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Early Neuronal and Glial Fate Restriction of Embryonic Neural Stem Cells

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The question of how neurons and glial cells are generated during the development of the CNS has over time led to two alternative models: either neuroepithelial cells are capable of giving rise to neurons first and to glial cells at a later stage (switching model), or they are intrinsically committed to generate one or the other (segregating model). Using the developing diencephalon as a model and by selecting a subpopulation of ventricular cells, we analyzed both in vitro, using clonal analysis, and in vivo, using inducible Cre/loxP fate mapping, the fate of neuroepithelial and radial glial cells generated at different time points during embryonic development. We found that, during neurogenic periods [embryonic day 9.5 (E9.5) to 12.5], proteolipid protein (plp)-expressing cells were lineage-restricted neuronal precursors, but later in embryogenesis, during gliogenic periods (E13.5 to early postnatal), plp-expressing cells were lineage-restricted glial precursors. In addition, we show that glial cells forming at E13.5 arise from a new pool of neuroepithelial progenitors distinct from neuronal progenitors cells, which lends support to the segregating model.

Key words: diencephalon; plp; mouse; in vivo genetic fate mapping; neuroblast; glioblast

Introduction

The question of how neurons and macroglial cells are generated during development in ventricular proliferative zones of the neural tube has a long history. Wilhelm His suggested the existence of two cell lines in the ventricular zone ("segregating model"), one giving rise to neurons ("neuroblasts") and the other to glia ("spongioblasts") (His, 1889). His’ model was soon contested by Schaper (1897) and later by Sauer (1935) who proposed that a single homogeneous proliferative cell population gives rise to both neurons and glia. Pulse-chase experiments led Fujita (Fujita and Fujita, 1963; Fujita and Nakai, 1966) to support and complement the hypothesis of a single progenitor population that generates neurons and glial cells sequentially ("switching" model). Over the years, reports have alternated in support of one model or the other (Briscoe et al., 2000; Anderson, 2001; Stiles, 2003; Noble et al., 2004). The segregating model has been supported by Rakic and coworkers, who provided evidence, at the ultrastructural level, of the coexistence of neuronal and glial precursor cells in the cerebral ventricular zone of the fetal monkey (Levitt et al., 1981). Clonal analyses have since shown that neuronal-restricted and glial-restricted progenitors, responsible for the production of only neurons or only glia, can be isolated from the embryonic spinal cord and cerebral cortex (Davis and Temple, 1994; Mayer-Proschel et al., 1997; Rao et al., 1998; Qian et al., 2000). Retroviral labeling and genetic fate-mapping experiments have also suggested a very early and regional segregation of neuronal and glial progenitor cells (Luskin et al., 1993; Mione et al., 1997; McCarthy et al., 2001; Lyons et al., 2003; Wu et al., 2006; Battiste et al., 2007). In contrast, the switching model is consistent with what is known about the origins of neurons and glia in invertebrates. In grasshopper and Drosophila, asymmetric division of neuroblasts produces another neuroblast and a precursor cell, which on division produces either two neurons, two glial cells, or one of each (Udolph et al., 1993; Doe et al., 1998). In vertebrates, the existence in the cerebral cortex of cells capable of generating both neurons and oligodendrocytes has been postulated based on retroviral labeling experiments and clonal analysis (Williams et al., 1991; He et al., 2001; Yung et al., 2002). Furthermore, neurons and glia emerge simultaneously in common territories and a number of intrinsic and extrinsic regulatory mechanisms are essential for both neurogenesis and gliogenesis, suggesting that they could be derived from a common progenitor (Bertrand et al., 2002; Rowitch, 2004; Deneen et al., 2006; Petryniak et al., 2007). Along the same line are the data reported by the group of Yi Sun,
showing that DNA methylation in neural progenitors leads to switching from neurogenesis to gliogenesis (Fan et al., 2005).

In the early embryo, the primitive neural tube is composed of neuroepithelial cells, which contact both the ventricular and the pial surface. Radial glia designate cells that have their soma in the ventricular zone, in which they contact the lumen of the ventricles and possess a long process that extends toward the pial surface (Rakic, 1971a,b). Both, neuroepithelial cells and radial glia express the immediate filament nestin (Lendahl et al., 1990). Radial glia, however, can be distinguished from neuroepithelial cells by the expression of an array of markers such as the murine radial glia-specific marker RC2 (Misson et al., 1988), the astrocyte-specific glutamate transporter (GLAST) (Shibata et al., 1997), and the brain-lipid-binding protein (BLBP) (Feng et al., 1994). It has long been accepted that radial glia are astroglial progenitors (Schmechel and Rakic, 1979; Leviit et al., 1981; Voigt, 1989). It has also been proposed that radial glia serve as oligodendrocyte progenitors (Choi and Kim, 1985, Hirano and Goldman, 1988). Finally, recent evidence from several laboratories has demonstrated that radial glia produce most of the cortical neurons (Malatesta et al., 2000; Hartfiuss et al., 2001; Miyata et al., 2001; Noctor et al., 2001; Tamamaki et al., 2001; Wu et al., 2005). The fact that neurons and glia are the progeny of a common progenitor pool that share intrinsic and extrinsic regulatory mechanisms is therefore generally agreed. However, whether neurons and glia are derived from separate progenitor cells (segmenting model) or whether they are generated from a progenitor common to both lineages (switching model), using asymmetric division, has remained unclear so far.

To gain additional insight into the lineage relationship between neurons and glia in the ventral diencephalon in vivo, we created a transgenic mouse line in which the CRE recombinase was cloned (EcoRI/BamHI) into pBluescript KS+ (Stratagene, La Jolla, CA), and the 1.26 kb 3’ fragment of Cre (harboring a simian virus 40 polyadenylation signal) was added by BamHI cloning. Next, by Clal/EcoRI partial digestion, a fragment containing 3.74 kb of the PLP 5’ flanking sequence, exon 1, and 1.96 kb of intron 1 was inserted. The construct was completed by subcloning of a 5.07 kb EcoRI fragment of intron 1 that was generated by EcoRI partial digestion. The entire construct was linearized with NotI, and the vector backbone was removed by partial digestion with KpnI. Pronuclear injection of transgene DNA (1–2 ng/μl) into mouse oocytes (DBA/2 × C57BL/6/N) was performed by standard procedures (Hogan et al., 1994). Routine genotyping was performed by PCR on tail genomic DNA using primers PLPI sense and PLPCre antisense. All the other transgenic mice were generated using the same plp expression cassette. The plp–shble–lacZ and the plp–gfp entire constructs were generated in DBA/2 × C57BL/6/N F1 and maintained homozygous in the C57BL/6/N strain (Spaak et al., 1998, 2001b). The plp–CreERT2 mouse (Leone et al., 2003) was generated using an improved tamoxifen (TM) inducible variant of the CRE recombinase (CreERT2) (Metzger and Chambon, 2001). This line was generated using DBA/2 × C57BL/6/N F1 females. Rat embryos were obtained from pregnant Wistar rats (Elevage, Janvier, France). For mouse and rat, the midpoint of the dark interval during which mating occurred was designated as day 0, and the embryos were considered to be E0.5 on the morning after fertilization. The rats were considered at the same developmental stage as the mice 1.5 d later.

Mouse breeding. The plp–shble–lacZ and plp–gfp mice were crossed with OF1 females (Iffa Credo, L’Arbresle, France), and animals used were therefore heterozygous. The plp–Cre and the plp–CreERT2 mice were crossed either with R26R (Soriano, 1999) or the Z/EG reporter mice (Novak et al., 2000). The difference observed between offspring generated from this two reporter transgenic mice was quantitative: more cells were generated from R26R than from Z/EG mice.

Tamoxifen treatment. TM (T5648, Sigma, St. Louis, MO) was dissolved in a DMSO/ethanol/sunflower oil (4:6:90) mixture at the concentration of 10 mg/ml. Pregnant mother mice were injected intraperitoneally with 1 mg of tamoxifen per 10 g of body weight. Embryos were then analyzed at birth.

Cell culture. The basal plate of the diencephalon of rat and mouse embryos was dissected from E9.5 to E14.5 in Ca2+/Mg2+-free HBSS (Invitrogen, Carlsbad, CA). Cells were dissociated by digestion with trypsin (Biological Industries, Beth Haemek, Israel) diluted at 2.5 ng/ml in 2 ml of HBSS. After 10 min at 37°C, 2% FCS (Invitrogen) was added, and the solution was completed to 8 ml with HBSS and centrifuged for 5 min at 1000 rpm. Before FACS, mouse cells were resuspended in 2 ml of PBS containing 2% FCS and filtered through a 70 μm nylon mesh (VWR, North American Region). The cells were cultured in a diencephalic environment for the sorted cells similar to that encountered during normal development, without the addition of specific growth factors that may bias the analysis by selecting for particular cell types (Williams et al., 1991; Lillien, 1997; Qian et al., 1998). To validate this system for clonal analysis, we first ensured that all cells derived from mouse diencephalon were labeled by the M2/M6 monoclonal antibodies (Z/EG; Sigma) and were counterstained with 4′,6-diamidino-2-phenylindole (DAPI; Sigma) to eliminate dead cells during sorting.

For the mouse coculture system, rat feeder cells were put at 1.105 cells in a 15-cm dish. Fifty sorted mouse cells were plated on each coverslip. Thirty minutes after sorting, 450 μl of Bottenstein and Sato (BS) medium (Bottenstein and Sato, 1979) supplemented with 2% FCS was added. The medium was then replaced every 3 d to maintain optimal concentration of factors synthesized by the rat feeder. This clonal coculture system provides a diencephalic environment for the sorted cells similar to that encountered during normal development, without the addition of specific growth factors that may bias the analysis by selecting for particular cell types (Williams et al., 1991; Lillien, 1997; Qian et al., 1998). To validate this system for clonal analysis, we first ensured that all cells derived from mouse diencephalon were labeled by the M2/M6 monoclonal antibodies (mAbs) (data not shown). Cells were allowed to grow for 10 d, and only clusters containing at least two cells were considered for analysis.

For neurosphere cultures, after bpd dissection as described above, GFP+ (plp+) cells were sorted by FACS as described above, and 50 or 10 GFP+ cells (at E9.5 and E13.5, respectively) were plated into a 96-well plate. Mouse cells were grown in DMEM/F-12 medium (Invitrogen).
The number of clones analyzed was 65 at E9.5 and 159 at E13.5. Cell sorting was performed with a MoFlo (DakoCytomation, Carpinteria, CA) in the single-cell mode at the appropriate sorting rate. At each stage and for each experiment, purity of the sorted cells was reassessed by flow cytometry of an aliquot and on average comprised between 95 and 99% (see Fig. 3B–D). In a second set of experiments, we verified the purity of our cells by immunocytochemistry 2 h after the sorting experiment, with the same results.

The clonal probability follows the Poisson distribution, indicating that we are clonal at E9.5, considering the number of negative coverslips. At E13.5, we were not properly clonal, so we calculated the probability of clonal superimposition as performed in retroviral experiments (Williams et al., 1991; Malatesta et al., 2000), and we found 7.4 × 10^−9%. Data were derived from at least two different batches. After 10 d in vitro (DIV), the mean number of clones (calculated from the values obtained per 12-mm-diameter coverslip containing M2/M6-positive clones) were 2.4 ± 0.5 and 3.1 ± 0.5, respectively, for cultures derived from E9.5 or E13.5, whereas the mean number of cells in each clone was 19.4 ± 2.7 and 7.7 ± 0.9 for cultures derived from E9.5 or E13.5, respectively. The number of clones analyzed was 65 at E9.5 and 159 at E13.5.

**Antibodies, immuno labeling, and detection of β-galactosidase enzymatic activity.** The following mouse mAbs were used: A2B5 [1:5 (Eisenbarth et al., 1979)], O4 [1:5 (Sommer and Schachner, 1981)], M2/M6 mAb [1:5 and 1:10 (gift from C. Lagenaur, Developmental Studies Hybridoma Bank)], anti-nestin (1:300; Invitrogen), anti-adenomatous polyposis coli (CC1, 1:40; Oncogene Sciences, Uniondale, NY), neuronal-specific nuclear protein (NeuN) (1:1000; Invitrogen), TuJ1 (1:500; Covance Research Products, Berkeley, CA), RC2 (1:50; gift from P. Leprince, Université de Liège, Liège, Belgium), anti-CRE–Z/EG–actin–lox–GFP (1:250 or 1:500; Euromedex, Mundolsheim, France), and rat mAb anti-GFP (1:1000; Nacalai, Osaka, Japan). Polyclonal antibodies raised in the rabbit were anti-BLBP (1:1000; gift from N. Heintz, The Rockefeller University, Liege, Belgium), anti-CRE-2D8-As or C7920 (1:250 or 1:500; Euromedex, Mundolsheim, France), and rat mAb anti-GFP (1:1000; gift from N. Heintz, The Rockefeller University, New York, NY), anti-GFAP (1:400; DakoCytomation), and anti-GFP (1:1000; Invitrogen); in guinea pig, they were anti-GLAST (1:1000; Chemicon, Temecula, CA); in the goat, they were anti-β-galactosidase (β-gal) (1:1000; Chemicon); and appropriate secondary antibodies were fluorescence-conjugated (Alexa, 1:1000; Invitrogen) or biotinylated for DAB and streptavidin peroxidase as substrate. We further used the ABC Vectastain kit for DAB detection as well as the Substrate Kit Vector SG (Vector Laboratories, West Grove, CA). Immunolabeling of cultures or tissue sections and detection of β-galactosidase enzymatic activity were as described previously (Spasky et al., 1998). Figures 1, 2, and 5 were obtained using a confocal laser microscope (Leica, Nussloch, Germany), and the software used was Leica lite, NIH Image, and Photoshop (Adobe Systems, San Jose, CA).

**Results**

**plp transgenes are expressed by both neuroepithelial and radial glial cells.**

Previous studies have shown that plp transcripts are expressed in the ventricular layer of the laterobasal plate of the diencephalon already at E9.5 (Ikenaka et al., 1992; Timsit et al., 1992, 1995). To identify plp-expressing cells, we took advantage of the plp transgenic mice, in which expression of the β-galactosidase or GFP reporter genes are driven by plp regulatory sequences (Spasky et al., 1998, 2001b; Thomas et al., 2000) (Fig. 1A, B). Coronal sections of E9.5 and E13.5 transgenic embryos were double immuno-ostained with anti-β-galactosidase (or anti-GFP; data not shown) and a battery of antibodies, specific for either neuroepithelial or radial glial cells. At E9.5, plp-expressing cells (β-galactosidase + or GFP +) were nestin + neuroepithelial cells (Fig. 1C). At this developmental stage, RC2, BLBP, and GLAST were not detectable (data not shown). At E13.5, plp + cells in the ventricular layer were radial glial cells coexpressing nestin, BLBP, RC2, and GLAST (Fig. 1D). At E9.5 and E13.5 plp-expressing cells in the laterobasal plate of the diencephalon have therefore distinguish neuroepithelial and radial glial cell phenotypes, respectively.

**plp-expressing cells generate all major types of neural cells.**

To label the entire progeny of plp-expressing cells, we exploited a recombinase-based fate-mapping technique that transforms transient gene expression into a permanent inheritable lineage marker (Dymecki and Tomasicwicz, 1998; Zinyk et al., 1998). This approach is based on the cell-type-specific expression of CRE recombinase that mediates the deletion of a stop cassette flanked by loxP sites. Four independent plp–CRE transgenic founder lines were generated. Plp–CRE mice were crossed to the Rosa26–lox–lacZ (R26R) or β-actin–lox–GFP (Z/EG) reporter strains, which have been shown to drive high levels of reporter expression in all embryonic tissues (Soriano, 1999; Novak et al., 2000). To ensure that our fate mapping reflects recombination in plp-expressing cells and not ectopic CRE expression, the recombination pattern in the plp–CRE;R26R double-transgenic mouse was compared with the pattern of β-galactosidase in the previously characterized plp–shble–lacZ mouse. At E10.5, whole-mount preparations or coronal sections of double-transgenic plp–CRE;R26R embryos stained for β-gal activity revealed a staining pattern similar to that observed in the plp–shble–lacZ mouse (compare Figs. 1A, B and 2A, B). We also verified on coronal sections across the diencephalon of E10.5 plp–CRE embryos that the localization of cells expressing CRE recombinase had a pattern superimposable on that of β-gal + cells in the plp–shble–lacZ line (data not shown). At E13.5, β-gal + cells in the ventricular zone expressed GLAST, suggesting that they were indeed radial glial cells (Fig. 2C).

To identify which populations are derived from plp-expressing cells, we next examined recombination in postnatal mice. Coronal sections across the diencephalon of P15 plp–CRE; Z/EG mice were immunolabeled with a combination of anti-GFP and cell-type-specific markers. GFP was consistently detected in E10.5 plp–CRE embryos that the localization of cells expressing CRE recombinase had a pattern superimposable on that of β-gal + cells in the plp–shble–lacZ line (data not shown). At E13.5, β-gal + cells in the ventricular zone expressed GLAST, suggesting that they were indeed radial glial cells (Fig. 2C).

**plp-expressing cells are neurosphere-forming cells in vitro.**

Fetal and adult neural stem cells have been defined by their ability to generate neurospheres in vitro (Doetsch et al., 1999; Uchida et al., 2000; Rietze et al., 2001). Neurospheres are floating aggregates of tripotent self-renewing cells that can differentiate into neurons, astrocytes, and oligodendrocytes (Reynolds and Weiss, 1992; Sirichai et al., 2000). Although E10.5 plp–CRE; Z/EG fetal cerebral cortices were the best source of neurosphere-forming cells, we were able to maintain neurosphere-forming activity from E13.5 plp–CRE; Z/EG fetal cerebral cortices for 10 d in vitro (DIV). The mean number of neurosphere-forming cells (calculated from the values obtained per 12-mm-diameter coverslip containing M2/M6-positive colonies) were 2.7 ± 0.5, respectively, for cultures derived from E9.5 or E13.5. The mean number of colonies was 65 at E9.5 and 159 at E13.5.

**FACS and clonal analysis.** Cells were sorted from the brain of plp–gfp mice at two ages: E9.5 and E13.5. Cell sorting was performed with a MoFlo (DakoCytomation, Carpinteria, CA) in the single-cell mode at the appropriate sorting rate. At each stage and for each experiment, purity of the sorted cells was reassessed by flow cytometry of an aliquot and on average comprised between 95 and 99% (see Fig. 3B–D). In a second set of experiments, we verified the purity of our cells by immunocytochemistry 2 h after the sorting experiment, with the same results.

The clonal probability follows the Poisson distribution, indicating that we are clonal at E9.5, considering the number of negative coverslips. At E13.5, we were not properly clonal, so we calculated the probability of clonal superimposition as performed in retroviral experiments (Williams et al., 1991; Malatesta et al., 2000), and we found 7.4 × 10^−9%. Data were derived from at least two different batches. After 10 d in vitro (DIV), the mean number of clones (calculated from the values obtained per 12-mm-diameter coverslip containing M2/M6-positive clones) were 2.4 ± 0.5 and 3.1 ± 0.5, respectively, for cultures derived from E9.5 or E13.5, whereas the mean number of cells in each clone was 19.4 ± 2.7 and 7.7 ± 0.9 for cultures derived from E9.5 or E13.5, respectively. The number of clones analyzed was 65 at E9.5 and 159 at E13.5.
To determine whether cells that express plp were neural stem cells, we thus examined their ability to form neurospheres. The bpd of plp–gfp embryos at E9.5 and E13.5 was microdissected, dissociated, and GFP-expressing cells isolated by preparative FACS (Fig. 3A, C). The number of GFP+ cells isolated per diencephalon was 1867 ± 39 (n = 4) and 3.8 × 10^4 ± 3.1 × 10^3 (n = 4) at E9.5 and E13.5, respectively. However, the proportion of GFP-expressing cells decreased with age, from 17% at E9.5 down to 8% at E13.5 (Fig. 3B, D). To characterize the identity of the labeled cells, GFP+ cells were immuno-stained with cell-type-specific markers 2 h after sorting. At E9.5, all the GFP+ cells are nestin+ and BLBP−, whereas at E13.5, 60 ± 5% (n = 5) of these cells were BLBP+. Of interest, 22 ± 2% (n = 4) of GFP+ cells isolated at E13.5 were already postmitotic NeuN+ neurons.

To test their self-renewal and differentiation potentialsities, isolated GFP+ cells were cultivated at clonal density to form neurospheres. Either 50 or 10 GFP+ cells (at E9.5 and E13.5, respectively) were sorted into wells of a 96-well plate containing serum-free medium and FGF-2 plus EGF as mitogens. After 9–10 d, the plates were inspected for the formation of neurospheres. The ratio of wells containing neurospheres was 35 and 68% for cells isolated at E9.5 and E13.5, respectively. To determine the differentiation capacity of neurospheres grown from plp–GFP+ cells isolated at each developmental stage, we plated dissociated cells obtained from neurospheres or individual neurospheres on poly-D-lysine-coated tissue culture dishes and withdrew mitogens (FGF-2 and EGF) from the culture medium. After 5 d, the cultures were fixed and triple labeled with a combination of antibodies specific for nestin, neuron-specific β-III-tubulin (TuJ1 mAb), GFAP, and O4 to detect neural progenitor cells, neurons, astrocytes, and oligodendrocytes, respectively.

Although the vast majority of cells were still undifferentiated nestin+ cells, all three differentiated cell types could be detected in E9.5-derived plp–GFP+ neurospheres: neurons (2.4 ± 0.4%), oligodendrocytes (2.9 ± 0.4%), and astrocytes (5.6 ± 0.8%).

Neurons, therefore, represented 20% of differentiated cells. We next investigated whether E13.5 plp–GFP+ cells could also form tripotent neurospheres in vitro. Surprisingly, the differentiation of E13.5-derived plp–GFP+ neurospheres was primarily limited to a glial fate [astrocytes (31 ± 3.2%) and oligodendrocytes (2.4 ± 0.4%)], whereas very few neurons were found (0.03 ± 0.02%). Neurospheres from E13.5 embryos were able to self-renew when clonally replated in the appropriate media for at least three times but were never able to generate neurons. To verify that the lack of neuronal differentiation among cells dissociated from E13.5 plp–GFP+ neurospheres was not an artifact resulting from the dissociation process, we repeated the same differentiation experiment without dissociating the neurospheres. Immunolabeling of nondissociated neurospheres (after withdrawal of FGF-2 and EGF) showed an absence of detectable TuJ1-positive cells in E13.5 plp–GFP+ neurospheres, which was in sharp contrast with the strong TuJ1 signal in E9.5 plp–GFP+ neurospheres (Fig. 3E–H). These data indicate that plp–GFP+ cells have the
Figure 2. *plp*-expressing ventricular cells give rise to all neural cell populations. A, Whole-mount of *plp–CRE;R26R* embryo stained with blue-gal at E10.5. The line indicates the plane of section in B. B, Coronal section across the bpd showing blue-gal-labeled cells (compare with Fig. 1B). C, Coronal section through the bpd of a *plp–CRE;Z/EG* at E13.5, double immunostained with GFP and GLAST antibodies showing that all GFP-positive cells in the ventricular zone are GLAST-positive radial glial cells. D–G, At later stages, *plp*-activated cells give rise to all (Figure legend continues.)
 capability to self-renew but that their potentiality to differentiate into neurons disappears between E9.5 and E13.5.

Ventricular diencephalic plp + progenitors in vitro are fate restricted
To determine the developmental potential of plp-expressing cells closer to physiological conditions, we used a clonal mouse–rat coculture system (Malatesta et al., 2000). GFP-expressing cells were FACs purified after dissection and dissociation of the bpd, as described above. After cell sorting, GFP + cells were seeded at a clonal density on a layer of rat diencephalic cells of the same developmental stage. Individual sorted cells and their progeny were identified using mouse-specific combined neural antibodies M2 and M6 (see Materials and Methods) (Lagenaur and Schachner, 1981). We then used triple immunostaining with cell-type-specific antibodies to identify the cellular composition of the M2/M6-immunoreactive clusters (Fig. 4A–H).

When isolated at E9.5, 75% of the M2/M6-positive cell clusters after 10 DIV consisted of a single cell type (Fig. 4I). The majority (70 ± 12%; n = 3) of M2/M6-positive clusters contained only neurons. Whereas just after sorting the M2/M6-positive cells were single cells, after 10 DIV, the clusters consisted mostly of 15–20 cells (mean clone size, 17 ± 3 cells; n = 3), indicating the proliferation of sorted plp–GFP + cells. These mixed clusters were composed of ~80% neurons and 20% glial cells. A more detailed examination of the glial phenotype in mixed clones generated from E9.5 plp–GFP + embryos showed that these glial cells expressed RC2, BLBP, or GLAST, indicating that they were radial glial cells, i.e., progenitor cells, rather than differentiated astrocytes or oligodendrocytes. In some experiments, cultures were maintained for 5 more days [15 DIV, corresponding to postnatal days 4/5 (P4/5) in vivo], and we did not observe any significant modifications in neither the proportion of mixed clones versus pure neuronal clones nor in the relative cell type composition of mixed clones. Notably, in the mixed clones, the glial components were still BLBP +, suggesting that they remained undifferentiated radial glia (Fig. 4G,H).

In contrast, when plp–GFP + cells were isolated from E13.5 brains, only few neuronal clusters were detected, and the majority (70 ± 4%; n = 4) of M2/M6-positive clusters analyzed after 10 DIV were pure and contained only glial cells (Fig. 4I). These results suggested that most plp progenitors have entered gliogenesis by E13.5. At this stage, the mean size of the glial clones comprised 7 ± 1 cells (n = 4), suggesting also that these cells did not have the same proliferation characteristics as those isolated at E9.5. At both ages, diencephalic plp–GFP + cells gave rise to the same proportion of mixed M2/M6-positive clusters, i.e., clones containing both neurons and glia (25 ± 12, n = 5 at E9.5; 25 ± 8, n = 4 at E13.5). The E13.5 mixed clusters were composed of 80% glia and 20% neurons.

It could have been possible that the restricted fate of cells isolated at E9.5 or E13.5 resulted from the environment in which the cells were cultivated. To address this question, we switched the developmental stage of the rat feeder layer. plp–GFP + cells sorted from E9.5 and E13.5 mice were cultivated on diencephalic cells dissociated from E15.5 and E11.5 rat embryos, respectively. These heterochronic culture conditions had no influence on the fate of differentiation of plp–GFP + cells (data not shown). Together, these results suggest that the potential of plp–GFP + cells is mostly neurogenic at E9.5 and becomes mostly gliogenic at E13.5 and that this change is primarily cell autonomous and independent of environmental conditions.

Diencephalic ventricular cells are restricted progenitors in vivo
To assess the fate of plp progenitors in vivo, we used a transgenic mouse expressing a TM-inducible variant of the CRE recombinase (CreERT2) under the control of the plp promoter (plp–CreERT2 mouse) (Leone et al., 2003). This transgenic mouse was crossed with the R26R or the Z/EG reporter lines. It has been...
shown previously that the CreER fusion protein translocates to the cell nucleus within 6 h of tamoxifen administration and that cells become marked in the following 12–24 h (Danielian et al., 1998). Therefore, by activating the CreERT2 with a single dose of tamoxifen at different embryonic stages, plp lineage cells were labeled at each stage, and their subsequent fate was determined in the young mouse brain (E0).

First, we verified that the plp–CreERT2 transgene was expressed during embryonic development exactly like the endogenous plp gene. We performed β-galactosidase histochemistry on whole-mount plp–CreERT2;R26R embryos and compared the pattern of 5-bromo-4-chloro-3-indolyl-β-d-galactopyranoside (X-gal) staining with the one in age-matched plp–Cre;R26R embryos. Pregnant plp–CreERT2;R26R females were injected at E9.5 with one dose of tamoxifen (typically 4 mg), and the embryos were collected 1 or 3 d later for β-galactosidase detection. Expression of the transgene was similar in plp–CreERT2;R26R and in plp–Cre;R26R embryos at E10.5 (Fig. 5A, B). We verified at E12.5 that the same cell populations were labeled on tissue sections (data not shown). On average, 144 ± 20 cells (n = 2) per diencephalon had recombinated at this stage after a single tamoxifen injection. Because ~1800 GFP + cells were FACs purified per E9.5 plp–GFP microdissected diencephalon, the efficiency of recombination of plp–CreERT2 can be estimated to be in the average of 8%.

To determine the identity of the progeny of E9.5 or E13.5 plp–expressing progenitors, we administered tamoxifen at either age to plp–CreERT2;Z/EG or plp–CreERT2;R26R pregnant mice and analyzed the labeled cells at birth after double immunolabeling with an anti-GFP or anti-β-gal and cell-type-specific antibodies. The same results were observed with either reporter mice, and the results were pooled. As predicted, the vast majority of cells labeled by tamoxifen induction at E9.5 were neurons (95 ± 1%; n = 9) (Fig. 5D). These neurons had mostly migrated away from the ventricular zone to contribute to the formation of diencephalic nuclei, which were mostly composed of calretinin+ neurons (Fig. 5F). Only a few GFAP+ astroglial cells (1 ± 1%; n = 9) were observed, most of them at a distance from the ventricular zone, near the pial surface. The remaining GFP-labeled cells (4 ± 1%; n = 9) were found in the ventricular layer and were identified as undifferentiated progenitors. In contrast, when tamoxifen induction was performed at E13.5, GFP-labeled cells were sparse, and the majority was double stained with anti-Olig-2 Ab (77 ± 5%; n = 13), whereas no neurons were found (Fig. 5E, arrows in G). The remaining GFP-expressing cells (22 ± 5%; n = 13) were localized in the ventricular layer and identified as undifferentiated progenitors (Fig. 5E, arrowhead in G).

In the diencephalon, the number of glial cells generated per brain was 3 ± 2 (n = 9) at E9.5, whereas at E13.5, the average number was 12 ± 2 (n = 13), which was statistically different (p < 0.001, Student’s t test). Assuming that the recombination efficiency was the same at each age, this difference showed that four times more glial cells were generated at E13.5 than at E9.5, which suggests that, by E13.5, a new population of plp–expressing cells has emerged that is committed to a glial fate.

To test whether plp is expressed in different progenitor cells at E9.5 and E13.5, we used the plp–CreZ/EG mouse and took advantage of the time lag between transgene expression (CRE-recombinase detection) and recombination (GFP detection). We reasoned that, at E13, all cells born between E9 and E12.5 should either express GFP only or both CRE and GFP, whereas the latest recombinase detection (GFP detection). We reasoned that, at E13, all cells born between E9 and E12.5 should either express GFP only or both CRE and GFP, whereas the latest group of cells (born at approximately E13) would express only CRE and still be GFP negative. Cross-sections of E13 plp–Cre;Z/EG embryos were double immunostained using anti-CRE and anti-GFP antibodies (Fig. 6A–C). Many CRE+/GFP+ cells had migrated out of the ventricular layer toward the marginal layer (Fig. 6A–C, arrowheads). In the ventricular and subventricular layers, a few cells were double labeled with the anti-GFP and anti-CRE antibodies (Fig. 6A–C). In addition, a large number of cells localized in the ventricular layer were CRE+/GFP−, suggesting that they had begun to express plp shortly before the embryos were fixed (Fig. 6A–C, arrows). These data demonstrate the emergence of approximately E13 of a new population of cells.
expressing *plp*. These cells are in the same lineage but genetically clearly distinct from the *plp*-expressing progenitor cells generated at E9.5. To determine whether *plp*-expressing progenitor cells segregated especially at E13.5 or continuously from E9.5 to E14.5, coronal sections of E9.5–E14.5 *plp*;CRE;Z/EG diencephalon were double immunostained with anti-CRE and anti-GFP antibodies (E9.5, *n* = 6; E10.5–E13.5, *n* = 4; E14.5, *n* = 2), and all CRE⁺/GFP⁻, CRE⁻/GFP⁺, and CRE⁻/GFP⁺ cells were counted along the ventricular and subventricular zone of the diencephalon. The CRE⁺/GFP⁻ and CRE⁻/GFP⁺ labeled cells were found at all ages almost exclusively in the ventricular and subventricular zone, respectively (Fig. 6D). The proportion of CRE⁺/GFP⁻ followed a clear biphasic pattern, high at E9.5–10.5, with a sharp decrease to 10% only at E11.5, an increase at E12.5, and a progressive decrease from E13.5 and E14.5. In contrast, the variation in the proportion of CRE⁻/GFP⁺ cells provided a mirror image to that of CRE⁺/GFP⁻. These data are compatible with the emergence of a new population of *plp*-expressing cells starting at E12.5.

**Discussion**

By specifically labeling *plp* progenitors and following their progeny in vivo, we demonstrate here that neurogenic *plp⁺* progenitor cells at E9.5 are distinct from gliogenic *plp⁺* progenitor cells at E13.5. The same observation applies to *plp⁺* progenitors when heterotopically grown in vitro. Using the tamoxifen-inducible Cre/loxP system, we further show that, at E13.5, based on their CRE⁺/GFP⁻ phenotype, *plp⁺* glioblasts are newly generated from a pool of (*plp⁻*) GFP⁻ radial glial cells. Diencephalic *plp⁺* progenitors are therefore a restricted and diverse population of progenitors. These findings have led us to propose that *plp⁺* neuroblasts and glioblasts are segregated early in the embryonic neuroepithelium.

This hypothesis is nevertheless confronted with alternative interpretations that support the opposing switching model. First, one can argue that *plp⁺* glioblasts might arise from *plp⁻*-negative neuroblasts, after a switch in the lineage specification of one single neural progenitor. Our in vitro data, however, indicate that this hypothesis is unlikely, because long-term cultures of E9.5 *plp* progenitors were never able to generate glial cells. A second contradictory interpretation would be that the same neural progenitors display various levels of *plp* expression during development, successively upregulating *plp* expression at E9.5, then downregulating before and upregulating again after E13.5. This possibility cannot be definitively discarded but is clearly not supported by the pattern of GFP expression in ventricular progenitors between E9.5 and E13.5. If *plp* expression was temporally regulated in ventricular progenitors, once *plp* expression has been downregulated, GFP expression should still be observed, because it remains in the cytoplasm long after *plp* has ceased to be expressed. However,
GFP-positive neuroblasts were rare in the ventricular zone between E10.5 and E12.5, indicating that plp lineage neuroblasts most probably do not remain in the ventricular zone but are exclusively dispersed in the parenchyme. Our conclusions for plp are therefore complementary to those made for Olig-1-expressing (Wu et al., 2006) and Ascl1-expressing (Battiste et al., 2007) progenitor cells in the developing spinal cord. Wu et al. (2006) used a conditional cell-ablation approach to provide in vivo evidence that motoneurons and oligodendrocytes are sequentially generated in the pmN domain and do not share a common lineage-restricted progenitor. Similarly, Battiste et al. (2007) used genetic fate-mapping experiments to show that dorsal horn interneurons are derived from Ascl1-early-expressing neuronal restricted progenitors and that oligodendrocytes are derived from Ascl1-late born expressing glial restricted progenitors. In the forebrain subventricular zone, neurogenesis and gliogenesis persist into adulthood, and recent clonal culture experiments show coexistence in the germinative zone of separate pools of neural stem cells (type B cells), generating either neurons or oligodendrocytes (Menn et al., 2006), thus providing an additional argument in favor of early segregation of neuroblasts and glioblasts. Moreover, by specifically targeting neural stem cells in the developing diencephalon, our results lend support to the model of neural stem cells (type B cells), generating either neurons or glioblasts. Moreover, by specifically targeting neural stem cells in vivo and in vitro. It has been reported previously that, from E12.5, the plp transcript allows the identification of a subpopulation of oligodendrocyte precursor cells (Spaskey et al., 1998, 2001a). Similar results, using Cre/loxP fate-mapping experiments, confirmed that radial glial cells mostly generate glial cells in the ventral telencephalon (Malatesta et al., 2003). Moreover, the transplantation experiments in which isolated Olig-2 cells from murine ventral spinal cord at E13.5 were directly transplanted to E2 chick spinal cord, demonstrated that these cells are intrinsically restricted to a glial fate (Mukoyama et al., 2006). Interestingly, the fate restriction of radial glial cells in the ventral diencephalon also seems to rely exclusively on cell-intrinsic cues. Indeed, the restricted gliogenic potential of plp+ radial glial cells was maintained whether the cells were cultivated on E10.5 or E14.5 rat feeder layer and was not influenced by treatment with EGF and FGF-2.

Coexistence of the two differentiation models

Altogether, our data provide compelling evidence that a subset of neurons and glial cells is sequentially generated from two different progenitors in the bpd. This does not exclude the possibility that, even in the same territory for other subsets of neural progenitors, neurons and glia share a common lineage. Because plp+ cells represent only 8–17% of the diencephalic basal plate, it is possible that other neighboring ventricular cells are bipotent neural-glial progenitors. This has been demonstrated in Drosophila, in which the dorsal neuroectoderm coexist progenitors with a restricted differentiation fate, either neuronal or glial, whereas others adopt a switching type of differentiation and produce neurons and glia (Schmidt et al., 1997). These authors have shown that each hemisegment of
the *Drosophila* gives rise to ~350 neurons and 30 glial cells during embryonic development. Neuroblasts from the dorsal neuroectoderm produce ~90% of the glial cells in the embryonic ventral ganglion. Two of the neuroblasts give rise to glial progeny exclusively, seven neuroblasts give rise exclusively to neurons, and five give rise to glia as well as neurons. Therefore, also in mammals, neurons and glia can most probably be generated by either model. The question that remains to be elucidated concerns the mechanisms controlling one or other of these processes of differentiation and whether this entails functional consequences for two apparently similar cells having been so generated. This could have consequences in terms of repair of certain pathologies such as multiple sclerosis, in which it is important to generate new oligodendrocytes, without also generating new neurons. In contrast, in the case of stroke, it may be of interest to generate in one step both neurons and glia.

**References**


