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Key role played by RhoA in the balance between planar and apico-basal cell divisions in the chick neuroepithelium

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Abstract

The cell division axis determines the position of daughter cells and is therefore critical for cell fate. During vertebrate neurogenesis, most cell divisions take place within the plane of the neuroepithelium (Das, T., Payer, B., Cayouette, M., and Harris, W.A. (2003). In vivo time-lapse imaging of cell divisions during neurogenesis in the developing zebrafish retina. *Neuron* 37, 597–609. Haydar, T.F., Ang, E., Jr., and Rakic, P. (2003). Mitotic spindle rotation and mode of cell division in the developing telencephalon. *Proc Natl Acad Sci U S A* 100, 2890–5. Kosodo, Y., Roper, K., Haubensak, W., Marzesco, A. M., Corbeil, D., and Huttner, W. B. (2004). Asymmetric distribution of the apical plasma membrane during neurogenic divisions of mammalian neuroepithelial cells. EMBO J. 23, 2314–24). The cellular constraints responsible for this preferential orientation are poorly understood. Combining electroporation and time-lapse confocal imaging of chick neural progenitors, the events responsible for positioning the mitotic spindle and their dependence on RhoA were investigated. The results indicate that the spindle forms with a random orientation. However, the final orientation of cell divisions is dependent on two main factors: (i) an early rotation of the spindle that aligns it within the plane of the neuroepithelium, and (ii) a specific limitation of spindle oscillations, despite free rotation around the apico-basal axis. Expressing a dominant-negative RhoA leads to apico-basal cell divisions after a correct initial rotation of the spindle. Our data reveal a specific role for RhoA in the maintenance of spindle orientation, prior to anaphase. Thus, RhoA could be a key player potentially regulated by the neurogenic program or by the neural stem cell environment to control the balance between planar and apico-basal divisions, during normal or pathological development.

Keywords: Chick; Cell division; Electroporation; Live imaging; Neural tube; Neuroepithelium; Progenitor; RhoA; RhoGTPase; Spindle

Introduction

The orientation of cell divisions is likely to be an important parameter for the generation of cellular diversity in the vertebrate central nervous system (CNS). Current models suggest that different fates can be adopted by sister cells that have asymmetrically inherited cytoplasmic or plasma-membrane-associated determinants during the division of neural stem cells (Fishell and Kriegstein, 2003; Roegiers and Jan, 2004; Wodarz and Huttner, 2003). In the vertebrate embryo,although a significant fraction of the cells undergo apico-basal cell divisions (ABcd) at late stages of neurogenesis (Chenn and McConnell, 1995), the vast majority of neuroepithelial (NE) cells divide within the plane of the neuroepithelium (planar cell divisions, Pcd; Das et al., 2003; Tibber et al., 2004). A partial shift from planar to apico-basal-oriented divisions in NE cells is associated with the period of neuronal differentiation (Das et al., 2003; Haydar et al., 2003; Kosodo et al., 2004). The constraints acting on the spindle to orient most cell divisions within the plane of the neuroepithelium, and its shift to an apico-basal orientation during neurogenesis, are poorly understood.

In *Drosophila* and *Caenorhabditis elegans*, the orientation of cell divisions is critically dependent on mechanisms that position the spindle, including early rotation events and

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maintenance of spindle orientation before anaphase (Cowan and Hyman, 2004; Glotzer, 2003; Wang and Chia, 2005). A number of studies have identified molecular regulators of spindle formation and positioning including, among others, small G-protein family members (Barros et al., 2003; Kaltschmidt et al., 2000; Sanada and Tsai, 2005; Zheng, 2004). An interesting candidate is the small RhoGTPase, RhoA. Although it is widely expressed in the neuroepithelium (Brouns et al., 2000; Liu and Jessell, 1998), its function there remains unknown. RhoA plays a central role in establishing cell polarity that requires asymmetric and ordered distribution of the signaling molecules and cytoskeletal proteins (Fukata et al., 2003). In *Drosophila*, the downstream effector of RhoA, Rho kinase, is required for such polarization in asymmetrically dividing neuroblasts (Barros et al., 2003). RhoA has been implicated in several important events in cell division. In fact, in other systems, RhoA is involved in cell rounding at metaphase (Maddox and Burridge, 2003). Precise regulation of cell shape could be, at least in principle, causal in orienting cell division (Thery et al., 2005). In addition, RhoA is a central regulator of the cytoskeleton and is involved in positioning the cleavage furrow (Piekny et al., 2005) and as such could regulate the orientation of cell divisions. Interestingly, although the function of RhoA is central for CNS morphogenesis and is implicated in early convergent extension motion in the neural plate (Keller, 2002) and later events of axons formation (Nikolic, 2002), its function in the neuroepithelium remains undetermined. This is due in part to the technical difficulty in knocking down its activity at the right time and place within the neuroepithelium.

Here, we have used a combination of in vivo electroporation of NE cells in the chick embryo and time-lapse videomicroscopy to monitor the dynamics of the spindle as it achieves correct planar orientation during NE cell division. Furthermore, using this electroporation strategy, we have addressed the specific roles of RhoA signaling by temporarily interfering with RhoA activity in NE progenitors.

Materials and methods

Plasmids

Plasmids used in this study include EB1-Green Fluorescent Protein (EB1–GFP, a gift from Franck Perez), pCAG–H2B–mRFP, coding for a fusion between the monomeric red fluorescent protein (mRFP) and the histone 2B (mRFP–H2B fusion a gift of Shahraghim Tajbaksh), YFP–α-tubulin (a gift from Edith Guoin), pCAGG–GFP–GPi expressing plasmids. RhoA or Rac1 dominant-negative cDNAs (a gift from Alan Hall) were subcloned into pCIG plasmid (a gift from Sean Megason).

Electroporation

Super-coiled plasmid DNA was injected into the neural tube of HH12-14 chicken embryos at a concentration of 2 μg/μl in PBS. Gold electrodes (Genetrodes, model 512), distanced 4 mm between anode and cathode, were placed parallel to the neural tube, and embryos were pulsed 5 times (25 V/50 ms duration), using a Electro Square Porator™ ECM830 (BTX). Embryos were incubated for 8 h and then harvested. The observed phenotypes with dominant-negative RhoA (dnRhoA) were dependent on both the length of the period of incubation after electroporation and on the concentration of the plasmid injected in the lumen of the neural tube. Embryos incubated for 24 h after electroporation using a concentration of 4 mg/ml of plasmid showed dramatic and complex phenotypes, including limited interkinetic movements, adhesion defects and an almost complete lack of cell divisions (data not shown). By using an intermediate concentration of DNA (2 mg/ml) and beginning the analysis 6–8 h after electroporation, it was possible to observe cell divisions.

Organotypic explants and videomicroscopy

Electroporated embryos were observed 6 h after electroporation under an epifluorescent microscope (Stemi SV11, Zeiss). Selected embryos were dissected in PBS to obtain the neural tube and adjacent somites and sectioned into 150-μm slices with a Tissue Chopper (Mickle Laboratory Engineering CO.LTD). Sections were placed in F12-HAM medium (Gibco-Invitrogen), L-glutamin (5 mM, Gibco-Invitrogen), sodium-pyruvate (1 mM, Gibco-Invitrogen), Fungizone AmphotericineB (5 mg/ml, Gibco-Invitrogen) and penicillin/streptomycin (100 mg/ml, Gibco-Invitrogen) solidified with 1% low melting point agarose (Nusive GTG Agarose, Cambert-Tebu-Bio), in a 35-mm Petri dish with a 14-mm microwell (MatTek corporation, ref P35G-1.5-14-C). Liquid medium was added on top of the gel. Mineral oil (SIGMA) was applied on the surface of the medium to avoid evaporation. The Petri dish was placed on an inverted microscope in a temperature-controlled chamber (Tempcontroller 37-2 digital, Zeiss), regulated to maintain 38°C at the level of the observed slice. We used a Zeiss Plan-NEOFLUAR 40X/1.3 oil objective. We also added an objective heater (6 W Objective Temperature Controller, Bioptech) adjusted at 37.5°C. Time-lapse videomicroscopy was performed with a Zeiss confocal inverted microscope (Axiovert 200M) and the LSM510 (3.2) software or with a Spinning Disk system on Zeiss Axiovert200 inverted microscope connected to a Hamamatsu Orca II ER camera and Perkin Elmer Ultraview RS acquisition software. The scan durations were 1 min or less on the confocal microscope. Average scanned depth was about 25–30 μm within the explants. Identical acquisitions were repeated automatically every 5 or 10 min. Movie duration ranged from 5 to 8 h.

Image analysis

Time-lapse experiments were analyzed using Zeiss LSM and Imaris software. Spinning Disk images were analyzed using Perkin Elmer Ultraview RS, OsiriX and Volocity software. The time-lapse measurements were started at the beginning of metaphase for each cell. To measure the angle between the spindle and the surface of the neuroepithelium, 4–6 stacks of images containing the dividing cell were extracted. Each *z*-stack, representing one time point, was reconstructed into three dimensions and then projected into a 180° series. The reconstructed stack was manually rotated until a maximal size of the spindle and/or the edge of the metaphase plate was visible (see Movie 2 and 4). A line was drawn along the spindle's main axis (joining the 2 microtubule asters) or through the middle of the metaphase plate. The angle of the spindle was calculated in reference to a straight line at the neuroepithelial surface. This procedure was repeated for every time point and for every dividing cell. When the spindle rotated around the apico-basal axis, it was sometimes not possible to measure accurately the angle and the corresponding time points were omitted. Movies published as supplementary information were made from series of flattened images (between 1 and 3 images) from each time point.

Immunohistochemistry

Immunohistochemistry was performed on 30-μm cryosections of embryos fixed in 4% PFA. Primary antibodies used were anti-γ-tubulin (Sigma antibody GTU-88), anti-β-tubulin (Sigma) and anti-RhoA (Santa-Cruz). The secondary antibodies used were Alexa Fluor 488 donkey anti-mouse (Molecular Probes) or biotinylated anti-mouse (Amersham Bioscience) revealed with TRITC conjugated streptavidin. DNA was stained with To-Pro3 (Molecular Probes) or DAPI, actin was stained with Texas-red phalloidin (Molecular Probes). Immunofluorescence with the neuronal marker TuJ1 was performed as described (Fior and Henrique, 2005).