Signalling and crosstalk of Rho GTPases in mediating axon guidance

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Axon extension during development of the nervous system is guided by many factors, but the signalling mechanisms responsible for triggering this extension remain mostly unknown. Here we have examined the role of Rho family small guanosine triphosphatases (GTPases) in mediating axon guidance by diffusible factors. Expression of either dominant-negative or constitutively active Cdc42 in cultured *Xenopus laevis* spinal neurons, at a concentration that does not substantially affect filopodial formation and neurite extension, abolishes the chemoattractive growth cone turning induced by a gradient of brain-derived neurotrophic factor that can activate Cdc42 and Rac in cultured neurons. Chemorepulsion induced by a gradient of lysophosphatidic acid is also abolished by the expression of dominant-negative RhoA. We also show that an asymmetry in Rho kinase or filopodial initiation across the growth cone is sufficient to trigger the turning response and that there is a crosstalk between the Cdc42 and RhoA pathways through their converging actions on the myosin activity essential for growth cone chemorepulsion.

During development of the nervous system, axon extension towards its target cell is guided by diffusible and membrane-bound factors[1]. Several guidance factors and their receptors have been identified, but the cytoplasmic signalling mechanisms that are responsible for triggering directed growth cone extension remain largely unknown[2,3]. Each guidance molecule must activate a cascade of cytoplasmic effectors that eventually results in the cytoskeleton rearrangement underlying directed axon extension. Proteins that regulate the assembly of the actin cytoskeleton, such as cofilin, gelsolin and profilin, have been shown to be downstream effectors of the Rho family of small GTPases, including Rho, Rac and Cdc42 (refs 4–6). These GTPases are thus likely to function as key mediators that link the guidance signal to cytoskeletal rearrangements[2,7].

Indeed, marked changes in the morphology, motility and pathfinding of axons have been observed after perturbation of the Rho family GTPases *in vitro* and *in vivo*[8–13]. In general, these studies suggest that Rac and Cdc42 are positive regulators that promote neurite extension, whereas Rho is a negative regulator that causes the inhibition or collapse of growth cones. Some known guidance factors, including slit, semaphorin, ephrin and netrin, have been shown to regulate intracellular Rho GTPase activity[14–17]. We therefore considered that different Rho GTPases might have distinct roles in mediating axon guidance by various guidance signals and that an asymmetry of Rho GTPase activity and consequent filopodial activity across the growth cone might cause the turning responses of the growth cone induced by extracellular guidance factors[7].

In this study, we used isolated *Xenopus* spinal neurons and a growth cone turning assay to examine the effects of expressing mutant forms of Cdc42 and RhoA on growth cone turning induced by brain-derived neurotrophic factor (BDNF) and lysophosphatidic acid (LPA). A gradient of BDNF can induce either the attraction or repulsion of growth cones through the receptor tyrosine kinase TrkB, depending on intracellular concentrations of Ca^{2+} and cyclic AMP[3]. The bioactive lipid LPA activates intracellular RhoA activity[18] and induces both inhibition of neurite extension and growth cone collapse[18]. It acts through specific G-protein-coupled, seven-transmembrane domain receptors, which are expressed in some developing neurons[18,19].

Here we show that BDNF can activate Cdc42 in developing neurons. Cdc42 directly mediates BDNF-induced chemoattraction and RhoA mediates the chemorepulsion induced by LPA. Furthermore, we show that there is crosstalk between the Cdc42 and RhoA pathways and that regulation of each of these two GTPases can trigger either attractive or repulsive turning, with a common pathway for repulsion mediated by myosin activity.

Results

Toxin B blocks growth cone turning induced by BDNF and LPA. Microscopic gradients of BDNF and LPA were applied to cultured *Xenopus* spinal neurons using a micropipette. Consistent with previous results[20], we observed a marked attractive turning of the growth cone towards the source of BDNF within 1 h after the initial application of the BDNF gradient (Fig. 1a, c), whereas no significant turning was observed when normal Ringer's solution was used in the micropipette. In contrast to the BDNF effect, we found that a gradient of LPA at a concentration that does not induce complete collapse of the growth cone induced repulsive turning of the growth cone (Fig. 1b, c, and see below).

To test whether Rho GTPases are involved in axon guidance by diffusible factors, we first examined the effect of toxin B, a specific inhibitor of Rho GTPases[21], on the turning response of the growth cone induced by BDNF or LPA. The average length of neurite extension, the number of filopodia per growth cone, and the percentage of neurites with a growth rate greater than 5 $\mu m\ h^{-1}$ were all reduced with increasing concentrations of toxin B in the culture medium (Fig. 2b). Most neurites showed retraction, leaving long hair-like protrusions without any motile filopodia when treated with 20 $ng\ ml^{-1}$ toxin B (Fig. 2a). Although a substantial growth and filopodial activity remained for many neurites at 10 $ng\ ml^{-1}$ toxin B, the attractive turning induced by the BDNF gradient and the repulsion induced by the LPA gradient at this concentration were totally abolished (Fig. 1).

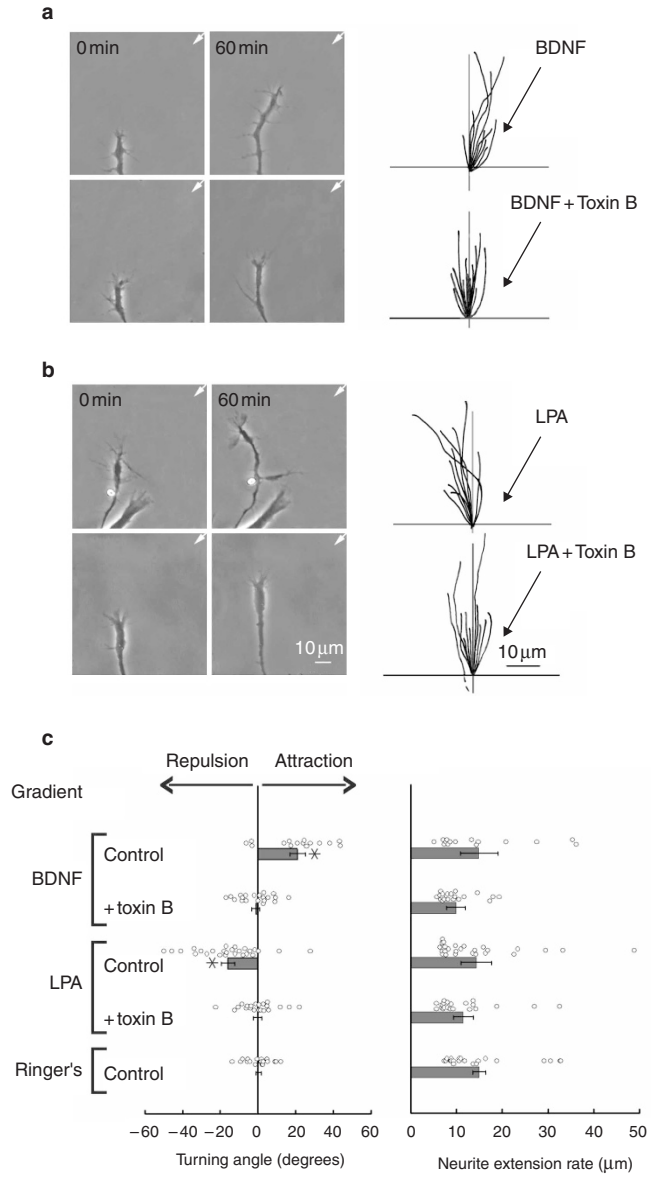

Figure 1 **Growth cone turning induced by a gradient of BDNF or LPA. a**, Top left, microscopic images of a neuron (cultured for 14–20 h) at the beginning (0 min) and the end (60 min) of a 1-h exposure to a BDNF gradient created by pulsatile application of a BDNF solution (50 μg ml^{-1} in the pipette). Top right, superimposed traces depict the trajectory of neurite extension during the 1-h period for all neurons examined. The origin is the centre of the growth cone at the beginning of the experiment and the original direction of growth was vertical. Arrow indicates the direction of the gradient. Bottom, neurons were incubated with toxin B (10 ng ml^{-1}) for 4 h before pulsatile application of BDNF. Scale bar, 10 μm. **b**, Experiments were done as in **a**, except that the pipette contained LPA (40 μM). **c**, Histograms represent the averaged turning angles (left) and extension rate (right) induced by a gradient of BDNF or LPA, with or without toxin B in the bath. Scattered points beside each column depict the individual turning angle or neurite extension during the 1-h assay for each experimental group. *$P < 0.01$, as compared with the groups incubated with toxin B (Kolmogorov–Smirnov test). Error bars, s.e.m.

We examined only neurons that showed a substantial amount of growth (>5 μm h^{-1}). Although the percentage of neurites that extended with a rate greater than 5 μm h^{-1} was reduced by toxin B treatment, the average neurite extension was not significantly different among the neurons for which turning assays were carried out (Fig. 1c). These results suggest that Rho GTPase activities are necessary for the growth cone turning induced by BDNF and LPA, and that the turning responses are much more sensitive to the activity of Rho GTPases than are neurite extension and filopodia initiation[13].

Expression of mutant Rho GTPases in *Xenopus* spinal neurons. To examine further the role of Rho family GTPases in mediating the growth cone turning response, we expressed fusion proteins of green fluorescent protein (GFP) and dominant-negative (DN) or constitutively active (CA) rat Rho GTPases in *Xenopus* spinal neurons by injecting complementary DNA into the embryos[22]. Expression of the rat GTPase–GFP in *Xenopus* cells was confirmed by western blots using antibodies against GFP, and the expressed proteins survived for at least 3 d after the injection (Fig. 3a, b). However, the expression of CA-RhoA–GFP in these embryos was consistently lower than in those injected with three other mutant GTPases. Very few neurite-bearing neurons survived in cultures prepared from embryos injected with vector containing the CA-RhoA–GFP-encoding gene. Thus, we did not use CA-RhoA–GFP in further studies.

In cultures of dissociated spinal neurons, the GFP fluorescence provided a reliable marker for identifying progeny cells derived from injected blastomeres (ref. 22 and Fig. 3c). The effects of expressing DN-Cdc42–GFP, CA-Cdc42–GFP or DN-RhoA–GFP on the neurite extension and filopodial activity of these neurons were quantitatively examined in cultures grown for 12 h. Expression of DN-Cdc42 caused a slight but significant reduction in the average length of neurites and the average number of growth cone filopodia, whereas expression of CA-Cdc42 reduced neurite extension without affecting the number of filopodia. By contrast, expression of DN-RhoA caused a slight increase in the filopodia number without affecting neurite extension. These findings are consistent with the notion that Cdc42 promotes filopodia initiation, whereas RhoA inhibits it[23].

Regulation of Rho GTPase activity by BDNF. To determine whether BDNF directly regulates Rho GTPase activity, we carried out biochemical assays[24] on cultures of cerebellar granule cells obtained from rats at postnatal day 5–6 (P5–P6). We observed a marked activation of Cdc42 and Rac within 3 min after the application of BDNF, whereas RhoA activity was not significantly affected (Fig. 4a). Notably, pre-incubation of the culture with the protein kinase A (PKA) inhibitor Rp-cAMPS (20 mM) converted the BDNF-triggered activation of Cdc42 and Rac to inhibition (Fig. 4b), whereas Rp-cAMPS did not affect RhoA activity. Rp-cAMPS alone could activate Rac and Cdc42 (Fig. 4b).

Thus, intracellular cAMP or PKA activity can switch the effects of BDNF on Cdc42 and Rac. High concentrations of cAMP favour activation, whereas low concentrations favour inhibition, although constitutive PKA activity in the absence of BDNF may cause some inhibition of Cdc42 and Rac.

Cdc42 mediates BDNF-induced chemoattraction. When we applied the BDNF gradient to neurons expressing either DN-Cdc42–GFP or CA-Cdc42–GFP, we found that the attractive turning of the growth cone was completely abolished, whereas attraction was not affected in neurons expressing GFP alone (Fig. 4c). Thus, an optimal level or pattern of Cdc42 activity seems to be required for BDNF-induced attraction.

By contrast, neurons expressing DN-RhoA–GFP showed normal attraction towards BDNF (Fig. 4c). Thus, BDNF-induced attraction is mediated by Cdc42 but is independent of RhoA activity. In addition, the endogenous functions of Cdc42 in growth cone turning seem to be more susceptible to disruption by mutant Cdc42 proteins than are its endogenous functions regulating filopodia formation and neurite growth.

BDNF-induced repulsion depends on Cdc42 and RhoA. When we applied the BDNF gradient to the growth cone in the presence of the PKA inhibitor Rp-cAMPS (20 μM), we observed significant repulsive turning, consistent with previous studies[20]. Unexpectedly,

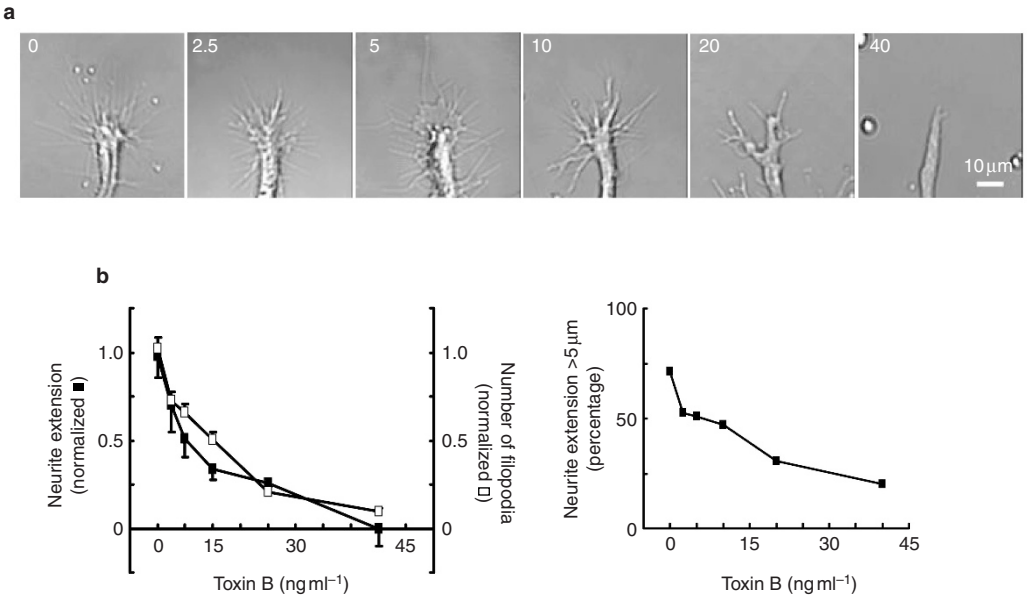

Figure 2 **Effect of toxin B on neurite extension and filopodial activity. a**, Microscopic images taken after growth cones had been incubated in increasing concentrations of toxin B for 4 h. Numbers indicate the toxin B concentration (ng ml^{-1}). Scale bar, 10 μm. **b**, Dose–response curves showing the effect of toxin B on the average rate of neurite extension, the total number of filopodia per growth cone and the percentage of cells with neurite extension greater than 5 μm h^{-1}, each normalized to values obtained in neurons grown in parallel cultures not exposed to toxin B. At least 50 neurites were assayed for each condition. Error bars, s.e.m.

expression of DN-Cdc42–GFP, CA-Cdc42–GFP or DN-RhoA–GFP converted the BDNF-induced repulsion into attraction (Fig. 4c). This finding indicates that the repulsive turning induced by the BDNF gradient depends on both Cdc42 and RhoA, perhaps through their converging actions in activating a common pathway responsible for the repulsive turning (see below).

This finding also suggests that, under conditions of PKA inhibition, the BDNF gradient can also activate an attractive pathway that is less dependent on Cdc42 and can be observed only when the more dominant Cdc42- and RhoA-dependent repulsive activity is blocked.

RhoA mediates LPA-induced chemorepulsion. Consistent with previous studies[18], we found that neurite growth was inhibited by LPA in a dose-dependent manner (Fig. 5a, b). This growth inhibition was blocked by the specific Rho kinase (ROCK) inhibitor Y-27632 (10 μM; ref. 25). Furthermore, this inhibitory effect was abolished in neurons expressing DN-RhoA–GFP, but was unaffected in neurons expressing DN-Cdc42–GFP (Fig. 5c). Thus, RhoA seems to mediate LPA-induced neurite retraction in these *Xenopus* spinal neurons.

When neurons expressing DN-RhoA–GFP were exposed to a gradient of LPA (at a concentration of about 40 nM at the growth cone), the growth cones showed no turning response. By contrast, expression of DN-Cdc42–GFP, which completely abolished BDNF-induced attraction, did not affect the repulsion induced by LPA (Fig. 5d). Thus, RhoA, rather than Cdc42, is required for LPA-induced repulsion. Unexpectedly, the same LPA gradient was markedly attractive to neurons expressing CA-Cdc42–GFP. As above, this suggests that there is crosstalk between the Cdc42 and RhoA pathways and that an LPA-induced attractive pathway exists, which can be observed when the repulsive pathway is blocked by CA-Cdc42.

Rho kinase is an effector of LPA-induced chemorepulsion. When the specific Rho kinase inhibitor Y-27632 at a concentration of 10 μM was applied to the culture 20 min before its exposure to the standard LPA gradient, the repulsive turning towards LPA was reduced significantly (Fig. 5d). The LPA-induced repulsion was converted to attraction when Y-27632 was increased to 100 μM (Fig. 5d). This suggests that the LPA gradient can activate a ROCK-independent signalling process that triggers the attractive turning when the repulsive pathway mediated by ROCK is completed blocked.

If RhoA activity directly mediates the guidance signal, then a gradient of RhoA or ROCK activity across the growth cone should be sufficient to trigger the turning response. Indeed, we found that an extracellular gradient of Y-27632 induced a marked attractive turning of the growth cone (Fig. 5d). A cytoplasmic gradient of inhibition of the constitutive ROCK activity seemed to result in a gradient of filopodial retraction, with less inhibition or retraction towards the source of Y-27632, thereby leading to the attractive turning. In addition, the attractive turning induced by the Y-27632 gradient was not affected by the expression of DN-RhoA–GFP, but was blocked by CA-Cdc42–GFP (Fig. 5d), consistent with the concept that RhoA functions upstream of ROCK and that there is crosstalk between the Cdc42 and RhoA pathways downstream from ROCK.

Filopodial asymmetry and growth cone turning. The above growth cone turning responses induced by the LPA and Y-27632 gradients suggested that an asymmetry of filopodia retraction across the growth cone is sufficient to trigger the turning response. We tested this idea by directly applying a gradient of cytochalasin B to the growth cone. As expected, the growth cones showed marked repulsive turning responses. By contrast, a gradient of BDM, an inhibitor of myosin ATPase[26], induced significant attractive turning (Fig. 6a).

Inhibition of actin polymerization by cytochalasin B is known to cause filopodia retraction[27], whereas inhibition of myosin contractile activity by BDM increases filopodia extension[28]. The attractive and repulsive effects of BDM and cytochalasin B gradients, respectively, can be therefore attributed to asymmetric filopodia retraction at the growth cone. To examine further whether filopodia asymmetry occurs before the turning response, we measured the number of filopodia on the side of the growth cone facing ('near') and away ('far') from the pipette[27] and calculated the ratio of filopodia number on the two sides (near:far) before and immediately after the initial

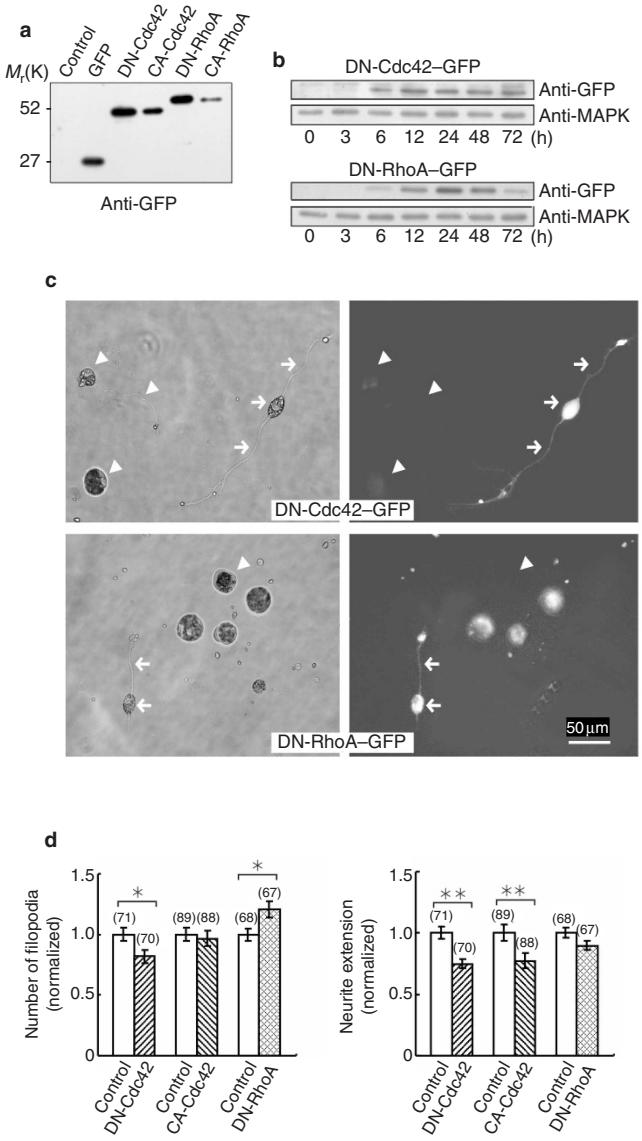

Figure 3 **Rho GTPase expression in *Xenopus* embryos and spinal neurons.** **a**, Western blot showing the expression of fusion proteins of GFP and Rho GTPases in *Xenopus* embryos injected with cDNA. **b**, Time course of DN-Cdc42–GFP and DN-RhoA–GFP expression in injected embryos, assessed by western blotting with antibodies against GFP. Numbers indicate the time (in h) after cDNA injection. Western blots using antibodies against MAPK were carried out as a control for protein loading. **c**, Microscopic images of *Xenopus* spinal neurons cultured in the same dish, shown in bright-field and GFP fluorescence. Arrows indicate neurons expressing the fusion protein; arrowheads indicate cells not expressing the fusion protein. **d**, Neurite extension and total filopodia number per growth cone of neurons expressing DN-Cdc42–GFP, CA-Cdc42–GFP or DN-RhoA–GFP. For neurite extension, the total length of the neurite trajectory of each neuron was measured and normalized to the mean value of control neurons (non-fluorescent neurons) in the same set of cultures. $*P < 0.05$ and $**P < 0.01$, as compared with the control group (Kolmogorov–Smirnov test). Error bars, s.e.m.

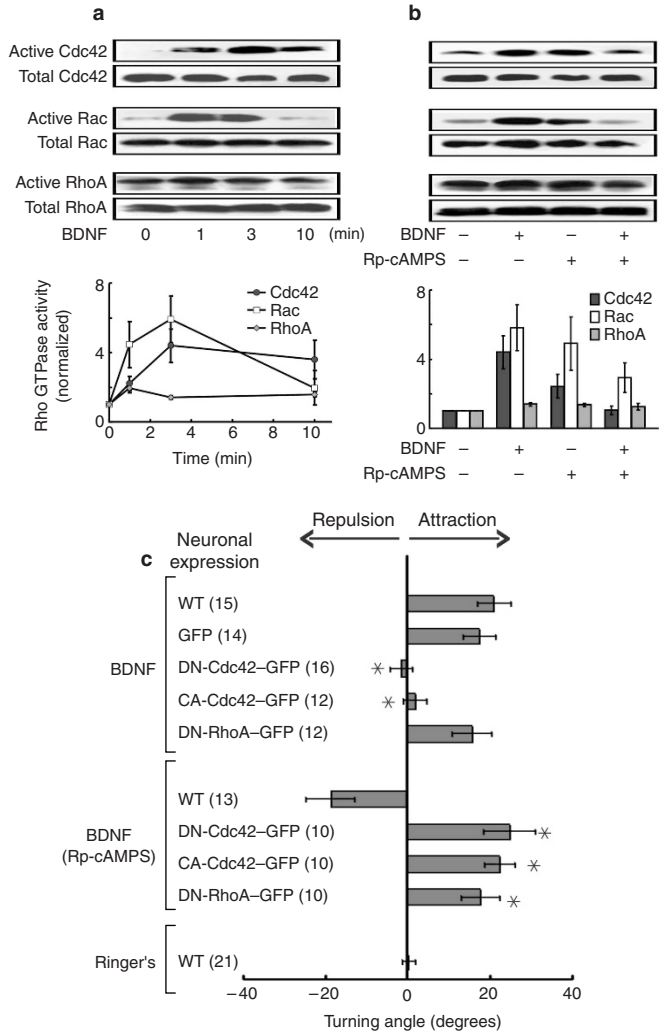

Figure 4 **Regulation of Rho GTPases by BDNF and effect of mutant Cdc42 and RhoA.** **a**, Western blots showing the time course of activation of three Rho GTPases in a serum-starved culture of cerebellar granule cells after treatment with BDNF (50 ng ml^{-1}). **b**, Effect of Rp-cAMPS (20 μM) on the BDNF-triggered activity of Rho GTPases. Active GTPases were pulled down by GST–PBD and GST–RBD (see Methods). Results are representative of at least three experiments and are expressed as the mean fold increase over the value of serum-starved cells not treated with BDNF. **c**, Histograms showing the averaged turning angles of *Xenopus* growth cones induced by a BDNF gradient (with or without 20 μM Rp-cAMP in the bath) under various experimental conditions. $*P < 0.01$, as compared with the uninjected (WT) group (Kolmogorov–Smirnov test). Error bars, s.e.m.

application of a gradient of cytochalasin B, BDM, LPA or BDNF.

We found that the average ratio of filopodia number in the control period (−5 to 0 min) was almost one, but became less than one within minutes after the application of the BDM or the BDNF gradient, before the 'palm' of the growth cone turned towards the gradient. An opposite change, that is, a ratio of more than one, was observed after the application of the cytochalasin B or the LPA gradient. At the concentrations of chemicals used in these experiments, the total filopodia number of the whole growth cone did not change significantly throughout the experiments (Fig. 6b).

Central role of myosin activity in chemorepulsion. Because myosin-II-mediated contractile activity has been proposed to induce growth cone retraction[23,29], we tested whether myosin II is involved in growth cone turning by examining the effects of the myosin ATPase inhibitor BDM and the myosin light chain (MLC) kinase inhibitor ML-7 (ref. 30). Although bath-application of ML-7 (5 μM) and BDM (10 μM) did not affect chemoattraction induced by the BDNF gradient, treatment with either drug converted the

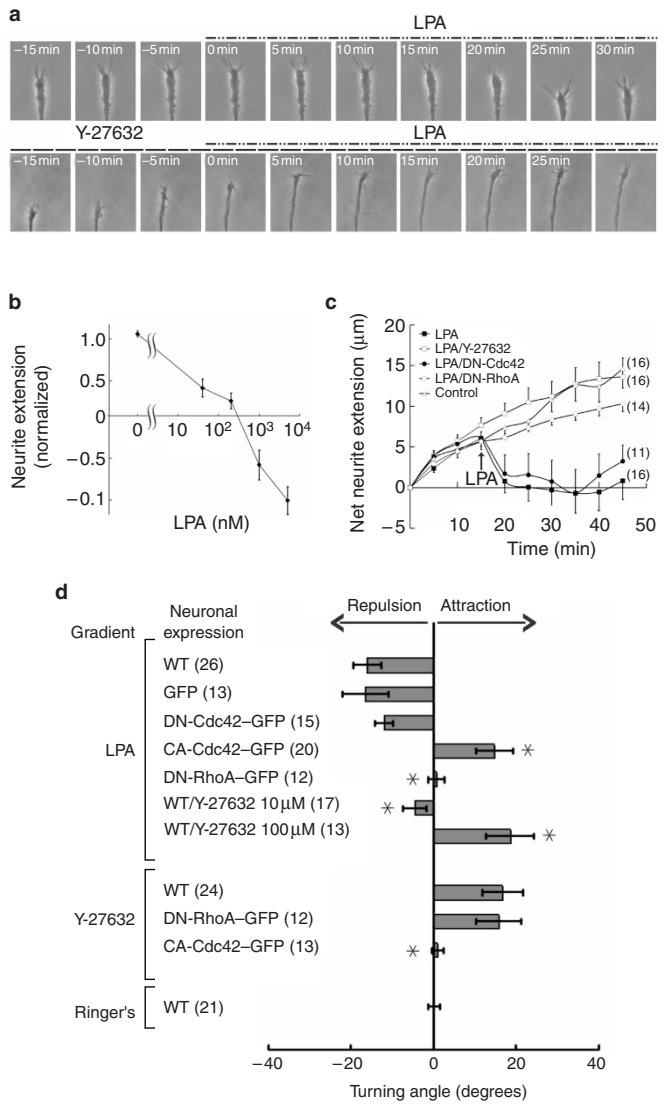

Figure 5 **RhoA- and ROCK-dependent neurite inhibition induced by LPA.** **a**, Microscopic images of a neurite before and at various times (in min) after the application of LPA (1 μM in the bath), in the absence or presence of Y-27632 (10 μM). Broken lines indicate the duration of drug treatment. **b**, Dose–response curve for the effect of LPA on neurite extension. Negative values represent the average length of neurite retraction. The number of neurons assayed for each dose was 25–40. **c**, Experiments in which neurite extension was monitored both before and after treatment with LPA (1 μM in the bath) in the absence or presence of Y-27632 (10 μM in the bath), for control neurons and for neurons expressing mutant DN-Cdc42 or DN-RhoA. Arrow indicates the start of LPA treatment. **d**, Histograms of averaged growth cone turning angles induced by a gradient of LPA or Y-27632 under various experimental conditions. *$P < 0.01$, as compared with the un-injected (WT) group for LPA-induced turning responses, or the Ringer's group in un-injected cells for Y-27632-induced responses. The bath concentrations of Y-27632 in the un-injected group were 10 μM and 100 μM, respectively. Error bars, s.e.m.

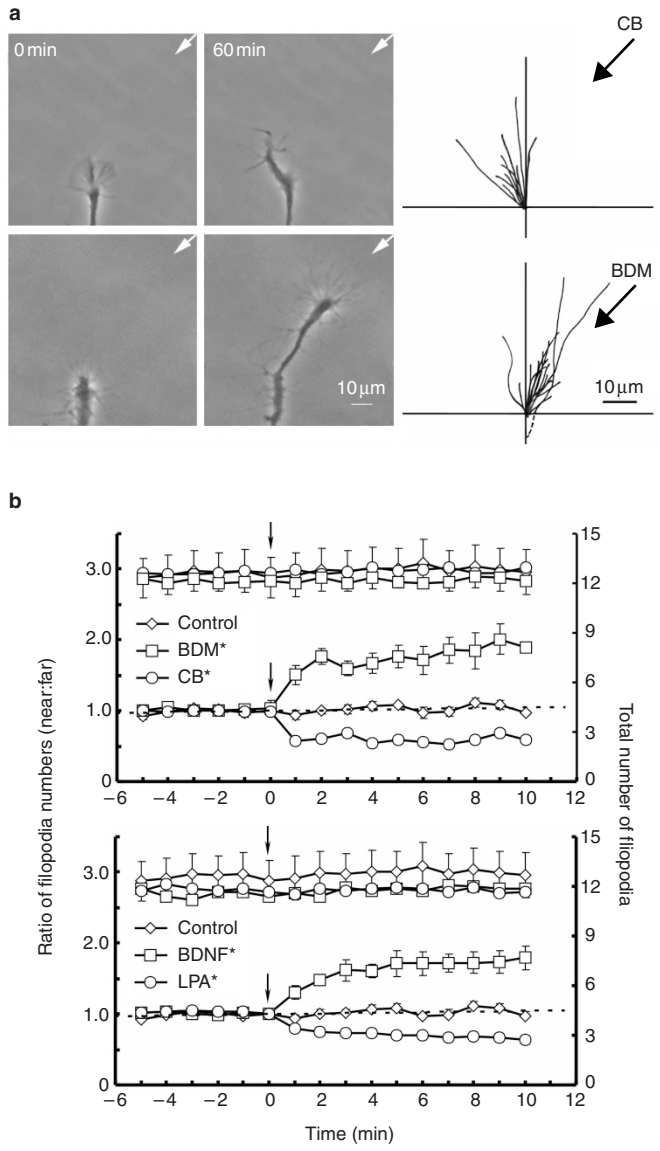

Figure 6 **Role of actin and myosin in filopodial dynamics.** **a**, Top left, microscopic images of a cultured *Xenopus* spinal neuron at the beginning (0 min) and the end (60 min) of a 1-h exposure to a cytochalasin B (CB) gradient created by pulsatile application of a cytochalasin B solution (10 μg ml^{-1} in the pipette). Top right, superimposed traces depict the trajectory of neurite extension during the 1-h turning assay for 12 randomly sampled neurons. Bottom, as in the top panels except that the pipette contained BDM (10 μM). Scale bar, 10 μm. **b**, Filopodia asymmetry induced by a gradient of cytochalasin B (10 μg ml^{-1} in the pipette, $n = 13$), BDM (10 μM, $n = 12$), BDNF (50 μg ml^{-1}, $n = 12$) or LPA (40 μM, $n = 12$). The ratio and total number were obtained from the recorded time-lapse images (1 frame per min) of filopodia before and after the start of the gradient. Arrow indicates the start of the gradient application. *$P<0.01$, as compared with the control (normal Ringer's solution in pipette, $n = 12$) after the start of the gradient application (Kolmogorov–Smirnov test). Error bars, s.e.m.

repulsion induced by the BDNF gradient (in the bath presence of Rp-cAMPS) to attraction (Fig. 7). Similarly, both drugs converted the repulsion induced by the LPA gradient to attraction. We also found that the attractive turning induced by a gradient of Y-27632 is mediated through myosin activity, as bath application of either ML-7 or BDM completely abolished the Y-27632-induced attractive turning (Fig. 7). These observations are consistent with the idea that myosin functions downstream of ROCK to mediate the turning responses induced by the asymmetric ROCK activity.

The existence of crosstalk between RhoA and Cdc42 pathways was shown further by studies using the ROCK inhibitor Y-27632. Bath application of 10 μM Y-27632 did not affect BDNF-induced attraction, but blocked the repulsion induced by BDNF (in the presence of Rp-cAMPS) or LPA (Fig. 7). Thus, ROCK activity contributes to the

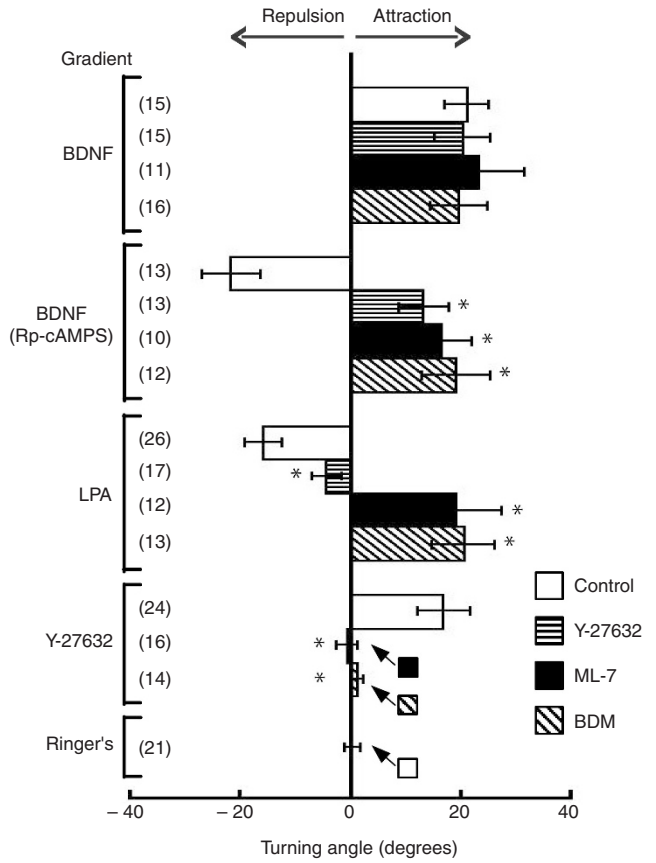

Figure 7 **Effects of Y-27632, ML-7 and BDM on growth cone turning responses.** The bath concentrations of Y-27632, ML-7 and BDM were 10, 5 and 10 μM, respectively. *$P < 0.01$, as compared with the control (no drug in the bath; Kolmogorov–Smirnov test). Error bars, s.e.m.

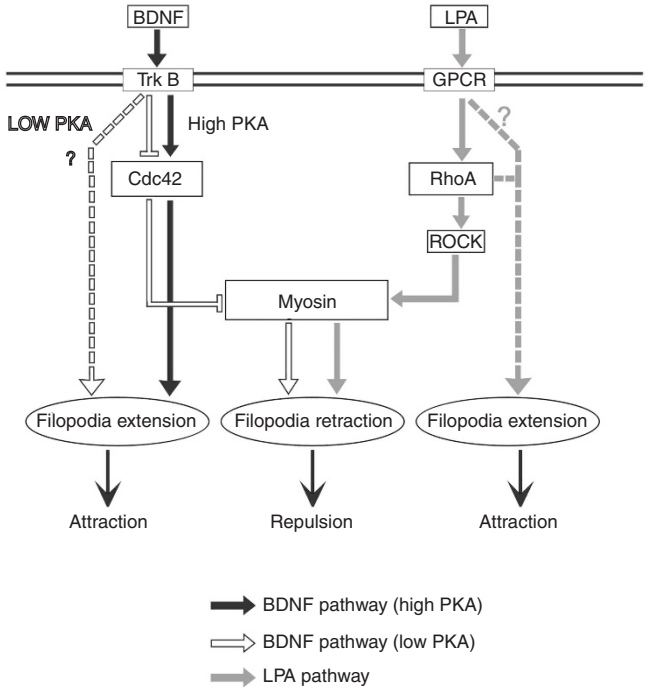

Figure 8 **Model of Cdc42- and RhoA-mediated growth cone guidance.** Under normal culture conditions, the effect of a BDNF gradient is mediated through the Cdc42 pathway, which is dominated by an increase in actin polymerization and filopodia extension, resulting in an attractive turning response. When the PKA activity is decreased, BDNF inhibits Cdc42, resulting in the increase in myosin activity, thereby causing filopodia retraction and a repulsive turning response. Our results also suggest the existence of an attractive pathway that is less dependent on Cdc42 at low concentrations of PKA (clear broken line). Under normal culture conditions, the dominant effect of RhoA activation by LPA is an increase in myosin activity, which leads to actomyosin contraction and filopodia retraction. RhoA might also increase actin polymerization through a secondary pathway when the RhoA–ROCK–myosin pathway is blocked (grey broken line). Disruption of actomyosin activity (by treatment with ML-7 or BDM, or by expression of CA-Cdc42) blocks chemorepulsion induced by either the Cdc42 or the RhoA pathway. GPCR, G-protein-coupled receptor.

repulsive responses induced by both of these gradients, possibly through its phophorylation of the MLC[31,32]. The effect of Y-27632 is similar to that of DN-RhoA, but differs from that of ML-7 and BDM, which were more effective in converting repulsion to attraction (Figs 4, 5 and 7).

Discussion

BDNF has been implicated as a chemoattractant for axon guidance *in vitro* and *in vivo*[20,33,34]. Our results have shown that BDNF can trigger the activation of Cdc42 and Rac in developing neurons. We have also shown that expression of either DN-Cdc42 or CA-Cdc42, at a concentration that does not severely affect neurite extension and growth cone filopodial activity, abolishes chemoattraction induced by BDNF. A uniformly high concentration of CA-Cdc42 might have swamped the instructive signal associated with a gradient of endogenous Cdc42 triggered by the BDNF gradient across the growth cone.

Neuronal Wiskott–Aldrich syndrome protein (N-WASP), which interacts with GTP-bound Cdc42, may function as a link between the guidance signal and actin polymerization[35]. An asymmetric filopodia initiation towards the side of the growth cone of higher Cdc42 activity (or the source of BDNF; see Fig. 6b) might lead to attractive turning. The blocking effect of both DN-Cdc42 and CA-Cdc42 suggests that an appropriate basal quantity of Cdc42 is required for the BDNF-induced gradient of Cdc42 activity to trigger an efficient amplification process necessary for robust turning response[36].

We also found that expression of DN-Rac and CA-Rac proteins in these neurons severely affected neurite development in culture, thus preventing examination of the role of Rac by the growth cone turning assay. In developing *Xenopus* retinal ganglion cell axons, Cdc42 was found to be more important in axon guidance, whereas Rac may influence axon extension[11]. Because the BDNF treatment triggers the activation of both Cdc42 and Rac, which share some common upstream and downstream effectors, our results on Cdc42 perturbations might also reflect in part the function of endogenous Rac.

In some cultured neurons BDNF causes growth cone collapse[20,37,38], suggesting that it may also have a repulsive function under some conditions. We found here that the PKA inhibitor Rp-cAMPS can switch BDNF-induced attraction to repulsion in the growth cone of *Xenopus* spinal neurons (Fig. 4c). This finding of the Rp-cAMPS-dependent switch of BDNF-induced Cdc42 and Rac activities suggests that the switching action of Rp-cAMPS on growth cone turning occurs through Rho GTPases. Our results also indicate that BDNF-triggered repulsion requires both Cdc42 and RhoA activity, because expression of either DN-Cdc42 or DN-RhoA (Fig. 4c), or bath application of the ROCK inhibitor Y-27632 (Fig. 7), abolished the repulsion and converted the response to attraction. These results lead to the following two conclusions.

First, there is crosstalk between the Cdc42 and RhoA pathways that is mediated through the converging actions of these two GTPases on myosin activity[31,32,39]. For BDNF-induced repulsion, basal activity of RhoA may be required to act synergistically with Cdc42 to regulate myosin activity. Second, when PKA is inhibited, a BDNF gradient can result in attractive turning through a pathway that is less dependent on either Cdc42 or RhoA.

We propose that inhibition of PKA activity by Rp-cAMPS leads to an inhibition of Cdc42 or Rac by BDNF, resulting in a decrease in actin polymerization and an increase in myosin activity, which in turn causes filopodia retraction and thus repulsive turning (Fig. 8). This is consistent with a previous report that BDNF can induce neurite retraction at low concentrations of PKA[37]. In addition, inhibition of MLC phosphorylation by ML-7 or by Y-27632 abolished this repulsive pathway, thereby 'unmasking' an attractive pathway that is less dependent on Cdc42. Because several signalling events can be triggered by the activation of TrkB[40], there are other potential links between TrkB activation and the regulation of filopodia activity in addition to Cdc42.

Uniform application of LPA to the culture resulted in neurite inhibition or growth cone collapse in a dose-dependent manner (Fig. 5). We found that a certain dose of LPA, when applied in a gradient across the growth cone, resulted in repulsive turning without significant changes in the rate of neurite extension. This suggests that growth cone repulsion can be induced by an asymmetric inhibition or collapse of filopodia across the growth cone, an idea that is supported further by the repulsion induced by a cytochalasin B gradient and the attraction induced by a BDM gradient (Fig. 6). The repulsive action of LPA depends on the activity of RhoA but not Cdc42, because neurons expressing DN-RhoA showed no turning response in the LPA gradient, whereas those expressing DN-Cdc42 still showed repulsive turning (Fig. 5).

An extracellular gradient of Y-27632 by itself could induce attractive turning (Fig. 5c), which implies that a gradient of ROCK activity is sufficient to act as an instructive signal for growth cone turning. A gradient of Y-27632 was also attractive for neurons expressing DN-RhoA–GFP (Fig. 5c), suggesting the existence of a constitutive ROCK activity in these neurons that is independent of RhoA. Such a RhoA-independent ROCK activation has been reported in fibroblasts or epithelial cells[41,42], and similar mechanisms may also exist in neurons. Because a high concentration of Y-27632 (100 μM) converted LPA-induced repulsion to attraction (Fig. 5c), we conclude further that LPA can activate an alternative ROCK-independent pathway that can presumably promote actin polymerization and growth cone attraction. This idea is supported by a study showing Rho-dependent axon attraction to the midline in the developing *Drosophila* nervous system[12]. A potential downstream effector of RhoA that is responsible for the attractive turning is Dia (diaphanous), which is known to have an opposite effect in modulating the cytoskeleton from that of ROCK[43]. In our system, this positive action may be normally masked by the dominant collapsing action of myosin.

Shape changes of neuroblasts in the ventricular zone during cerebral cortical neurogenesis seem to be mediated by LPA through the activation of Rho[43,44]. In addition, postmitotic neurons can produce extracellular LPA, which may act as a signal from postmitotic neurons to proliferating neuroblasts[43,44]. Our results show that a gradient of LPA can induce growth cone turning. Whether LPA can be generated in appropriate amounts and locations in the developing nervous system and can function as a guidance factor for the growth cone remain to be determined.

Because activation of PAK (p21-activated kinase) by Cdc42 results in a decrease in myosin activity[39] and activation of RhoA results in an increase in myosin activity, either directly by ROCK phosphorylation of MLC[31] or indirectly by ROCK-induced inhibition of MLC phosphatase[32,45], we propose that myosin is a site for crosstalk between the Cdc42 and RhoA pathways in these *Xenopus* growth cones (Fig. 8). An increase in actomyosin contractility results in growth cone collapse in neurons[23,29]. This seems to be the dominant effect of RhoA activation triggered by LPA. Because inhibition of Cdc42 and activation of RhoA have similar effects on myosin activity, the gradient in myosin activity across the growth cone induced by a gradient of either Cdc42 inhibition or RhoA activation may result in an asymmetric filopodial retraction, and thus in the repulsive turning response (Fig. 8). In neurons expressing DN-Cdc42, PAK activation was prevented without affecting either myosin activity induced by the LPA-dependent RhoA pathway or the resultant repulsion of the growth cone. However, expression of CA-Cdc42 may prevent myosin function or disrupt actin–myosin interactions[39,46], thereby abolishing LPA-induced repulsion (Fig. 5) or the attractive turning induced by the Y-27632 gradient (Fig. 5).

Finally, we note that the crosstalk between the Cdc42 and RhoA pathways may also be mediated by GTPases[47–49]. There is evidence that Cdc42, Rac and Rho may exist in a hierarchical cascade wherein Cdc42 activates Rac, which in turn activates Rho[47]. But there is also evidence that Cdc42 and Rac downregulate Rho activity[49]. Our results are not consistent with a model involving crosstalk through only GTPases. Instead, crosstalk between downstream effectors of these GTPases is more likely to have a dominant role in these spinal neurons. Our results also suggest that one axon guidance factor may 'switch' or 'mask' the effect of another through crosstalk between their intracellular signalling pathways. □

Methods

Culture preparation and growth cone turning assay

Cultures of *Xenopus* spinal neurons were prepared from 1-day-old *Xenopus* embryos as described[20]. The culture medium comprised 50% (v/v) Leibovitz medium (Gibco-BRL, Gaithersburg, MD), 1% (v/v) fetal bovine serum (HyClone, Logan, UT) and 49% (v/v) Ringer's solution (115 mM NaCl, 2 mM $CaCl_2$, 2.5 mM KCl and 10 mM HEPES, pH 7.4). The cells were used for turning assays 15–20 h after plating at room temperature (20–22 °C). We produced microscopic gradients of chemicals as described[20].

To assay growth cone turning, the pipette tip was placed 100 μm from the centre of the growth cone of an isolated neuron and at an angle of 45° with respect to the initial direction of neurite extension (indicated by the last 10-μm segment of the neurite). We defined the turning angle as the angle between the original direction of neurite extension and a straight line connecting the positions of the growth cone at the beginning and the end of the 1-h period. Theoretical analysis and direct measurements of the gradient using fluorescent dyes have shown that, at a distance of 100 μm from the pipette tip, the concentration gradient across the growth cone (typical width 10 μm) is in the range of 5–10% and the average concentration at the growth cone is about 10^3-fold lower than that in the pipette. Microscopic images of neurites were captured with a CCD (charge-coupled device) camera (TK-C1381; JVC, Yokohama, Japan) attached to a phase-contrast microscope (CK-40, Olympus, Tokyo, Japan) and stored in a microcomputer for subsequent analysis using Scion Image programs. To determine the total length of neurite extension, the whole trajectory of the neurite at the end of the 1-h period was measured with a digitizer. Only those growth cones with a net extension of more than 5 μm over the 1-h period were included in the analysis of turning angles.

All experiments were carried out at room temperature in modified Ringer's solution (140 mM NaCl, 2.5 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$ and 10 mM HEPES, pH 7.4). BDNF was obtained from Promega (Madison, WI). LPA (Avanti Polar Lipids; Sigma, St Louis, MO) was prepared in pure water. For pharmacological treatments, chemicals were added to the culture medium 20 min before the gradient was applied and were present throughout the experiments, unless otherwise indicated. Rp-cAMPs and foskolin were from Calbiochem (San Diego, CA). Y-27632 was a gift of Yoshitomi Pharmaceuticals (Osaka, Japan). Data are reported as the means ± s.e.m. We analysed statistical significance by Kolmogorov–Smirnov test. The use and care of animals were carried out in accordance with the guidelines of the Shanghai Institutes of Biological Sciences Animal Research Advisory Committee.

Expression of Rho-family GTPases by cDNA injection

We used GFP–S65T as a marker of the expression of GTPases. GFP fusion constructs of L61 CA-Cdc42, L63 CA-RhoA, N17 DN-Cdc42 and N19 DN-RhoA were subcloned into pCS2 (a gift from D. Turner, University of Michigan, Ann Arbor, MI) at the *Stu* site. The DN mutants are competitive inhibitors that bind irreversibly to guanine nucleotide-exchange factors, which are upstream regulators of GTPases. The CA mutants are unresponsive to inactivation mediated by GTPase-activating proteins. The cDNAs were a gift from G. Bokoch (Scripps Research Institute, La Jolla, California). We used the Endofree Plasmid Maxi kit (Qiagen, Hilden, Germany) to purify the cDNA clones. The final concentration of cDNAs for injection was 0.2 μg μl^{-1} for DN-Cdc42, CA-Cdc42 or CA-RhoA, and 0.5 μg μl^{-1} for DN-RhoA and GFP.

About 1.5 ng of DN-Cdc42, CA-Cdc42 or CA-RhoA, and 4 ng of DN-RhoA or EGFP were injected into one of the blastomeres of *Xenopus* embryos at the two- or four-cell stage using a Eppendorf transjector 5246 (Eppendorf, Hamburg, Germany). The embryos were kept in 10% Ringer's solution at room temperature (20–22 °C) for 24 h before culture preparation.

Western blotting

We collected five *Xenupus* embryos at each time point after *in vitro* fertilization and homogenized them by sonication in 0.2 ml of lysis buffer (0.1% SDS, 1% Nonidet P-40, 1% glycerin, 50 mM HEPES,

pH 7.4, 2 mM EDTA and 100 mM NaCl). The homogenates were centrifuged at 12,879*g* for 5 min. The supernatant was mixed with equal volume of 1,1,2-trichlorotrifluoroethane and centrifuged again to remove the yolk. 15 μg of total protein was loaded in each lane, resolved by 12% SDS–PAGE and blotted onto poly(vinylidene difluoride) membrane. The blots were blocked for 1 h at room temperature in 0.2% block-1 (Tropix, Bedford, MA) and incubated overnight at 4 °C with a polyclonal antibody raised against GFP (1:1,000 dilution; Santa Cruz Biotechnology, Santa Cruz, CA). Membranes were rinsed and incubated for 1 h with a horseradish-peroxidase-conjugated goat antibody against rabbit IgG (1:20,000 dilution; Biorad, Hercules, CA). Chemiluminent detection was done with the ECL kit (Pierce, Rockford, IL). To verify equal protein loading in each lane, we washed the membranes in stripping buffer at 70 °C for 30 min, and rinsed and probed them with rabbit antibodies against mitogen-activated protein kinase (MAPK; 1:2,500 dilution; Sigma).

Assay for GTPase activity

We measured GTPase activity as described[24]. Briefly, lysates of cultured cerebellar granule cells were pre-incubated with glutathione–Sepharose 4B (Amersham Pharmacia, Buckinghamshire, UK) for 1 h and then incubated with the bacterially produced glutathione *S*-transferase (GST)-fused Rac/Cdc42-binding domain of Pak (GST–PBD; 5 μg) and the Rho-binding domain of Rhotekin (GST–RBD; 20 μg) bound to glutathione-coupled Sepharose beads, at 4 °C overnight with constant rocking. The beads and proteins bound to the fusion protein were washed five times with modified RIPA buffer at 4 °C, eluted in SDS sample buffer, and analysed for bound Cdc42, Rac1 or RhoA by western blotting using monoclonal antibodies against Cdc42, rabbit polyclonal antibodies against Rac1 or monoclonal antibodies against RhoA (all from Santa Cruz). The quantities of Rho GTPase activation were measured by densitometry analysis of the blots.

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COMPETING FINANCIAL INTERESTS

The authors declare that they have no competing financial interests.