

Nonselective Sister Chromatid Segregation in Mouse Embryonic Neocortical Precursor Cells

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We have investigated whether the precursor cells that give rise to the neurons of the neocortex during mouse embryonic development segregate sister chromatids nonrandomly upon mitosis, as would be predicted by the immortal strand hypothesis. Using various protocols of 5-bromo-2-deoxyuridine (BrdU) labeling and chase, we were unable to detect BrdU label-retaining neocortical precursor cells at any of the embryonic stages analyzed, even when the entire brain was analyzed by serial sectioning. Analysis of mitotic neuroepithelial and radial glial cells revealed BrdU-labeled sister chromatid segregation to both nascent daughter cells, which showed a mirror-symmetrical pattern in the first and a non-mirror-symmetrical pattern in the second division after BrdU labeling. Taken together, our data are incompatible with embryonic neocortical precursor cells segregating the sister chromatids selectively to one daughter cell upon mitosis and hence argue against the existence of immortal DNA strands in these cells. In light of the previously reported existence of immortal DNA strands in adult neural stem cells, we discuss that either 1) embryonic and adult neural stem cells in the cortex are distinct or 2) that most, if not all, of the embryonic precursor cells to neocortical neurons are progenitor cells rather than true neural stem cells.

Keywords: BrdU, immortal strand hypothesis, neocortex, neural precursor cells

Introduction

Neurogenesis during the development of the mammalian brain involves neural precursors with stem cell-like properties, that is, neuroepithelial cells and the radial glial cells they transform into (Kriegstein and Götz 2003; Götz and Huttner 2005; Merkle and Alvarez-Buylla 2006). Likewise, neural stem cells are thought to participate in the neurogenesis that persists in certain regions of the adult mammalian brain, specifically, the wall of the lateral ventricle and the dentate gyrus of the hippocampus (Alvarez-Buylla et al. 2001; Dietrich and Kempermann 2006; Merkle and Alvarez-Buylla 2006). Embryonic and adult neural stem cells share certain features, such as epithelial cell polarity and the expression of certain molecular markers (Alvarez-Buylla et al. 2001; Merkle and Alvarez-Buylla 2006).

However, the neural stem cells in the embryonic and adult mammalian brain also differ from each other, notably with regard to the extent to which they undergo self-renewing divisions. Thus, during development, the size of the mammalian neocortex increases to reach a plateau, after which net growth ceases, and the generation of neocortical neurons occurs only during the early phase of cortical development (Rakic 1985; Bayer and Altman 1991; Spalding et al. 2005; Bhardwaj et al. 2006). This implies a limited number of self-renewing divisions of the embryonic neural stem cells involved. By contrast, during

adult mammalian neurogenesis, the neural stem cells in the wall of the lateral ventricle and in the dentate gyrus of the hippocampus are thought to continuously, that is, throughout life, undergo self-renewing divisions leading to the generation of neurons (Dietrich and Kempermann 2006; Merkle and Alvarez-Buylla 2006).

A single neural stem cell, like any other stem cell, can self-renew and generate differentiated progeny only by an asymmetric (as opposed to symmetric) division (Götz and Huttner 2005). Such asymmetric divisions of neural stem cells have been observed in the embryonic rodent neocortex by time-lapse imaging (Miyata et al. 2001, 2004; Noctor et al. 2001, 2004, 2008; Haubensak et al. 2004; Attardo et al. 2008; Konno et al. 2008). Moreover, to address the cell biological basis of embryonic neural stem cell self-renewal by asymmetric division, the differential distribution of cellular constituents between the daughter stem cell and the daughter cell fated to differentiate has been investigated (Miyata et al. 2001; Kosodo et al. 2004; Konno et al. 2008). The key molecules of the cellular substructures examined in these studies, notably the apical membrane and cell cortex, the adherens junctions, and the basal process, have been proteins, not nucleic acids.

An important concept in the context of stem cell self-renewal by asymmetric division is the “immortal strand” hypothesis (Cairns 1975). This hypothesis proposes that in repeating asymmetric self-renewing divisions of a stem cell, upon sister chromatid separation, the chromatids containing the original template DNA strands all end up in one daughter cell, that is, the daughter stem cell. Although appealing as a potential mechanism to further the ability of the repeatedly dividing stem cells to correct replication errors, the immortal strand hypothesis has been controversial (Lansdorp 2007; Rando 2007; Lew et al. 2008).

A major line of evidence in favor of the immortal strand hypothesis is the nonrandom segregation of labeled DNA to the daughter cells (Potten et al. 2002; Karpowicz et al. 2005; Shinin et al. 2006; Conboy et al. 2007). With regard to neural stem cells, such evidence has been reported for cells isolated from the adult lateral ventricle and grown in vitro (Karpowicz et al. 2005). Given that embryonic and adult neural stem cells share certain properties but differ with regard to other features, notably the extent to which they undergo self-renewing divisions, an important question arising is whether or not nonrandom segregation of DNA occurs during the divisions of neural stem cells in the developing brain in vivo. Here we have addressed this question by investigating the fate of labeled DNA in neural precursor cells of the embryonic mouse neocortex.

Materials and Methods

BrdU Labeling

Mouse embryos of strain C57BL/6 were obtained from natural overnight matings of adult mice. The developmental stage of the embryos was defined as embryonic day (E) 0.5 at noon of the following day. For BrdU labeling of mouse embryos, single or repeated daily intraperitoneal injections of BrdU into pregnant mice (20–30 g weight, 1 mg BrdU per injection) were performed at noon, as indicated in the figures.

Immunohistochemistry

Mouse embryos (E9.5–E11.5) or dissected embryonic brains (E12.5–E16.5) were fixed in 4% paraformaldehyde in 120 mM phosphate buffer overnight at 4 °C. BrdU immunohistochemistry was performed on 10- to 14- μ m cryosections as described previously (Calegari et al. 2005). Stained cryosections were analyzed using an Olympus epifluorescence microscope or a Zeiss confocal laser-scanning microscope (Zeiss LSM510).

Quantifications

Confocal images obtained with a 40 \times objective were used. Typically, one image was taken per cryosection, 2 cryosections were analyzed per embryo, 2 embryos per pregnant mouse, and 1–2 pregnant mice per time point and independent experiment. The proportion of BrdU-labeled precursor cells was determined by counting the number of BrdU-immunoreactive nuclei in comparison to the number of 4',6-diamidino-2-phenylindole (DAPI)-stained nuclei in the ventricular zone (VZ) and, when present, subventricular zone of the dorsal telencephalon in a given entire image. The amount of BrdU incorporated per single nucleus was determined by first defining the nuclear area from the DAPI-stained image and then measuring the mean intensity of the pixels in this area in the BrdU-immunostained image, using ImageJ software.

Results

Mouse Neocortical Precursor Cells Do Not Retain BrdU Label during Development

In a first set of experiments, we labeled mouse embryos by a single administration of BrdU (which is incorporated during DNA replication in S-phase) at E9.5 and analyzed the cortical wall of the dorsal telencephalon for BrdU-labeled cells at various time points thereafter, until E13.5 (Fig. 1A). This period of cortical development was chosen because it includes the onset of neurogenesis (Bayer and Altman 1991; Haubensak et al. 2004), the transformation of neuroepithelial cells into radial glial cells (Kriegstein and Götz 2003; Götz and Barde 2005), and the switch of these precursor cells to stem cell-like asymmetric divisions (Miyata et al. 2001, 2004; Noctor et al. 2001, 2004, 2008; Haubensak et al. 2004; Attardo et al. 2008; Konno et al. 2008). Consistent with the known cell cycle parameters of cortical precursor cells, in particular the proportion of cells in S-phase (Takahashi et al. 1995; Calegari and Huttner 2003; Calegari et al. 2005), >60% of the cells in the cortical wall, virtually all of which are precursor cells at this stage of development (Bayer and Altman 1991; Calegari and Huttner 2003; Haubensak et al. 2004), were labeled 6 h after

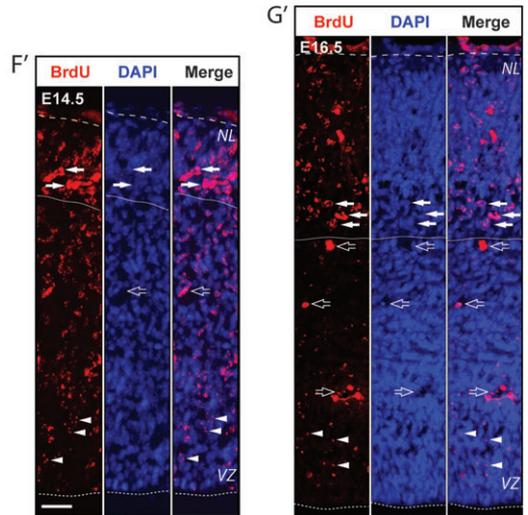
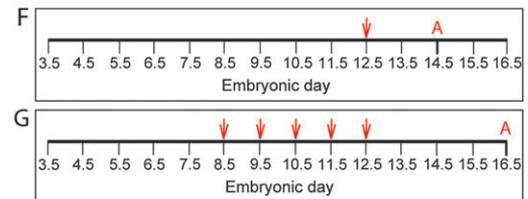
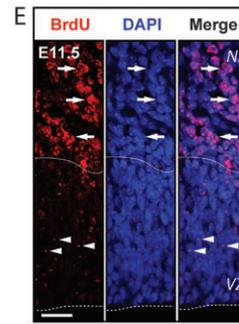
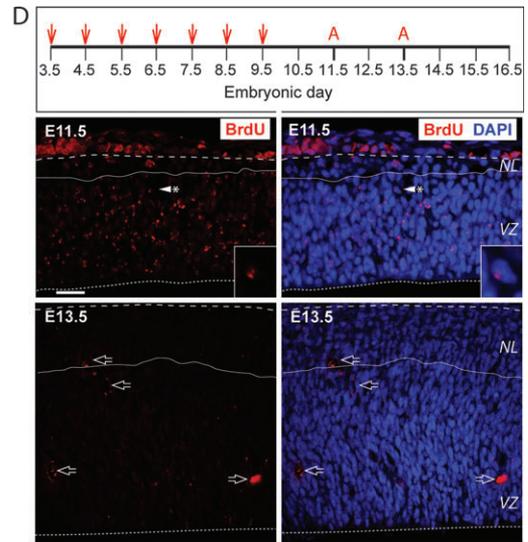
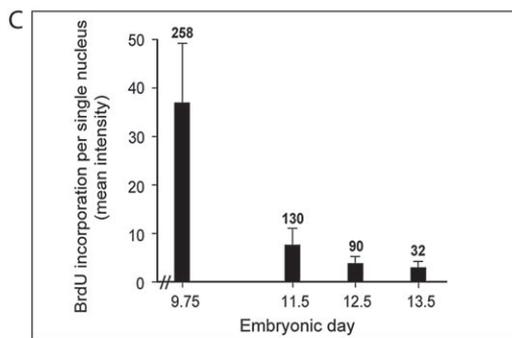
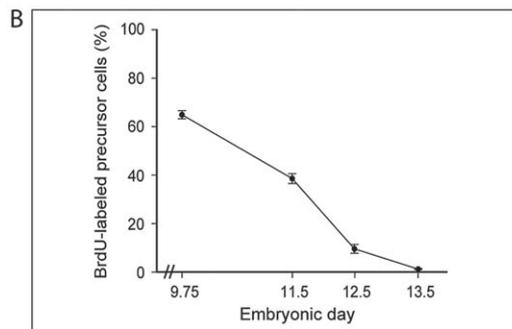
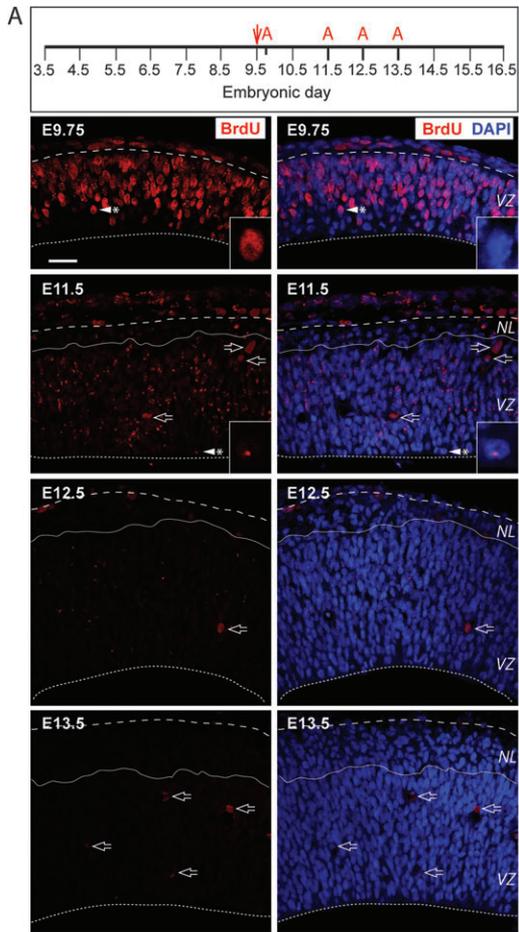
BrdU injection (E9.75). In many cells, BrdU immunoreactivity was found to be broadly distributed throughout the DAPI-stained interphase nucleoplasm (Fig. 1A, top row insets). Chasing the BrdU label for 2–4 days (E11.5–E13.5) resulted in a virtually complete loss of BrdU label from the precursor cells by E13.5, as revealed by analysis of the VZ (Fig. 1A,B). This loss was preceded by a marked reduction in the abundance of BrdU immunoreactivity in the nucleoplasm, which was evident already at E11.5 (Fig. 1A, second row and insets; Fig. 1C). Thus, with regard to E9.5 precursor cells and their progeny, these data are consistent with the notion that in the ensuing cell divisions the sister chromatids were distributed randomly, rather than nonrandomly, between the 2 daughter cells, resulting in the stepwise reduction in the proportion of BrdU-labeled chromosomes per nucleus and, eventually, the increasing appearance of progeny with unlabeled nuclei.

It could be argued that the synthesis of immortal DNA strands occurred earlier in development than E9.5. If so, their possible selective inheritance by only 1 of the 2 daughter cells, that is, the self-renewed stem cell, in the precursor cell divisions occurring between E9.5 and E13.5 might not have been detected in the above experiments—the respective other daughter cells would have divided in a non-stem cell manner, with random sister chromatid distribution and, consequently, lack of retention of BrdU label. We investigated this possibility by labeling mouse embryos by daily administration of BrdU during the period E3.5–E9.5, followed by analysis of the cortical wall for BrdU label-retaining precursor cells at E11.5 and E13.5 (Fig. 1D). However, the results were very similar, if not identical, to the data obtained after single BrdU administration at E9.5 (Fig. 1A–C). Specifically, the BrdU immunoreactivity in the nucleoplasm of precursor cells was already sparse at E11.5 (Fig. 1D, top row and insets), and virtually no BrdU label-retaining cells could be detected by E13.5 (Fig. 1D, bottom row).

To corroborate this lack of BrdU label-retaining cells, one of the E13.5 brains was subjected, in its entirety, to consecutive serial sectioning, and each of the 332 sagittal 12- μ m-thick cryosections was immunostained for BrdU-labeled cells and examined. Upon inspection of the precursor cell-containing layers, not a single cell with BrdU immunoreactivity distributed through most of its nucleoplasm (i.e., similar to the staining shown in Fig. 1A, top row insets), or at least showing multiple BrdU-immunoreactive spots, was detected neither in the dorsal telencephalon nor in the more caudal parts of the brain (down to the hindbrain). Rather, the precursor cell-containing layers in the cryosections lacked BrdU immunoreactivity as shown in the representative example in Figure 1D, bottom row.

As a positive control that BrdU label-retaining cells can be detected in the above experiments, we examined neurons. In the E13.5, dorsal telencephalon of embryos that had received the last, or only BrdU administration at E9.5, BrdU-labeled cells

Figure 1. Lack of BrdU label-retaining precursor cells in the mouse embryonic neocortex. Mouse embryos were subjected to BrdU labeling as indicated in the panels in (A, D, F, G) depicting the timescale of embryonic development; red arrows indicate single BrdU injections and red letters “A” time points of analyses. (A, D–G') BrdU immunostaining (red) and DAPI staining (blue), shown alone or merged as indicated, of coronal cryosections of dorsal telencephalon (A, D, F', G') and hindbrain (E; BrdU labeling as in D) at the indicated stages of embryonic development. Arrowheads with asterisks, single nuclei shown at higher magnification in the insets (note that the top right inset in A is DAPI staining only); open arrows, autofluorescent blood vessels; arrowheads, precursor cell nuclei with sparse BrdU immunoreactivity; arrows, neuronal nuclei with abundant BrdU immunoreactivity; dotted lines, ventricular surface; dashed lines, basal lamina; solid lines, boundary between the germinal layers and the NL. Scale bars, 20 μ m. (B) Quantitation of BrdU-immunoreactive precursor cells in the VZ and, when present, subventricular zone after BrdU labeling as in (A). Data are expressed as a percentage of total cells present in the area of quantification, as determined by DAPI staining, and are the mean of 12 (E9.75), 6 (E11.5), 4 (E12.5), and 6 (E13.5) images; bars indicate standard deviation (SD). (C) Quantitation of the BrdU incorporation per single precursor cell nucleus after BrdU labeling as in (A). Data indicate the average intensity per nuclear area and are the mean of the indicated number of nuclei; bars indicate SD.



in the neuronal layers (NL) were detected only very rarely (and if so, showing only sparse dots of immunoreactivity; Fig. 1A,D, NL). This presumably reflected the fact that the neurogenic progenitors had already lost most of the BrdU label by E11.5 (Fig. 1A,D). By contrast, in the E11.5 hindbrain of these embryos, where neurogenesis is known to start earlier than in the dorsal telencephalon (Iacopetti et al. 1999; Haubensak et al. 2004), we did observe plenty of neurons with abundant BrdU label, whereas the precursor cells in the VZ showed only sparse BrdU immunoreactivity, indicating that they had lost most of their BrdU label between E9.5 and E11.5 (Fig. 1E). This difference in BrdU label retention between neurons (abundant label) and precursor cells (sparse dots) was also observed in the dorsal telencephalon at later stages of development when precursor cell cycle lengthens and neurogenesis is more advanced, specifically, when BrdU was administered at E12.5 and the tissue analyzed at E14.5 (Fig. 1F,F') or when BrdU was administered daily between E8.5 and E12.5 and the tissue analyzed at E16.5 (Fig. 1G,G').

Taken together, our observations indicate that the neural precursor cells that give rise to the neurons of the mouse neocortex do not retain BrdU label during embryonic development.

Mitotic Neocortical Precursor Cells Do Not Distribute BrdU-Labeled DNA Asymmetrically

Whereas normally the sister chromatids of the various chromosomes are segregated, during mitosis, randomly to the 2 daughter cells (Fig. 2A, left), the immortal strand hypothesis predicts that the sister chromatids containing the immortal DNA strands all end up in one daughter cell only (Fig. 2A, right). In the latter case, on BrdU pulse-chase labeling, analysis of mitotic figures should show segregation of BrdU-labeled DNA into one of the daughter cells, which would be either the immortal strand-inheriting or the noninheriting cell depending on whether the BrdU labeling took place when the immortal strands were first synthesized (not illustrated in Fig. 2A) or during a subsequent round of replication (Fig. 2A, right). Although the lack of BrdU label-retaining precursor cells (Fig. 1) already rendered the existence of nonrandom sister chromatid segregation very unlikely, we nonetheless directly addressed this issue by analyzing neocortical precursor cells dividing at the ventricular surface for the distribution of BrdU-labeled DNA in anaphase/telophase. For this analysis, E12.5–13.5 embryos were chosen because asymmetric self-renewing divisions of neural stem cells are known to occur abundantly at this stage (Miyata et al. 2001, 2004; Noctor et al. 2001, 2004, 2008; Haubensak et al. 2004; Attardo et al. 2008; Konno et al. 2008).

To this end, mouse embryos received a single BrdU administration at E12.5, followed by analysis either 5–6 h thereafter, that is, when in light of the known cell cycle parameters of neocortical precursor cells (Takahashi et al. 1995; Calegari et al. 2005) a BrdU-labeled mitotic figure would represent the first M-phase after BrdU incorporation (Fig. 2B, see also panel A), or 22–24 h thereafter, that is, when BrdU-labeled mitotic figures would represent precursor cells undergoing their second M-phase after the BrdU labeling (Fig. 2C, see also panel A). Analysis 5–6 h after BrdU labeling revealed that the BrdU immunoreactivity was mirror symmetrically distributed over the segregating sister chromatids (Fig. 2B); the 4 cases shown are representative of >50 mitotic BrdU-labeled cells analyzed. Analysis of >200 mitotic BrdU-labeled cells 22–24 h after administration of the tracer showed that, in every single case, the BrdU immunoreactivity was associated in

similar intensity with both daughter cells (see Fig. 2C for 4 representative examples). Interestingly, the BrdU-immunoreactive spots in the nascent daughter cells were not found in a mirror-symmetrical pattern but in a distinct pattern when comparing the 2 sets of sister chromatids (Fig. 2C). These observations are indicative of random sister chromatid segregation during the division of mouse neocortical VZ precursor cells and are not compatible with the selective segregation behavior expected for immortal DNA strands.

Discussion

We have studied the fate of BrdU-labeled DNA during the division, and in the progeny, of the precursor cells to neocortical neurons in the developing embryonic mouse brain. Collectively, our data provide evidence against the selective segregation of sister chromatids upon precursor cell mitosis that is predicted by the immortal strand hypothesis. Such selective segregation has been reported for neural stem cells isolated from the adult lateral ventricle and grown in vitro (Karpowicz et al. 2005). Unless one assumes that nonrandom segregation of sister chromatids only occurs in isolated neural stem cells dividing in vitro, our data leave us with 2 possible interpretations as to the nature of the precursor cells that give rise to neocortical neurons during embryonic development.

First, neural stem cells participating in embryonic and adult neurogenesis are different from one another with regard to sister chromatid segregation, with selective segregation taking place in adult, but not embryonic, neural stem cells. As mentioned in the Introduction, during neurogenesis in the developing embryonic neocortex, the neuroepithelial and radial glial precursor cells that have been regarded as neural stem cells and shown to undergo asymmetric self-renewing divisions (Miyata et al. 2001, 2004; Noctor et al. 2001, 2004, 2008; Haubensak et al. 2004; Attardo et al. 2008; Konno et al. 2008) do so only for a limited number of divisions, until the neurons of the neocortex have been generated. By contrast, the neural stem cells in the wall of the lateral ventricle and in the dentate gyrus of the hippocampus from which neurons are derived during adult neurogenesis are thought to keep on dividing throughout life (Alvarez-Buylla et al. 2001; Dietrich and Kempermann 2006; Merkle and Alvarez-Buylla 2006). If one extrapolates from the selective sister chromatid segregation observed for isolated adult neural stem cells in vitro (Karpowicz et al. 2005) and assumes that such selective segregation also occurs upon division of neural stem cells in the wall of the lateral ventricle and dentate gyrus of the hippocampus in adult mammals in vivo, an appealing concept to reconcile the previous (Karpowicz et al. 2005) and present observations would be that adult neural stem cells undergoing a virtually unlimited number of divisions distribute their DNA after replication according to the immortal strand hypothesis, whereas the neural stem cells in the embryonic neocortex, undergoing only a limited number of divisions, do not.

However, even if future investigations should provide evidence supporting this concept for neural stem cells, it is clear that it is not applicable in general. Studies investigating the immortal strand hypothesis in nonneural tissues indicate that not all stem cells in adult tissues that divide continuously throughout life, or have the capacity to do so, distribute their DNA after replication according to the immortal strand hypothesis. Thus, whereas observations consistent with this hypothesis have been reported for certain tissue stem cells, for example, isolated muscle satellite cells in vitro (Shinin et al.

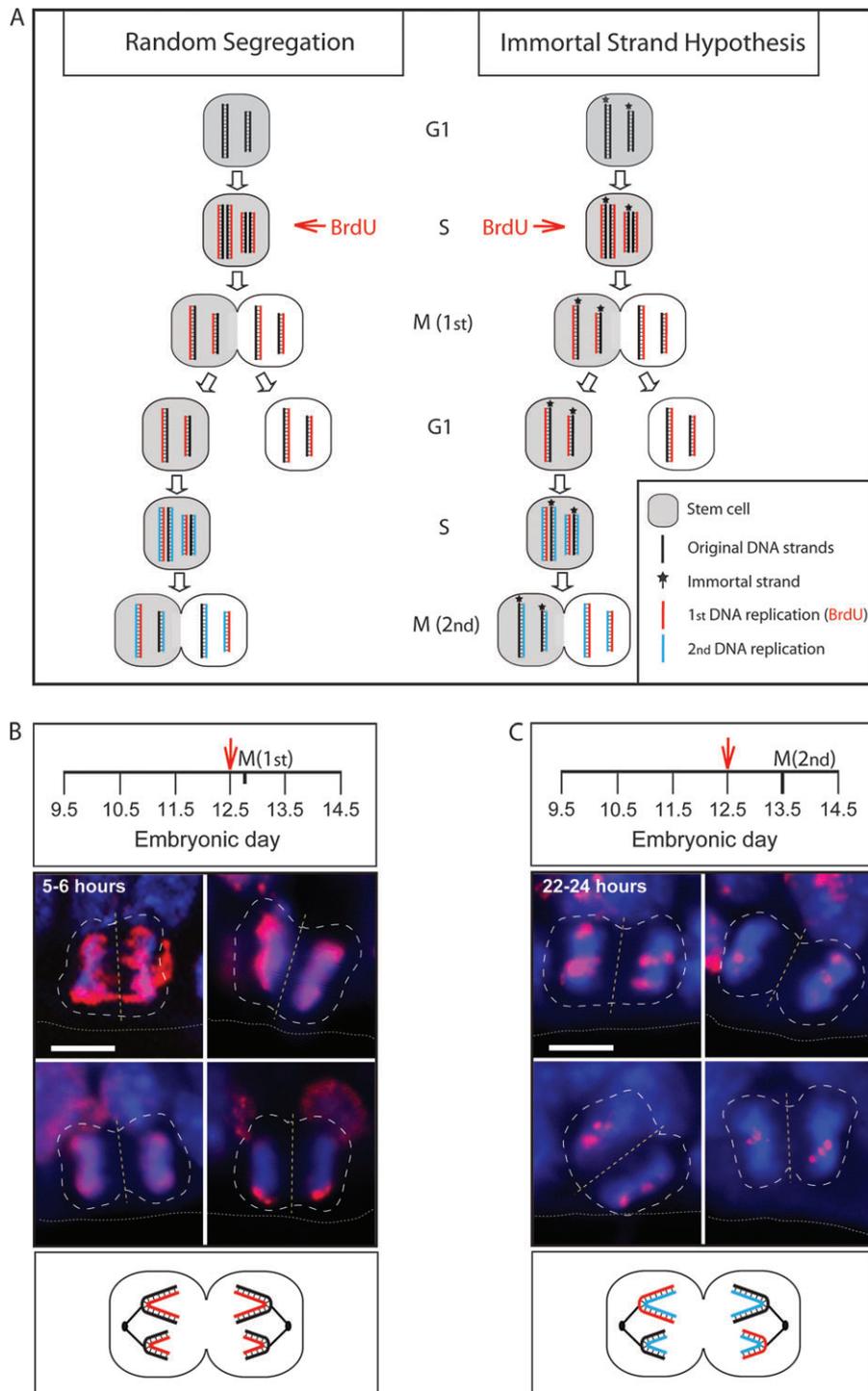


Figure 2. Lack of asymmetric distribution of BrdU-labeled DNA to the nascent daughter cells of mitotic neocortical precursor cells. (A) Cartoon illustrating the random segregation of BrdU-labeled sister chromatids to both daughter cells during the second M-phase after BrdU labeling of a stem cell (left) and the segregation into one daughter only upon inheritance of the unlabeled immortal DNA strands by the other daughter (right). For simplicity, only 2 chromosomes (1 long and 1 short) are depicted. For details, see key. (B, C) Mouse embryos were subjected to BrdU labeling as indicated in the panels in (B, C) depicting the timescale of embryonic development; red arrows indicate single BrdU injections; “M(1st)” and “M(2nd)” indicate the time point of analysis 5–6 h after BrdU labeling (B), that is, the first M-phase after BrdU incorporation (see panel A), and 22–24 h after BrdU labeling (C), that is, the second M-phase after BrdU incorporation (see panel A), respectively. BrdU immunostaining (red) combined with DAPI staining (blue) of coronal cryosections of dorsal telencephalon is shown. White dashed lines outline the mitotic cell; yellow dashed lines indicate the cleavage plane; white dotted lines, ventricular surface. Note that irrespective of the amount of BrdU incorporation, which is different for the 4 cells in each set, the pattern of distribution of BrdU-labeled DNA to the nascent daughters is mirror symmetrical in (B) but not in (C), as also illustrated in the cartoons at the bottom (red DNA strands, see also panel A). Scale bars, 5 μ m.

2006; Conboy et al. 2007), evidence against the immortal strand hypothesis has been reported for hematopoietic stem cells (Kiel et al. 2007), one of the canonical somatic stem cells.

A second possible interpretation of our observations is that most, if not all, of the neuroepithelial and radial glial precursor cells during embryonic neocortical development in vivo, rather

than being true neural stem cells, are progenitor cells whose self-renewal over a limited number of asymmetric divisions is more apparent than real. This interpretation is not contradicted by the findings that these cells can form neurospheres in vitro (Reynolds and Weiss 1996; Tropepe et al. 1999), as this could be the result of the in vitro conditions used, and the predictive value of the neurosphere assay for the occurrence of neural stem cells is limited (Reynolds and Rietze 2005).

Be this as it may, the selective sister chromatid segregation in adult neural stem cells (Karpowicz et al. 2005), but not in embryonic neocortical precursor cells (this study), has an interesting implication. Given that the adult neural stem cells in the wall of the lateral ventricle arise from embryonic neocortical precursor cells (Merkle et al. 2004; Merkle and Alvarez-Buylla 2006), our data raise the possibility that DNA strands can acquire an immortal fate as adult neural stem cells emerge during late embryonic and/or postnatal development.

Funding

Deutsche Forschungsgemeinschaft (DFG) (SPP 1109, Hu 275/7-3; SPP 1111, Hu 275/8-3; SFB/TR 13, B1; SFB 655, A2) to WBH; DFG-funded Center for Regenerative Therapies Dresden; Fonds der Chemischen Industrie; Bundesministerium für Bildung und Forschung, Nationales Genomforschungsnetz.

Notes

We thank Christiane Haffner for the rhodamine-conjugated anti-BrdU antibody, Jeremy Pulvers for help with data analysis, and the Light Microscopy and Animal Facilities of Max Planck Institute of Molecular Cell Biology and Genetics (MPI-CBG) for excellent support. J-FF was a member of the International Max Planck Research School for Molecular Cell Biology and Bioengineering. *Conflict of Interest:* None declared.

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