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Radial Glia Serve as Neuronal Progenitors in All Regions of the Central Nervous System

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Summary

Radial glial cells function during CNS development as neural progenitors, although their precise contribution to neurogenesis remains controversial. Recent work has argued that regional differences may exist regarding the neurogenic potential of radial glia. Here, we show that the vast majority of neurons in all brain regions derive from radial glia. Cre/*loxP* fate mapping and clonal analysis demonstrate that radial glia throughout the CNS serve as neuronal progenitors and that radial glia within different regions of the CNS pass through their neurogenic stage of development at distinct time points. Thus, radial glial populations within different CNS regions are not heterogeneous with regard to their potential to generate neurons versus glia.

Introduction

Radial glial cells function during central nervous system development as both neural progenitors (Malatesta et al., 2000, 2003; Miyata et al., 2001; Noctor et al., 2001, 2002) and as a scaffolding upon which nascent neurons migrate (Rakic, 1971a, 1971b, 1972; Sidman and Rakic, 1973). As progenitors, radial glia give rise to many, if not all, of the neurons generated within the cerebral cortex (Noctor et al., 2002; Malatesta et al., 2003). However, it has remained unclear whether radial glia also function as progenitors for most neurons in other CNS structures. For example, Cre/*loxP* fate mapping done in transgenic mice using the human GFAP (hGFAP) promoter to trace radial glial progeny resulted in large numbers of radial glial-derived neurons generated in the dorsal, but not the ventral, telencephalon (Malatesta et al., 2003). This led to the suggestion that ventral telencephalic radial glia might not pass through a neurogenic stage of development, and instead give rise solely to glial cells. Furthermore, expression profiling has also raised the possibility that radial glial cells may have varying potential even within a single developing brain region. Immunohistochemical staining of neocortical sections suggested that radial glial markers are not uniformly expressed, and analysis of dissociated radial glia has shown that they can be divided into multiple antigenically distinct subpopulations that differ in their cell cycle kinetics, with each subpopulation changing in a characteristic manner during development (Hartfuss et al., 2001). While these results support models proposing that the heterogeneity of mRNA and protein expression in radial glia reflect differences in their potential as neural progenitor cells, they are also consistent with models suggesting that the heterogeneity observed in radial glial populations reflects temporal aspects of radial glial development rather than underlying differences in their potential.

One approach toward addressing these issues is to analyze the expression of markers that are dynamically regulated in radial glia and determine the potential of the progenitors expressing them using genetic methods for lineage tracing in vivo. Brain Lipid Binding Protein (BLBP) is a nervous system-specific member of the large family of hydrophobic ligand binding proteins and is exclusively expressed in radial glial cells and astrocytes throughout the developing CNS (Feng et al., 1994; Kurtz et al., 1994; Hartfuss et al., 2001). Interestingly, BLBP has been shown to be expressed in a subset of forebrain radial glial cells early in neurogenesis and is upregulated in the remainder as neurogenesis proceeds (Hartfuss et al., 2001). Whether or not this heterogeneity reflects distinct lineal properties of BLBP-expressing radial glia has remained unclear.

The dynamic expression of BLBP in radial glia in all regions of the developing CNS suggested that studies defining the potential of BLBP-expressing radial glia could contribute to our knowledge of neural progenitor function. We report here that virtually all neuronal populations in the mouse brain derive from BLBP-expressing progenitors and that the vast majority of telencephalic neurons are derived from $BLBP^{+}$ radial glia. Furthermore, we show that radial glia of the ganglionic eminences complete their neurogenic stage of development earlier than their counterparts in the neocortex. Taken together with spatiotemporal analyses of BLBP expression in the developing forebrain, these studies demonstrate that radial glia function as progenitors for the majority of CNS neurons and that the neurogenic stage of radial glial development is temporally and spatially dynamic.

Results

Radial Glial Antigenic Heterogeneity Reflects Spatiotemporal Differences in Developmental Stage

Expression analysis has demonstrated that forebrain radial glia can be segregated into multiple antigenically distinct subpopulations that change in a stereotypical manner as development proceeds (Hartfuss et al., 2001). One of the genes found to be heterogeneously expressed was BLBP, which was detected in only 40% of proliferating progenitors in the E12 neocortex. As the previous quantitative analyses were done by dissociating cells

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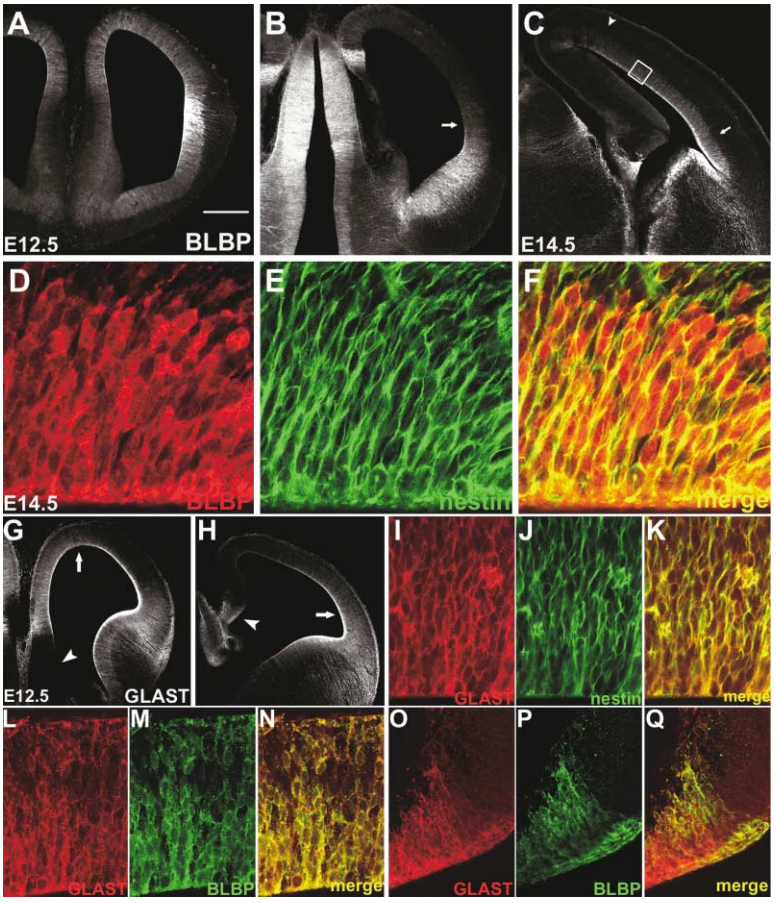

Figure 1. Radial Glial Antigenic Heterogeneity Reflects Developmental Changes Occurring in All Radial Glial Cells

Immunofluorescent staining for BLBP (A–C, D, M, and P), nestin (E and J), and GLAST (G–I, L, and O); merged images of double labeled cells are shown in (F), (K), (N), and (Q). Analysis of the onset of neocortical BLBP expression shows that it is induced in and progresses in the same spatiotemporal pattern as neurogenesis. At E12.5, BLBP staining is seen in radial glia throughout the rostral neocortex (A), but only in laterally positioned cells in caudal areas (arrow in B). (C) By E14.5, BLBP has been induced in medially situated radial glia in the caudal neocortical neuroepithelium, and a gradient of expression is evident, with higher levels laterally (arrow) and lower levels medially (arrowhead). Furthermore, radial glia uniformly express BLBP once it has been induced in a particular region. This is clear from BLBP/Nestin double labeling; the boxed area in (C) is shown at high magnification in (D)–(F), where it can be seen that all radial glia in BLBP-expressing regions are BLBP$^+$. Similar to that observed for BLBP, GLAST expression at E12.5 is high in radial glia throughout the rostral neocortex (G), but only in the most lateral cells in more caudal regions (arrows in G and H). However, all radial glia in these regions are GLAST$^+$; double labeling for GLAST and Nestin of caudolateral radial glia is shown in (I)–(K). Note that similar to BLBP, GLAST expression is weak in the septum and strong in the cortical hem (arrowheads in G and H, respectively). Double labeling for BLBP and GLAST in the neocortex (L–N) and cortical hem (O–Q) confirms that these two genes mark the same radial glial population. Scale bar equals 250 μm in (A)–(C), (G), and (H), 15 μm in (D)–(F), 26 μm in (I)–(K), 35 μm in (L)–(N), and 50 μm in (O)–(Q).

from large brain regions and counting immunopositive cells in vitro, we sought to determine how BLBP$^+$ radial glia are distributed in vivo. Examination of the onset of BLBP in the developing neocortex demonstrates a high correlation with the initiation of neurogenesis and radial glial directed neuronal migration, which is known to commence and progress in a rostrolateral to caudomedial gradient in this region (Angevine and Sidman, 1961; Hicks and D'Amato, 1968; Bisconte and Marty, 1975; McSherry, 1984; McSherry and Smart, 1986; Bayer and Altman, 1991; Takahashi et al., 1999). At E10.5, when the preplate is being formed and immediately prior to the start of neurogenesis, BLBP protein is virtually undetectable in sections and acutely dissociated cells using standard immunofluorescent labeling (data not shown). By E12.5, staining is seen in radial glia throughout the rostral neocortical neuroepithelium (Figure 1A), but only in the most caudolaterally situated radial glia (Figure 1B). However, by E14.5, BLBP has been induced in more medially situated radial glia, and a clear lateral-to-medial gradient of staining is evident (Figure 1C). Finally, by E16.5, BLBP is highly expressed in radial glia through the entire mediolateral extent of the caudal neocortex (data not shown). The correlation between BLBP onset and the neurogenetic gradient has also been observed in the spinal cord (Feng et al., 1994) and midbrain (Kurtz et al., 1994). These results suggested that all radial glia might express BLBP at a particular developmental time point. This idea is supported by double labeling with nestin, which is expressed in all radial glial cells. This analysis confirmed that essentially all radial glia in BLBP-expressing regions (where BLBP has already been induced) are BLBP$^+$, regardless of their position along the mediolateral axis of the neocortex (Figures 1D–1F). This was also found to be the case in several other areas of the developing CNS, including basal ganglia, midbrain, hindbrain, and spinal cord (data not shown). Taken together, these data support the idea that BLBP expression does not distinguish radial glial subtypes in any particular region, but instead defines developmental changes occurring in all radial glial cells in a spatiotemporal pattern that parallels neurogenesis.

These observations appear to indicate that the heterogeneous expression of BLBP in radial glial is a reflection of regional developmental differences. To examine if this is true for other markers of radial glial heterogeneity, we looked at the in vivo distribution of neocortical radial glia expressing the astrocyte-specific glutamate trans-

porter (GLAST); this is another molecule previously found to be contained in a subpopulation of dissociated radial glia (Hartfuss et al., 2001). Relatively homogenous GLAST expression was seen in radial glia throughout the E12.5 rostral neocortex (Figure 1G). However, as observed for BLBP, GLAST staining was highest in laterally situated cells in caudal neocortical regions (Figure 1H), weak in the E12.5 septum (arrowhead in Figure 1G), and strong in the cortical hem (arrowhead in Figure 1H). In addition, essentially all rostral (not shown) and caudolateral (Figures 1I–1K) radial glial cells are GLAST$^+$. Moreover, GLAST expression was found to be upregulated in caudomedial radial glia by E16.5 (data not shown). As with BLBP, these results indicate that all radial glia pass through similar stereotyped patterns of gene expression during development and that distinct antigenic profiles are characteristic of particular developmental stages. Since double labeling for BLBP and GLAST confirmed that they are induced in the same spatiotemporal pattern and in the same cells (Figures 1L–1Q), our data are consistent with the notion that induction of these two genes defines a single developmental change that takes place in all radial glia. As it is well established that unique spatiotemporal gradients of neurogenesis exist within different developing brain regions, these shifts in radial glial gene expression may reflect changes in mode of cell division (symmetric versus asymmetric) and/or types of progeny generated (neurogenic versus gliogenic radial glia).

1.6 kb of BLBP 5′ Flanking Genomic Sequence Recapitulates Endogenous BLBP Expression in Transgenic Mice

Extensive analysis of BLBP expression in the developing CNS has confirmed that it is restricted to radial glia and immature astrocytes and also distinguishes radial glia from earlier neuroepithelial cells (Kurtz et al., 1994; Hartfuss et al., 2001; Malatesta et al., 2003). As we have now shown that BLBP is expressed in all radial glia (Figure 1), these findings together indicate that lineage tracing BLBP$^+$ cells is a sensitive and specific means of determining the neurogenic potential of all radial glia cells in all CNS regions. To accomplish this, we used Cre/*loxP* fate mapping to mark the progeny of BLBP$^+$ radial glia. This approach requires two mouse strains: one that expresses the Cre recombinase under the regulation of the BLBP promoter (the cells to be fate mapped), and a second that uses a promoter active in most cell types to drive a *loxP*-stop-of-transcription/translation-*loxP*-LacZ cassette (a Cre-reporter line) (Dymecki and Tomasiewicz, 1998; Zinyk et al., 1998). Analysis of double transgenic mice allows determination of the progeny of BLBP$^+$ radial glia, as only cells in which Cre has been expressed undergo recombination to remove the STOP cassette and express LacZ. If BLBP is induced in neurogenic radial glia, many neurons should be LacZ$^+$, whereas if induction occurs only in gliogenic radial glia, few if any neurons would express LacZ.

To generate transgenic mice expressing Cre in BLBP$^+$ radial glia, we used the 1.6 kb BLBP promoter. This sequence has previously been shown to direct expression of reporter genes to radial glia and astrocytes throughout the CNS of transgenic mice in a manner that recapitulates endogenous BLBP expression (Feng and Heintz, 1995). Thorough analysis of transgenic mice expressing GFP under the regulation of the 1.6 kb BLBP promoter demonstrates that the onset and pattern of GFP expression in the developing forebrain is essentially identical to endogenous BLBP and that GFP expression is restricted to radial glia (Figures 2A–2M). Furthermore, both BLBP and GFP are confined to radial glia within the ventricular zone (VZ) of the striatum and are not detected in striatal subventricular zone (SVZ) progenitors (Figures 2A, 2B, and 2H–2J). Postnatally, GFP is expressed exclusively in BLBP$^+$ astroglial cells throughout the brain, including neocortical gray matter astrocytes (Figures 2N–2P) and Bergmann glia (data not shown). The restriction of GFP expression to radial glia and astrocytes establishes that Cre/*loxP* fate mapping using the 1.6 kb BLBP promoter to drive *Cre* is useful for determining which neuronal populations derive from radial glial cells.

Recombination in BLBP-Cre/R26R Mice Occurs in the Same Spatiotemporal Pattern as Onset of Endogenous BLBP Expression

Four independent BLBP-Cre transgenic founder lines (transgenic mice expressing Cre under the regulation of the 1.6 kb BLBP promoter) were generated for comparison. The fate mapping data presented below were obtained from one of these lines, but all other lines gave the same results. BLBP-Cre mice were crossed to the Rosa26 Cre reporter strain (R26R), which has been shown to drive high levels of reporter expression in all embryonic tissues (Soriano, 1999).

To ensure that our fate mapping reflects recombination in radial glia and not spurious or ectopic Cre expression, we analyzed the embryonic onset of recombination in BLBP-Cre;R26R double transgenic mice. At E9.5, recombination was virtually undetectable in the midbrain and forebrain but had begun in the hindbrain and spinal cord (Figures 3A and 3B). By E10.5, recombination had begun to take place in the midbrain and forebrain (Figure 3C). Similar to what was observed for BLBP and promoter-driven GFP expression, recombination in the forebrain took place in a rostral to caudal pattern (arrows in Figures 3A and 3C).

Given that the E9.5 brain is highly enriched for neuroepithelial cells, the lack of recombination at this time provides direct genetic evidence that BLBP is not expressed in neuroepithelial cells and indicates that onset of BLBP expression coincides with radial glial differentiation. To further establish that the earliest BLBP$^+$ cells are radial glia, we double labeled the E11.0 forebrain for BLBP and GLAST, another marker believed to distinguish radial glia from neuroepithelial cells. In agreement with the pattern of Cre recombination, BLBP expression is strongest in the rostral forebrain at this time point (Figure 3D). Moreover, all BLBP$^+$ cells also express GLAST (Figures 3E–3G). Thus, both genetic data and immunostaining demonstrates that BLBP expression defines the transition from neuroepithelial cells to radial glial cells.

The tight correlation between Cre recombination and BLBP onset was also seen by β-gal immunostaining of the E12.5 forebrain of BLBP-Cre;R26R double transgenic embryos (Figures 3H–3J). As seen with BLBP expression (Figure 1C), recombination in more caudal por-

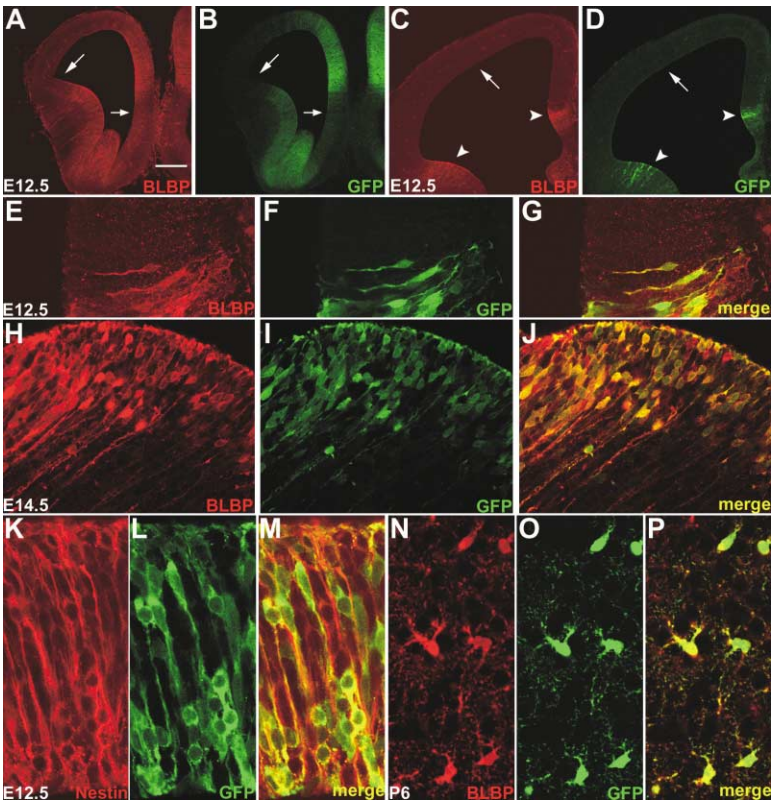

Figure 2. The 1.6 kb BLBP Promoter Drives Specific Expression in BLBP$^+$ Radial Glia and Astrocytes in Transgenic Mice

Double immunofluorescence for promoter-driven GFP and endogenous BLBP or nestin; merged images are indicated. Staining patterns for BLBP and promoter-driven GFP in the rostral (A and B) and caudal (C and D) neocortex are essentially identical. In particular, low levels of expression for both are seen in the septum, lateral LGE rostrally, and caudal neocortex (arrows in A–D), whereas intense staining for both is seen in the cortical hem and caudal LGE (arrowheads in C and D). In addition, note that staining for both proteins is restricted to radial glia within the VZ of the LGE. Higher magnification of the cortical hem (E–G) and LGE (H–J) confirms that labeling is in the same radial glial populations. (K–M) Double labeling with nestin confirms that neocortical GFP expression is confined to radial glia. (N–P) As has been previously described for BLBP, promoter-driven GFP is restricted to astrocytes in the postnatal neocortex. Scale bar equals 200 μm in (A) and (B), 150 μm in (C) and (D), 25 μm in (E)–(G), 35 μm in (H)–(J), and 10 μm in (K)–(P).

tions of the neocortex has occurred by E14.5 (Figure 3K). Double labeling for β-gal and the neuronal marker β-III Tubulin demonstrates that neurons of the cortical plate, as well as those in the marginal zone and subplate, are recombined (Figures 3L and 3M); these latter two neuronal populations are derived from the preplate, which contains the earliest born neurons in the neocortex (Marin-Padilla, 1971, 1978).

Radial Glia in All Developing Brain Regions Pass through a Neurogenic Stage

To identify which neuronal populations are derived from radial glia, we next examined recombination in postnatal mice. The major limitation in these experiments is the activity of the promoter used for the reporter line, as it must drive expression in the cell types of interest for a recombination event to be detected. All of the previously published Cre reporter strains used one of two promoters, β-actin or Rosa26. Since β-actin was known to be neuron specific in the brain (Kaech et al., 1997), we used R26R mice. To determine the cell types that express Rosa26 in the postnatal brain (and therefore which are able to be followed in our fate mapping), we analyzed the gtrosa26 line; these mice express the β-gal(LacZ)/neo fusion protein β-geo from the Rosa26 genomic locus in a Cre-independent manner (Friedrich and Soriano, 1991). Although X-Gal staining was extensive in the P45 brain of gtrosa26 mice (Figure 4A), detectable expression was largely restricted to neurons: essentially all LacZ$^+$ cells were labeled by the neuronal marker NeuN (Figure 4B). Conversely, labeling with the astroglial markers S100 or GFAP showed very few astrocytes that contained LacZ activity in most brain regions (Figures 4C–4E). Thus, fate mapping using R26R is of limited utility for tracing astroglial lineages, but does allow the primary question of interest to be addressed, namely whether or not radial glia in all developing brain regions pass through a neurogenic stage.

X-Gal histochemical staining showed that extensive recombination had taken place in all brain regions of P45 BLBP-Cre;R26R double transgenic mice (Figure 4F). β-gal/NeuN and β-gal/calbindin double immunofluorescence confirmed that essentially all of these LacZ$^+$ cells were neurons; examples of recombined neurons from the neocortex, hippocampus, and cerebellum are shown in Figures 4G–4I. To quantitate the levels of recombination, we counted the percentages of NeuN$^+$ neurons (or Calbindin$^+$ Purkinje cells) that were also β-gal$^+$. The vast majority of neurons in these populations had recombined in BLBP-Cre;R26R double transgenic mice: neocortex, 98.7% (n = 1237); hippocampal pyramidal neurons, 91.3% (n = 492); and cerebellar Purkinje neurons, 79.5% (n = 205).

In contrast to the fate mapping results obtained using the hGFAP promoter (Malatesta et al., 2003), BLBP-Cre;R26R double transgenic mice showed extensive recombination in neuronal populations that are generated in the ventral telencephalon. The majority of neocortical interneurons originate in the subpallium and subsequently migrate tangentially into the dorsal telencephalon where they become local circuit neurons (Marin and Rubenstein, 2001). Double labeling for β-gal and several markers of interneuron subpopulations demonstrated that most of these ventrally generated neocortical cells underwent recombination in BLBP-Cre;R26R mice (Fig-

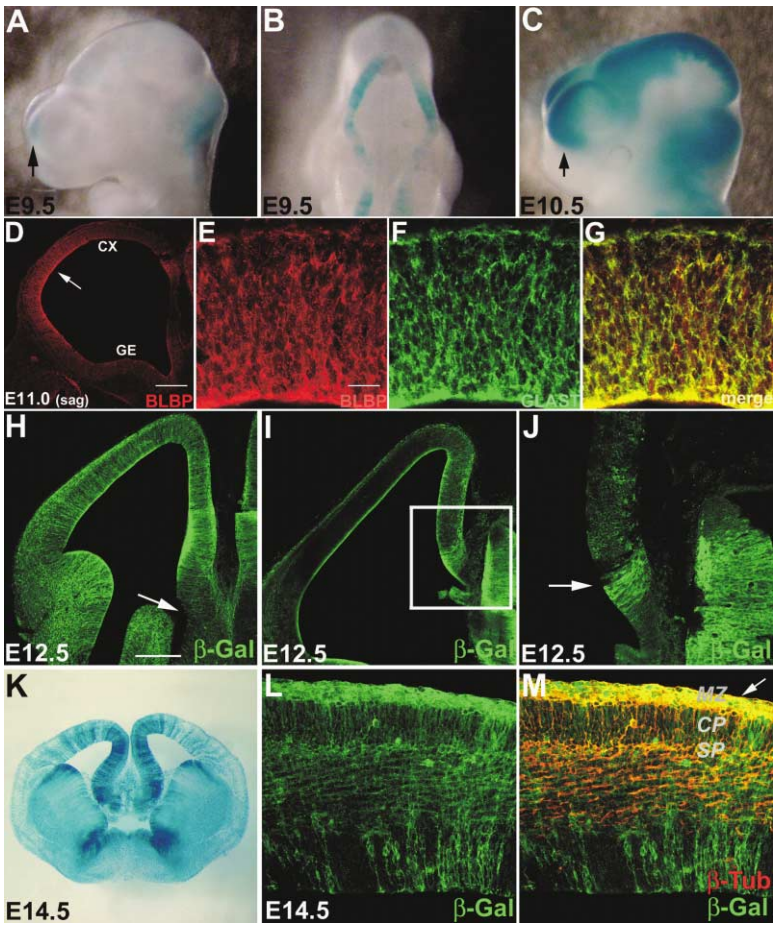

Figure 3. Onset of Recombination in BLBP-Cre;R26R Embryos Parallels Onset of Endogenous BLBP Expression

X-Gal histochemical staining (A–C and G) and immunofluorescent labeling for β-gal (D–F, H, and I) and β-III Tubulin (I) of BLBP-Cre;R26R embryos, and double immunofluorescent labeling for BLBP (D, E, and G) and GLAST (F and G) of wild-type embryos; sections in (D)–(G) are sagittal, whereas those in (H)–(M) are coronal. At E9.5, significant recombination is not yet detected in the midbrain or forebrain (A) but has already taken place in the hindbrain and spinal cord (B). By E10.5, recombination throughout the brain has begun (C). Note that X-Gal staining is only detected within the brain and is not seen in nonneural tissue. As seen for both BLBP and promoter-driven GFP, recombination in the forebrain occurs in a rostral to caudal gradient. The most rostral portion of the forebrain is the only X-Gal stained area of the E9.5 midbrain/forebrain and is the most intensely stained region in the E10.5 forebrain (arrows in A and C). Staining for BLBP protein reveals an identical pattern at E11.0 (D), and all BLBP$^+$ cells are GLAST$^+$ (E–G). The correlation between BLBP expression and recombination was also observed in the E12.5 forebrain. Extensive recombination was detected in the rostral forebrain but was minimal in the septum (arrow in H); this was also the case for BLBP and promoter-driven GFP (compare with Figures 2A and 2B). Furthermore, recombination in the E12.5 caudal neocortex (I; boxed region shown at higher magnification in J) was minimal, but intense in the cortical hem (arrow in J; compare with Figures 2C and 2D). By E14.5, X-Gal staining is observed in caudal areas of the neocortex (K). Double labeling for β-gal and β-tub in this region (L and M) shows that most neurons of the marginal zone (arrow in M), subplate, and cortical plate have been recombined. FACS-sorted BLBP$^+$ radial glia generated significant numbers of neurons in vitro, establishing that this recombination occurred in radial glial cells (Figure 6). Scale bar equals 200 μm in (D), (H), and (I), 25 μm in (E)–(G), 100 μm in (J), and 50 μm in (L) and (M).

ures 4J and 4K): Calbindin$^+$ interneurons, 87.2% (n = 86); Calretinin$^+$ interneurons, 80.7% (n = 114); and Parvalbumin$^+$ interneurons, 92.5% (n = 134). Furthermore, X-Gal histochemical staining demonstrated that considerable recombination had taken place in neurons within the ventral telencephalon itself (Figure 4L). Particularly noteworthy was the extensive LacZ staining observed in the basal ganglia and thalamus, two regions in which virtually no recombination occurred in hGFAP-Cre;R26R mice. Double labeling for β-gal and NeuN confirmed that LacZ$^+$ cells in both of these regions are neurons (Figures 4M and 4N), and quantitating the percentages of recombined NeuN$^+$ cells demonstrates that the majority of neurons in these regions derive from radial glia: basal ganglia NeuN$^+$ neurons, 71.4% (n = 916); and thalamic NeuN$^+$ neurons, 98.1% (n = 515). Moreover, this recombination occurs in both projection neurons and interneurons, as most Choline Acetyltransferase$^+$ and Parvalbumin$^+$ striatal interneurons were β-gal$^+$ (data not shown).

These fate mapping data demonstrate that radial glia from both the dorsal and ventral telencephalon pass through a neurogenic stage during development and that radial glia give rise to the majority of neurons in the brain.

SVZ Progenitors of the Ganglionic Eminences Are Derived from Radial Glia

BLBP is expressed in the ventral telencephalon from as early as E10 (Kurtz et al., 1994), with high levels seen by E12.5. Immunostaining confirms that this expression is specific to radial glia of the ventricular zone (VZ) (Figures 5A–5C). Importantly, we did not detect any nonradial BLBP$^+$ cells within the subventricular zone (SVZ) (Figures 5B and 5C). This is noteworthy, as precursors within the SVZ of the ganglionic eminences are thought to be a source of ventrally generated neurons. Thus, given that our fate mapping results showed that ventrally generated neurons are extensively recombined in BLBP-Cre;R26R mice and that BLBP expression is restricted to radial glia residing in the ventricular zone, SVZ precursors most likely arise directly from radial glia. Analysis of E12.5 BLBP-Cre;R26R embryos supports this conclusion, as most SVZ progenitors are recombined (Figures 5D–5G).

The Shift from Neurogenesis to Gliogenesis Occurs Earlier in Radial Glia of the Ganglionic Eminences than in Neocortical Radial Glia

The strong correlation between the onset of BLBP expression and initiation of radial glial neurogenesis, to-

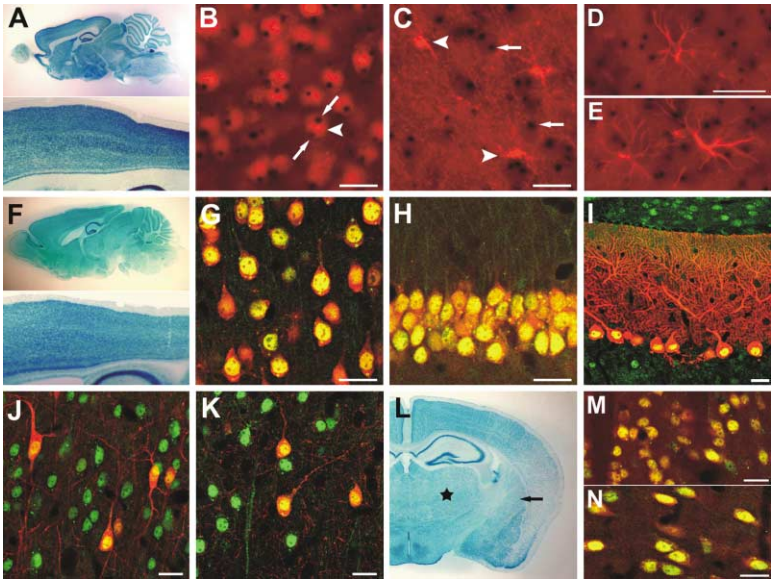

Figure 4. Radial Glia in All Developing Brain Regions Pass through a Neurogenic Stage of Development

The Rosa26 reporter is detectable in neurons but not astrocytes, and Cre/*loxP* fate mapping of BLBP$^+$ radial glia.

(A) X-Gal histochemical staining of the gtrosa26 line shows that the Rosa26 promoter is active throughout the brain and in all neocortical layers.

(B) Immunofluorescent labeling (red) of X-Gal-stained sections demonstrates that almost all β-gal activity (arrows) is contained in cells expressing the neuronal marker NeuN (arrowhead). Note that the gtrosa26 line expresses β-geo, which is a fusion of the β-gal and neo proteins. This fusion protein aggregates in vivo, hence the punctate labeling seen here; control sections show no blue labeling or aggregates at all (data not shown).

(C–E) Cells stained by the astroglial markers S100 (C) or GFAP (D and E) show virtually no colocalization with β-gal aggregates in most brain regions. In (C), arrows point to β-gal aggregates that do not colocalize with S100$^+$ cells within the neocortex (arrowheads); (D) and (E) are striatal astrocytes. Cre/*loxP* fate mapping of BLBP$^+$ radial glia is shown in the remaining panels.

(F) BLBP-Cre;R26R double transgenic mice show extensive recombination throughout the brain and in all neocortical layers.

(G–N) Double labeling for β-gal plus: NeuN (G, H, M, N), Calbindin (I and J), or Parvalbumin (K) demonstrates that recombination has taken place in multiple classes of neurons. In all of these images, β-gal staining is green and neuronal marker staining is red; note that the R26R line expresses nuclear β-gal, not β-geo. Cell types shown are neocortical neurons (G), hippocampal pyramidal neurons (H), cerebellar Purkinje cells (I), neocortical interneurons (J and K), striatal neurons (M), and thalamic neurons (N). X-Gal histochemical staining (L) shows that considerable recombination has taken place in neurons of the striatum (arrow) as well as in the thalamus (star), two regions that were not recombined in hGFAP-Cre;R26R mice. Scale bars equal 20 μm.

gether with the observation that BLBP is detected at high levels ventrally days earlier than it is dorsally, suggested that regional differences exist regarding the timing of radial glial neurogenesis. To test this possibility, we used fluorescence-activated cell sorting (FACS) to purify radial glia at E11.5 or E14.5 from the ganglionic eminences (GE) and neocortices of transgenic mice expressing EGFP under the regulation of the BLBP promoter; the gating parameters used enabled us to obtain highly enriched populations of RC2$^+$ radial glia, as shown in Figures 6A and 6D. To determine the neurogenic potential of the different radial glial populations, we followed the progeny of individual radial glial cells by growing the FACS-sorted cells at clonal density. Feeder layers from the same regions/time points were added to Transwell inserts; these inserts have porous membranes that allow diffusion of soluble factors but provide a physical barrier between the feeder layer and the clones.

At E11.5, radial glia from both the neocortex and GE generated considerable numbers of neurons (Figure 6G). Due to the purity of the FACS-sorted cells (93% [n = 154] of GE cells and 96% [n = 341] of neocortical cells were RC2$^+$), these data directly demonstrate that the vast majority of radial glia from both the dorsal and

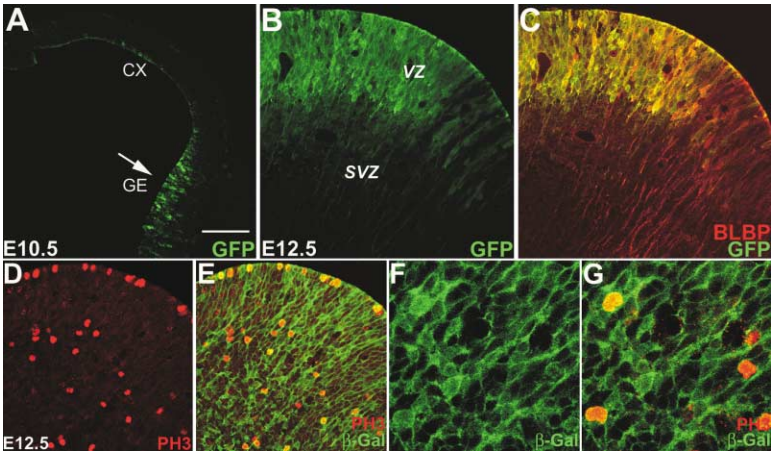

Figure 5. BLBP Expression in the Ganglionic Eminences Is Detected as Early as E10.5 and Is Restricted to Radial Glial Cells in the VZ

Immunostaining for BLBP promoter-driven GFP (A–C), endogenous BLBP staining (C), PH3 (D, E, and G), and β-gal (E–G); merged images are shown in (C), (E), and (G). Both endogenous BLBP (C) and reporter expression driven by the BLBP promoter (A and B) are detected at high levels in radial glia of the ganglionic eminences from as early as E10. At all stages of GE development, BLBP and GFP are restricted to radial glia of the ventricular zone and are never detected in cells within the SVZ. (D–G) Staining sections from E12.5 BLBP-Cre;R26R embryos for the mitotic marker PH3 demonstrates that most proliferating progenitors of the LGE SVZ are recombined, indicating they were generated by radial glia of the VZ. Scale bar equals 100 μm in (A), 60 μm in (B) and (C), 50 μm in (D) and (E), and 20 μm in (F) and (G).

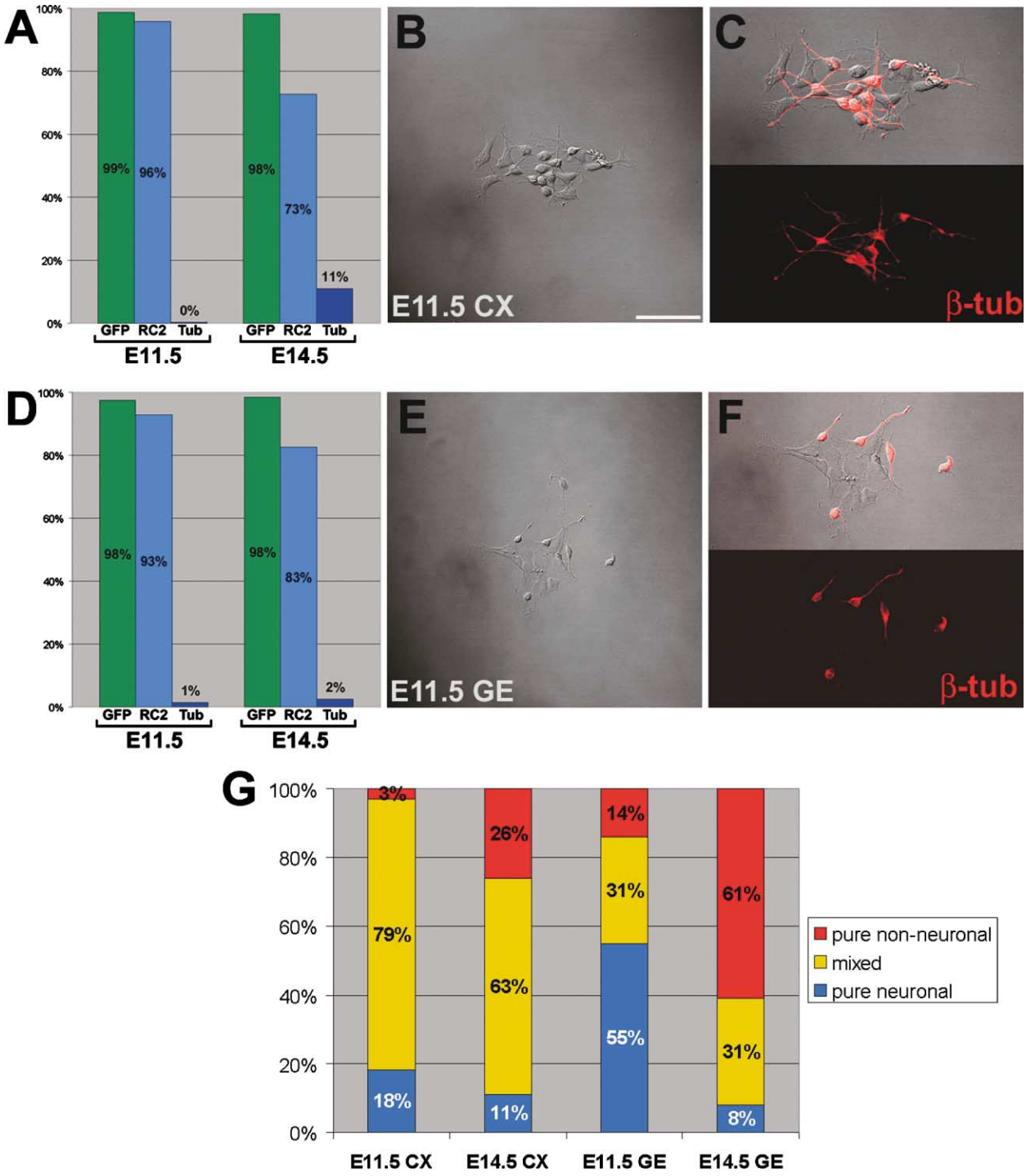

Figure 6. Radial Glia of the Ganglionic Eminences Complete Their Neurogenic Stage of Development Earlier than Neocortical Radial Glia

To assay the potential of neocortical (CX) and ganglionic eminence (GE) radial glial populations at different developmental time points, these regions were dissected from E11.5 and E14.5 BLBP-GFP mice and FACS sorted. (A and D) Purity of sorted fractions was determined by immunostaining aliquots of sorted cells for GFP, RC2 (radial glial marker), and β-tubulin (neuronal marker); a minimum of 150 cells was counted per marker. All fractions were significantly enriched for $RC2^{+}$ radial glia. Following FACS, sorted cells were plated at clonal density and cultured for 3 days, after which their neurogenic potential was determined by staining for β-tubulin. Examples of mixed clones (containing both neuronal and nonneuronal cells) from the E11.5 neocortex (B and C) and GE (E and F) are shown as DIC images (B and E), DIC plus β-tubulin fluorescence (C and F; top), and β-tubulin fluorescence alone (C and F; bottom). (G) Analysis of clone composition demonstrates that radial glia from both the neocortex and GE are neurogenic at E11.5. However, whereas most neocortical radial glia are still generating neurons at E14.5, the majority of GE radial glia have completed neurogenesis by this time. Number of clones analyzed: E11.5 CX, 248; E14.5 CX, 285; E11.5 GE, 244; E14.5 GE, 288. Scale bar equals 50 μm in (G) and 65 μm in (I).

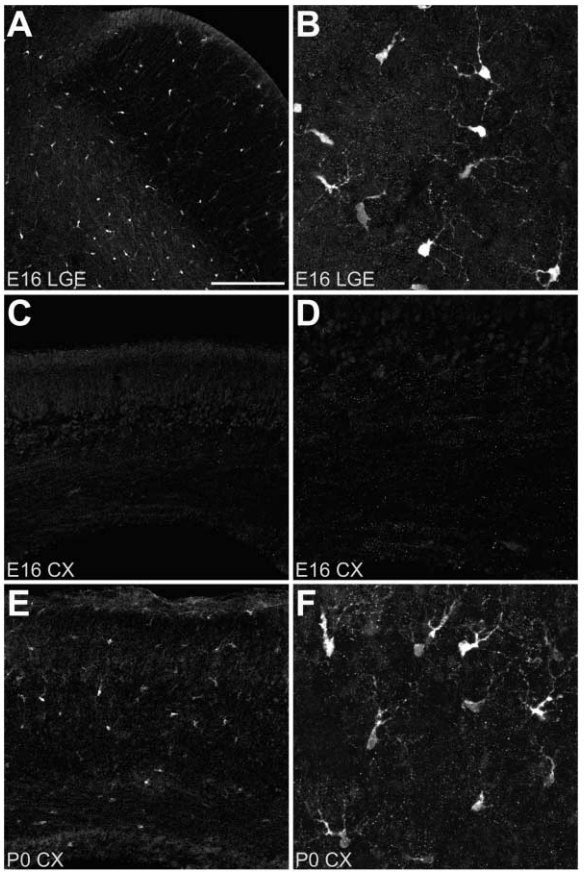

Figure 7. S100$^+$ Astrocytes Are Detected in the Ventral Telencephalon Days Earlier than in the Neocortex

Immunofluorescent staining for S100 at E16 (A–D) and P0 (E and F). Sections in (A), (C), and (E) are shown at higher magnification in (B), (D), and (F). Although numerous S100$^+$ astrocytes are observed in the E16 LGE (A and B) and other ventral telencephalic structures (not shown), they are not abundant in the neocortex (CX) until P0 (C–F). Scale bar equals 200 μm in (A), 50 μm (B), 120 μm in (C) and (E), 60 μm in (D), and 40 μm in (F).

ventral telencephalon can generate neurons. Interestingly, whereas most neocortical radial glia gave rise to large mixed clones (79%, n = 196), the majority of neurogenic GE radial glia (55%, n = 135) produced pure neuronal clones that contained ~4–5 neurons. This result suggests that a subset of GE radial glia become restricted to a neuronal lineage at the start of neurogenesis.

At E14.5, neocortical radial glia continued to generate large mixed clones containing numerous neurons (63%, n = 180). In contrast, 61% (n = 175) of GE clones at E14.5 produced pure nonneuronal clones. Moreover, of the mixed GE clones that were observed (31%, n = 89), almost 60% of them contained only one neuron (58%, n = 52). This indicates that most mixed GE clones were generated by progenitors that had reached the end of neurogenesis and suggests that up to 80% of GE radial glia have entered gliogenesis by E14.5. The staining pattern of the astroglial marker S100 provides additional evidence for regional differences in the timing of radial glial neurogenesis. Whereas significant numbers of S100$^+$ astrocytes are already present in the E16 LGE (Figures 7A and 7B), comparable staining is not seen in the neocortex until P0 (Figures 7C–7F). Taken together, the results from clonal analysis and staining for the onset of glial expression in vivo establish that ventral telencephalic radial glia complete neurogenesis and begin gliogenesis earlier than radial glia of the dorsal telencephalon.

Discussion

In this paper, we report three major results that are directly relevant to discussions of the role of radial glial cells as neuronal progenitors in the developing CNS. First, we have shown that both BLBP and GLAST are induced in essentially all neocortical radial glia in a spatiotemporal pattern paralleling the neurogenetic gradient. Second, we have shown that radial glia in all brain regions pass through a neurogenic stage of development and that most neurons are derived from these progenitors. Third, we have demonstrated that ventral telencephalic radial glia complete their neurogenic stage of development earlier than dorsal telencephalic radial glia.

The finding that radial glia appear to be the primary neuronal precursors in the neocortical ventricular zone suggested that these cells might serve as the major source of neurons throughout the CNS (Noctor et al., 2002). Our data are consistent with this idea and support a model in which radial glial cells in all brain regions pass through a neurogenic stage. This contrasts with the model of Malatesta et al. (2003), which proposed that neurons derived from the ventral telencephalon do not arise from radial glial progenitors. The apparent discrepancy between these models is likely explained by the timing of Cre expression in the fate mapping studies using either the BLBP (this study) or the hGFAP (Malatesta et al., 2003) promoter. In particular, BLBP is expressed in radial glia several days earlier than hGFAP in the ventral telencephalon. This difference in the timing of Cre expression in ventral telencephalic radial glia is critical, since it is well established that neocortical radial glia follow a developmental progression in which they serve as neuronal progenitors early in development and glial progenitors later. It seems likely, therefore, that the failure of lineage tracing experiments using the hGFAP promoter to detect neurons derived from ventral telencephalic radial glia is due to the fact that this promoter is not active ventrally until E14.5, a time point when most of these cells are no longer neurogenic.

Our data is consistent with previous neuronal birthdating studies that have demonstrated considerable regional differences in the timing of neurogenesis. For example, whereas generation of spinal motor neurons is already peaking by E9.5 (Nornes and Carry, 1978), neocortical neurogenesis does not even begin until E10 (Bayer and Altman, 1991; Takahashi et al., 1999). It must follow that radial glia, which we have now shown to be neuronal precursors throughout the CNS, are also temporally heterogeneous with respect to when they are neurogenic. Accordingly, recombination in BLBP-Cre;R26R embryos took place in the same spatiotemporal patterns as known neurogenetic gradients. For exam-

ple, recombination was already detected in the spinal cord by E9.5 (Figure 3B) but not in the neocortex until E10.5 (Figures 3A and 3C). In addition, recombination in the neocortex took place in a rostral to caudal pattern. Given that essentially all neocortical neurons were recombined in P45 BLBP-Cre;R26R mice, our results suggest that induction of BLBP expression marks the onset of radial glial neurogenesis.

Radial glial differentiation has been defined as the acquisition by neuroepithelial cells of differentiated glial characteristics, including BLBP and GLAST (Doetsch, 2003; Kriegstein and Gotz, 2003). The absence of BLBP from neuroepithelial cells is directly demonstrated by our fate mapping data, as no recombination was seen in the E9.5 neocortex (Figure 3), a time when this region is comprised almost exclusively of symmetrically dividing neuroepithelial cells (Takahashi et al., 1999; Qian et al., 2000). We note that BLBP and GLAST are not expressed in neuroepithelial cells but are co-expressed in radial glia, and both progress in a spatiotemporal pattern paralleling neurogenesis. Taken together, these data support the idea that the maturation of neuroepithelial cells to radial glia and the onset of radial glial neurogenesis are concurrent.

Our results further suggest that radial glia may give rise to neurons in one of two ways. For example, according to the model proposed by Noctor et al. (2001) for the generation of neocortical projection neurons, we suggest that radial glia can divide to generate a radial glial progenitor and a postmitotic neuron. Alternatively, radial glia may give rise to neuroblasts that form secondary proliferative layers, which subsequently produce postmitotic neurons. This appears to be the case in the ventral telencephalic SVZ, as the radial glial-derived neuroblasts in this region express the proneural gene *Mash1* and the *Dlx-1* and *Dlx-2* homeobox genes (Porteus et al., 1994; Eisenstat et al., 1999), and mice lacking these latter two genes exhibit defects in the development of the striatal SVZ and have few GABA-expressing interneurons in the neocortex (Anderson et al., 1997a, 1997b). It is interesting to note that granule neurons of the cerebellum (another interneuron population generated in a secondary proliferative zone [Hatten and Heintz, 1995]) were recombined in both hGFAP-Cre;R26R (Zhuo et al., 2001) and BLBP-Cre;R26R mice, suggesting that they also descend from radial glia. This raises the interesting possibility that while radial glia serve as progenitors for all neuronal classes, generation of interneuron populations throughout the brain may require the establishment of secondary proliferative zones as a necessary step in their development.

Our data illustrate the need to distinguish between lineally versus developmentally distinct radial glial cells. The expression patterns of multiple markers and Cre/*loxP* fate mapping has shown that while all radial glia appear to progress through the same developmental stages, they do so at different time points, depending upon where in the CNS they reside. For this reason, detailed expression information is likely a prerequisite for determining whether a candidate radial glial gene is involved in regulating lineage restriction or developmental progression and for further defining the precise stages through which radial glia pass during their maturation.

Experimental Procedures

Constructs and Generation of Mice

The BLBP-Cre and BLBP-GFP transgenes were made by PCRing the BLBP promoter from −1.6 kb to +53 (Feng and Heintz, 1995) using Expand High Fidelity PCR system (Roche) and cloning the product in front of an intron-Cre(or EGFP)-polyA cassette. Transgene DNA was prepared for injection by digestion to remove vector sequences, and then isolated from low melting gels using Qiaquick columns (Qiagen) followed by further purification using Elutip-d columns (Schleicher and Schuell). Transgenic mice were generated using standard protocols (Hogan et al., 1994). The gtrosa26 (Friedrich and Soriano, 1991) and R26R (Soriano, 1999) lines were obtained from The Jackson Laboratory. All mice were typed by PCR of DNA isolated from yolk sacs (embryos) or tails (adults).

Immunofluorescence

Tissues were either immersion fixed (embryos) or perfused (postnatal/adult) with 4% PFA and postfixed overnight at 4°C. Tissues were then washed in PBS and cut either on a cryostat (15 μm) or vibratome (75 μm). The following antibodies were used: rabbit α-BLBP (1:2000; Feng et al., 1994); mouse α-Nestin/rat-401 (1:4, Developmental Hybridoma Bank); rabbit α-GFAP and rabbit α-S100A/B (1:500, DAKO); mouse α-Calbindin and mouse α-Calretinin (1:500, Swant); rabbit α-β-galactosidase (1:500, ICN); goat α-GFP (1:500, US Biological); guinea pig α-GLAST, mouse α-NeuN, and mouse α-Parvalbumin (1:5000, 1:100, and 1:500, Chemicon); mouse α-β-III Tubulin (1:1000, Sigma). All secondary antibodies (Cy2 and Cy3) were from Jackson Immunoresearch and were used at 1:500. Sections were preblocked in 5% donkey serum, 0.1% Triton X-100 in PBS and incubated with primary antibodies overnight at 4°C. After washing, sections were incubated with secondary antibodies generated in donkeys for 2 hr in the same solution at RT. Confocal imaging was done on an LSM 510 Axioplan (Zeiss).

X-Gal Histochemistry

Embryos were fixed with 4% paraformaldehyde by immersion for 10 min at RT and incubated up to 1 hr at 37°C in X-Gal solution: 1 mg/ml X-Gal (Sigma), 20 mM $K_3Fe(CN)_6$, 20 mM $K_4Fe(CN)_6$, 2 mM $MgCl_2$, and 0.02% NP-40 in PBS. Adults were perfused with 4% PFA and postfixed for 30 min in the same solution. 70 μm sections were cut on a vibratome and developed for up to 1 hr in X-Gal solution.

FACS and Clonal Analysis

Cerebral cortices and ganglionic eminences were dissected from E11.5 or E14.5 embryos expressing EGFP under the regulation of the BLBP promoter. Cells were dissociated using a Papain dissociation kit (Worthington) according to the manufacturer's instructions and FACS sorted using a Coulter Epics Elite Cell Sorter. Purity of sorted cells was determined by immunostaining for GFP, RC2, and β-III Tubulin (see Figure 6). Sorted cells were plated at clonal density onto PLL and laminin-coated coverslips in antibiotic-free basal serum-free culture medium containing 10 ng/ml bFGF (as described in Qian et al., 2000). Feeder layers from the same brain region/time point were added to 0.4 μm Transwell chamber inserts (Costar), and clones were allowed to grow for 3 days. Clones were then fixed and stained for β-III Tubulin to assess their neurogenicity. For counting purposes, clones were defined as isolated clusters of 2 or more cells, although very few clones were observed (in any of the conditions tested) that contained fewer than 4 cells.

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