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The Subventricular Zone Is the Developmental Milestone of a 6-Layered Neocortex: Comparisons in Metatherian and Eutherian Mammals

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The major lineages of mammals (Eutheria, Metatheria, and Monotremata) diverged more than 100 million years ago and have undergone independent changes in the neocortex. We found that adult South American gray short-tailed opossum (*Monodelphis domestica*) and tammar wallaby (*Macropus eugenii*) possess a significantly lower number of cerebral cortical neurons compared with the mouse (*Mus musculus*). To determine whether the difference is reflected in the development of the cortical germinal zones, the location of progenitor cell divisions was examined in opossum, tammar wallaby, and rat. The basic pattern of the cell divisions was conserved, but the emergence of a distinctive band of dividing cells in the subventricular zone (SVZ) occurred relatively later in the opossum (postnatal day [P14]) and the tammar wallaby (P40) than in rodents. The planes of cell divisions in the ventricular zone (VZ) were similar in all species, with comparable mRNA expression patterns of *Brn2, Cux2, NeuroD6, Tbr2, and Pax6* in opossum (P12 and P20) and mouse (embryonic day 15 and P0). In conclusion, the marsupial neurodevelopmental program utilizes an organized SVZ, as indicated by the presence of intermediate (or basal) progenitor cell divisions and gene expression patterns, suggesting that the SVZ emerged prior to the Eutherian–Metatherian split.

Keywords: basal progenitors, cortical neurogenesis, cortical unit column, evolutionary biology of cerebral cortex, intermediate progenitors, *Monodelphis domestica*

Introduction

The hallmark of mammalian brain evolution is the emergence of a 6-layered neocortex. Although overall lamination and basic neuronal cell types are largely conserved, mammalian neocortices show dramatic variation of cortical and laminar thickness, cortical neuron number and density, and cortical field number and connectivity (Brodmann 1909; Kaas 2006; Rakic 2008). In one of the most influential and puzzling observations in comparative studies of the adult mammalian cerebral cortex, Rockel et al. (1980) argue that the number of neurons in a 30-μm wide "unit column" in cerebral cortex is constant across mammals (ca. 110 per radial strip in 25-μm sections), despite a diversity of cortical thicknesses and relative proportion of layers . However, this observation does not include marsupials, and recent studies contend that neuronal number does differ across cortical areas and between the species analyzed by Rockel et al. (1980) (Haug 1987; Cheung et al. 2007; Herculano-Houzel et al. 2008; Rakic 2008).

Mammalian cortical neurons originate from 2 germinal areas. Projection neurons are born locally along the neuroepithelium in the dorsal pallium, whereas cortical interneurons are mainly generated in the medial ganglionic eminence (MGE), lateral ganglionic eminence (LGE), and caudal ganglionic eminence (CGE) in the subpallium and travel tangentially to the dorsal pallium (Parnavelas and Nadarajah 2001; Marín and Rubenstein 2003). Tangential migration has been postulated in the equivalent circuit hypothesis as part of the mammalian–reptilian transformation (Karten 1969, 1997), but these tangentially migrating neurons are exclusively inhibitory in mammals and sauropsids (Cobos et al. 2001; Tuorto et al. 2003; Métin et al. 2007; Moreno et al. 2008). As evidence for tangential migration of projection neurons from the subpallium to the cortex in vertebrates is scant, the radial expansion from a 3 to a 6-layered neocortex and subsequent tangential enlargement of cortical surface areas are more likely due to increased progenitor population (Fish et al. 2008) and neural production from the cortical neuroepithelium.

Although neuroepithelial cells (which later turn into radial glial cells [RGCs]) in the ventricular zone (VZ) are universal to all vertebrates, the rate at which they produce progenitors and the length of time over which neurogenesis occurs can vary (Rakic 1995; Dehay and Kennedy 2007). These differences can account for the tremendous variability observed in the size of the cortical sheet in different species. Furthermore, a distinct embryonic mammalian progenitor compartment called the subventricular zone (SVZ) is present, where it is believed the number of neurons is amplified by increasing the rate and duration of neurogenesis. Instead of producing one neuron, RGCs may undergo asymmetric division to produce another RGC progenitor and one intermediate progenitor cell (IPC, or basal progenitor cell) that subsequently migrates to the SVZ. In the SVZ, IPCs undergo 1–3 symmetric divisions to amplify neuron production (Haubensak et al. 2004; Miyata et al. 2004; Noctor et al. 2004). The 2-step pattern of neurogenesis could facilitate the formation of a 6-layered cortex with a larger surface area (Martínez-Cerdeño et al. 2006; Pontious et al. 2007). Conversely, sauropsids have a 3-layered cortex lacking an organized SVZ (Molnár, Tavare, and Cheung 2006; Cheung et al. 2007). These studies suggest that a cortical SVZ is an exclusive hallmark of mammalian neurogenesis. However, this assumption arises from a limited number of species, and generalizations could be premature.

Metatheria (marsupials) have evolved independently from eutheria (often referred incorrectly as "placental" mammals) for approximately 173–190 million years (Kumar and Hedges

2002; Murphy et al. 2004) and represent an interesting group for 2 reasons. First, although they possess a 6-layered neocortex, it contains considerably fewer neurons than eutheria (Haug 1987). Second, morphological studies (Saunders et al. 1989) have hinted that a secondary proliferative zone (the SVZ) is not seen in the dorsal pallium of the South American gray short-tailed opossum (*Monodelphis domestica*), although such zone has been described in tammar wallaby (*Macropus eugenii*) (Reynolds et al. 1985). If an organized SVZ is not present in the opossum, this suggests that the SVZ is not universally required for the generation of a 6-layered neocortex in all mammals. Is it possible that these species have adopted a different strategy for cortical neurogenesis?

In this study, we compared the number of cortical neurons and glia in a unit column of adult opossum, wallaby, and mouse. We examined the pattern of dividing cells and the orientation of mitotic spindles in the VZ of opossum and wallaby. The expression pattern of selected genes with known cortical VZ and SVZ expression in mouse and opossum was also compared. Our data demonstrate that although there are differences in the number of neurons in a standardized column unit of cortex (affecting both infragranular and supragranular layers equally), the general organization of the SVZ has been conserved across all studied mammals.

Materials and Methods

Animals

All animal experiments were conducted in accordance with the UK Animals (Scientific Procedures) Act (1986). All protocols for opossum were approved by the Institutional Animal Care and Use Committee, the University of Melbourne Animal Ethics Committee, the National Institutes of Health, and the National Health and Medical Research Council Australia guidelines.

South American gray short-tailed opossum (*M. domestica*): Brains from postnatal days (P) 6, 7, 9, 10, 12, 14, 16, and 20, which covered early, mid, and late stages of neurogenesis (Table 1), and adult were obtained from N. Saunders (University of Melbourne) and L. Krubitzer (University of California–Davis). The mothers were anesthetized with isoflurane, whereas the pups ($n = 3$ for each stage) were collected from their teats. The pups were terminally anesthetized with isoflurane, decapitated, and fixed in 4% paraformaldehyde (PFA) for 3 days.

Tammar wallaby (*M. eugenii*): Paraffin sections of Bouin's fixed brains from P9, 15, 19, 34, 40, and 50 were obtained from the colony at the Commonwealth Scientific and Research Organization, Division of Wildlife and Rangelands Research, Lynham, ACT, Australia, and described in earlier studies (Dziegielewska et al. 1988). The ages represent early, mid, and late stages of cortical neurogenesis (Table 1). Three adult Tammar wallaby was perfused with 4% PFA (see details below in section on cell quantification in a unit column).

Rat (*R. norvegicus*): The proliferation patterns in opossum and tammar wallaby were compared with rat. Our group has previously described in detail the distribution of phosphohistone H3-immunoreactive cells in rat (Carney et al. 2007). Timed pregnant Wistar rats were obtained from Harlan Laboratories, UK, and maintained in the Oxford University Animal Facility. Brains from embryonic days (E) 13, 17, and 19 were fixed by immersion in 4% PFA for 7–10 h, and coronal sections were cut at 50 μm using a Vibroslicer (Leica VT1000S) for phosphohistone H3 immunohistochemistry.

Mouse (*M. musculus*): Timed pregnant C57/BL6 mice were obtained from the Oxford University Animal Facility. Brains from E15 and P0 ($n = 3$ for each stage) were collected for in situ hybridization.

Cell Quantification in a Unit Column

Three adult mouse, opossum, and wallaby brains were used for quantification. Animals were perfusion fixed with 4% PFA, and coronal sections of mouse and opossum brains were cut at 40 μm using a Vibroslicer (Leica VT1000S). Three interspaced sections (200 μm) within the primary somatosensory area were stained with cresyl violet. The mouse and opossum brains and sections were processed in identical fashion. For wallaby, brains were cut at 60 μm using a cryostat. Cresyl violet–stained sections within the primary visual area were kindly provided by L. Marotte (The Australian National University). Section shrinkage in all specimens, as measured by scanning the *z*-plane using confocal microscopy (Zeiss 710), was 75% (40 to 10 μm, 60 to 15 μm; data not shown). Using Neurolucida 8 (MBF Bioscience, Magdeburg, Germany), an arbitrary 100-μm wide area (a unit column) was defined in the primary somatosensory/visual area spanning layers 1–6 (Fig. 1). Neurons were identified by cytoplasmic and nucleolar staining, whereas neuroglia stain only the nucleus. Cells touching the bottom (the boundary between white matter and layer 6) and left boundaries were counted directly under the microscope, and those touching the top (the pial surface) and right boundaries were excluded. Neuronal and glial cell counts from individual layers were obtained by focusing up and down in the *z*-plane of the section and expressed as mean ± standard error of the mean (SEM). As the section of the wallaby cortex was thicker than that of mouse and opossum, the wallaby data represented in Figure 1*D* have been adjusted to two-thirds of the original counts.

Immunohistochemistry and Cell Quantification

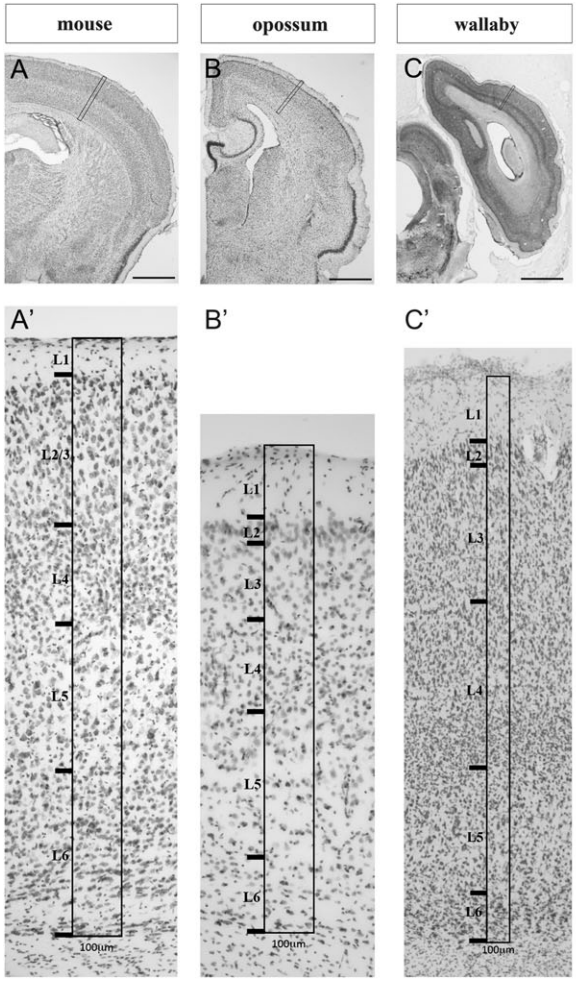
Phosphohistone H3 immunohistochemistry was used to reveal the mitotic division pattern because it is more amenable to quantification and comparisons. Opossum brains at P10, P14, and P16 ($n = 5$ for each stage) were cut coronally at 40 μm using a Vibroslicer, incubated overnight at 4 °C in rabbit polyclonal antiphosphohistone H3 antibody (1:500, Millipore, Livingston, UK) prior to incubation in Alexa546-conjugated goat antirabbit secondary antibody (1:500, Invitrogen, Paisley, UK) for 2 h at room temperature. For wallaby, 8-μm paraffin sections at P15, P40, and P50 ($n = 4$ for each stage) were used.

Due to the small size of the brains, the total number of H3-immunoreactive mitotic figures in the VZ, SVZ, and extraventricular (EV, which include intermediate zone [IZ], cortical plate [CP], and marginal zone [MZ]) regions of the entire dorsal cortex of opossums and tammar wallabies was counted from 3 levels along the rostrocaudal axis of the cortex (rostral, intermediate, and caudal), averaged, and expressed as mean ± SEM. Our quantification was performed on the germinal zone of the entire cortical sector, extending between the cingulate sulcus and the rhinal fissure. In contrast, data for Wistar rat were quantified by sampling 2 regions in the dorsal cortex of E13, 17, and 19 pups ($n = 8$, for each stage, Figs 1*B* and 2 in Carney et al. 2007). In brief, H3-immunoreactive mitotic figures in the VZ, SVZ, and EV were counted using an ocular reticule of 66 000 μm^2 under a ×40 objective lens in the dorsolateral and dorsomedial cortex from 3 levels along the rostrocaudal axis.

In a recent study, it has been demonstrated that 2 dense vascular plexi exist in the VZ and SVZ during cortical development, especially at E14

Table 1
Comparisons of the gestational ages at similar cortical developmental stages for the species used in this study. (Cheung and Kondo et al.)

	Gestational period (days)	Developmental stage				References
		Initial stage (no SVZ)	Early stage (small SVZ)	Later stage (large SVZ)	Final stage (small SVZ)	
Mouse	19–20	Before E12	E13–E15	E16–P0	P1	Takahashi et al. (1995)
Opossum	14	Before P6	P7–P12	P14–P24	P64–P100	Saunders et al. (1989)
Wallaby	27	Before P2	P5–P15	P20–P50	P67–P213	Reynolds et al. (1985)



D

	Mouse		Opossum		Wallaby	
	Neurons	Glia	Neurons	Glia	Neurons	Glia
Total	**743±9**	**117±12**	**418±29**	**89±7**	**544±13**	**130±8**
L1	13±3	5±3	14±4	7±2	15±3	5±3
L2/3	183±5	22±5	122±17	14±2	72±23	22±5
L4	186±5	15±5	105±9	14±2	204±11	15±5
L5	130±3	31±3	96±13	27±3	102±8	31±3
L6	233±7	44±7	81±7	27±5	82±13	44±7

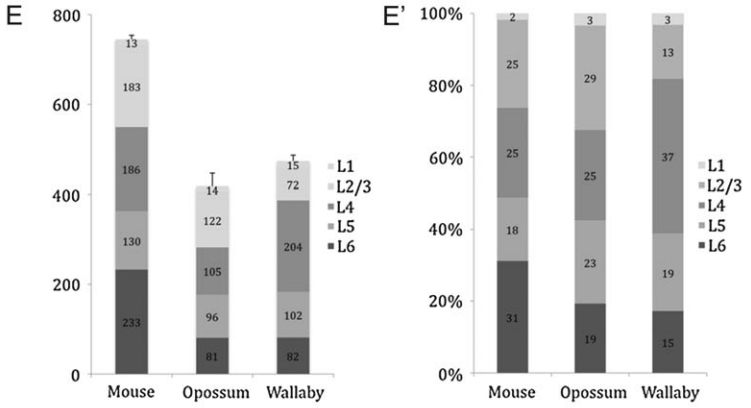


Figure 1. Quantification of the number of neurons in different species. (*A–C*) Cresyl violet–stained sections of adult (*A*) mouse, (*B*) opossum, and (*C*) tammar wallaby. An arbitrary "unit column" (a 100-μm wide, 40-μm thick region [60-μm thick for wallaby] spanning from layer 1 to 6) was marked in the primary somatosensory/visual area (boxed areas in *A–C*, higher magnification in *A'–C'*). The number of neurons and glia was quantified in each layer and expressed as mean ± SEM in (*D*). (*E*) The mean number of neurons present in each cortical layer, showing that the number of neuron in a unit column is not constant between different infraclass within mammals. (*E'*) The proportion of neurons in each cortical layer. Scale bar: *A–B* = 500 μm, *C* = 1 mm.

and 15, when neurogenesis is at its peak (Stubbs et al. 2009). To examine the neurovascular pattern in opossum, 10-μm thick cryostat sections from P6, 12, 14, and 16 brains ($n = 2$) were incubated overnight at 4 °C in fluorescein isothiocyanate-conjugated Griffonia simplifolica isolectin B4 (1:100, Vector Laboratories Inc, Burlingame, CA).

Mitotic Spindle Orientation

Mayer's hematoxylin and eosin (H & E) were used to reveal the angle of mitotic spindle orientation of dividing RGCs. For opossum, a total of 67 cells from 8-μm thick paraffin sections of P7 ($n = 2$, 8 sections) and P9 animals ($n = 2$, 5 sections) were measured. For tammar wallaby, a total of 44 cells from 8-μm thick paraffin sections of P9 ($n = 1$, 2 sections), P15 ($n = 1$, 3 sections), P19 ($n = 1$, 3 sections), P34 ($n = 1$, 1 section), P40 ($n = 1$, 1 section), and P50 ($n = 1$, 1 section) were measured. All sections are interspaced, and the slides were observed under a Leica DMR upright microscope, and images were captured using a Leica DFC500 camera and Firecam software. The relative angle between a line bisecting the 2 chromosome sets and the apical surface (Fig. 3*C*) was measured and scored into six 15° bins as described previously (Konno et al. 2008).

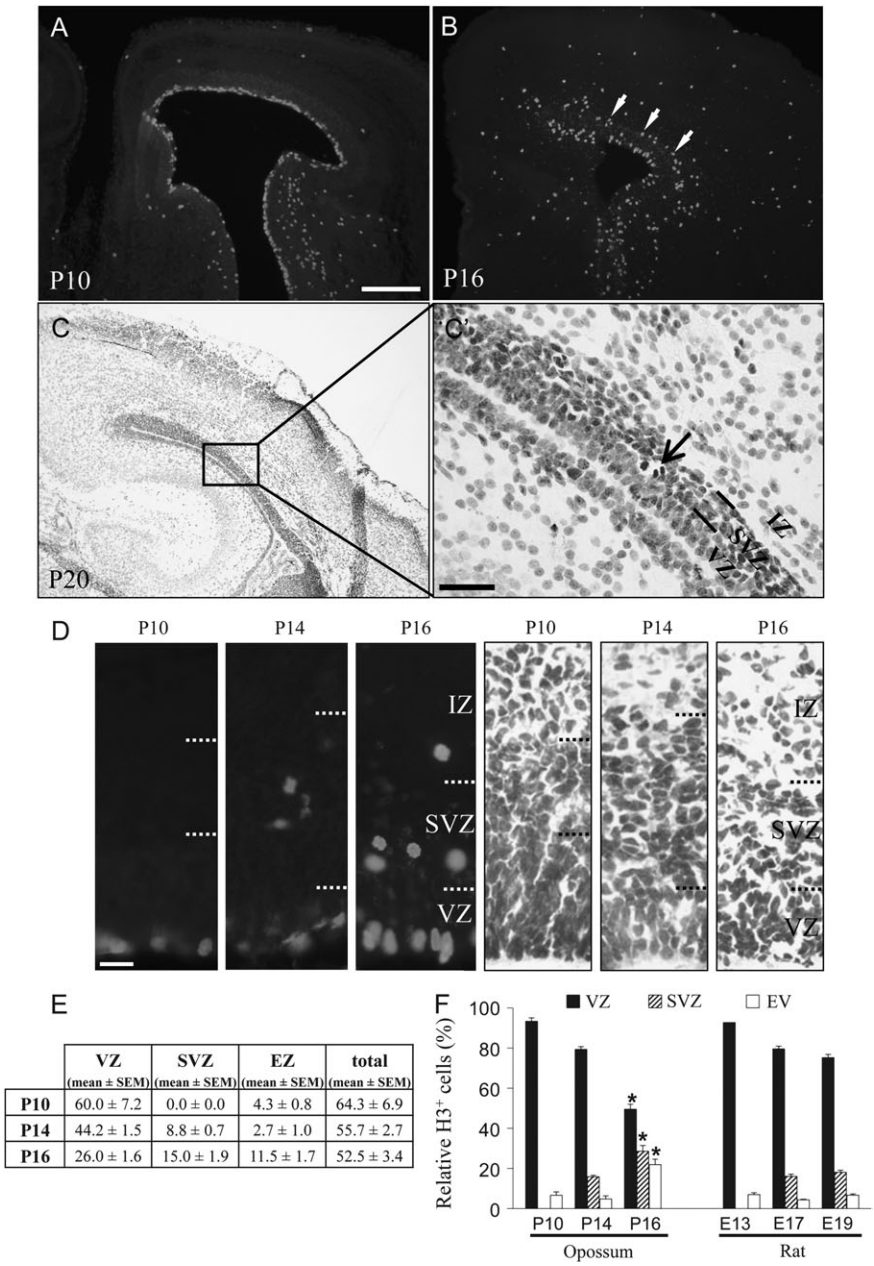
Probe Fragment Isolation and In Situ Hybridization

E15 and P0 mouse brains and P12 and P20 opossum brains were embedded and flash frozen in Tissue Tek O.C.T. compound. Sections were cut at 14 μm using a cryostat (Leica Jung CM3000) and stored at –80 °C. Tissues were then processed for in situ hybridization with digoxigenin-labeled riboprobes for the following genes: *Brn2* (NM_008899), *Cux2* (NM_007804), *NeuroD6* (NM_009717), *Tbr2* (NM_010136), and *Pax6* (NM_013627). Primers for amplification of mouse probe fragments were designed to sequences retrieved from GenBank. Opossum orthologs of the above genes were identified by basic alignment search tool of the opossum genome assembly (The Broad Institute, Cambridge, MA) on the UCSC and Ensembl Genome browsers. The identified opossum fragments were then aligned with known mammalian and chicken sequences using Clustal X (Thompson et al. 1997) and primers designed to regions of conservation outside gene family motifs using Primer3 v0.4.0 (Supplementary Table 1). To define the VZ, SVZ, IZ/subplate (SP), CP, and the MZ, mouse and opossum brain sections stained with cresyl violet were compared.

Results

The Number of Neurons in a Standardized Unit Column of Adult Marsupials Is Fewer than Mouse

To compare the number of neurons per unit volume in the cerebral cortex, the number of neurons of adult mouse,



E

	VZ (mean ± SEM)	SVZ (mean ± SEM)	EZ (mean ± SEM)	total (mean ± SEM)
P10	60.0 ± 7.2	0.0 ± 0.0	4.3 ± 0.8	64.3 ± 6.9
P14	44.2 ± 1.5	8.8 ± 0.7	2.7 ± 1.0	55.7 ± 2.7
P16	26.0 ± 1.6	15.0 ± 1.9	11.5 ± 1.7	52.5 ± 3.4

Figure 2. Distribution of dividing cells in the developing opossum cortex. (*A*, *B*) At P10, the majority of H3-immunopositive (H3+) cells were in the VZ, although a few H3+ cells were in the extraventricular zone (EZ, which includes IZ, CP, and MZ). At this stage, there were no H3+ cells in the cortical SVZ; however, there were numerous SVZ divisions in the subpallium. Only at P14 onward were H3+ cells located in the SVZ (arrows). (*C*, *C*') Cell division in the SVZ could be not only detected by H3 immunohistochemistry but also on Nissl-stained sections (arrow). (*C*') is a higher magnification of the boxed region in (*C*). (*D*, *E*) The anatomical boundary for VZ, SVZ, and EZ region at P10, 14, and 16 was defined using Nissl-stained sections (*D*, right 3 panels), and the number of H3+ cells in each region (*D*, left 3 panels) was then quantified and expressed as mean ± SEM in (*E*). (*F*) The relative ratio of H3+ cells in different anatomical compartments of opossum. Whereas the total number of cell division decreases as development progresses, cell divisions in the SVZ become more prominent, a feature very similar to rat and wallaby (Fig. 4*E*). Asterisks represent a significant difference between opossum and rat ($P < 0.05$, Student's *t*-test). Scale bar: *A*–*C* = 200 μm; *C*' = 30 μm; *D* = 20 μm.

opossum, and wallaby was counted (Fig. 1). There was a significant difference in the number of neurons between the 3 species ($P < 0.05$, one-way analysis of variance). On average, there were 743 ± 9 neurons in mouse; however, there were fewer neurons in both marsupials. Wallaby has 544 ± 13 neurons, whereas opossum has even fewer (418 ± 29), although it could be attributed to different cortical areas selected for quantification. These data indicate that although marsupials have a very similar neocortical organization, they have lower neuronal numbers overall and different neuron/glia ratios than observed in a mouse unit column. It has been suggested that the population of IPCs might be responsible for the increased neuronal numbers in mammalian cerebral cortex (Kriegstein et al. 2006; Molnár, Métin, et al. 2006; Molnár, Tavare, and Cheung 2006; Pontious et al. 2007; Fish et al. 2008). Therefore, differences in adult cortical neuronal numbers suggest that the structure and organization of the germinal zone differs between marsupials and mouse.

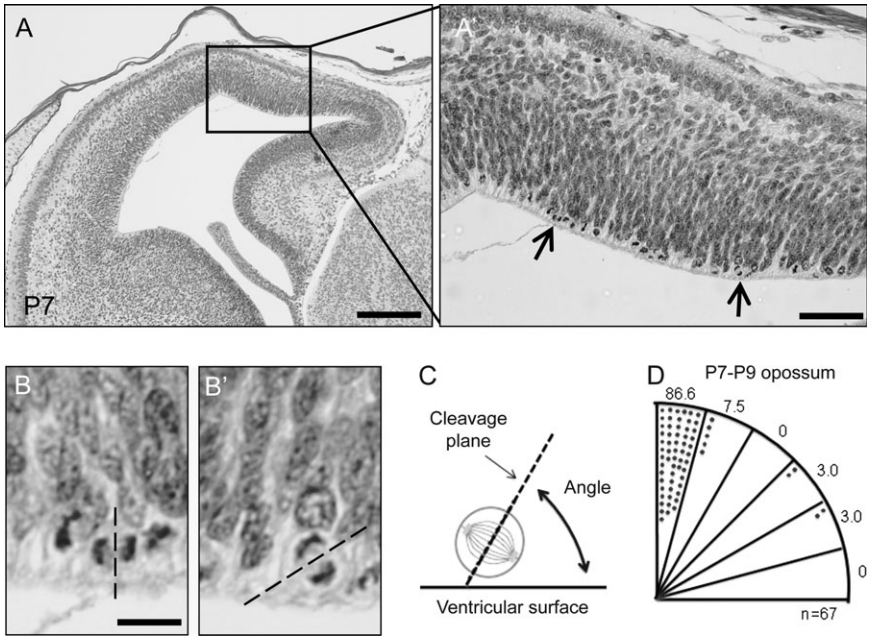


Figure 3. Orientation of mitotic spindle in the developing opossum cortex. (*A*, *B*) H & E was used to reveal neuroepithelial cells that were in anaphase (arrows) in order to measure the angle between the cleavage plane and the ventricular surface. (*A'*) is a higher magnification of the boxed region in (*A*). (*B*) A dividing cell with typical planar division, whereas (*B'*) shows a dividing cell with an oblique spindle orientation. Dotted lines represent the cleavage plane. (*C*) Schematic diagram of a mitotic neuroepithelial cell. The cleavage plane was defined as the orthogonal plane bisecting the line between the center of each chromosome (adapted from Konno et al. 2008). (*D*) Distribution of the cleavage plane orientation for mitotic neuroepithelial cells measured at P7 and P9 and averaged. Similar to mouse, most planar divisions were within 15° of the apicobasal axis. Scale bar: *A* = 200 μm; *A'* = 50 μm; *B* = 10 μm.

Dividing Cells Appear in SVZ at a Later Stage of Cortical Development in Opossum

In P10 opossum brains, the majority of phosphohistone H3–immunoreactive mitotic (H3+) cells were in the VZ. A few H3+ scattered cells located in the CP and IZ, but no H3+ cells were found in the SVZ (Fig. 2*A*). Starting at P14 and by P16, much later than expected, a number of H3+ cells were present in the SVZ (Fig. 2*B,E*). The average number of H3+ cells in different anatomical compartments was calculated in the entire dorsal cortex of opossums, and the relative ratio of H3+ cells in SVZ between opossum and rat was shown in Figure 2*F*. Although the total number of H3+ cells gradually decreases as development progresses, the proportion of SVZ divisions increases during development in both species (Carney et al. 2007). There was no clear difference in the pattern of VZ and SVZ division between opossum and rat (see Fig. 2 legends for statistical comparisons).

The Spindle Orientation of Neuroepithelial Cells of Opossum Is Similar to Mouse

Given the differences in gestational length and total neuronal output between mouse and marsupials, it is likely that the neural stem cell pool size is different. We hypothesized that mitotic spindle orientation, an indicator for neuroprogenitors undergoing symmetric or asymmetric division, might also differ at the early stages of brain development (Fig. 3*A,B*). The angle between the cleavage plane and the apicobasal axis (Fig. 3*C*) was scored into six 15° bins (Fig. 3*D*). The majority (86.6%) of dividing cells of the opossum dorsal cortex were planar divisions in which cleavage planes were within 15° of the apicobasal axis. This percentage of planar division was similar to that of E13.5–E15.5 mouse data reported previously (Kosodo et al. 2004; Konno et al. 2008). These results suggest that despite over 100 million years of independent evolution, there is no difference in the proportion of spindle orientation of neuroprogenitor cells between opossum and mouse.

The Developmental Organization of VZ and SVZ Division Is Similar between Wallaby and Opossum

In another marsupial, the tammar wallaby, the distribution of H3+ mitotic cells in the dorsal cortex was examined at P15, P40, and P50 (Fig. 4*A–C*). Similar to opossum, the number of H3+ cells in the VZ, SVZ, and EZ was counted (Fig. 4*D*). No H3+ cells were detected in the SVZ until P40 (arrows in Fig. 4*B*). Although the total number of H3+ cells has gradually decreased, the SVZ has shown an increase of H3+ cells at P50. The relative proportion of H3+ cells present in VZ, SVZ, and EV of wallaby is presented in Figure 4*E*. As observed in opossum and rat (Fig. 2*F*), the proportion of H3+ cells in the SVZ of the wallaby dorsal cortex gradually increased throughout brain development. We also analyzed the spindle orientation of neuroepithelial cells in P9–P50 wallaby and observed that the percentage of planar divisions (Fig. 4*F,G*) was similar to opossum (Fig. 3*D*) and mouse (Kosodo et al. 2004; Konno et al. 2008). These results suggest that the proportion of IPCs in the SVZ and the dividing neuroepithelial cells in the VZ are very similar in rat, opossum, and wallaby (see Fig. 4 legend for statistical analysis) despite considerable difference in neuronal numbers in an arbitrary unit cortical column and the much more protracted period of cortical development of marsupials.

mRNA Expression Pattern of VZ and SVZ Marker Genes in Opossum Is Similar to Mouse

To confirm that the SVZ of opossum is similar to that of other mammals, in situ hybridization for *Pax6* (VZ marker), *Brn2,*

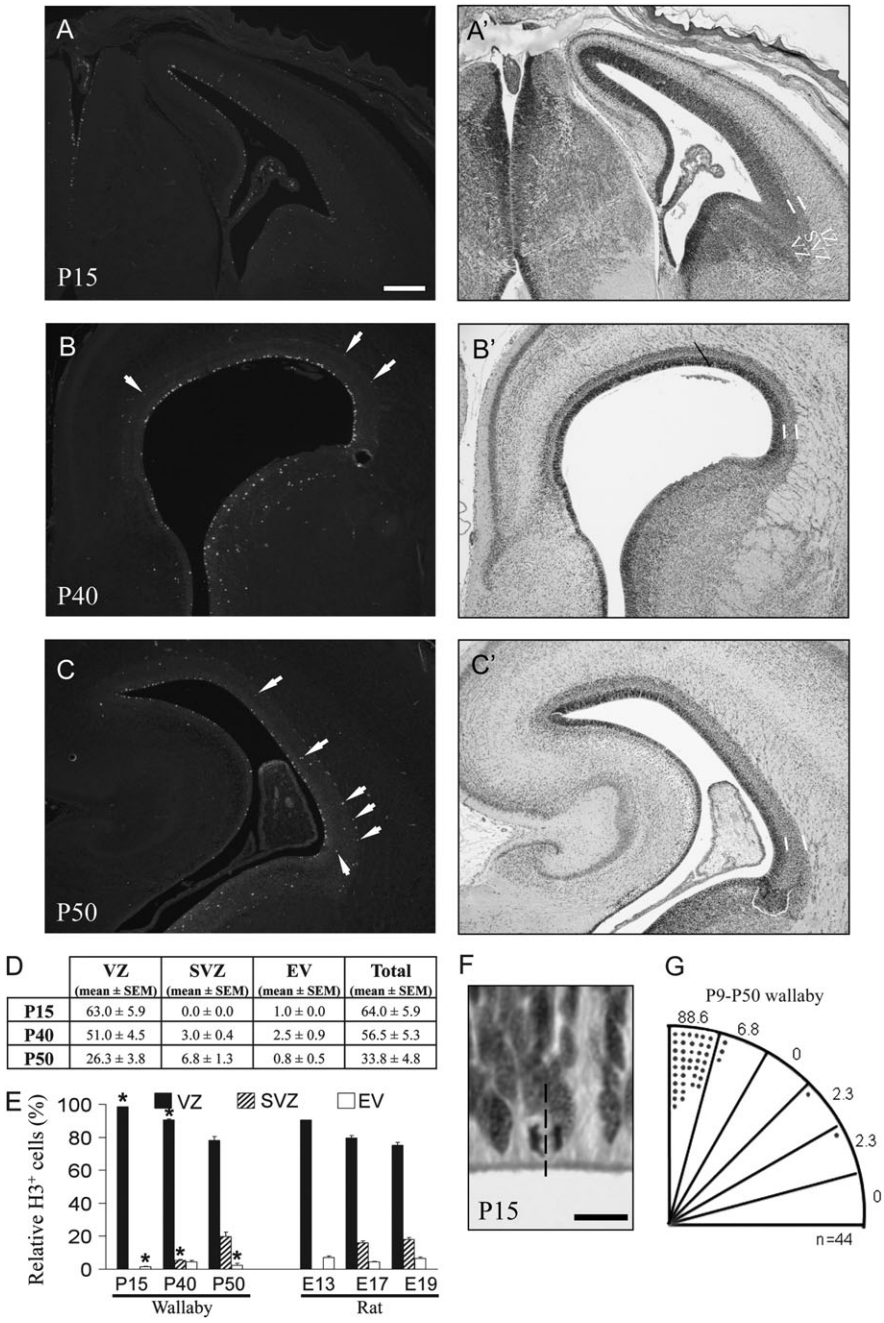


Figure 4. Distribution of dividing cells and mitotic spindle orientation in the developing wallaby cortex. (*A–C*) At P15, the majority of H3+ cells were in the VZ, although a few H3+ cells were in the IZ. There was no H3+ division in the SVZ until P40 (arrows in B), and by P50, numerous H3+ cells were located in the SVZ. The anatomical boundary for VZ, SVZ, and EZ was defined using H & E–stained sections (*A'–C'*), and the number of H3+ cells in each region was then quantified and expressed as mean ± SEM in (*D*). (*E*) The relative ratio of H3+ cells in different anatomical regions of wallaby. Although the total number of cell division decreases as development progresses, cell divisions in the SVZ become more prominent, a feature very similar to opossum and rat. Asterisks represent that there is a significant difference between wallaby and rat ($P < 0.05$, Student's *t*-test). (*F*) A dividing cell with a typical planar division. Dotted lines represent the cleavage plane used to measure the orientation of mitotic spindle. (*G*) Distribution of the cleavage plane orientation for mitotic neuroepithelial cells measured at P9, 15, 40, and 50 and averaged. Similar to opossum, most planar divisions were within 15° of the apicobasal axis. Scale bar: *A–C* = 300 μm; *F* = 10 μm.

Cux2, NeuroD6, and *Tbr2* (SVZ markers) was carried out. Of note, attempts at immunohistochemistry were not successful with Cux1 (1:1000 Santa Cruz Biotechnology, Santa Cruz, CA), Tbr2 (1:2000 generous gift from R. Hevner, University of Washington), and Pax6 (1:100 Developmental Studies Hybridoma Bank, University of Iowa) antibodies. At the earlier stage of brain development (E15 mouse and P12 opossum), both the SVZ and VZ marker genes were expressed in corresponding compartments of the germinal zone in a similar fashion (Fig. 5*B–F*, *N–R*). Whereas the mRNAs of *Pax6* and *Tbr2* were more homogeneously expressed within VZ and SVZ (Fig. 5*B*,*F*,*N*, and *R*), *Cux2*, *NeuroD6*, and *Brn2* were more abundant in the SVZ of both mouse and opossum (Fig. 5*C–E*, *O–Q*). At the later stage of brain development (P0 mouse and P20 opossum), *Cux2*,

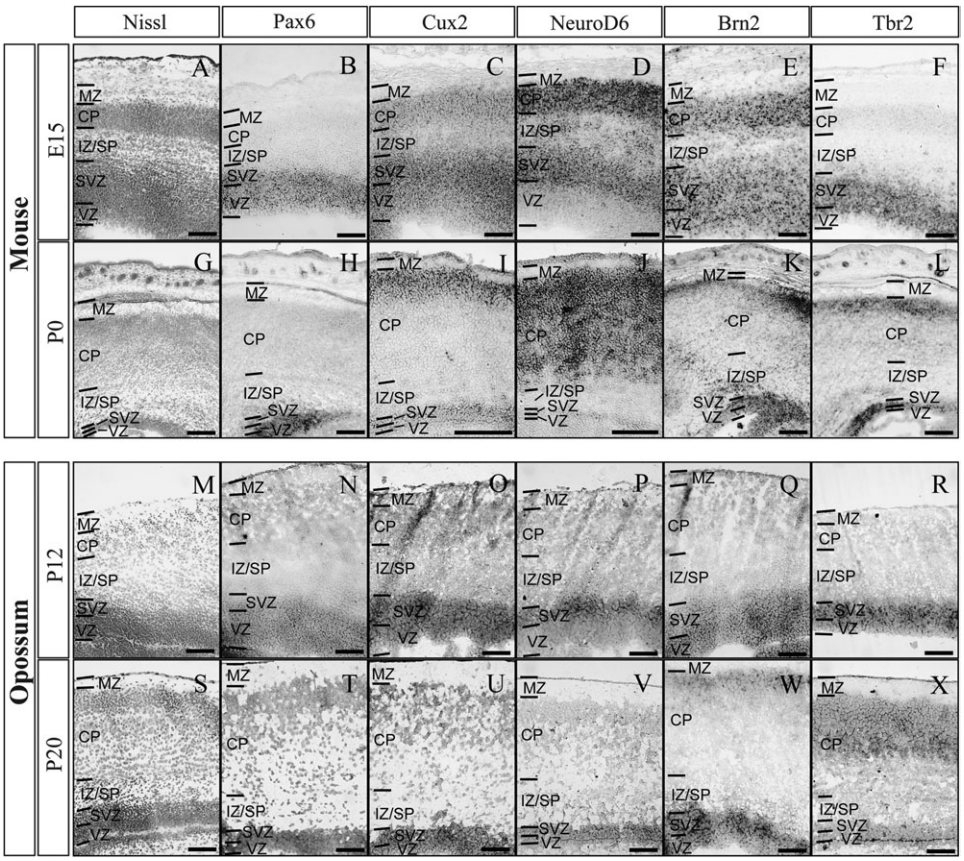


Figure 5. Comparison of mRNA patterns of VZ (*Pax6*) and SVZ (*Cux2*, *NeuroD6*, *Brn2*, and *Tbr2*) marker genes in mouse and opossum dorsal cortex at early (E15 and P12) and later stages (P0 and P20) of neurogenesis. The expression patterns of VZ and SVZ marker genes in opossum (*N–R*, *T–X*) are similar to those of the mouse (*B–F*, *H–L*). (*A*, *G*, *M*, *S*) Nissl-stained sections were used to identify VZ, SVZ, IZ or subplate, CP, and MZ. Scale bar: *A–F*, *M–X* = 100 μm; *G–L* = 200 μm.

Brn2, and *Tbr2* mRNAs were expressed in the upper layer of the cortex (Fig. 5*I*,*K*,*L*,*U*,*W*, and *X*). The proportion of the upper CP that expresses these varied, for example, *Tbr2* in the P0 mouse is only at the very top of the CP, whereas in the opossum, it takes up about the top half of the CP. These differences might be genuine or due to the slightly different stages selected for comparisons. Nevertheless, these results suggest that the overall VZ and SVZ marker gene expression is highly conserved and that these transcription factors may have a similar function in both mouse and opossum.

The Neurovasuclar Pattern Is Similar between Mouse and Opossum

Our previous observations in mouse revealed that cortical blood vessels form a characteristic plexus in the SVZ during cortical development (Stubbs et al. 2009). We performed similar staining in opossum at various developmental stages and observed identical patterns of vascular plexi in the SVZ (Supplementary Figure 1). Analyses on both the anatomical and molecular levels suggest that marsupials do have a distinct SVZ, with dividing IPCs.

Discussion

Although the process of cortical development is believed to be fundamentally similar in all mammals (Rakic 1988; Bystron et al. 2008), the data on which this supposition is based have been obtained from only a handful of species such as mouse, rat, hamster, cat, ferret, and macaque. These studies have excluded orders of mammals such as marsupials and monotremes where substantially less is known about cortical development. The metatherian (marsupial) lineage split from the last common ancestor of eutherian mammals more than 100 million years ago (Murphy et al. 2004). They are a highly diverse order of mammals and, like eutherian mammals, have undergone dramatic radiations and have diversified to fill a variety of environmental niches. For example, marsupials possess fewer neurons in their cortex (Haug 1987; Saunders et al. 1989), have a large anterior commissure instead of a corpus callosum (Reynolds et al. 1985), and have a more protracted period of cortical development (Reynolds et al. 1985; Saunders et al. 1989; Mark and Marotte 1992; Molnár et al. 1998) with an apparent absence of a distinct motor cortex (Karlen and Krubitzer 2007). Furthermore, it has been suggested that their cortical germinal zone organization might differ (Saunders et al. 1989; Abdel-Mannan et al. 2008) from their eutherian counterparts. To determine whether cortical development, as observed in rodents and primates, is conserved among all mammals, as opposed to a derivation of eutherian mammals, we compared several aspects of organization and development in 2 representatives from each group.

Reduced Neuronal Numbers in all Cortical Layers in Adult Marsupial Cortex Compared with Mouse

Marsupials possess a 6-layered cerebral cortex with similar basic organization to eutherian mammals (Northcutt and Kaas

1995); however, there are some marked differences in marsupial cortex. Haug (1987) first compared the neuronal numbers in several mammalian species, including opossum. Our data confirmed his observation that the number of cortical neurons arranged in a unit column is fewer in opossum and extends to tammar wallaby as well. This suggests that even with a protracted period of neurogenesis, marsupials still have less cell division during cortical development. It has been reported that several eutherian mammals possess the same number of neurons (congruent to 112) in a 30-μm wide unit column, except area 17 of primates, which contains 2.5 times more neurons (Rockel et al. 1980). However, it remains unclear whether all marsupials maintain a constant, albeit fewer, neuronal number. Our data suggest that opossum has fewer neurons than wallaby. We only had access to visual cortical sections from wallaby, and we acknowledge that such difference could be due to the cortical area selected for counting. The primary visual area of wallaby is clearly demarcated by the Line of Gennari (Vidyasagar et al. 1992), a feature also present in primates. Thus, it is likely that the primary visual area of wallaby is another exception in that it possesses more neurons than other cortical areas.

The Basic Organization of the Cortical Germinal Zone Is Similar in all Mammals

It has been suggested that the elaboration of the germinal zone might compensate for the time constraints imposed on the rapid development of the cerebral cortex in mammals (Smart et al. 2002; Fish et al. 2008) and birds (Striedter and Charvet 2008). Although the elaboration of the dorsal cortical SVZ is one of the hallmarks of the mammalian dorsal cortex, there are several observations that suggest that further elaboration of the SVZ may be the driving force of an expanded cortical sheet observed in some groups of mammals. Before the demonstration of the presence of the SVZ in marsupials, it was an interesting possibility that the lower neuronal numbers and the extended cortical development in marsupials correlated with an altered germinal zone. However, our study has shown that the spatial and temporal organization of mitotic figures in the developing cerebral cortex of marsupials, such as opossum and wallaby, are fundamentally similar to previously studied eutherian species such as rodents and primates (Lukaszewicz et al. 2005; Kriegstein et al. 2006; Bystron et al. 2008). We used several independent approaches to investigate the SVZ divisions: 1) H3 staining to look for mitotic cells corresponding to an SVZ, 2) morphological differences based on Nissl and H & E staining and quantitative analysis of the mitotic spindle orientation in VZ, 3) VZ/SVZ–specific mRNA expression of selected genes, and 4) patterns of vascular plexi in the SVZ. The results obtained from these experiments all suggest that like other studied mammalian species with 6-layered dorsal cortex, opossum and wallaby have a SVZ with IPCs during cortical development. Although the number of neurons was proportionally lower in both upper and lower layers in a standard unit column, these species achieve the same cortical arrangement with similar gene expression within a comparable structure.

Does the Evolution of a 6-layered Mammalian Cerebral Cortex Coincide with the Appearance of the Organized Cortical SVZ during Cortical Development?

In the telencephalon of reptiles, birds, and mammals, embryonic neurogenesis occurs in the VZ along the lateral ventricle and in abventricular divisions at various distances from the VZ (Martínez-Cerdeño et al. 2006). It is believed that developmental mechanisms in the mammalian telencephalon are distinguished from those in reptiles and birds by the presence of a neuron-generating SVZ in the germinal zone of dorsal cortex (Cheung et al. 2007). Previous analysis of the variation in mitotic cell frequency with distance from VZ in chicken brains demonstrated a second proliferation peak corresponding to an organized SVZ in the basal ganglia, nidopallium, and mesopallium at E8 and E10 but was absent in the hyperpallium (equivalent to mammalian dorsal cortex) (Cheung et al. 2007). Mammals also have a large SVZ with scattered mitotic profiles throughout the depth of the LGE, MGE, and CGE of the subpallium (Bhide 1996). Mitotic cells are abundant at similar levels throughout the bulged ganglionic eminences, but they do not line up into a discrete band parallel to the ventricular surface (Bhide 1996; Carney 2005). The subpallial SVZ is believed to produce tangentially migrating interneurons (Anderson et al. 2001), whereas the IPCs in the cortical SVZ generate excitatory neurons (Haubensak et al. 2004; Miyata et al. 2004; Noctor et al. 2004). In marsupials, dorsal pallial abventricular proliferation was infrequent and less organized at early stages, although the ratio of abventricular to ventricular H3+ cells is not significantly different at P10 in opossum and P15 in wallaby between the pallium and subpallium. In the turtle, VZ mitosis peaks in earlier stages (S18 and 20) before shifting to an increasingly abventricular site of proliferation (Molnár, Tavare, and Cheung 2006; Cheung et al. 2007). However, abventricular division remains infrequent and scattered in the turtle dorsal cortex, whereas they align along a distinct zone at P14 in opossum and P40 in wallaby, and this is consistent with the hypothesis that IPCs in the organized pallial SVZ contribute to the expansion of the cortex from sauropsids to mammals. By demonstrating the existence of organized pallial SVZ in the developing marsupial cortex, we strongly support the notion that the evolution of a 6-layered mammalian cerebral cortex coincides with the appearance of the organized cortical SVZ. However, our study included representative species from only 1 of the 2 subclasses of mammals, that is, Theria, which includes the infraclasses Metatheria and Eutheria. The other subclass of mammals—Prototheria (monotremes)—has not been examined. This egg-laying group of mammals may represent a more primitive condition and thus may very well not follow the pattern of SVZ development common to Eutherians and the Metatherians examined in this study. It will be important to carry out similar work on Prototheria.

The Role of Intermediate Progenitors

Currently, the developmental or evolutionary role of IPCs is not known. The expansion in cortical surface area accompanying mammalian evolution might have arisen from an increase in the intermediate progenitor compartment (intermediate progenitor hypothesis; Tarabykin et al. 2001; Haubensak et al. 2004; Kriegstein et al. 2006). IPCs were further implicated in the generation of upper cortical layers (upper layer hypothesis; e.g., Tarabykin et al. 2001; Wu et al. 2005). IPCs and upper layers indeed share several genes. A precise molecular expression sequence for the transition of neuroepithelial cells to RGCs to IPCs to neurons might have played a major role in the diversification of the mammalian telencephalon. In mammals, several transcription factors label the pallial SVZ during neurogenesis including *Svet1, Cux1* and *Cux2*, and *Tbr2*

(Tarabykin et al. 2001; Nieto et al. 2004; Englund et al. 2005, respectively), and upper layer differentiation defects were reported in *Cux2* (Cubelos et al. 2008) and *Tbr2* (Arnold et al. 2008; Sessa et al. 2008) knock-outs. However, there are several observations that question the IPC –upper layer hypothesis. Haubensak et al. (2004) reported on the appearance of neurogenic IPCs in the mouse cortex as early as E10.5, implying that the role of IPC cannot be confined to upper layer neuron production only. Moreover, Kowalczyk et al. (2009) recently demonstrated that IPCs contribute pyramidal projection neurons to all layers in the mouse cerebral cortex. Conditional inactivation of *Tbr2* in the central nervous system of mice selectively reduces the pool of IPCs (Arnold et al. 2008). Although the differentiation of upper layer neurons is more prominently affected in the *Tbr2* conditional knock-out, the layer thickness of both supra- and infragranular layers are abridged and the number of both supra and infragranular neurons are equally reduced (AFP Cheung, S Arnold, M Groszer, Z Molnár, unpublished data). Consistent with the fundamental radial unit concept of Rakic (1988), Farkas et al. (2008) have shown that in the *Insm1* knock-out mouse, there is cortical expansion in the lateral dimension concomitant with the reduction in IPCs and radial thickness. Hevner and colleagues suggest that changes in IPC abundance alter cortical thickness and not necessarily cortical surface area in various mouse mutants and propose a modified radial unit hypothesis (Pontious et al. 2007), where IPCs would act as radial amplifiers for the neuronal output from the ontogenic units (Rakic 1988).

Further Elaboration of the Cortical Germinal Zone in Mammals

Although our study emphasizes the universal presence of SVZ in all mammalian cerebral cortices, we would like to stress the considerable differences in the elaboration of the cortical mitotic compartments. The elaboration of the germinal zone is more apparent in macaque (Smart et al. 2002; Lukaszewicz et al. 2005) with 3 distinct zones: VZ, inner SVZ, and outer SVZ (OSVZ), each with a characteristic gene expression pattern (Smart et al. 2002; Fish et al. 2008). Recently, the interesting "epithelial progenitor hypothesis" was proposed (Fish et al. 2008). This hypothesis argues that evolutionary changes, which promote the maintenance of epithelial features in neural progenitors, including OSVZ progenitors, have been instrumental in the expansion of the cerebral cortex in primates. Therefore, by extrapolation, it is plausible that the mitotic spindle orientation of neuroepithelial cells in marsupials (which has a less complex cortical organization than primates) is not as tightly regulated as in primates.

More species with gyrencephalic and lissencephalic brains should be investigated to be able to correlate the presence or the absence of sulci and gyri with the compartmentalization of the germinal zones (Cheung et al. 2007). However, our result suggests that spindle orientation of neuroepithelial cells of marsupials was similar to that of mouse, thus extending the concept to other mammalian infraclass. Further studies are needed to clarify the role of spindle orientation in regulating sibling cell fate. Future experiments with electroporation of green fluorescent protein-expressing plasmids into the pallium for time-lapse microscopy of cell dynamics in various vertebrates could shed light on the intrinsic and environmental factors involved in regulation of cell division in the SVZ.

Neuron Numbers in Mammalian Cerebral Cortex

The marsupial cortex has a protracted development that would seemingly reduce pressure on the timing of neuronal production compared with some small eutherians such as mouse. Our current study revealed the relatively late appearance of the SVZ with slightly altered proportions of VZ and SVZ cell division especially at early stages of marsupial cortical development. The prolonged cell cycle duration and the increased number of cell divisions of cortical progenitors in macaque when compared with rodents are hypothesized to underlie the evolutionary expansion of the neocortex (Rakic 1995). Interestingly, the protracted development in opossum and wallaby does not result in an expanded cortical sheet. It would be interesting to examine other marsupials, such as the striped possum, which have an encephalization quotient that rivals that of many primates to determine if similar mechanisms account for the expansion of the cortical sheet. Because comparative developmental neurobiology depends on observations across numerous species, additional marsupial, eutherian, and monotreme species will need to be examined to determine the differential role of the pallial SVZ division.

Conclusion

Our description of the organized IPC in the SVZ, the comparable orientation of mitotic spindles in the VZ, and similar expression of selected genes with known cortical VZ and SVZ expression in mouse, opossum, and tammar wallaby further support the hypothesis that a cortical SVZ is a hallmark of 6-layered cortex in all mammals. The role of the SVZ as a key mitotic compartment in mammalian cortical evolution is still not fully understood, but SVZ divisions might be necessary for the generation of extra neurons for both radial and tangential expansion of the mammalian cerebral cortex.

Supplementary Material

Supplementary Table 1 and Figure 1 can be found at: http://www.cercor.oxfordjournals.org/.

Notes

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