Adaptation in the chemotactic guidance of nerve growth cones

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Pathfinding by growing axons in the developing nervous system may be guided by gradients of extracellular guidance factors. Analogous to the process of chemotaxis in microorganisms, we found that axonal growth cones of cultured *Xenopus* spinal neurons exhibit adaptation during chemotactic migration, undergoing consecutive phases of desensitization and resensitization in the presence of increasing basal concentrations of the guidance factor netrin-1 or brain-derived neurotrophic factor. The desensitization is specific to the guidance factor and is accompanied by a reduction of Ca^{2+} signalling, whereas resensitization requires activation of mitogen-associated protein kinase and local protein synthesis. Such adaptive behaviour allows the growth cone to re-adjust its sensitivity over a wide range of concentrations of the guidance factor, an essential feature for long-range chemotaxis.

The formation of specific connections between nerve cells in the developing nervous system depends on the ability of growing axons to reach appropriate target cells, a process known as pathfinding[1,2]. Intrigued by the amoeboid morphology of axonal growth cones, Ramón y Cajal suggested more than a century ago that growth cones might be guided by gradients of chemical substances secreted from the target tissue in a manner similar to the chemotaxis of leukocytes[3]. There is now substantial evidence that axon pathfinding in many regions of the developing brain is indeed guided by diffusible guidance cues[1,2,4–7]. For long-range guidance based on the detection of chemoattractant gradients, the growth cone must be able to respond reliably to small gradients of guidance cues across its surface. This may be achieved by amplification of guidance signals through cellular transduction processes[8]. In addition, as the growth cone migrates in an environment in which the basal concentration of the guidance cue varies by many orders of magnitude, it may also need to constantly re-adjust its sensitivity, a process commonly referred to as adaptation[8–11]. The cellular mechanisms underlying the amplification and adaptation of guidance signals are largely unknown. In this study, we have examined quantitatively the phenomenon of adaptation in the chemotaxis of nerve growth cones *in vitro*. When exposed to high levels of netrin-1 or brain-derived neurotrophic factor (BDNF), the growth cones of cultured *Xenopus* spinal neurons undergo consecutive phases of desensitization (or interference) and resensitization (or adaptation) in their ability to detect gradients of netrin-1 or BDNF. This process is reminiscent of the adaptation in bacterial chemotaxis towards a chemoattractant[12]. These cyclic phases of desensitization and resensitization are also reflected in the 'zig-zag' path of alternating attractive and repulsive turning of fast-advancing growth cones in their migration towards the source of these two guidance cues (netrin-1 and BDNF). Further studies indicate that desensitization occurs at a level upstream from the signalling effector(s) shared by netrin-1 and BDNF, and is accompanied by a loss of Ca^{2+} signalling in response to the gradient of the guidance factor. The resensitization, on the other hand, requires the activation of mitogen-associated protein kinase (MAPK), which is induced by both netrin-1 and BDNF. Surprisingly, we found that inhibition of protein translation, but not gene transcription, blocked the resensitization of the growth cone to the netrin-1 gradient, suggesting that MAPK-dependent initiation of protein synthesis is required for the resensitization process. This idea is further supported by the observation that inhibition of protein synthesis in a growing neurite severed from the cell body abolished the chemotaxis of its growth cone in a netrin-1 gradient.

Adaptation in growth-cone chemotaxis

Growth cones of isolated *Xenopus* spinal neurons in culture exhibit chemotactic turning responses when exposed to a gradient of netrin-1 (ref. 13)—a diffusible guidance cue for developing axons[4] and migrating neuroblasts[14]. The gradient was produced by repetitive pulsatile ejection of picolitres of solution containing netrin-1 ($5\,\mu g\,ml^{-1}$) from a micropipette[15–18], which was positioned at a 45° angle with respect to the direction of initial neurite extension and a distance of 100 μm away from the centre of the growth cone. As shown in Fig. 1a, marked chemotactic turning of the growth cone was observed 30 min after application of this standard netrin-1 gradient. To examine the role of the basal level of receptor activation on the turning response of the growth cone, we uniformly applied netrin-1 at different concentrations to the culture immediately before the onset of the turning assay. We found that the overall chemotactic response, as determined by the average turning angle of growth cones at the end of the 30-min assay, was significantly reduced in the presence of $0.5\,ng\,ml^{-1}$ netrin-1 in a bath solution, and completely abolished when the bath concentration was increased to 1 or $5\,ng\,ml^{-1}$ (Figs 1b, d and 2a). The presence of netrin-1 in the bath interfered and desensitized the ability of the growth cone to sense the standard netrin-1 gradient, probably owing to uniform activation of netrin-1 receptors before exposure to the gradient. This dose-dependent desensitization of chemotactic turning was also observed for BDNF, a neurotrophin known to be a chemoattractant for some growth cones *in vitro*[17,18] and *in vivo*[19]. As shown in Fig. 2c, BDNF at a bath concentration of $20\,ng\,ml^{-1}$ completely desensitized the attractive response induced by the standard gradient of BDNF ($50\,\mu g\,ml^{-1}$ in the pipette). The desensitization induced by either netrin-1 or BDNF required the continuous presence of the factor. Removal of netrin-1 from the medium after 30 min pre-incubation resulted in the recovery of the turning response within 30 min (Fig. 2c), the minimal time required for the turning assay. At the threshold bath concentration of netrin-1 ($1\,ng\,ml^{-1}$), the lack of response towards the standard netrin-1 gradient represents a reduction rather than complete loss of netrin-

1 sensitivity, as increasing the gradient of netrin-1 by putting the netrin-1 pipette at 70 μm from the growth cone resulted in a significant turning response in the presence of 1 ng ml^{-1} of netrin-1 (Fig. 2a).

In the continuous presence of netrin-1 applied to the bath (at 1 or 5 ng ml^{-1}), which desensitized the ability of the growth cone to detect the standard netrin-1 gradient, the growth cone regained its sensitivity to the gradient after a prolonged period of incubation. When we applied a netrin-1 gradient at 90 or 120 min after bath-application of netrin-1 (1 or 5 ng ml^{-1}), robust attractive turning of the growth cone was found (Figs 1c, d and 2c). This recovery of sensitivity was not due to degradation of netrin-1 in the bath, as the medium containing 1 ng ml^{-1} netrin-1 remained fully effective in desensitizing the growth cone when introduced into fresh cultures (see Methods). Furthermore, when the growth cone recovered its sensitivity towards the standard netrin-1 gradient after 90 min incubation of 1 ng ml^{-1} in the bath, increasing netrin-1 in the bath to 5 ng ml^{-1} again desensitized the growth cone (Fig. 2c). Attractive turning in the standard gradient of BDNF also recovered in the continuous presence of 20 ng ml^{-1} BDNF, with normal attractive turning observed when the turning assay began at 60 min after application of BDNF to the bath (Fig. 2d). Thus the growth cone is capable of re-adjusting its sensitivity towards a guidance factor after a change in the basal concentration of the factor.

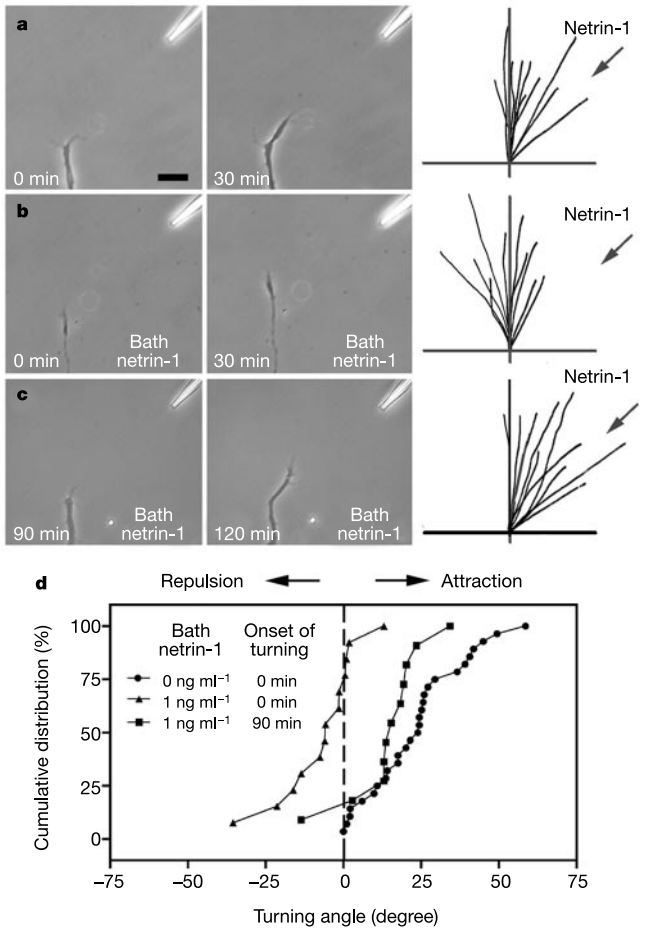

Figure 1 Turning of nerve growth cones induced by a gradient of netrin-1. **a**, Images of a growth cone at the onset (left) and the end (right) of a 30-min exposure to the standard netrin-1 gradient (arrow). Superimposed traces depict ten samples of neurite trajectories at the end of the assay (diagram). The origin is the centre of the growth cone at the onset of the assay, and the original direction of growth was vertical. Scale bar, 20 μm for images and 5 μm for diagram. **b**, **c**, As in **a**, except that netrin-1 (1 ng ml^{-1}) was added to the culture immediately before (**b**) or 90 min before (**c**) the onset of the netrin-1 gradient. **d**, The distribution of turning angles. For each experimental condition, angular positions of all growth cones at the end of 30-min exposure to a netrin-1 gradient are plotted. The per cent value refers to the percentage of growth cones with turning angle less than or equal to a given angular value. Data are from experiments similar to those shown in **a**–**c**.

Zig-zag chemotaxis of the growth cone

Does the adaptive process revealed by bath application of guidance cues have a role in growth-cone chemotaxis in a gradient of the guidance cue? To address this question, we further examined the behaviour of the growth cone as it migrates up the gradient of netrin-1 or BDNF. As shown in Fig. 3, we observed that fast-advancing growth cones consistently exhibited a zig-zag pattern of migration towards the source of netrin-1 or BDNF, with alternating periods of attractive and repulsive responses. Whereas the behaviour of filopodia initiation and the path of growth-cone migration showed a zig-zag pattern, the trajectory of the neurite became smoother with time. Thus the zig-zag pattern was often not detectable for slowly advancing neurites at the end of the turning assay. The average interval for the zig-zag was 20.0 ± 2.5 min (s.e.m., $n = 5$) in the standard netrin-1 gradient and 22.8 ± 2.0 min (s.e.m., $n = 5$) in the standard BDNF gradient. In the absence of the guidance factor, there is no regular zig-zag and the growth cone advances in an irregular, unpredictable manner. This irregular path is probably determined by variations in substrate adhesion and other random variables in the culture condition. As discussed below, this zig-zag pattern of extension is consistent with cycles of rapid and dose-dependent desensitization and slower resensitization/adaptation processes at the growth cone. Asymmetry in the extent of desensitization across the growth cone, with a higher level of desensitization on the side facing the gradient may be the cause of repulsion after each attractive turning. The average

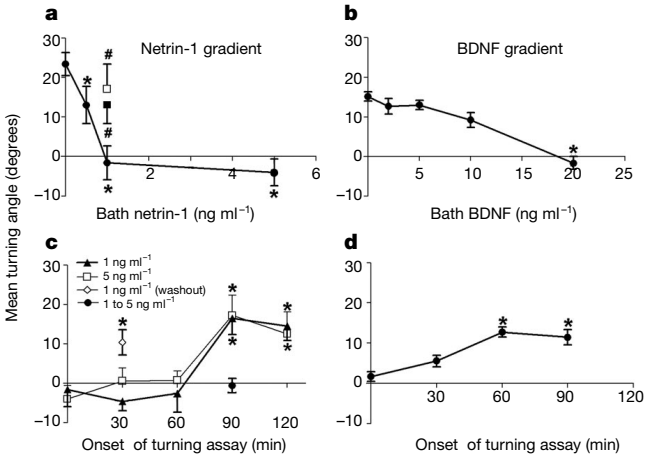

Figure 2 Desensitization and resensitization of turning responses. **a**, **b**, Dose-dependent desensitization of growth-cone responses in a standard gradient of netrin-1 (**a**) or BDNF (**b**). The concentration refers to netrin-1 and BDNF added in the saline before the turning assay. The data shown are mean turning angles (±s.e.m, $n = 10$–30). An asterisk indicates values significantly different from controls (without netrin-1 or BDNF in the bath; $P < 0.01$). Also shown is turning induced by a higher netrin-1 gradient (pipette at 70 μm) in the absence (open square) or presence (filled square) of bath netrin-1 (1 ng ml^{-1}). Hash symbols indicate values significantly different from controls (standard netrin-1 gradient with 1 ng ml^{-1} bath netrin-1). **c**, **d**, Time course of resensitization. The mean turning angle was measured at various times ($n = 10$–30) after addition of netrin-1 (1 and 5 ng ml^{-1}, in **c**) or BDNF (20 ng ml^{-1}, in **d**) to the bath. An asterisk indicates values significantly different from the control ($t = 0$ min, $P < 0.01$). Washout, bath netrin-1 (1 ng ml^{-1}) was washed out after 30 min; 1 to 5, bath netrin-1 was changed from 1 to 5 ng ml^{-1} after 90 min incubation before the turning assay.

interval between each zig-zag was shorter than the time required for resensitization described above. Owing to the shallow gradient (pipette at 100 μm) used in the standard turning assay, the time course of resensitization found in the bath incubation experiments may represent an underestimate of the normal adaptive responses of the growth cone when it faces progressively steeper gradients during its migration towards the pipette.

Heterologous desensitization

The specificity of growth-cone adaptation was further studied by examining heterologous cross-desensitization between netrin-1 and BDNF. For transduction of the directional signal triggered by a gradient of receptor activation at the growth cone, a gradient of activity of cytoplasmic effector(s) must be generated[8]. As these two guidance cues share common cytosolic transduction mechanisms, for example, activation of phosphoinositide 3 kinase (PI(3)K) and phospholipase Cγ (PLC-γ, ref. 20), and also Ca^{2+} signalling[13,17,18,21], uniform bath application of one factor at concentrations that cause saturated activation of the shared effector(s) should desensitize the response of the growth cone towards a gradient of the other factor. In agreement with a previous report[20], we found that the growth cone exhibited no turning response towards the standard netrin-1 gradient when 100 ng ml^{-1} BDNF was present in the bath (Fig. 4a). Conversely, a high bath concentration of netrin-1 (10 ng ml^{-1}) desensitized the turning response of the growth cone towards the standard BDNF gradient (Fig. 4b). Thus, saturated activation of guidance signalling mechanisms indeed results in heterologous desensitization of the growth cone. However, when the bath concentration of BDNF was lowered to the threshold level (20 ng ml^{-1}) for inducing homologous desensitization towards the standard BDNF gradient, we found significant turning response still occurred towards the standard netrin-1 gradient, although the average turning angle was lower than that found in the absence of BDNF in the bath (Fig. 4a). Conversely, bath application of 1 ng ml^{-1} netrin-1 resulted in no heterologous desensitization of the growth cone towards the BDNF gradient (Fig. 4b). Thus homologous desensitization induced by bath application of netrin-1 or BDNF at the threshold concentration did not cause saturated activation of their respective surface receptors nor their shared cytoplasmic effector(s). This is consistent with the finding that after homologous desensitization of the growth cone towards the standard netrin-1 gradient at the threshold bath concentration of netrin-1 (1 ng ml^{-1}), increasing the slope of the netrin-1 gradient resulted in significant attractive turning (Fig. 2a). The absence of heterologous desensitization described above also suggests that homologous desensitization of netrin-1 and BDNF responses at threshold concentrations is specific to the factor and occurs upstream from the shared cytoplasmic effector(s), possibly at the level of membrane receptors.

Cytosolic Ca^{2+} signalling

Cytosolic Ca^{2+} ($[Ca^{2+}]_i$) regulates neurite outgrowth[22–25] and mediates growth-cone turning responses induced by acetylcholine[16] and netrin-1 (refs 13, 21). We monitored changes of growth cone $[Ca^{2+}]_i$ induced by the standard netrin-1 gradient, using the Ca^{2+}-sensitive fluorescence indicator Oregon green BAPTA-1 conjugated to dextran (Fig. 5a, b). Elevation of $[Ca^{2+}]_i$ at the growth cone was consistently observed within minutes after the onset of the gradient, with a transient gradient of Ca^{2+} across the growth cone detected in some cases[21]. Moreover, we found that bath application of netrin-1 at a desensitizing concentration (5 ng ml^{-1}) caused a persistent $[Ca^{2+}]_i$ elevation (Fig. 5d) and the standard netrin-1 gradient failed to induce significant $[Ca^{2+}]_i$ elevation during the period of desensitization (Fig. 5a, c, e). This elevation of $[Ca^{2+}]_i$ induced by bath-applied netrin-1 was not responsible for triggering the desensitization. We found that similar $[Ca^{2+}]_i$ elevation induced by perfusing the culture with medium containing 2.5 mM Ca^{2+} and

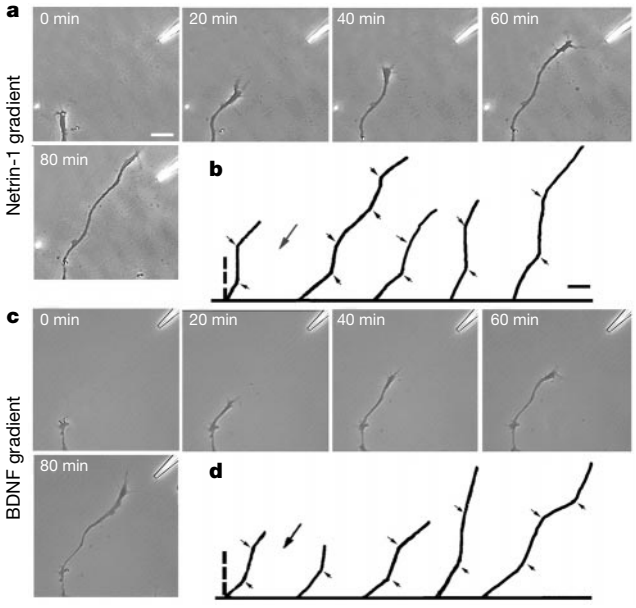

Figure 3 Zig-zag pathfinding of the growth cone. **a**, **b**, Images of fast-advancing growth cones in a standard gradient of netrin-1 at different times after the onset of the gradient (**a**). Note the zig-zag pattern of the neurite trajectory, reflecting alternating attractive and repulsive turning of the growth cone. Sample tracings of the migratory path of growth cones examined in similar fashion are indicated (**b**). The original direction of growth was vertical and the large arrow marks the direction of the gradient (shown only for one growth cone). Note that the tracing depicts the actual position of five different growth cones during chemotaxis, not the neurite trajectory at the end of the experiment. Small arrows indicate turning points of the zig-zag. Scale bar, 20 μm for images and 5 μm for traces. **c**, **d**, Similar to **a**, **b**, except that a standard BDNF gradient was used.

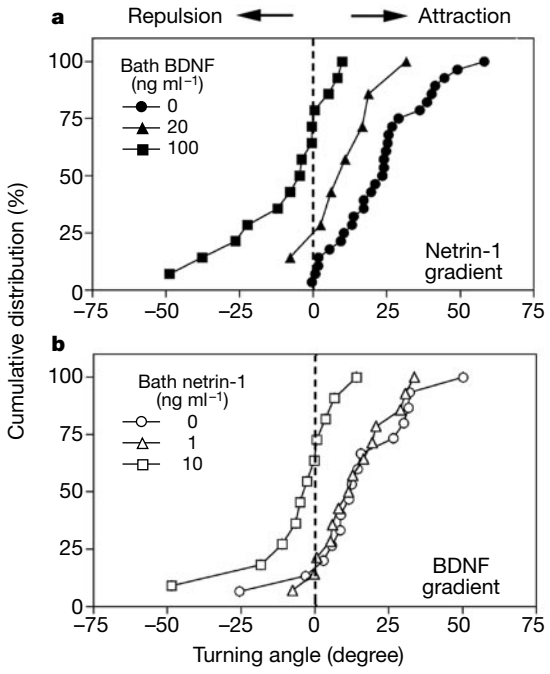

Figure 4 Heterologous desensitization between netrin-1 and BDNF. **a**, **b**, Growth-cone turning induced by the standard gradient of netrin-1 or BDNF, in the presence of different concentrations of BDNF or netrin-1 in the bath, respectively. Note significant heterologous desensitization at the high but not at low concentrations of BDNF and netrin-1 ($P < 0.01$).

12.5 mM K^+ had no significant effect on the attractive turning of the growth cone in the netrin-1 gradient (data not shown). Thus, the growth-cone sensitivity to the netrin-1 gradient correlates with Ca^{2+} signalling, but $[Ca^{2+}]_i$ elevation by itself is not sufficient to trigger desensitization of the growth cone.

After 90 min incubation with netrin-1 in the bath, when resensitization of the growth cone had occurred, we found that the standard netrin-1 gradient was able to induce further $[Ca^{2+}]_i$ elevation, which was superimposed on top of the elevated basal $[Ca^{2+}]_i$ (Fig. 5b, c, e). Similar results were obtained with ratio imaging of fluorescence from co-injected Oregon green and Texas red (see Methods), making it unlikely that these results were due to fluctuations in the growth-cone thickness or changes in focal plane that may have occurred during the experiment. Taken together, these findings indicate that Ca^{2+} signals correlate with the sensitivity of the growth cone, and that netrin-1-induced desensitization/resensitization of the turning responses results from transduction failure/restoration at the level(s) upstream from Ca^{2+} signalling.

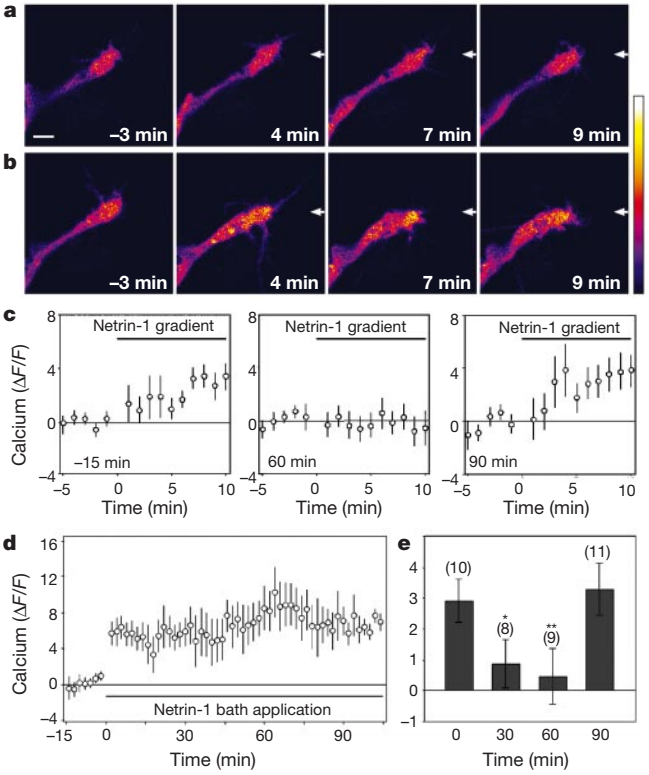

Figure 5 Calcium signals induced by a netrin-1 gradient correlate with adaptation. **a**, **b**, Fluorescence images of a *Xenopus* growth cone loaded with a Ca^{2+}-sensitive fluorescent indicator (Oregon green BAPTA-1-dextran; images show Oregon green BAPTA fluorescence normalized to Texas red). The neuron was incubated with netrin-1 (5 ng ml^{-1}) in the bath 30 min (**a**) or 90 min (**b**) before exposure to a netrin-1 gradient (arrows). Images show Oregon green fluorescence at different times (min) before and after the onset of the gradient. Pseudocolour indicates Ca^{2+} levels: white is highest and black is lowest. Scale bar, 10 μm. **c**, Percentage changes in fluorescence ($\Delta F/F$) with time of the growth cone induced by the netrin-1 gradient (bar) 15 min before (left), and 60 min (middle) and 90 min (right) after addition of netrin-1 (5 ng ml^{-1}) to the bath. The fluorescence values (1-min bins) were normalized to the mean value during the 5-min period before applying the gradient for each growth cone. Data points represent mean ± s.e.m. ($n = 9$–11). **d**, Ca^{2+} changes in the growth cone with time before and after uniform exposure to 5 ng ml^{-1} netrin-1 in the bath solution. **e**, Summary of Ca^{2+} changes induced by a netrin-1 gradient after pre-incubation with netrin-1 (5 ng ml^{-1}) in the bath for 0, 30, 60 and 90 min. The mean fluorescence change in the growth cone measured during the last 5 min of a 10-min exposure to a netrin-1 gradient was normalized to the mean pre-gradient value over a 10-min period. Data significantly different from the control (no netrin-1 in the bath) are marked by a single ($P < 0.05$) or double ($P < 0.01$) asterisk, using the Mann–Whitney rank sum test. Values in parentheses indicate total number of growth cones examined in this condition.

Resensitization requires MAPK activation

To further explore the intracellular events involved in the resensitization of chemotactic responses, we examined the role of MAPK signalling, which is known to be activated by neurotrophins[26]. We treated *Xenopus* spinal cord explant cultures with BDNF (10 ng ml^{-1}) for various times and examined MAPK activity by monitoring phosphotransferase activity of immunoprecipitated MAPK towards myelin basic protein, a standard substrate for examining MAPK activity[27]. We found that BDNF induced a robust increase in MAPK activity that persisted for at least 60–90 min (Fig. 6). Interestingly, MAPK activity was also activated by netrin-1 (20 ng ml^{-1}) by 4–5-fold, peaking within 5–10 min after the onset of the treatment. Similar results were obtained by immunoblot analysis using a specific antibody directed against dually phosphorylated MAPK (Fig. 6c). Furthermore, the MAPK activation was blocked by U0126 (10 μM), which is a potent and selective inhibitor of MEK1, the upstream activator of MAPK[28].

Is MAPK activation by netrin-1 required for resensitization of the turning response? We found that bath application of 10 μM U0126 or PD98059 (ref. 29)—two structurally different inhibitors for MEK1—for the first 60 min completely abolished resensitization of the turning response when tested at 90 min after bath application of netrin-1 (1 ng ml^{-1}, Fig. 6d). Furthermore, in the absence of any bath-applied netrin-1, the attractive turning response induced by the standard netrin-1 gradient was significantly attenuated when the turning assay was performed 20 min after addition of these MEK1 inhibitors into the culture (Fig. 6e). Pre-incubation with U0126 for 60 min before the turning assay, however, completely abolished the chemotaxis in the netrin-1 gradient (Fig. 6e). These results suggest that the effects of MAPK inhibition may proceed with a slow time course, resulting in a lack of resensitization required for effective chemotaxis of the growth cone.

The role of local protein synthesis

The MAPK pathway has been implicated in a wide range of cellular processes, including stimulation of protein synthesis in response to growth factors[30]. We thus examined whether resensitization of the growth cone response to gradients of netrin-1 requires protein synthesis. Neurons were incubated for 60 min with netrin-1 (1 ng ml^{-1}) and cycloheximide (10 μg ml^{-1}), a reversible protein synthesis inhibitor[31]. They were then perfused with fresh saline containing 1 ng ml^{-1} netrin-1 for 30 min and assayed for the turning response at 90 min, a time normally sufficient for resensitization. We found that the growth cones exhibited no recovery in their sensitivity towards the standard netrin-1 gradient (Fig. 7a). Similar results were also observed with anisomycin (30 μM, Fig. 7a), another reversible protein synthesis inhibitor[32]. In contrast, when 5,6-dichlorobenzimidazole riboside (DRB, 50 μM), a reversible inhibitor of gene transcription[33], was used instead of cycloheximide or anisomycin in the incubation saline, we observed normal recovery of chemoattraction when assayed at 90 min (Fig. 7a). Importantly, the control experiments using cultures not incubated with netrin-1 in the bath showed that such a 60-min treatment with cycloheximide or DRB did not significantly affect the attractive turning response induced by the netrin-1 gradient examined 30 min after washing away the drug (Fig. 7a). Thus, protein synthesis but not gene transcription during the first 60 min after the treatment of a desensitizing concentration of netrin-1 is required for resensitization to take place.

The role of protein synthesis was further examined for chemotaxis of the growth cone under the standard netrin-1 gradient in the absence of any bath-applied netrin-1. As shown in Fig. 7b, after

20 min pre-incubation of cycloheximide (10 μg ml^{-1}), the growth cone consistently failed to exhibit chemoattraction in the standard netrin-1 gradient; instead, repulsive turning was observed in most growth cones. Similar results were found when cultures were treated with 30 μM anisomycin or 100 μM emetine[34], two other specific protein synthesis inhibitors. On the other hand, treatment with 10 ng ml^{-1} actinomycin[35], or 50 μM DRB, two specific transcriptional inhibitors, had no effect on chemoattraction induced by netrin-1 (Fig. 7b). These observations are consistent with the idea that in order for continuous chemotaxis to be maintained, protein synthesis is necessary for resensitization of the growth cone towards netrin-1. As discussed below (see Fig. 8), we attribute the repulsive turning to an asymmetric desensitization across the growth cone in the netrin-1 gradient. In the absence of resensitization, the growth cone may extend in the direction that was set after the first cycle of desensitization, leading to an overall repulsive response.

Protein synthesis responsible for the resensitization of the growth cone may be carried out locally in the neurite. To test this hypothesis, we transected the neurite from the neuronal soma and examined the turning response of the growth cone in a gradient of netrin-1. Out of all isolated neurites, a fraction of them (18 out of 75) recovered their growth-cone motility in culture. These isolated growing neurites responded to the gradient of netrin-1 with attractive turning (Fig. 7c). However, when cycloheximide (10 μg ml^{-1}) was added to the bath after the transection, these growth cones failed to exhibit chemotaxis in the netrin-1 gradient and instead showed repulsion (Fig. 7d). This repulsion is consistent with a lack of resensitization or adaptation after the initial asymmetric desensitization, causing a persistent state of repulsion at the end of the first zig-zag (see Fig. 8). Thus, local protein synthesis in the neurite appears to be responsible for resensitization of the growth cone towards netrin-1, a process that is required for effective long-range chemotaxis.

Discussion

Adaptive behaviour in growth-cone guidance has been observed in retinal ganglion axons, which can adjust their sensitivity according to the basal concentration of the tectal membrane repellents so that they grow up the gradient of repellents for a fixed increment of concentration before stalling[10,11]. Adaptation in both chemotaxis and chemorepulsion requires a modulation in the gain of guidance-signal transduction[8]. In analogy to the desensitization of β-adrenergic receptors[36], the guidance cue may induce a modification of its receptors or receptor-interacting proteins, resulting in changes in the ligand affinity or the efficacy of signal transduction. In response to increasing basal concentrations of netrin-1 or BDNF, the receptors may also be downregulated in a manner similar to that found for many growth factors[37]. Subsequent recovery of functional receptors, in a MAPK- and protein synthesis-dependent manner[38], may lead to resensitization. In addition to changes at the receptor

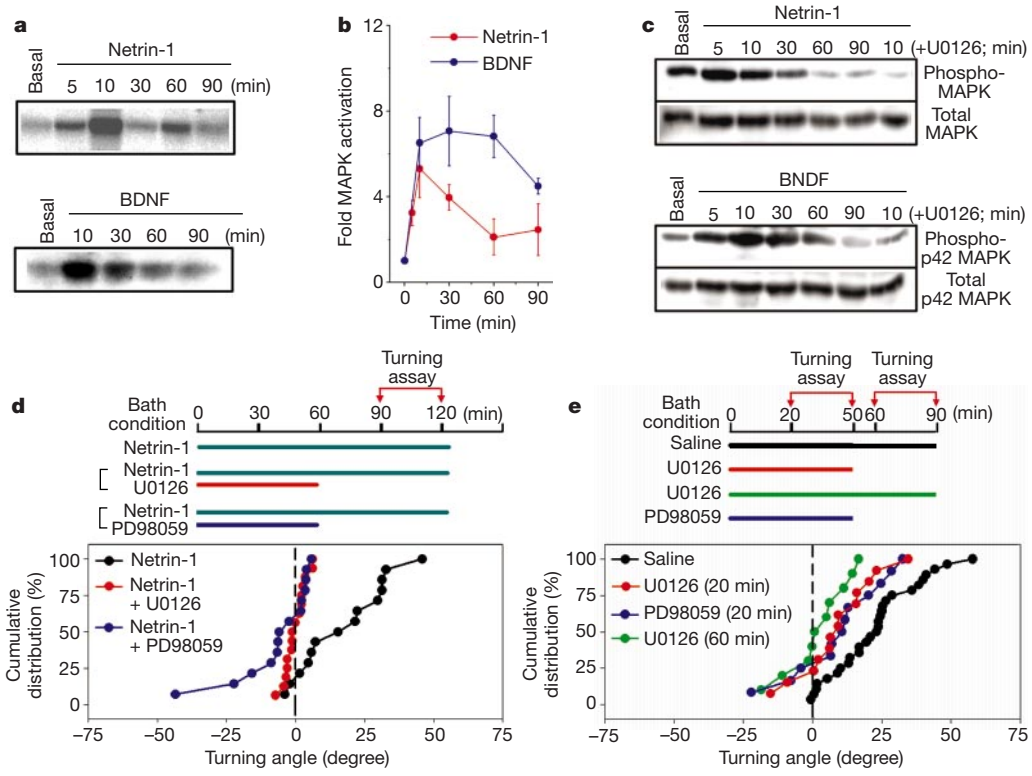

Figure 6 Activation of MAPK by netrin-1 and BDNF, and involvement of MAPK in growth-cone chemotaxis. **a**, Time course of netrin-1- and BDNF-stimulated MAPK activity towards myelin basic protein. Activities at given time points were visualized by separation on 10% SDS–polyacrylamide gel electrophoresis gels followed by autoradiography. **b**, Radioactivity from excised gel bands as shown in **a** was quantified by dividing the increase in c.p.m. (counts per minute) at each time point by the basal c.p.m. levels. All data are means ± s.e.m. from at least three experiments. **c**, MAPK activation in response to netrin-1 and BDNF was confirmed by immunoblot analysis with phospho-specific antibodies directed against the active form of MAPK at different time points as indicated. U0126 (10 μM), a specific MEK1 inhibitor, blocked MAPK activation. **d**, Effects of MAPK inhibitor on resensitization/adaptation of the growth cone. U0126 (10 μM) or PD98059 (10 μM) was applied during the first 60 min of bath incubation with netrin-1 (1 ng ml^{-1}), and the turning assay was performed at $t = 90$–120 min. Data shown are distributions of turning angles for growth cones induced by the standard gradient of netrin-1 under different conditions. The distributions of turning angle in the presence of inhibitors were significantly different from that observed in the absence of the drugs ($P < 0.01$). **e**, Effects of MAPK inhibitors on the netrin-1-induced turning response. Shown are the distributions of turning angles for growth cones induced by the standard netrin-1 gradient in the absence or presence of MAPK inhibitor U0126 (10 μM) or PD98059 (10 μM). These inhibitors were present 20 or 60 min before and during the turning assay. The distributions of turning angle in the presence of inhibitors were significantly different from that observed in the absence of the inhibitors ($P < 0.01$).

level, modulation of downstream cytoplasmic effectors may alter the sensitivity of the growth cone to the guidance cue[8,39]. For example, elevation of cytosolic cyclic AMP can boost the chemotactic response of the growth cone in a gradient of low concentration of netrin-1 (refs 13, 40) or BDNF[17]. The absence of heterologous desensitization at a threshold bath concentration of the factor that is sufficient for homologous desensitization suggests that homologous desensitization may occur upstream from cytoplasmic effector(s) shared by these two guidance cues, an idea further supported by results from our Ca^{2+} imaging studies.

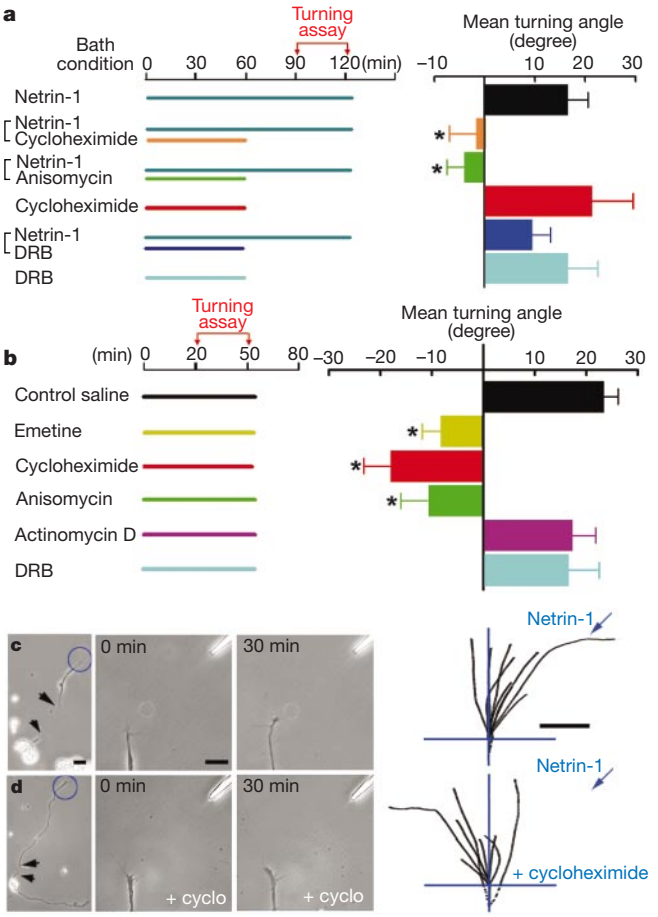

Figure 7 Effects of protein synthesis inhibition. **a**, Reversible inhibitors of protein synthesis (cycloheximide, 10 μg ml^{-1} or anisomycin, 30 μM) or gene transcription (DRB, 50 μM) were applied during the first 60 min of incubation with netrin-1 (1 ng ml^{-1} in the bath), and the mean turning angle (±s.e.m. $n = 8$–15) was determined by the standard netrin-1 gradient at $t = 90$–120 min. Data are also shown for turning assayed at 90 min with bath application of netrin-1 (1 ng ml^{-1} in the bath) alone, or after 60-min treatment with the inhibitor in the absence of bath netrin-1. An asterisk indicates values significantly different from the control with a bath application of netrin-1 alone ($P < 0.01$). **b**, Mean turning angles (±s.e.m., $n = 8$ to 15) of growth cones in the standard netrin-1 gradient in the absence or presence of protein synthesis inhibitors emetine (100 μM), cycloheximide (10 μg ml^{-1}) and anisomycin (30 μM), or transcription inhibitors actinomycin D (10 ng ml^{-1}) and DRB (50 μM). Each drug was present for 20 min before and throughout the turning assay. An asterisk indicates values significantly different from the control group (no drugs in the bath; $P < 0.01$). **c**, Neurites exhibit normal chemotaxis in the standard netrin-1 gradient, after being mechanically transected near the cell body (arrowheads). The growth cone of the severed neurite (blue circle) was exposed to the netrin-1 gradient; images are shown at 0 and 30 min after the onset of the gradient. Scale bar, 20 μm. The superimposed traces show neurite trajectories of all isolated neurites that resumed growth ($n = 9$; scale bar, 5 μm). **d**, Similar to **c**, except that cycloheximide (10 μg ml^{-1}) was added after neurite transection, at 20 min before and during the turning assay.

The resensitization of growth cones towards netrin-1 and BDNF was inhibited by two structurally different MEK1 inhibitors, suggesting that MAPK activation is critical for resensitization. Activation of MAPK seems to provide either an enhanced recruitment or a *de novo* synthesis of chief components required for resensitization or adaptation. Although it is well known that MAPKs are direct downstream effectors of BDNF TrkB signalling[26], how netrin-1 activates MAPK is unclear. The MAPK-dependent adaptation towards chemoattractants seems to be a phylogenetically conserved mechanism. In amoeboid chemotaxis in *Dictyostelium*, MAPK is also required for adaptation of the cells to high concentrations of the chemoattractant cAMP, but not for the process of locomotion itself[41]. The effect of MAPK inhibition on growth-cone chemotaxis is a slow process (Fig. 6e), consistent with a blockade of resensitization, although some effects or immediate turning response may also be present. Our findings on the effect of translation inhibitors suggest that constitutive protein synthesis in the neurite is critical for resensitization. As MAPK activity can initiate protein translation[42], and netrin-1 and BDNF can induce MAPK activation (Fig. 6) and trigger protein synthesis in cell cultures[43,44], it is likely that MAPK-dependent local protein synthesis in the neurite[45] is responsible for the resensitization process.

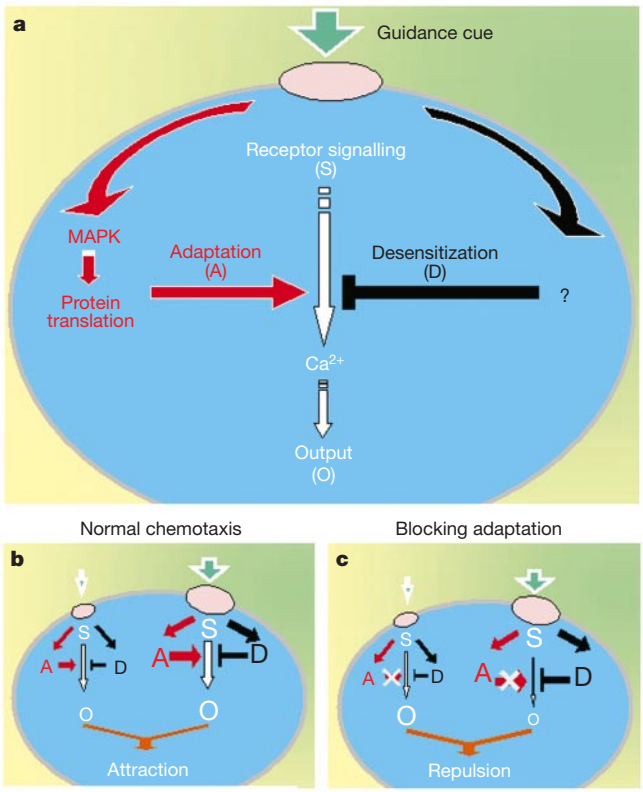

Figure 8 A model of growth-cone adaptation in a gradient of guidance cues. **a**, The guidance cue triggers a cytoplasmic signal (S) and two parallel processes of desensitization (D) and resensitization or adaptation (A), which regulate the output (O) of the signalling cascade, mediated through elevation of Ca^{2+}, leading to cytoskeletal rearrangements associated with attractive or repulsive responses. **b**, Asymmetry in the output determines the direction of growth. With time, the effect of resensitization or adaptation compensates for that of desensitization, leading to chemotaxis. **c**, Assuming the onset of desensitisation precedes that of resensitization or adaptation, the asymmetric desensitization (higher towards the gradient) before the onset of resensitization or adaptation results in repulsive turning after the initial attraction, hence the zig-zag trajectory of growth (Fig. 3). Blocking resensitization or adaptation by protein synthesis inhibitors results in a persistent asymmetric output after the initial densensitization, leading to the end result of repulsion (Fig. 7b, d).

To account for the zig-zag pattern of growth-cone migration and the repulsive turning in the presence of translation inhibitors, we propose that, in the presence of a netrin-1 gradient, the difference in receptor activation on two sides of the growth cone leads to an asymmetry in the receptor activation for cytoplasmic signalling and in the rate or the extent of desensitization (Fig. 8). Owing to differences in the time course of desensitization and resensitization, such asymmetry in desensitization in fast-advancing growth cones may result in a repulsive turning response before adaptation, leading to a zig-zag path of chemotaxis. In the absence of resensitization due to inhibition of protein synthesis, such asymmetry in desensitization persists after the first cycle of desensitization, leading to an overall repulsive response.

Previous studies have shown that dendrites and axons contain both messenger RNAs and the machinery for local protein translation[45–47]. In cultured retinal ganglion cells, both netrin-1 and semaphorin 3A (Sem3A) were shown to stimulate protein synthesis in neurites severed from cell bodies, and inhibition of translation resulted in a loss of growth-cone turning induced by a gradient of netrin-1 or Sema3A[44]. Our results suggest that translational inhibition affects the resensitization/adaptation of the growth cone, rather than initial growth-cone turning responses, resulting in the final repulsive turning in the netrin-1 gradient after the initial cycle of desensitization. Such repulsive turning was in fact observed for a substantial fraction of retinal ganglion axons after inhibition of translation[44]. Whether long-range chemorepulsion of growth cones by gradients of repellants also requires adaptive changes in the sensitivity of the growth cone remains to be determined.

Guidance of growth cones by extracellular gradients of guidance cues involves a complex sequence of cellular events that re-adjust the sensitivity of signalling pathways to the guidance cue. Although the immediate turning of the growth cone can be achieved by rearrangement of the pre-existing cellular components, as shown by rapid asymmetric filopodia initiation within minutes after the onset of the gradient[13,15], successful chemotaxis of the growth cone in the gradient of guidance cues requires activation of MAPK and initiation of protein translation, a more protracted process that allows adaptive changes in the sensitivity of the growth cone. Although it remains to be determined whether adaptation is a general phenomenon of axon guidance in the developing nervous system, the existence of the adaptive behaviour in growth-cone chemotaxis described here exemplifies Ramón y Cajal's imagery of the growth cone as a chemotaking amoeba, and suggests common mechanisms underlying all forms of directed cell motility. □

Methods

Culture preparation

Cultures of *Xenopus* spinal neurons were prepared from one-day-old embryos by methods described previously[13,15–17,48]. The experiments were carried out with 14–20 h cultures at 20–22 °C in saline containing (in mM): 140 NaCl, 2.5 KCl, 1 $MgCl_2$, 1 $CaCl_2$ and 10 HEPES, pH 7.4. For MAPK assays, explant cultures were prepared from stage 22–23 embryos. Neural tubes were treated with collagenase/dispase (0.1%, Sigma) for 10 min and triturated to isolate the spinal cord. They were then cultured in the presence of 50 μM GM6001 (Chemicon) for 14–16 h before biochemical assays (each with 10–12 explants) to ensure optimal expression of netrin receptor DCC[49].

Growth cone turning assay

Microscopic gradients of guidance factors were produced by methods described previously[15–18]. The standard gradient used 5 or 50 μg ml^{-1} netrin-1 or BDNF, respectively, in the micropipette. Previous analyses[15,16] have shown that, under standard pulsing conditions, the average concentration of the factor at the growth cone is about 10^3-fold lower than that in the pipette, and the concentration gradient across the growth cone at a distance of 100 μm from the pipette tip is 5–10%. For the growth-cone turning assay, the tip of the pipette containing the factor was placed 100 μm away from the centre of the growth cone at an angle of 45° with respect to the initial direction of neurite extension (set 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 centre of the growth cone at the onset and the end of the 30 min period. Only those growth cones of isolated neurons with net neurite extension greater than 5 μm over the 30-min period were included for analysis. Significance was assessed using the Kolmogorov–Smirnov test unless otherwise noted. In quantitative assays of growth-cone adaptation after bath incubation of the guidance cue, the degradation of netrin-1 and BDNF in the bath solution over the duration of these experiments was negligible. A bath solution containing 1 ng ml^{-1} netrin-1 was fully active in causing desensitization when repeatedly used in other fresh cultures within 10 h, whereas 50% reduction in netrin-1 (0.5 ng ml^{-1}) resulted in significant reduction in desensitization (see Fig. 2a). This was further confirmed by a titration experiment: a 5 ng ml^{-1} netrin-1 bath solution was used in a desensitization experiment for 90 min. The solution was then diluted five times and examined for its effect on fresh, new cultures. Full desensitization (turning angle $-0.4 \pm 3.1°$, s.e.m., $n = 11$) was found, suggesting that the degradation of netrin-1, if it occurs at all, must be less than 50%.

Calcium imaging

We carried out calcium imaging as reported previously[21]. In some experiments, the Ca^{2+} indicator Oregon green 488 BAPTA-1 conjugated to dextran (relative molecular mass of 70,000; Molecular Probes) was co-injected with Texas Red-conjugated dextran (300 μM; Molecular Probes), and Ca^{2+} imaging was done using a Leica confocal imaging system, using a × 63 objective (HCX Pl Apo; NA, 1.32) and separate Kr and Ar gas lasers. The Oregon green BAPTA-dextran and Texas Red-dextran were excited at 488 and 568 nm, respectively, and the respective emission signals were collected at 495–550 nm and 595–680 nm. Fluorescence images were collected sequentially in pairs every 20–30 s and analysed with NIH Image Software. We also collected transmission images to monitor laser output. Mean fluorescence intensity was measured over a fixed square area that covered the entire growth cone. Ratios of normalized Oregon green and Texas red fluorescence were used to control for fluctuations in growth-cone thickness or changes in focal plane that may have occurred during experiments.

MAPK assays

Myelin basic protein (MBP) assay was performed as previously described[27]. Spinal cord explants were treated with netrin-1 (20 ng ml^{-1}) or BDNF (10 ng ml^{-1}) and stopped by ice cold RIPA buffer containing 50 mM Tris at pH 8, 50 mM NaF, 5 mM EDTA, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 10 μg ml^{-1} leupeptin, 50 μg ml^{-1} phenylmethylsulphonyl fluoride (PMSF), 2 μg ml^{-1} aprotinin, 1 mM ortho-vanadate and 50 μM GM6001 (Chemicon). The protein content for each sample was determined as described[50] and normalized. After clarification of the lysates with sepharose (Amersham-Pharmacia), MAPK was immuno-precipitated with 1.5 μg anti-MAPK antibody (Promega) pre-absorbed to protein A sepharose (Amersham-Pharmacia) for 2 h at 4 °C. The beads were washed twice with ice-cold RIPA and once with kinase assay buffer (KAB (in mM): 25 HEPES, pH 7.4, 25 β-glycerophosphate, 25 $MgCl_2$, 0.1 Na ortho-vanadate, 0.5 dithiothreitol), then incubated with 30 μl of 'hot' assay mix (KAB, 5 μg MBP, 20 μM ATP, 10 μCi [γ-^{32}P]ATP) for 15 min and the reaction stopped by adding 5 μl EDTA (0.5 M). Twenty-five microlitres from each reaction were subjected to SDS–polyacrylamide gel electrophoresis. The radioactive bands were exposed to X-ray film and then excised for scintillation counting. We performed immunoblot assays for MAPK according to the manufacturer's protocols (Promega).

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1. Tessier-Lavigne, M. & Goodman, C. S. The molecular biology of axon guidance. *Science* **274,** 1123–1133 (1996).
2. Mueller, B. K. Growth cone guidance: first steps towards a deeper understanding. *Annu. Rev. Neurosci.* **22,** 351–388 (1999).
3. Ramón y Cajal, S. *Histology of the Nervous System* (trans. Swanson, N. & Swanson, L. W.) Vol. 1, 532–537 (Oxford Univ. Press, New York, 1995).
4. Tessier-Lavigne, M., Placzek, M., Lumsden, A. G., Dodd, J. & Jessell, T. M. Chemotropic guidance of developing axons in the mammalian central nervous system. *Nature* **336,** 775–778 (1988).
5. Pini, A. Chemorepulsion of axons in the developing mammalian central nervous system. *Science* **261,** 95–98 (1993).
6. Lumsden, A. G. & Davies, A. M. Earliest sensory nerve fibres are guided to peripheral targets by attractants other than nerve growth factor. *Nature* **306,** 786–788 (1983).
7. Keynes, R. *et al.* Surround repulsion of spinal sensory axons in higher vertebrate embryos. *Neuron* **18,** 889–897 (1997).
8. Song, H.-J. & Poo, M.-M. The cell biology of neuronal navigation. *Nature Cell Biol.* **3,** E81–E88 (2001).
9. Baier, H. & Bonhoeffer, F. Axon guidance by gradients of a target-derived component. *Science* **255,** 472–475 (1992).
10. Rosentreter, S. M. *et al.* Response of retinal ganglion cell axons to striped linear gradients of repellent guidance molecules. *J. Neurobiol.* **37,** 541–562 (1998).
11. Loschinger, J., Weth, F. & Bonhoeffer, F. Reading of concentration gradients by axonal growth cones. *Phil. Trans. R. Soc. Lond. B Biol. Sci.* **355,** 971–982 (2000).
12. Falke, J. J., Bass, R. B., Butler, S. L., Chervitz, S. A. & Danielson, M. A. The two-component signalling pathway of bacterial chemotaxis: a molecular view of signal transduction by receptors, kinases, and adaptation enzymes. *Annu. Rev. Cell Dev. Biol.* **13,** 457–512 (1997).
13. Ming, G. L. *et al.* cAMP-dependent growth cone guidance by netrin-1. *Neuron* **19,** 1225–1235 (1997).
14. Yee, K. T., Simon, H. H., Tessier-Lavigne, M. & O'Leary, D. M. Extension of long leading processes and neuronal migration in the mammalian brain directed by the chemoattractant netrin-1. *Neuron* **24,** 607–622 (1999).
15. Lohof, A. M., Quillan, M., Dan, Y. & Poo, M.-M. Asymmetric modulation of cytosolic cAMP activity induces growth cone turning. *J. Neurosci.* **12,** 1253–1261 (1992).
16. Zheng, J. Q., Felder, M., Connor, J. A. & Poo, M.-M. Turning of nerve growth cones induced by neurotransmitters. *Nature* **368,** 140–144 (1994).
17. Song, H-j., Ming, G-l. & Poo, M.-M. Cyclic-AMP-induced switching in turning direction of nerve growth cones. *Nature* **388,** 275–279 (1997).
18. Ming, G., Lohof, A. M. & Zheng, J. Q. Acute morphogenic and chemotropic effects of neurotrophins on cultured embryonic *Xenopus* spinal neurons. *J. Neurosci.* **17,** 7860–7871 (1997).

19. Tucker, K. L., Meyer, M. & Barde, Y. A. Neurotrophins are required for nerve growth during development. *Nature Neurosci.* **4,** 29–37 (2001).
20. Ming, G. *et al.* Phospholipase C-γ and Phosphoinositide 3-kinase mediate cytoplasmic signalling in nerve growth cone guidance. *Neuron* **23,** 139–148 (1999).
21. Hong, K., Nishiyama, M., Henley, J., Tessier-Lavigne, M. & Poo, M. Calcium signalling in the guidance of nerve growth by netrin-1. *Nature* **403,** 93–98 (2000).
22. Kater, S. B., Mattson, M. P., Cohan, C. & Connor, J. Calcium regulation of the neuronal growth cone. *Trends Neurosci.* **11,** 315–321 (1988).
23. Gu, X. & Spitzer, N. C. Distinct aspects of neuronal differentiation encoded by frequency of spontaneous Ca^{2+} transients. *Nature* **375,** 784–787 (1995).
24. Gomez, T. M. & Spitzer, N. C. *In vivo* regulation of axon extension and pathfinding by growth-cone calcium transients. *Nature* **397,** 350–355 (1999).
25. Gomez, T. M., Robles, E., Poo, M. & Spitzer, N. C. Filopodial calcium transients promote substrate-dependent growth cone turning. *Science* **291,** 1983–1987 (2001).
26. Huang, E. J. & Reichardt, L. F. Neurotrophins: roles in neuronal development and function. *Annu. Rev. Neurosci.* **24,** 677–736 (2001).
27. Seger, R. *et al.* Overexpression of mitogen-activated protein kinase kinase (MAPKK) and its mutants in NIH 3T3 cells. Evidence that MAPKK involvement in cellular proliferation is regulated by phosphorylation of serine residues in its kinase subdomains VII and VIII. *J. Biol. Chem.* **269,** 25699–25709 (1994).
28. DeSilva, D. R. *et al.* Inhibition of mitogen-activated protein kinase kinase blocks T cell proliferation but does not induce or prevent anergy. *J. Immunol.* **160,** 4175–4181 (1998).
29. Dudley, D. T., Pang, L., Decker, S. J., Bridges, A. J. & Saltiel, A. R. A synthetic inhibitor of the mitogen-activated protein kinase cascade. *Proc. Natl Acad. Sci. USA* **92,** 7686–7689 (1995).
30. Sonenberg, N. & Gingras, A. C. The mRNA 5′ cap-binding protein eIF4E and control of cell growth. *Curr. Opin. Cell Biol.* **10,** 268–275 (1998).
31. Waring, P. DNA fragmentation induced in macrophages by gliotoxin does not require protein synthesis and is preceded by raised inositol triphosphate levels. *J. Biol. Chem.* **265,** 14476–14480 (1990).
32. Kochi, S. K. & Collier, R. J. DNA fragmentation and cytolysis in U937 cells treated with diphtheria toxin or other inhibitors of protein synthesis. *Exp. Cell Res.* **208,** 296–302 (1993).
33. Mittleman, B., Zandomeni, R. & Weinmann, R. Mechanism of action of 5,6-dichloro-1-beta-D-ribofuranosylbenzimidazole. II. A resistant human cell mutant with an altered transcriptional machinery. *J. Mol. Biol.* **165,** 461–473 (1983).
34. Burhans, W. C. *et al.* Emetine allows identification of origins of mammalian DNA replication by imbalanced DNA synthesis, not through conservative nucleosome segregation. *EMBO J.* **10,** 4351–4360 (1991).
35. Martin, S. J., Lennon, S. V., Bonham, A. M. & Cotter, T. G. Induction of apoptosis (programmed cell death) in human leukemic HL-60 cells by inhibition of RNA or protein synthesis. *J. Immunol.* **145,** 1859–1867 (1990).
36. Hausdorff, W. P., Caron, M. G. & Lefkowitz, R. J. Turning off the signal: desensitization of beta-adrenergic receptor function. *FASEB J.* **4,** 2881–2889 (1990).
37. Sorkin, A. & Waters, C. M. Endocytosis of growth factor receptors. *Bioessays* **15,** 375–382 (1993).
38. Meyer-Franke, A. *et al.* Depolarization and cAMP elevation rapidly recruit TrkB to the plasma membrane of CNS neurons. *Neuron* **21,** 681–693 (1998).
39. Song, H.-J. & Poo, M.-M. Signal transduction underlying growth cone guidance by diffusible factors. *Curr. Opin. Neuroiol.* **9,** 355–363 (1999).
40. Ming, G., Henley, J., Tessier-Lavigne, M., Song, H. & Poo, M. Electrical activity modulates growth cone guidance by diffusible factors. *Neuron* **29,** 441–452 (2001).
41. Wang, Y., Liu, J. & Segall, J. E. MAP kinase function in amoeboid chemotaxis. *J. Cell Sci.* **111,** 373–383 (1998).
42. Chang, L. & Karin, M. Mammalian MAP kinase signalling cascades. *Nature* **410,** 37–40 (2001).
43. Takei, N., Kawamura, M., Hara, K., Yonezawa, K. & Nawa, H. Brain-derived neurotrophic factor enhances neuronal translation by activating multiple initiation processes. *J. Biol. Chem.* **276,** 42818–42825 (2001).
44. Campbell, D. S. & Holt, C. E. Chemotropic responses of retinal growth cones mediated by rapid local protein synthesis and degradation. *Neuron* **20,** 1013–1026 (2001).
45. Aakalu, G., Smith, W. B., Nguyen, N., Jiang, C. & Schuman, E. M. Dynamic visualization of local protein synthesis in hippocampal neurons. *Neuron* **30,** 489–502 (2001).
46. Steward, O. & Schuman, E. M. Protein synthesis at synaptic sites on dendrites. *Annu. Rev. Neurosci.* **24,** 299–325 (2001).
47. Job, C. & Eberwine, J. Localization and translation of mRNA in dendrites and axons. *Nature Rev. Neurosci.* **2,** 889–898 (2001).
48. Spitzer, N. C. & Lamborghini, J. E. The development of the action potential mechanism of amphibian neurons isolated in culture. *Proc. Natl Acad. Sci. USA* **73,** 1641–1645 (1976).
49. Pierceall, W. E. *et al.* Expression of a homologue of the deleted in colorectal cancer (DCC) gene in the nervous system of developing *Xenopus* embryos. *Dev. Biol.* **166,** 654–665 (1994).
50. Hill, H. D. & Straka, J. G. Protein determination using bicinchoninic acid in the presence of sulfhydryl reagents. *Anal. Biochem.* **170,** 203–208 (1988).

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Competing interests statement

The authors declare that they have no competing financial interests.

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