Distict roles of Rac1/Cdc42 and Rho/Rock for axon outgrowth and nucleokinesis of precerebellar neurons toward netrin 1

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Summary
During embryonic development, tangentially migrating precerebellar neurons emit a leading process and then translocate their nuclei inside it (nucleokinesis). Netrin 1 (also known as netrin-1) acts as a chemoattractant factor for neurophilic migration of precerebellar neurons (PCN) both in vivo and in vitro. In the present work, we analyzed Rho GTPases that could direct axon outgrowth and/or nuclear migration. We show that the expression pattern of Rho GTPases in developing PCN is consistent with their involvement in the migration of PCN from the rhombic lips. We report that pharmacological inhibition of Rho enhances axon outgrowth of PCN and prevents nuclei migration toward a netrin 1 source, whereas inhibition of Rac and Cdc42 sub-families impair neurite outgrowth of PCN without affecting migration. We show, through pharmacological inhibition, that Rho signaling directs neurophilic migration through Rock activation. Altogether, our results indicate that Rho/Rock acts on signaling pathways favoring nuclear translocation during tangential migration of PCN. Thus, axon extension and nuclear migration of PCN in response to netrin 1 are not strictly dependent processes because: (1) distinct small GTPases are involved; (2) axon extension can occur when migration is blocked; and (3) migration can occur when axon outgrowth is impaired.

Movies available online

Key words: Nuclear translocation, Hindbrain, Chemotropic molecules, Rho GTPases, Mice, Collagen assays, In situ hybridization, GST-RBD-Rhotekin

Introduction
During the development of the central nervous system, young postmitotic neurons migrate over long distances from the ventricular zone to the position they occupy in the mature brain (Rakic, 1990). Precerebellar neurons (PCN) follow neurophilic tangential migrations from various regions of the alar ventricular zone of the rhombencephalon to their final destinations. The neurons that will form the external cuneatus nucleus, the lateral reticular nucleus and the inferior olivary nucleus originate from the germinative neuroepithelium (rhombic lip) in the caudal hindbrain (Altman and Bayer, 1987). Although there is some spatial and temporal overlap, neurons that will form distinct nuclei of PCN have distinct temporal patterns of birthdates (Altman and Bayer, 1980; Altman and Bayer, 1987; Bourrat and Sotelo, 1988; Bourrat and Sotelo, 1991). Their migratory route also differs because the migration of inferior olivary neurons (ION) occurs through the submarginal stream (Altman and Bayer, 1980; Altman and Bayer, 1987; Bourrat and Sotelo, 1988; Bourrat and Sotelo, 1991), whereas neurons that will form the lateral reticular nucleus and the external cuneatus nucleus migrate through the marginal stream. During their tangential migration, all PCN first emit a leading process that leads the way, imposing the circumferential migratory route until reaching the floor plate (Bourrat and Sotelo, 1988), and the nuclei translocate inside the leading process. However, although all the leading processes cross the ventral midline, the behavior of the soma of PCN differs at the floor plate. The cell bodies of external cuneatus neurons (ECN) and of lateral reticular neurons (LRN) cross the midline and continue their translocation until reaching their presumptive territories in the contralateral rhombencephalon. By contrast, cell bodies of ION stop before crossing the floor plate and aggregate to form club-shaped masses (Altman and Bayer, 1987; Bourrat and Sotelo, 1990). Thus, the ION axon and cell body do not respond identically to signals from the intermediate target, the floor plate. During their migratory process, PCN respond to various guidance molecules, including chemotropic factors. In previous studies, we showed that netrin 1 was involved both in vivo and in vitro in the migration of ION (Bloch-Gallego et al., 1999; Causeret et al., 2002), and, more generally, netrin 1 was also shown to direct the tangential migration of other PCN, such as pontine neurons and LRN (Yee et al., 1999; Alcantara et al., 2000). The netrin
1 receptor deleted in colorectal cancer (Dcc) is implicated in PCN responses to netrin 1 gradient (Yee et al., 1999) (E.B.-G., unpublished).

Intracellular signaling cascades involved in axon guidance and neuronal migration remain poorly characterized. In vivo, RhoB has been involved in the migration of neural crest cells (Liu and Jessell, 1998; del Barrio and Nieto, 2002), whereas Rac and Cdc42 GTPases have been previously implicated in various axon growth and guidance processes in the nematode (Lundquist et al., 2001) and Drosophila (Hakeda-Suzuki et al., 2002; Kim et al., 2002; Kim et al., 2003). The function of Rho GTPases has been most extensively studied in fibroblasts in vitro, showing that Cdc42, Rac1 and RhoA are key modulators of the cytoskeletal dynamics that occur after a cell adhesion event, and/or during cell migration (Clark et al., 1998; Hall, 1998; Nobes and Hall, 1999; Kaibuchi et al., 1999). Remodeling of the cytoskeleton in response to netrin 1 has been studied in, among others, Swiss 3T3 fibroblasts. When expressing exogenous Dcc, addition of netrin 1 triggers actin reorganization and a lasting activation of Cdc42 and Rac1 in these cells (Li et al., 2002a).

We have attempted to characterize small Rho GTPases that might participate in axon guidance and nuclei migration of developing PCN. We have combined in vivo analysis of Rho proteins with PCN, using LRN/ION explants. This model allows the analysis of the initiation of neurophilic migration, a process that can be divided into two distinct steps: axon outgrowth and nuclear translocation (i.e. cell body migration). We report that RhoB is strongly expressed in the marginal and submarginal migratory streams in the dorsal hindbrain, whereas Rhoa, Rac1 and Cdc42 all show a very similar expression pattern, with a high expression in the ventricular and subventricular zones. Pharmacological blockades in vitro, showing that Cdc42, Rac1 and RhoA are key modulators of the cytoskeletal dynamics that occur after a cell adhesion event, and/or during cell migration (Clark et al., 1998; Hall, 1998; Nobes and Hall, 1999; Kaibuchi et al., 1999). Remodeling of the cytoskeleton in response to netrin 1 has been studied in, among others, Swiss 3T3 fibroblasts. When expressing exogenous Dcc, addition of netrin 1 triggers actin reorganization and a lasting activation of Cdc42 and Rac1 in these cells (Li et al., 2002a).

In Materials and methods Embryo processing For detailed methods, see Bloch-Gallego et al. and Causseret et al. (Bloch-Gallego et al., 1999; Causseret et al., 2002). Briefly, mouse embryos (Janvier, Le Genest St Isle, France) were fixed in 4% paraformaldehyde in phosphate buffer (PB), cryoprotected overnight with 10% sucrose solution and embedded in 7.5% gelatin/10% sucrose. They were then frozen and serially sectioned in the frontal plane using a cryostat.

In situ hybridization and RNA probes In situ hybridization was carried out on cryosections according to Myers et al. (Myat et al., 1996). Murine Cdc42, Rac1, Rhoa, Rbho, Rock1 and Rock2 IMAGE cDNA clones were obtained from HGMP (Cambridge, UK). Brn3b and Tag1 cDNA clones have been described previously (Bloch-Gallego et al., 1999; Backer et al., 2002). No signal was obtained when using the sense probes.

Immunohistochemistry and antibodies Immunohistochemistry on cryosections was performed in phosphate buffered saline (PBS) containing 2 g/l gelatin, 0.05% Triton X-100 and 0.2 mg/ml sodium azide, and immunohistochemistry on collagen assays was performed in PBS, 1% normal goat serum (NGS), 0.1% Triton X-100 and 0.2 mg/ml sodium azide. The following primary antibodies were used in collagen assays: rabbit polyclonal anti-GFP (1:400; Molecular Probes, Eugene, OR) and mouse monoclonal anti-class III β-tubulin (TuJ1; 1:2000; Jackson ImmunoResearch, West Grove, PA). These primary antibodies were revealed using secondary antibodies raised from goat, directed against mouse or rabbit IgG and conjugated to Alexa 488 (1:400; Molecular Probes) or Cy3 (1:200; Jackson ImmunoResearch).

In situ detection of active Rho GTPases GST (glutathione S-transferase)-RBD (Rho binding domain)-Rhotekin was provided by M. A. Schwartz (State University of New York) and prepared as described in Li et al. (Li et al., 2002b). After fixation and cryosectioning of embryos, active Rhoa/B/C activity was detected through the binding of their GST-RBD-Rhotekin effector, as described by Li et al. (Li et al., 2002b) with some modifications. Cryosections were saturated in 0.3% Triton X-100 and 5% NGS for 10 hours, and GST-proteins, which had been previously detached from the beads with 0.05 M Tris (pH 8) and 3 mg/ml Glutation, were incubated overnight at 4°C. After post-fixation with 2% paraformaldehyde for 10 minutes and several washes in PBS/0.3% Triton X-100, sections were saturated with 0.1 M lysine and incubated with a polyclonal anti-GST antibody (Oncogene). As a control of the binding of GST-coupled proteins, we used a non-relevant protein corresponding to the extracellular domain of an adhesion molecule that is upregulated during differentiation and fusion of muscle cells, GST-M-Cadherin. The EcoRV fragment of M-cadherin corresponding to nucleotides 750-1765 was cloned in the pGEX5X vector. The GST-M-cadherin fragment was produced as described in Li et al. (Li et al., 2002b) at least three animals were examined for each condition tested.

To evaluate RhoA activity on explants of brainstem, E11 explants of rhombic lips were incubated for 2 hours in presence of netrin 1, then washed and lysed in 50 mM Tris (pH 7.2), 1% Triton X-100, 500 mM NaCl, 10 mM MgCl2, 1 mM PMSF and protease inhibitors cocktail (Sigma-Aldrich, St Louis, MO). Cleared lysate was incubated with 25 μg of GST-fusion protein, containing the RhoA-binding domain of Rhotekin (GST-RBD-Rhotekin), attached to beads (Sigma) for 40 minutes at 4°C. The beads were then washed four times in 50 mM Tris (pH 7.2), 1% Triton X-100, 150 mM NaCl, 10 mM MgCl2 and 0.1 mM PMSF and protease inhibitors cocktail before the addition of Laemmli buffer. Fractions were analyzed by western blotting with RhoA antibody (1:400; Santa Cruz Biotechnology, Santa Cruz, CA). Owing to poor antibody specificity in western blots, RhoB presence could not be tested.

Western blots Lysates were prepared from E11 or E12 rhombic lip, and E13 ventromedial parts excluding floor plate, and used as samples. Freshly dissected tissues were dissociated in lysis buffer [50 mM Tris (pH
Collagen assays
Collagen assays were performed as previously described (Causseret et al., 2002), using rhomic lip explants from E11 or E12 mouse embryos facing cN etrin 1 -secreting cells (Kennedy et al., 1994). After 60-72 hours in a 5% CO₂, 37°C, 95% humidity incubator, collagen assays were fixed in 4% paraformaldehyde and immunolabeled with TuJ1 antibody for visualization of neuronal processes. Cell nuclei in explants and migrating cells were visualized with DAPI (1 μg/ml, Vector). F-actin staining was performed with rhodamine-conjugated phalloidin (1:200, Molecular Probes) (Gallo and Letourneau, 1998).

Drug application
Several drugs were used to inhibit specific pathways involving precise Rho GTPases. Lethal toxin from Clostridium sordelli strain VP9048 [LT-9048 (Humeau et al., 2002)] was used at a final concentration of 1 ng/ml to inhibit Rac1 and Cdc42. TAT-C3 [a kind gift from Jacques Bertoglio (Sauzeau et al., 2001)] was used at a final concentration of 1 μg/ml in 25 μM to inhibit Rock1 and Rock2 effectors. These drugs were diluted in the collagen culture medium and applied from the first day in culture. We checked, by western blot, that none of them affected netrin 1 secretion by quantifying netrin 1 protein in the supernatant of cells cultured in presence of working concentrations of drugs.

In vitro ADP-ribosylation
Control rhomic lips treated in vivo with TAT-C3 were washed with PBS, and then lysed with ADP-ribosylation buffer [50 mM triethanolamine/HCl (pH 7.5), 100 mM KCl, 5 mM MgCl₂, 10 mM dithiothreitol, 1 mM EDTA, 10 mM GDP and 10 mM thymidine] containing leupeptin (1 μg/ml), pepstatin (1 μg/ml), 1 mM PMSF and 0.5% Triton X-100 (Marvaud et al., 2002). The extracts were centrifuged (1000 g, 5 minutes) and the supernatant (50 μg of total protein) was ADP-ribosylated in vitro in a final volume of 20 μl ADP-ribosylation buffer containing 2.5 mM of [³²P]NAD (NEN-Du Pont de Nemours, Boston, MA; 20,000 dpm/ml) and 10⁻⁷ M TAT-C3. After incubation at 37°C for 30 minutes, samples subjected to SDS-PAGE, and the radioactive Rho bands were visualized and quantified by PhosphorImager (Amersham) and the IQMac program.

Scoring of cell migration and quantification of leading process outgrowth
For quantification of cell migration and axon outgrowth in collagen assays, pictures of each explant were taken using a Leica fluorescent microscope and a Photometrics coolsnap fx monochrome CCD camera (Roper Scientific, Duluth, GA). The blue channel allowed visualization of cell nuclei staining with DAPI, the red channel allowed visualization of TuJ1 staining with a Cy3-conjugated secondary antibody and the green channel allowed visualization of anti-GFP staining with an Alexa 488-conjugated secondary antibody. Thereafter, the surface covered by TuJ1 immunostaining outside of the explant was measured using the Metamorph Area Analysis program (Universal Imaging Corporation, Downingtown, PA). We only considered proximal and distal regions of the explan (as defined in Fig. 2A). For quantification of nuclear migration we measured the surface covered by DAPI labeling outside the explant toward the netrin 1 source. We also evaluated the number of nuclei leaving the explants, using standard area count in the Integrated Morphometry Analysis function of Metamorph. In some cases we also calculated the migration/outgrowth ratio, which corresponds to the surface covered by migrating nuclei, divided by the surface covered by growing axons in the proximal quadrant for each explant. Areas were expressed in mm² and migration/outgrowth ratios were presented using arbitrary units and normalized to one in control conditions. Averages and distributions were analyzed. Differences were considered as significant when P<0.05 using a non-parametric Mann-Whitney test.

To get a quantitative analysis of axon morphology, we measured the mean deviation between the axon trajectory and a theoretical rectilinear path (regression line), and values were expressed using arbitrary units (normalized to one in control).

Electroporation and videomicroscopy experiments
E12 rhomic lip explants were transfected with pEFGFP-N1 (Clontech, Palo Alto, CA), as a reporter construct, using electroporation. Using a microcapillary glass pipette, ~2 μl of plasmid DNA [2.5 μg/μl in 25 μM EDTA and 1 mM Tris (pH 8)] tied with 0.025% fast-green dye, was injected into the rhombencephalon (before dissection), and three pulses of 100 V were then applied to each side of the injected hindbrain using an Intracell electroporator and CUY610 electrodes (Nepa Gene, Chiba, Japan). Videomicroscopy experiments were performed using an inverted Zeiss microscope and a Hamamatsu camera. Collagen assays were performed in phenol red-free medium. Images were acquired from the second day of culture and at a rate of one every 8 minutes. Quantification of axons and nuclei velocities was obtained using the tracking function in Metamorph.

Confocal microscopy
Confocal fluorescence microscopy was performed by using a Leica Microsystem confocal microscope (SP2) equipped with a 40×Fluar oil immersion objective (numerical aperture 1.25), He-Ne (ex=543 nm) lasers.

Results
Expression of Rho GTPases during the migration of PCN in mice
To assess the role of small Rho GTPases during the development of PCN, we first analyzed their expression pattern in the developing hindbrain. We focused on studying embryos at E11, to analyze the initiation of migration from the rhomic lip of PCN when they enter the migratory stream (Taber-Pieche, 1973; de Diego et al., 2002). We also analyzed the expression of Rho GTPases at E13, when ION stop and locate close to the floor plate, whereas LRN migrate and cross the floor plate ventro-marginally to locate contralaterally to their birthplace. We first delineated the cartography of Rho GTPases expression by in situ hybridization on hindbrain cryosections. ION were identified through their expression of Brn3b transcripts (Turner et al., 1994; Bloch-Gallego et al., 1999) and LRN were identified through Tag1 expression (Backer et al., 2002) (data...
not shown). At E11, when PCN migration is initiated, *Cdc42* and *Rac1* transcripts were expressed in the ventricular (vz) and subventricular (svz) zones of the hindbrain, although some expression was also observed in neighboring structures in the hindbrain (Fig. 1A,A'). No expression was detected in the marginal (mz) and submarginal (smz) migratory zones. At E11, *Cdc42* transcripts were highly expressed in the migrating LRN and ION masses (Fig. 1B). *Rac1* transcripts were similarly expressed in the LRN but showed a lower expression in ION (Fig. 1D). At E11, *Rhoa* expression (Fig. 1E) was similar to that of *Cdc42* and *Rac1*, whereas, by contrast, *Rhob* transcripts were absent in the ventricular and subventricular zones but were highly expressed in the early migratory stream (Fig. 1G,G'). At E13, *Rhoa* and *Rhob* transcripts were both strongly expressed in the marginal stream that contains migrating LRN that cross the floor plate ventro-marginally (Fig. 1F,H), whereas ION expressed *Rhoa* to a lesser extent (Fig. 1I) than *Rhob* transcripts (Fig. 1H). *Rhoc* transcript localization was coincident with that observed for *Rhoa* transcripts, but the intensity was much lower (data not shown). By western blot analysis, we revealed the presence of *Rac1*, *Cdc42* and *Rhoa* proteins in the rhombic lips at E11 and E12 (Fig. 1I). At E13, *Rac1*, *Cdc42* and *RhoA* proteins were detected in the medioventral part (ION plus migrating LRN) of the hindbrain (Fig. 1J).

As shown schematically in Fig. 1K, *Cdc42*, *Rac1* and *Rhoa* transcripts were expressed in the ventricular and subventricular zones at E11, whereas *Rhob* transcripts were located in the migratory streams. At E13, *Cdc42*, *Rac1* and *Rhoa* transcripts were expressed in LRN, and *Cdc42* and *Rhob* transcripts were additionally expressed in ION that were compacted close to the floor plate.

**Cdc42/Rac1 are required for initial extension of the PCN leading process whereas nuclear migration depends on RhoA/B/C activity**

To study the role of Cdc42, Rac and RhoA/B/C GTPases in both axon outgrowth and nuclear translocation of PCN, we used pharmacological approaches in a collagen assay that had been previously set up (Causeret et al., 2002). In this assay, E11 or E12 rhombic lip explants were faced with netrin 1-secreting cells in collagen gel matrix. Under those conditions, initial extension of neurites out of the explant toward the netrin 1 source occurred from the first day in culture (DIC). Nuclear...
migration developed on the second DIC, in the same orientation as axons (i.e. toward the netrin 1 source). Cultures were maintained for 3 DIC, and then both axon outgrowth and nuclear migration were analyzed (Fig. 2A,C) (Causeret et al., 2002). Quantifications revealed the surfaces covered by Tuj1-positive neurites and by migrating nuclei toward the netrin 1 source after 3 DIC.

We tested the effects of blocking Cdc42, Rac and RhoA/B/C GTPases to establish whether they could be involved in axon outgrowth and/or nuclei migration of PCN. We first tested the ability of various concentrations of lethal toxin (LT-9048) to inhibit Rac1 and Cdc42 in PCN explants, and determined that an application of 1 ng/ml was an optimal concentration to analyze the effects of LT-9048 without any toxicity (as was observed at 10 ng/ml, data not shown). Application of 1 ng/ml LT-9048 in culture resulted in a 54% reduction of axon outgrowth.

![Image of control and lethal toxin conditions for axon outgrowth and nuclear migration](image_url)

**Fig. 2.** Rac and Cdc42 GTPases play an essential role in axon outgrowth of PCN toward a netrin 1 source. E11 rhombic lip explants were faced with netrin 1-secreting cells (Net) and cultured for 3 days. Axon outgrowth and nuclear migration were then analyzed after Tuj1 and DAPI staining, respectively. In control conditions, axon growth developed mainly toward the netrin 1 source (A) and nuclear migration occurred almost exclusively within the netrin 1-attracted neurites (C). Addition of 1 ng/ml of lethal toxin, to specifically inhibit Rac and Cdc42, resulted in a severe impairment of axon growth (B) but did not affect nuclear migration (D). Measurement of the surface covered by migrating nuclei (E), or quantification of their number (F), represented as cumulative distributions and histograms (mean±s.e.m.), revealed no significant difference between control (n=24) and drug-treated (n=24) explants. (G) Axon outgrowth toward (proximal) and away (distal) from the netrin 1 source (proximal and distal quadrants are represented in A; *P<0.001 compared with control; error bars represent s.e.m.). (H) Migration/outgrowth ratio in control and drug-treated explants (*P<0.001). (I,J) High magnification of axons stained with rhodamine-conjugated phalloidin to visualize F-actin structures. Whereas control growth cones showed F-actin enrichment (arrows in I), lethal toxin-treated axons lacked phalloidin staining at their distal tip (arrows in J). Scale bars: A, 500 μm (for A,B); C, 200 μm (for C,D); I,J, 10 μm.
outgrowth at E11 after 3 DIC (compare proximal quadrants in Fig. 2A with 2B; quantifications in Fig. 2G; \( P < 0.001, n = 24 \) control and treated explants). The outgrowth showed a 63% decrease at E12 (data not shown; \( P < 0.001; n = 21 \) for control; \( n = 19 \) for LT-treated explants). In addition, phalloidin staining was used to visualize F-actin in control and LT-9048-treated explants. Whereas control growth cones revealed F-actin-rich structures by labelling with rhodamine-conjugated phalloidin (Fig. 2I), these actin structures were severely affected in LT-9048-treated axons that lacked phalloidin staining at their distal tip (Fig. 2J).

Interestingly, the surface covered by migrating nuclei upon lethal toxin treatment represented 101% of control at E11, and 113% at E12 (Fig. 2C,D,E; not significant, \( P = 0.94 \) and \( P = 0.53 \), respectively). The number of migrating nuclei was also evaluated using the Integrated Morphometry Analysis function (Metamorph) at E11 (243±27 in control and 281±21 in the presence of 1 ng/ml lethal toxin, \( P = 0.28 \); Fig. 2F). As a consequence, the migration/outgrowth ratio was significantly different in control explants and in explants treated with lethal toxin (Fig. 2H; 82% increase, \( P < 0.001 \)). Altogether, these data show that Rac/Cdc42 inhibition through application of 1 ng/ml lethal toxin specifically altered axon outgrowth without modifying nuclear migration toward the netrin 1 source.

To assess the specific requirement of RhoA/B/C in PCN neuronal migration and axon outgrowth, we treated rhombic lip explants in collagen assays with the toxin C3 fused with a TAT domain in order to optimize intracellular penetration of the toxin (Sauzeau et al., 2001). We first controlled TAT-C3 efficiency on ION explants through ADP-ribosylation assays (see Materials and methods, and Fig. 3A). In vitro ADP-ribosylation of neurons pre-treated with TA T-C3 showed that the radiolabeled Rho band was markedly decreased, indicating that TA T-C3 efficiently modified Rho substrate in neurons. Application of 20 \( \mu \)g/ml of TA T-C3 in a collagen assay resulted in a significant reduction of nuclear migration outside E11 (Fig. 3C,D) or E12 (data not shown) rhombic lip explants (at E11, 75% reduction, \( n = 19 \) control explants, \( n = 13 \) TA T-C3-treated explants, \( P < 0.001 \); at E12, 71% reduction, \( n = 12 \) control explants, \( n = 17 \) TA T-C3-treated explants, \( P < 0.001 \)), combined with a potentiation of axon outgrowth (Fig. 3B,E,F). Tuj1-positive neurites appeared less fasciculated (compare Fig. 3B with Fig. 2A); they lost their preferential orientation, extending similarly towards and away from the netrin 1 source (Fig. 3E,
and compare proximal and distal quadrants in Fig. 3B). In addition, TAT-C3 treatment affected axonal morphology. A detailed analysis of axonal morphology was performed after GFP electroporation that allowed the visualization of individual processes. Control axons showed a straight morphology (Fig. 4A,C), whereas TAT-C3-treated explants exhibited tortuous axons (Fig. 4B,E). The deviation from a straight line was measured to quantify the trajectories of the axons from the drawings of 11 control and TAT-C3-treated axons (Fig. 4D).

Taken together, these data suggest that Rac, and/or Cdc42, is involved in mediating axon outgrowth of PCN explants, and RhoA/B/C would control axon fasciculation and polarity of PCN facing a netrin 1 source. In addition, we show for the first time that the RhoA/B/C signaling pathway regulates PCN nuclear translocation.

RhoA/B/C are in a GTP-bound active state in the rhombic lips and in the early migratory stream of PCN

We have focused on the regulation of the migratory process during development of PCN that appeared, from expression patterns and inhibition experiments, to be regulated by RhoA/B/C GTPases. As the Rho GTPases cycle between a GDP-bound inactive state and a GTP-bound active state (Ridley, 1997), we aimed to measure Rho GTPase activation. We first analyzed the amount of active RhoA GTPase in E11 rhombic lips using pull-down assays. Rhombic lips contained a low but detectable amount of active RhoA-GTPase (Fig. 5A). We aimed to get a precise localization of active RhoA/B/C GTPases during PCN migration. For this purpose, we adapted the assay initially developed for studying in situ Rho GTPase activation in optic tectal cells (Li et al., 2002b). We analyzed the localization of active RhoA/B/C GTPases at key stages of the migration, when ION and LRN initiate their migration (at E11 and E12, respectively), and at E13, when ION stop their migration ventrally, whereas LRN continue migrating and cross the floor plate. In situ binding assays, with the Rho-binding domain (RBD) of the specific Rho effector Rhotekin fused to glutathione-S-transferase (GST) (which could only bind active RhoA/B/C-GTPases), were performed on cryosections at different stages of embryonic development (Fig. 5B,C,D). Activated Rho was detected at E11 in the ventricular zone (vz), in the most dorsal part of the hindbrain (arrow, Fig. 5B), as well as in the initial migratory pathway of PCN (mz and smz; Fig. 5B). Scattered GST-RBD-Rhotekin-positive cells (arrowheads, Fig. 5C) were observed when leaving the ventricular zone (vz; large arrow, Fig. 5C) toward the early migratory stream in the periphery of the dorsal hindbrain (short arrows, Fig. 5C). At E12, active RhoA/B/C-GTPases could be detected in throughout the migratory pathways of caudal hindbrain, with GST-RBD-Rhotekin binding in the migratory streams (data not shown). At E13, GST-RBD-Rhotekin proteins stained the whole marginal stream that contains LRN and ECN migrating neurons (arrowhead, Fig. 5D). Lower levels of Rhotekin binding could be detected when ION ended their migration in the vicinity of the floor plate, when ION stop their migration. A decreasing gradient of activated RhoA/B/C was observed from ventrolateral ION to ventromedially stopped ION (asterisk, Fig. 5D). However, significant levels of Rhob mRNA were still expressed in ION ending their migration (Fig. 1H). At E11 and E13, no binding of a GST-tagged non-relevant protein (M-Cadherin) was observed (Fig. 5E,F).

Altogether (Fig. 5G), the data show that activated Rho GTPases can be detected at E11 in the rhombic lips and in the migratory streams of PCN. At E13, RhoA/B/C activity was decreased in ION that had achieved their migration and reached the floor plate, whereas migrating LRN still strongly expressed active RhoA/B/C-GTPases when they cross the floor plate.

Rock1/2 are implicated downstream RhoA/B/C during PCN nuclei migration in response to netrin 1

Once the in situ localization of active Rho GTPases had been determined, we aimed to characterize the Rho target(s) involved in nuclear migration. The main effector proteins of RhoA GTPases are Rock1 (also named ROKα) (Amano et al., 2000) and Rock2 (also named ROKβ) (Gan et al., 2001; Matsui et al., 1996; Nakagawa et al., 1996).

First, we analyzed Rock1/2 expression in the rhombic lips, and in the migratory pathways of ION and LRN in vivo. In situ RNA hybridization on hindbrain cryosections revealed a clear labeling of neurons located in the vz and subventricular zone (svz) at E11 for both Rock1 (Fig. 6A) and Rock2 (data not

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Fig. 4. TAT-C3 treatment affects axonal morphology. The individual morphology of neuronal processes from E12 explants could be visualized after GFP electroporation. Control neurons showed straight axons (A), whereas TAT-C3-treated neurons exhibited tortuous axons (B). For quantification analysis, the trajectory of entire axons in control (C, n=11) and TAT-C3 treated explants (E, n=11) was drawn, and the mean deviation from a straight line measured (D). Data are presented using arbitrary units; *P<0.001. Scale bar in A: 100 μm for A,B.
shown). Rock1 transcripts were also detected in the initial migratory streams (Fig. 6A). At E13, a developmental stage allowing visualization and discrimination of both LRN/ECN and ION, Rock1 transcripts (Fig. 6B), as well as Rock2 transcripts (not shown), were strongly expressed in the marginal migratory stream that contains LRN and ECN, and at a lower level in late migrating and stopping ION.

Thus, at E11, Rock1/2 were expressed in the vz and svz that also express Rhoa, and Rock1/2 were also expressed in the migratory stream from the rhombic lips, similar to Rhob (compare Fig. 6A,A' with Fig. 1E,G). At E13, Rock1/2 showed strong expression in ION and LRN, as was observed for Rhob transcripts (compare Fig. 6B with Fig. 1H). In addition, the presence of Rock1/2 proteins was visualized by western blotting of E11 and E12 rhombic lip explants, and E13 ION, altogether with LRN located below ION in the marginal stream. As shown in Fig. 6C,D, significant levels of Rock proteins could be detected at all three stages.

We further investigated the Rho downstream signal transduction pathway that leads to PCN nucleokinesis triggered by netrin 1 using a specific inhibitor of Rock1/2 kinases, Y-27632 (Uehata et al., 1997). This pharmacological reagent is a cell-permeant compound, which is highly specific and efficient for inhibition of the catalytic activity of Rock1/2 (Ishizaki et al., 2000). We faced E11 and E12 rhombic lip explants with netrin 1-secreting cells in the presence of 5-100 μM of Y-27632. We analyzed axon pathfinding and nuclear migration in fixed collagen assays to quantify both processes after 3 DIC. Quantification of nuclear migration toward the netrin 1 source revealed a gradual inhibition from 5 to 100 μM (Fig. 7C). We then chose to apply 20 μM Y-27632, which allowed a strong inhibition of nuclei migration and was consistent with doses generally used in the literature (e.g. Wylie and Chantler, 2003). At 20 μM, the migration showed an 85% decrease at E11 (n=42 control, n=24 Y-27632-treated, P<0.001; Fig. 7B,E) and a 74% decrease at E12 (n=24 control, n=18 Y-27632-treated, P<0.001; data not shown). Neurons treated with 20 μM Rock inhibitor exhibited a loss of fasciculation (compare Fig. 7A with Fig. 2A) and morphological abnormalities similar to those observed in TAT-C3-treated PCN (compare Fig. 7A with Fig. 3B). Analyzing axon polarity revealed that Y-27632 treatment also mimicked the effects of RhoA/B/C inhibition but to a lesser extent. Although axon growth was significantly increased in the distal quadrant (opposite to netrin 1-secreting cells), compared with control conditions (distal quadrants in Fig. 7A and Fig. 2A), it did not reach the values of axon outgrowth in the presence of TAT-C3 (compare Fig. 7A,D and Fig. 3B,E). Thus, Rock inhibition impaired but did not completely abolish the orientation of PCN axon outgrowth toward netrin 1.

To obtain a dynamic view of the morphological changes induced by Y-27632 treatment, video-microscopy experiments were performed using PCN from E12 in collagen assays. In control conditions, axon pathfinding appeared as a continuous process whereas nuclear migration occurred through saltatory...
translocation (see Movie 1 at http://dev.biologists.org/supplemental). Migrating neurons presented a long monopolar process, and often a small transient trailing process. PCN exposed to 20 µM Y-27632 failed to extend straight axons toward the netrin 1 source, but appeared to grow faster and in various orientations; no nuclear migration occurred (see Movie 2 at http://dev.biologists.org/supplemental). Quantification revealed that axons in control conditions grew at a mean velocity of 27.0±1.1 µm/hour (n=7, 359 frames corresponding to 47 hours 52 minutes of tracking), whereas Y-27632-treated axons grew at 38.2±1.8 µm/hour (n=4, 349 frames corresponding to 46 hours 32 minutes, P<0.001). Cell bodies in control conditions moved at 17.5±1.2 µm/hour (n=4, 374 frames corresponding to 49 hours 52 minutes).

Taken together, these results suggest that Rock1/2 act downstream RhoA/B/C to direct migration of PCN nuclei toward a netrin 1 source. In addition, Rho/Rock activation is required to allow correct attraction and fasciculation of axons.

**Discussion**

Recent evidence suggests that cues, in particular chemotropic factors governing axon outgrowth may also be involved in neuronal migration (Hu and Rutishauser, 1996; Wu et al., 1999; Bloch-Gallego et al., 1999; Alcantara et al., 2000). Whether identical or distinct intracellular cascades are required for each process in response to a common diffusible factor remains to be determined. We have focused on the migration of PCN whose migratory process occurs in repeated sequences of axon outgrowth followed by neuronal migration through nuclear translocation. We aimed to characterize small GTPases that regulate both processes during tangential neuronal migration, in order to analyze whether both events were strictly dependent on, and/or regulated through, the same or distinct pathways. We show here that Rac1, Cdc42 and Rhoa/b GTPases are expressed by migrating PCN (ION and LRN). The two processes that govern tangential migration, i.e. axon outgrowth and nuclear translocation, can be uncoupled and depend on distinct pathways. Pharmacological inhibition of Rac and Cdc42 affected leading process elongation but allowed nuclear migration of both ION and LRN. Conversely, inhibition of RhoA/B/C GTPase or of their effector kinases (Rock1/2) blocked nuclear migration, whereas axon outgrowth was enhanced and presented morphological abnormalities.

**Rac1/Cdc42 activity is necessary for axon outgrowth, and RhoA/B/C is implicated in axon morphology/orientation and nuclear migration of PCN in vitro**

We show that Cdc42 and Rac inhibition by lethal toxin results in severe impairment of PCN axon outgrowth. Interestingly, 1 ng/ml lethal toxin did not affect migration because migration occurred normally in shortened neurites, indicating that it is not strictly dependent on previous long axon extension. By contrast, PCN axon outgrowth could develop independently of nucleokinesis as, following RhoA/B/C blockade with TAT-C3, axon outgrowth extensively developed whereas nuclear migration toward netrin 1 was completely blocked.

In addition, we report that TAT-C3 totally abolished the tropic effect of netrin 1 on axon attraction, as axon outgrowth developed extensively and in a similar fashion all around the explant. Thus, RhoA/B/C activity is absolutely required to maintain an oriented axonal outgrowth of PCN toward a netrin 1 source. The requirement of RhoA/B/C for oriented axon outgrowth in response to a chemoattractant may be specific to a particular biological system or to a tropic molecule such as netrin 1. For instance, in cultured Xenopus laevis spinal neurons, chemoattraction toward a source of brain-derived neurotrophic factor is Cdc42 dependent, whereas chemorepulsion by lysophosphatidic acid is RhoA dependent (Yuan et al., 2003).

Altogether, these data indicate that: (1) axon outgrowth and migration of PCN are not strictly dependent on each other because a shortened axon extension allows nuclear

**Fig. 6.** Expression of Rock in mouse at E11-E13. At E11 (A, A’), Rock1 mRNA could be detected by in situ hybridization in the ventricular (short arrow) and subventricular zone (large arrow) of the rhombic lip. Note the presence of Rock1-positive cells in the early migratory pathway (arrowheads in A’). At E13 (B), Rock1 expression could be detected in ION (asterisk) and in migrating LRN (arrowhead). The floor plate (fp) showed strong Rock1 expression (A, B). (C, D) Western blot illustrating the presence of Rock1 and Rock2 proteins in rhombic lip at E11 and E12 (C), as well as in the LRN and ION domains at E13 (D). Scale bars: A, 180 µm; A’, 90 µm; B, 100 µm.
translocation and axon outgrowth can develop without nucleokinesis; (2) Rac1/Cdc42 are necessary for axon growth of PCN; and (3) RhoA/B/C are necessary for nuclear migration, axon fasciculation and tropism in response to netrin 1. During tangential migration of PCN, the activity of both Rac and Rho has to be locally controlled in order to get coordinated axon and nuclei pathfinding. This cellular process probably requires crosstalk between Rho and Rac pathways, which could regulate spatio-temporally growth cone and cell bodies dynamics, as has been reported in fibroblasts to determine cellular morphology and migratory behavior (Sander et al., 1999), or for chemorepulsion of spinal neurons from *Xenopus laevis* (Yuan et al., 2003).

**Rock1/2 act as key effectors in the RhoA/B/C signaling pathway for nuclear migration**

We report evidence for the involvement of Rock1/2 in the regulation of neuronal migration in response to netrin 1. We show here that upon pharmacological blockade of Rock1/2 by Y-27632, the aspect of Y-27632-treated axons was similar to the one observed after TAT-C3 treatment. Thus, upon TAT-C3 or Y-27632 treatments, initiation of axon outgrowth and axon fasciculation of PCN were modified, suggesting that Rho/Rock played a role in the control of growth cone dynamics of various neurons, including PCN, cultured chick neurons from dorsal root ganglion (Fournier et al., 2003) and cerebellar granule neurons (Bito et al., 2000).

Pharmacological experiments revealed that inhibition of Rock1/2 abolishes PCN nuclear migration toward a netrin 1 source in collagen assays, confirming that Rock1/2 are involved in nucleokinesis. Our data are consistent with previous reports that describe Rock proteins as critical modulators of Rho-mediated actin dynamics in several migratory processes in nematodes (Spencer et al., 2001), mammalian leukocytes and neural crest cells, and cancer cells (Liu and Jessell, 1998; Itoh et al., 1999; Alblas et al., 2001); however, intracellular cascades involved in nucleokinesis were not characterized. Nucleokinesis had been previously reported to be a process dependent on the microtubule network, which would involve proteins interacting with microtubules such as Lis1 and doublecortin (Lambert de Rouvroit and Goffinet, 2001). From mutant mice analyses, it has been proposed that the reelin signaling pathway and Cdk5 could also regulate nucleokinesis (for a review, see Walsh and Goffinet, 2000). We report here the possible involvement of Rock in nucleokinesis during tangential migration. Rocks are serine/threonine kinases, with multiple functional domains involved in actomyosin assembly. Several reports have analyzed the respective role of the different domains of Rho-kinase in various cell lines (Amano et al., 1999; Chen et al., 2002; Riento et al., 2003). It will be informative to overexpress various mutant Rock proteins in PCN to further characterize the involvement of their sub-domains in the migratory process.

Interestingly, Y-27632 treatment less severely affected axon attraction by the netrin 1 source than did TAT-C3 treatment. The latter directly inhibits RhoA/B/C GTPases, whereas Y-27632 acts as a specific inhibitor of one of the targets of RhoA/B/C, the Rho effector Rock family of kinases (Rho-kinase/Rok/Rock) (Uehata et al., 1997). Several other proteins have been isolated as putative Rho effectors on the basis of their selective interaction with the GTP-bound form of Rho. These include, in addition to the Rock family comprising p160Rock (Rock-I) (Ishizaki et al., 1996) and Rok/Rho-kinase/Rock-II (Leung et al., 1995; Matsui et al., 1996; Nakagawa et al., 1996), protein kinase PKN (Amano et al., 1996; Watanabe et al., 1996), citron kinase (Madaule et al., 1995; Madaule et al., 1998; Madaule et al., 2000), and mammalian diaphanous homologs mDia1 and mDia2.
Requirement of a dynamic regulation of Rho GTPase activity for the proper development/positioning of PCN

During their migration, PCN repeatedly extend a leading process, translocate their nucleus inside this leading process and retract their trailing process. Consequently, cell bodies move through a series of jumps, as reported in various neuronal systems (Gilthorpe et al., 2002; Polleux et al., 2002). This could result from a direct fast regulation of Rock activity, or it may be due to the alternate presence of active/non active Rho proteins. The activity of RhoA/B/C GTPases can be regulated either by downregulation of downstream effectors such as Rock, or, more directly, by guanine nucleotide-exchange factors (GEFs) and GTPase-activating proteins (GAPs). GEFs exchange the GDP on an inactive GTPase for a GDP, whereas GAPs increase the intrinsic GTPase activity, thus converting GTP-bound forms to GDP-bound forms (Etienne-Manneville and Hall, 2002). During development, PCN migration can be divided into successive steps, including the initiation of migration from the rhombic lips, nucleokinesis through the migratory stream, and stopping of cell bodies at their final location in the hindbrain. Thus, it remains to be established which GAPs or GEFs are expressed and involved in regulating the various phases of the migratory process.

For ION, contrary to other PCN populations, cell bodies do not cross the floor plate. The intracellular mechanism that leads to the ending of nuclear migration in the vicinity of the floor plate remains to be established, but it possibly involves downregulation of Rho GTPases activity. It is noteworthy that, whereas transcripts of Rho GTPases are present in ION located near the floor plate, low levels of active Rho GTPases are detected in situ in ION that reach the floor plate and stop. The extinction of Rho GTPase activity could be due to the spatio-temporally restricted expression of a specific GAP when ION reach the vicinity of the floor plate, or possibly could be due to the loss of a GEF expression that would silence RhoA/B/C activity. Whether specific regulators of Rho GTPase activity are expressed when PCN reach their appropriate final location remains to be established. An interesting candidate would be the GEF Trio. In C. elegans, both UNC-40/Dcc and UNC-73/Trio participate in a signaling system that orients and polarizes neuroblast migration (Honigberg and Kenyon, 2000). In addition, Trio is able to link RhoA and Rac/Cdc42 pathways (Bellanger et al., 1998; Blangy et al., 2000), and thus could be an appropriate coordinator of both axon outgrowth and nucleokinesis during PCN migration.

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