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RhoE stimulates neurite-like outgrowth in PC12 cells through inhibition of the RhoA/ROCK-I signalling

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Abstract

Neurite formation involves coordinated changes between the actin cytoskeleton and the microtubule network. Rho GTPases are clearly implicated in several aspects of neuronal development and function. Indeed, RhoA is a negative regulator of neurite outgrowth and its effector Rho-kinase mediates the Rho-driven neurite retraction. Considering that RhoE/round protein (Rnd3) acts antagonistically to RhoA and it is also able to bind and inhibit rho kinase-I (p160ROCK) – ROCK-I, it is tempting to speculate a role of RhoE in neurite formation. We show for the first time that, in the absence of nerve growth factor (NGF), RhoE induces neurite-like outgrowth. Our results demonstrate that over-expression of RhoE decreases the activity of RhoA and reduces the expression of both ROCK-I and the phosphorylated myosin light chain phosphatase (MLCPp). Conversely, over-expression of either active RhoA or ROCK-I abolishes the RhoE-promoted neurite outgrowth, suggesting that RhoE induces neurite-like formation through inhibition of the RhoA/ROCK-I signalling. We also show that Rac and Cdc42 have a role in RhoE-induced neurite outgrowth. Finally, the present data further indicate that RhoE may be involved in the NGF-induced neurite outgrowth in PC12 cells, as depletion of RhoE by siRNA reduces the neurite formation induced by NGF. These findings provide new insights into the molecular mechanism implicated in neuronal development and may provide novel therapeutic targets in neurodegenerative disorders.

Keywords: nerve growth factor, neurite outgrowth, PC12, rho kinase-I (p160ROCK), RhoA, RhoE/Rnd3.

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The nervous system functions as a highly organized neuronal network based in specific pattern of connections between neurons. Neurites extend from the cell body, branching to form a complex tree-like structure that receives and processes inputs from other neurons (Govek *et al.* 2005). The outgrowth of neurites involves coordinated changes between the actin cytoskeleton and the microtubule network.

Rho GTPases are a family of proteins implicated in several cellular functions as actin cytoskeleton organization and microtubule dynamics (Heasman and Ridley 2008). Most Rho proteins act as molecular switches, cycling between an active, GTP-bound conformation and an inactive, GDP-bound conformation. However, there are several atypical members, as the round (Rnd) proteins, that are predominantly GTP-bound and therefore are considered constitutively activated. RhoE/Rnd3 is a member of the Rnd subfamily that acts antagonistically to RhoA (Riento *et al.* 2005a) and induces stress fiber disassembly (Guasch *et al.* 1998; Nobes *et al.* 1998). RhoE inhibits RhoA signalling via inactivation of RhoA, by binding to p190 GTPase-activating protein for Rho (RhoGAP) and reducing the RhoA-GTP levels (Wennerberg *et al.* 2003), and/or regulating the RhoA effector, rho kinase-I (p160ROCK), ROCK-I (Riento *et al.* 2003). It has recently been proposed that phosphorylation of RhoE by ROCK-I forms part of a feedback mechanism to regulate RhoA signalling (Komander *et al.* 2008). RhoE binds to and inhibits ROCK-I, preventing the phosphorylation of its downstream target, myosin light chain phosphatase (MLCP) (Riento *et al.* 2003), and RhoE itself is regulated by ROCK-I mediated phosphorylation on multiple sites (Riento *et al.* 2005b). This small GTPase is regulated at the level of gene expression (Hansen *et al.* 2000; Ongusaha *et al.* 2006),

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Abbreviations used: GFP, green fluorescent protein; MLCP, myosin light chain phosphatase; NGF, nerve growth factor; N-WASP, neural Wiskott-Aldrich syndrome protein; RhoGAP, GTPase-activating protein for Rho; Rnd, round proteins; ROCK-I, rho kinase-I (p160ROCK); TBS, Tris-buffered saline; WAVE, WASP-family verprolin-homologous.

phosphorylation and protein degradation (Riento *et al.* 2005b).

Increasing evidences indicate that the Rho family of proteins play an important role in several aspects of neuronal development, including neurite outgrowth and differentiation, axon pathfinding, and dendritic spine formation and maintenance (Govek *et al.* 2005). Rho activation causes the collapse of the growth cone and neurite retraction, and activation of Rac and Cdc42 induces the formation of lamellipodia and filopodia at the growth cone (Negishi and Katoh 2002), regulating dendritic branch dynamics and growth. Other Rho GTPases, such as Rnd 1 and 2, play a role in neurite formation and retraction. Rnd1 induces neurite extension (Ishikawa *et al.* 2003) by a Rac-dependent mechanism involving disruption of cortical actin filaments (Aoki *et al.* 2000). Rnd2 promotes dendrite branching in PC12 cells and primary hippocampal neurons via its effector Rapostlin (Fujita *et al.* 2002). However, the effects of RhoE/Rnd3 on neuronal development and the underlying molecular mechanisms have not yet been investigated.

Here, we have examined the role of RhoE/Rnd3 in neuronal differentiation in PC12 cells. This cell line is a useful model system for studying neuronal differentiation and signal transduction. Treatment of these cells with nerve growth factor (NGF) results in growth arrest and the formation of neurites resembling that of sympathetic neurons (Greene and Tischler 1976). Our results show that RhoE by itself stimulates neurite-like outgrowth in PC12 cells, through the inhibition of the RhoA/ROCK-I signalling pathway. We further demonstrate that Rac and Cdc42 play a role in RhoE-induced neurite formation.

Material and methods

Cell culture

Rat pheochromocytoma PC12 cells were purchased from European Collection of Cell Cultures. Cells were cultured in RPMI (Roswell Park Memorial Institute) medium containing 10% heat-inactivated horse serum, 5% foetal bovine serum, 1% glutamine and 1% penicillin–streptomycin, all purchased from Gibco-Invitrogen (Paisley, UK) and cultured under humidified conditions in 95% air and 5% CO_2 at 37°C. Tissue culture plates and coverslips were coated with collagen type IV (Sigma-Aldrich, Steinheim, Germany) for at least 6 h and then left to dry in the incubator the day before being used. To induce differentiation of PC12 cells, they were seeded at a density of 1×10^5 cells/mL on collagen-coated plates, and cultured in complete medium to allow them to settle. Then the medium was replaced with RPMI (Roswell Park Memorial Institute) supplemented with 1% horse serum (serum-reduced medium) and cells were starved for 18 h. Stimulation of PC12 cells was performed by addition of NGF-2.5S (Sigma-Aldrich) at a final concentration of 100 ng/mL.

Transient transfection of PC12 cells

PC12 cells were seeded on collagen-coated glass coverslips (12 mm) in 24-well plates at a density of 2.5×10^5 cells/well and cultured for 15–18 h so that the cells were 90–95% confluent at the time of transfection. Cells were transfected with LipofectAMINE 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Briefly, cells were incubated in normal growth medium without antibiotics. 2 μL of LipofectAMINE 2000 were mixed with the plasmids of interest (0.8 μg of DNA) and incubated for 20 min at room temperature, whereupon complexes were added to each well. Transfected cells were fixed 48 h after transfection and examined for neurite outgrowth. Cells were transfected with vectors expressing the following proteins: green fluorescent protein (GFP) (pQBI25, Quantum Biotechnologies, Montreal, Canada), wild-type RhoE (pCMV5-RhoE) (Guasch *et al.* 2007), constitutively active RhoA (pEXV3-V14RhoA), constitutively active ROCK-I, aROCK-I, (pCAG-ROCK-Δ1), an inactive mutant of ROCK-I, iROCK-I (pCAG-ROCK-Δ5) (Ishizaki *et al.* 1997) and constitutively active Rac1 (pEXV3-V12Rac1). pEXV3-V14RhoA and the ROCK-I constructs were kindly provided by Dr Anne Ridley and Dr Shuh Narumiya, respectively. For immunoblot studies, 8 μg of pCMV5-FLAG-RhoE together with 0.8 μg of GFP were used. In the case of cotransfection experiments, double immuno-stainning (anti-myc and anti-flag) was performed to check for single- and double-transfected cells. Double immuno-labeled quantification gave high percentages of cotransfected cells. Results were: Wt-RhoE + ROCKI-Δ1 = 92.89% ± 25.05, Wt-RhoE + ROCKI-Δ5 = 73.07% ± 6.06, Wt-RhoE + V14RhoA = 87.91% ± 17.59 and Wt-RhoE + V12Rac1 = 99.41% ± 32.25.

RhoE knock-down

To suppress endogenous RhoE expression, we used a pool of the following oligonucleotides (siGENOME SMARTpool siRNA from Dharmacon Research, Inc., Lafayette, CO, USA): GGCAGACUCCUGUGUCAUA, CAAAGCGGAUUUCACACAU, AAUCGACACACAAAGAAUA, GUGAAAUGCAAGAUAGUAG. The non-targeting control was also purchased from Dharmacon. PC12 cells were transfected with LipofectAMINE 2000 (Invitrogen) following the manufacturer's protocol with 200 pmol of siRNA together with 0.8 μg of GFP and then siRNA-transfected cells were stimulated with NGF 8 h after transfection and analyzed 96 h after transfection.

Western blotting

PC12 cells were washed with phosphate-buffered saline (PBS) and lysed for 30 min on ice in cold lysis buffer [1% NP40, 20 mM Tris–HCl, pH 8, 130 mM NaCl, 10 mM NaF, 10 μg/ml aprotinin, 40 μM leupeptin, 1 mM dithiothreitol (DTT), 1 mM Na_3VO_4 and 10 mM phenylmethylsulfonyl fluoride (PMSF)]. Total cell lysates were clarified by centrifugation at 13 500 *g* for 15 min at 4°C. Next, equal amounts of proteins were resolved on sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) 12% gels, transferred to polyvinylidene fluoride (PVDF) membranes (Immobilon-P, Millipore, Billerica, MA, USA) and blocked for 1 h with non-fat milk 5% in Tris-buffered saline (TBS) containing 0.1% Tween 20 (T-TBS) to prevent non-specific binding. After blocking, blots were incubated for 2 h at room temperature or at 4°C overnight with the primary antibodies diluted in blocking solution. The following antibodies were used: anti-RhoE (1 : 2000, Upstate, Lake Placed, NY, USA), anti-Rac1 (1 : 15 000, clone 23A8, Upstate), anti-RhoA (1 : 750, Santa Cruz Biotechnologies, Santa Cruz, CA, USA), anti-

Cdc42 (1 : 250, Santa Cruz Biotechnologies), anti-pMLCP/phospho-myosin light chain phosphatase (MYPT1) (1 : 400, Thr696 Upstate), anti-ROCK-I [1 : 100, C-19, Santa Cruz (sc-6055)], anti-ROCK-I [1 : 400, H-85, Santa Cruz (sc-5560)], anti-paxillin (1 : 30 000, BD Transduction Laboratories, Lexington, KY, USA), anti-Neurofilament 68 (1 : 1000, Sigma-Aldrich), anti-actin (1 : 2000, Sigma-Aldrich), anti-N-Wasp (1 : 200, Santa Cruz Biotechnologies), anti-Wave (1 : 1000, Santa Cruz Biotechnologies), anti-pCofilin (1 : 1000, Santa Cruz Biotechnologies), anti-p190-B RhoGAP (1 : 500, Cell Signalling Technology, Inc., Danvers, MA, USA) and anti-Rho GDIα (1 : 100, Santa Cruz Biotechnologies) and anti-PI3 Kinase p85 (1 : 1000, Cell Signaling). After three washes with T-TBS, blots were incubated for 1 h at room temperature with the following horseradish peroxidase-conjugated anti-IgG antibodies: anti-mouse (1 : 1000, Santa Cruz Biotechnologies) for RhoE, Rac1, RhoA, paxillin, and N68, anti-rabbit (1 : 20 000, Sigma-Aldrich) for Cdc42, pMLCP/phospho-myosin light chain phosphatase (MYPT1), ROCK-I (H85), actin, N-Wasp, Wave and PI3K, and anti-goat (1 : 20 000, Sigma-Aldrich) for ROCK-I (C19). Finally, blots were developed using the enhanced chemiluminescent system ECL Plus (Amersham Pharmacia Biotech, Little Chalfont, UK).

RhoA, Rac1 and Cdc42 activity assays

The Rho Activation Assay Kit (Minambres *et al.* 2006) and the Rac1/Cdc42 Activation Assay Kit (both from Upstate) were used according to the manufacturer's instructions. PC12 cells were seeded at a density of 2.5×10^6 cells/60 mm dish, cultured for 18 h and transfected with wt-RhoE for 48 h. Lysates were incubated with either 10 μg of Rac/Cdc42 Assay Reagent (PAK-1 PBD, agarose) for Rac1 and Cdc42 or 40 μg of Rho Assay Reagent (Rhotekin Rho-Binding Domain, agarose) for RhoA. Proteins retained on the beads were resolved on sodium dodecyl sulfate–polyacrylamide gel electrophoresis 12% gels. Bound Rho proteins were detected by western blot using the anti-RhoA, anti-Rac1, or anti-Cdc42 antibodies. The amount of GTP-bound protein was normalized relative to the total amount of RhoA, Rac1 or Cdc42 in the cell lysates, respectively. Every pull-down assay was repeated 4-6 times.

Immunofluorescence

PC12 cells were seeded on collagen-coated glass coverslips (12 mm) in 24-well plates at a density of 2.5×10^5 cells/well and transfected with the appropriate construct along with the GFP expressing plasmid. Forty-eight hours after transfection, cells were fixed and permeabilised as previously described (Guasch *et al.* 2003). Neurofilament N200 was detected by using the anti-Neurofilament 200 antibody (1 : 400, Sigma-Aldrich) followed by incubation with Cy3-conjugated donkey anti-rabbit IgG (1 : 200, Jackson Immuno-Research, West Grove, PA, USA). Actin was detected by incubating cells for 45 min with 0.1 μg of tetramethyl rhodamine isothiocyanate (TRITC)-labelled phalloidin per ml (Sigma). Cells were mounted in Mounting medium (Dakocytomation, Carpinteria, CA, USA). Confocal images were acquired with a Leica TCS SP2 AOBS (Leyca Microsystems Heidelberg GmbH, Mannheim, Germany) inverted laser scanning confocal microscope using a HCXPLAPOibd.BL 63.0 × 1.40 oiI objective for immunofluorescence and a HCXPLAPOcs 40.0 × 1.25 oil uv objective for phase-contrast images. The excitation wavelengths for fluorochromes were 488 nm (argon laser) for GFP and 561 nm (DPSS laser) for tetramethyl rhodamine isothiocyanate (TRITC). Two-dimensional pseudo colour images (255 colour levels) were gathered with a size of 1024 × 1024 pixels. Confocal images were acquired using the same settings and analyzed with the MetaMorph image analysis software (Universal Imaging Corporation TM, Molecular Devicer, Downingtown, PA, USA).

Neurite outgrowth analysis

For quantification of various parameters of neurite outgrowth, fluorescent images from a Zeiss microscope (Axioskop 2) were taken, and morphological measurements were performed using the MetaMorph software (Universal Imaging). A neurite was defined as a process that measured at least the length of one cell body and stained positively for neurofilament protein. Total length of neurites is defined as the sum of the neurite length per cell. Finally, cells with different neurite numbers (none, one, two or more than two) were scored as a percentage of the total number of transfected cells. At least 100 cells were counted in each experiment, and the experiments were repeated 4–6 times, therefore, no less than 400 cells were analysed in each experiment.

Statistical methods

Data are shown as the mean ± standard error. Student's *t*-test for unpaired variables was used to test for differences elicited by RhoE expression and differences were considered statistically significant at $p < 0.05$.

Results

RhoE induces neurite-like outgrowth in PC12 cells

It is known that PC12 cells differentiate in response to NGF and show neurite outgrowth similar to that of sympathetic neurons. Our investigation started when we realized that NGF-stimulated PC12 cells showed a 3.5-fold increase in the protein levels of RhoE (Fig. 1a). This prompted us to examine whether the small GTPase RhoE was involved in neuronal differentiation. For this purpose, PC12 cells were cotransfected with an expression vector containing a Flag-tagged wild type RhoE together with a plasmid expressing the GFP. Transfected cells were visualized in green and actin was detected by phalloidin staining (red). As illustrated in Fig. 1b, PC12 cells transfected with the GFP plasmid alone as a control, showed a rounded morphology, like that of untransfected cells. However, transfection of the wild type RhoE protein resulted in an increase in the number of cells showing neurite-like outgrowth, in the absence of NGF. We also quantified the number of neurites per cell as well as the total length of these processes per cell. Figure 1c shows that overexpression of RhoE significantly increased those parameters.

To further confirm the implication of RhoE in neurite outgrowth, we used RhoE siRNA to knock-down the endogenous RhoE protein. Western blot analysis of several experiments indicated that PC12 cells transfected with control siRNA (200 pmol) for 96 h did not show different

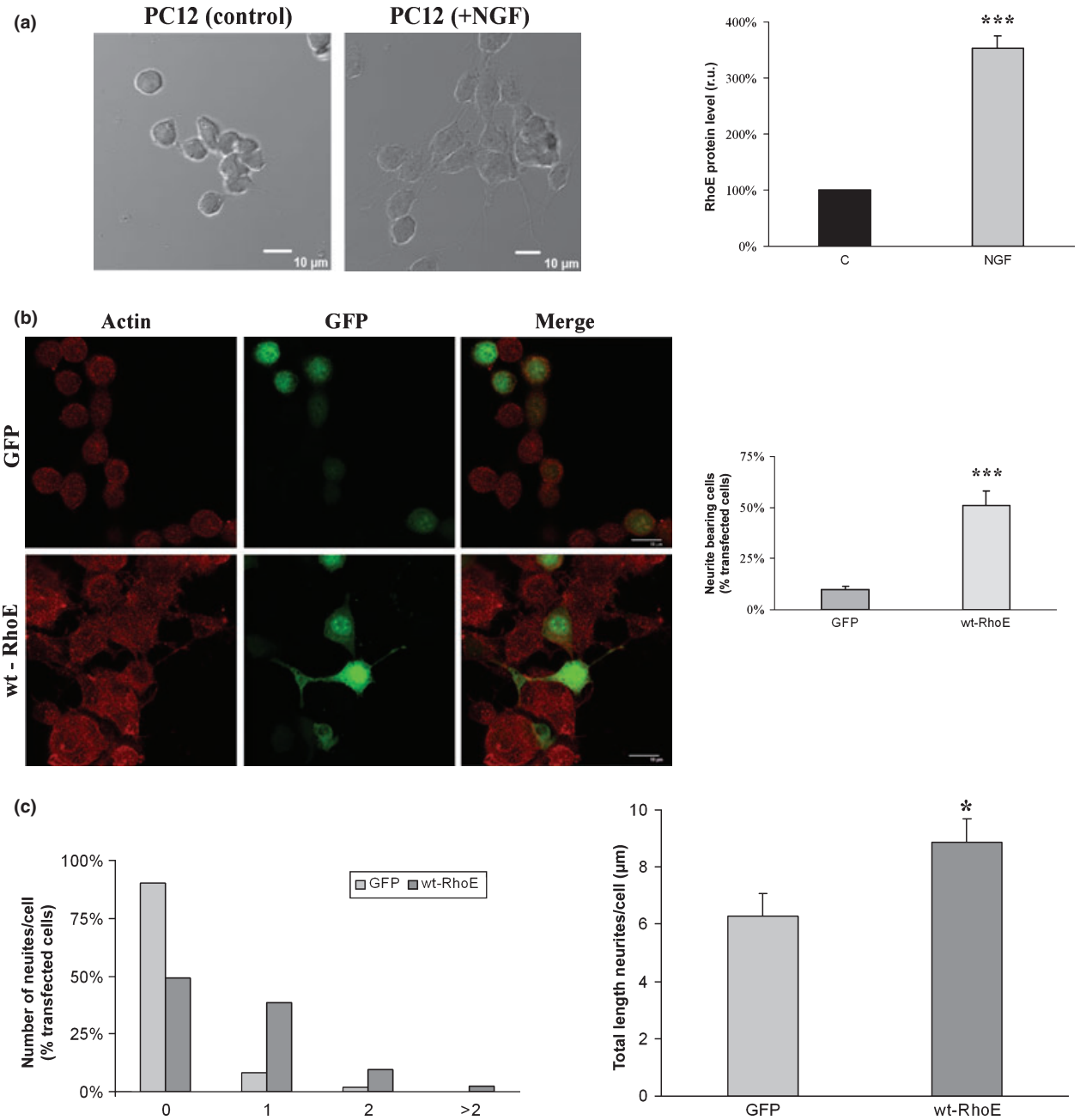

Fig. 1 RhoE promotes the formation of neurite-like processes in PC12 cells. (a) NGF increases the levels of RhoE protein in PC12 cells. (b) Neurite-like formation in RhoE-transfected cells. Confocal images showing PC12 cells transfected only with a plasmid expressing GFP to identify transfected cells or together with a plasmid expressing wt-RhoE. Panels show actin staining with phalloidin and GFP expression. Neurite definition was specified in Mat. and Met. (c) RhoE-transfected cells show an increase in the number of neurites per cell as well as in the total length of the neurites. (d) Inhibition of RhoE expression reduces the neurite formation induced by NGF in PC12 cells. RhoE expression levels were analyzed by western blotting in cells transfected with control siRNA and RhoE siRNA at different conditions. Quantification of the RhoE knockdown after 96 h of transfection with either control or RhoE siRNA (both at 200 pmol) was performed. The graph displays the number of cells with neurites and the total length of the processes in cells transfected with either control or RhoE siRNA and stimulated with NGF for 3–4 days. (e) RhoE increases the expression of both neurofilaments N68 and N200. Cells transfected with RhoE show an increase in the N68 protein levels. A representative western blot and the quantification of the results are shown. Confocal images showing both GFP and RhoE transfected cells stained for the neurofilament N-200 are also shown. Mean ± SEM is shown for a total of 8–10 western blots and 4–6 experiments in neurite analysis. *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$ in a Student's t test. Bars, 20 μm.

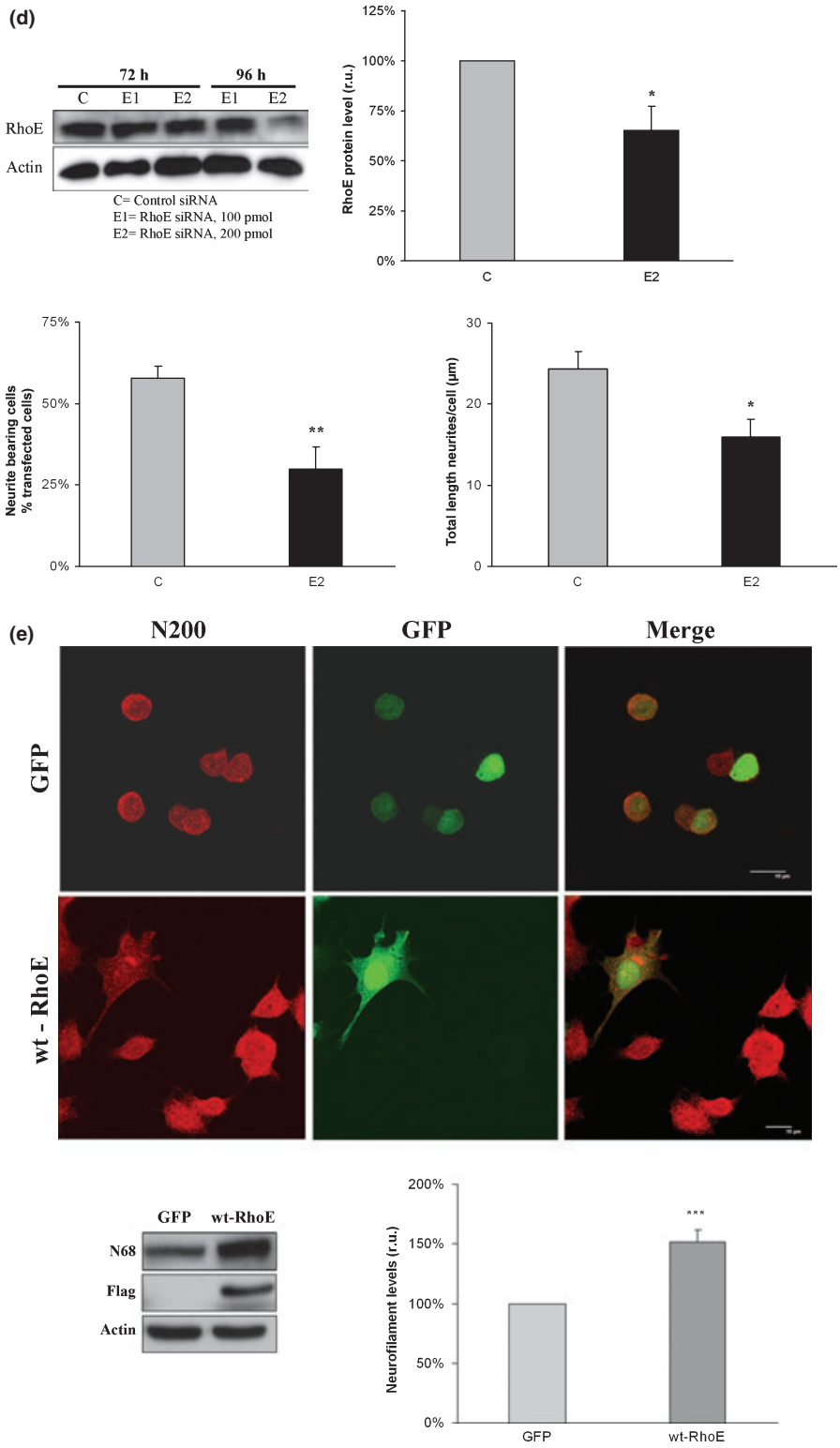

Fig. 1 *(Continued)*

RhoE levels, whereas transfection with RhoE siRNA lowered endogenous protein levels by 65% (Fig. 1d). Cells were then transfected with either RhoE siRNA or control siRNA and then stimulated with NGF for 3–4 days to promote the formation of neurites. Our results show that depletion of RhoE by RhoE siRNA in PC12 cells caused a reduction in

the NGF-stimulated neurite outgrowth, suggesting that RhoE is involved in the process of neurite extension.

To study whether this effect of RhoE is related to neuronal differentiation, we investigated the expression of both the neurofilament N68 and N200, which are intermediate filaments associated with differentiation. RhoE-expressing cells showed an increase in the levels of N68, as assessed by western blotting analysis. In addition, RhoE-induced neurite-like processes also contain the neurofilament N200, as detected by immunofluorescence (Fig. 1e). These results suggest that RhoE promotes the formation of neuritic processes which correlates with the induction of differentiation-associated neurofilament proteins.

Signalling induced by RhoE in PC12 cells

To investigate the involvement of other Rho GTPases in the RhoE-mediated neurite-like outgrowth, we studied the expression levels of several proteins in PC12 cells. For this purpose, cells were transfected with the Flag-RhoE expression vector and the expression of several proteins was analysed by western blotting (Fig. 2a). As RhoE induces disassembly of the actin cytoskeleton and associated focal adhesions (Guasch *et al.* 1998), we first investigated the effect of RhoE over-expression on the protein levels of paxillin, a marker of focal adhesions. We found that RhoE expressing cells showed a decrease in the content of paxillin, in agreement with our previous results in other cell types (Guasch *et al.* 2007).

As RhoE opposes RhoA, at least in part, by binding to and inhibiting ROCK-I from phosphorylating its downstream target MLCP (Riento *et al.* 2003), we also investigated the effect of RhoE over-expression on these two proteins. Figure 2a shows that PC12 cells transfected with RhoE showed a decrease in the expression levels of ROCK-I. Furthermore, this decrease was observed by using the C-19 antibody, which recognizes the C-terminus of ROCK-I, and by using the H-85 antibody, which binds to an internal region of ROCK-I, as previously demonstrated in cortical astrocytes (Minambres *et al.* 2006). In addition, we observe that RhoE expressing PC12 cells showed a decrease in the phosphorylation of MLCP. Our results also show that RhoE over-expression induced a decrease in the expression levels of RhoA and Cdc42. More importantly, a decrease in the GTP form of RhoA, Rac and Cdc42 was detected in cells transfected with RhoE (Figs 3a and 5a), which reflects the activation state of these three small GTPases. Our results further show that RhoE induced a decrease in the phosphorylated Cofilin, an actin depolymerising factor which is associated with neurite extension.

It is well established that the NGF stimulates the formation of neurites in neuronal cell lines (Negishi and Katoh 2002), and this effect occurs through the modulation of Rho GTPases. However, it has also been demonstrated that there is a spatio-temporal regulation of the induction of Rac/Cdc42 activity by NGF (Aoki *et al.* 2004). Therefore, we decided to study the effect of NGF on the Rho proteins in our experimental conditions. We first assessed that PC12 cells stimulated with NGF for 4 days induced extension of neurites (Fig. 1a) and then observed a decrease in RhoA expression levels (Fig. 2b), in line with the known effect of RhoA on neurite retraction (Govek *et al.* 2005). We also examined the involvement of the Rho signalling pathway by studying the levels of ROCK-I and MLCPp. NGF-stimulated PC12 cells showed a decrease in ROCK-I levels, as measured with both the C-19 and the H-85 antibodies. In addition, there was a decrease in the phosphorylation of MLCP. Our results also show that NGF induced a decrease in Cdc42 expression levels and an increase in the expression of the Rac protein. Finally, PC12 cells treated with NGF showed a dephosphorylation of Cofilin, as expected for a mediator of neurite extension. All these results indicate that RhoE and NGF stimulate similar signalling pathways related to Rho GTPases.

RhoE regulates neurite-like outgrowth via inhibition of the RhoA/ROCK-I signalling pathway

To further investigate whether RhoE-induced neurite outgrowth affects the RhoA signalling, we first measured the activation of RhoA in RhoE transfected PC12 cells by using a pull-down assay. This assay makes use of the rhotekin Rho-binding domain as a glutathione S-transferase (GST) fusion protein which specifically binds to and isolates the GTP-bound RhoA. Expression of RhoE in PC12 cells resulted in a decrease of the amount of GTP-bound RhoA protein (Fig. 3a).

These results suggest that RhoE-induced neurite outgrowth is accompanied by a suppression of RhoA activation. We also asked whether coexpression of wild-type RhoE and constitutively active RhoA would abolish the RhoE-promoted neurite development. Fig. 3b shows that in PC12 cells cotransfected with Flag-RhoE and V14RhoA the number of neurite bearing cells was significantly decreased. To further confirm this effect, the number of neurites per cell and the total length of these processes were also quantified in cells expressing both RhoE and V14RhoA (Fig. 3c). Our results indicate that constitutively active RhoA suppressed most of the neuritic processes induced by RhoE in these cells. We also wondered whether RhoE reduced the levels of GTP-RhoA through p190GAP, as previously demonstrated (Wennerberg *et al.* 2003). Western blot analysis showed no significant differences on the p190GAP levels between control and RhoE transfected cells (Fig. 3d), suggesting that this protein does not appear to be involved in the RhoE-induced inactivation of RhoA. Similarly, no changes were detected on the expression levels of Rho-GDI, when comparing control and RhoE transfected cells.

As Rho-kinase is a major downstream target of RhoA and we had observed that RhoE induced a decrease in the levels of ROCK-I in PC12 cells (Fig. 2a), we decided to

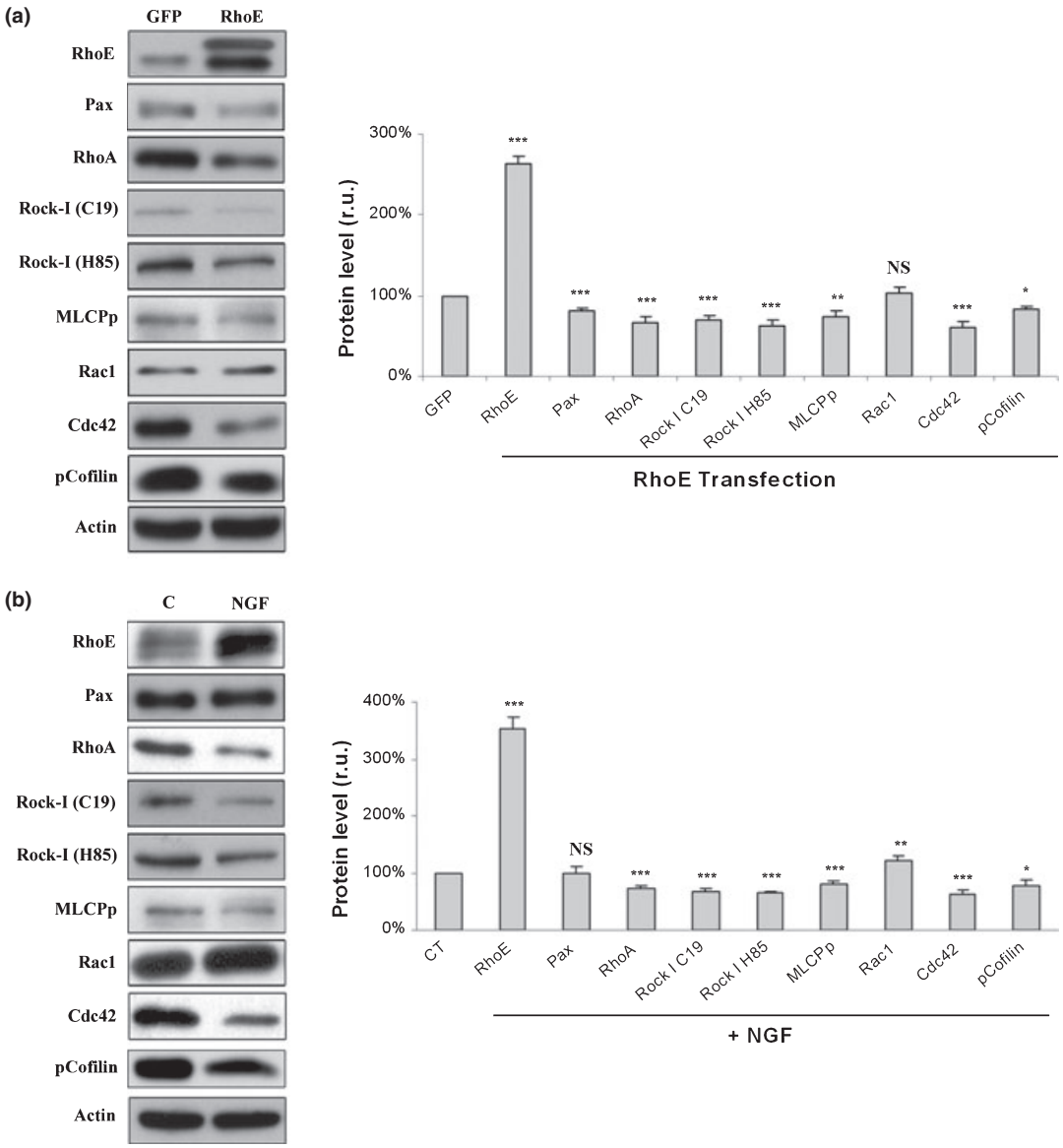

Fig. 2 RhoE and NGF affect the Rho signalling pathway. (a) RhoE over-expression alters the signalling of other Rho GTPases. Neuronal cells expressing RhoE show a decrease in Paxillin, RhoA, ROCK-I, MLCPp, Cdc42 and pCofilin. PC12 cells were transfected with wt-RhoE together with a plasmid expressing GFP for 48 h. A representative western blot from lysates of transfected cells is shown. Quantification of the levels of each protein is also shown. (b) NGF induces alterations in the Rho signalling pathway. PC12 cells were treated with NGF for 4 days and western blotting analysis was performed. NGF induced a decrease in RhoA, ROCK-I, MLCPp, Cdc42 and pCofilin protein levels. Mean ± SEM is shown for a total of 8–10 different experiments. $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$ in a Student's *t*-test.

further investigate whether this kinase was involved in the RhoE-induced neurite-like outgrowth. For this purpose, PC12 cells were transfected with an expression vector encoding a constitutively active C-terminally truncated ROCK-I, ROCKI-Δ1 (Ishizaki *et al.* 1997), together with the Flag-RhoE expressing vector. The expression of this ROCK-I mutant (active ROCK-I, aROCK-I) in PC12 cells was detected by western blot analysis (Fig. 4). Our results show that expression of aROCK-I completely abolished the RhoE-stimulated neurite-like development, as revealed by counting the number of neurites bearing cells (Fig. 4). This effect was also observed when the total length of neurites per cell was analyzed as well as when the number of processes per cell was counted (Fig. 4). In addition, PC12 cells were also transfected with a ROCK-I-truncated mutant, ROCKI-Δ5, that closely corresponds to the kinase domain and is considered inactive (Ishizaki *et al.* 1997; Minambres *et al.* 2006), together with the wild type RhoE

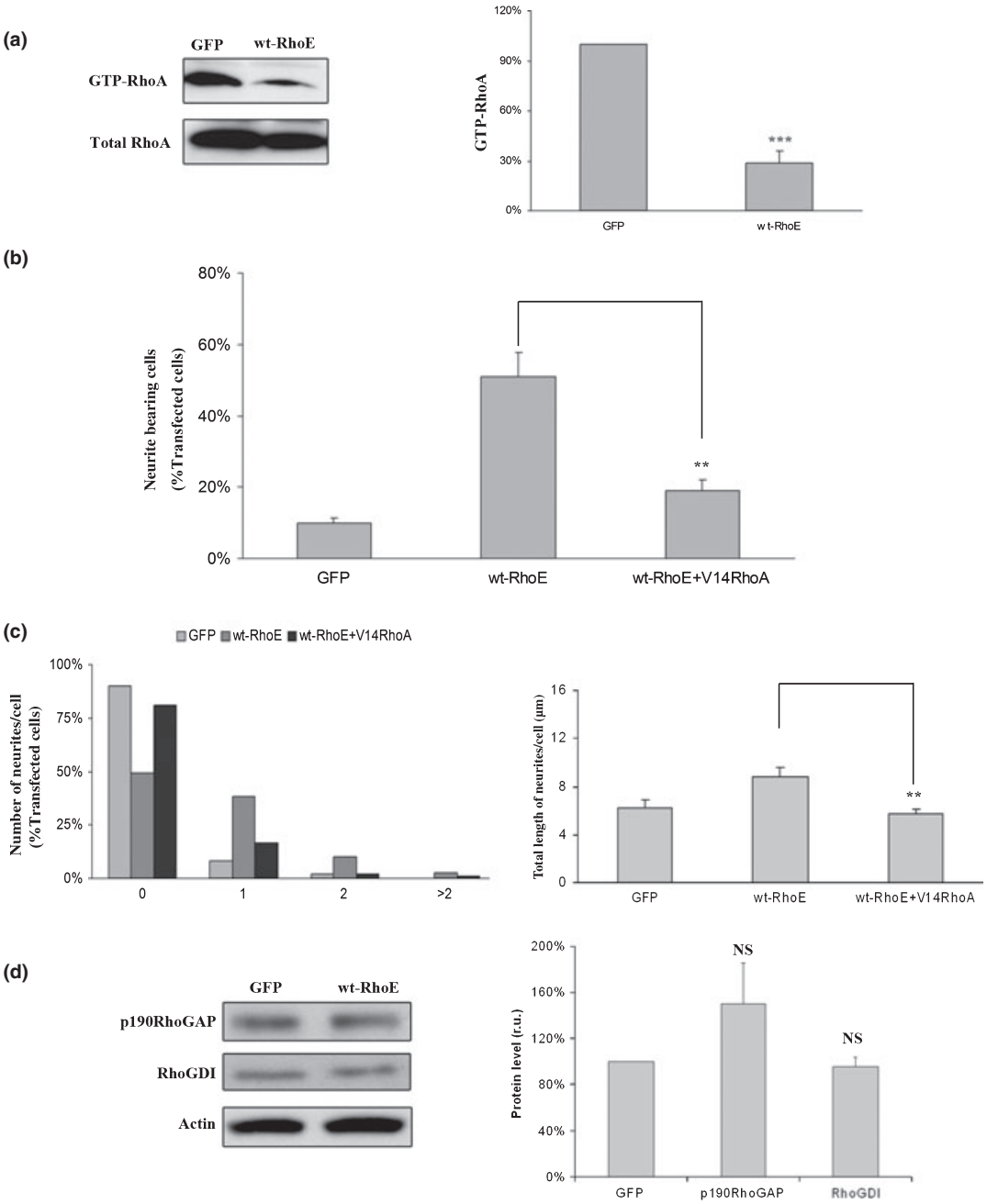

Fig. 3 RhoA is involved in the RhoE-induced neurite-like outgrowth. (a) Over-expression of RhoE in PC12 cells inactivates endogenous RhoA. Cells were transfected with either GFP alone or together with wt-RhoE for 48 h. Activated RhoA was pulled down by TRBD (Rho-binding domain from Rhotekin) on beads and analyzed by western blotting. GTP-RhoA was normalized relative to the total amount of RhoA. Quantification of the results is also shown. (b) Constitutively active RhoA inhibits the formation of neurites induced by RhoE. Cells were transfected with either wt-RhoE alone or together with V14RhoA and neurites were counted, as specified in Material and methods. (c) Constitutively active RhoA decreases the number of neurites per cell and the total length of the processes. (d) Over-expression of RhoE does not affect either p190GAP or RhoGDI expression levels, as detected by western blotting analysis. Mean ± SEM is shown for a total of 4–6 experiments. $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$ in a Student's *t*-test.

protein. The inactive ROCK-I (iROCK-I) did not alter the RhoE-induced neurite-like outgrowth, as observed by the number of cells with dendrites (Fig. 4). The same observation was found when the total length of neurites and the number of neurites per cell were counted. All these results suggest that RhoE induces neurite-like outgrowth through the inhibition of the RhoA/ROCK-I signalling pathway.

Involvement of Rac/Cdc42 in the RhoE-induced neurite-like outgrowth

To examine whether the Rac/Cdc42 activity is required for RhoE to induce neurite-like outgrowth in PC12 cells, we measured the amount of GTP-bound Rac/Cdc42 in RhoE-transfected cells using a pull-down assay. Our results show that transient expression of wild type RhoE in PC12 cells resulted in a decrease in both the endogenous GTP-Rac and GTP-Cdc42 (Fig. 5a). To further study the involvement of these Rho GTPases, we also performed coexpression experiments of wt-RhoE and constitutively active Rac. PC12 cells cotransfected with wt-RhoE + V12Rac1 showed a decrease in the number of cells with neurites and in the number of neurites per cell (Fig. 5b and c), but we did not observe any significant variation in the total length of the processes (Fig. 5c).

We also examined the effect of RhoE on effectors that are specific for each GTPase, N-Wasp (for Cdc42) and Wave (for Rac), which have been implicated in neurite extension and spine formation (Takenawa and Suetsugu 2007). RhoE expressing cells showed a decrease in both the levels of N-Wasp and Wave, as detected by western blotting analysis (Fig. 6). In addition, we studied the effect of RhoE on PI3K, a molecule that has been involved in Rac activation in PC12 cells (Nusser *et al.* 2002). Over-expression of RhoE induced a decrease in the levels of PI3K protein (Fig. 6). All of these data together suggest that RhoE promotes inactivation of Rac and Cdc42 when inducing neurite-like formation in PC12 cells and also suggest that Rac is probably involved in the process of neurite initiation.

Discussion

Rho proteins play essential roles in the remodeling of neuronal protrusions important for guidance and synaptic plasticity (reviewed in (Govek *et al.* 2005). Rnd proteins, which include Rnd1, Rnd2 and Rnd3/RhoE, are atypical Rho members that are considered constitutively active (Chardin 2006). The functions of Rnd proteins have been studied in several cell types and they antagonize the action of RhoA in the actin cytoskeleton organization. It has been shown that in neurons RhoA stimulates neurite retraction whereas Rnd1 is involved in neurite extension (Ishikawa *et al.* 2003) and Rnd2 induces dendrite branching (Fujita *et al.* 2002). However, the effects of RhoE/Rnd3 on neuronal development and its associated molecular mechanism have not yet been investigated. Here, we show that RhoE stimulates neurite-like outgrowth in PC12 cells, in the absence of NGF, through inhibition of the RhoA/ROCK-I signalling pathway. In addition, we have observed that Rac and Cdc42 have a role in this effect.

In the present study, we demonstrate for the first time that RhoE by itself not only induces an increase in the number of neurite bearing cells but also in the number of neurites per cell and in the length of the processes. Our results further support the implication of RhoE in neurite extension because depletion of RhoE with siRNA reduces the formation of neurites induced by NGF in PC12 cells. In agreement with these data, a recent study shows that modulation of RhoE/Rnd3 by some drugs is likely to be associated with changes in dendritic branching and neurite outgrowth (Marie-Claire *et al.* 2007). Other Rnd proteins have also been shown to be involved in different aspects of neuronal development. Rnd1 has been shown to promote spine elongation but it does not increase the number of spines in neurons, suggesting that Rnd1 promotes elongation of pre-existing protrusions but does not initiate new protrusions (Ishikawa *et al.* 2003). Rnd1 also induces processes formation in PC12 cells, but neurofilaments were not detected in these processes (Aoki *et al.* 2000). In this regard, our results demonstrate that RhoE not only increases the neurofilament N68 expression levels, but also induces the number of N200-positive neurites. However, we have noticed that the RhoE-induced neurite-like extensions are not as long as the ones stimulated by NGF. This fact suggests that RhoE is able to stimulate neurite-like outgrowth in the absence of NGF, but other pathways are likely to be required for the final extension of the developing neurites.

It is known that RhoA has a negative role in neurite formation (Govek *et al.* 2005). Because RhoE acts antagonistically to RhoA in several cellular events (Chardin 2006), it was tempting to hypothesize that the RhoA/ROCK signalling pathway was involved in the RhoE-induced neurite-like outgrowth. Indeed, our findings show that over-expression of RhoE in PC12 cells suppressed the endogenous activation of RhoA. This is in line with many studies showing that RhoA inactivation allows for the extension of dendrites, as demonstrated in several model systems including neurons from *Drosophila* (Lee *et al.* 2000), *Xenopus* (Li *et al.* 2000) and mouse (Ahnert-Hilger *et al.* 2004). Our results also show that expression of a constitutively active mutant of RhoA decreased the number of cells with neurites induced by RhoE. Moreover, the active version of RhoA induced a reduction in both the length of the extensions and in the number of neurites induced by RhoE. These findings suggest that RhoA inactivation is involved in the RhoE-induced neurite-like outgrowth. Interestingly, it has recently been proposed that the mechanism of RhoE over-expression forms part of a feedback loop to regulate RhoA signalling (Komander *et al.* 2008). Indeed, we have recently shown that over-expression of RhoE in astroglial cells induced a decrease in RhoA levels (Guasch *et al.* 2007). We have also found that RhoE over-expression does not affect the p190GAP levels, suggesting that p190GAP does not seem to be implicated in the reduction of GTP-RhoA levels induced by RhoE. Therefore, other RhoE effectors may be responsible for this RhoA inactivation.

To further support the abovementioned hypothesis, we investigated the effect of RhoE on ROCK-I, a major target of

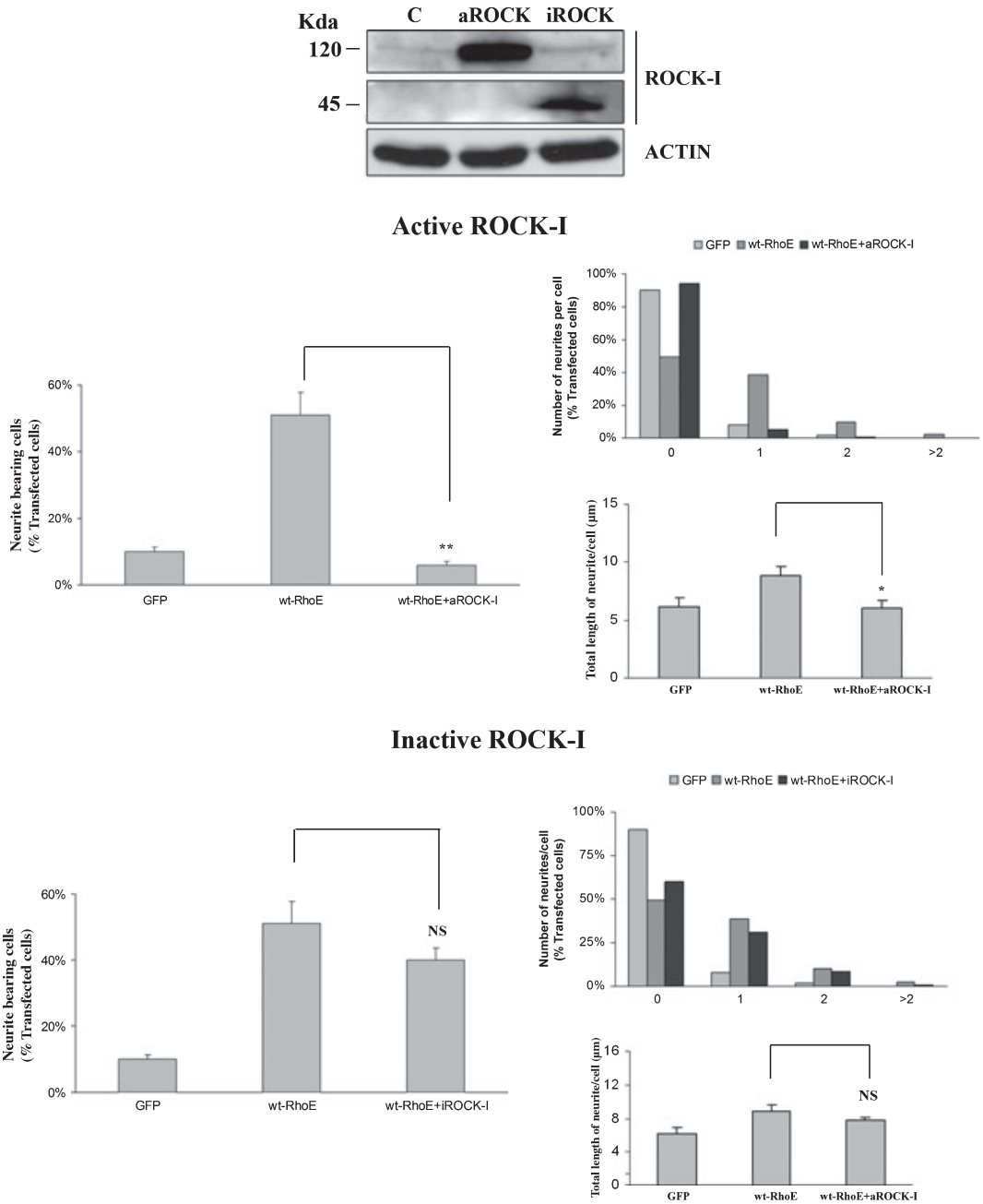

Fig. 4 Effect of ROCK-I expression on RhoE-induced neurite-like outgrowth. PC12 cells were transfected with wt-RhoE alone or together with either active ROCK-I (aROCK-I) or inactive ROCK-I (iROCK-I) for 48 h. The expression of these two mutants was detected by western blotting analysis. Active ROCK-I prevents the formation of neurites induced by RhoE, whereas inactive ROCK-I does not block the RhoE-induced processes formation. Quantification of neurites was performed as indicated in Material and methods. Total length of neurites and number of processes per cell are also shown. Mean ± SEM is shown for a total of 4–6 different experiments. $^{*}p < 0.05$ and $^{**}p < 0.001$ in a Student's *t*-test.

RhoA which has been demonstrated to be involved in Rho-mediated neurite retraction (Amano *et al.* 1998; Hirose *et al.* 1998). Our results show a reduction in the expression levels of ROCK-I when RhoE was over-expressed in PC12 cells. In addition, expression of active ROCK-I inhibited the RhoE-induced neurite outgrowth whereas the inactive ROCK-I did not affect the extension of processes stimulated by RhoE. These results indicate that ROCK-I inhibition is required for RhoE-induced neurite outgrowth. In agreement with these findings, it has been shown that constitutively active Rho-kinase promoted the retraction of neurites induced by several factors (Katoh *et al.* 1998; Da Silva *et al.* 2003). Our results

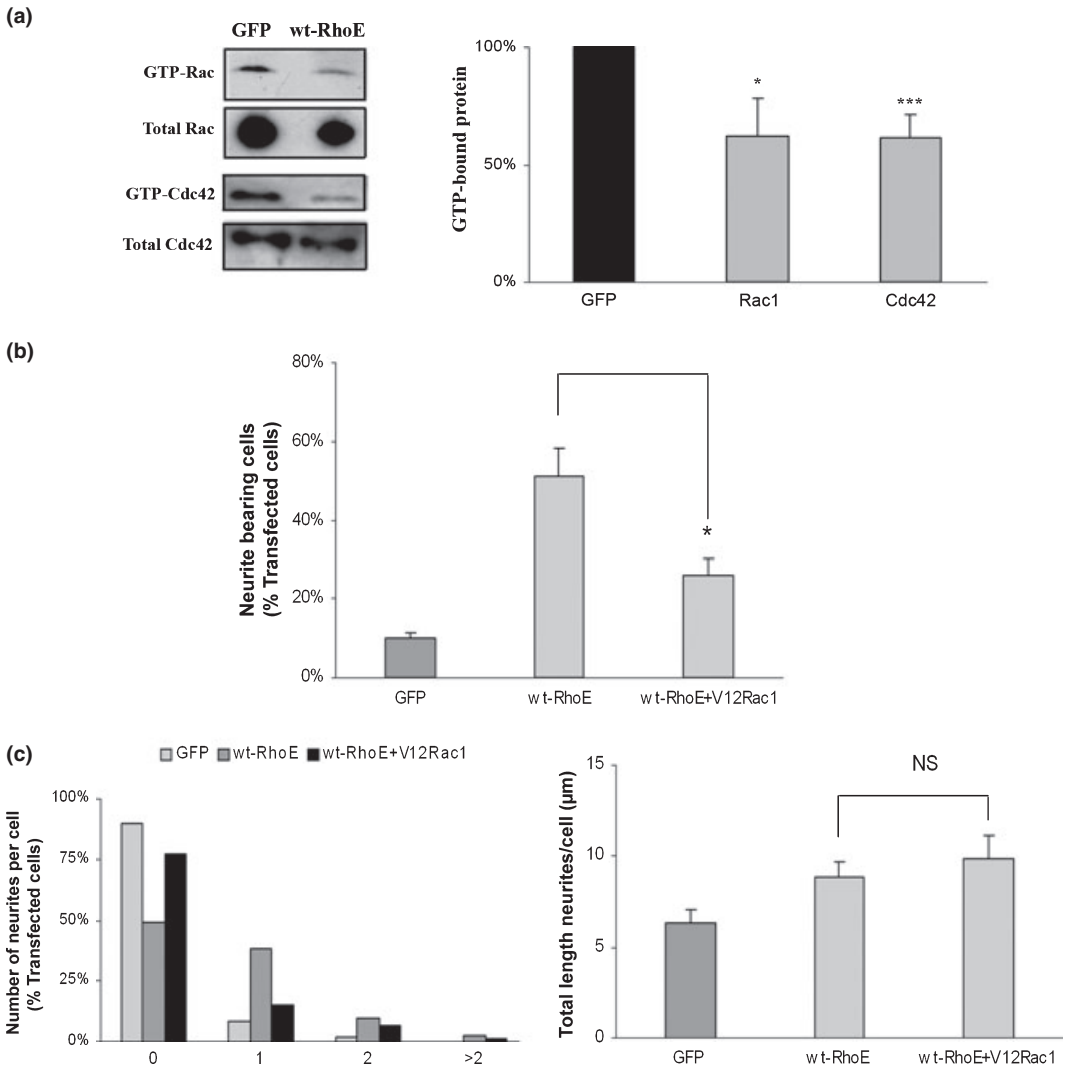

Fig. 5 Effect of RhoE on Rac and Cdc42 GTPases. (a) RhoE reduces the activation of both Rac and Cdc42. PC12 cells were transfected with GFP alone or together with wt-RhoE for 48 h. Activated Rac and Cdc42 were normalized relative to their total amount of these GTPases. A representative western blot of each pull-down as well as quantification of the results are shown. (b) Constitutively active Rac1 inhibits the formation of neurites induced by RhoE. Cells were transfected with either wt-RhoE alone or together with V12Rac1 and neurites were counted, as specified in Material and methods. (c) Constitutively active Rac decreases the number of neurites per cell but it does not affect the total length of the processes. Mean ± SEM is shown for a total of 4–6 different experiments. $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$ in a Student's *t*-test.

are also in line with other studies where Rho-kinase inhibition rescued the stimulation of neurite elongation in hippocampal neurons (Da Silva *et al.* 2003). Interestingly, recent studies point to an inhibition of ROCK as a strategy to induce neurite outgrowth and stem cell neuronal differentiation (Dottori *et al.* 2008; Pacary *et al.* 2008).

Our results show that in the absence of NGF RhoE decreased the activation of both Rac and Cdc42, suggesting that inactivation of these GTPases may be involved in the RhoE-induced neurite outgrowth. We have also observed that over-expression of V12Rac + RhoE reduced the neurite outgrowth induced by RhoE alone. This coexpression not only reduced the number of cells with neurites but also decreased the number of neurites per cell. In addition, we have demonstrated that RhoE over-expression induced a decrease of PI3K, a molecule that activates Rac in PC12 cells (Nusser *et al.* 2002). Taken together, in the absence of NGF Rac activation does not seem to be involved in the RhoE-induced neurite outgrowth. Accordingly, our results show that the expression of active Rac + RhoE did not cause an additive effect on the length of the neurites when compared to RhoE alone.

We also observe a RhoE-induced reduction of two effectors, N-Wasp and Wave, which have been previously

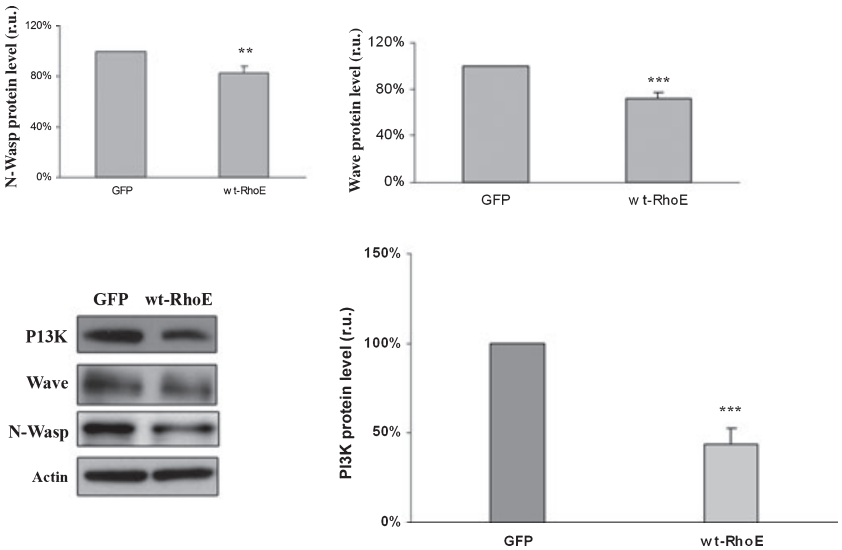

Fig. 6 RhoE affects Rac and Cdc42 signalling. RhoE decreases the levels of N-Wasp, Wave and PI3K. Cells were transfected for 48 h with RhoE and the levels of these proteins were detected by western blotting analysis. Mean ± SEM is shown for a total of 4–6 different experiments. $*p < 0.05$, $**p < 0.01$ and $***p < 0.001$ in a Student's *t*-test.

involved in neurite extension and spine formation (Takenawa and Suetsugu 2007). In agreement with these findings, we have recently demonstrated that RhoE reduces Rac expression in primary astrocytes (Guasch *et al.* 2007) and several studies have shown a role of Rac as a negative regulator of neurite extension (Allen *et al.* 2000; Fournier *et al.* 2003). Our results also agree with previous studies where active mutants of Cdc42 do not produce neurites (Daniels *et al.* 1998; Katoh *et al.* 2000) and decrease dendrite number and length (Ruchhoeft *et al.* 1999), and they are also consistent with studies showing that a Cdc42 knock-down does not affect the number and length of minor processes (Schwamborn and Puschel 2004). Differences in species, cell types and age of cells or organisms used may account for the different roles of Rac and Cdc42 in neurite extension (Govek *et al.* 2005). RhoE may also inactivate these two GTPases through either downstream effectors of RhoE or by direct binding to Rac/Cdc42 GAPs, as it has been demonstrated for p190 RhoGAP (Wennerberg *et al.* 2003).

Finally, our results further support the notion that RhoE is involved in the NGF-induced neurite outgrowth in PC12 cells. Indeed, knock-down of RhoE decreases the neurite extension induced by NGF in these cells. In addition, we show that NGF increases the levels of RhoE and also stimulates a similar pattern of the Rho signalling pathway to that obtained by RhoE over-expression. However, over-expression of RhoE does not change the TrkA levels (data not shown), suggesting that this kinase is not downstream of the RhoE signalling. Our findings also demonstrate that RhoE reduces the levels of phsphorylated Cofilin similarly to what has been demonstrated in NGF-stimulated PC12 cells (Meberg *et al.* 1998). However, given the tight regulation of the pathways implicated in neurite initiation and outgrowth, other signals are likely to be involved in this process.

How does RhoE induce neurite-like outgrowth? It has been proposed that the effect of RhoA and Rho-kinase on neurite retraction is probably caused by an increase in the actomyosin contractility (Govek *et al.* 2005). Activation of Rho-kinase contributes to this contractility by direct phosphorylation of MLC (Amano *et al.* 1996) and also by inhibition of MLCP through its phosphorylation (Kimura *et al.* 1996). Our findings show that ROCK-I is implicated in the RhoE-induced neurite formation and also that RhoE induces a decrease in the phosphorylation levels of MLCP, which would contribute to a decrease in the MLC phosphorylation. Therefore, a decrease in the cortical actomyosin force would facilitate the neurite-like extension induced by RhoE. In this regard, it has previously been shown that NGF and C3 treatment of PC12 cells decreases MLC phosphorylation (Jalink *et al.* 1994). Rho-kinase is also necessary for lysophosphatidic acid (LPA)-induced MLC phosphorylation in neuronal cells (Hirose *et al.* 1998). In addition, previous studies have shown that RhoE directly binds to and inhibits ROCK-I, preventing it from phosphorylating MLCP and thus leading to reduced actomyosin contractility (Riento *et al.* 2003). This signalling pathway may also contribute to the extension of the processes induced by RhoE in PC12 cells. In conclusion, we demonstrate that RhoE by itself induces neurite-like outgrowth in PC12 cells through inhibition of the RhoA/ROCK signalling pathway. The modulation of the RhoE signalling pathway may have a significant impact in injury within the nervous system and may provide a novel candidate for neurodegeneration therapy.

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References

Ahnert-Hilger G., Holtje M., Grosse G., Pickert G., Mucke C., Nixdorf-Bergweiler B., Boquet P., Hofmann F. and Just I. (2004) Differential effects of Rho GTPases on axonal and dendritic development in hippocampal neurones. *J. Neurochem.* **90**, 9–18.

Allen M. J., Shan X. and Murphey R. K. (2000) A role for Drosophila Drac1 in neurite outgrowth and synaptogenesis in the giant fiber system. *Mol. Cell. Neurosci.* **16**, 754–765.

Amano M., Ito M., Kimura K., Fukata Y., Chihara K., Nakano T., Matsuura Y. and Kaibuchi K. (1996) Phosphorylation and activation of myosin by Rho-associated kinase (Rho-kinase). *J. Biol. Chem.* **271**, 20246–20249.

Amano M., Chihara K., Nakamura N., Fukata Y., Yano T., Shibata M., Ikebe M. and Kaibuchi K. (1998) Myosin II activation promotes neurite retraction during the action of Rho and Rho-kinase. *Genes Cells* **3**, 177–188.

Aoki J., Katoh H., Mori K. and Negishi M. (2000) Rnd1, a novel rho family GTPase, induces the formation of neuritic processes in PC12 cells. *Biochem. Biophys. Res. Commun.* **278**, 604–608.

Aoki K., Nakamura T. and Matsuda M. (2004) Spatio-temporal regulation of Rac1 and Cdc42 activity during nerve growth factor-induced neurite outgrowth in PC12 cells. *J. Biol. Chem.* **279**, 713–719.

Chardin P. (2006) Function and regulation of Rnd proteins. *Nat. Rev. Mol. Cell Biol.* **7**, 54–62.

Da Silva J. S., Medina M., Zuliani C., Di Nardo A., Witke W. and Dotti C. G. (2003) RhoA/ROCK regulation of neuritogenesis via profilin IIa-mediated control of actin stability. *J. Cell Biol.* **162**, 1267–1279.

Daniels R. H., Hall P. S. and Bokoch G. M. (1998) Membrane targeting of p21-activated kinase 1 (PAK1) induces neurite outgrowth from PC12 cells. *EMBO J.* **17**, 754–764.

Dottori M., Leung J., Turnley A. M. and Pebay A. (2008) Lysophosphatidic acid inhibits neuronal differentiation of neural stem/progenitor cells derived from human embryonic stem cells. *Stem Cells* **26**, 1146–1154.

Fournier A. E., Takizawa B. T. and Strittmatter S. M. (2003) Rho kinase inhibition enhances axonal regeneration in the injured CNS. *J. Neurosci.* **23**, 1416–1423.

Fujita H., Katoh H., Ishikawa Y., Mori K. and Negishi M. (2002) Rapostlin is a novel effector of Rnd2 GTPase inducing neurite branching. *J. Biol. Chem.* **277**, 45428–45434.

Govek E. E., Newey S. E. and Van Aelst L. (2005) The role of the Rho GTPases in neuronal development. *Genes Dev.* **19**, 1–49.

Greene L. A. and Tischler A. S. (1976) Establishment of a noradrenergic clonal line of rat adrenal pheochromocytoma cells which respond to nerve growth factor. *Proc. Natl Acad. Sci. USA* **73**, 2424–2428.

Guasch R. M., Scambler P., Jones G. E. and Ridley A. J. (1998) RhoE regulates actin cytoskeleton organization and cell migration. *Mol. Cell. Biol.* **18**, 4761–4771.

Guasch R. M., Tomas M., Minambres R., Valles S., Renau-Piqueras J. and Guerri C. (2003) RhoA and lysophosphatidic acid are involved in the actin cytoskeleton reorganization of astrocytes exposed to ethanol. *J. Neurosci. Res.* **72**, 487–502.

Guasch R. M., Blanco A. M., Perez-Arago A., Minambres R., Talens-Visconti R., Peris B. and Guerri C. (2007) RhoE participates in the stimulation of the inflammatory response induced by ethanol in astrocytes. *Exp. Cell Res.* **313**, 3779–3788.

Hansen S. H., Zegers M. M., Woodrow M., Rodriguez-Viciana P., Chardin P., Mostov K. E. and McMahon M. (2000) Induced expression of Rnd3 is associated with transformation of polarized epithelial cells by the Raf-MEK-extracellular signal-regulated kinase pathway. *Mol. Cell. Biol.* **20**, 9364–9375.

Heasman S. J. and Ridley A. J. (2008) Mammalian Rho GTPases: new insights into their functions from *in vivo* studies. *Nat. Rev. Mol. Cell Biol.* **9**, 690–701.

Hirose M., Ishizaki T., Watanabe N., Uehata M., Kranenburg O., Moolenaar W. H., Matsumura F., Maekawa M., Bito H. and Narumiya S. (1998) Molecular dissection of the Rho-associated protein kinase (p160ROCK)-regulated neurite remodeling in neuroblastoma N1E-115 cells. *J. Cell Biol.* **141**, 1625–1636.

Ishikawa Y., Katoh H. and Negishi M. (2003) A role of Rnd1 GTPase in dendritic spine formation in hippocampal neurons. *J. Neurosci.* **23**, 11065–11072.

Ishizaki T., Naito M., Fujisawa K., Maekawa M., Watanabe N., Saito Y. and Narumiya S. (1997) p160ROCK, a Rho-associated coiled-coil forming protein kinase, works downstream of Rho and induces focal adhesions. *FEBS Lett.* **404**, 118–124.

Jalink K., van Corven E. J., Hengeveld T., Morii N., Narumiya S. and Moolenaar W. H. (1994) Inhibition of lysophosphatidate- and thrombin-induced neurite retraction and neuronal cell rounding by ADP ribosylation of the small GTP-binding protein Rho. *J. Cell Biol.* **126**, 801–810.

Katoh H., Aoki J., Ichikawa A. and Negishi M. (1998) p160 RhoA-binding kinase ROKalpha induces neurite retraction. *J. Biol. Chem.* **273**, 2489–2492.

Katoh H., Yasui H., Yamaguchi Y., Aoki J., Fujita H., Mori K. and Negishi M. (2000) Small GTPase RhoG is a key regulator for neurite outgrowth in PC12 cells. *Mol. Cell. Biol.* **20**, 7378–7387.

Kimura K., Ito M., Amano M. *et al.* (1996) Regulation of myosin phosphatase by Rho and Rho-associated kinase (Rho-kinase). *Science* **273**, 245–248.

Komander D., Garg R., Wan P. T., Ridley A. J. and Barford D. (2008) Mechanism of multi-site phosphorylation from a ROCK-I:RhoE complex structure. *EMBO J.* **27**, 3175–3185.

Lee T., Winter C., Marticke S. S., Lee A. and Luo L. (2000) Essential roles of Drosophila RhoA in the regulation of neuroblast proliferation and dendritic but not axonal morphogenesis. *Neuron* **25**, 307–316.

Li Z., Van Aelst L. and Cline H. T. (2000) Rho GTPases regulate distinct aspects of dendritic arbor growth in Xenopus central neurons *in vivo*. *Nat. Neurosci.* **3**, 217–225.

Marie-Claire C., Salzmann J., David A., Courtin C., Canestrelli C. and Noble F. (2007) Rnd family genes are differentially regulated by 3,4-methylenedioxymethamphetamine and cocaine acute treatment in mice brain. *Brain Res.* **1134**, 12–17.

Meberg P. J., Ono S., Minamide L. S., Takahashi M. and Bamburg J. R. (1998) Actin depolymerizing factor and cofilin phosphorylation dynamics: response to signals that regulate neurite extension. *Cell Motil. Cytoskeleton* **39**, 172–190.

Minambres R., Guasch R. M., Perez-Arago A. and Guerri C. (2006) The RhoA/ROCK-I/MLC pathway is involved in the ethanol-induced apoptosis by anoikis in astrocytes. *J. Cell Sci.* **119**, 271–282.

Negishi M. and Katoh H. (2002) Rho family GTPases as key regulators for neuronal network formation. *J. Biochem.* **132**, 157–166.

Nobes C. D., Lauritzen I., Mattei M. G., Paris S., Hall A. and Chardin P. (1998) A new member of the Rho family, Rnd1, promotes disas-

sembly of actin filament structures and loss of cell adhesion. *J. Cell Biol.* **141**, 187–197.

Nusser N., Gosmanova E., Zheng Y. and Tigyi G. (2002) Nerve growth factor signals through TrkA, phosphatidylinositol 3-kinase, and Rac1 to inactivate RhoA during the initiation of neuronal differentiation of PC12 cells. *J. Biol. Chem.* **277**, 35840–35846.

Ongusaha P. P., Kim H. G., Boswell S. A., Ridley A. J., Der C. J., Dotto G. P., Kim Y. B., Aaronson S. A. and Lee S. W. (2006) RhoE is a pro-survival p53 target gene that inhibits ROCK I-mediated apoptosis in response to genotoxic stress. *Curr. Biol.* **16**, 2466–2472.

Pacary E., Petit E. and Bernaudin M. (2008) Concomitant inhibition of prolyl hydroxylases and ROCK initiates differentiation of mesenchymal stem cells and PC12 towards the neuronal lineage. *Biochem. Biophys. Res. Commun.* **377**, 400–406.

Riento K., Guasch R. M., Garg R., Jin B. and Ridley A. J. (2003) RhoE binds to ROCK I and inhibits downstream signaling. *Mol. Cell. Biol.* **23**, 4219–4229.

Riento K., Villalonga P., Garg R. and Ridley A. (2005a) Function and regulation of RhoE. *Biochem. Soc. Trans.* **33**, 649–651.

Riento K., Totty N., Villalonga P., Garg R., Guasch R. and Ridley A. J. (2005b) RhoE function is regulated by ROCK I-mediated phosphorylation. *EMBO J.* **24**, 1170–1180.

Ruchhoeft M. L., Ohnuma S., McNeill L., Holt C. E. and Harris W. A. (1999) The neuronal architecture of Xenopus retinal ganglion cells is sculpted by rho-family GTPases *in vivo*. *J. Neurosci.* **19**, 8454–8463.

Schwamborn J. C. and Puschel A. W. (2004) The sequential activity of the GTPases Rap1B and Cdc42 determines neuronal polarity. *Nat. Neurosci.* **7**, 923–929.

Takenawa T. and Suetsugu S. (2007) The WASP–WAVE protein network: connecting the membrane to the cytoskeleton. *Nat. Rev. Mol. Cell Biol.* **8**, 37–48.

Wennerberg K., Forget M. A., Ellerbroek S. M., Arthur W. T., Burridge K., Settleman J., Der C. J. and Hansen S. H. (2003) Rnd proteins function as RhoA antagonists by activating p190 RhoGAP. *Curr. Biol.* **13**, 1106–1115.

