Glial progenitors of the neonatal subventricular zone differentiate asynchronously, leading to spatial dispersion of glial clones and to the persistence of immature glia in the adult mammalian CNS

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Abstract

The subventricular zone (SVZ) of the developing mammalian forebrain gives rise to astrocytes and oligodendrocytes in the neocortex and white matter, and neurons in the olfactory bulb in perinatal life. We have examined the developmental fates and spatial distributions of the descendants of single SVZ cells by infecting them in vivo at postnatal day 0–1 (P0–1) with a retroviral “library”. In most cases, individual SVZ cells gave rise to either oligodendrocytes or astrocytes, but some generated both types of glia. Members of glial clones can disperse widely through the gray and white matter. Progenitors continued to divide after stopping migration, generating clusters of related cells. However, the progeny of a single SVZ cell does not differentiate synchronously: individual clones contained both mature and less mature glia after short or long intervals. For example, progenitors that settled in the white matter generated three types of clonal oligodendrocyte clusters: those composed of only myelinating oligodendrocytes, of both myelinating oligodendrocytes and non-myelinating oligodendrocytes, or of only non-myelinating cells of the oligodendrocyte lineage. Thus, some progenitors do not fully differentiate, but remain immature and may continue to cycle well into adult life.

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Keywords: Glia; Astrocytes; Oligodendrocytes; SVZ; Cell lineage

Introduction

The subventricular zone (SVZ) of the mammalian forebrain contains mitotically active, migratory progenitors that give rise to many of the astrocytes and oligodendrocytes of the neocortex, subcortical white matter, and deep gray matter (Altman, 1966; Kakita and Goldman, 1999; Levison and Goldman, 1993; Luskin and Mc Dermott, 1994; Parnavelas, 1999; Paterson et al., 1973; Privat, 1975). Retroviral lineage studies indicate that progenitors generate tightly knit clonal clusters of glia in the neocortex (Grove et al., 1993; Levison and Goldman, 1993; Luskin and Mc Dermott, 1994; Luskin et al., 1993; Parnavelas, 1999), indicating that a given cell continues to divide after it has stopped migrating. The majority of these clusters have appeared homogeneous with respect to glial type, although a smaller proportion contain both astrocytes and oligodendrocytes. However, these studies would not have identified glial clones, the members of which were separated in space by more than the confines of a “cluster”. Thus, the full extent of spatial dispersion and the degree of cellular heterogeneity of glial clones have not been thoroughly explored.

Another issue not approached in previous studies concerns whether the descendants of a single progenitor differentiate synchronously or not. Is a given clone made up entirely of mature astrocytes or myelinating oligodendrocytes? The asynchronous differentiation of progenitors would result in clonal mixtures of mature and immature glia, leading to a plausible explanation for the continued presence of immature cells in the adult CNS (Gensert and Goldman, 1996, 2001; Levison et al., 1999; McCarthy and Leblond, 1988; Wolswijk and Noble, 1989).
In the present study, we address the issue of clonal dispersion and asynchronous differentiation by genetically labeling early postnatal SVZ progenitors in vivo with a retroviral library (Walsh and Cepko, 1992), so that their progeny could be identified later in development regardless of their location in the brain. Our results indicate that SVZ progenitors can give rise to clones that disperse spatially and that progenitors can generate both homogeneous and heterogeneous glial populations, although the former predominate. The descendants of a single SVZ cell do not always differentiate synchronously in vivo. Thus, some members of oligodendrocyte clones do not differentiate into myelinating oligodendrocytes, but rather remain as non-myelinating cells of the oligodendrocyte lineage. In fact, some clones were composed entirely of non-myelinating cells or mixtures of myelinating and non-myelinating cells as much as 2 months after retroviral labeling.

**Materials and methods**

**Retroviral labeling of SVZ cells in vivo by stereotactic injection**

SVZ labeling by stereotactic injection into Sprague–Dawley rats (on P0–1, the day of birth being P0) was performed as described (Zerlin et al., 1995). Protocols were approved by the University Animal Institute Care Committee. At the appropriate times after injection, animals were anesthetized and euthanized by intracardiac perfusion of tissue culture medium containing heparin followed by paraformaldehyde. Brains were sectioned at 100-μm intervals and X-gal histochemistry was performed (Levison and Goldman, 1993; Zerlin et al., 1995). Before PCR analysis, maps of each of the brains were constructed indicating the exact location of each of the labeled cells and each cell identified by morphology and photographed.

**Low titer labeling of SVZ cells in vivo to obtain brains for PCR analysis**

Different dilutions of the retrovirus library were used to label SVZ cells in vivo in P0–1 rats to determine the appropriate dilution that consistently would label only a few cells per brain. One to 2 days after the injection, the animals were perfused and the brains processed for X-gal histochemistry (Levison and Goldman, 1993; Zerlin et al., 1995). Either 1:60 or 1:90 dilutions of the library (corresponding to titers of $8 \times 10^3$ and $5 \times 10^3$ CFU/ml, respectively, as determined by infection of NIH 3T3 cells in the absence of polybrene) labeled only a few, on average no more than five, SVZ cells per brain. These dilutions were alternated in labeling experiments to obtain brains labeled at low titer to use in the PCR analysis. To control for experimental variation, half of the injected animals in every labeling experiment (comprised of 10–14 animals) were analyzed 1 day later to monitor the number of X-gal-labeled SVZ cells per brain. If these brains contained only a few labeled cells, then the rest of the injected animals would be analyzed after either a few days or 4–7 weeks.

**PCR analysis of retrovirally infected cells**

PCR analysis was carried out essentially as previously described (Walsh, 1995; Walsh and Cepko, 1992) with certain modifications. Dissected X-gal-labeled cells were digested overnight at 55–60°C in 12 μl digestion buffer containing 0.4 mg/ml proteinase K (Sigma) in 10 mM Tris–HCl pH 8.3, 0.5% Tween 20. Digested samples were then incubated at 85°C for 20 min, subsequently microwaved at full power for 3 min, and then chilled on ice before proceeding with the first PCR amplification reaction (PCR I): 85°C for 2 min, then the addition of 1 unit of AmpliTaq DNA polymerase (Perkin-Elmer) per sample in 20 μl of PCR I reaction mix containing 10 mM Tris–HCl (pH 8.3), 50 mM KCl, 0.1% gelatin, 2.5 mM MgCl$_2$, 1 μM each of the external primers PBR-5 and BND-4, 200 μM each of dA, dC, dG, and dTTP; then the reaction continued at 94°C for 30 s, 50°C for 3 min, 72°C for 2 min (45 cycles), and 72°C for 7 min. A second PCR reaction (PCR II) was carried out using the products of the first PCR as templates. Each of the PCR I reactions was diluted one in a hundred in 40 μl of PCR II reaction mix containing 10 mM Tris–HCl (pH 8.3), 50 mM KCl, 0.5 mM MgCl$_2$, 0.01% gelatin, 0.2 μM each of the internal nesting primers PBR-4 and BND-3, 10% glycerol, 150 μM each of dA, dC, dG, and dTTP, and 0.025 units/μl (1 unit/reaction) of AmpliTaq DNA polymerase (Perkin-Elmer) and amplified at 94°C for 30 s, 55°C for 3 min, 72°C for 2 min (30 cycles), and then 72°C for 7 min. Products from PCR II amplification were characterized by restriction enzyme digestion and agarose gel electrophoresis of the digestion products. All incubations were performed under mineral oil. In each experiment, 13% of the samples were represented by negative controls containing unlabeled tissue located several millimeters away from X-gal-labeled cells to monitor the specificity of the PCR amplification.

**Characterization of the retrovirus library with regard to its complexity**

Appropriate use of the retroviral library strategy requires that all members of the library are approximately equally represented. That is, no one or a few members are highly overrepresented and therefore more likely to occur in any random selection. To investigate insert representations in the library, we examined labeled cells selected at random in brains that were labeled at a titer at least 50 times higher than the one used in brain B5. These brains also contained mixtures of different retroviral integrates. A total of 46 labeled cells were selected at random from four of these brains (out of many hundreds of X-gal-labeled cells) for PCR analysis and products were recovered from 32 cells. We
recovered four cells, each having a different insert from the first brain, seven cells representing six different inserts from the second, 14 cells representing nine different inserts from the third, and three cells, each with a different insert, from the fourth. In addition, as noted above, inserts recovered from B5 were all different from each other and different from those recovered from M1, B3, and B4. Thus, we have no indication that the library is skewed toward a few members.

Immunocytochemistry

In a separate series of experiments, we injected a green-fluorescent protein (GFP) encoding retrovirus into the same region of the SVZ as we labeled with the “library”. The distribution and characteristics of cells labeled with this virus are the same as those labeled with the beta-galactosidase encoding virus used in our previous studies and in this study (for comparison, see Kakita and Goldman, 1999; Suzuki and Goldman, 2003). Five to 7 weeks after the injection of the GFP retrovirus, animals were euthanized and perfused as described above, and brains were immersed in 4% paraformaldehyde overnight and transferred to 20% sucrose for cryoprotection. The GFP-labeled cells were characterized further on frozen sections using the antibodies to CNPase (diluted 1:100, Sternberger Monoclonals Incorporated, MD), NG2 (1:400 dilution, a gift from Dr. Stallcup), PDGFRα (1:100, Santa Cruz, CA), and the Yp form of glutathione-S-transferase (1:50, Biotrin). Sections were incubated overnight at 4°C in primary antibodies diluted in 5% normal goat serum and 0.25% Triton X-100 in PBS. Sections were washed in PBS and appropriate fluorescent secondary antibodies were applied. The secondary antibodies used were conjugated to TRITC or Cy5 (Chemicon International Inc., Temecula, CA). After completing the immunolabeling, sections were mounted and examined on an LSM 510 META laser scanning confocal microscope attached to the Zeiss Axiovert 200 inverted microscope (Carl Zeiss, Inc., Thornwood, NY), as described before (Milosevic and Goldman, 2002). Images were processed in Adobe PhotoShop 7.0.

Results

Classification of retrovirally labeled glia

To classify the various glial types in the clones, we relied on both morphology and antigen markers. Mature astrocytes were easily identified by their bushy appearance and contacts with blood vessels or the pial surface (Figs. 2c and 4d; for many examples of retrovirally labeled astrocytes, see Kakita and Goldman, 1999; Levison and Goldman, 1993; Levison et al., 1999; Luskin and Mc Dermott, 1994; Parnavelas, 1999). Immature astrocytes were discerned because of their early blood vessel contacts (Zerlin and Goldman, 1997). Myelinating oligodendrocytes displayed the β-galactosidase or GFP in long thin, usually parallel processes because the reporter protein is found in the oligodendrocyte loops of myelin sheaths (Figs. 2f and 4a and the above-noted references). Myelinating oligodendrocytes were also immunolabeled for CNPase (Fig. 1a). We also found oligodendrocytes...
in an early stage of myelination, in which a round cell body was attached to thin processes, some of which branched in a “T” form (Fig. 2e). As described below, however, many of the oligodendrocyte clones contained non-myelinating cells. These fit into several morphological categories: (1) cells with round somas and many thin, branching processes, often arranged radially, giving the cell a “lacy” appearance (Figs. 1b and 4c, and see Levison et al., 1999); (2) cells with somas larger than myelinating oligodendrocytes, but with very few, short processes (Fig. 1a, arrow, and see Levison et al., 1999); and (3) unipolar or bipolar cells that looked like immature glia (Fig. 1c).

It would have been technically problematic to perform β-galactosidase staining, immunostaining for several different antigens, and then PCR on the same cells, so we used another strategy to help define these non-myelinating cells. We injected the GFP-encoding retrovirus into the same region of the lateral SVZ at P0–1, fixed tissue after the same interval (5–7 weeks), and then analyzed the labeled cells by immunofluorescence. We were able to perform double labeling for antigens in addition to visualizing the cell’s morphology clearly with the GFP by confocal microscopy. The large majority (96%, 23/24) of the “lacy” cells were labeled with the NG2 antibody (Fig. 1b). As noted before, these cells are characterized by a round nucleus and an extensive network of many, delicate, branching processes (see Figs. 2B, 3C, and 4C in Levison et al., 1999). There is current controversy over the nature of these cells—they have been called “polyandrocytes” (Nishiyama et al., 2003) or “syn-antocytes” (Berry et al., 2002; Butt et al., 2002), and considered by some investigators to represent a stable glial population in the mammalian CNS. They are thought to belong to the oligodendrocyte lineage. For our present purposes, however, we will forgo a specific terminology and function, and characterize them by morphology and NG2 expression, and call them “lacy” oligodendrocytes.

In contrast, the cells with few processes were neither NG2 nor CNPase-positive (Fig. 1a, arrow), but some were labeled with the antibodies to the Yp form of glutathione-S-transferase or PDGFRα (data not shown). They probably represent some form of immature oligodendrocyte (see below). Neither the “lacy” nor the larger cells with few processes were labeled with antibodies to astrocyte markers, GFAP and S-100 β (and see Levison et al., 1999). The bipolar or unipolar cells did not stain with any of these antibodies, and we consider them “immature” and draw no conclusions as to the specific lineages to which these cells belong. They comprise but a few percent of the total labeled population.

Thus, the category of “non-myelinating oligodendrocytes” used in the text includes immature oligodendrocytes as well as “lacy” NG2-positive cells. The proportion of these two classes of oligodendrocytes is presented in the later sections.

**Labeling SVZ cells in vivo for clonal analysis**

A library of 100 different LacZ retrovirus vectors, each carrying a unique PCR-amplifiable sequence tag (Walsh, 1995; Walsh and Cepko, 1992), was used to label SVZ cells in newborn rats (Levison and Goldman, 1993; Zerlin et al., 1995). Their brains were analyzed later during early adulthood and the progeny of infected cells was identified by X-
gal histochemistry. Retroviral integrates within these cells were characterized by restriction enzyme digestion of PCR products that were synthesized using primers specific to sequences flanking the sequence tags (Walsh, 1995). Thus, members of a clone were identified independent of their locations in the brain because they possessed identical tags.

Brains were labeled at very low titers, which assured a low probability that two different SVZ progenitors were independently labeled by two retroviruses carrying the same tag. We determined, in vivo, the viral titers that gave only a few labeled cells in the SVZ and used them to obtain the brains for PCR analysis (see Materials and methods). Out of 14 such brains with low titer injections, nine contained astrocytes, myelinating oligodendrocytes, “lacy”, and immature cells, two contained myelinating oligodendrocytes, “lacy”, and immature cells, and three contained only myelinating oligodendrocytes.

**SVZ cells generate both homogeneous and heterogeneous clones**

Results from PCR analysis of four brains performed at 4–7 weeks postinfection are presented in Table 1. The number of cells in a “clone” was defined as the number of labeled cells from which the same PCR product was recovered. Recovery varied, however, from 33% to 100%. Thus, we are underestimating the actual sizes of clones (e.g., if some members of a clone did not amplify), but we are assured that those cells from which we did recover the same PCR product have a high chance of belonging to the same clone.

Brain M1 contained a single, dispersed clone containing two astrocytes, four early myelinating oligodendrocytes, and an immature cell in the striatum, and a cluster of myelinating oligodendrocytes in the overlying white matter. Clone members were distributed in the striatum and the overlying white matter within 500 μm in the anteroposterior plane around the injection site. This brain contained 21 retrovirus-labeled cells; we recovered PCR products from nine individual cells and from two groups of two adjacent oligodendrocytes. All PCR products were identical (Fig. 2). In some cases, cells were located so close to each other (see Fig. 2f) that we had to dissect them out in groups of two. Thus, for these pairs, we do not know if the insert was recovered from only one cell or both cells. However, we never recovered more than one insert from any small group of adjacent cells. Furthermore, in all of our experiments, we invariably recovered only a single PCR product from tightly knit oligodendrocyte clusters, regardless of the percentage of cells from which product was recovered, suggesting strongly that each cluster is clonal (see below).

Brains B3 and B4 contained four and six clones, respectively. Each of these brains contained a dispersed clone containing both glial types in addition to homogeneous clones of either astrocytes or oligodendrocytes. Clone 1 in brain B4 was located in the cortex with clonal members (six astrocytes, one non-myelinating oligodendrocyte, and one immature cell) distributed within 500 μm in the anteroposterior plane. In brain B3, the members of clone 2 consisted of myelinating oligodendrocytes in the white matter and astrocytes at the pial surface in a position that was dorsal to the oligodendrocyte cluster. Here, the distance between clone members was represented by the whole thickness of the cortex, about 3 mm. Other clones appeared homogeneous with respect to glial type. However, clone 1 in brain B3 contained two myelinating oligodendrocytes and one non-myelinating oligodendrocyte (see below), thus representing a clone in which related cells did not develop synchronously into myelinating oligodendrocytes.

Brain B5 (Table 1) contained 12 different viral inserts, all of which were different from the inserts in brains M1, B3, and B4. Groups of cells with the same insert were distributed in coronal planes with little anterior–posterior mixing. In this brain, all clones were homogeneous, except for clone 12, which contained 38 non-myelinating oligodendrocytes and one astrocyte; of these, 28 oligodendrocytes and one astrocyte were amplified by PCR to give the same insert. The members of this clone spanned the entire thickness of the lateral cortex and were contained within four consecutive 100-μm sections (Fig. 3).

If the progeny of a single SVZ cell differentiated synchronously, we would expect to find clones composed of cells that were homogeneous with respect to stage of development. We examined the morphologies of the cells in the 23 clones characterized and presented in Table 1. Of these, only 44% (10 of 23) were composed entirely of mature astrocytes or myelinating oligodendrocytes. Some of the clones contained mixtures of these mature cell types and other forms (17%), and some contained neither mature astrocytes nor myelinating oligodendrocytes (39%). The majority (96%) of the cells that were neither mature astrocytes nor myelinating oligodendrocytes were either immature astrocytes, judged by the contact with blood vessels, or non-myelinating oligodendrocytes, either “lacy” cells or cells with large, round to oval nuclei with little perinuclear cytoplasm and a few thin processes that did not appear to contact myelin sheaths—some of the latter type displayed a cytoplasmic “dot” of X-gal reaction (see Fig. 4a1). The remaining 4% of cells displayed a unipolar or bipolar morphology, and we called these simply immature, unidentified cells (Figs. 1c and 2d).

**Size and composition of oligodendrocyte clonal clusters in white matter**

A number of investigators have reported tightly knit clusters of glia in the white matter and cortex after retroviral labeling in vivo (see Introduction) and have suggested that these clusters are clonal, each representing the proliferation a progenitor undergoes after it stops migrating. We examined the size and composition of these clusters to gain insights into the synchrony of clonal oligodendrocyte differentiation and into the numbers of times a progenitor divides after it
stops migrating. We determined the average size of oligodendrocyte clusters in the white matter of brains labeled at P0–1 and processed for X-gal histochemistry at 4–7 weeks post-retrovirus infection. Neither oligodendrocyte nor astrocyte cluster size, nor proportions of mature vs. immature cells, varied as a function of age during this interval. To evaluate cluster size, we counted labeled oligodendrocytes in the white matter that clustered within one section or clustered in two to five adjacent (100 µm thick) sections (Fig. 4, Table 2). We chose a limit of five sections because this represented the maximum dispersion of clones defined by PCR. For many of these clusters we were able to recover PCR

Table 1
In vivo clonal analysis of forebrain glia

<table>
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<th>Brain (age)</th>
<th>No clones per brain</th>
<th>Cell no per clone</th>
<th>Percent PCR</th>
<th>Section no</th>
<th>WM</th>
<th>Cortex</th>
<th>Striatum</th>
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</table>

A retrovirus library was introduced into the dorsal SVZ of P0–1 rats by stereotactic injection at the level of the septal nuclei, unilaterally, and at low titer. Brains were processed for X-gal histochemistry and PCR analysis 4–7 weeks later.

Abbreviations: the following abbreviations in this table, and subsequent tables, are used to designate location and glial cell types: WM, white matter; non–mol, this category includes oligodendrocytes with immature morphology, ol(i), as well as ‘‘lacy’’ ol. The location and proportion of these two cell types are presented in Tables 2 and 3; mol, myelinating oligodendrocyte; a, astrocyte; a(i), immature astrocyte; u(i), immature glial cell that possessed a simple morphology with very few processes (sometimes unipolar or bipolar) and that was unable to be unequivocally placed into an oligodendrocyte or astrocyte lineage.

a Clones are designated as C1 to Cn where ‘‘n’’ represents the total clone number per brain. A clone is defined as a group of β-gal-positive cells that carry identical retrovirus sequence tags, determined by PCR analysis (see Materials and methods).

b A portion of β-gal-positive cells in some clusters did not yield a PCR product. Cells that contained amplified tags, however, are shown here as a percent of the total number of β-gal-positive cells per cluster.

c Sections (100 µm each) are represented by Roman numerals. Section I represents the first section that contained β-gal-positive cells recovered by PCR in each brain.
d Analysis of this brain is shown in Fig. 1.
e Members of this clone were extensively dispersed along the lateral white matter.
f C10 was comprised of a single cell. Single cells were always analyzed by PCR because they could have been part of dispersed clones whose siblings are in distant locations in the forebrain.
products, although the percentage of cells in each cluster from which we recovered a product varied widely, from 0% to 83%. For the analysis we chose clusters in which every member was within 100 μm of its nearest neighbor and considered all cells in each of such clusters highly likely to be related to each other for two reasons. First, in all such clusters from which PCR products were recovered, only a single PCR product was found, regardless of the percentage of cells from which a product was recovered. Second, a previous in vivo clonal analysis performed by labeling SVZ cells with a mixture of two recombinant retroviruses (of higher titer than used in the present study), each with a different reporter gene, concluded that there was a 96% chance that each tightly knit cluster was in fact clonal (Levison and Goldman, 1993).

We examined a total of 44 oligodendrocyte clusters for size and cell type composition (Table 2). Twenty-four of these clusters came from brains whose PCR analyses are presented in Table 1: M1, B3, B4, and B5. The other 20 clusters came from seven brains that were not included in that table, but that contained oligodendrocyte clusters in the white matter. Three brains of the total of 14 brains analyzed did not contain such clusters.

We divided clusters into three groups: (1) those that contained only myelinating oligodendrocytes; (2) those that contained both myelinating oligodendrocytes and non-mye-
linating or early myelinating oligodendrocytes; and (3) those that contained only non-myelinating oligodendrocytes (Table 2, Fig. 4). We observed only rare “lacy” oligodendrocytes in the white matter after retroviral injection. The average size of clusters that contained only myelinating oligodendrocytes (5.8 ± 0.9 (±SEM) cells, n = 31 clusters) was similar to that of clusters containing mixtures of myelinating and non-myelinating cells (7.8 ± 3.3 cells, n = 4 clusters). Both of these were appreciably larger than clusters that contained only non-myelinating cells (2.2 ± 0.4 cells; n = 9 clusters). The size of myelinating oligodendrocyte clusters varied widely from 1 to 23 cells. Each mixed cluster contained between 1 and 14 myelinating oligodendrocytes as well as two or three non-myelinating cells, so that the majority of cells in these mixed clusters were myelinating oligodendrocytes. The clusters containing only non-myelinating oligodendrocytes were always small, containing from one to five cells.

Despite the mixture of myelinating and non-myelinating oligodendrocytes, the population as a whole was heavily skewed toward myelinating cells. When we counted the total number of cells in all of the tightly knit clusters, we found 87% (201/231) were myelinating oligodendrocytes and 13% (30/231) were non-myelinating cells (Table 2).

Size and composition of oligodendrocyte clonal clusters in the cortex and striatum

Clonal clusters in the cortex and striatum also contained myelinating and non-myelinating oligodendrocytes. In contrast to their relative rarity in the white matter, “lacy” oligodendrocytes, characterized by a round nucleus and an extensive network of delicate branching processes, appeared frequently in the cortex and striatum (see Figs. 1b and 4c). In a previous study (Levison et al., 1999) and in the present work, we found that the large majority of the cells with that characteristic morphology express the NG2 proteoglycan (see above). Some of these clonal clusters are represented in Table 1. Thus, in brain B4, clone 5 contained five “lacy” oligodendrocytes in the striatum, and clones 2 (in cortex) and 7 (in striatum) in brain B5 contained six and four “lacy” oligodendrocytes, respectively. In addition, clone 1 in B4 and clone 12 in B5 also contained one and five “lacy” oligodendrocytes, respectively. These two latter groups of cells had to be defined as clonal by PCR because some members resided farther than the 100-μm distance from other cells.

We examined 26 oligodendrocyte clusters for size and cell type composition (Table 3); 17 of them came from brains presented in Table 1: M1, B3, B4, and B5. The other nine clusters came from five brains not included in that table. The proportions of different types of oligodendrocyte clonal clusters are as follows: 42% were composed only of myelinating oligodendrocytes (11/26) (Fig. 4b); 8% were composed of myelinating, early myelinating, and non-myelinating oligodendrocytes (5/26) (Fig. 2e); and 31% were composed of “lacy” oligodendrocytes (8/26) (Fig. 4c). One cortical clone (B5, clone 12, see Fig. 3) was composed of one astrocyte and 38 non-myelinating oligodendrocytes, including both “lacy” cells and immature oligodendrocytes (PCR products were recovered from the one astrocyte and 23 oligodendrocytes; all products were identical). The average size of clusters that contained only myelinating oligodendrocytes (3.7 ± 2.1 cells; n = 11 clusters) was similar to that of clusters containing mixtures of myelinating and non-myelinating cells (2.5 average; n = 31 clusters). Both of these were similar to clusters that contained only “lacy” cells or immature cells (3.8 ± 0.9 cells; n = 13 clusters), with “lacy” and immature clusters approximately equivalent in size. The size of myelinating oligodendrocyte clusters varied widely from 1 to 24 cells. The clusters containing only “lacy” or immature cells ranged from 1 to 14 cells.

### Table 2

<table>
<thead>
<tr>
<th>Cluster type</th>
<th>Number of clusters (%)</th>
<th>Cluster size (Range)</th>
<th>Total cells (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>mol</td>
<td>31 (70)</td>
<td>5.8 ± 0.9 (1–23)</td>
<td>mol: 201 (87)</td>
</tr>
<tr>
<td>mol + ol(i)</td>
<td>4 (9)</td>
<td>7.8 ± 3.3 (4–16)</td>
<td>ol(i): 30 (13)</td>
</tr>
<tr>
<td>ol(i)</td>
<td>21 (9)</td>
<td>2.2 ± 0.4 (1–5)</td>
<td></td>
</tr>
</tbody>
</table>

A retrovirus library, at low titer, was injected into the dorsal SVZ of P0–1 rats 4 – 7 weeks later by X-gal histochemistry. Clusters of β-galactosidase-positive oligodendrocytes whose members were in contact or close proximity to each other were included in this analysis. The distance between any member of a cluster and its nearest neighbor was <100 μm.

### Table 3

<table>
<thead>
<tr>
<th>Cluster type</th>
<th>Number of clusters (%)</th>
<th>Cluster size (Range)</th>
<th>Total cells (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>mol</td>
<td>11 (42)</td>
<td>3.7 ± 2.1 (1–24)</td>
<td>mol: 43 (45)</td>
</tr>
<tr>
<td>mol + ol(i)</td>
<td>2 (8)</td>
<td>2.5 (2, 3)</td>
<td></td>
</tr>
<tr>
<td>ol(i)</td>
<td>5 (19)</td>
<td>3.8 ± 0.9 (1–14)</td>
<td>ol(i) and “lacy” ol: 52 (55)</td>
</tr>
</tbody>
</table>

A retrovirus library, at low titer, was injected into the dorsal SVZ of P0–1 rats. Brains were analyzed 4 – 7 weeks later by X-gal histochemistry. Clusters of β-galactosidase-positive oligodendrocytes whose members were in contact or close proximity to each other were included in this analysis. The distance between any member of a cluster and its nearest neighbor was <100 μm.

*Cluster size: mean number of cells per cluster ± SEM.
*Range: minimum to maximum cell number per cluster.
*Total of mol and ol(i) plus “lacy” ol in all the 26 clusters analyzed.
In contrast to the white matter, where the population as a whole was heavily skewed toward myelinating oligodendrocytes, at this age in cortex and striatum, the population was more evenly split. When we counted the total number of labeled cells of the oligodendrocyte lineage, we found 45% (43/95) were myelinating oligodendrocytes and 55% (52/95) were either immature oligodendrocytes or “lacy” oligodendrocytes (Table 3).

Size and composition of astrocyte and mixed astrocyte/oligodendrocyte clusters

A total of 20 astrocyte clusters (16 in cortex, 4 in striatum) were analyzed. Ten of these clusters came from brains included in Table 1: B4, B3, and B5. The other 10 came from brains not included in that table. Labeled astrocytes were rarely found in the white matter, as previously noted (Levison and Goldman, 1993). Clusters composed only of astrocytes averaged 2.2 ± 0.2 cells \( (n = 20) \) in size and ranged from one to five cells. We found one cluster of astrocytes plus immature oligodendrocytes (four cells total) and three dispersed clones containing both astrocytes and immature oligodendrocytes or “lacy” cells (9, 16, and 39 cells). The last two of these clones correspond to clone 1 from brain B4 and clone 12 from brain B5 (Table 1 and Fig. 3).

Clonal dispersion during early stages of gliogenesis

We considered that examining the early stages of clonal growth might reveal further insights into the clonal dispersion and differentiation patterns of glial progenitors. Therefore, brains were examined 4–5 days after retroviral labeling. An example is shown in Fig. 5, in which two intracortical radial arrays of immature cells were found in two consecutive 300-μm sections. All were analyzed by PCR and inserts, all identical, were isolated from three cells. Because these X-gal-positive cells were the only labeled cells in the entire brain, it is likely that cells in these arrays were clonal.

These cells showed morphological heterogeneity. Several looked immature (unipolar), while others exhibited a more complex appearance, contacting and beginning to wrap around blood vessels, indicative of early astrocytic differentiation (Zerlin and Goldman, 1997; Zerlin et al., 1995). Two immature cells are not radially aligned, but tangentially oriented. This is likely due to tangential dispersion, as recently visualized in living tissue slices (Kakita and Goldman, 1999).

A second brain analyzed 4 days postinjection showed a single radial array of 13 cells, dispersed from the white matter–gray matter border to the pial surface. We recovered identical inserts from four to seven of these cells (three analyses were performed on pairs of closely situated cells, so we do not know whether the insert represented one or two cells). All of the cells in this radial array also displayed either immature morphology or early astrocyte characteristics (not shown).

Discussion

SVZ cells generate both homogeneous and heterogeneous clones and both dispersed and clustered clones

Single SVZ cells generate dispersed and clustered clones, most of them homogeneous in lineage type, but others composed of both astrocytes and oligodendrocytes. Some clones were composed of widely dispersed clusters, indicating that related cells do not necessarily remain together after division, while others were composed of a single cluster or groups of clusters near each other. Several mechanisms could generate these various clonal patterns. Homogeneous clones could have been generated from SVZ cells that became restricted to a single lineage before they...
migrated out of the SVZ. However, they could have also originated from progenitors that became committed to a lineage during their migration through the CNS, or even after migration ceased. Heterogeneous clones would presumably arise from progenitors that are not restricted to a specific lineage before they emigrate from the SVZ, but diverge in lineage during migration. Heterogeneous clonal clusters would arise from a progenitor that gave rise to astrocytic and oligodendrocytic lineages during continued cell divisions after migration had ceased.

While it is possible that a heterogeneous clonal cluster could have been generated by a group of related SVZ cells that migrated together and then stopped at exactly the same place, this seems highly unlikely because there is extensive and constant progenitor mixing within the SVZ, where cells appear undirected in their movements (Kakita and Goldman, 1999). Thus, it seems unlikely for sibling cells to take identical migratory paths. Furthermore, when we have observed division of migrating cells in white or gray matter in living slices, the two progeny invariably migrate away from the site of division in different directions (Suzuki and Goldman, unpublished observations).

Several previous observations argue for some degree of fate restriction during progenitor migration. First, SVZ cells, labeled in vivo with retrovirus and then placed in culture within 24 h, generated a much higher proportion of mixed, astrocyte/oligodendrocyte clones (30–70%) than that which we found in vivo (Levison and Goldman, 1997; Levison et al., 1993). This suggests that, as a population, retrovirally-labeled SVZ cells display a far greater developmental plasticity while residing in the SVZ than they show after they migrate. Second, during postnatal development, the large majority of SVZ cells that colonize the subcortical white matter become oligodendrocytes, while the large majority that migrate into the neocortex and differentiate into neurons eventually generates neurons as well as glia (Levison and Goldman, 1997).

At this point, we favor a developmental model in which some SVZ cells begin to differentiate into oligodendrocytes or astrocytes before or during their migration, while others retain a bipotency during migration. It is likely that these SVZ cells correspond to a type of glial-specified (or glial fate-restricted) progenitor. Whether or not they are similar or identical to progenitors isolated from the embryonic spinal cord that appear restricted to a glial fate (Rao et al., 1998) bears further study. However, our clonal observations in vivo strongly suggest that at least some of the SVZ cells become specified to a glial fate but still retain the ability to differentiate into either astrocytes or oligodendrocytes.

The process of lineage restriction may in fact be a stochastic one in which a given progenitor has a certain probability of beginning astrocytic or oligodendrocytic differentiation during its migration. Those that “escape” such restriction can generate mixed clonal clusters. It is likely that fate specification of some oligodendrocytes occurs even before the generation of the large perinatal SVZ because there is evidence that at least some oligodendrocytes of the dorsal telencephalon appear to be specified more ventrally at much earlier embryonic times (reviewed in Woodruff et al., 2001).

The neonatal forebrain SVZ is the source of both glia and neurons, the former migrating radially and developing into glia, the latter migrating anteriorly to the olfactory bulb, where they develop into interneurons (Luskin et al., 1993). We were unable in this study, however, to conclude one way or another whether the labeled glial cells were lineally related to olfactory interneurons. We have seen both glia and neurons generated from retroviral injections at all coronal levels of the forebrain (Suzuki and Goldman, 2003). However, the relative numbers of olfactory interneurons vs. glia is a function of several variables. First, SVZ cells labeled with retrovirus at any coronal level will produce neurons and glia, but one sees many more neurons with injections into the anterior aspect of the SVZ and relatively fewer with injections at more posterior levels. The injections in our clonal study were performed at the coronal level of the septal nuclei, posterior to the most anterior SVZ. Second, we have observed that with very low titers of injected retrovirus at the level of the septal nuclei or further posteriorly, we only see glia, while with high titers, we always observe both neurons and glia. We are not sure why this is the case—perhaps different cell cycle times could play a role. However, we seem to label glioblastoid population(s) preferentially with the low titer injections. In the present clonal study, we were constrained to use very low titer injections because of the statistical arguments.

There are “potentially” multipotent cells in the SVZ at this time, however. When we injected retrovirus into the early postnatal SVZ and then placed the cells in culture, we observed clones containing both neurons and glia (Levison and Goldman, 1997). Thus, there are dividing cells in the
Fig. 6. A model for the generation of dispersed glial cell clones. (a) The progeny of a single SVZ progenitor can colonize the entire thickness of the cerebral hemisphere. A progenitor emigrates from the SVZ during neonatal life, enters and divides in white matter. An offspring remains in the white matter while the other migrates into the cortex, where it divides again. One offspring stops migrating while the other ascends toward the pial surface. This process repeats itself until members of the clone reach the pial surface (far left corner of panel a). The events depicted in each successive, schematic segment of the developing forebrain can be accomplished within a day. Thus, by 4–6 days postinjection, a clone could have dispersed through the whole cortical thickness (Zerlin et al., 1995). Cells that have stopped migrating continue to divide, forming mainly homogeneous clusters. Some cells in each cluster may remain immature, while others differentiate into mature, postmitotic glia. Those that reside in the white matter mostly form oligodendrocytes and those in the cortex form astrocytes and oligodendrocytes. As the brain enlarges during postnatal growth, clusters become more widely separated. (b) Cell death, by eliminating some of the members of a clone, could contribute to larger distances between clonal relatives and also may result in homogeneous clones (if, for example, a cell differentiating into an astrocyte was to die but other cells in the clone developed into oligodendrocytes). (c) Tangential migration can disperse clones further. Symbols: ●, differentiating and early myelinating oligodendrocytes; *, astrocytes; ●, myelinating oligodendrocytes; ◊, migrating immature cells; ○, non-migrating immature cells; +, cell death.
postnatal SVZ that have the capacity to generate both neurons and glia, but we have no evidence to date that they do so in vivo. A clear determination would require use of a library with many more variants, however, so that we could increase the injection titer.

Patterns of glial progenitor colonization of the forebrain

The progeny of an individual SVZ cell could colonize the forebrain to produce clonal clusters and dispersed clones (Fig. 6). In this model, progenitors continue to divide after they emigrate from the SVZ. Siblings disperse if they take different paths following division, or if one sibling stops migrating, while the other continues. Dispersion of progeny might promote the generation of heterogeneous clones (Fig. 6a), such as oligodendrocytic in the white matter and astrocytic in the cortex. Cell death and tangential migration will further disperse clonal relatives (Figs. 6b and c). Progenitors that continue to divide after they stop migrating are likely to generate homogeneous clonal clusters (Fig. 6d), the sizes of which vary. We observed clusters of myelinating oligodendrocytes up to 24 cells in size (as previously noted by Grove et al., 1993), requiring that a progenitor go through at least five divisions after stopping migration. Notably, astrocyte clusters were smaller than oligodendrocyte clusters, although we do not know why that is so. Perhaps oligodendrocyte lineage cells divide more times, or there is more cell death in the astrocyte lineage.

Caveats to in vivo clonal analysis

There are several caveats in interpreting clonal composition, related to the retroactive nature of the analysis. If a clone is composed of only one glial type, we cannot conclude definitively that the initially labeled progenitor was restricted to that lineage before leaving the SVZ. This progenitor could have been initially multipotent and restricted later on to an astrocyte or oligodendrocyte lineage. Furthermore, any cell death during the growth of a clone could skew the distribution of glial cell types within a clone, giving a false impression of uniformity of fates. Finally, it is possible that silencing of the reporter gene expression (either randomly or preferentially in a specific cell type) will skew the distribution of labeled cells (see Gaiano et al., 1999, for discussion).

Clonal relatives do not differentiate synchronously and some progenitors remain immature

Two observations indicate that the progeny of an SVZ cell does not differentiate synchronously. First, several clonal clusters of oligodendrocytes include both myelinating cells and non-myelinating, immature cells (Fig. 7). Second, the radial clones we studied a few days postinjection contain immature cells as well as those that have begun to display astrocyte features.

Myelinating and non-myelinating cells of the oligodendrocyte lineage were represented quantitatively in very different numbers, however. Clonal clusters composed of myelinating oligodendrocytes were larger than those containing either “lacy” or immature oligodendrocytes or immature unidentified glia. Furthermore, those clusters that contained both myelinating and immature oligodendrocytes always exhibited a smaller immature component. This observation could be a consequence of survival and mitogenic signals provided by axons to oligodendrocyte progenitors (Barres and Raff, 1993, 1994). Thus, as oligodendrocytes myelinate axons they survive as stable cells. Many of the oligodendrocytes that do not myelinate axons die, but some oligodendrocyte lineage cells survive as NG2-positive, “lacy” cells or as immature cells. By some mechanism, perhaps the availability of survival factors (Barres et al., 1993), the size of immature clusters is regulated to average in the range of 3–4 cells.

We noted that myelinating and “lacy” oligodendrocytes tended to appear in separate clusters. This suggests a diversion in the lineage before a cell’s stopping migration. Much more commonly, we observed myelinating oligodendrocytes sharing clusters with immature (non-“lacy”) cells, suggesting that the immature cells might represent oligodendrocyte precursors. Whether “lacy”, NG2-positive cells are precursors of myelinating oligodendrocytes have been a controversial point (see above). Our observations do not allow a clear conclusion either way, but further clonal work might help resolve this issue.

This average size of clusters appears to remain relatively stable for many months. We infer this from previous studies in which we labeled dividing cells in the white matter of the adult rat forebrain with recombinant retrovirus to follow the fates of such cells (Gensert and Goldman, 1996). The majority of these cycling cells appear to belong to the oligodendrocyte lineage. Although they express O4, but not O1 (Gensert and Goldman, 2001), only about 25% are NG2-positive (Mason and Goldman, unpublished observations). One month after retroviral labeling, the cells had not differentiated into myelinating oligodendrocytes (with very few exceptions) but remained apparently immature. While
the retrovirus initially labeled individual, non-clustered cells, after 1 month, the cells were distributed in clusters. We have argued that these clusters most likely represented local, clonal expansion of some of the initially labeled cells. The average size of these clusters (about three cells, with a range of 1–15) is very close to the average size of non-myelinating oligodendrocyte clusters we describe in this paper. These observations do not, however, rule out the generation of white matter oligodendrocytes from residual SVZ cells in these adult brains. The nature and heterogeneity of these immature cells in adult white matter are not fully understood. Some of them will differentiate into myelinating oligodendrocytes in vivo after a demyelinating lesion (Gensert and Goldman, 1997). Most will develop along an oligodendrocyte pathway in vitro (Gensert and Goldman, 2001; Mason and Goldman, 2002). However, the adult mammalian white matter does contain immature cells capable of differentiating in vitro into astrocytes (Gensert and Goldman, 2001) or even neurons (Nunes et al., 2003).

Most of the progeny of SVZ cells that settled in the white matter developed into myelinating oligodendrocytes. The ratio of (labeled) myelinating oligodendrocytes to non-myelinating cells in the subcortical white matter was about 6.7:1 (Table 2). We assume that this ratio is representative of the progeny of all the SVZ cells that emigrated out of the SVZ at that time, not just the retroviral-labeled cells, and have no a priori reason to think otherwise. In a previous study, we analyzed the phenotypes of the progeny of SVZ cells identified by retrovirus at P0 but then not studied, we analyzed the phenotypes of the progeny of SVZ cells that settled in the white matter after a demyelinating lesion (Gensert and Goldman, 1996). To keep the numbers of such immature cells constant, the number of labeled retrovirus-infected cells was kept constant throughout the experiments. Thus, an apparent consequence of the asynchronous differentiation of SVZ cells is the continued presence of immature cells in the adult white matter. We suggest that the neonatal SVZ is the source of at least some of the immature, cycling glia that populate the adult white matter (see Gensert and Goldman, 1996; Levison et al., 1999; McCarthy and Leblond, 1988). To keep the numbers of such immature cells relatively constant despite proliferation, cell division must be balanced by cell death.

Acknowledgments

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References


