Development 119, 611-622 (1993)
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The migrational patterns and developmental fates of glial precursors in the rat subventricular zone are temporally regulated

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SUMMARY

Postnatal gliogenesis in the rodent forebrain was studied by infecting subventricular zone cells of either neonates or juvenile rats with replication-deficient retroviruses that encode reporter enzymes, enabling the migration and fate of these germinal zone cells to be traced over the ensuing several weeks. Neither neonatal nor juvenile subventricular zone cells migrated substantially along the rostral-caudal axis. Neonatal subventricular zone cells migrated dorsally and laterally into hemispheric gray and white matter and became both astrocytes and oligodendrocytes. Juvenile subventricular zone cells migrated into more medial areas of the subcortical white matter and on occasion appeared in the white matter of the contralateral hemisphere, but rarely migrated into the neocortex. Juvenile subventricular zone cells almost exclusively differentiated into oligodendrocytes. Thus, the migratory patterns and the developmental fates of subventricular zone cells change during the first 2 weeks of life. When either neonatal or juvenile subventricular zone cells were labeled in vivo and then removed and cultured, some generated homogeneous clones that contained either astrocytes with a 'type 1' phenotype or oligodendrocytes, but some generated heterogeneous clones that contained both glial types. These results provide additional evidence for a common progenitor for astrocytes and oligodendrocytes and strongly suggest that temporally and spatially regulated environmental signals control the destiny of glial progenitors during postnatal development.

Key words: lineage, migration, differentiation, rat oligodendrocyte, astrocyte, retroviral vectors

INTRODUCTION

During the perinatal period of rodent development, the subventricular zone (SVZ) is the major source of neuroglial cells in the mammalian forebrain. Like neurons, which are produced earlier, neuroglial precursors emigrate from this spatially restricted germinal zone to colonize the hemispheres. Much less is known about the migration of glial precursors than of immature neurons, however. Initial studies on SVZ cell migration and maturation were performed by administering pulses of [^{3}H]thymidine to label dividing cells and then observing the distribution and cell type of the labeled cells during subsequent development. These studies demonstrated that many of the SVZ cells were mitotically active and that, with time, the number of strongly labeled cells residing in the SVZ decreased. Since cells that morphologically resembled SVZ cells were observed outside of this germinal zone, it was concluded that the SVZ cells migrated out of the germinal zone. Based on both light and electron microscopic observations of labeled cells, the SVZ was regarded as a source of astrocytes and oliogodendrocytes (Smart, 1961; Lewis 1968; Privat and Leblond, 1972; Paterson et al., 1973; Paterson, 1983). However, thymidine uptake by glial cells dividing outside of the SVZ and dilution of label with successive mitoses makes a clear interpretation of these experiments difficult. In addition, pathways of SVZ cell migration are difficult to assess because the [^{3}H]thymidine labels cells throughout the germinal matrix zone. For example, the relative contributions of coronal and sagittal migration to the eventual dispersion of glial cells cannot be determined.

We have re-examined the developmental fates of cells in the postnatal SVZ using replication-deficient retroviruses that encode heritable markers. Unlike [^{3}H]thymidine labeling, retrovirally labeled cells (and their progeny) are permanently labeled. Furthermore, by labeling precursor cells by stereotactically injecting the retrovirus into a spatially restricted region of the germinal zone the migration of precursor cells can be readily visualized. We have previously demonstrated that both astrocytes and oligodendrocytes of the rat forebrain are descendants of SVZ progenitors labeled 2 days after birth (P2) (Levison and Goldman, 1993). SVZ cells appeared to migrate through adjacent white matter and into adjacent gray matter to colonize neocortex, white matter and striatum. Those glial progenitors that remained in white matter largely became oligodendrocytes, while those that settled in gray matter developed into both glial types. Some individual progenitors

appeared to give rise to both astrocytes and oligodendrocytes. These observations suggested that SVZ cells are bipotential progenitors whose fates are determined in part by environmental cues.

These observations were reminiscent of the in vitro differentiation of the bipotential glial progenitor, designated the O-2A progenitor, which can differentiate into either an oligodendrocyte or a type 2 astrocyte depending upon the culture conditions (for review, see Miller et al., 1989). Studies using immunological markers for glial lineages support a model of gliogenesis in which type 1 astrocytes emerge prenatally, and O-2A progenitors appear late in gestation to generate oligodendrocytes around birth and then type 2 astrocytes during the second postnatal week (Williams et al., 1985; Lillien et al., 1988). Since both astrocytes and oligodendrocytes were generated by neonatal SVZ cells, we were interested in determining whether their developmental potential is restricted later in development.

In this report, we show that both the fates and migration patterns of SVZ cells are restricted during the first two postnatal weeks. In contrast to the progeny of neonatal SVZ cells, those of juvenile SVZ cells (infected at P14) do not colonize gray matter but remain in subcortical white matter where they develop into oligodendrocytes. This developmental fate restriction cannot be attributed to lineage restriction since SVZ cells from both neonatal and juvenile animals exhibit similar developmental potentials in vitro. When labeled in vivo and allowed to proliferate and differentiate in culture, both neonatal and juvenile SVZ cells generated some clones that were homogeneous, containing either astrocytes with 'type 1' phenotypes or oligodendrocytes, but some generated heterogeneous clones that contained both glial types. Altogether, these results provide evidence that neonatal and juvenile SVZ cells have similar developmental potentials and that environmental signals play major roles in fate determination in vivo.

MATERIALS AND METHODS

Stereotactic surgery and retrovirus injections

Concentrated BAG and DAP retroviral stocks were produced and titered as outlined in Levison and Goldman (1993).

Labeling neonatal SVZ cells: Sprague-Dawley rat pups (P2-3; the day of birth being P0) were anesthetized by immersion in ice water for 7 minutes. They were positioned in a stereotactic apparatus modified for neonatal rats (Heller et al., 1979) and kept cold with ice packs. For migration and fate mapping studies using the BAG retrovirus, the scalp was deflected, and 1 μl of retroviral stock (containing 7 CFU of BAG virus) was injected unilaterally with a 10 μl Hamilton syringe at a rate of 0.2 μl/minute. Stereotactic coordinates (relative to bregma) were anterior 0.8 mm and lateral 2.0 mm, at a depth of 2 mm. For the migration study employing two retroviral vectors, BAG was injected as above and 70 CFU of DAP was injected at coordinates 0.75 mm posterior, 3.0 mm lateral, at a depth of 1.8 mm (relative to bregma). The scalp incision was closed with Vetbond tissue adhesive (3M Products, St Paul, MN), the pup was warmed and it was returned to its mother.

Labeling juvenile SVZ cells: Sprague-Dawley rats (P14) were anesthetized with a mixture of ketamine (Aveco, Ft Dodge, IO; 50 mg /kg) and xylazine (Mobay Co. Shawnee, KS; 10 mg/kg). The rat's head was shaved and positioned in a stereotactic apparatus (David Kopf, Tujunga, CA). A mid-sagittal incision was made in the scalp to expose the skull and a burr hole was made using a dental drill (either unilaterally or bilaterally). 1 μl of retroviral stock (containing 70 CFU of BAG) was injected through a glass micropipet mounted on a 10 μl Hamilton syringe. Stereotactic coordinates (relative to bregma) were anterior 0.5 mm, lateral 1.8 mm, at a depth of 3.2 mm. After withdrawing the micropipet, a piece of gel foam (Upjohn, Kalamazoo, MI) was placed in the hole and the scalp incision was closed with no. 5 polyethylene sutures.

Tissue fixation and histology

Experimental animals were killed by intracardiac perfusion at 2, 7, 14, 21, or 28 days postinjection (dpi). Animals were anesthetized with a mixture of ketamine (200 mg/kg) and xylazine (40 mg/kg) prior to perfusion with RPMI culture medium containing 6 units/ml heparin followed by 3% paraformaldehyde in 0.1 M phosphate buffer, pH 7.35. The brains were removed and fixed overnight at 4°C prior to transfer to PBS. The brains were sectioned either coronally or sagittally at 100 μm intervals on a vibratome (Technical Products International, series 1000) and processed for X-gal histochemistry or alkaline phosphatase histochemistry as described (Levison and Goldman, 1993).

Cell culture

Bilateral injections of single retrovirus were made into the SVZ at the more anterior site as described above. In experiments to assess clonal boundaries, the BAG and DAP retroviruses were mixed in a ratio of 1:5 and 1 μl (containing a total of 55 CFU) injections were made bilaterally. One day later the animals were deeply anesthetized and decapitated and their brains removed. The SVZ was excised with fine forceps and minced. Tissue from neonatal animals was enzymatically and mechanically dissociated as described by Levison and McCarthy (1990), while tissue from juvenile animals was enzymatically dissociated with papain, collagenase and trypsin, and then mechanically dissociated as described by Armstrong et al. (1992). The cells were filtered through 33 μm Nitex mesh (Tetco, Elmsford, NY) and collected by centrifugation at 100 ***g***. Cells were plated at 65,000 trypan blue excluding cells per 40 μl drop onto poly-L-lysine-coated glass coverslips. 2 hours later, 1 ml of culture medium was added to each well. Neonatal SVZ cells were maintained in Minimum Essential Medium (Gibco, Long Island, NY) containing 5% fetal bovine serum (Gibco), 2 mM glutamine, 100 μg/ml streptomycin and 100 U/ml penicillin. Juvenile SVZ cells were maintained in either the above medium or Eagle's Basal Medium with Earle's salts (BME) containing 10% fetal bovine serum, 2 mM glutamine, 100 μg/ml streptomycin and 100 U/ml penicillin. Cultures maintained in BME had fewer microglia. The cultures were maintained in a humidified atmosphere with 5% CO_2 in air at 37°C. Culture medium was changed every 3 days. β-galactosidase histochemistry and triple-label immunocytochemistry was performed as described (Vaysse and Goldman, 1990).

RESULTS

Migration of immature cells from the SVZ

To follow the migration of SVZ cells, we stereotactically injected retroviruses, carrying either the *E. coli* β-galactosidase gene (BAG) or the human placental alkaline phosphatase gene (DAP), into the SVZ of neonatal or juvenile rats (P2 or P14). When the neonatal brains were examined 2 days postinjection (dpi), 80% of the X-gal-labeled cells were restricted to the SVZ (Table 1). The remainder of the cells were located adjacent to the SVZ. Almost all of the cells (95%) had an immature morphology, characterized by

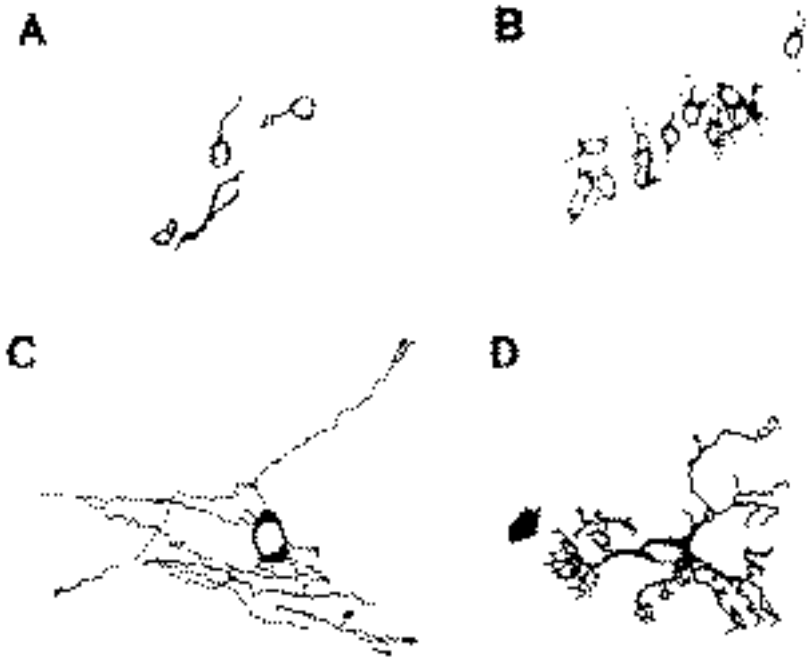

Fig. 1. Camera-lucida drawings of X-gal-labeled cells. (A) SVZ cells with the characteristic small size and simple morphology, seen 2 dpi after infection at P14. Sometimes labeled cells were caught during mitotic division. ×1000. (B) A cluster of immature oligodendrocytes at 21 dpi after P14 infection. ×820. (C) A myelinating white matter oligodendrocyte at 21 dpi after P2 infection. ×1280. (D) A neocortical astrocyte with protoplasmic features that has an endfoot on a blood vessel (arrow) seen 7 dpi after P2 infection. ×1000.

Table 1. Developmental loss of labeled cells from the SVZ

Days	% Total cells	s.e.m.	*n*
2	80	3	6
7	22	8	4
14	1.5	–	2
21	0.73	0.6	4

The BAG retrovirus (7 CFU's/μL) was stereotactically injected unilaterally into the SVZ of P2-P3 rat pups. On the indicated days postinjection, the rats were perfused intracardially with phosphate-buffered fixative prior to processing the brains for β-gal histochemistry. The results are averages ± s.e.m. of the number of cells in the SVZ as a percentage of the total cells in each brain.

small, round to oval nuclei, scant cytoplasm, and usually a single, thin cytoplasmic process (Levison and Goldman, 1993). The morphology and distribution of the labeled cells of juvenile rats at 2 dpi was similar (Fig. 1 and unpublished observations). We never observed labeled cells with a radial glial morphology. With increasing times after injections, the number of labeled cells in the SVZ decreased (expressed either as a percent of total labeled cells or as the absolute number of labeled SVZ cells per brain) (Table 1). By 21 dpi, few labeled cells remained in the SVZ, despite the presence of a residual SVZ. As cell numbers in the SVZ decreased, similar immature cells appeared in the white matter adjacent to the SVZ in larger and larger numbers. With time, cells with the morphologies of oligodendrocytes appeared in white matter and oligodendrocytes and astrocytes appeared in gray matter (see below). Since the virus is non-replicating and is given in a single dose, we infer that we are documenting the migration and differentiation of SVZ cells in these experiments.

Glial progenitor migration in a coronal plane

Neonatal SVZ cells migrated extensively in a coronal plane, with labeled cells appearing in cortex dorsal to the SVZ, as well as in ventrolateral cortex, white matter and striatum (Figs 2, 3). The migration patterns of the juvenile SVZ cells were different. They rarely migrated into the neocortex and fewer settled in the striatum, with the vast majority colonizing subcortical white matter (Figs 2, 3). Furthermore, juvenile SVZ cells migrated differently within the subcortical white matter than those in neonatal rats. To quantitate this difference, we divided the white matter into three regions relative to the corners of the lateral ventricle, establishing medial, central and lateral areas (see inset, Fig. 4). Neonatal SVZ cells migrated chiefly into the 'lateral' white matter, with few cells entering the callosum and none the contralateral hemisphere. In contrast, juvenile SVZ cells predominantly populated the 'central' area of white matter, the remainder being evenly distributed between 'medial' and 'lateral' areas (Figs 2, 4). A greater percentage of juvenile cells migrated medially, and some cells even migrated into the contralateral hemispheric white matter. In one experiment, SVZ cells were labeled at P2 with the BAG virus, and (in the same animals) at P14 with the DAP virus and perfused at P42. Blue cells (from the BAG virus injection) were observed in more lateral white matter, while the purple cells (from the DAP virus) were observed primarily in the medial white matter (data not shown).

Glial progenitor migration in a sagittal plane

While glial progenitors migrated extensively in a coronal plane, neither the neonatal nor juvenile SVZ cells migrated as far in a sagittal plane. The extent of anteroposterior migration was assessed by counting the total number of cells per 200 μm coronal slice and displaying the data as a histogram (Fig. 5). In the neonatal animals, the distance over which the cells were distributed along an anteroposterior axis was greater after several weeks than after 2 dpi (Fig. 5A). However, this difference can be largely accounted for by the 2-fold increase in brain size over the corresponding

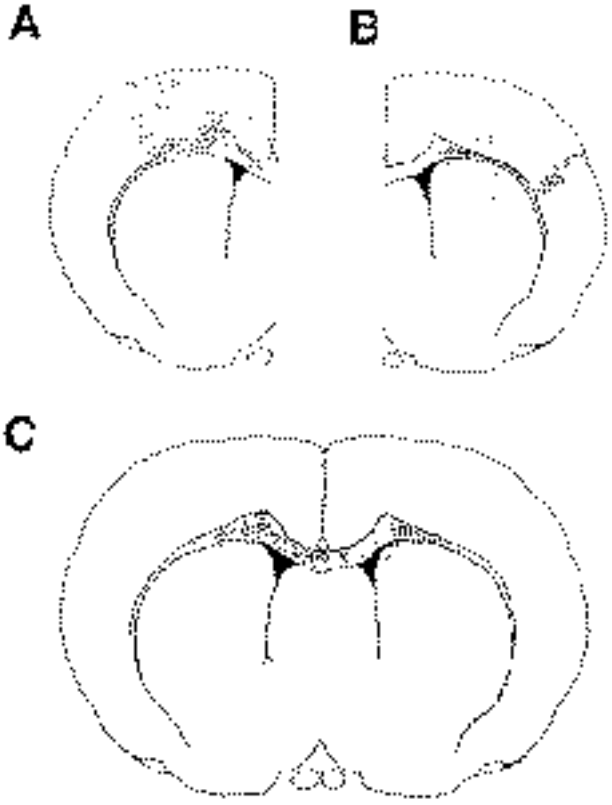

Fig. 2. Migratory patterns in the coronal plane. The distribution of labeled cells in the forebrain of rats injected at either 2 days (A,B; unilateral injections) or 2 weeks (C; bilateral injection) of age. Labeled cells were counted in every third (A) or every other (B,C) 100 μm section and charted on a coronal map of the rat forebrain at the level of the septal nuclei. Each dot represents a single labeled cell. Rostral-caudal spread for each panel was 3.6 mm (A), 1.6 mm (B) and 1.1 mm (C).

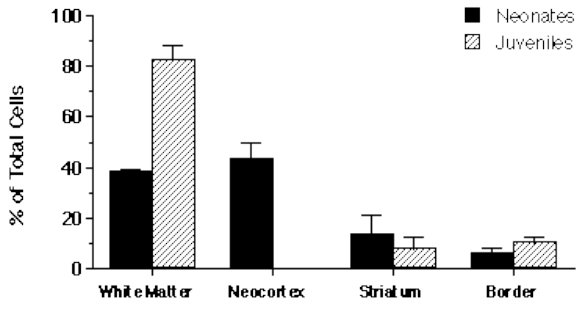

Fig. 3. Regional distribution of SVZ progeny. The location of labeled SVZ progeny was tabulated 21 dpi for infections at P2 (solid bars) and P14 (hatched bars). Labeled cells could be found in white matter, neocortex, striatum or along the border of the white matter and neocortex or striatum. Results are presented as the average ± s.e.m. from 6 neonatal and 4 juvenile rats.

period of development. The profile for the juvenile cells was similar at 2 and 21 dpi (Fig. 5B).

A separate experiment was performed to illustrate the extent of anteroposterior migration. Two regions of the forebrain SVZ, separated by 1.75 mm along the anteroposterior axis, were labeled simultaneously in the left hemispheres of four neonatal rats. The BAG virus was injected into the more anterior of these two SVZ regions (at the level of the septal nuclei, where all previous injections had been targeted) and the DAP retrovirus was injected 1 mm lateral and 1.75 mm posterior to the first site (to reach a more posterior region of the SVZ). 28 days later, sagittal sections were cut and processed for both β-galactosidase and alkaline phosphatase. Although labeled cells had migrated into white matter and cortex adjacent to the two labeling sites, there was only a small amount of mixing observed between the sites (Fig. 6).

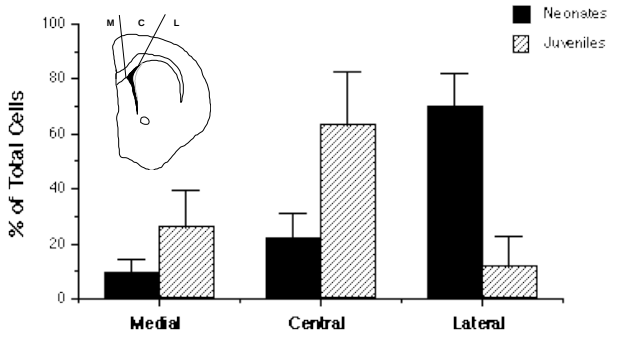

Fig. 4. Distribution of SVZ progeny within the white matter. The distribution of cells within the white matter were quantified for infections of neonates (solid bars) or juveniles (hatched bars). The white matter was divided into medial, central and lateral domains relative to the corners of the lateral ventricles (see inset). Results are plotted as the percentage of total white matter cells per animal and presented as the average ± s.e.m. of 6 neonatal and 4 juvenile rats.

Neonatal and juvenile SVZ cells have different developmental fates

Labeled cells were classified 21 dpi as either astrocytes or oligodendrocytes using morphological criteria (Fig. 1). We have shown previously that cells meeting these morphological criteria also express antigenic markers appropriate to their classification (Levison and Goldman, 1993). A small number of cells were characterized as 'transitional.' These either displayed characteristics of both glial types or were insufficiently stained with X-gal to delineate their morphologies adequately. Labeled cells with microglial morphology were never seen. Regardless of whether the SVZ cells were infected at P2 or P14, the large majority of cells in white matter displayed the morphologies of oligodendrocytes. Many, though not all, had processes continuous with myelin sheaths. Myelin sheaths were especially obvious when cells were visualized using alkaline phosphatase histochemistry after infection by the DAP retrovirus; however, oligodendroglial somas were not simultaneously evident, presumably because the alkaline phosphatase is largely translocated into the cell's processes (Fields-Berry et al., 1992), ie. the cytoplasmic loops of their myelin sheaths.

SVZ cells labeled at P2 migrated into both neocortex and striatum, where they developed into astrocytes and oligodendrocytes (Levison and Goldman, 1993). SVZ cells

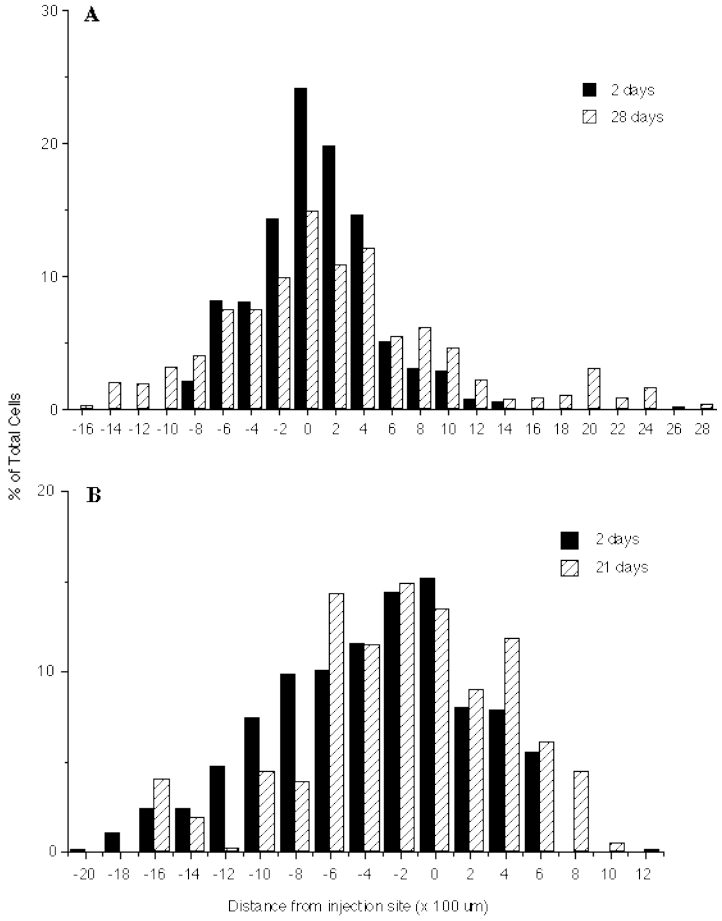

Fig. 5. Migration in a sagittal plane. The extent of anterior-posterior migration of SVZ cells was determined at 2 dpi (solid bars) or several weeks later (hatched bars) for rats infected as neonates (A) or juveniles (B). Each bar represents the number of labeled cells in a 200 μm coronal slice expressed as the percentage of the total cells per animal. The injection site was designated 0 with positive coordinates indicating anterior sections. The apparent migration in a sagittal plane seen after the P2 infections reflects the doubling in brain size that occurs during this developmental period. The observed distribution likely reflects diffusion of the virus from the injection site rather than migration.

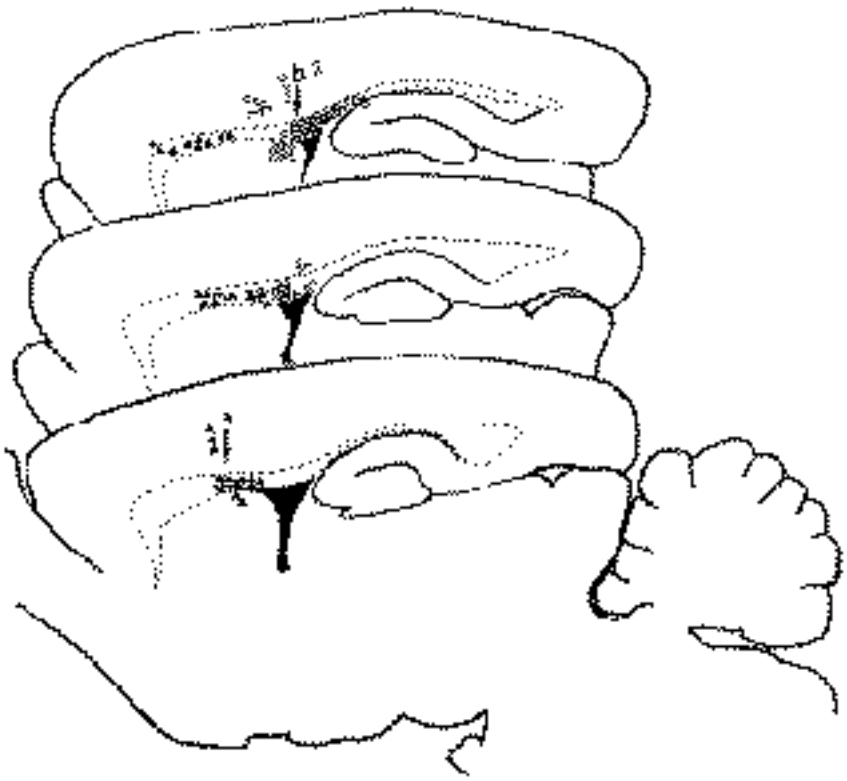

Fig. 6. Labeling distant SVZs with separate retroviruses reveals limited anterior-posterior migration. The BAG retrovirus was injected into the neonatal rat SVZ at the level of the septal nuclei (site a) while the DAP retrovirus was injected in the same animals into the SVZ 1.75 mm caudally and 1 mm laterally (site b). Plotted are the locations of the X-gal$^+$ cells (triangles) and the AP$^+$ cells (circles) from a representative animal. Every other section was sampled and plotted on the most appropriate coronal section from 1.9 mm to 2.9 mm lateral to bregma (most medial section in foreground).

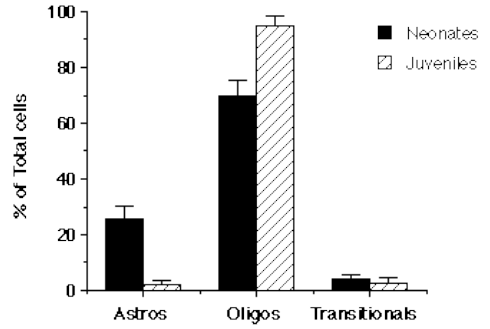

Fig. 7. Developmental fates of SVZ cells in vivo. The types of cells were assessed in brains infected as neonates (solid bars) or juveniles (hatched bars) 21 dpi. Labeled cells were characterized morphologically as either astrocytes, oligodendrocytes or transitional cells as described (Levison and Goldman, 1993). The graph presents data as the average ± s.e.m. from 6 animals infected as neonates and 4 animals infected as juveniles.

labeled in juveniles, however, did not migrate into gray matter, but remained in white matter, where they differentiated almost exclusively into oligodendrocytes (Fig. 7). Within the striatum of animals injected as neonates, 43% of the cells differentiated into astrocytes (mean value from 4 animals); whereas, in the striata of animals injected as juveniles, only 12% of the progeny differentiated into astrocytes (mean value from 2 animals), the remainder differentiating into oligodendrocytes.

SVZ cell differentiation in vitro

The observed migration and fate restriction that occurred by postnatal day 14 could have occurred by lineage restriction, in that the migratory progenitors were committed oligodendroblasts destined to colonize white matter, or by epigenetic interactions that nurtured bipotential progenitors to develop into oligodendrocytes and limited their transit to white matter (see Discussion). To assess the differentiation potential of neonatal and juvenile SVZ cells, we labeled cells in vivo by stereotactic injection, prepared cultures one day later and maintained these cells for 6-8 days in vitro (div).

In cultures established from both ages, the labeled cells continued to proliferate, forming small clusters of cells that expressed the reporter enzyme encoded in the vector (Fig. 8). To determine if each cluster represented the (clonal) progeny of a single infected cell, we injected BAG and DAP in the same 1 μl volume. Clusters composed of the progeny of two or more infected cells would contain both BAG- and DAP-labeled cells with a probability dependent upon the relative viral titers (Table 2). When we defined a cluster as a group of cells in which each member lay within 150 μm of another member of that cluster, then no clusters contained both histochemical markers (Fig. 8; Table 2). Thus, the cells in each cluster were related to each other. The chances of two clusters overlapping obviously increases with the

Table 2. Defining clonal boundaries

Total clusters	No. only Blue	No. only Purple	No. bicolored
224	37	187	0

The experiment described in Fig. 8 was analyzed quantitatively. Clusters were defined as groups of labeled cells in which each member was within 150 μm of at least one other member of the group. Data represent the total number of clusters counted on 13 coverslips. We used a mixture containing a ratio of 5:1 (AP:β-gal); therefore, if each clone were the result of two infections then the number of bicolored clones should have been 2(pPB)×224 or 2(0.167×0.83)×224=63 (Fields-Berry et al., 1992). If only 40% of the clones had resulted from two infections (to account for the mixed phenotype clones we report in this study), then we should still have observed 25 bicolored clones. That we did not observe any clusters containing both blue and purple cells, indicates that the criteria employed successfully defines clonal boundaries with high probability.

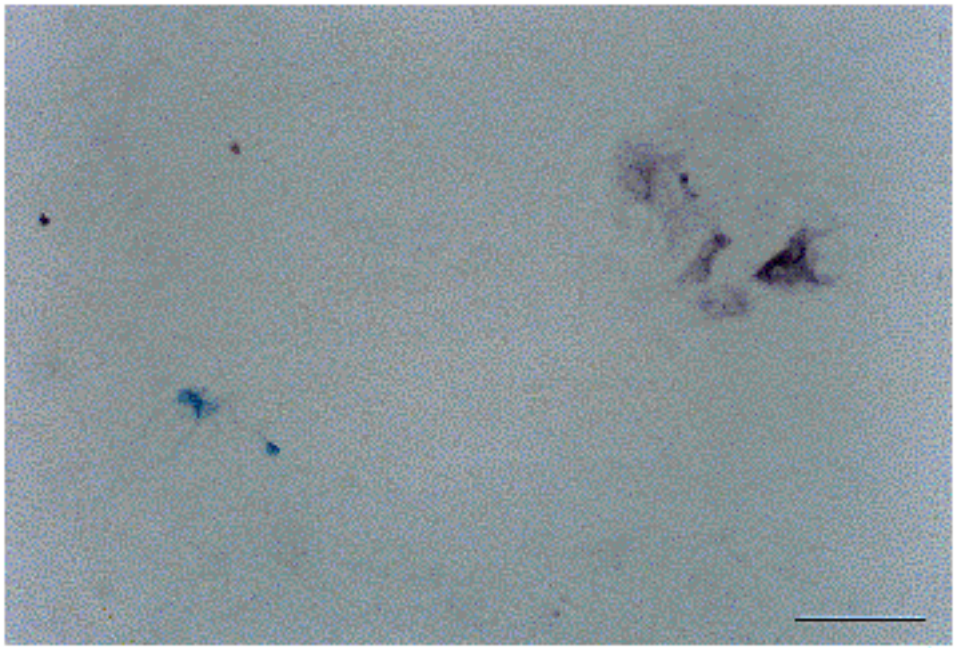

Fig. 8. The figure illustrates two clusters at 7 div. The β-galactosidase$^+$ cluster (blue) contains both polygonal astrocytic cells and a small process-bearing cell. The alkaline phosphatase$^+$ cluster (purple) contains polygonal astrocytic cells. These clusters are encompassed by a confluent glial monolayer of unlabeled cells. Bar represents 150 μm.

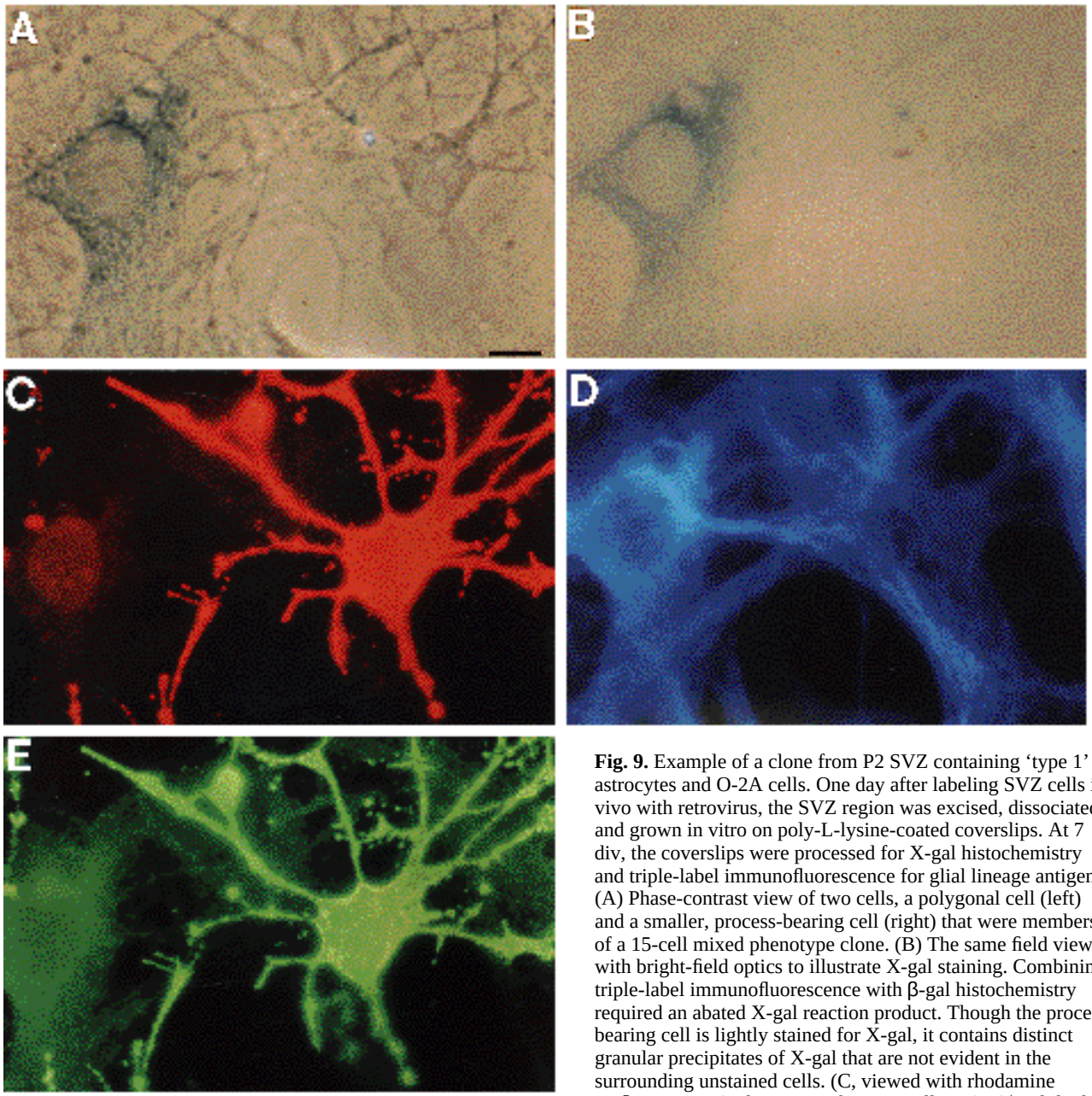

Fig. 9. Example of a clone from P2 SVZ containing 'type 1' astrocytes and O-2A cells. One day after labeling SVZ cells in vivo with retrovirus, the SVZ region was excised, dissociated and grown in vitro on poly-L-lysine-coated coverslips. At 7 div, the coverslips were processed for X-gal histochemistry and triple-label immunofluorescence for glial lineage antigens. (A) Phase-contrast view of two cells, a polygonal cell (left) and a smaller, process-bearing cell (right) that were members of a 15-cell mixed phenotype clone. (B) The same field viewed with bright-field optics to illustrate X-gal staining. Combining triple-label immunofluorescence with β-gal histochemistry required an abated X-gal reaction product. Though the process-bearing cell is lightly stained for X-gal, it contains distinct granular precipitates of X-gal that are not evident in the surrounding unstained cells. (C, viewed with rhodamine epifluorescence) The process-bearing cells is $GD3^+$, while the polygonal cell is $GD3^-$. (D) The same field viewed with coumarin epifluorescence to show that the polygonal $GD3^-$ cell is $GFAP^+$, and, therefore, a 'type 1' astrocyte. (E) The same field viewed with fluorescein epifluorescence to show that the $GD3^+$ process-bearing cell is $O4^+$ and, therefore, an oligodendrocyte progenitor. Bar represents 10 μm.

number of infections, so we adjusted the titer in the viral inoculum such that the number of clusters per coverslip was small (Table 3).

The β-galactosidase activity in infected cells could be visualized using X-gal histochemistry 24 hours after establishing the cultures. At this time, the labeled cells appeared either apolar or unipolar with cell bodies approximately 8 μm in diameter (not shown). This morphology is similar to that of labeled SVZ cells in vivo (Fig. 1 and Levison and Goldman, 1993), SVZ cells during migration in forebrain white matter (unpublished observations) and O-2A cells in the early postnatal optic nerve (Fulton et al., 1992). After 1 week in vitro, the labeled cells had proliferated to form clusters that contained up to 65 cells (Table 3). There was no consistent difference in the size of P2 versus P14 SVZ cell clones after 1 week in vitro.

These clusters were characterized by morphology and with a panel of antibodies (R24, specific for GD3 ganglioside; anti-glial fibrillary acidic protein (GFAP); monoclonal antibodies (mAbs) O4 and O1) that recognize developmentally regulated antigens expressed on SVZ cells, O-2A progenitors, oligodendrocytes and astrocytes (for review, see Goldman, 1992). When stained for X-gal and with antibodies to ganglioside GD3 and GFAP, and with O4 at 7-8

Table 3. Distribution of clonal types after 1 week in vitro

			Homogeneous			Heterogeneous	
Postnatal age	No. of clones	Avg no. of clones/cs	No. of type 1 $GD3^-/O4^-/GFA^+$	No.of O-2A lineage $GD3^+/O4^+/GFA^\pm$	No. unclassified	No. of Clones with Type 1 + O-2A	% Mixed clones
P2	26	4.3	3	0	6	11 (9)	42
P2	10	3.3	1	0	1	7 (7)	70
P14	32	8	4	7	9	12 (12)	38
P14*	13	3.3	0	0	9	4 (4)	31

BAG retrovirus (70 CFU/μl) was bilaterally injected into the SVZ of P2 or P14 rat pups. One day later, high density dissociated cultures enriched in SVZ cells were prepared (see methods). Cultures were fixed and processed after 1 week in vitro for X-gal histochemistry and triple label immunocytochemistry using the mAb to GD3, mAb O4 and rabbit antibodies to GFAP. Homogeneous clones were grouped into: 'type 1' astrocytes, O-2A lineage cells, or unclassified (cells unstained for all three markers, or cells obscured because they lay within aggregated clumps). Mixed clones contained at least one 'type 1' astrocyte and at least one O-2A lineage cell in addition to variable numbers of 'unclassified' cells. Values in parentheses indicate the number of clones that contained at least one $O4^+$ cell. Columns for mixed clones that contained at least one unclassified cell and either 'type 1' astrocytes (4 clones) or O-2A cells (3 clones) were not included in the Table. Results from two separate experiments at each age are presented. P2 cultures had a mean of 5.2±5.3 (SD) cells per clone, n=67, with a range of 1-25; P14 cultures had a mean of 8.6±10 (SD) cells per clone, n=31, with a range of 1-65.

*Only O4 and GFA staining was performed in this experiment, therefore O-2A progenitors would not have been detected.

days in vitro, we observed clusters of labeled cells that contained only cells of the oligodendrocyte lineage (small, process-bearing $O4^+/GFAP^-$) or only polygonal astrocytes of the 'type 1' variety ($GD3^-/O4^-/GFAP^+$), but approximately 40% of the clusters were morphologically and antigenically heterogeneous (Table 3; Figs 9, 10). These mixed clusters contained variable numbers of $GD3^-/O4^-/GFAP^+$ polygonal cells ('type 1' astrocytes); small, round $GD3^+/O4^-/GFAP^-$ cells (presumably precursors); $O4^+/GFAP^-$ process-bearing cells (immature oligodendrocytes); and $GD3^+/GFAP^+$ process-bearing cells ('type 2' astrocytes). Clusters containing both 'type 1' astrocytes and galactocerebroside positive (O1+) oligodendrocytes were also observed (Fig. 10). Thus, the developmental potential of the neonatal and juvenile SVZ cells appear to be similar in that they can produce both astrocytes and oligodendrocytes in vitro, even though the juvenile cells only produce oligodendrocytes in vivo.

No labeled cells with the morphology or antigen expression (neurofilament+, MAP-2+) of neurons were observed, even in serum-free medium supplemented with epidermal growth factor, brain-derived neuronotrophic factor, 15 mM potassium, or combinations (data not shown), even though (unlabeled) neurons did grow in the cultures. Since postnatal SVZ cells can produce neurons and astrocytes in vivo and in vitro (Levison and Goldman, 1993; Lois and Alvarez-Buylla, 1993), it is possible that different culture medium supplements could support neuronal differentiation.

DISCUSSION

In the present study, we have characterized the migration and differentiation of rat SVZ cells at two postnatal ages. Stereotactically injected, replication-deficient retroviruses containing reporter genes selectively and permanently labeled proliferating SVZ cells, allowing their routes of migration and the fate of their progeny to be determined in vivo. In addition to following their development in situ, we challenged their differentiation potential by removing them from their normal environments and allowed them to proliferate and differentiate in vitro.

Patterns and pathways of glial progenitor migration

The retroviral labeling paradigm employed in the present study has been described in greater detail (Levison and Goldman, 1993). 80% of the labeled cells were within the SVZ at P4 (2 dpi). Those few labeled, immature cells that were observed outside of the SVZ at 2 dpi probably represented cells that began to migrate from the SVZ within 48 hours after infection, rather than dividing glia outside the SVZ. We did not perform cell counts prior to 2 dpi since, in our hands, the levels of β-gal do not reach detectable levels prior to this time. SVZ cells, labeled at P2, migrated significant distances and colonized both gray and white matter throughout the coronal plane at the level of injection (Fig. 2, and see Levison and Goldman, 1993). In contrast, SVZ cells, labeled at P14 showed a restricted migration - settling largely in central and medial white matter within the coronal plane at the level of injection (Fig. 2). What might account for this time-dependent difference in migrational routes?

Early in neocortical development, a radial glial scaffolding is established to assist the movement of migrating neural progenitors. Immunohistochemical studies using monoclonal antibodies to these radial cells (the RC1 and RC2 mAbs) indicate that these pathways are not strictly radially aligned, especially in mediodorsal and ventrolateral neocortex (Misson et al., 1988; Edwards et al., 1990). Neuronal precursors migrate from a germinal zone surrounding the ventricles along radial glia and settle in specific cortical laminae dictated by their birthdate (Rakic, 1972; Berry and Rogers, 1965; Bayer et al., 1991; McConnell, 1991). The coronal pattern of neonatal SVZ cell colonization that we observed closely resembles that described for neurons during late prenatal development (Bayer et al., 1991). This similarity leads us to conjecture that SVZ glial progenitors that migrate in early postnatal life may follow the same pathways that neuroblasts took before them. Several lines of evidence support this hypothesis. (1) SVZ progenitors look like retrovirally labeled neuroblasts (Misson et al., 1991a), with a single process oriented along the glial fiber system of the nascent white matter. (2) During the first week of postnatal life, the radial glial processes that neuroblasts used previously for their migration through

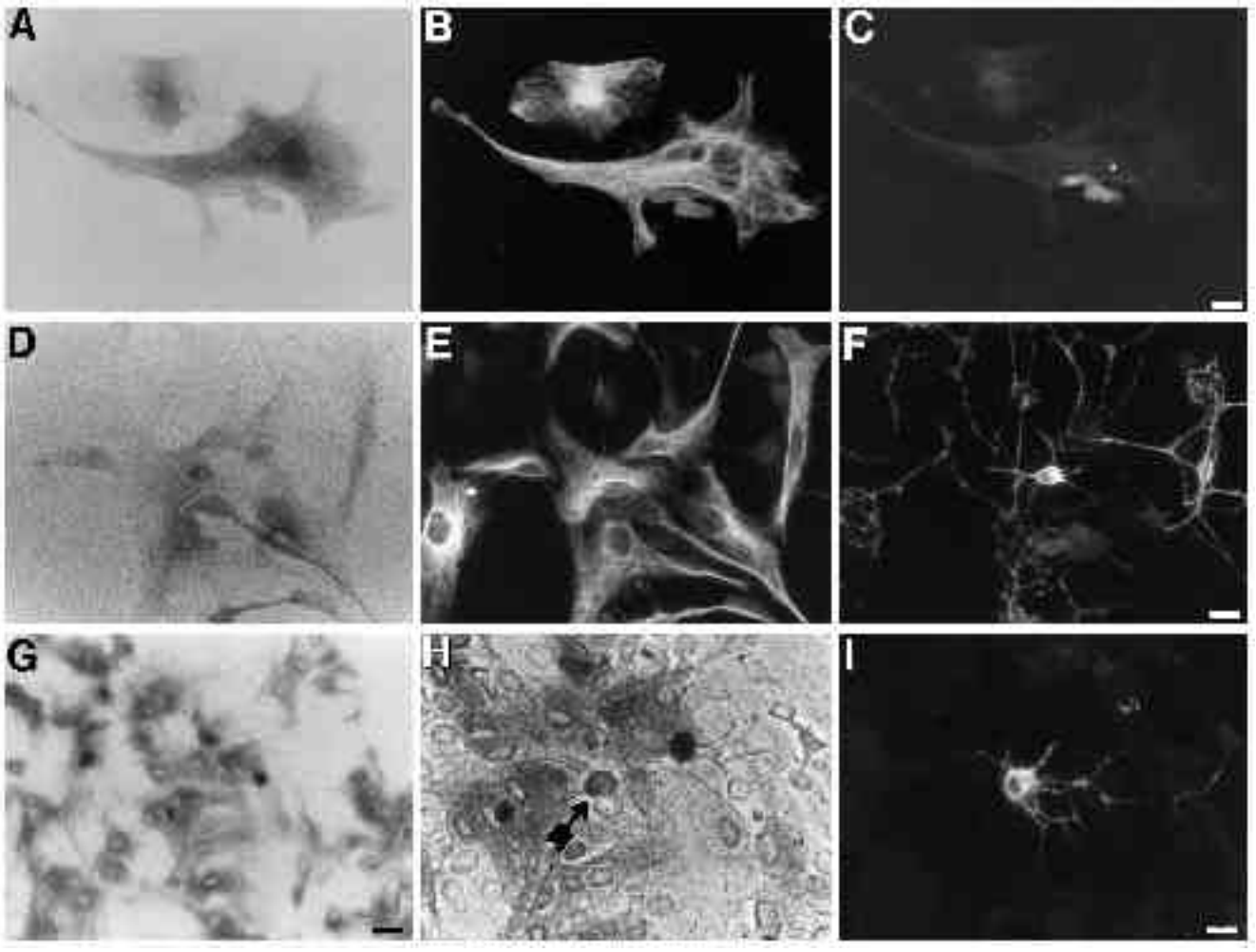

Fig. 10. Developmental fates of P14 SVZ cells in vitro. (A-C) A 4-cell clone as seen by X-gal staining (A) that contained two polygonal, $GFAP^+$ (B), $GD3^-$ (C) 'type 1' astrocytes and two $GFAP^-/GD3^-$ cells of undetermined nature (the small cells show a low level cytoplasmic fluorescence, not the bright surface labeling exhibited by cells that we consider positive for ganglioside GD3). (D-F) An X-gal-stained clone (D) that contained several polygonal, $GFAP^+$ 'type 1' astrocytes (E), as well as two $O4^+$ process-bearing, immature oligodendrocytes (F). The $O4^+$ cell on the right overlies an $O4^-/GFAP^+$ astrocyte that is also X-gal labeled. (G-I) Part of a very large X-gal-stained clone (G) that contained many polygonal 'type 1' astrocytes as well as several small process-bearing cells, one of which was galactocerebroside positive as detected by the O1 mAb (I). This lightly X-gal-positive oligodendrocyte is indicated by the arrow in the phase-contrast photomicrograph (H). Bars represent 20 μm in all panels except panel G, where the bar represents 40 μm.

white matter and into cortex are still present. (3) We have observed columns of labeled cells that are oriented perpendicularly to the pial surface (Fig. 2B).

If glial precursors use radial systems to support their migration, then why does glial migration become restricted to white matter by P14? The answer to this question may lie in the fact that cortical radial glia begin to transform into astrocytes late in the first postnatal week (LeVine and Goldman, 1988; Misson et al., 1991b) and largely disappear by P14, coincident with the restriction of SVZ glial progenitors to white matter pathways. If the orientation of radial glial processes determines the migration routes of SVZ cells, then one would expect that glial processes oriented towards the corpus callosum would be the last to disappear (if they ever disappear). However, to our knowledge, this observation has yet to be reported. Another prediction from this hypothesis is that SVZ glial progenitors will migrate along radial glia in vitro, as do granule cells and hippocampal neuroblasts (Hatten and Mason, 1990).

However, SVZ glial precursors may use other migrational aids. Alternative cues include axonal tracts (Gray and Sanes, 1991), endothelial cells and precursors (Chan-ling and Stone, 1991), chemoattractants (Armstrong et al., 1990) or extracellular matrix molecules (Hynes, 1990). At the present time, each of these remains a viable addition or alternative to radial glia in directing glial progenitor migration.

A number of transplantation studies have shown extensive migration of oligodendrocytes and astrocytes, largely through white matter tracts. For example, oligodendrocyte progenitors move from the thalamus into the brain stem and cerebellum when normal, neonatal CNS tissue is transplanted into the myelin basic protein-deficient shiverer mouse (Lachapelle et al., 1984; Gansmuller et al., 1991). Although extensive migration in an anterior-posterior plane

has been commonly observed in such studies, we have observed little migration along the A-P axis. One might argue that a myelin-deficient nervous system might allow more extensive migration than would the normal CNS. Alternatively, some of the oligodendrocytes seen in caudal regions of the shiverer CNS could have migrated within the ventricular system, since some transplanted cells were found in the choroid plexus and ventricle (Lachapelle et al., 1984). Yet, migration in an anterior-posterior axis has also been observed when glial precursors are transplanted into normal rodent CNS, although the extent and pattern of migration have not been characterized in detail (Espinosa de los Monteros et al., 1992; Warrington et al., 1992). Clearly, much remains to be learned about the migratory behavior of glial precursors in situ.

Assessing fate determination

Since neonatal SVZ cells that develop in gray matter generated both oligodendrocytes and astrocytes although cells that settled in white matter almost uniformly developed into oligodendrocytes, we suggest that white matter contains signals that promote oligodendrocyte differentiation and/or inhibit astrocyte differentiation. This hypothesis is supported by the demonstration that neonatal and juvenile SVZ cells have equivalent developmental potentials in vitro, yet juvenile cells only produce oligodendrocytes in vivo and this fate restriction coincides with their (restricted) colonization of cerebral white matter. While these studies do not elucidate the mechanism responsible for this restriction, these observations strongly suggest that epigenetic signals are important in determining an SVZ cell's fate in vivo.

These SVZ cells do not appear to be identical to any progenitors yet described. They do not appear to be self-renewing stem cells, at least not a population of stem cells that stay in the SVZ (Table 1), nor do they have the properties of neuroblasts, astroblasts, O-2A progenitors or neuron/glial progenitors (Temple, 1989; Reynolds et al., 1992; Luskin et al., 1988; Cattaneo and McKay, 1990; Raff, 1989; Williams et al., 1991). Furthermore, they appear to be at an earlier stage of differentiation than any previously described glial progenitor. A large number of in vitro lineage studies, including several reports in which retroviruses were used, have supported the two lineage model of gliogenesis that was based on studies of rat optic nerve cultures (Raff, 1989), because clones containing both type 1 astrocytes and oligodendrocytes were never observed (Vaysse and Goldman, 1990; Luskin et al., 1988; Williams et al., 1991; Lubetzki et al., 1992). However, mixed clones represented approximately 40% of the total clones that we observed. In the present study, we injected the retrovirus directly into the germinal zone while, in previous studies, the infections were performed after the brain cells had been dissociated and cultured. That less complex clones were observed in previous studies suggests that the multipotential state is transient and that a committed progenitor (or progenitors) is produced soon after placing cells in vitro. Alternatively this multipotential glial progenitor may elude retroviral infection in vitro.

We have observed both astrocytes and oligodendrocytes within clones descended from postnatal SVZ cells in vivo (Levison and Goldman, 1993), a finding that supports the existence of bipotential progenitors. Our present results, however, do not allow us to conclude whether the astrocytes that arise postnatally from SVZ cells in vivo correspond to a specific astrocyte 'type' because both 'types 1 and 2' are produced in vitro from SVZ cells.

In contrast to our inference that some SVZ cells are uncommitted precursors, several in vivo retroviral lineage studies performed on rodent forebrain have concluded that there are separate, determined, progenitors for oligodendrocytes, astrocytes and neurons that diverge prenatally (Luskin et al., 1988, 1993; Price and Thurlow, 1988; Grove et al., 1993). Though a few clones containing more than one cell type were observed, the authors focussed on the predominance of homogeneous clones. In the above noted studies, retroviruses were introduced prenatally into the ventricular fluid. Where the initially labeled cell populations have been localized, they have been found largely in the ventricular zone (VZ) (Halliday and Cepko, 1992; Acklin and van der Kooy, 1993), although such localization has not generally been determined. In our studies, we injected the vectors directly into the postnatal SVZ. Since available evidence suggests that the SVZ is derived from the VZ (Halliday and Cepko, 1992), one might expect prenatal VZ infections to give rise to mixed glial-neuronal clones if progenitor fate is not entirely lineage-determined. However, such speculation supposes that derivatives of a labeled VZ cell persist in the germinal matrices into postnatal life, initially producing neurons and then glia during this developmental period - a possibility not explored in much detail. In contrast, distinct bands of thymidine-labeled cells have been observed in the forebrain germinal zone and cells in these bands appear to be clonally distinct (Altman and Bayer, 1990; Acklin and van der Kooy, 1993). These observations suggest that there is heterogeneity in the germinal zone, and thus it is possible that we have been labeling a different progenitor population in the postnatal SVZ than others have labeled by prenatal ventricular injection.

Restraint is advised before concluding that homogeneous clusters of related cells indicates lineage restriction, especially in light of recent studies demonstrating that clonally related cells within the forebrain migrate tangentially (Walsh and Cepko, 1993; Fishell et al., 1993; O'Rourke et al., 1992). In the present in vitro clonal analysis, mixed clones were observed at least three times more frequently than in vivo (Levison and Goldman, 1993). It is likely that the clonal boundaries that we employed in our in vivo clonal analysis were so restrictive that single clones were split into several smaller more homogeneous clones, thus reducing the observed frequency of heterogeneity. Furthermore, without experimentally challenging the developmental fate of an individual progenitor, either by using heterochronic transplantation or in vitro studies, no conclusion can be drawn regarding the potential of that precursor. As we demonstrated, SVZ cells from a two week rat forebrain only produce white matter oligodendrocytes in vivo, which might lead one to conclude that they are determined oligodendroblasts, yet when placed in vitro, these precursors show the same developmental plasticity as their neonatal counterparts and are able to generate 'type 1' astrocytes. Thus, characterizing the final cellular composition in a cluster of

related cells in situ does not provide information on the developmental potential of their precursors.

Migration and myelination

It has long been known that phylogenetically older white matter tracts myelinate prior to more recently evolved tracts and that the corpus callosum is one of the last regions to myelinate (Jacobson, 1963). We observed that SVZ cells from an early postnatal rat migrate laterally through white matter, while SVZ cells later in development prefer to migrate medially towards the corpus callosum. These results support the large body of data from [^{3}H]thymidine-labeling studies demonstrating that, in young adult rats, the majority of thymidine-labeled cells appeared to migrate into the corpus callosum, where they differentiate into oligodendrocytes (Altman, 1966; Imamoto et al., 1978; Ling et al., 1973; Paterson et al., 1973). Indeed, the temporal sequences and spatial dispersion of glial progenitor migration, in conjunction with the kinetics of glial differentiation, must determine the sequence of myelination. The regional acquisition of myelin may also serve to restrict the pathways taken by growing neurites, preventing them from entering previously formed, and therefore inappropriate, pathways (Schwab and Schnell, 1991). Our results indicate that the migratory routes taken by glial precursors are not random, but are developmentally orchestrated. Further research on the nature of the signals that direct their migration may provide insights into the early events required for myelination.

The authors would like to thank Connie Cepko and Shawn Fields-Berry for generously providing the DAP retroviral helper cell line, Matthew Goldman for assistance with the camera-lucida drawings,Ray Manson in the pathology department and Sagrario Lugo at Rainbow 1 Hour Photo, NY, NY for printing the photomicrographs, and JoAnn Gensert and Sharon Newman for their comments on the manuscript. This work was supported by NS17125, MH 15174 and AG 00189 and was presented in part at the Annual Society for Neuroscience Meeting (Chuang et al. 1992 and Levison et al., 1992).

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(Accepted 23 August 1993)