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Subventricular Zone Astrocytes Are Neural Stem Cells in the Adult Mammalian Brain

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Summary

Neural stem cells reside in the subventricular zone (SVZ) of the adult mammalian brain. This germinal region, which continually generates new neurons destined for the olfactory bulb, is composed of four cell types: migrating neuroblasts, immature precursors, astrocytes, and ependymal cells. Here we show that SVZ astrocytes, and not ependymal cells, remain labeled with proliferation markers after long survivals in adult mice. After elimination of immature precursors and neuroblasts by an antimitotic treatment, SVZ astrocytes divide to generate immature precursors and neuroblasts. Furthermore, in untreated mice, SVZ astrocytes specifically infected with a retrovirus give rise to new neurons in the olfactory bulb. Finally, we show that SVZ astrocytes give rise to cells that grow into multipotent neurospheres in vitro. We conclude that SVZ astrocytes act as neural stem cells in both the normal and regenerating brain.

Introduction

Neurons and macroglia (astrocytes and oligodendrocytes) arise from precursors in the germinal layers of the developing brain, the ventricular and subventricular zones (VZ and SVZ, respectively). Multipotential neural stem cells are present in these regions during development (Temple, 1989; Walsh and Cepko, 1992; Davis and Temple, 1994; Johe et al., 1996; Weiss et al., 1996b; Qian et al., 1998). However, separate precursors for glial cells and neurons have also been described (Raff et al., 1983; Luskin et al., 1988; Price and Thurlow, 1988; Mayer-Proschel et al., 1997; Rao et al., 1998; Shi et al., 1998). It has long been thought that lineages for neurons and macroglia become progressively segregated during development (Jacobson, 1991), suggesting that multipotential stem cells are only present early on during brain histogenesis. Neurogenesis and gliogenesis, however, continue throughout life, and multipotent stem cells can be isolated from the adult brain (reviewed in Gage et al., 1995; Weiss et al., 1996b; McKay, 1997; Temple and Alvarez-Buylla, 1999). In nonneural epithelia and the hematopoietic system, the continued production of new cells throughout adulthood has permitted experimental manipulations that led to the identification of stem cells in several tissues (Potten and Morris, 1988; Sprangrude et al., 1988; Cotsarelis et al., 1989).

The generation of large numbers of new neurons destined for the olfactory bulb throughout adult life (Altman, 1969; Lois and Alvarez-Buylla, 1994; Doetsch and Alvarez-Buylla, 1996) provides an experimental opportunity for the identification of their in vivo primary precursors. These new neurons are born in the SVZ, a thin layer of dividing cells that persists along the lateral wall of the lateral ventricles. A layer of epithelial cells known as ependymal cells separates the SVZ from the lateral ventricles. SVZ neuroblasts migrate as a network of tangentially oriented chains (Doetsch and Alvarez-Buylla, 1996; Lois et al., 1996) that converge on the rostral migratory stream (RMS) to reach the olfactory bulb, where they differentiate into local interneurons (Luskin, 1993; Lois and Alvarez-Buylla, 1994; Doetsch and Alvarez-Buylla, 1996). Within the SVZ, the migrating neuroblasts (type A cells) advance as chains through tubes defined by the processes of slowly proliferating SVZ astrocytes (type B cells). Clusters of rapidly dividing immature precursors (type C cells) are scattered along the network of migrating chains (Doetsch et al., 1997). The identification of the in vivo stem cells will allow elucidation of the molecular mechanisms for their proliferation and differentiation. Furthermore, the targeted manipulation of these cells in vivo and the isolation of specific pools of adult-derived precursors may be of use for brain repair.

Neural stem cells that have the capacity to self-renew and differentiate into neurons and glia can be cultured from the adult SVZ (Morshead et al., 1994; Gritti et al., 1999). These cells grow as spherical floating clusters (neurospheres) in the presence of epidermal growth factor (EGF) or basic fibroblast growth factor (bFGF) (Reynolds and Weiss, 1992; Gritti et al., 1995). Previous work has suggested that SVZ neural stem cells correspond to a rare population of relatively quiescent cells (Morshead et al., 1994). More recently, it has been suggested that ciliated ependymal cells correspond to the neural stem cells (Johansson et al., 1999).

We show here that SVZ astrocytes (type B cells) are the in vivo precursors of new neurons. SVZ astrocytes divide to regenerate the SVZ network of migrating neuroblasts after elimination of all migrating neuroblasts and type C cells. In untreated mice, only SVZ astrocytes, and not ependymal cells, remain labeled in the SVZ following long survivals after administration of proliferation markers. Furthermore, following the targeted introduction of a retrovirus into glial fibrillary acidic protein (GFAP)-positive cells of untreated mice, retrovirally labeled migrating neuroblasts and fully differentiated neurons were observed in the olfactory bulb. Finally, we show that SVZ astrocytes give rise to cells that grow into multipotent neurospheres in vitro. These findings identify SVZ astrocytes, or a subpopulation of these cells, as the in vivo precursors of newly generated neurons in the adult mammalian brain and suggest that they act as stem cells in this region.

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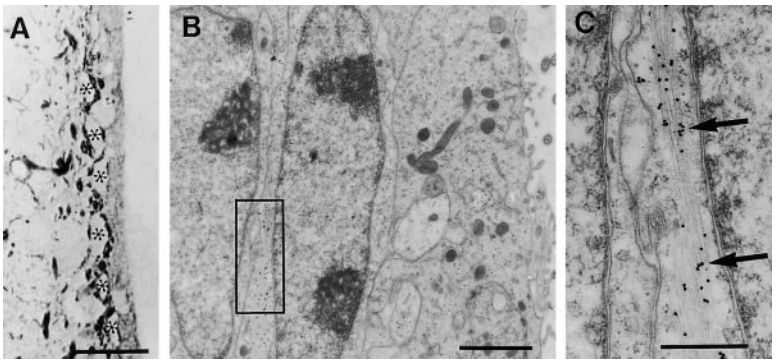


Figure 1. Cells Remaining in the SVZ after Ara-C Treatment Are GFAP Positive

GFAP-postembedding immunostaining of the SVZ in 1.5 μm semithin (A) and ~70 nm ultrathin sections (B and C).

(A) SVZ cells immediately after Ara-C are GFAP positive. Asterisks show cell bodies of labeled cells.

(B) Silver-enhanced immunogold labeling for GFAP immediately after Ara-C treatment. At the EM, these cells are characterized by dense bundles of intermediate filaments that are immunostained by GFAP antibodies.

(C) Detail of immunostained intermediate filaments shown in (B) (rectangle). Scale bars: (A), 20 μm; (B), 1 μm; (C), 0.25 μm.

Results

In work to be presented elsewhere, we show that the SVZ of the adult mouse brain completely regenerates after elimination of migrating neuroblasts (type A cells) and precursor cells (type C cells) with the antimitotic drug cytosine-β-D-arabinofuranoside (Ara-C) (F. D. et al., unpublished data). Immediately after Ara-C treatment, SVZ astrocytes (type B cells) and ependymal cells remain; 2 and 4 days later, type C and A cells appear consecutively. These findings suggest that either SVZ astrocytes or ependymal cells divide to regenerate the SVZ.

Type B Cells Divide Early during Regeneration of the SVZ

In order to confirm the identification of type B cells in the SVZ immediately after Ara-C treatment, we performed immunostaining for GFAP, a marker of SVZ astrocytes. At the light microscope (Figure 1A), all cells appeared GFAP positive. However, since GFAP-negative cells may appear labeled if surrounded by GFAP-positive processes, we used postembedding immunogold staining for GFAP to identify labeled cells at the electron microscope (EM). This analysis confirmed that after Ara-C treatment, remaining cells in the SVZ were labeled by GFAP antibodies. GFAP immunostaining was localized to the bundles of intermediate filaments that characterize type B cells (Figures 1B and 1C). Type B cells after Ara-C treatment were also lightly stained for S100β and vimentin (not shown).

Dividing cells reappear scattered throughout the SVZ 0.5 days after termination of Ara-C treatment (F. D. et al., unpublished data). To identify which cells divided after Ara-C treatment, mice were injected systemically with [^{3}H]thymidine, a marker of DNA synthesis, 0.5 days after Ara-C treatment and killed 1 hr later. [^{3}H]thymidine-labeled cells in both the injected and contralateral hemispheres were identified at the EM (Figure 2A and Table 1). Of 154 cells studied, 152 corresponded to type B cells and 2 to microglia. None corresponded to ependymal cells. Some [^{3}H]thymidine-labeled cells were very close to the ependymal layer but were always separated from the ventricle by the cytoplasm of an ependymal cell.

Dividing Type B Cells Give Rise to Type C Cells and Neuroblasts

To test whether type B cells divide to give rise to other cell types, we injected [^{3}H]thymidine systemically 0.5 days after Ara-C treatment to label proliferating type B cells. Mice were then killed 1.5 or 5.5 days after [^{3}H]thymidine injection. After processing for autoradiography, [^{3}H]thymidine-labeled cells were identified at the EM. One and a half days after [^{3}H]thymidine injection, more than one-third of the labeled cells corresponded to type C cells (Figure 2B and Table 1). These results indicate that proliferating type B cells divided to give rise to type C cells. In mice pulse labeled at 12 hr and killed 5.5 days later, labeled type A cells were identified, indicating that they too are derived from type B cells (Table 1). Labeled type A cells, however, had only a few grains overlying their nuclei, suggesting multiple divisions of their progenitors.

To confirm that type A cells were derived from proliferating type B cells, three different replication-incompetent retroviruses, which are stably inherited by the progeny of dividing cells without dilution upon division, were used to infect proliferating type B cells 0.5 days after Ara-C treatment. A retrovirus encoding human placental alkaline phosphatase (DAP) (Fields-Berry et al., 1992) was used to analyze the morphology of retrovirally labeled cells. Since the DAP precipitate could not be localized at the EM, we used a retrovirus encoding β-galactosidase (β-gal) (Price et al., 1987) to characterize labeled cells at the EM. Finally, a retrovirus encoding green fluorescent protein (GFP) (LZRS-CA-gapEGFP; Okada et al., 1999) was used to follow live cells in culture (see below).

At different survivals after retroviral infection with DAP following Ara-C treatment, whole mounts were dissected and processed for alkaline phosphatase (AP) histochemistry, and the morphology of AP-positive cells was analyzed. One day after infection with the DAP retrovirus, AP-positive stellate cells with multiple processes were observed (Figure 2C). This branched morphology suggested that these cells correspond to type B cells (Doetsch et al., 1997). In order to confirm the identity of retrovirally labeled cells, 11 cells that were infected with a β-gal-encoding retrovirus were studied at the EM 1 day after infection. All 11 corresponded to type B cells (e.g., Figures 2F and 2G). By 4 days after infection with the DAP retrovirus, tight clusters of AP-positive cells were observed (Figure 2D). These clusters contained globular and branched cells. Five and a half days after infection, many smaller AP-positive cells with the morphology of migrating neuroblasts (type A cells) (Kishi, 1987; Wichterle et al., 1997) were present. Their cell bodies were round or elongated, with a thin leading

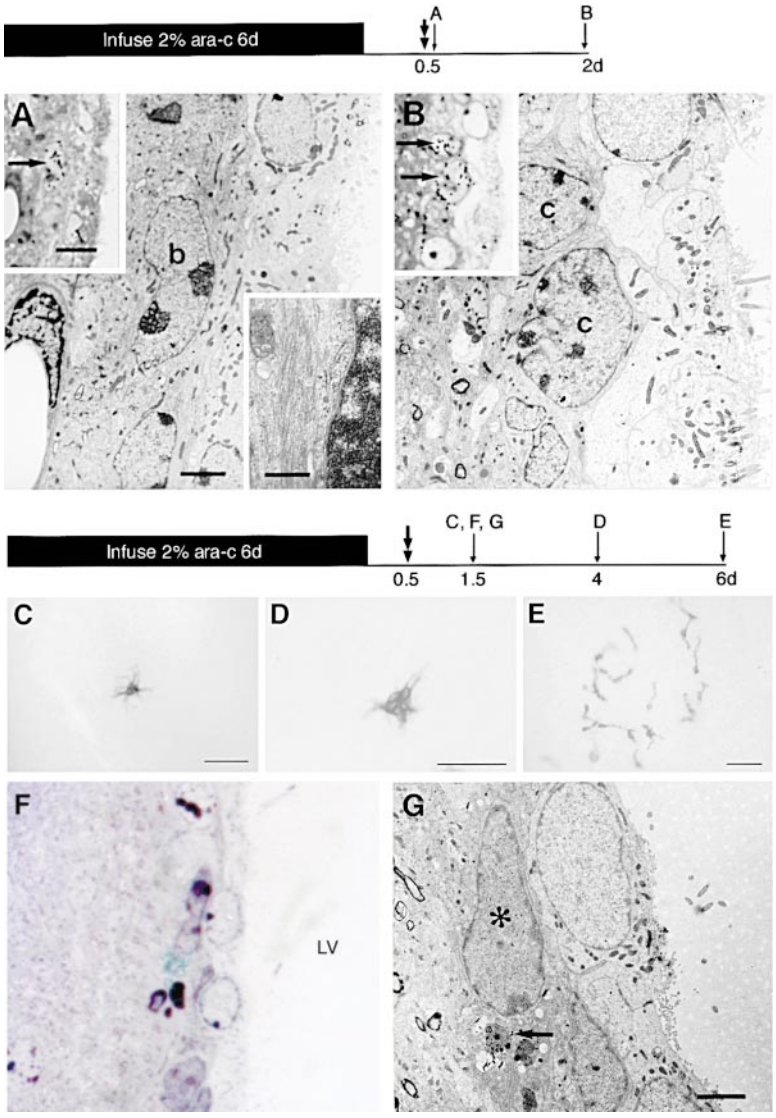


Figure 2. Proliferating Type B Cells Give Rise to Type C Cells and Neuroblasts

Time lines illustrate the duration of Ara-C treatment and time of pulse labeling (double arrow) with [^{3}H]thymidine (top panels) or retrovirus (bottom panels). Thin arrows indicate the time points at which the SVZ was analyzed with respect to the end of Ara-C treatment.

(A) In [^{3}H]thymidine-injected mice killed 1 hr after pulse labeling, type B cells (b) were observed with the EM; upper inset shows the same cell with silver grains overlying its nucleus in a semithin section (see Table 1). Lower right inset shows a bundle of intermediate filaments, characteristic of type B cells, in the [^{3}H]thymidine-labeled cell.

(B) Two days after pulse labeling proliferating astrocytes, type C cells (c) were observed in addition to type B cells. Inset shows silver grains overlying the nuclei of the two cells identified at the EM as type C cells.

(C) Alkaline phosphatase–positive cells with a stellate morphology were observed 1 day after infection with DAP retrovirus.

(D) At 4 days, small clusters of tightly associated cells are present.

(E) At 6 days, many cells with the morphology of migrating neuroblasts (type A cells) were observed.

(F) Semithin section of β-galactosidase retrovirally labeled cell (blue precipitate) 1.5 days after Ara-C.

(G) This same cell analyzed at the EM corresponds to a type B cell. Scale bars: (A) and (B), upper inset, 10 μm; photomicrograph, 2.5 μm; lower inset, 0.5 μm; (C), 30 μm; (D), 40 μm; (E), 20 μm; (F), 10 μm; (G), 2 μm.

process tipped by a growth cone and occasionally with a trailing process (Figure 2E). Some type A cells were dispersed, suggesting that they had already migrated tangentially.

We analyzed the composition of retrovirally infected clusters in whole-mount preparations at different survivals after Ara-C treatment (Table 2). AP-positive cells within each cluster were classed as branched, globular, or migratory based on their morphology (Table 2). Initially, all of the clusters comprised only one or two cells, and all contained branched cells. Over time, more mixed clusters with both branched and globular morphologies were observed, and then clusters containing branched, globular, and migratory morphologies appeared. Six

Table 1. Cell Types Labeled with [^{3}H]thymidine after Pulse Labeling at 12 Hr after Ara-C Treatment

	Survival	Type E (Ependymal)	Type B (Astrocyte)	Type C (Precursor)	Type A (Neuroblast)	Microglia	n Cells
Injected	0.5 days + 1 hr	0	97.6% (82)	0	0	3.2% (2)	84
	0.5 days + 1.5 days	0	63.6% (7)	36.4% (4)	0	0	11
	0.5 days + 5.5 days	0	18.9% (7)	29.7% (11)	48.6% (18)	2.7% (1)	37
Contralateral	0.5 days + 1 hr	0	100% (70)	0	0	0	70
	0.5 days + 1.5 days	0	41.7% (10)	54.2% (13)	0	4.2% (1)	24
	0.5 days + 5.5 days	0	46.7% (7)	20% (3)	33.3% (5)	0	15

[^{3}H]thymidine-labeled cell types at different survivals following pulse labeling at 12 hr after Ara-C treatment. [^{3}H]thymidine was injected at 0.5 days after Ara-C treatment and mice killed 1 hr or 1.5 or 5.5 days later. Percentage of each cell type labeled with [^{3}H]thymidine of total labeled cells analyzed at the EM (absolute numbers of cells in brackets).

Table 2. Characterization of Cellular Composition of Retrovirally Infected Clusters after Ara-C Treatment

	Morphology of Retrovirally Infected Cells # of Clusters (# Cells/Cluster)					
						
Survival	Branched	Branched/ Globular	Globular	Branched/Globular/ Migratory	Globular/ Migratory	Migratory
1.5 days	9 (1) 3 (2)	1 (2)				
2.5 days	2 (1) 12 (2) 2 (4) 1 (6)	1 (5)	2 (1)			
4 days	2 (1) 6 (2)	7 (4) 2 (5) 2 (6) 2 (7) 1 (10)		1 (8–9) 1 (12) 1 (19)	1 (5)	1 (2) 1 (3)
6 days	1 (1)		1 (2)	1 (5) 3 (11) 1 (14–15) 1 (20) 1 (22) 1 (24) 1 (50)		2 (1)

Analysis of morphologies of retrovirally infected cells expressing alkaline phosphatase found in individual clusters at different survivals after Ara-C treatment. Cells were classed as branched, globular, or migratory. Representative camera lucida drawings of the three morphologies are shown.

days after termination of Ara-C treatment, most of the clusters contained the three cell morphologies, indicating that many of the single infected cells we observed at one and a half days eventually formed part of neurogenic clusters.

The above results suggest that type B cells give rise to neurons via an intermediate globular cell type. In order to directly follow the production of neuroblasts from type B cells, we used a retrovirus encoding GFP injected in vivo 0.5 days after termination of Ara-C treatment. One day after infection, GFP-labeled cells with the branched morphology of type B cells were observed in whole-mount preparations of the SVZ (Figure 3A). These whole mounts were sectioned and double stained for GFAP. The processes of GFP-positive cells were stained for GFAP (Figures 3B–3D), confirming that they were SVZ astrocytes.

To follow the fate of individual live GFP-labeled cells over time, dividing cells were infected 0.5 days after Ara-C treatment in vivo. One day after infection, fragments of the SVZ were dissected and cultured in a collagen gel. Individual GFP-positive cells were marked and photographed daily to follow their progression (Figures 3E–3K). Some of the branched cells divided to give rise to tight clusters of globular cells (Figure 3F). The tight clusters dispersed after 4 days (Figure 3G), and cells with a small round or elongated cell body and a leading process appeared (Figure 3H). Some of these cells were TuJ1 positive, confirming their neuronal identity (Figures 3I–3K). These results directly show that dividing type B cells give rise to neurons after Ara-C treatment via an intermediate globular cell type.

Type B Cells Are the Only Label-Retaining Cells in the Adult SVZ

Although SVZ astrocytes regenerate the network of chains of neuroblasts after Ara-C treatment, these cells may behave as neuronal precursors only during regeneration. A recent paper has suggested that under normal conditions, ependymal cells are the in vivo precursors of new neurons (Johansson et al., 1999).

Cells that remain labeled in germinal layers for extended periods after [^{3}H]thymidine injection are considered candidate stem cells (Potten and Morris, 1988; Cotsarelis et al., 1989). We therefore studied which dividing cell types in the SVZ of normal adult mice remained labeled at long survivals after BrdU or [^{3}H]thymidine treatment. Single injections of [^{3}H]thymidine had previously revealed that migrating neuroblasts (type A cells), type C cells, and SVZ astrocytes (type B cells) are proliferating. However, more slowly proliferating cells may not be labeled with single injections. We therefore used the same protocol as Johansson et al. (1999), in which BrdU was administered continuously to mice in their drinking water for 2 weeks. Sections were double stained for both BrdU and mCD24 (Calaora et al., 1996) or S100β (Didier et al., 1986), both highly expressed by ependymal cells. In mice killed immediately after treatment, many BrdU-labeled cells were encountered in the SVZ, but none corresponded to ependymal cells (not shown). In mice killed 1 week after the end of treatment, the number of BrdU-positive nuclei was greatly reduced. Again some labeled nuclei were found close to the ventricular surface, but no labeled ependymal cells were observed. Strongly labeled S100β (not shown) or mCD24

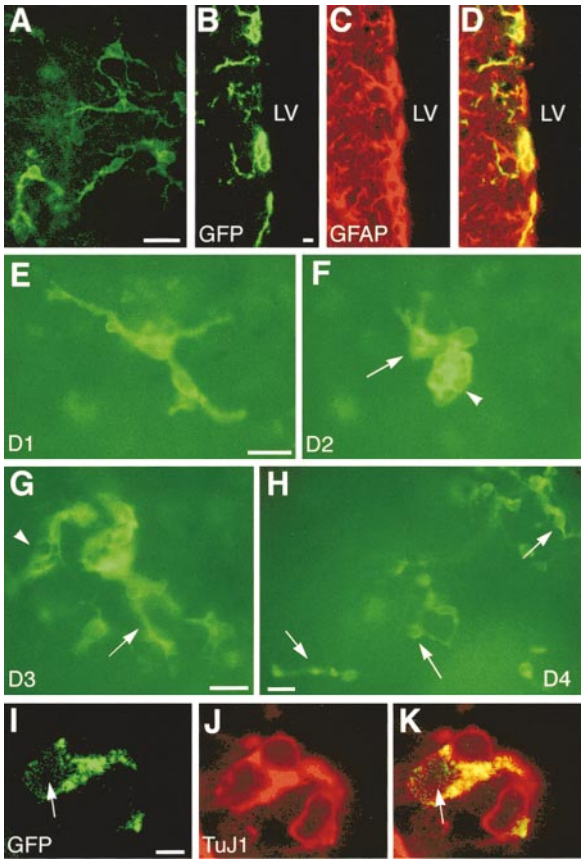


Figure 3. GFAP-Positive Cells Give Rise to Neuroblasts In Vitro

(A) Whole-mount dissection of the lateral wall of the lateral ventricle 1 day after infection with a retrovirus encoding GFP. The retrovirus was injected 0.5 days after the end of Ara-C treatment. Only cells with a branched morphology were present at this survival. These whole mounts were sectioned transversely and stained with an anti-GFAP mAb.

(B) One micrometer confocal optical slice showing the GFP-labeled cells.

(C) GFAP staining of this same section.

(D) GFP-positive cells are GFAP positive. The ependymal layer in (B–D) came off during immunocytochemical processing. Ependymal cells were not labeled by the reporter gene.

(E–K) Direct observation of the production of neuroblasts by type B cells. Proliferating type B cells were infected as in (A). One day after infection, whole mounts were dissected, embedded in a collagen gel, and cultured to follow the fate of GFP-labeled cells daily. (E) Two GFP cells with a branched morphology after 1 day in culture. (F) The lower of the two branched cells divided and generated a tight cluster of globular cells (arrowhead). The upper cell also divided into branched (arrow) and unbranched cells. (G) By 3 days, the lower cluster began to disperse, and some cells with a leading process and a growth cone typical of type A cells (arrow) were present. By this time, the upper cell had also generated a tight cluster of globular cells (arrowhead). (H) One day later, several type A cells were present (arrows), and some had moved away from the clusters. (I–K) At this time, the cultures were fixed and stained for TuJ1, revealing that some GFP-positive cells (arrow) were TuJ1 positive. (E–H) are photomicrographs of live three-dimensional cultures. (B–D) and (I–K) are confocal 1 μm optical sections through fixed cultures. Scale bars: (A–D), 10 μm; (E–K), 5 μm.

(Figure 4A) processes separated these nuclei from the ventricular cavity.

Since SVZ cells and ependymal cells are so tightly associated, electron microscopy is required to confirm the membrane boundaries of labeled cells. We infused [^{3}H]thymidine into the lateral ventricle continuously for 12 days with a mini-osmotic pump. We then analyzed [^{3}H]thymidine-labeled cells close to the ventricle (Figure 4B). ^{3}H-labeled cells were identified in 2 μm autoradiograms and analyzed at the EM. One hundred and fifteen cells were studied, none of which corresponded to ependymal cells. In a separate experiment, [^{3}H]thymidine was injected systemically four times and mice allowed to survive 15 or 30 days. Twenty-four ^{3}H-labeled cells were analyzed at the EM. With the exception of 1 microglial cell, the remaining 23 cells all corresponded to type B cells. Figure 4C shows a labeled type B cell adjacent to a chain of neuroblasts.

The above results indicate that type B cells remain labeled with proliferation markers in the SVZ after long survivals under normal conditions. In contrast, we did not observe labeled ependymal cells after continuous BrdU administration for 2 weeks or after 12 days of continuous infusion of [^{3}H]thymidine into the lateral ventricle. The finding that type B cells were the only label-retaining cells in the SVZ at long survivals suggests that these cells may have divided asymmetrically and that one of the daughter cells generated progeny that had died or migrated away. Alternatively, this labeling could simply reflect endogenous glial turnover.

GFAP-Positive Cells in Untreated Mice Give Rise to New Neurons in the Olfactory Bulb

To determine whether type B cells are also neuronal precursors under normal conditions, a retrovirus encoding AP was specifically targeted into GFAP-expressing cells using transgenic mice engineered to express the receptor for avian leukosis virus under the GFAP promoter (GFAP-Tva mice) (Holland and Varmus, 1998). Mammalian cells do not normally express the receptor for the avian leukosis virus and therefore cannot be infected by these viruses. However, when replication-competent avian leukosis virus encoding alkaline phosphatase (RCAS-AP) is injected into the brain of GFAP-Tva mice, GFAP-expressing cells undergoing division become infected (Holland and Varmus, 1998) and express the marker gene alkaline phosphatase. This allows the progeny of infected GFAP-expressing cells to be followed.

When RCAS-AP was injected into nontransgenic mice, no AP-positive cells were observed, confirming that RCAS-AP does not infect mammalian cells. When DAP retrovirus is injected into nontransgenic mice, AP-positive migrating cells, globular cells, and branched cells are present in the SVZ 1 day later. In contrast, 1 day after infection of GFAP-Tva mice with the RCAS-AP retrovirus, only labeled cells with branched morphologies were observed ($n = 5$, 8–20 cells per hemisphere) (Figure 5A); no AP-positive migrating cells or globular cells were observed at this short survival. Furthermore, no labeled ependymal cells were observed, substantiating the above finding that ependymal cells do not divide in vivo. This confirms that the RCAS-AP virus is selectively infecting type B cells. In GFAP-Tva mice allowed to survive 3.5 days ($n = 2$) after infection, AP-positive migrating neuroblasts were encountered in the

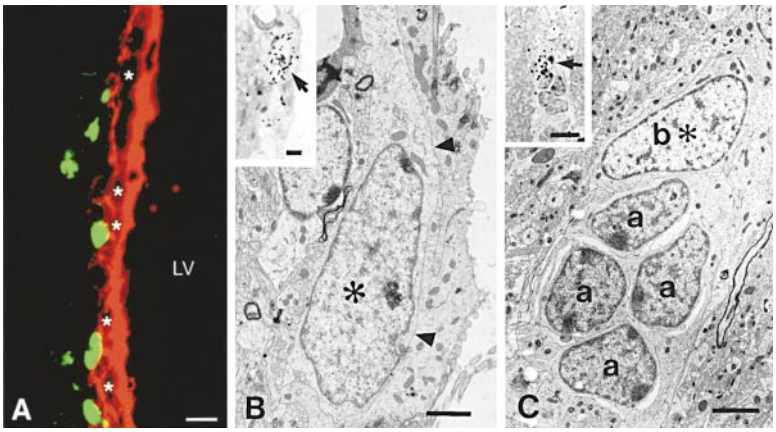


Figure 4. Type B Cells in the SVZ Are Labeled at Long Survivals after BrdU Administration or [^{3}H]Thymidine Injections

BrdU was administered continuously in the drinking water for 2 weeks, and sections were double stained for BrdU (green) and mCD24 (red).

(A) One week after treatment, no BrdU-positive mCD24 ependymal cells were observed. While BrdU-labeled nuclei were closely associated with ependymal cells, these nuclei were not surrounded by mCD24 staining. Ependymal nuclei (arrows) were surrounded on all sides by mCD24.

(B) Upper inset shows silver grains overlying the nucleus (arrow) of a ^{3}H-labeled cell in the SVZ after [^{3}H]thymidine was infused into the lateral ventricles for 12 days. Labeled nuclei that at the light microscope appeared to be in the ependymal layer were studied at the EM. These cells did not correspond to ependymal cells but had the ultrastructural characteristics of type B cells. Thin ependymal cell processes separate most of these cells from the ventricle.

(C) Upper inset shows silver grains overlying the nucleus (arrow) of a ^{3}H-labeled cell in the SVZ 30 days after four [^{3}H]thymidine injections. Ultrastructural analysis of this labeled cell (asterisk) reveals that it is a type B cell (b) adjacent to a chain of type A cells (a). Scale bars: (A), 10 μm; (B–C), inset, 10 μm; photomicrograph, 2 μm. LV, lateral ventricle.

SVZ and RMS. The latter were oriented in the direction of the olfactory bulb (Figure 5B). By 14 days (n = 5) after infection, many cells had reached the olfactory bulb and differentiated into granular and periglomerular neurons (Figures 5C and 5D). Interestingly, infected cells remained labeled in the SVZ at 14 days, suggesting that stem cells had been originally infected. At this time, the SVZ contained both solitary branched cells and clusters that contained a mixture of the globular, branched, and migratory neurons (Figure 5E). These results show that astrocytes in the SVZ produce new neurons under normal conditions in untreated mice.

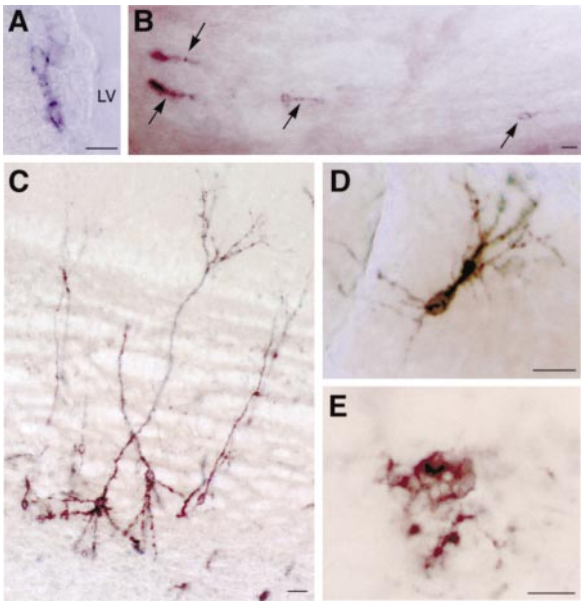


Figure 5. GFAP-Expressing Cells Generate Olfactory Bulb Neurons under Normal Conditions

Alkaline phosphatase–positive cells observed after the targeted infection of GFAP-positive cells using GFAP-Tva mice.

(A) Section through an AP-positive branched cell in the SVZ 1 day after infection. At this time, no globular or migrating neuroblasts were encountered.

(B) Three and a half days after infection, AP-positive migrating cells were observed in the RMS en route to the olfactory bulb (arrows).

(C and D) Fourteen days after infection, many AP-labeled cells were found in the olfactory bulb. These cells had the typical morphology of granule (C) and periglomerular neurons (D).

(E) In the SVZ of these mice, clusters containing branched globular and migratory cells were found. Scale bars: (A–E), 20 μm.

Neurospheres Are Derived from SVZ Cells

The above results indicate that SVZ astrocytes are the primary precursors for new neurons in vivo. Neural stem cells, which grow in vitro as neurospheres (Reynolds and Weiss, 1992), can be isolated from the adult SVZ (Morshead et al., 1994). A recent study suggests that the majority of neurospheres are derived from ciliated ependymal cells (Johansson et al., 1999). However, the long-term BrdU and [^{3}H]thymidine administration experiments described above indicate that ependymal cells do not divide in vivo. We tested whether ependymal cells lining the walls of the lateral ventricles could give rise to neurospheres. We injected DiI into the lateral ventricle to vitally label the ependymal cells (Figure 6A). Neurospheres were prepared from the contralateral hemisphere to avoid contamination of cells labeled along the injection track. After 10 days in the presence of EGF, 780 neurospheres grew, of which 191 contained DiI-labeled cells (Figures 6B and 6C). However, we noticed that uninternalized DiI or DiI-labeled cellular debris was present in these cultures. Since the labeling of the neurospheres was not homogeneous, it is possible that DiI may have labeled some neurospheres during or after dissociation. In addition, DiI may have been transferred between cells in vivo.

We therefore repeated this experiment using rhodamine fluorescent beads, which, unlike DiI, are internalized and not transferred between cells. When these beads were injected intraventricularly into one hemisphere, ependymal cells in the contralateral hemisphere

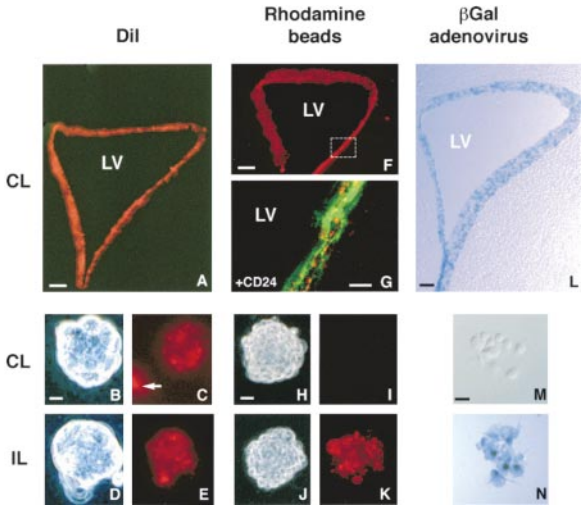


Figure 6. Ependymal Cells Do Not Give Rise to Neurospheres

(A) Frontal section showing the labeling of the ependymal layer in the contralateral hemisphere of a mouse that received an intraventricular injection of DiI.

(B–E) Phase contrast (B and D) and epifluorescent (C and E) images of two neurospheres cultured from the lateral wall of the lateral ventricle of DiI-injected mice. Both the contralateral (B and C) and the ipsilateral hemispheres (D and E) gave rise to neurospheres containing DiI-positive cells. However, we noticed that these cultures contained unincorporated DiI (arrow) and patchy DiI labeling of neurospheres.

(F) Section showing the labeling of the ependymal layer in the contralateral hemisphere of a mouse that received an intraventricular injection of rhodamine beads.

(G) Confocal image showing the region marked by a rectangle in (F) at higher magnification. Rhodamine-positive cells are also immunopositive for mCD24 (green), which labels ependymal cells.

(H–K) Phase contrast (H and J) and epifluorescent (I and K) images of two neurospheres cultured from the lateral wall of the lateral ventricle of rhodamine bead–injected mice. Neurospheres arising from the contralateral hemisphere did not show any rhodamine labeling (H and I), whereas some neurospheres from the ipsilateral hemisphere were fluorescent (J and K).

(L) Section showing the labeling of the ependymal layer in the contralateral hemisphere of a mouse that received an intraventricular injection of an adenovirus carrying LacZ.

(M and N) Nomarski images of five DIV neurospheres from the lateral wall of the lateral ventricle of β-gal adenovirus-infected mice. Neurospheres grown from the contralateral hemisphere (M) are X-gal negative, whereas some neurospheres from the ipsilateral hemisphere are X-gal positive (N). These small neurospheres were allowed to attach to poly-L-ornithine for 5 hr for staining. Scale bars: (A), 100 μm; (B–E), 25 μm; (F), 100 μm; (G), 10 μm; (H–K), 50 μm; (L), 100 μm; (M and N), 25 μm. LV, lateral ventricle; CL, contralateral; IL, ipsilateral.

were homogeneously labeled (Figure 6F). Double staining with mCD24 antibodies that strongly label the ependymal cells (Calaora et al., 1996) showed that the rhodamine bead labeling was restricted to the ependymal layer (Figure 6G). From the contralateral hemispheres of rhodamine bead–injected mice, 936 neurospheres grew in EGF. None of these neurospheres were labeled with fluorescent beads (Figures 6H and 6I). Brightly labeled fluorescent cells could be identified immediately after plating, but after 1 day, most of them had died and by 2 days could not be detected. It could be argued that rhodamine beads were not incorporated into stem cells or that beads were cytotoxic to the stem cells. However, from the ipsilateral side in which both SVZ cells and ependymal cells were labeled with rhodamine beads, 146 of 1022 neurospheres grown in EGF were fluorescently labeled (Figures 6J and 6K). Moreover, similar numbers of neurospheres were obtained from noninjected animals, indicating that the rhodamine beads did not kill neurosphere precursors.

We further tested whether ependymal cells could give rise to neurospheres by using a replication-defective adenovirus carrying β-galactosidase as a reporter gene. This virus infects both dividing and nondividing cells, and when injected intraventricularly in one hemisphere, it resulted in labeling of the ependymal wall of the contralateral hemisphere (Figure 6L). As with the above experiments, in order to avoid contamination of cells that became labeled along the injection track, we used the contralateral noninjected hemisphere. Seven hundred and thirty-two neurospheres grew with EGF from the contralateral hemisphere. These neurospheres were cultured for 4 days and stained with X-gal for detection of β-gal activity. None of the neurospheres were X-gal positive (Figure 6M). In control experiments, we used the ipsilateral side, where the virus infected some SVZ cells. In this latter case, 17 neurospheres out of 652 were X-gal labeled. This indicates that SVZ cells and not ependymal cells give rise to neurospheres. However, neurospheres from the contralateral side may have diluted the viral reporter gene below detection after repeated division. To follow the fate of virally labeled cells during the initial plating procedure, an adenovirus carrying GFP was used. This virus resulted in similar ependymal cell labeling as its β-gal counterpart (not shown). After 10 days in vitro (DIV), 422 neurospheres from the contralateral hemisphere grew with EGF, of which none were GFP positive. We followed the fate of fluorescently labeled cells in culture during the initial period after plating. Soon after dissociation, brightly fluorescent cells could be detected, but 1 day later, no GFP-labeled cells were present in our cultures. This suggests that ependymal cells, under these conditions, die soon after plating. Control experiments using the ipsilateral side, where the GFP adenovirus infected the SVZ, resulted in GFP-labeled cells that gave rise to neurospheres. In this latter case, 12 GFP-labeled neurospheres out of 387 were observed. Taken together, the above experiments suggest that ependymal cells are not the precursors for neurospheres.

We repeated the above experiments using bFGF+EGF to grow neurospheres after labeling the ependymal cells with adenovirus and fluorescent beads. We found no evidence for ependymal cells giving rise to neurospheres when grown with bFGF+EGF (not shown).

Neurospheres Are Derived from Type B Cells

In order to vitally label type B cells in the SVZ, we constructed an adenoviral vector expressing GFP under the control of the GFAP promoter (Yoon et al., 1996; Zolotukhin et al., 1996; Baba et al., 1997) (GFAPp-GFP AV). When this virus was injected into the SVZ and animals allowed to survive for 48 hr, type B cells and adjacent astrocytes in the striatum and corpus callosum became selectively labeled (Figures 7A–7F, 7H, and 7K).

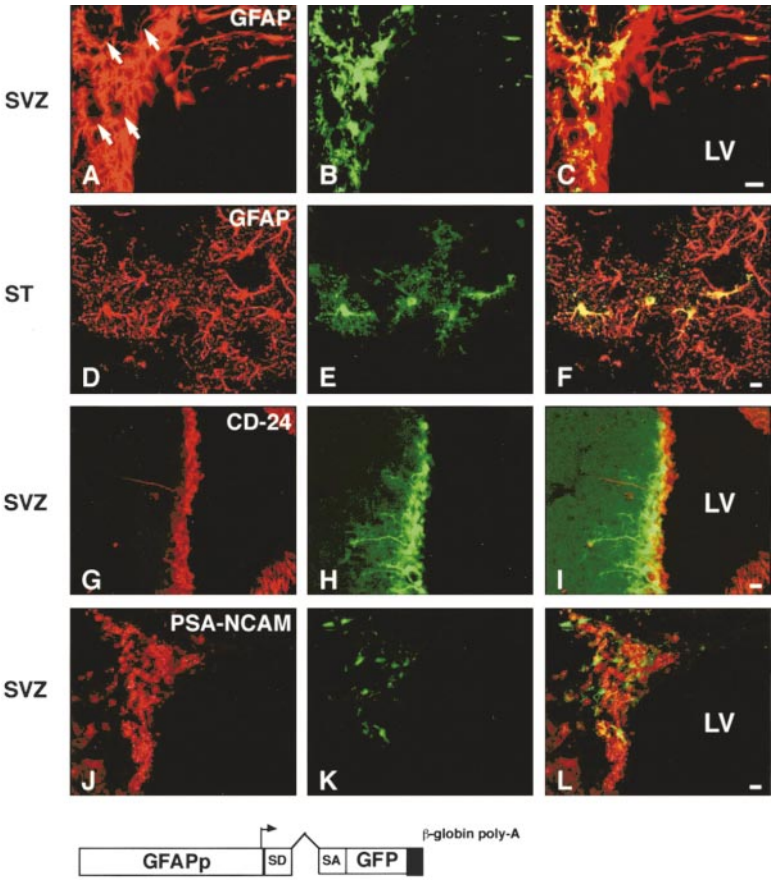


Figure 7. GFAPp-GFP Adenovirus Selectively Labels Astrocytes

All panels are 1 μm confocal optical sections of frontal sections through the SVZ (A–C and G–L) and striatum (D–F) of mice infected with the GFAPp-GFP adenovirus 48 hr earlier. The rhodamine channel is shown in the left column and the FITC channel (GFP) in the middle column, and the two are merged in the right column.

(A–F) GFP-positive cells in the SVZ (B) and striatum (E) are GFAP positive (A and D and C and F). Arrows in (A) indicate the GFAP-negative regions that correspond to chains of migrating neuroblasts in the SVZ.

(G–I) CD24-positive cells in the ependymal wall (G) were not GFP positive (H and I). The GFP-positive cells correspond to SVZ cells.

(J–L) PSA-NCAM-positive cells ([J], the migrating neuroblasts, type A cells) were not GFP positive (K and L). Instead, astrocytes around PSA-NCAM-positive migrating neuroblasts were positive. Scale bars: 10 μm. LV, lateral ventricle. The schematic at the bottom shows the GFAP promoter reporter construct used for the adenoviral vector.

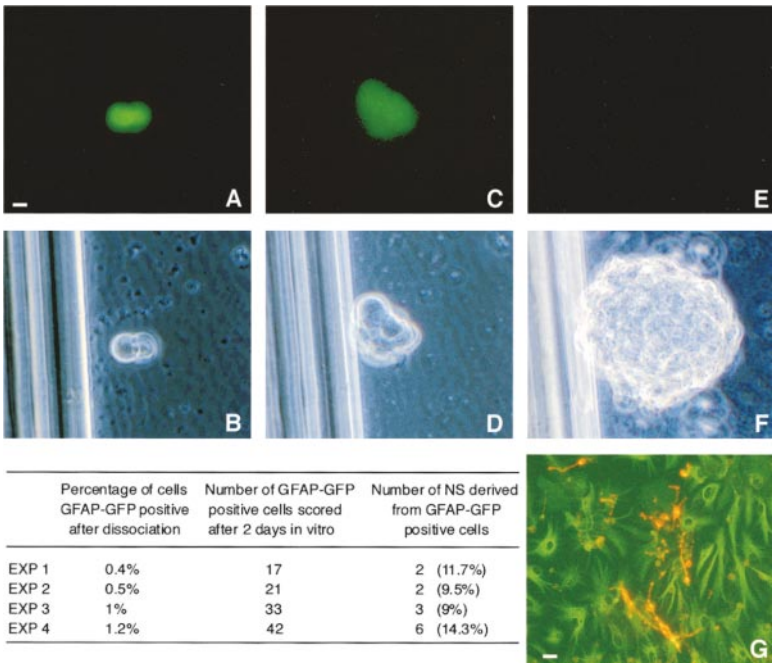
Double labeling for GFAP confirmed that the reporter gene was specifically expressed in GFAP-containing cells (Figures 7A–7F). Using cell-specific markers, we showed that the reporter gene was not expressed in ependymal cells or in type A cells (Figures 7G–7L). Labeling of type B cells with GFP was confirmed by preembedding anti-GFP immunogold staining and EM (not shown). Ependymal cells were not labeled.

After dissociation of the walls of the ventricle in mice that were injected with the GFAP-GFP adenovirus, 0.4%–1.2% of the total cells were brightly fluorescent. Wells containing single GFP-positive cells were marked and these fluorescent cells followed on a daily basis (Figures 8A–8F). Results for four independent experiments are shown in Figure 8. Of the fluorescently labeled cells scored, 9%–14.3% gave rise to neurospheres. Half of the neurospheres derived from GFP-expressing cells were plated for differentiation and shown to generate neurons and glia. In addition, the other half of the neurospheres differentiated into neurons and glia after forming secondary neurospheres (Figure 8G). This experiment indicates that neurospheres can be derived from GFAP-expressing cells in the SVZ.

Discussion

By taking advantage of the ability of the SVZ to regenerate following elimination of dividing cells, we have identified SVZ astrocytes as the primary precursors for new neurons generated in the adult rodent brain. Three observations identify type B cells as the primary precursors during generation: (1) with the exception of microglial cells, only type B cells incorporated [^{3}H]thymidine shortly after Ara-C treatment; (2) pulse labeling of proliferating type B cells with [^{3}H]thymidine or retrovirus shortly after Ara-C treatment resulted in labeled type C and A cells, consecutively; (3) we followed the generation of migrating neuroblasts from individual retrovirally GFP-labeled type B cells in vitro. Interestingly, dividing type B cells gave rise to clusters of globular cells that remain tightly associated until neuroblasts appeared. Similar clusters of type C cells are present in vivo in untreated animals (Doetsch et al., 1997; García-Verdugo et al., 1998). These results suggest that type B cells divide to give rise to type C cells, which in turn divide repeatedly, forming tight clusters that then generate type A cells (Figure 9).

Our experiments using GFAP-Tva transgenic mice show that type B cells are also the primary precursors of new neurons under normal conditions. Specific infection of dividing SVZ cells expressing GFAP shows that SVZ astrocytes give rise to new granule and periglomerular neurons in the olfactory bulb. At long survivals after infection, clusters of branched and globular cells were still present in the SVZ, suggesting that cells initially infected had undergone self-renewal. These results provide direct evidence that GFAP-positive type B cells are neurogenic.



	Percentage of cells GFAP-GFP positive after dissociation	Number of GFAP-GFP positive cells scored after 2 days in vitro	Number of NS derived from GFAP-GFP positive cells
EXP 1	0.4%	17	2 (11.7%)
EXP 2	0.5%	21	2 (9.5%)
EXP 3	1%	33	3 (9%)
EXP 4	1.2%	42	6 (14.3%)

Figure 8. GFAP-GFP-Positive Cells Give Rise to Neurospheres

(A–F) SVZ cells were infected in vivo with the GFAPp-GFP adenovirus; 48 hr later, cells were dissociated and cultured in multiwell plates. Epifluorescent (A, C, and E) and phase contrast (B, D, and F) images of a GFP-positive cell monitored as it generated a neurosphere. The GFP fluorescence is progressively reduced as the neurosphere grows due to dilution of the adenovirus or to promoter inactivation (E). (A and B): 3DIV; (C and D): 5DIV, (E and F): 8DIV.

(G) Double staining with Tuj1 (red) and GFAP (green) of a secondary neurosphere derived from the neurosphere shown in (F) differentiated into neurons and glia upon removal of growth factor and plating onto an adhesive substrate. Scale bars: (A–F), 25 μm; (G), 50 μm. The table shows the percentage of GFP-positive cells after dissociation and the proportion of these cells that gave rise to neurospheres in four independent experiments.

A recent report suggests that ependymal cells divide and are the in vivo neural stem cells (Johansson et al., 1999). Our results do not support this conclusion. Following continuous BrdU or [^{3}H]thymidine treatment for 14 and 12 days, respectively, we detected no labeled ependymal cells. Some labeled cells were very closely associated with the ependymal layer. However, analysis of [^{3}H]thymidine-labeled cells at the EM did not reveal any labeled ependymal cells. Likewise, during regeneration, when many stem cells are likely recruited, we did not detect mitoses or [^{3}H]thymidine-labeled ependymal cells. This suggests that ependymal cells do not divide under normal conditions or during regeneration of the SVZ. We cannot exclude the possibility that ependymal cells may divide extremely rarely or under certain conditions. It will be interesting to determine whether the conditioned media used in the study of Johansson et al. (1999) may induce ependymal cells to divide in vitro and form neurospheres. Our intraventricular labeling experiments with fluorescent latex beads or adenovirus suggest that ependymal cells are not the precursors for the majority of neurospheres. We provide direct evidence that GFAP-expressing SVZ astrocytes specifically labeled with an adenovirus carrying GFP give rise to EGF-responsive stem cells in vitro. As 48 hr is necessary for sufficient GFP to accumulate in these cells before culturing, it is possible that some of the fluorescent cells that gave rise to neurospheres had already transformed into type C cells. Thus, while our data suggest that the primary precursors of neurospheres are astrocytes, we do not know which are the immediate precursors. It is possible that multiple populations of cells in the SVZ have the capacity to give rise to neurospheres.

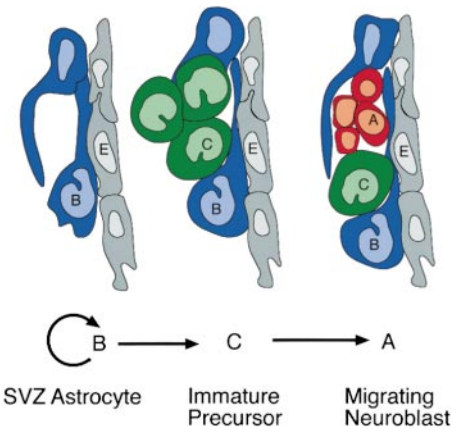


Figure 9. Model of Neurogenesis in the SVZ of the Adult Mouse Brain

SVZ astrocytes (type B cells, blue) are the primary neuronal precursors in the SVZ. These cells divide to give rise to clusters of type C cells (green), which in turn generate migrating neuroblasts (type A cells, red). Ependymal cells (gray) line the wall of the ventricles and in our experiments did not divide.

We have observed that during regeneration and in normal mice, type B cells occasionally contact the ventricle. However, type B cells contacting the ventricle are morphologically very different from ependymal cells. Most importantly, these cells do not have multiple long cilia characteristic of ependymal cells but occasionally display a single short cilium (our unpublished observation) similar to that described in neuronal precursors in the embryonic neuroepithelium (Sotelo and Trujillo-Cenóz, 1958) and in adult birds (Alvarez-Buylla et al., 1998). In order for type B cells to become neurogenic, they may need to contact the ventricle as neuroepithelial cells normally do during development.

Type B cells express glial markers and have the ultrastructural characteristics of astrocytes, that is, bundles of intermediate filaments, dense bodies in the cytoplasm, gap junctions, and multiple processes (Peters et

al., 1991). Based on these characteristics, we and others (Smart, 1961; Paterson et al., 1973) have identified these cells as SVZ astrocytes. We do not know whether only a subpopulation of astrocyte-like cells in the SVZ has the capacity to act as in vivo neuronal precursors or whether many can but are inhibited from following a pathway toward neuronal production by local environmental signals.

Many astrocytes express EGF and bFGF receptors (Nieto-Sampedro et al., 1988; Yazaki et al., 1994; Gomez-Pinilla et al., 1995). Interestingly, using these growth factors, neural stem cells can be isolated in vitro not only from the adult SVZ (Morshead et al., 1994) but also from other brain regions (Palmer et al., 1995; Johe et al., 1996), including the spinal cord (Weiss et al., 1996a), diencephalon (Weiss et al., 1996a), and hippocampus (Palmer et al., 1997). Our results raise the intriguing possibility that neural stem cells that have been cultured from other brain regions may actually be derived from astrocyte-like cells in vivo. Astrocytes encompass a heterogeneous population of cells (Wilkin et al., 1990) that are widely distributed in the adult brain and continue to divide in situ (Messier et al., 1958; Korr et al., 1973; Eckenhoff and Rakic, 1988). After injury, astrocytes proliferate to form glial scars (Cavanagh, 1970; Latov et al., 1979) but not neurons. It is possible that a neurogenic potential is latent in many astrocytes throughout the CNS but that inhibitory signals may suppress these cells from producing neurons. Neurogenic factors may only be present close to the brain ventricles or within the adult SVZ. Alternatively, SVZ astrocytes that act as stem cells in vitro and generate neurons in vivo may correspond to a fundamentally different cell type that resembles astrocytes and expresses GFAP and other glial markers. If this is the case, then our work suggests that use of markers such as GFAP, a hallmark of glial cells (Bignami et al., 1972), may not be a reliable indication of fully differentiated glia. Further work is required to characterize those SVZ astrocytes that can act as primary neuronal precursors and to determine how they differ from other astrocytes within the SVZ and the rest of the brain.

During neural development, astrocytes are derived from radial glia (Schmechel and Rakic, 1979; Voigt, 1989). Adult cortical astrocytes assume the characteristics of radial glia upon exposure to embryonic brain extracts (Hunter and Hatten, 1995). Interestingly, radial glia divide and have been hypothesized to function as neuronal precursors (Frederiksen and McKay, 1988; Alvarez-Buylla et al., 1990; Gray and Sanes, 1992). The identification of SVZ astrocytes as neuronal precursors in the adult brain further suggests that what has classically been considered an astrocytic lineage, including radial glia, may in fact correspond to embryonic and adult neuroepithelial cells that retain some of the properties of neural stem cells.

The present results demonstrate that SVZ astrocytes are the in vivo primary precursors for new neurons during regeneration and under normal conditions. Furthermore, neurospheres in vitro can be derived from SVZ astrocytes labeled in vivo, indicating that these cells can act as neural stem cells. Exploitation of the regenerative capacity and neurogenic potential of SVZ astrocytes may have powerful implications for brain repair.

Experimental Procedures

Ara-C Infusions

All animal care was in accordance with institutional guidelines. Cytosine-β-D-arabinofuranoside (2%; Ara-C, Sigma) in 0.9% saline or vehicle alone was infused onto the surface of the brain of adult CD-1 male mice (2–3 months) with a mini-osmotic pump (Alzet Model 1007D flow rate 0.5 μl/hr, 7 days). Cannulas were implanted stereotaxically onto the surface of the brain at the following coordinates: anterior (A), 0; lateral (L), 1.1; depth (D), 0.1 (relative to Bregma and the surface of the brain). After 6 days of infusion, the pump was removed and mice were killed at the indicated survivals.

Immunohistochemistry

For preembedding immunostaining for EM, brains were perfused with 3% paraformaldehyde and 0.5% glutaraldehyde, postfixed overnight, and immunostained for GFAP (1:500 DAKO), vimentin (1:1 40E-C [Alvarez-Buylla et al., 1987]) as described before (Doetsch et al., 1997) or GFP (1:500 Clontech) as described (Caillé et al., 1996). For postembedding immunostaining, brains were perfused with 3% paraformaldehyde and 1% glutaraldehyde, embedded in araldite (Durcupan), and immunostained for GFAP (1:300 Dako [polyclonal] or 1:200 [monoclonal, Boehringer Mannheim]) and S100β (Dako 1:200) in 1.5 μm semithin or 50 nm ultrathin sections (Blasco-Ibáñez et al., 1998). Frontal sections containing the entire dorsoventral lateral wall of the lateral ventricle were analyzed for GFAP immunoreactivity.

For light microscopy, brains were fixed by perfusion with 3% paraformaldehyde, postfixed overnight, and 50 μm thick sections cut with a vibratome. Differentiated neurospheres were fixed by immersion in 3% paraformaldehyde for 15 min at room temperature. After three rinses in phosphate-buffered saline (PBS; pH 7.4), neurospheres or sections were incubated for 1 hr in PBS containing 10% normal goat serum and 0.1% Triton X-100. The following primary antibodies were used: anti-Tuj1, mouse monoclonal IgG, dilution 1:1000 (Babco); anti-PSA-NCAM, mouse monoclonal IgM (Rougon et al., 1986), dilution 1:1000; anti-CD24, rat IgG, dilution 1:1000 (both gifts from Dr. G. Rougon, Université de Marseilles); and anti-GFAP, rabbit polyclonal IgG (DAKO), dilution 1:1000. Incubations in primary antibodies were carried out at 4°C overnight for neurospheres or for 48 hr for sections. Neurospheres or sections were washed in PBS three times and incubated with fluorescent secondary antibodies. For detection of Tuj1, Cy3-conjugated anti-mouse IgG (Sigma) diluted 1:1000 was used. For the detection of PSA-N-CAM, TRITC-conjugated anti-mouse IgM (Jackson Laboratories) diluted 1:200 was used. A biotinylated anti-rat IgG diluted 1:200 (Vector Laboratories) followed by Cy2 streptavidin diluted 1:1000 (Jackson Laboratories) was used for detection of CD24. A fluorescein-conjugated anti-rabbit IgG diluted 1:200 (Vector Laboratories) was used to detect GFAP. All secondary antibodies were incubated for 1 hr at room temperature. Sections or neurospheres were rinsed three times in PBS and coverslipped. Double-labeled sections were observed with a Zeiss LSM510 confocal microscope.

BrdU Administration and Detection

BrdU (1 mg/ml, Sigma) was given to mice in their drinking water for 2 weeks. Mice were killed immediately after treatment or 1 week later and their brains dehydrated and embedded in polyethylene glycol (Alvarez-Buylla et al., 1987). Three micron sections were cut on a rotating microtome and mounted on silanated slides (Sigma). Double staining for S100β and BrdU was performed by digesting with pepsin (0.25% in 0.1 N HCl) for 2 min at 37°C followed by blocking in 10% goat serum. Sections were incubated overnight at 4°C in a 1:1000 dilution of rabbit anti-S100β (Dako) and revealed with biotinylated secondaries (1:200, Vector) followed by streptavidin Cy3 (1:2000, Jackson Immuno). Sections were then processed for BrdU immunostaining by incubating 20 min at 37°C in 2 N HCl followed by blocking in 10% horse serum. Sections were incubated overnight in 1:200 mouse anti-BrdU (Dako) in blocking solution and revealed with anti-mouse Cy2 (1:500, Jackson Immuno). Thirty micrometer floating vibratome sections were stained sequentially for mCD24 (1:1000) and BrdU as for S100β, except that no pepsin was used.

[^{3}H]Thymidine Autoradiography and EM Analysis

For pulse-labeling experiments, mice were injected with 50 μl of 6.7 Ci/mmol [^{3}H]thymidine 0.5 days after pump removal and were killed either 1 hr later or 2 or 6 days after pump removal and brains processed for autoradiography and EM analysis as previously described (Doetsch et al., 1997). Briefly, cells overlaid by nine or more silver grains in three consecutive semithin sections were considered labeled. These sections were detached from the slide and reembedded in Araldite for ultrathin sectioning and analysis of the [^{3}H]thymidine-labeled cells at the EM. For long survival experiments, mice were injected four times every 4 hr with [^{3}H]thymidine and then allowed to survive 15 or 30 days. For continuous infusion of [^{3}H]thymidine into the lateral ventricle, mini-osmotic pumps (Alzet 2002, 0.5 μl/hr) filled with 200 μl of [^{3}H]thymidine (6.7 Ci/mmol) were implanted with the aid of a stereotaxic apparatus at A: 0, L: 1.1, D: 2 mm for 12 days.

Retroviral Injections for In Vivo Studies

Replication-incompetent retroviruses encoding either the marker gene human placental alkaline phosphatase (DAP) or β-gal (BAG) were harvested from the psi2 DAP cell line (ATCC 1949-CRL) or from the psi2 BAG cell line (ATCC 9560-CRL), concentrated, titered, and tested for helper virus (Cepko, 1992). The titer was 10^8 colony-forming units/ml. Twelve hours after Ara-C pump removal, 2 μl of retrovirus and 8 μg/ml of polybrene were injected stereotaxically into each lateral ventricle (above coordinates). Control injections of retrovirus into one lateral ventricle resulted in no retrovirally labeled cells in the contralateral SVZ. Mice were killed 1.5, 2.5, 4, or 6 days after pump removal and whole mounts of the lateral wall of the lateral ventricle dissected (Doetsch and Alvarez-Buylla, 1996), fixed for 3–4 hr in 3% paraformaldehyde, and enzyme histochemistry for alkaline phosphatase performed as described (Fields-Berry et al., 1992). Whole mounts were analyzed for the presence and morphology of labeled cells. β-gal-infected cells were analyzed at the EM as described (Wichterle et al., 1999).

For production of RCAS-AP, chicken embryo fibroblasts (CEFs) were prepared from the trunk of E10 embryos. After two passages, CEFs were calcium phosphate transfected with 5 μg of a plasmid encoding RCAS-AP. Three hours after transfection, cells were glycerol shocked for 90 s (Cepko, 1992). The cells were carried until the entire dish was infected, as assayed by histochemical staining for alkaline phosphatase. RCAS-AP was harvested and concentrated as described (Fekete and Cepko, 1993). RCAS-AP retroviruses (0.2 μl or 0.5 μl) were injected into the SVZ at three coordinates (A, L, and D: 0, 1.4, 1.6; 0.5, 1.1, 1.7; 1, 1, 2.3) of GFAP-Tva mice. Whole mounts (n = 5 for 1 day after infection, n = 2 for 3.5 days after infection, and n = 5 for 14 days after infection) were dissected and stained for AP as described above. Fifty micrometer vibratome sections were cut through the olfactory bulb and stained for AP as above.

Pseudotyped retrovirus encoding green fluorescent protein as a marker (LZRS-CA-gapEGFP; gift of R. Lansford and S. Fraser) was injected (0.5 μl into the lateral ventricle). One day after infection, whole mounts were dissected. Whole mounts processed for double staining were fixed for 2 hr in 3% paraformaldehyde, sectioned on a vibratome at 50 μm, blocked in 10% horse serum, incubated with 1:200 dilution of GFAP monoclonal antibody (Boehringer Mannheim) for 24–48 hr, and revealed using 1:1000 Cy3-anti-mouse secondary antibodies (Jackson Immuno). Sections were analyzed with an LSM510 Zeiss confocal microscope.

Whole-Mount Cultures

For whole-mount cultures, regions of the SVZ with GFP-positive cells were identified and small fragments (~1 mm) dissected. Thirty-five millimeter dishes (Falcon) were prepared as in Wichterle et al. (1997). Whole-mount fragments were placed with the ventricular surface facing down embedded in collagen gels (Wichterle et al., 1997) and covered with 1 ml of Neurobasal medium. Dishes were incubated at 37°C in a tissue culture incubator with constant slow rocking on a Varimix mixer that had been modified to rock at 1 rpm. Cultures were photographed daily on an inverted Olympus IX70 microscope. The experiment was repeated in eight Ara-C-treated mice (one to three cultures/mouse). When GFP-positive cells with the morphology of neurons were observed, cultures were fixed for 30 min in 3% paraformaldehyde washed extensively and stained for TuJ1 (1:1000 Babco) and revealed using 1:1000 Cy3 anti-mouse secondary antibody (Jackson Immuno). Six cultures containing GFP-labeled cells gave rise to neurons. Immunostained cultures were studied with an LSM510 Zeiss confocal microscope.

Neurosphere Preparation and Differentiation

Adult CD1 mice were killed by cervical dislocation (n = 5 per preparation) and their brains removed and placed into Pipes buffer (20 mM Pipes, 25 mM glucose, 0.12 M NaCl, 0.5 mM KCl [pH 7.4]). The lateral walls of the lateral ventricle were dissected, collected in Pipes buffer, and incubated in a mixture containing 1 ml of activated papain and 4 ml of Pipes buffer. Papain was activated half an hour in advance by placing 3 mg of papain (Worthington Biochemical) in 1 ml of 1.1 mM EDTA, 5.5 mM cysteine HCl. The pieces of tissue were incubated in papain solution at 37°C on a rocking platform for 1 hr, collected by centrifugation at 200 g for 2 min, and resuspended in 1 ml of DMEM-F12 (GIBCO) containing 0.7 mg of ovomucoid inhibitor and 1 mg of DNase. The cells were dissociated using a fire-polished pasteur pipette, and cells were collected by centrifugation at 300 g for 7 min. The cellular pellets were resuspended in 1 ml of DMEM-F12 (GIBCO) supplemented with N2 (GIBCO), 2 mM glutamine, 0.6% (w/v) glucose, 0.02 mg/ml insulin, and 15 mM HEPES. The cells were counted and plated in uncoated 35 mm dishes (50,000 cells per dish) in the same medium containing 20 ng/ml of EGF. Neurospheres were passaged by harvesting them by centrifugation (200 g for 5 min) and triturating them in 100 μl of medium with an automatic pipetter (P200 Gilson). For differentiation, neurospheres that had been grown for 8 to 12 days in vitro were plated onto laminin-coated (10 μg/ml) Lab-Tek 16-well chamber slides (Nunc) in the medium described above without EGF for 5–8 days.

Adenoviral Vectors

Three different replication-defective adenoviruses were used: one expressing LacZ as a reporter gene (Ad5-CMV-LacZ, 1.9×10^{11} pfu/ml) (Yoon et al., 1996), one expressing GFP as a reporter gene (Ad5-CMV-GFP, 1.3×10^{12} viral particles/ml), and a third one that we produced in which GFP was driven by the GFAP promoter.

Production of pAd5-GFAPp-GFP Adenoviral Vector

The mouse 2.5 kb GFAP promoter region was removed from GFAP 1L/PIP200 (Baba et al., 1997) (a kind gift of Dr. H. Baba) with a HindIII digest, filled in, and ligated into subcloning plasmid pSL at the StuI site. The GFAP promoter was then released from pSL with NotI and XbaI and ligated into adenoviral transfer plasmid pAd5(HS)V (Yoon et al., 1996) that had been digested with NotI and XbaI to remove the CMV promoter. The humanized GFP cDNA (Zolotukhin et al., 1996) was isolated from pGFP-neo with NotI, filled in, and ligated into a filled-in SalI site 3′ of the GFAP promoter start site in the adenoviral transfer plasmid, resulting in the clone pAd5-GFAPp-GFP. The adenoviral vector was generated by cotransfection of the pAd5-GFAPp-GFP and the pJM17 adenovirus genome plasmids into 293 human embryonic kidney cells. pJM17 contains the Ad5 genome with an insertion in the E1 region that prevents viral packaging. Recombination between pJM17 with pAd5-GFAPp-GFP produces an adenoviral genome that can be efficiently packaged. 293 cells provide the E1 gene products for complementation. The adenoviral vector Ad5-GFAPp-GFP was recovered after transfection, plaque purified twice, and verified by both PCR and restriction enzyme analysis of HIRT-extracted viral DNA. A viral stock was purified by cesium chloride banding twice. The titer was 1×10^{12} viral particles/ml.

Adenoviral Injections into the SVZ

The GFAPp-GFP adenovirus was injected stereotaxically into the SVZ. Two hundred nanoliters of a viral suspension was injected bilaterally into the SVZ at the following coordinates A, L, D: 1, 1, 2.2; 0.5, 1.1, 1.7; 0.5, 1, 2.1; 0, 1.4, 1.6; 0, 1.3, 1.8. The mice were sacrificed for neurosphere preparation 48 hr after infection. The efficiency of infection was verified by whole-mount dissections of the lateral wall of the lateral ventricle (Doetsch and Alvarez-Buylla, 1996). Three control mice, having received GFAPp-GFP adenovirus

infections, were perfused and their brains processed for immunohistochemistry as described above.

Labeling of the Ependymal Layer with Vital Dyes

Labeling of the ependymal layer was performed by intraventricular stereotaxic injections (A: −0.5; L: 1.1; D: 2) of vital dyes. For each type of injection, two mice were perfused and their brains sectioned to assess the specificity of the ependymal labeling. The ipsilateral and contralateral hemispheres were processed independently for neurosphere preparation.

Fluorescent Tracers

For DiI labeling, mice were injected with 2 μl of a 1% solution of DiI (1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate; Molecular Probes) in 300 mM saccharose and sacrificed 4 hr after injection. For rhodamine bead labeling, mice were injected with 2 μl of a 1:100 suspension of rhodamine beads (rhodamine-labeled latex microspheres; Lumafluor, NY) in PBS and sacrificed 16 hr later. Neurospheres were prepared from these labeled walls and cells plated and scored as described below.

Adenoviral Tracers

Mice were injected with 2 μl of an adenovirus expressing LacZ or 10 μl of an adenovirus expressing GFP and sacrificed 48 hr after injection. In the case of the β-gal adenovirus, neurospheres were grown for 4 days and allowed to attach onto poly-L-ornithine for 5 hr before fixing with 3% paraformaldehyde for 10 min, rinsed in PBS, and incubated at 37°C overnight in X-gal solution (2 mM $MgCl_2$, 4 mM potassium ferrocyanide, 4 mM potassium ferricyanide, 1 mg/ml 5-bromo-4-chloro-3-indolyl β-d-galactoside in PBS [pH 7.3]).

Scoring of Vitally Labeled Cells and Neurospheres

After fluorescent labeling of the ependymal cells with DiI, rhodamine beads, or GFP adenovirus and of SVZ cells with GFAP-GFP adenovirus, cells were dissociated, counted, and plated in clonal isolation dishes (10 cm diameter hybridoma tissue culture dishes, Marsh) at a density of 100,000 cells per dish. Fluorescent cells were then identified under an inverted Olympus IX70 microscope and their position scored with an objective marker (Nikon). The survival and growth of fluorescently labeled cells were monitored daily.

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