Characterization of the lateral diffusion barrier established in neural progenitor cells during embryonic cortical development

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Abstract

Development of the cerebral cortex occurs via successive divisions of neural progenitor cells (NPCs) generating distinct neocortical layers that build up the mammalian brain. It has been shown during development in mice that a subset of NPCs exhibits a diffusion barrier in the endoplasmic reticulum (ER) that may be critically involved in the establishment of asymmetric cell division. However, it remains unclear if the ER-diffusion barrier is dynamically regulated in the course of embryonic cortical development. We here used fluorescence loss in photobleaching (FLIP) experiments in organotypic slice cultures of the developing mouse brain to analyze the presence and strength of the ER diffusion barrier in apical progenitors (APs) and basal progenitors (BPs) throughout cortical development. Our data indicate that a substantial fraction of APs and BPs establish an ER-diffusion barrier during cell divisions and that the strength of the ER barrier is decreased in BPs compared to APs. In addition, we show that during development, APs' diffusion barrier strength reaches a peak around e15-e16, while the strength of the BP diffusion barrier is reduced with advancing developmental stages. Thus, our findings may suggest that the ER diffusion barrier is associated with the asymmetric division of NPCs during brain development that may contribute to the functional and cellular diversity of brain tissues.

Introduction

The formation of the central nervous system is a highly complex process that requires the proper patterning and subsequent specification of a large variety of neuronal and glial cell types. After the regionalization of the neural tube, one of the three vesicles to be formed will give rise to the forebrain [1]. The forebrain will be further subdivided into the diencephalon and telencephalon forming structures that include the cerebral cortex. During the first stages of cortical development, the pseudostratified neuroepithelium of the telencephalon is comprised of neuroepithelial cells (NECs). During the onset of neurogenesis, NECs symmetrically divide and give rise to neural progenitor cells (NPCs) called radial glia cells (RGCs) [1]. RGCs – or apical progenitors (APs) – amplify their pool by undergoing symmetric proliferative divisions and then generate neurons of all cortical layers in the brain, first directly through asymmetric cell divisions and then through intermediate/ basal progenitors (BPs), leading to cortical specification and expansion [2] [3] [4]. Throughout divisions in the subventricular zone (SVZ), BPs show a more restricted potential, giving rise mainly to neurons (rodents) or undergoing a few proliferative divisions (primate) before their terminal division [5] [6] [7]. In contrast, RGCs are also the source of glial cells (e.g., astrocytes and oligodendrocytes) during later stages of neurogenesis [8].

Part of the mechanism involved in the generation of cell-type a diversity from RGCs may involve asymmetric segregation of cargoes, i.e., cellular components. Asymmetric partition of cargoes can generate progeny with distinct fates by segregating fate determinants to only one daughter cell. In addition, other structural components of cells (e.g., cellular structures such as basal process, midbody, or primary cilium and organelles such as the Golgi apparatus or centrosomes) can be unequally inherited by daughter cells and thus shape the fate and behavior of daughter cells. Thus, the asymmetric segregation of cellular components represents a mechanism to regulate fate but also to perpetuate stemness by maintaining cellular fitness and through the elimination of damaging or aging factors [9] [10] [11] [12] [13].

An organelle that seems to have a critical impact on the segregation of cargoes is the endoplasmic reticulum (ER). It has been shown that ER stress can activate unfolded protein response (UPR), favoring direct neurogenesis from RGCs and impairing the generation of intermediate progenitors [14]. Additionally, a lateral diffusion barrier in the membrane of the ER has been correlated with asymmetric segregation of damaged proteins in NPCs, suggesting a mechanism to segregate age and to maintain stemness [11]. To investigate how the ER-diffusion barrier is developmentally regulated, we here used fluorescence loss in photobleaching (FLIP) experiments to assess the exchange of luminal and membrane-bound ER proteins between daughter cells during AP and BP divisions. We used green fluorescent protein (GFP)-tagged reporters fused either to an ER luminal protein (KDEL; LumER-GFP) or to an ER membrane protein (Sec61a; MemER-GFP). We performed FLIP experiments in progenitor cells of the mouse embryonic brain in the developmental stages embryonic day 14 (e14) until e17.

Objective

To determine how the ER-diffusion barrier is regulated in NPCs during mammalian brain development.

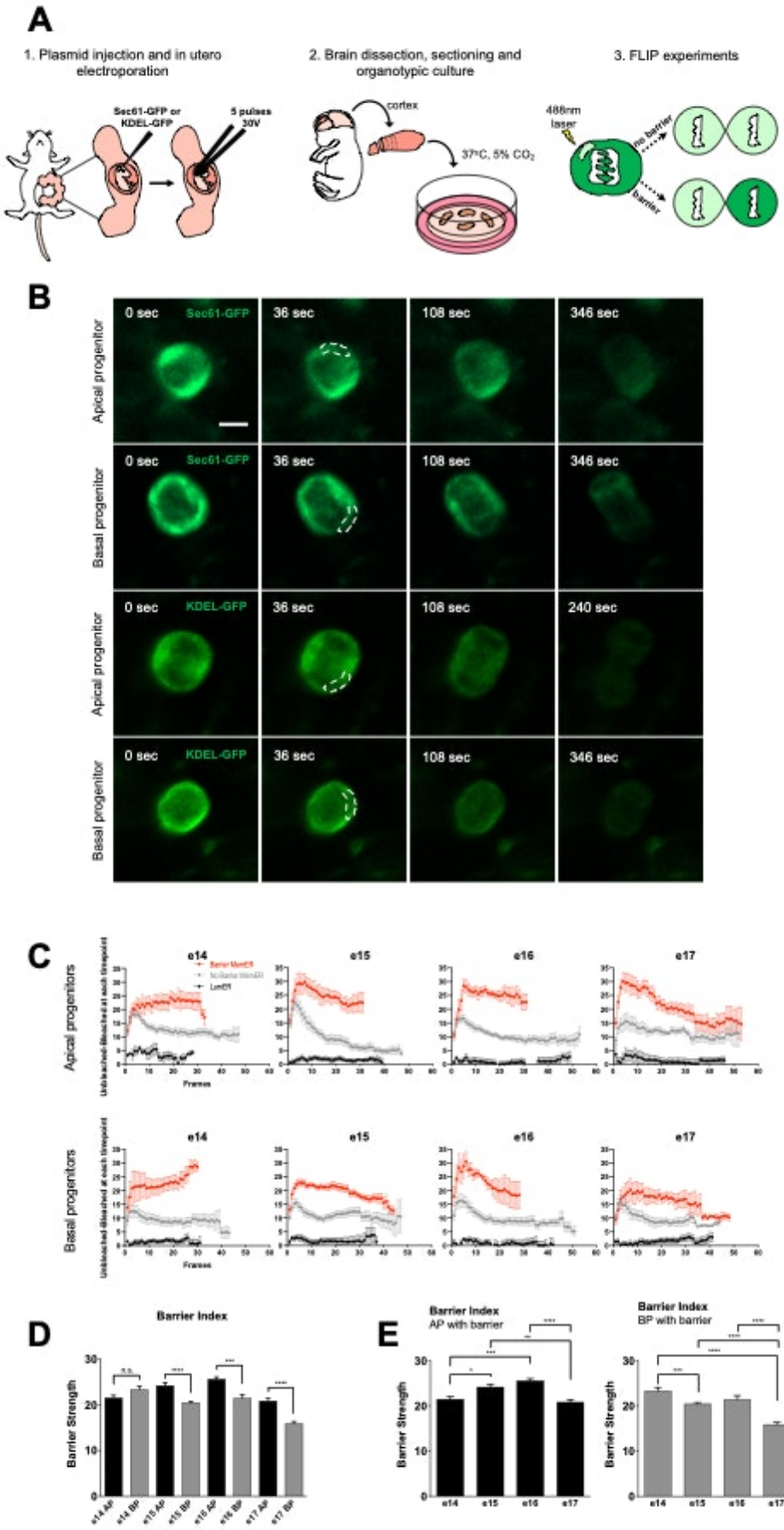
A
1. Plasmid injection and in utero electroporation
Sec61-GFP or KDEL-GFP
5 pulses 30V
2. Brain dissection, sectioning and organotypic culture
cortex
37°C, 5% CO2
3. FLIP experiments
488nm laser
no barrier
barrier
B
Apical progenitor
Basal progenitor
Apical progenitor
Basal progenitor
0 sec
Sec61-GFP
KDEL-GFP
36 sec
108 sec
346 sec
240 sec
C
Apical progenitors
Basal progenitors
e14
e15
e16
e17
Frames
D
Barrier Index
Barrier Strength
E
Barrier Index
AP with barrier
BP with barrier
Barrier Strength

Figure Legend

Figure 1. A subpopulation of NPCs in the developing mouse brain cortex establishes an ER-diffusion barrier during mitosis that is maintained throughout the onset of neurogenesis.

(A) Schematic representation of the sample preparation evidencing (1) plasmid injection (Sec61-GFP or KDEL-GFP) and electroporation performed in the embryos *in utero*, (2) brain dissection and organotypic slice culture preparation, and (3) fluorescence loss in photobleaching (FLIP) experiments being performed during anaphase by repeatedly bleaching a small region of interest during time-lapse imaging, leading to a retention of fluorescence in the unbleached daughter cell with a loss in the bleached daughter cell overtime when a diffusion barrier is present.

(B) Time-course of fluorescence loss during FLIP of Sec61a-GFP and KDEL-GFP in the apical and basal progenitor of the developing mouse brain cortex. FLIP in the Sec61a-GFP was performed at e17 (IUE at e16) for the apical progenitor and at e15 (IUE at e13) for the basal progenitor. FLIP in the KDEL-GFP were both performed at e15 (IUE at e14). Scale bar: 5 mm.

(C) Averaged single-cell traces of the difference in fluorescence intensity of Sec61a-GFP (MemER-GFP) between unbleached and bleached compartments during FLIP for each imaged cell reveal subpopulations that possess a barrier (red lines, Barrier MemER, Sec61a-GFP) and those that do not possess a barrier (gray lines, No barrier MemER, Sec61a-GFP) relative to LumER FLIP traces (black lines, KDEL-GFP). The number of cells for each group is depicted in supplementary figure 1.

(D & E) Quantification of barrier strength derived from one-phase association fitted curves created from **(C)** and comparing **(D)** Barrier AP X Barrier BP in the different embryonic stages (e14 to e17), and **(E)** only the APs or BPs with barrier among themselves. (One-way ANOVA, mean ± SEM, $^{****}p < 0.0001$).

Results & Discussion

Here we used organotypic slice cultures from embryonic *in utero* electroporated mouse brains and performed FLIP experiments to assess the dynamics of the ER-diffusion barrier throughout cortical development (Fig. 1A). APs and BPs transfected with Sec61a-GFP (MemER-GFP) or KDEL-GFP (LumER-GFP) were imaged and a small cellular region was repetitively photobleached from anaphase until cytokinesis was completed in order to assess the diffusion in the ER throughout cell division (Fig. 1B). We systemically collected FLIP data from the APs and BPs in the developmental stages e14, e15, e16, and e17. Further, we measured the fluorescence intensity in each time point from both daughter cells. We then calculated how much fluorescence was lost in each compartment over the period observed using the first time point as a reference (100%). Photobleaching of LumER-GFP resulted in a similar loss of fluorescence between the bleached and unbleached daughter cells (Fig. 1B and Movie 1). Similar results were observed in a subset of MemER-GFP cells (Movies 3, 5). In contrast, FLIP of MemER-GFP showed a robust fraction of APs (Movie 2) and BPs (Movie 4) with an unequal loss of fluorescence between both compartments, consistent with previous reports, and suggesting the presence of a diffusion barrier in the membrane of the ER during NPC division [11].

To systematically classify the establishment of an ER-diffusion barrier, we subtracted the bleached from the unbleached fluorescence intensities in LumER-GFP single-cell traces and used the standard deviation of the highest difference as a reference (for details, see *Methods* section). We identified approximately 30% of all APs and BPs establishing a diffusion barrier during cellular division throughout the observed time points of cortical development (e14-e17) (Fig. 1C and Suppl. Fig. 1; Percentages: AP: 31.25% e14, 26.67% e15, 20% e16, and 38.46% e17. BP: 27.78% e14, 48.57% e15, 15% e16, and 31.25% e17, Suppl. Fig. 1B). From the subset of cells that established an ER-diffusion barrier, we next determined the barrier strength (barrier index). To perform this classification, we used the individual traces from the difference of the normalized values between

bleached and unbleached compartments to make an average nonlinear fitted curve and the numbers obtained were used to calculate the barrier index. These analyses were based on the previous reports for similar experiments [11] [15]. APs showed stronger barrier at e15 (p <0.0001), e16 (p <0.001), and e17 (p <0.0001) compared with BP at the same developmental stage, with no difference in the strength observed at e14 (Fig. 1D). The diffusion barrier strength of BPs decreased during neurogenesis, while the strength of the barrier in APs peaked between e15-e16 (Fig. 1E). Since APs are more committed to self-renewal than BPs, the finding of a stronger barrier in APs supports the hypothesis that an ER-diffusion barrier may act as a mechanism to segregate aging/ damaged factors in order to maintain stemness [11].

Additionally, considering that the identity of newborn neurons is spatially but also temporally encoded in the brain cortex, the variation of the barrier strength at the different developmental stages observed among apical progenitor cells may represent an additional mechanism to contribute to the fate specification. Finally, another particular feature of apical progenitor cells is their polarity, and the asymmetric segregation of structural components such as basal process or apical domain can contribute to the fate of daughter cells. In this regard, it would be relevant to investigate if there is a correlation between the diffusion barrier establishment and the orientation of the mitotic cleavage plane.

Limitations

Although the identification of an ER-diffusion barrier during proliferation of NPCs suggests a mechanism to promote asymmetry, further experimental evidence is required to investigate its biological composition and properties, among others the establishment of the ER barrier, and its regulation and association with other cellular events (e.g., fate specification and division history) during brain development.

Additionally, future experiments using long-term imaging will be required to track the bleached-unbleached pair of cells and identify their fate, helping to provide evidence of a potential association between diffusion barrier strength and cellular asymmetry (in terms of fate as well as of cellular fitness/ stemness). However, it will be necessary to track NPCs long enough to observe cellular maturation, which is technically challenging since the bleached cells lose their marker (GFP reporter) and the embryonic brain has limited viability in an *ex vivo* set up [16].

The establishment of an ER-diffusion barrier during cellular proliferation as a mechanism to promote asymmetric segregation of cargoes may be a mechanism to ensure cellular diversity and maintenance of stem cell activity in the nervous system. Considering that neurogenesis is potentially preserved through adulthood in areas such as neocortex and hippocampus [17] [18], understanding how the diffusion barrier is regulated through consecutive divisions and in different niches may provide novel insights how somatic stem cells enable lifelong tissue homeostasis and repair.

Alternative Explanations

Additional Information

Methods

Animals and *in utero* electroporation

Timed-pregnant C57BL/6JRj mice were anaesthetized by intraperitoneal (i.p.) injection of saline solution containing fentanyl (0.05 mg/kg), midazolam (5 mg/kg), and medetomidine (0.5 mg/kg). Plasmids pCAG-KDEL-sfGFP and pCAG-Sec61a-EGFP were diluted in PBS to reach a final concentration of 0.25 mg/mL and 3 mg/mL respectively and injected in the lateral ventricle of the brain of embryos from e12 to e16. Each plasmid was

injected with Fast Green (2.5 mg/mL, Sigma) and electroporated using Electro Square Porator ECM830 (Harvard Apparatus) and tweezertrodes of 3 mm (Harvard Apparatus) targeting the brain cortex. Pulses were applied 5 times at 30 V for 50 ms at 950 ms intervals. Anaesthesia was reversed using i.p. injection of saline solution containing atipamezole (2.5 mg/kg) and flumazenil (0.5 mg/kg) plus buprenorphine (0.1 mg/kg). Animals were allowed to recover in a warm pad and kept under observation. The electroporated embryos were dissected as described by Shitamukai and colleagues [19]. Briefly, 24 h after electroporation, slices of dorsal cortex were cut (approximately thickness of 300 mm) using an ophthalmic microsurgical knife (Alcon), embedded into collagen matrix (Nitta Gelatin, Cell Matrix-type A) and placed in a cell culture insert with 0.4 mm pore membrane (Millicell, 30 mm, Millipore). The insert containing the tissues was placed in a bottom glass dish containing DMEM and F12 (Sigma D6434) with supplements (FBS, HS, N2, B27 minus vitamin A, PSF, Glucose, Glutamax, FGF, EGF). The slices were cultured for 2 h (AP) or 16 h (BP) in cell culture incubator (37°C/ 5% CO_2) before the FLIP experiments start.

FLIP experiments

Using a confocal laser-scanning microscope (Zeiss LSM 800, Carl Zeiss) with a water-immersion 40X objective lens (LUMPlanFl NA0.8, Olympus), heating stage and incubation chamber, brain slices were screened for cells in metaphase, apparent by spherical shape and condensed DNA. Apical mitoses were defined by location at the ventricular surface (apical progenitors, APs) and discriminated from basal mitoses, which occur at the subventricular zone (basal progenitors, BPs).

All cells were imaged in a picture frame size of 512×512 pixels and a scanning speed of 7 ms per pixel. At anaphase, a ROI was drawn in one of the poles avoiding the condensed DNA. After an initial single plane picture was taken, the ROI was bleached for approximately 3 s with the 488 nm laser line at 7% laser power ("bleaching"), 30 iterations. The alternating cycles of "bleaching" and "image acquisition" for FLIP were repeated with an interval of 12 s until cytokinesis was finished, apparent by the time point when an obvious darker line separated the two newly formed daughter cells. The laser settings were kept consistent between experiments, with laser power in the 488 nm wavelength at 4% (Sec61a-GFP) and 2.4% (KDEL-GFP) (580–750 V). A photobleaching control to determine the loss of fluorescence due to repeated imaging was carried out by measuring the loss of fluorescence in the cells that did not receive the ROI for photobleaching.

To analyze the experiments, ROIs were created in ImageJ based on the future cleavage plane in order to separate the bleached and unbleached compartments. At each frame, the mean gray value (the sum of the gray values of all the pixels in the selection divided by the number of pixels) was obtained for each compartment. The analysis was followed by normalizing fluorescence values to the first image that was taken for that compartment. After that, the normalized fluorescence value of the bleached compartment was taken at each timepoint and subtracted from the unbleached compartment. Thus, one value could be given at each time point for each cell, and all cells compared within the same graph. This analysis shows the relationship between the 2 compartments over time, such that the greater the difference of the fluorescence intensity between the two compartments (for example, a strong barrier), the larger the value. A line that remains fairly flat (for example, KDEL-GFP, LumER-GFP) would have little differences between the unbleached and bleached compartments. Cells were separated for analysis into either apical or basal progenitors. Single-cell analysis revealed different populations of cell behaviors, thus further binning was performed to characterize these specific cell populations.

To distinguish between the cells with and without a diffusion barrier, we created 3 criteria. We took the highest value of fluorescence intensity from the averaged LumER-GFP single-cell traces (specific to the type of progenitors analyzed), and then using 6 times its standard deviation, created the first criteria to discriminate between the 2 different populations of Sec61+ cells. Since the different cell population might possess distinct bleaching behavior, we establish two additional criteria (and a cell must to fit 2 out of 3 to be considering possessing a barrier). The three criteria were: I) have at least 1 time

point higher than 6 times its standard deviation; II) the difference between the average of plateau (frames 10 to 25) need to be kept higher than 4 times its standard deviation; and III) the difference between the average of the peak (frames 3 to 7) and the average of the plateau cannot be higher than 30%. Using these criteria, we separate those which had a barrier, and those that did not. The single-cell traces of these 2 distinct bins were then averaged separately to demonstrate the different behaviors of these populations. Statistical analyses to determine Barrier strength were performed at each time point as following: the first image (pre-bleach) was attributed to be 100%, and all measurements after were normalized to this initial image. Then, to combine the data from all cells, the normalized fluorescence of either the unbleached or the bleached compartment at each frame is averaged together from all cells at the same frame. Graphs represent data for all the timepoint where there are at least 3 cells that still have movie frames present. Videos' scale bar: 5 mm.

To perform statistical analysis on these curves, the traces from all cells were used to make an average non-linear fitted curve. A one-phase association was used with Yo=0, and the K and Plateau unconstrained. The numbers obtained from all of these analyses provided the barrier index ("plateau" from the best-fit values) and the standard error ("plateau" from the standard error, SE, values). This Barrier Index (barrier strength) and SE were then entered into Prism for statistical analyses.

Statistical analyses

Analyses were performed using Excel (Microsoft) or Prism (Graphpad). For comparisons of averages among three or more groups, ordinary one-way ANOVA followed by Tukey's multiple comparison test was performed. Differences were considered significant at p <0.05.

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Ethics Statement

All experimental procedures were conducted in accordance with the ethical principles and guidelines for animal experiments of the Veterinary Office of Switzerland and were approved by the Cantonal Veterinary Office in Zurich.

Citations

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