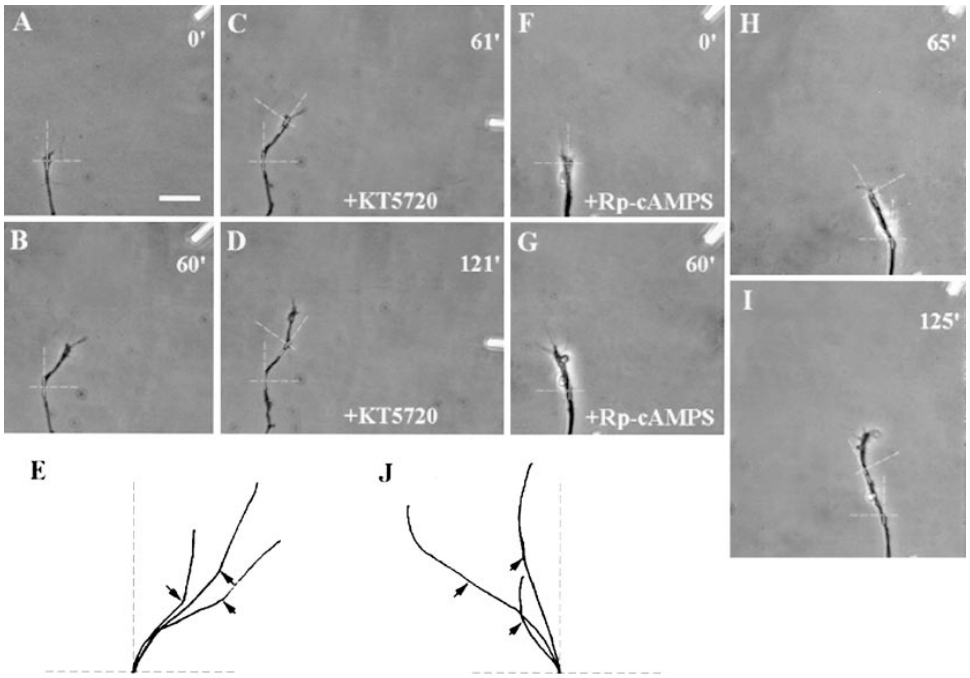

Figure 5. Dual Turning Experiments

(A–D) Images of a growth cone that was exposed to a netrin-1 gradient (the same as that in Figure 1) for 1 hr (A and B), followed by the exposure to the same gradient for another hour (C and D) in the presence of KT5720 (200 nM). The pipette was repositioned to a site 100 μm away and oriented 45° with respect to the new neurite direction at the end of the first hour. The number refers to the time (in minutes) after the onset of the experiment. Dashed lines depict the coordinates for pipette positioning at the beginning of the first and the second hour, respectively. Scale bar: 10 μm.

(E) Superimposed traces of the trajectory of neurite extension for three neurons examined in the manner described in (A–D). The arrow marks the end of the first hour.

(F-J) Experiments similar to that described in (A–E), except that Rp-cAMPS (10 μM) was in the medium during the first hour and was removed by extensive perfusion of the culture at the end of the first hour.

extension toward the pipette showed a "zig-zag" pattern, with alternating periods of attractive and repulsive responses. As illustrated by the example shown in Figure 6, the growth cone showed repeated turning responses as it grew toward the pipette. Near the pipette, the average concentration of netrin-1 was likely to be two to three orders of magnitude higher than that at a distance of 100 μm (Lohof et al., 1992; Zheng et al., 1994), and all netrin receptors on the growth cone are likely to be fully occupied. The extracellular gradient of netrin-1 may thus become ineffective in triggering a gradient of cellular activity across the growth cone at these distances. For promotion of neurite outgrowth from spinal neurons and retinal neurons in culture, high concentrations of netrin-1 were previously found to be ineffective (Serafini et al., 1994; de la Torre et al., 1997). The continuing attractive response of these growth cones as they moved up the gradient indicates that adaptation of the transduction mechanism might be occurring to recover the sensitivity of the growth cone toward the netrin-1 gradient.

Dependence on Ca^{2+}

Cytosolic calcium is known to play an important regulatory role in growth cone motility (Kater et al., 1988; Bandtlow et al., 1993; Gu and Spitzer, 1995) and to regulate the dynamics of actin polymerization and depolymerization (Lankford and Letourneau, 1989), processes necessary for local changes of the cytoskeleton associated with growth cone turning. We have examined the involvement of cytosolic Ca^{2+} in netrin-1-induced turning by manipulating extracellular Ca^{2+} ($[Ca^{2+}]_o$). As shown in Figure 7, reduction of $[Ca^{2+}]_o$ to 1 μM significantly increased the rate of neurite extension by about 2-fold, consistent with previous results (Bixby and Spitzer, 1984b). However, it completely abolished both the attractive turning toward a netrin-1 source and the repulsive turning in the presence of Rp-cAMPS (20 μM), with the median turning angles reduced to 2.0° and 0.6°, respectively. This result suggests that in the cascade of transduction events induced by netrin-1, the cAMP-dependent switch is downstream of a Ca^{2+}-dependent step. Using the calcium indicator fluo-3, we did not detect a change in cytosolic Ca^{2+} during netrin-1-induced turning (data not shown), although small or very local alterations in Ca^{2+} may have escaped our detection. We noted that the reduction of $[Ca^{2+}]_o$ and/or the increased growth rate in low Ca^{2+} medium does not block the ability of the growth cone to sense extracellular gradients, since these growth cones still exhibit attractive turning responses toward a gradient of neurotrophin-3 (NT-3) under the same low $[Ca^{2+}]_o$ conditions (Song et al., 1997).

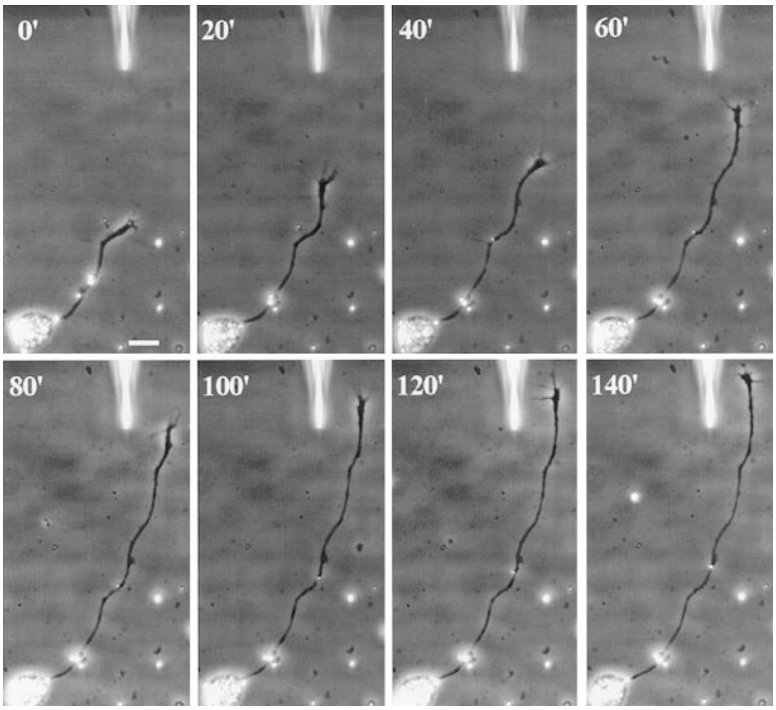

Figure 6. Long-Term Growth in Gradients

Images of a growth cone in the presence of a netrin-1 gradient (the same as that in Figure 1). The number refers to the time in minutes following the onset of the gradient. Note the zig-zag pattern of the trajectory, reflecting alternating attractive and repulsive turning of the growth cone. Bar = 20 μm.

Discussion

Previous studies have shown that netrin-1 may serve as either an attractant or a repellent for different populations of axons, effects that are thought to be mediated by distinct receptor types. The findings described here indicate that the same population of axons may exhibit opposite turning responses to netrin-1, depending on the status of cytosolic cAMP-dependent activity. Cellular effects of a number of extracellular signaling molecules, including hormones, neuropeptides, and neurotransmitters, are known to be mediated by changes in the cytosolic concentration of cAMP (Laufer and Changeux, 1987; Cooper et al., 1996; Hempel et al., 1997). In addition to diffusible factors, cytosolic cAMP activity may also be modulated by cell–cell or cell–substratum interactions. The action of a guidance cue may thus depend critically on other coincident signals received by the neuron, which can vary dynamically as the growth cone encounters different environments along its pathway of extension. For example, one can imagine scenarios in which a growth cone is attracted to a "guidepost" cell by a secreted factor, then contact with the guidepost cell results in inhibition of PKA activity, which leads to repulsion of the growth cone by the same secreted factor and its continued journey to the next target.

Receptors Mediating the Netrin-Induced Responses

Two types of netrin receptors, belonging to the DCC and UNC-5 families, have been identified (Leung-Hagesteijn et al., 1992; Chan et al., 1996; Keino-Masu et al., 1996; Kolodziej et al., 1996; Ackerman et al., 1997; Leonardo et al., 1997). In C. elegans, UNC-40 is required for the presumed attractive actions of the netrin UNC-6 (Hedgecock et al., 1990). Similarly, an antibody to DCC selectively blocked netrin-1-dependent outgrowth of rat commissural axons in vitro (Keino-Masu et al., 1996). However, it was not clear whether the expression of DCC is necessary to mediate all actions of netrins, including the turning of commissural axons toward a source of netrin. In the present study, the effects of netrin-1 on the direction of growth cone extension were abolished by the antibody against DCC, indicating that these neurons express a Xenopus homolog of DCC (Pierceall et al., 1994) and, more importantly, that this receptor is required for both attractive and repulsive turning of growth cones induced by netrin-1. These results are consistent with that found for the netrin-1-induced turning of retinal axons (de la Torre et al., 1997). In C. elegans, the repulsive action of UNC-6 depends primarily on a putative receptor encoded by the *unc-5* gene, but also to a lesser extent on the receptor encoded by *unc-40* (Hedgecock et al., 1990). This has raised the possibility that UNC-5 may form a heteromeric complex with UNC-40. Recently, vertebrate homologs of UNC-5 have been identified, which show specific netrin binding (Ackerman et al., 1997; Leonardo et al., 1997), thus qualifying them as candidate netrin receptors. The gene encoding one of these, *Unc5h1*, is strongly expressed in the developing ventral spinal cord, including motor neurons. Whether the cultured Xenopus spinal neurons studied here also express UNC-5-like proteins and whether the latter are required, in addition to DCC, for the repulsive response to netrin-1 remains to be determined.

It should be noted that neurons in these Xenopus cultures are known to be heterogeneous in cell types, exhibiting differential responses to various neurotransmitters (Bixby and Spitzer, 1984a) and large variation in the efficacy of ACh release upon muscle cell contact (Xie and Poo, 1986; Evers et al., 1989). It was thus surprising that these neurons exhibited rather uniform attractive responses to netrin-1 in normal culture medium

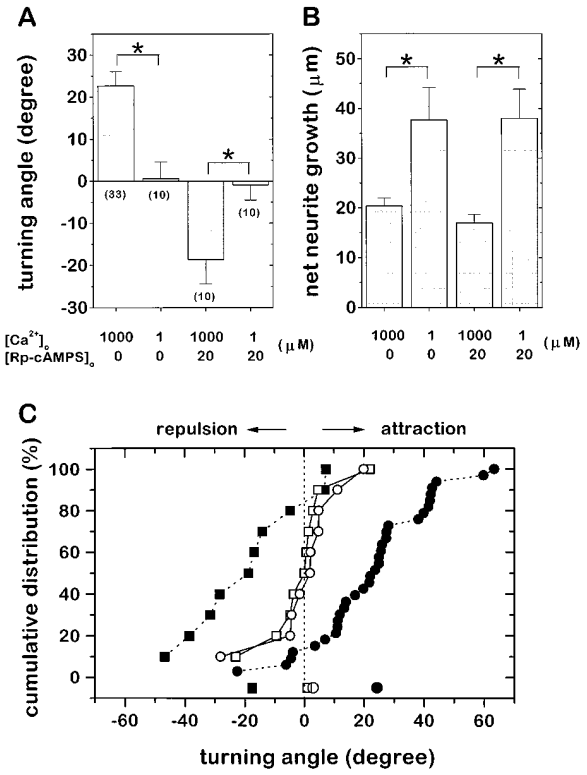

Figure 7. The Dependence of Netrin-1-Induced Turning on Extracellular Ca^{2+} ($[Ca^{2+}]_o$)

(A and B) The average turning angles and net neurite extension of growth cones, respectively, for netrin-1-induced attractive and repulsive responses, in the presence of normal (1 mM) and low (1 μM) $[Ca^{2+}]_o$. The error bar refers to SEM, and the number associated with the bar refers to the total number of growth cones examined. (C) The distribution of turning angles for turning in normal $[Ca^{2+}]_o$ medium (closed circles), in normal $[Ca^{2+}]_o$ medium containing Rp-cAMPS (20 μM; closed squares), in low $[Ca^{2+}]_o$ medium (open circles), and in low $[Ca^{2+}]_o$ medium containing Rp-cAMPS (20 μM; open squares). The same data set as that shown in (A) and (B) was used. Isolated data points along the abscissa are median values for corresponding data shown above.

and uniform repulsive responses after inhibition of PKA. It is possible that the culture system used here has simplified and homogenized the environmental factors that normally endow various spinal neurons with different levels of PKA activity, which are conditions for the diverse guidance behaviors of spinal neurons observed in vivo.

Signal Transduction Mechanisms

Similar to what has been found for gradients of BDNF or ACh (Song et al., 1997), growth cone turning induced by netrin-1 gradients depends on the level of cAMP and exhibits the same requirement for $[Ca^{2+}]_o$. Thus, these three molecules may utilize similar signaling cascades to trigger the turning response. In contrast, gradients of NT-3, another member of the neurotrophin gene family, were shown to attract growth cones of these Xenopus neurons, but the attractive response was independent of $[Ca^{2+}]_o$ and unaffected by PKA inhibitors (Song et al., 1997). These findings indicate that there exist alternative intracellular signal pathways for axon guidance. One surprising implication of our findings is that guidance triggered by completely unrelated molecules (BDNF and netrin-1) may share one pathway, while closely related molecules (BDNF and NT-3) may utilize divergent signaling mechanisms.

Previous studies on BDNF-induced turning of growth cones suggest that Ca^{2+} signaling lies upstream from the cAMP-dependent step in the cascade of events, since attractive turning induced by a forskolin gradient was not affected by removal of extracellular Ca^{2+} (Song et al., 1997). A netrin-induced Ca^{2+} influx may trigger a rise in cAMP through activation of Ca^{2+}-dependent adenylate cyclases (Yovell et al., 1992; Wayman et al., 1994), thus creating a cAMP gradient within the growth cone, a condition known to result in an attractive turning response of these Xenopus growth cones (Lohof et al., 1992). It is possible that a gradient of cytosolic Ca^{2+} induced by netrin-1 is responsible for triggering the repulsive response of the growth cone, but the effect is normally overridden by the attractive response due to a cAMP gradient generated by the Ca^{2+} gradient. Inhibition of cAMP-dependent processes by Rp-cAMPS or KT5720 may thus unmask the repulsive action of the cytosolic Ca^{2+} gradient.

In principle, cAMP and the cAMP-dependent protein kinase pathway could regulate either the receptors for different diffusible guidance cues or the activity of the downstream effector molecules activated by these receptors. The cAMP/cAMP-dependent protein kinase may thereby act as a gating mechanism (Iyengar, 1996), being differentially permissive for a receptor-induced signaling cascade, dependent on its functional status. Various neurotrophic factors, including BDNF, fail to support the survival of retinal ganglion cells in culture, unless the cytosolic cAMP levels are raised by forskolin, glutamate, or high concentrations of potassium, an example of a cAMP-mediated gating mechanism for neurotrophin transduction (Meyer-Franke et al., 1995). Alternatively, cAMP may serve as a molecular switch. For example, while EGF alone stimulates the proliferation of PC12 cells, elevating the cytosolic cAMP level causes PC12 cells to differentiate in the presence of EGF (Mark et al., 1995; Yao et al., 1995). In the case of BDNF-induced growth cone turning, the cAMP/cAMP-dependent protein kinase pathway appears to operate not merely as a gating or switching mechanism. Elevating cytosolic cAMP levels by treatment with forskolin or Sp-cAMP caused a potentiation of the attractive response of the growth cones to BDNF gradients created at low concentrations, which normally were ineffective in attracting the growth cone (Song et al., 1997). In the present study, we did not find significant enhancement of the turning response to netrin-1 gradients by addition of Sp-cAMP (Figures 2 and 4). This finding, together with the narrow range of Rp-cAMPS by which the turning response is changed from an attractive to a repulsive response, suggests that the action of cAMP-dependent activity resembles more closely a switch.

One group of potential downstream targets of PKA is small GTP-binding proteins of the rho family, e.g., rhoA, rac1, and cdc42, which are known to mediate morphological changes by regulating the actin cytoskeleton and

to play a role in growth cone turning (Luo et al., 1997). There is evidence that rhoA is involved in mediating growth cone collapse and neurite retraction (Kozma et al., 1997). It is known that PKA can phosphorylate rhoA, leading to the translocation of membrane-associated rhoA to the cytoplasm and thus providing an additional mechanism for its inactivation. Laudanna et al. (1997) have shown recently that cAMP functions as a gating element on the chemoattractant-induced rho-dependent signaling pathway in leukocytes, since elevation of cAMP inhibits rhoA activation and integrin-dependent adhesion in these cells. It would thus be of interest to examine whether netrin-1 exerts its effect on growth cones of these Xenopus spinal neurons through the local modulation of members of the rho family.

The observation that lowering PKA activity converts netrin-1-induced attraction into repulsion suggests the intriguing possibility that activation of an UNC-5-like protein may down-regulate PKA. For example, UNC-5 may inhibit adenylate cyclase activity or stimulate phosphodiesterase, which lowers the cAMP level and consequently PKA activity. Alternatively, the cAMP/cAMP-dependent protein kinase pathway may determine the permissiveness for netrin signaling through UNC-5. Xenopus spinal neurons may express an UNC-5 homolog, and its activation may lead to growth cone repulsion. In this case, the efficacy of UNC-5 signaling must be regulated by the intracellular milieu, e.g., the status of the cAMP/cAMP-dependent protein kinase pathway, otherwise netrin-1 would be inducing repulsion of these growth cones under normal conditions. Such regulation could be achieved either at the level of downstream effector proteins of the signaling cascade or at the level of the receptor itself. In the latter case, some modification of UNC-5 or an associated molecule may inhibit signaling through this receptor, for example, by preventing the recruitment of UNC-5 to a heteromeric receptor complex with DCC/UNC-40. Regardless of the mechanism through which modulation of the cAMP pathway produces its effects in this experimental setting, however, our results support the idea that the pathways involved in netrin-mediated attraction and repulsion are mechanistically related.

Experimental Procedures

Cell Cultures

Cultures of Xenopus spinal neurons were prepared from the neural tube tissue of 1-day-old Xenopus embryos by methods previously described (Tabti and Poo, 1991). The tissue was dissociated in Ca^{2+}–Mg^{2+}-free solution (115 mM NaCl, 2.5 mM KCl, 10 mM HEPES, and 0.5 mM EDTA [pH 7.4]) for 20 min and plated on the surface of clean glass coverslips. The cultures were incubated at room temperature (20°C–22°C) for 14 hr before use for the experiment. The culture medium consisted of 49% (v/v) Leibovitz medium (GIBCO, Gaithersburg, MD), 1% (v/v) fetal bovine serum (HyClone, Logan, UT), and 50% (v/v) Ringer's solution (115 mM NaCl, 2 mM $CaCl_2$, 2.5 mM KCl, and 10 mM HEPES [pH 7.4]). The experiments were carried out in culture medium. For Rp-cAMPS and KT5720 experiments, drugs were added to the culture medium 30 min before and were present during the experiments. For antibody blocking experiments, anti-DCC antibody (an IgG1 mouse monoclonal antibody, clone AF5, Oncogene Sci., used at 1 μg/ml) was added to the culture medium 30 min before and present during the experiment. Saline of 1 μM $[Ca^{2+}]_o$ was modified Ringer's solution containing 140 mM NaCl, 1 mM $MgCl_2$, 0.9 mM $CaCl_2$, 5 mM KCl, 4 mM EGTA, and 10 mM HEPES (pH 7.4).

Production of Netrin-1 Gradients

Chick recombinant netrin-1 was purified by heparin-affinity chromatography (Serafini et al., 1994) from medium conditioned by a human embryonic kidney 293 cell line secreting recombinant netrin-1 (Shirasaki et al., 1996). Microscopic gradients of netrin-1 were produced by methods previously described (Lohof et al., 1992; Song et al., 1997). Repetitive pressure injection of picoliter volumes of solutions containing purified recombinant chick netrin-1 protein was applied through a micropipette with a tip opening of ~1 μm. The pressure was applied with an electrically gated pressure application system (Picospritzer, General Valve, Fairfield, NJ). A standard pressure pulse of 3 psi in amplitude and 20 ms in duration was applied to the pipette at a frequency of 2 Hz using a pulse generator (SD9, Grass Instruments, Quincy, MA). By measuring the size of droplets in mineral oil after 50 pulses of repetitive ejection with the same pressure pulse parameters, the average volume of ejected solution per pulse was estimated to be ~1.5 pl. Theoretical analysis (Lohof et al., 1992) and direct measurements of the gradient using fluorescent dyes (Zheng et al., 1994) have shown that, using our standard pulsing parameters, the average concentration of the chemical is $\sim 10^3$-fold lower at the growth cone than that in the pipette, and the concentration gradient across the width of a typical growth cone (10 μm) at a distance of 100 μm from the pipette tip is in the range of 5%–10%. The total volume of the saline in the culture dish was 5 ml. The final average concentration of netrin-1 at the end of the experiment was about 0.01–0.02 ng/ml.

Measurements of Neurite Extension and Growth Cone Turning

Phase-contrast inverted microscopes (Nikon Diaphot or TMS) were used to observe the neurite growth. Microscopic images of neurites were recorded with a CCD camera (Toshiba IK-541RA) attached to the microscope and stored in videotapes for later analysis. To determine the total length of neurite extension, the entire trajectory of the neurite at the end of the 1 hr period was measured with a digitizer. For assaying growth cone turning, the tip of the pipette containing the chemical was placed 100 μm away from the center of the growth cone and at an angle of 45° with respect to the direction of initial direction of neurite extension. The initial direction of the neurite was determined 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 hr period. Only growth cones with net extension >5 μm over the 1 hr period were included for analysis.

Acknowledgments

This work was supported by grants from the National Institutes of Health to M.-M.P. and to C.E.H. B.B. is supported by a fellowship of the Human Frontier Science Program Organization. M.T.-L. is an Investigator of the Howard Hughes Medical Institute.

Received September 19, 1997; revised October 27, 1997.

References

Ackerman, S.L., Kozak, L.P., Przyborski, S.A., Rund, L.A., Boyer, B.B., and Knowles, B.B. (1997). The mouse rostral cerebellar malformation gene encodes an UNC-5-like protein. Nature *386*, 838–842.

Bandtlow, C.E., Schmidt, M.F., Hassinger, T.D., Schwab, M.E., and Kater, S.B. (1993). Role of intracellular calcium in NI-35-evoked collapse of neuronal growth cones. Science *259*, 80–83.

Bixby, J.L., and Spitzer, N.C. (1984a). The appearance and development of neurotransmitter sensitivity in Xenopus embryonic spinal neurons in vitro. J. Physiol. *353*, 143–155.

Bixby, J.L., and Spitzer, N.C. (1984b). Early differentiation of vertebrate spinal neurons in the absence of voltage-dependent Ca^{2+} and Na^+ influx. Dev. Biol. *106*, 89–96.

Chan, S.S.-Y., Zheng, H., Su, M.-W., Wilk, R., Killeen, M.T., Hedgecock, E.M., and Culotti, J.G. (1996). UNC-40, a C. elegans homolog of DCC (Deleted in Colorectal Cancer), is required in motile cells responding to UNC-6 netrin cues. Cell *87*, 187–196.

Colamarino, S.A., and Tessier-Lavigne, M. (1995). The axonal chemoattractant netrin-1 is also a chemorepellent for trochlear motor axons. Cell *81*, 621–629.

Cooper, J.R., Bloom, F.E., and Roth, R.H. (1996). The Biochemical Basis of Neuropharmacology, 7th Ed. (New York: Oxford University Press).

Deiner, M.S., Kennedy, T.E., Fazeli, A., Serafini, T., Skaenes, W.C., Tessier-Lavigne, M., and Sretevan, D.W. (1997). Netrin-1 and DCC mediate axon guidance locally at the optic disc: loss of function leads to optic nerve hypoplasia. Neuron *19*, 575–589.

de la Torre J.R., Höpker, V.H., Ming, G.-L., Poo, M.-M., Tessier-Lavigne, M., Hemmati-Brivanlou, A., and Holt, C.E. (1997). Turning of retinal growth cones in a netrin-1 gradient mediated by the netrin receptor DCC. Neuron, this issue, *19*, 1211–1224.

Evers, J., Laser, M., Sun, Y.A., Xie, Z.P., and Poo, M.-M. (1989). Studies of nerve-muscle interactions in Xenopus cell culture: analysis of early synaptic currents. J. Neurosci. *9*, 1523–1539.

Fazeli, A., Dickinson, S.L., Hermiston, M.L., Tighe, R.V., Steen, R.G., Small, C.G., Stoeckli, E.T., Keino-Masu, K., Masu, M., Rayburn, H., et al. (1997). Phenotype of mice lacking functional deleted in colorectal cancer (DCC) gene. Nature *386*, 796–804.

Gu, X., and Spitzer, N. (1995). Distinct aspects of neuronal differentiation encoded by frequency of spontaneous Ca^{2+} transients. Nature *375*, 784–787.

Hamelin, M., Zhou, Y., Su, M.-W., Scott, I.M., and Culotti, J.G. (1993). Expression of the UNC-5 guidance receptor in the touch neurons of C. elegans steers their axons dorsally. Nature *364*, 327–330.

Harris, R., Sabatelli, L.M., and Seeger, M.A. (1996). Guidance cues at the Drosophila CNS midline: identification and characterization of two Drosophila Netrin/UNC-6 homologs. Neuron *17*, 217–228.

Hedgecock, E.M., Culotti, J.G., and Hall, D.H. (1990). The *unc-5*, *unc-6*, and *unc-40* genes guide circumferential migrations of pioneer axons and mesodermal cells on the epidermis in C. elegans. Neuron *2*, 61–85.

Hempel, C.M., Vincent, P., Adams, S.R., Tsien, R.Y., and Selverston, A.I. (1997). Spatio-temporal dynamics of cyclic AMP signals in an intact neural circuit. Nature *384*, 166–169.

Iyengar, R. (1996). Gating by cyclic AMP: expanded role for an old signaling pathway. Science *271*, 461–463.

Kase, H., Iwahashi, K., Nakanishi, S., Matsuda, Y., Yamada, K., Takahashi, M., Murakata, C., Sato, A., and Kaneko, M. (1987). K-252 compounds, novel and potent inhibitors of protein kinase C and cyclic nucleotide-dependent protein kinases. Biochem. Biophys. Res. Commun. *142*, 436–440.

Kater, S.B., Mattson, M.P., Cohan, C., and Conner, J.A. (1988). Calcium regulation of the neuronal growth cone. Trends Neurosci. *11*, 317–323.

Keino-Masu, K., Masu, M., Hinck, L., Leonardo, E.D., Chan, S.S., Culotti, J.G., and Tessier-Lavigne, M. (1996). Deleted in colorectal cancer (DCC) encodes a netrin receptor. Cell *87*, 175–185.

Kennedy, T.E., Serafini, T., de la Torre, J.R., and Tessier-Lavigne, M. (1994). Netrins are diffusible chemotropic factors for commissural axons in the embryonic spinal cord. Cell *78*, 425–435.

Kolodziej, P.A., Timpe, L.C., Mitchell, K.J., Fried, S.R., Goodman, C.S., Jan, L.Y., and Jan, Y.N. (1996). frazzled encodes a Drosophila member of the DCC immunoglobulin subfamily and is required for CNS and motor axon guidance. Cell *87*, 197–204.

Kozma, R., Sarner, S., Ahmed, S., and Lim, L. (1997). Rho family GTPases and neuronal growth cone remodelling: relationship between increased complexity induced by cdc42Hs, Rac1, and acetylcholine and collapse induced by rhoA and lysophosphatidic acid. Mol. Cell. Biol. *17*, 1201–1211.

Lankford, K.L., and Letourneau, P.C. (1989). Evidence that calcium may control neurite outgrowth by regulating the stability of actin filaments. J. Cell Biol. *109*, 1229–1243.

Laudanna, C., Campbell, J.J., and Butcher, E.C. (1997). Elevation of intracellular cAMP inhibits RhoA activation and integrin-dependent leukocyte adhesion induced by chemoattractants. J. Biol. Chem. *272*, 24141–24144.

Laufer, R., and Changeux, J. (1987). Calcitonin gene-related peptide elevates cyclic AMP levels in chick skeletal muscle: possible neurotrophic role for a coexisting neuronal messenger. EMBO J. *6*, 901–906.

Leung-Hagesteijn, C., Spence, A.M., Stern, B.D., Zhou, Y., Su, M.W., Hedgecock, E.M., and Culotti, J.G. (1992). UNC-5, a transmembrane protein with immunoglobulin and thrombospondin type 1 domains, guides cell and pioneer axonmigrations in C. elegans. Cell *71*, 289–299.

Leonardo, E.D., Hinck, L., Masu, M., Keino-Masu, K., Ackerman, S.L., and Tessier-Lavigne, M. (1997). Vertebrate homologs of C. elegans UNC-5 are candidate netrin receptors. Nature *386*, 833–838.

Lohof, A.M., Quillan, M., Dan, Y., and Poo, M.-M. (1992). Asymmetric modulation of cytosolic cAMP activity induces growth cone turning. J. Neurosci. *12*, 1253–1261.

Luo, L., Jan L.Y., and Jan Y.-N. (1997). Rho family small GTP-binding proteins in growth cone signalling. Curr. Opin. Neuro. *7*, 81–86.

Mark, M.D., Liu, Y., Wong, S.T., Hinds, T.R., and Storm, D.R. (1995). Stimulation of neurite outgrowth in PC12 cells by EGF and KCl depolarization: a Ca^{2+}-independent phenomenon. J. Cell Biol. *130*, 701–710.

Meyer-Franke, A., Kaplan, M.R., Pfrieger, F.W., and Barres, B.A. (1995). Characterization of the signaling interactions that promote the survival and growth of developing retinal ganglion cells in culture. Neuron *15*, 805–819.

Mitchell, K.J., Doyle, J.L., Serafini, T., Kennedy, T.E., Tessier-Lavigne, M., Goodman, C.S., and Dickson, B.J. (1996). Genetic analysis of Netrin genes in Drosophila: Netrins guide CNS commissural axons and peripheral motor axons. Neuron *17*, 203–215.

Pierceall, W.E., Reale, M.A., Candia, A.F., Wright, C.V.E., Cho, K.R., and Fearon, E.R. (1994). Expression of a homolog of the Deleted in Colorectal Cancer (DCC) gene in the nerve system of developing Xenopus embryos. Dev. Biol. *166*, 654–665.

Rothermel, J.D., and Parker Botelho, L.H. (1988). A mechanistic and kinetic analysis of the interactions of the diastereoisomers of adenosine 3′,5′-(cyclic)phosphorothioate with purified cyclic AMP-dependent protein kinase. J. Biochem. *251*, 757–762.

Serafini, T., Kennedy, T.E., Galko, M.J., Mirzayan, C., Jessell, T.M., and Tessier-Lavigne, M. (1994). The netrins define a family of axon outgrowth-promoting proteins homologous to C. elegans UNC-6. Cell *78*, 409–424.

Serafini, T., Colamarino, S.A., Leonardo, E.D., Wang, H., Beddington, R., Skarnes, W.C., and Tessier-Lavigne, M. (1996). Netrin-1 is required for commissural axon guidance in the developing vertebrate nervous system. Cell *87*, 1001–1014.

Shirasaki, R., Mirzayan, C., Tessier-Lavigne, M., and Murakami, F. (1996). Guidance of circumferentially growing axons by netrin-dependent and -independent floor plate chemotropism in the vertebrate brain. Neuron *17*, 1079–1088.

Song, H.-J., Ming, G.-L., and Poo, M.-M. (1997). cAMP-induced switching in turning direction of nerve growth cones. Nature *388*, 275–279.

Tabti, N., and Poo, M.-M. (1991). Culturing spinal cord neurons and muscle cells from Xenopus embryos. In Culturing Nerve Cells, G. Banker and K. Goslin, eds. (Cambridge, MA: MIT Press), pp. 137–154.

Tessier-Lavigne, M., and Goodman, C.S. (1996). The molecular biology of axon guidance. Science *274*, 1123–1133.

Varela-Echavarria, A., Tucker, A., Puschel, A.W., and Guthrie, S. (1997). Motor axon subpopulations respond differentially to the chemorepellents netrin-1 and semaphorin D. Neuron *18*, 193–207.

Wadsworth, W.G., Bhatt, H., and Hedgecock, E.M. (1996). Neuroglia and pioneer neurons express UNC-6 to provide global and local netrin cues for guiding migrations in C. elegans. Neuron *16*, 35–46.

Wayman, G.A., Impey, S., Wu, Z., Kindsvogel, W., Prichard, L., and Storm, D.R. (1994). Synergistic activation of the type I adenylyl cyclase by Ca^{2+} and Gs-coupled receptors in vivo. J. Biol. Chem. *269*, 25400–25405.

Xie, Z.P., and Poo, M.-M. (1986). Initial events in the formation of neuromuscular synapse: rapid induction of acetylcholine release from embryonic neuron. Proc. Natl. Acad. Sci. USA *83*, 7069–7073.

Yao, H., Labudda, K., Rim, C., Capodieci, P., Loda, M., and Stork, P.J. (1995). Cyclic adenosine monophosphate can convert epidermal growth factor into a differentiating factor in neuronal cells. J. Biol. Chem. *270*, 20748–20753.

Yovell, Y., Kandel, E.R., Dudai, Y., and Abrams, T.W. (1992). A quantitative study of the Ca^{2+}/calmodulin sensitivity of adenylyl cyclase in Aplysia, Drosophila, and rat. J. Neurochem. *59*, 1736–1744.

Zheng, J.Q., Felder, M., Connor, J.A., and Poo, M.-M. (1994). Turning of nerve growth cones induced by neurotransmitters. Nature *368*, 140–144.

Zheng, J.Q., Wan, J.-J., and Poo, M.-M. (1996). Essential role of filopodia in chemotropic turning of nerve growth cone induced by a glutamate gradient. J. Neurosci. *16*, 1140–1149.