

Cytokinesis of neuroepithelial cells can divide their basal process before anaphase

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Neuroepithelial (NE) cells, the primary stem and progenitor cells of the vertebrate central nervous system, are highly polarized and elongated. They retain a basal process extending to the basal lamina, while undergoing mitosis at the apical side of the ventricular zone. By studying NE cells in the embryonic mouse, chick and zebrafish central nervous system using confocal microscopy, electron microscopy and time-lapse imaging, we show here that the basal process of these cells can split during M phase. Splitting occurred in the basal-to-apical direction and was followed by inheritance of the processes by either one or both daughter cells. A cluster of anillin, an essential component of the cytokinesis machinery, appeared at the distal end of the basal process in prophase and was found to colocalize with F-actin at bifurcation sites, in both proliferative and neurogenic NE cells. GFP-anillin in the basal process moved apically to the cell body prior to anaphase onset, followed by basal-to-apical ingression of the cleavage furrow in telophase. The splitting of the basal process of Mphase NE cells has implications for cleavage plane orientation and the relationship between mitosis and cytokinesis.

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Introduction

A hallmark of neuroepithelial (NE) cells, the primary stem and progenitor cells to all neurons and macroglial cells of the mammalian central nervous system, is their apical-basal polarity. This polarity is evident, for example, from the organization of their plasma membrane, with the apical surface facing the lumen of the neural tube and the basal surface contacting the basal lamina. During embryonic development, the apical-basal polarity of NE cells is thought to influence their division. Initially, these cells divide in a symmetric, proliferative manner, such that polarized cell constituents, notably critical apical components (Kosodo et al, 2004), are equally distributed to both NE daughter cells (Huttner and Brand, 1997; Fishell and Kriegstein, 2003; Wodarz and Huttner, 2003; Götz and Huttner, 2005). At the onset of neurogenesis, NE cells switch to asymmetric, neurogenic divisions that yield a NE daughter cell and a neuronal daughter cell or neuronally committed basal progenitor (Haubensak et al, 2004; Miyata et al, 2004; Noctor et al, 2004). Here, the daughter cells now generated are thought to receive different amounts of the polarized constituents (Huttner and Brand, 1997; Fishell and Kriegstein, 2003; Wodarz and Huttner, 2003; Götz and Huttner, 2005). Concomitant with this switch, NE cells transform into radial glial cells, which retain key features of apical-basal polarity and which continue to generate neurons by asymmetric division (Kriegstein and Götz, 2003; Rakic, 2003; Götz and Huttner, 2005).

Symmetric proliferative divisions of NE/radial glial cells typically show a cleavage plane orientation parallel to the apical-basal cell axis, with the cleavage furrow ingressing in the basal-to-apical direction (Chenn and McConnell, 1995; Das et al, 2003; Haydar et al, 2003; Kosodo et al, 2004; Fish et al, 2006; Wilcock et al, 2007). How NE cells orient their cleavage plane parallel to their long apical-basal axis and how the cleavage furrow ingression is set in the basal-toapical direction remain unknown. Several proteins implicated in spindle pole positioning have been recently shown to control this cleavage plane orientation (Feng and Walsh, 2004; Huttner and Kosodo, 2005; Sanada and Tsai, 2005; Zigman et al, 2005; Fish et al, 2006; Morin et al, 2007; Konno et al, 2008). Alternatively, subcellular structures other than the mitotic spindle may also have a role in determining cleavage plane orientation and furrow ingression in these cells.

Recent time-lapse fluorescence imaging studies on the generation of neurons from radial glial cells in the embryonic rodent brain (Miyata et al, 2001; Noctor et al, 2001) and from retinal progenitors in mouse (Cayouette and Raff, 2003; Saito et al, 2003) and zebrafish (Das et al, 2003) have demonstrated that these apically dividing cells retain a very thin radial process contacting the basal lamina (also referred to as basal process; Das et al, 2003) throughout M phase (reviewed in Fishell and Kriegstein, 2003; Wodarz and Huttner, 2003).

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When these progenitors retain their basal process through M phase, it is thought that only one of the daughter cells inherits it (Miyata et al, 2001; Cavouette and Raff, 2003; Das et al, 2003; Saito et al, 2003). This implies that their division is asymmetric in cell biological terms (Fishell and Kriegstein, 2003; Wodarz and Huttner, 2003). Consistent with this, the asymmetric inheritance of the basal process has been proposed to correlate with fate differences between the daughter cells (Miyata et al, 2001; Fishell and Kriegstein, 2003). Whether it is the neuron (Miyata et al, 2001) or the radial glial progenitor (Fishell and Kriegstein, 2003) that inherits the basal process remains controversial (Huttner and Kosodo, 2005). However, asymmetric inheritance of the basal process has also been recently reported for symmetric divisions of retinal NE cells (Saito et al, 2003) and radial glial cells (Miyata et al, 2004) that generate two progenitor cells. In this context, it must be pointed out that the fate of the extremely thin mitotic basal process has proved to be very challenging to follow with light microscopy (Cayouette and Raff, 2003; Das et al, 2003). Therefore, it remains unclear whether the inheritance of this process is always asymmetric and, if it is, whether it always influences daughter cell fate. An emerging alternative explanation is that this inheritance may not be asymmetric in all divisions.

To clarify this issue, we have here investigated the possibility that the basal process may be split on NE cell division. Such splittings may have previously remained undetected by fluorescence microscopy, due to the close apposition of the two daughter processes. We have therefore combined fixedand live-cell imaging to analyse the components and dynamics of the cytokinesis machinery within the basal process, together with electron microscopy (EM) to study the structure of the basal process at high resolution.

Results

Bifurcating basal processes of M-phase NE cells visualized by two independent fluorescent markers

To identify the basal process of NE cells specifically in M phase, we tested the monoclonal antibody MPM-2 (Davis et al, 1983). This antibody recognizes the phosphoserine/ phosphothreonine-proline epitope, present in many proteins from a wide variety of species, phosphorylated at the G2/M transition by cdc2 (Westendorf et al, 1994) or POLO kinase (Logarinho and Sunkel, 1998) and dephosphorylated at the end of M phase (Doonan and Morris, 1989). In cryosections of mouse E10.5 telencephalon, MPM-2 stained many round NE cell bodies at the apical surface of the ventricular zone (Figure 1A), consistent with them being in M phase. Furthermore, MPM-2 staining fully overlapped with phosphohistone H3 immunostaining (an established mitotic marker), and all these cells had chromosomes with mitotic morphology, as seen with DAPI (Supplementary Figure S1 and Supplementary Table S1). Importantly, MPM-2 immunostaining revealed processes extending radially from the basal side of many MPM-2-stained cell bodies through the ventricular zone (Figure 1A). Thus, MPM-2 immunostaining can be used to visualize the basal process of those NE cells that are in M phase.

Furthermore, the MPM-2 staining of NE cells in M phase, especially those in prometaphase and metaphase, fully extended from the apical ventricular surface all the way to the

basal lamina. This was revealed by immunostaining of prominin-1 (CD133) (Figure 1B), an apical plasma membrane protein of NE cells (Weigmann *et al*, 1997) and laminin α 1, a basal lamina marker (Figure 1Ca). Remarkably, in some of these cases, the MPM-2 staining suggested that a single basal process emerging from an M-phase NE cell body had undergone bifurcation (Figure 1Bb, arrowhead and Figure 1Cb, arrows). This was supported by the analysis of the corresponding consecutive optical sections (Supplementary Movies S1 and S3). This strongly suggested that the MPM-2 staining was confined to a single bifurcating basal process (Supplementary Movie S2), rather than the basal processes of two fully overlapping mitotic NE cells. As MPM-2 also recognizes phosphorylated MAP2 (Sanchez et al, 2000), a marker of post-mitotic neurons, there was massive staining of neuronal cell bodies and processes in the developing cortex at later stages (after E12.5, data not shown). This precluded the use of the MPM-2 antibody to identify the basal processes of radial glial cells at later stages of neurogenesis.

Bifurcation of the basal process could be reliably detected by MPM-2 immunostaining in prometaphase and metaphase as mentioned above. In later phases, the immunoreactivity remained reliably detectable in the cell body (Supplementary Table S1), but not along the entire process (Figure 1Dd; Supplementary Figure S2d). To investigate the structure of the basal process in anaphase and telophase, we expressed plasma membrane-targeted GFP in single E10.5 mouse telencephalic NE cells after 24 h of whole-embryo culture. In anaphase, a pair of adjacent basal processes could be seen to extend from the basal centre of the cell body (Figure 1D, arrowheads). In telophase, each daughter cell body showed a basal bulge from which a very thin process emerged (Supplementary Figure S2, arrowheads). However, during this final step of mitosis, the fate of the entire basal processes became difficult to visualize (at least in mouse telencephalic NE cells), even when using membrane GFP labelling.

Ultrastructure of the basal process of M-phase NE cells

As the fine structure of the basal process bifurcations of M-phase NE cells is beyond the resolution of light microscopy, we investigated it by EM. Changes in the shape of the NE cell body during the cell cycle have been described in previous EM studies (Hinds and Ruffett, 1971; Seymour and Berry, 1975). In interphase, the cell body has a radially oriented, elongated bipolar shape along the apical-basal axis and for most of the time it is not located at the apical surface, whereas in M phase at the apical surface, the cell body rounds up, adopting first a so-called pyriform, then conical and finally globular shape (Hinds and Ruffett, 1971; Seymour and Berry, 1975). Consistent with these studies, we also observed pyriform (Figure 1Ca), conical (Figure 1B) and globular (Figure 1A, arrowhead) shapes for the M-phase NE cell bodies stained by MPM-2 antibody at E10.5. Importantly, the MPM-2-stained NE cell bodies with such shapes were virtually always located directly at the apical surface of the ventricular zone, as indicated by prominin-1 immunostaining (Figure 1B). Taken together, these observations imply that Mphase NE cells can be identified by EM even without MPM-2 staining by their shape and location at the ventricular surface.

We explored various EM approaches, including scanning EM (SEM), conventional thin-section transmission EM and thick-section high-voltage EM (HVEM) (Hama *et al*, 1994),

and found SEM and HVEM to be most suitable to trace long processes in dense tissue. To reveal the basal process of M-phase NE cells in HVEM, Golgi silver impregnation (Pannese, 1999) was found to be superior in resolution to MPM-2 immunoperoxidase. Golgi-stained NE cells were first examined in a 100- μ m Epon section under the light microscope to identify a cell in M phase, by morphology and direct localization at the ventricular surface. Then 5- μ m sections containing the M-phase cell of interest were prepared and analysed by HVEM.

Stereo observation of HVEM images revealed that the process emerging from an identified M-phase NE cell body in the basal direction can consist of one (12 cases), two (5 cases) or, as previously seen by SEM (Seymour and Berry, 1975), even multiple branches (3 cases) (Table I). Figure 2A and B shows two examples of an apical round-shaped NE cell body extending a pair of very thin processes in the basal direction. A tomographic reconstruction (data not shown) corroborated the existence of the pair of basal processes shown in Figure 2Bb-b^{''}.

Similarly, we observed split basal processes of M-phase NE cells by SEM (Figure 2C–F), applying modified sample preparation steps to improve the preservation of thin processes

Figure 1 Basal processes of NE cells in M phase can be visualized by MPM-2 immunostaining or expression of membrane-targeted GFP. (A) Overview of the ventricular zone. A frozen section of E10.5 mouse telencephalic neuroepithelium was immunostained with MPM-2 antibody. Eight 1-µm optical sections were processed into one stacked image. The apical surface is down and the basal side is up. Note that the MPM-2 immunostaining reveals not only the M-phase NE cell bodies but also their basal processes extending radially towards the basal side of the neuroepithelium. Arrowhead, a globular-shaped M-phase NE cell body. The asterisk indicates ectodermal cells, which were stained by the secondary anti-mouse antibody. V, ventricle; VZ, ventricular zone. $Bar = 5 \mu m$. (B) Presence of the M-phase NE cell body directly at the ventricular surface of the neuroepithelium. (a) E10.5 mouse telencephalic neuroepithelium was double immunostained with MPM-2 antibody (red) and prominin-1 antibody to reveal the apical plasma membrane of NE cells (green). Note the MPM-2-immunostained basal process extending from the apical cell body. Bar = $5 \,\mu m$. The image is a stack of five consecutive 0.2-µm optical sections selected from a total of 25. See also Supplementary Movie S1. (b) 3D reconstruction from the stacked 25 consecutive 0.2 µm optical sections (see Supplementary Movie S1) of the MPM-2-stained cell in (a). The image shows 1 of the 15 views obtained on rotation (see Supplementary Movie S2). The triangle indicates the bifurcation point of the basal process. (C) Bifurcation of the basal process extending from an M-phase NE cell body. (a) E10.5 mouse telencephalic neuroepithelium was double immunostained with MPM-2 antibody (red) and laminin $\alpha 1$ antibody (green). Note the contact of the basal process with the basal lamina (yellow). The image is one of six consecutive 1-µm optical sections. See also Supplementary Movie S3. (b) Magnified image of the basal region of (a). Note the bifurcation of the MPM-2-immunostained basal process (arrows) and the basal lamina contact of both branches. $Bar = 5 \mu m$. (D) Plasmid encoding membrane-targeted GFP was electroporated into E10.5 mouse telencephalic neuroepithelium followed by wholeembryo culture for 24 h. Frozen sections were stained with DAPI (DNA, a, c blue) and MPM-2 antibody (d) and observed by confocal microscopy (1-µm optical section). Note that the GFP-expressing NE cell (b, c green) in anaphase extends two basal processes (arrowheads) from its cell body. These two processes become indistinguishable when they are adjacent to each other (double arrowheads). By this stage of mitosis, MPM-2 immunostaining of the basal process has become weak (d). Arrows in (b) indicate the plasma membrane of GFP-expressing NE cells in interphase. Apical surface is down. Bar = $5 \,\mu m$.

when compared with previous studies (see Materials and methods). Figure 2C and D shows two examples (of a total number of 17 cases observed) in which an apical round-shaped NE cell body extended two (or occasionally more) adjacent, very thin processes in the basal direction, similar to the HVEM observations (compare Figure 2Ab-b" and Cb). Figure 2E and F shows two examples (of a total number of



Table I Frequency of basal process bisection in mouse E10.5 M-phase neuroepithelial cells

MPM-2/anillin double immunoste	ining		
MPM-2-stained cell bodies ^a 125 (100%)	Basal processes with anillin spots ^a 20 (16%)		
MPM-2-stained basal processes	Processes with anillin spots	Processes with bifurcations	Bifurcated processes with anillin spots
69 (100%)	23 (33%)	17 (25%)	5 (7%)
HVEM			
M-phase cell bodies with process(es)	One process arising from cell body	Two processes arising from cell body	>2 processes arising from cell body
20 (100%)	12 (60%)	5 (25%)	3 (15%)

^aData from the legend of Figure 5C. Note that only a fraction of the MPM-2-stained cell bodies show a basal process in the single optical section analysed, which explains why the percentage of anillin spots in basal processes is lower when expressed per MPM-2-stained cell bodies than when expressed per MPM-2-stained basal processes.

five cases observed) in which two processes emerge at a distance (>2 μm) to each other from a single apical round-shaped NE cell body.

It should be noted that tight pairs of basal processes such as the one shown in Figure 2Ab-b" or Cb would not be resolved by conventional confocal microscopy. These highresolution observations confirm that the thin basal processes of M-phase NE cells can exist as a paired structure. This is consistent with these processes having undergone splitting during cell division.

Time-lapse live imaging of basal process splitting and inheritance during NE cell division in mouse neocortex and zebrafish spinal cord

To directly observe basal process splitting during M phase, we imaged single NE/radial glial cells in organotypic slice cultures of mouse E14.5 neocortex labelled with DiI, by epifluorescence microscopy (Miyata et al, 2001; Weissman et al, 2003). This revealed the migration of the branch point of the basal process towards the cell body in the basal-to-apical direction (Figure 3A, 0-60 min, arrowheads; Figure 3B, red curve). Remarkably, the arrival of the branch point to the basal tip of the cell body was followed by the typical mitotic rounding up of the cell body at the apical surface (Figure 3A, 60-108 min; Figure 3B, black curve), consistent with the splitting of the basal process down to its origin at the cell body (Figure 3A, 108 min, triangles) prior to anaphase onset. These two events were in turn followed by the cleavage of the cell body (Figure 3A, 120 min, asterisks; see also Supplementary Movie S4).

The zebrafish embryo offers unique advantages to observe neural progenitors in the developing central nervous system by live imaging (Das et al, 2003; Lyons et al, 2003; Tawk et al, 2007). In a separate line of investigation, we studied the behaviour of the basal processes during the division of NE cells of the anterior spinal cord of 24 h.p.f. zebrafish embryos expressing membrane GFP and nuclear RFP, using time-lapse microscopy. This stage of zebrafish development represented an early neurogenic stage that allowed us to study the first divisions after the neural tube is formed, when some progenitors divide to generate two progenitors, others divide to generate a progenitor and a neuron and a few divide to generate two neurons (PA and JDWC, unpublished results). We found the basal process splits during NE cell division in 19/22 cases. In many cases when the basal process splits, the daughter processes very quickly re-associated with one an-

other, and their inheritance by the daughter cell bodies was often impossible to determine (10/19 cases). However, in nine cases we found that the divided processes were either symmetrically inherited by the daughter cells (4/9; Figure 4A; Supplementary Movie S5), or asymmetrically (5/9; Figure 4B and C, right cell; see also Supplementary Movies S6 and S7). Splitting was initiated at the most basal end of the process and occurred approximately 10-15 min ahead of cleavage in the cell body. In 3/22 cases, we could not detect any splitting of the basal process (Figure 4C, left cell). These observations demonstrate that NE cell division in the developing zebrafish central nervous system is frequently accompanied by basal process splitting. This, however, is not necessarily followed by inheritance of the split processes by both daughters (symmetric inheritance), as interestingly the split processes may also be inherited by only one of the daughters (asymmetric inheritance).

The cytokinesis protein anillin is concentrated in the basal process of M-phase NE cells at the site of bifurcation

In the light of the splitting of the NE cell basal process during cell division, we asked whether the M-phase basal process contains components of the cytokinesis machinery. We analysed anillin, an actin-binding and -bundling protein that is known to be contained in the nucleus in interphase (Field and Alberts, 1995; Oegema et al, 2000; Kinoshita et al, 2002) but becomes a constituent of the contractile ring (Field and Alberts, 1995; Oegema et al, 2000; Kinoshita et al, 2002), also in NE cells (Kosodo et al, 2004). During M phase, anillin was found as expected in the apically located NE cell bodies, where it was concentrated on the contractile ring in telophase (Figure 5Aa, arrow). Importantly, it also localized to a discrete spot-like structure in the MPM-2-immunostained basal process of mitotic NE cells (Figure 5Aa, arrowhead). The specificity of the affinity-purified anti-anillin antibody was supported by a single immunoblot band in E10.5 mouse brain total homogenate (data not shown). Furthermore, the spot-like anillin immunoreactivity was observed only in basal processes of NE cells in M phase, it was distinct in size and appearance from the pattern of anillin immunostaining of interphase nuclei and telophase cell bodies (Figure 5Aa and b, arrowheads versus asterisk and arrow), and it was not associated with mitotic basal/intermediate progenitors (Haubensak et al, 2004; Miyata et al, 2004; Noctor et al,

2004) (Figure 5Ac, arrowhead). We shall refer to this structure as 'anillin spot'.

Actin staining by phalloidin together with anillin and MPM-2 immunostaining showed the expected concentration of F-actin at the apical cortex of NE cells (Figure 5Bc), and also showed an F-actin spot in the basal process of M-phase NE cells that closely colocalized with the anillin spot (Figure 5Ba–d, arrowheads). This strongly suggests that the anillin spot in the basal process indeed marks a specific F-actin cytokinetic structure within this process during M phase.

Figure 5Ca-d and e-h show two examples of the anillin spot being localized to the branching point of the M-phase basal process. We also observed anillin spots (i) in M-phase basal processes at sites more proximal to the cell body (Supplementary Figure S3, arrowheads), but, importantly, never more distal, than the apparent site of bifurcation and (ii) in M-phase basal processes that did not appear to bifurcate (for quantification, see Table 1). Given the limited resolution of fluorescence microscopy mentioned above, the former observation can be explained by the true site of bifurcation being more proximal than the site where the duality of the process could be resolved. Along this line, the latter observation may reflect (besides a true lack of bifurcation) cases in which the two branches of a bifurcated process could not be resolved by light microscopy. Therefore, the percentage of bifurcations we present can be interpreted as a lower estimate.

The anillin spot is observed in NE cells undergoing both proliferative and neurogenic divisions

In the E10.5 mouse telencephalon, NE cells have begun to switch from proliferative to neurogenic divisions, which have been shown to coexist in the same region of the ventricular zone (Haubensak *et al*, 2004). It was therefore important to determine whether the anillin spot in the basal process

Figure 2 Ultrastructure of the basal process extending from an apical NE cell body as revealed by HVEM following Golgi impregnation, or by SEM. (A) An apical round-shaped NE cell body extending a pair of very thin processes. (a) Low-power HVEM view of a Golgi-stained NE cell in a 5-µm Epon section. The NE cell body indicated by the asterisk is located at the ventricular surface and hence the cell was considered to be in M phase (see Results). (b-b") High-power HVEM views of the processes shown in the black rectangle in (a). Stereo-paired (0° , $\pm 16^\circ$) images were obtained by tilting the microscope stage. Note the pair of twisted thin processes (arrows) arising from the cell body. Bars = $1 \,\mu m$. (B) Another example of an apical round-shaped NE cell body extending a pair of very thin processes. (a) Low-power HVEM view of a Golgi-stained NE cell in a 5-µm Epon section. The NE cell body is located at the ventricular surface and hence the cell was considered to be in M phase. (b-b") High-power HVEM views of the processes shown in the black rectangle in (a). Three images at the indicated angles were selected from a series of 61 (-60° to $+60^{\circ}$, 2° increments). Note the pair of thin processes (arrows) arising from the cell body, and the short branch (asterisk) emerging from one of them. Bars = $1 \mu m$. (C-F) SEM images of apical round-shaped NE cell bodies extending thin basal processes in E9.5 mouse brains. The NE cell body indicated by the asterisk is located at the ventricular surface and hence the cell was considered to be in M phase (see Results). Note thin basal processes (arrows) arising from the cell body. Bubble-like structures (double arrowheads) were often identified at the basal pole of the round-shaped cell bodies as described earlier (Seymour and Berry, 1975). Low-power (Ca) and high-power (Cb) views of the same basal processes. Bars = $1 \mu m$.

occurred in both proliferative and neurogenic divisions, or in only one of these two sub-populations. To distinguish between these types of divisions, we used heterozygous embryos of the Tis21-GFP knock-in mouse line, which express nuclear GFP in the neurogenic, but not the proliferating, sub-population of NE cells (Haubensak et al, 2004). Figure 6Aa and c, and Ba and c shows two examples of adjacent M-phase NE cells in the E10.5 telencephalon. In each example, one cell was Tis21-GFP negative (proliferative division; Figure 6Ae and Be, grey arrowheads) and the other one was Tis21-GFP positive (neurogenic division; green arrowheads). Anillin spots were observed in the basal process of both types of cells (Figure 6Ab and c, and Bb and c, white arrowheads). On quantitation, 80% of cells with anillin spots in their basal process underwent proliferative divisions and 20% underwent neurogenic divisions (Figure 6C, left columns) at E10.5. This proportion was almost identical to that of all M-phase NE cells present in the same images (i.e. irrespective of the detection of a basal process and anillin spot in the 1-µm





Figure 3 Basal-to-apical branch point movement in the basal process precedes cell body division. (**A**) Mouse E14.5 neocortex was subjected to DiI labelling, and single labelled NE/radial glial cells were analysed by live imaging in organotypic slice culture using epifluorescence microscopy, acquiring a stack of images every 12 min (19 images per stack, $2.5 \,\mu m z$ -steps), as indicated at the top of the panels. The panels show selected single images in which the two branches of the basal process were most conspicuous. DiI fluorescence is shown in black (inverted colour). The apical surface is down. Arrows, position of the branch point at 0 min; arrowheads, position of the branch point at the respective time; triangles, the most proximal part of the basal process, which appears to be split; asterisks, prospective daughter cell bodies; bar in right panel, $10 \,\mu m$. See also Supplementary Movie S4. (**B**) Distance between branch point and cell body (red curve) and ratio width/length of the cell body (black curve) of the cell depicted in (A).

optical section, Figure 6C, right columns; 83 and 17%, respectively). Thus, the cytokinesis machinery is present in the basal process of both, NE cells undergoing proliferative and neurogenic divisions.

The anillin spot in the basal process undergoes basal-to-apical migration towards the cell body during NE cell division

Other epithelial cells also show cleavage furrow ingression in the basal-to-apical direction (Reinsch and Karsenti, 1994). Given the localization of the anillin spot at the bifurcation site of the basal process in M-phase NE cells, and the general role of anillin in cytokinesis (Field and Alberts, 1995; Oegema et al, 2000; Echard et al, 2004), we asked whether the division of the entire cell body actually begins in the basal process. If this were the case, mitotic progression should be accompanied by the basal-to-apical migration of the anillin spot, relative to the extension of the basal process. To test this, we performed double immunostaining of MPM-2 and anillin in combination with DAPI labelling to determine the mitotic stage. Figure 7 shows representative examples of anillin localization in NE cells at the various stages of mitosis. In prophase, the anillin spot was observed near the basal end of the basal process (Figure 7a and a', arrowheads), with no other particular concentration of anillin immunoreactivity in the cell body. In prometaphase, the anillin spot was found at various intermediate positions along the basal process (Figure 7b and b', arrowheads). In metaphase, we typically observed clustered anillin immunoreactivity at the origin of the basal process in the cell body (Figure 7c and c', arrowheads), often accompanied by another cluster at the apical cell cortex (Figure 7c and c', arrows). As expected, in anaphase (Figure 7d-d'') and telophase (Figure 7e-e''), there was strong anillin staining associated with the contractile ring (Figure 7d and d', dashed lines), which narrowed in the basal-to-apical direction (Figure 7e and e', double arrowheads). Importantly, no anillin immunoreactivity was detectable in the basal process in these late mitotic stages, consistent with a single event of exclusively basal-to-apical ingression of the cleavage furrow.

To corroborate these observations, we expressed GFPtagged mouse anillin (GFP-anillin) in NE cells by *in ovo* electroporation into Hamburger-Hamilton (HH) stages 10

and 11 chick spinal cord neuroepithelium, along with cytoplasmic mRFP to identify cell bodies and processes of targeted M-phase cells. Time-lapse imaging was carried out 24 h after electroporation (HH stages 18 and 19), when chick NE cells were in a similar state with regard to neurogenesis (onset) as those in the mouse E10.5 telencephalon. Three representative examples of time-lapse imaging are shown in Figure 8 (see also the corresponding Supplementary Movies S8-S10). GFP-anillin localized as expected to the nucleus during interphase (Figure 8Aa-Ca, asterisks), as did endogenous anillin in mouse NE cells (Figure 5Aa, asterisk). When the cell body reached the ventricular surface (by interkinetic nuclear migration; Figure 8Aa, double arrowhead), the nuclear envelope started to breakdown (Figure 8Ab and Ca, double arrowheads) and the anillin spot appeared at a distal site in the basal process (Figure 8Ab, Ba and Cb, arrowheads). The anillin spot then migrated exclusively in the basal-to-apical direction within the basal process until it reached the cell body located at the ventricular surface (Figure 8Ab-g, Ba-e and Cb-g, arrowheads). Anillin then accumulated at the basal pole of the cell body and associated with the contractile ring (Figure 8Ah, Bf and g, and Ch, arrows), which then narrowed (Figure 8Bh and Ci-l, arrows). After completion of cytokinesis, anillin re-localized to the nuclei of the daughter cells (Figure 8Cm and n, pairs of double arrowheads). It should be noted that the spatial resolution obtained in the live imaging of chick spinal cord NE cells did not allow for reliable correlation of the migrating anillin spot with bisection of the basal process (Supplementary Figure S4). Nonetheless, these fixed and live-tissue observations together demonstrate that a key component of the vertebrate cytokinesis machinery operates in the basal-to-apical direction within the basal process of M-phase NE cells.

Discussion

Taken together, our results on the structure and dynamics of the basal process, and the observations on the dynamics of the cytokinesis protein anillin, demonstrate that the basal process of vertebrate NE cells can be bisected during M phase.



Figure 4 Zebrafish NE cells can split their basal processes prior to cell body cytokinesis, and the multiple basal processes can be symmetrically or asymmetrically inherited by the two daughter cells. (**A-C**) Selected frames of dividing NE cells in 24 h.p.f. zebrafish embryos from the Supplementary Movies S5–S7, respectively. NE cells express membrane EGFP and nuclear RFP. Bars = $10 \,\mu$ m. (A) In this cell, the basal process (arrow) begins to split approximately 8 min ahead of cleavage of the cell body. In this case, the original process has several short side branches along its length and splits into four as the cell body approaches cytokinesis. The four processes quickly become two pairs of two, and each daughter cell (asterisks) inherits a pair of basal processes (symmetric inheritance). Arrowheads show the ingression of cytoplasm and cell membrane from basal towards the apical surface. Images were acquired every 80 s. (B) In this cell, the basal process (arrows) begins to split into two approximately 15 min ahead of cleavage of the cell body, and both basal processes are inherited by only one of the daughter cells (asterisk, asymmetric inheritance). Arrowheads show the ingression of cytoplasm and cell membrane from basal towards the apical surface. Images were acquired every 80 s. (B) In this cell, the basal process (arrows) begins to split into two approximately 15 min ahead of cleavage of the cell body, and both basal processes are inherited by only one of the daughter cells (asterisk, asymmetric inheritance). Arrowheads show the ingression of cytoplasm and cell membrane from basal towards the apical surface apparent by begins to split at time point 70′, approximately 15 min ahead of cleavage of the cell body. In this case, both basal processes are inherited by one daughter (asterisk, asymmetric inheritance). In both divisions, the daughter that does not inherit the process quickly re-grows its own basal process, which reaches the basal surface apparently by climbing along its sister's basal process or

Frequency of basal process bisection

We observed a split basal process in at least a quarter to a third of all E10.5 mouse NE cells during M phase. However,

several factors indicate that this number may be an underestimation of the real frequency of bisection. First, not all MPM-2-stained basal processes are expected to show anillin spots because, by metaphase, the anillin clusters have moved from the basal process into the cell body. Second, light microscopy is unlikely to resolve all bifurcations in bisected basal processes, particularly when they are tightly pressed against each other in the very dense telencephalic tissue. Third, an unbisected process, as revealed by HVEM, may reflect an M-phase NE cell in prophase, shortly before bisection starts. Consistent with these considerations, basal process splitting was observed in the vast majority of cases for zebrafish NE cells.

Basal process bisection in NE versus radial glial cells, and implications for cell fate

The bisection of the basal process has been previously considered a possibility for the division of NE cells (Wodarz and Huttner, 2003). Instead, previous studies of radial glia divisions using time-lapse fluorescence imaging reported that their substantially longer basal process is inherited by only one of the two daughter cells (Miyata et al, 2001; Fishell and Kriegstein, 2003). Our observations, mainly with NE cells in the E10.5 mouse, when radial glia have not yet appeared, are therefore consistent with these views. This suggests that the much longer basal process of radial glial cells is less likely to undergo bisection than that of NE cells. In this context, the observed splitting of the basal process at E14.5 (Figure 3), a stage well after the onset of transformation of NE cells into radial glial cells (Kriegstein and Götz, 2003; Götz and Huttner, 2005), may have been from the recently characterized 'short neuronal progenitors' (Gal et al, 2006), the basal process of which is shorter than that of radial glial cells. Alternatively, it may also be possible that some radial glial basal processes are also bisected, but that this could not be revealed by the limited resolution of light microscopy. Indeed, our EM analyses of the basal process showed that

Figure 5 The basal process of M-phase NE cells contains anillin, which is concentrated in a spot-like structure. (A) Association of anillin with the contractile ring in the cell body and a spot-like structure in the basal process of M-phase NE cells. A frozen section of E10.5 mouse telencephalic neuroepithelium was triple stained with MPM-2 antibody (a, red), anillin antibody (a, green and b) and DAPI (DNA, c). Strong anillin immunoreactivity was observed on the contractile ring in the M-phase cell body undergoing cytokinesis (arrow in a) at the apical surface (down), and in a discrete spot within an MPM-2-labelled basal process extending from another Mphase cell body (arrowhead in a, corresponding region is also indicated in b and c). Note the anillin immunoreactivity in the nucleus of NE cells in interphase (asterisk). Bar = $5 \mu m$. (B) F-actin is concentrated at the anillin spot in the basal process of M-phase NE cells. E10.5 mouse telencephalic neuroepithelium was triple stained with MPM-2 antibody (a and d, red), anillin antibody (b and d, green) and phalloidin to label F-actin (c and d, blue). Note the Factin staining of the anillin spot in the MPM-2-labelled basal process (arrowheads) and on the apical surface of the neuroepithelium (down). Bar = $5 \mu m$. (C) The anillin spot is localized to the bifurcation site of the basal process of M-phase NE cells. E10.5 mouse telencephalic neuroepithelium was double immunostained with MPM-2 (a, b, e, f; d and h, red) and anillin (c, g; d and h, green) antibodies. (a and e) Low-power views of two MPM-2-labelled NE cells, the cell bodies of which are located directly at the ventricular surface of the neuroepithelium (bottom) and which extend a basal process to the basal lamina (top). (b-d and f-h) High-power views showing the localization of the anillin spots at the bifurcation sites of the MPM-2-labelled basal processes (arrowheads in a-d and e-h). Bars = $5 \mu m$. All images are 1- μm optical sections.

the diameter of the individual branches of a basal process can be less than 100 nm and their distance to each other minimal. Such dual branches with a combined thickness below the theoretical resolution of conventional light microscopy may thus appear as one process.

The splitting of the NE basal process during M phase has implications for the relationship between inheritance of this process and progeny fate. Our observations with zebrafish spinal cord NE cells, showing that at the onset of neurogenic divisions the inheritance of the split basal processes by the daughter cells may be either symmetric or asymmetric, highlight the potential complexity of this relationship. With regard to mouse cortical NE cells, our HVEM data (Table I) show that the basal process is split in 40% (25+15%) of cases. Although this may be an underestimate (see above considerations on frequency), it nonetheless raises the





Anillin spots Mitotic cells

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Figure 6 Both NE cells undergoing proliferative and neurogenic divisions contain anillin spots in their basal process. (A, B) Examples of anillin spots in the basal process of NE cells undergoing either proliferative or neurogenic divisions. Frozen sections of E10.5 heterozygous Tis21-GFP knock-in mouse telencephalic neuroepithelium (e) were triple stained with MPM-2 antibody (a and c, red), anillin antibody (b and c, green) and DAPI (DNA, d). Single 1-µm optical sections are shown. The M-phase NE cells in (A, B) indicated by the grey arrowheads in (e) do not express Tis21-GFP, indicative of proliferative division, whereas the cells in (A, B) indicated by green arrowheads in (e) express Tis21-GFP, indicative of neurogenic division. White arrowheads in (b and c), anillin spots in the basal process of the M-phase cells. Apical surface is down. $Bar = 5 \mu m.$ (C) Quantitation of anillin spots in the basal process of NE cells undergoing either proliferative or neurogenic divisions. Left columns: Tis21-GFP expression was analysed in 20 M-phase NE cells in the E10.5 telencephalon that showed a basal process with an anillin spot, as exemplified in (A, B), and the proportion of M-phase Tis21-GFP negative (grey column) and Tis21-GFP positive (green column) NE cells showing a basal process with an anillin spot is expressed as a percentage of the sum of both cell populations. Right columns: in each of the fields, the absence (grey columns) or presence (green column) of Tis21-GFP expression was also determined for all MPM-2-labelled NE cells (i.e. irrespective of whether or not the single optical section showed a basal process with an anillin spot; n = 125), and the data are expressed as a percentage of the sum of Tis21-GFP-negative plus Tis21-GFPpositive cells.



Figure 7 The anillin spot appears at the distal end of the basal process at the onset of mitosis and translocates towards the NE cell body as mitosis proceeds. E10.5 mouse telencephalic neuroepithe-lium was triple stained with MPM-2 antibody (a–e, red), anillin antibody (a–e green; the relevant regions are shown as single staining in a'–e') and DAPI to determine the stage of mitosis from chromosome structure (a"–e"). Representative examples for the relationship between the position of the anillin spot and the stage of mitosis are shown: (a, a', a") prophase, (b, b', b") prometaphase, (c, c', c'') metaphase, (d, d', d'') anaphase and (e, e', e'') telophase. Arrowheads, anillin spot in the basal process; arrow, apical anillin cluster; dashed line and double arrowhead, contractile ring at different stages. (a–e) Apical surface is down. Bars = 5 μ m. All images are 1- μ m optical sections.

possibility that not all symmetric NE cell divisions at E10.5, which constitute the vast majority of all NE cell divisions at this early stage, necessarily include basal process splitting. In addition, the similar frequency of occurrence of anillin spots in the basal process of NE cells undergoing symmetric proliferative divisions and NE cells undergoing asymmetric neurogenic divisions suggests that basal process splitting is not a specific feature of symmetrically dividing NE cells. Moreover, as basal process splitting is not necessarily followed by symmetric inheritance by both daughter cells, a range of scenarios of basal process inheritance and daughter cell fate can be envisioned. Further investigations are needed to explore these issues.

A novel temporal relationship between cytokinesis progression and anaphase onset with implications for cleavage plane orientation

Our observations on the localization and dynamics of anillin in NE cells during M phase suggest an unexpected temporal relationship between the beginning of cell cleavage and the onset of sister chromatid segregation. In NE cells, anillin is contained in the nucleus during interphase and released on



Figure 8 Time-lapse imaging of GFP-anillin reveals basal-to-apical migration of the anillin spot in the basal process during M phase of NE cells. Plasmids encoding GFP-anillin and mRFP were electroporated into NE cells of chick spinal cord, and organotypic slices were observed by confocal laser scanning microscopy, acquiring an image every 100 s. (**A**-**C**) Sequences of selected images for GFP-anillin showing three examples of M-phase NE cells (for the entire sequence of GFP-anillin images, see Supplementary Movies S8–S10; for mRFP staining, see Supplementary Figure S4). (A) NE cell entering M phase (b; note onset of nuclear envelope breakdown) and progressing to the beginning of formation of the contractile ring (h). (B) NE cell in M phase progressing to cytokinesis in the cell body (f-h; note the decrease in the size of the contracile ring, which is oriented parallel to the optical section). (C) NE cell entering M phase (b, onset of nuclear envelope breakdown) and progressing through cytokinesis (note the two daughter cell nuclei in n). (A–C) Time of time-lapse is indicated in minutes and seconds at the bottom of each panel. Double arrowheads, nuclei/cell bodies; arrowheads, anillin spot in the basal process; white dashed lines, basal process (identified by mRFP expression, not shown; for an example, see Supplementary Figure S4); arrows, contractile ring in the cell body at different stages; asterisks, GFP-anillin-containing interphase nuclei. Apical surface is down.

nuclear envelope breakdown, consistent with reports for other animal cells (Field and Alberts, 1995; Oegema *et al*, 2000). In these other cells, which lack processes, the first anillin-containing, cytokinesis-related structure that forms is the contractile ring at anaphase/telophase, which typically starts to divide the cell body only after the sister chromatids have been segregated (Field and Alberts, 1995; Oegema *et al*, 2000). However, in M-phase NE cells, the first anillin structure found was the spot at the very end of the basal process, detected already in prophase/prometaphase. This anillin spot, colocalized with F-actin, was localized to the bifurcation sites of the dividing basal process, moved within this process together with mitotic progression and reached the cell body before the start of sister chromatid segregation. These dynamics are very likely to constitute the bisection of the basal process. This in turn implies that cytokinesis involving the basal process occurs before the onset of sister chromatid segregation in anaphase, and then proceeds in the cell body via the contractile ring during anaphase/telophase. Consistent with this, basal process bifurcation in the zebrafish NE cells is observed up to 15 min ahead of sister chromatid segregation.

NE cells undergoing symmetric divisions usually show a cleavage plane orientation parallel to their apical-basal axis (Smart, 1973; Landrieu and Goffinet, 1979; Chenn and McConnell, 1995; Das et al, 2003; Haydar et al, 2003; Kosodo et al, 2004; Fish et al, 2006; Wilcock et al, 2007), and there is increasing evidence, in particular for the early stages of neurogenesis, that this also the case for the vast majority of asymmetric divisions (Das et al, 2003; Kosodo et al, 2004; Konno et al, 2008). This orientation is remarkable, given that NE cells are very elongated (Huttner and Brand, 1997) and would be expected to cleave perpendicular to their long axis (Thery and Bornens, 2006), which is also how invertebrate neural progenitors cleave (reviewed in Huttner and Brand, 1997; Fishell and Kriegstein, 2003; Wodarz and Huttner, 2003; Götz and Huttner, 2005). The early basal-toapical movement of the anillin spot may provide the mechanistic explanation for this intriguing parallel orientation of the plane in mammalian neural progenitors. cleavage Specifically, the arrival of the anillin spot at the origin of the basal process before anaphase onset could be a nucleation cue for the formation of the contractile ring within the cell body. This association of the cytokinesis machinery with the origin of the basal process, a defining structure of the apical-basal cell axis, could orient the cleavage plane parallel to this axis. In short, the maintenance of a basal process in M phase, through which the cytokinesis machinery migrates, could set the stage for the ingression of the cleavage furrow along the basal-to-apical axis.

Materials and methods

Immunostaining

Immunostaining on cryosections of mouse brains was performed according to standard procedures (Kosodo *et al*, 2004). For details, see Supplementary data.

DNA constructions, electroporation and mouse whole-embryo culture

Plasmids were constructed according to standard protocols. Proteins were expressed by plasmid electroporation into mouse NE cells followed by whole-embryo culture. For details, see Supplementary data.

Golgi impregnation and HVEM observation

Heads of E10.5 NMRI mouse embryos were fixed overnight at 4°C in 4% paraformaldehyde, 0.1% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4. Golgi impregnation of the fixed brains was performed using the FD Rapid GolgiStain Kit (FD NeuroTechnologies) according to the manufacturer's instructions. After the silver impregnation step, brains were dehydrated in a graded series of alcohol, infiltrated with propylene oxide and embedded in Epon–Araldite.

HVEM was performed as described earlier (Kosaka *et al*, 2001), with modifications. Epon–Araldite-embedded Golgi-impregnated

heads were cut into 100-µm-thick sections. NE cells that, judging from the shape of the cell body and its location directly at the ventricular surface, appeared to be in M phase were identified under a light microscope using a × 40 objective (Olympus BX61). Selected sections were cut further into 5-µm-thick sections and examined with a high-voltage electron microscope (Hitachi H-1250M; at the National Institute for Physiological Sciences, Okazaki, Japan) at an accelerating voltage of 1000 kV. Stereo-paired photomicrographs were prepared by tilting the specimen stage $\pm 8^\circ$. For electron tomography analysis, a series of tilted images was recorded from -60° to $+60^\circ$ with 2-5° increments along the y axis.

SEM

E9.5 NMRI mouse embryos were fixed overnight at 4° C in 2.5% glutaraldehyde and 1% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4. Heads of fixed embryos were subjected to freeze fracturing to expose the lateral surface of NE cells suitable for scanning. Fractured heads were re-fixed in 2.5% glutaraldehyde and 1% paraformaldehyde, post-fixed in 2% osmium tetroxide in 0.1 M phosphate buffer, dehydrated in ascending concentrations of ethanol (50, 70, 90, 95 and 100%), immersed in *t*-butyl alcohol at room temperature, cooled on ice and subjected to freeze drying under continuous vacuum. The dried samples were mounted on brass stubs, coated with platinum in a sputter coater (JEOL JFC-1600), and viewed in a scanning electron microscope (JEOL JSM-5600LV) at an accelerating voltage of 10 kV.

Time-lapse imaging of Dil-labelled cells

Isolated E14.5 C57BL/6 mouse neocortices were incubated for 15-30 min on ice in DMEM/F12 containing 1 µg/ml of extremely fine crystals of DiI C18(3) (1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate; D-282; Molecular Probes, Eugene, OR, USA) (Miyata et al, 2001; Saito et al, 2003). Coronal slices (200 µm thick) were cut using a vibratome (Leica VT1000S) and embedded in collagen (Type I-A, Cellmatrix; Nitta Gelatin, Japan), prepared according to the manufacturer's instructions to a concentration of 1.5 mg/ml in DMEM/F12, using a glass bottom Petri dishes (Bioscience Tools), such that the apical-basal axis of the tissue was parallel to the observation plane. Slice culture was performed in DMEM/F12 supplemented with $1 \times N2$ (Invitrogen), $1 \times B27$ (Invitrogen) and 100 U/ml penicillin-streptomycin, at 37°C in an atmosphere of 40% O2, 5% CO2 and 55% N2, with recirculation of the medium. Time-lapse recording was started 2 h after initiating the culture. Images were acquired using a Zeiss Axiovert 200 epifluorescence microscope equipped with a $\times 40$ C-Apochromat water immersion objective (NA 1.2), at 12-min intervals, collecting 19 images at 2.5-µm z-steps using automated image acquisition software (Metamorph). Images were processed using Imaris software (Bitplane AG).

Time-lapse imaging in zebrafish

Wild-type zebrafish embryos were injected at the 64–120 cell stage with mRNAs coding for membrane EGFP and nuclear RFP to obtain mosaic expression. Confocal time-lapse imaging was started at 24 h.p.f. The embryos were anaesthetized and mounted dorsal side up in 1.5% low-melting point agarose (Sigma). Long-working distance $\times 40$ or $\times 63$ water immersion objectives were used. Embryos were maintained throughout the time lapse at 28.5°C in embryo medium containing anaesthetic and 0.003% 1-phenyl-2-thiourea to prevent pigmentation. Images were taken every 1–4 min, collecting a series of *z*-levels between 1 and 2 µm apart at each time point.

Chick electroporation and time-lapse imaging of GFP-anillin

Chick *in ovo* electroporation was performed as described (Dubreuil *et al*, 2000). HH stage 10 and 11 chick embryos were coelectroporated at the level of the spinal cord with pCAGGS-GFPanillin (0.3–1 mg/ml) and pCAGGS-mRFP (Campbell *et al*, 2002) (0.3 mg/ml), applying five pulses of 25 V for 30 ms each. Spinal cord slices (\sim 500 µm) were prepared 24 h after electroporation (HH stages 18 and 19) and cultured according to Haubensak *et al* (2004) in Leibovitz medium supplemented with 10% chicken serum, 5% fetal calf serum and penicillin–streptomycin. Dual colour time-lapse recording (100s intervals, \sim 60s for scanning one set of 10–15 \sim 1.2-µm-thick optical sections) was performed with an Olympus FluoView 1000 confocal microscope. Images were processed using Imaris software (Bitplane AG).

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (http://www.embojournal.org).

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