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# EGF Converts Transit-Amplifying Neurogenic Precursors in the Adult Brain into Multipotent Stem Cells

**Fiona Doetsch,[1,2,5] Leopoldo Petreanu,[2] Isabelle Caille,[2] Jose-Manuel Garcia-Verdugo,[3] and Arturo Alvarez-Buylla[2,4,5]**

[1]Department of Molecular and Cellular Biology
Harvard University
16 Divinity Avenue
Cambridge, Massachusetts 02138

[2]Rockefeller University
1230 York Avenue
New York, New York 10021

[3]University of Valencia
Burjassot 46100
Spain

[4]Brain Tumor Research Center
University of California, San Francisco
10 Kirkham Street K-127
San Francisco, California 94143

## Summary

**Neural stem cells in the subventricular zone (SVZ) continue to generate new neurons in the adult brain. SVZ cells exposed to EGF in culture grow to form neurospheres that are multipotent and self-renewing. We show here that the majority of these EGF-responsive cells are not derived from relatively quiescent stem cells in vivo, but from the highly mitotic, Dlx2$^+$, transit-amplifying C cells. When exposed to EGF, C cells downregulate Dlx2, arrest neuronal production, and become highly proliferative and invasive. Killing Dlx2$^+$ cells dramatically reduces the in vivo response to EGF and neurosphere formation in vitro. Furthermore, purified C cells are 53-fold enriched for neurosphere generation. We conclude that transit-amplifying cells retain stem cell competence under the influence of growth factors.**

## Introduction

Many, if not all, organs of the adult animal contain stem cells that retain the capacity to generate multiple cell types. Stem cells are often relatively quiescent cells that, upon activation, divide to generate rapidly cycling transit-amplifying cells with a limited proliferation and differentiation potential. The transit-amplifying cells then generate restricted progenitor cells that ultimately undergo terminal differentiation (Potten and Loeffler, 1990).

Neural stem cells in restricted regions of the adult vertebrate brain constantly generate new neurons (reviewed in McKay, 1997; Gage, 2000; Temple, 2001). The largest germinal region in the adult mammalian brain is the subventricular zone (SVZ), which extends along the length of the lateral wall of the lateral ventricle. Stem cells in the adult rodent SVZ generate inhibitory neurons destined for the olfactory bulb (reviewed in Alvarez-Buylla and Garcia-Verdugo, 2002). Recently we have identified SVZ astrocytes as the neural stem cells in the adult SVZ and characterized the lineage of olfactory bulb interneurons (Doetsch et al., 1999b). SVZ astrocytes (Type B cells) function as the primary precursors of rapidly dividing transit-amplifying Type C cells (secondary precursors), which generate neuroblasts (Type A cells) destined for the olfactory bulb. From sites of birth throughout the SVZ, neuroblasts join an extensive tangential network of pathways for chain migration that feeds into the rostral migratory stream (RMS) leading to the olfactory bulb (Doetsch and Alvarez-Buylla, 1996). The chains of neuroblasts migrate through glial tunnels formed by the processes of astrocytes (Lois et al., 1996; Doetsch et al., 1997; Peretto et al., 1997; Wichterle et al., 1997). Within the olfactory bulb, the young neurons migrate radially and differentiate into granule and periglomerular neurons (Luskin, 1993; Lois and Alvarez-Buylla, 1994; Doetsch and Alvarez-Buylla, 1996; Petreanu and Alvarez-Buylla, 2002).

Multipotent EGF-responsive neural stem cells can be isolated from the SVZ and grown as neurospheres (Reynolds and Weiss, 1992; Morshead et al., 1994). These EGF-responsive cells are believed to arise from relatively quiescent neural stem cells in vivo (Morshead et al., 1994). Cells expressing the EGF receptor (EGF-R) have been detected in vivo in the SVZ (Morshead et al., 1994; Seroogy et al., 1995; Weickert et al, 2000), and infusion of EGF or transforming growth factor $\alpha$ (TGF-$\alpha$) into the lateral ventricle of adult mice results in a dramatic expansion of dividing cells in the SVZ, concomitant with a decrease in the number of cells migrating to the olfactory bulb (Craig et al., 1996; Kuhn et al., 1997; Fallon et al., 2000). Furthermore, signaling through the EGF-R plays a role in vivo as proliferation in the SVZ is reduced in mice null for TGF-$\alpha$, an endogenous ligand of the EGF-R (Tropepe et al., 1997). However, the identity of EGF-responsive cells and the relationship between the in vivo stem cells and neurosphere-forming cells is still unclear.

Here, we show that, contrary to the commonly accepted hypothesis that EGF-responsive cells derive from a relatively quiescent population, the majority of EGF-responsive cells in the adult SVZ correspond to the rapidly dividing transit-amplifying C cells. This work suggests that transit-amplifying cells retain stem cell characteristics when induced to continually proliferate by the addition of exogenous growth factors.

## Results

### Dlx2 Is Expressed by C Cells and Migrating Neuroblasts

Previous studies identified markers for SVZ astrocytes and neuroblasts (see Table 1 and Figure 1; Doetsch et al., 1997), but no marker is known for C cells. Dlx2 is a homeobox-containing transcription factor implicated in

[5]Correspondence: doetsch@fas.harvard.edu (F.D.), abuylla@itsa.ucsf.edu (A.A-B.)

Table 1. Markers for the Different SVZ Cell Types

| | Neuroblast (Type A) | Transit Amplifying (Type C) | SVZ Astrocyte (Type B) | Ependymal | References |
|---|---|---|---|---|---|
| PSA-NCAM | + | − | − | − | Doetsch et al., 1997 |
| Dlx2 | + | + | − | − | Present study |
| GFAP (mono) | − | − | + | − | Present study |
| GFAP (poly) | − | − | + | + | Doetsch et al., 1997 |
| mCD24 (HSA) | + | − | − | + | Calaora et al., 1996 |

The cell types in the SVZ can be distinguished by immunostaining for the markers in this table.

the development of GABA-ergic neurons and oligodendrocytes in the embryo (reviewed in Panganiban and Rubenstein, 2002), which is also expressed in the adult SVZ (Porteus et al., 1994). We performed pre-embedding immunostaining in combination with electron microscopy to identify which cells in the adult SVZ express Dlx2 (Figure 1). Dlx2$^+$ cells were present throughout the SVZ and along the RMS. C cells, identified by their abundant free ribosomes, deeply invaginated nucleus, and absence of intermediate filaments (Doetsch et al., 1997), were stained by the Dlx2 antibody, as were migrating neuroblasts (Figure 1A). Consistent with the high mitotic index of C cells, Dlx2 staining was frequently associated with cells undergoing mitosis (Figures 1B and 1C). Dlx2 was symmetrically distributed to both daughter cells in mitotic figures as seen in the embryonic brain (Eisenstat et al., 1999). In contrast, SVZ astrocytes and ependymal cells did not express Dlx2 (Figure 1A). Interestingly, a few Dlx2$^-$ mitotic figures were encountered that had ultrastructural characteristics of SVZ astrocytes (Figure 1C). We confirmed that Dlx2 is expressed in C cells and neuroblasts by EM analysis of mice in which tau-LacZ was targeted to the Dlx2 locus (Corbin et al., 2000; data not shown).

The above results show that Dlx2 is highly expressed in C cells and in their descendents, the migrating neuroblasts. C cells do not express the polysialylated neural cell adhesion molecule (PSA-NCAM) in contrast to migrating neuroblasts (Doetsch et al., 1997). Therefore, C cells (Dlx2$^+$/PSA-NCAM$^-$) can be distinguished from migrating neuroblasts (Dlx2$^+$/PSA-NCAM$^+$) by double immunostaining.

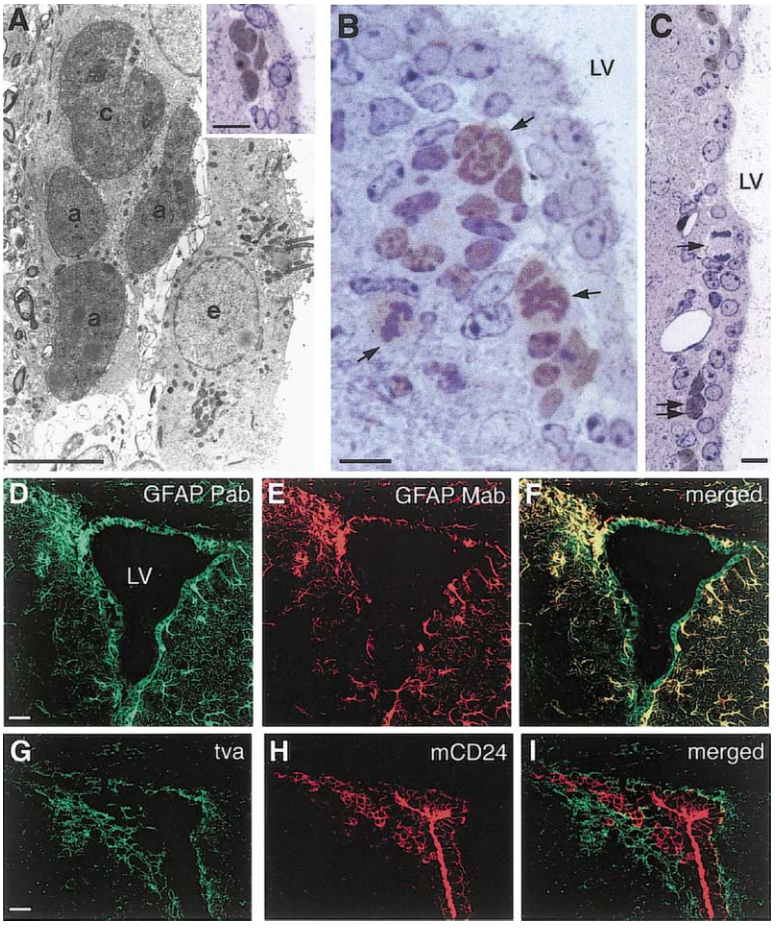


Figure 1. Dlx2 and GFAP Expression in the Adult Mouse SVZ

(A) Inset shows semithin section immunostained for Dlx2 and counterstained with toluidine blue. Electronmicrograph of the same section reembedded, cut, and analyzed at the EM. Both C cells and migrating neuroblasts (a) express Dlx2 in their nuclei. Ependymal cells (e) and SVZ astrocytes are immunonegative for Dlx2. Scale bar equals 5 μm, inset scale bar equals 10 μm.

(B) Dlx2 expression is maintained during mitosis (arrows).

(C) Both labeled (double arrow) and unlabeled (single arrow) mitoses are present in the same section. The unlabeled mitosis likely corresponds to a diving SVZ astrocyte. Scale bars for (B) and (C) equal 10 μm.

(D–F) Ependymal cells do not express GFAP or the tva receptor. Confocal optical sections of coronal sections of the SVZ coimmunostained with antibodies against GFAP (polyclonal in green and monoclonal in red). The polyclonal GFAP antibody recognizes ependymal cells, whereas the monoclonal does not. In contrast, the underlying SVZ astrocytes were stained with both antibodies and appear yellow in the merged images.

(G–I) Confocal optical sections of the SVZ of a GFAP-tva mouse, which expresses the receptor for the avian leukosis retrovirus under the GFAP promoter (Holland and Varmus, 1998), double immunostained for the tva receptor (green) and for mCD24 (red). The tva receptor is not expressed in ependymal cells or neuroblasts. LV, lateral ventricle.

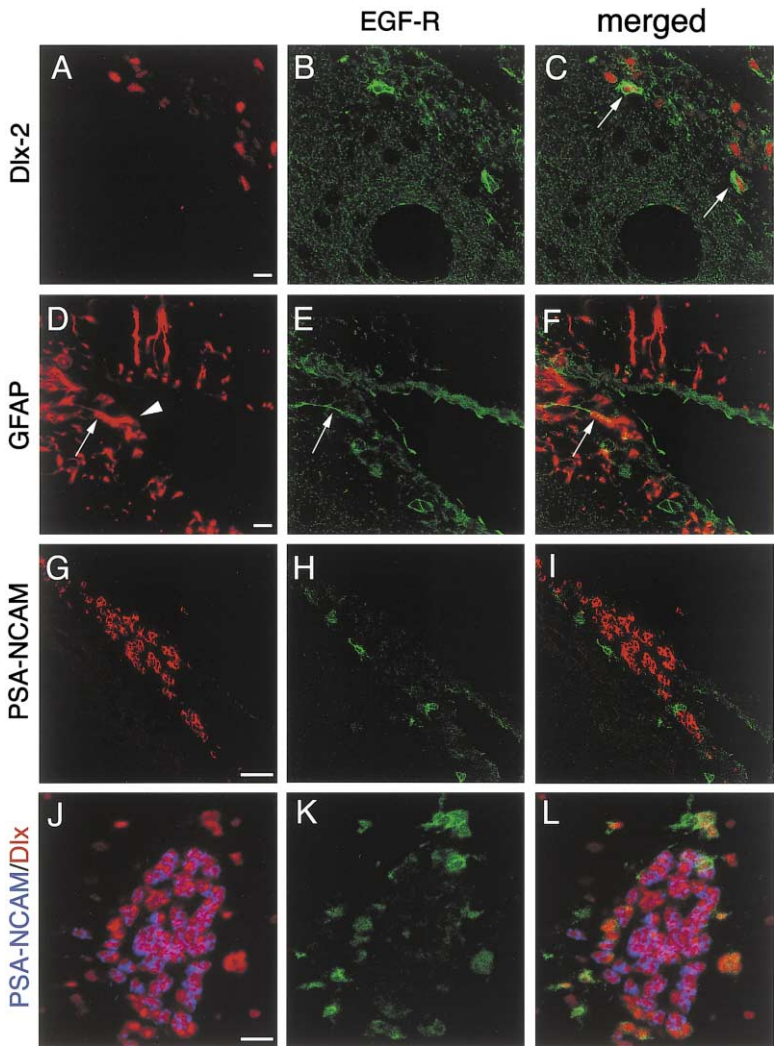


Figure 2. SVZ Cell Types Expressing the EGF Receptor

Confocal optical sections of coronal sections of the SVZ immunostained for the EGF-R (green) and markers for the different SVZ cell types (red).

(A–C) The EGF-R is expressed by C cells (Dlx2 immunostaining). White arrows show double-labeled cells. The more lightly immunostained Dlx2 cells are neuroblasts. The EGF-R is not expressed in neuroblasts (see G–L).

(D–F) Some EGF-R double-labeled GFAP$^+$ processes (arrow) contact the lateral ventricle (arrowhead).

(G–L) The EGF-R is not expressed by migrating neuroblasts (PSA-NCAM immunostaining) in the SVZ (G–I) or in the RMS (J–L). Occasional EGF-R$^+$/DII$^+$/PSA-NCAM$^-$ C cells are present in the RMS. Scale bars equal 10 μm in (A)–(F) and 25 μm in (G)–(L).

### C Cells Express the EGF Receptor

We analyzed sections immunostained for the EGF-R and SVZ cell type-specific markers (Table 1) at the confocal microscope to identify which cells expressed the EGF-R in the adult mouse SVZ. EGF-R$^+$ cells were present in both the SVZ and the RMS (Figure 2). C cells, identified by Dlx2 immunostaining, expressed the EGF-R (Figures 2A–2C). Although most SVZ astrocytes did not express the EGF-R, a small subset of SVZ astrocytes, stained with GFAP or S100, did. Interestingly, some GFAP/EGF-R$^+$ astrocytes apparently contacted the lateral ventricle (Figures 2D–2F). EGF-R was not detected in the PSA-NCAM$^+$ neuroblasts in the SVZ (Figures 2G–2I) or in the RMS (Figures 2J–2L). Thus, the majority of EGF-R$^+$ cells in the adult SVZ correspond to the rapidly dividing C cells. Occasionally, we observed EGF-R$^+$/Dlx2$^+$/PSA-NCAM$^-$ cells in the RMS (Figures 2J–2L), suggesting that some C cells may also be found in the RMS, although the majority of C cells are localized to the SVZ.

### Short Infusions of EGF Induce C Cell Proliferation and Astrocytic Rearrangement

To investigate how SVZ cells respond to EGF stimulation in vivo, we used miniosmotic pumps to infuse EGF into the lateral ventricle for 7 hr. This short infusion was used to identify which SVZ cells responded to EGF before any changes in cell fate or large-scale expansion and disorganization of the SVZ occurred (Craig et al., 1996; Kuhn et al., 1997). EM analysis revealed that after only 7 hr of EGF infusion, the proportion of C cells and of cells undergoing mitosis doubled compared to saline-infused controls (Table 2). In contrast, the number of neuroblasts was reduced to approximately one-half of that in control animals (Table 2). Although the number of SVZ astrocytes was similar to controls, 2.79 times more SVZ astrocytes contacted the lateral ventricle (Figure 3 and Table 2) in mice infused with EGF ($n = 4$) compared to control mice ($n = 4$). These cells frequently had a single cilium with an associated centriole at its base (Figure 3), a structure also found in neuroepithelial cells in the embryo (Sotelo and Trujillo-Cenóz, 1958), during regeneration of the adult mouse SVZ (Doetsch et al., 1999a), and in neuronal precursors in adult songbirds (Alvarez-Buylla et al., 1998), and may correspond to activated astrocytes.

To determine which cell types were dividing after 7 hr of EGF infusion, we injected mice with [$^3$H]-thymidine (for analysis at the EM) or bromodeoxyuridine (BrdU)

Table 2. Effect of Seven Hours of EGF Infusion on SVZ Cell Types

| | Saline | EGF | EGF/Saline |
|---|---|---|---|
| Neuroblast (Type A) | 28.37 ± 1.41 | 12.72 ± 1.25 | 0.45 |
| SVZ astrocyte (Type B) | 26.45 ± 0.75 | 27.76 ± 0.92 | 1.05 |
| SVZ astrocyte contacting ventricle | 1.44 ± 0.11 | 4.01 ± 0.39 | 2.79 |
| Type C | 10.19 ± 1.00 | 22.49 ± 0.78 | 2.21 |
| Ependyma | 28.98 ± 1.56 | 28.45 ± 0.59 | 0.98 |
| Neuron | 1.14 ± 0.31 | 1.14 ± 0.19 | 0.99 |
| Microglia | 0.39 ± 0.11 | 0.05 ± 0.06 | 0.13 |
| Mitosis | 0.41 ± 0.19 | 0.87 ± 0.15 | 2.12 |
| Unidentified | 2.31 ± 0.15 | 1.64 ± 0.46 | 0.71 |

The SVZs of mice infused with EGF or saline for 7 hours were analyzed at the EM. Number of each SVZ cell type/mm in EGF- and saline-infused mice. The last column indicates the change in the proportion of cell types between experimental and control animals.

(for quantification at the light microscope) 3 hr prior to perfusion. Many C cells were labeled with [$^3$H]-thymidine. To quantify the effect of EGF exposure on proliferation of C cells, we analyzed sections triple-immunostained for BrdU, Dlx2, and PSA-NCAM at the confocal microscope. A significantly greater number of C cells (Dlx2$^+$/PSA-NCAM$^-$) were undergoing cell division in EGF-infused mice (1.28-fold increase, 72.63% ± 1.39% versus 56.75% ± 1.63%, $p = 0.0003$). We also determined a shift in the distribution of cell types incorporating BrdU. BrdU$^+$ C cells (BrdU$^+$/Dlx2$^+$/PSA-NCAM$^-$) were slightly but significantly increased (1.18-fold increase, $p = 0.00083$) over saline-infused animals, whereas the proportion of BrdU$^+$ neuroblasts (BrdU$^+$/Dlx2$^+$/PSA-NCAM$^+$) was 2.3 times lower than in saline-infused animals ($p = 0.00018$). This reduction in the proportion of neuroblasts is consistent with the 2.2-fold reduction in the number of neuroblasts observed at the EM.

Seven hours of exogenous EGF had three effects in the SVZ: (1) an increase in the number of SVZ astrocytes touching the ventricle, without an increase in [$^3$H]-thymidine-labeling of these cells; (2) an increase in the proliferation and number of C cells; and (3) a decrease in the number of neuroblasts. These observations suggest that a short infusion of EGF induces C cells to undergo self-renewing divisions at the expense of neuroblast production.

### EGF Induces SVZ Cells to Invade the Brain and Arrests Neuroblast Production

We infused EGF into the lateral ventricles for 6 days to observe longer-term effects on SVZ cells. Chains of PSA-NCAM$^+$ neuroblasts, visualized in whole-mount preparations, were largely abolished (Figures 4A and 4B). Despite the increased division of C cells observed after 7 hr of EGF infusion, Dlx2 expression was greatly downregulated in the SVZ after 6 days of EGF infusion (400 ng/day) (Figures 4C and 4D). Instead, tenascin, an extracellular matrix molecule normally found associated with astrocytes in this region (Jankovski and Sotelo, 1996; Thomas et al., 1996), was upregulated and extended into the regions of SVZ expansion (Figures 4E and 4F), suggesting that SVZ cells were adopting a more glial phenotype. Interestingly, infusion of lower concentrations of EGF does not result in downregulation of Dlx2 (F.D. and A.A.-B., unpublished data).

To determine the effect of longer-term EGF exposure on the SVZ lineage of olfactory bulb neurons, we labeled

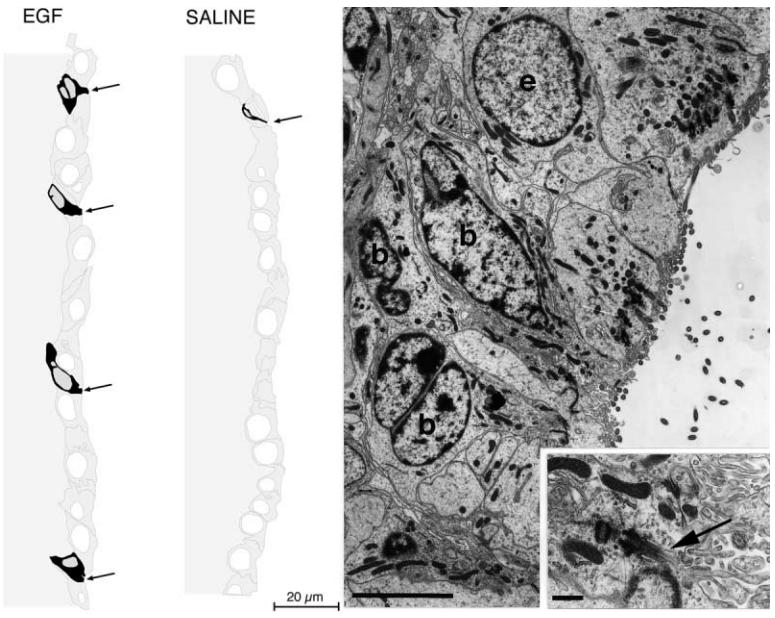


Figure 3. SVZ Astrocytes Contact the Lateral Ventricle after Short Infusions of EGF

Reconstruction of the SVZ of mice infused with EGF or saline for 7 hr. Only ependymal cells (light gray) and SVZ astrocytes contacting the lateral ventricle (black) are depicted. Astrocytic contacts with the ventricle are rare in saline-infused controls (arrow, right), but common (arrows, left) in the EGF-infused animals.

Electronmicrograph of two SVZ astrocytes in contact with the lateral ventricle. Inset shows detail of the single cilium typical of SVZ astrocytes in contact with the lateral ventricle (arrow). Note that the clumped chromatin of SVZ astrocytes (b) is easily distinguishable from the more diffuse chromatin of ependymal cells (e). Also note the elongated shape of the SVZ astrocytes in contact with the lateral ventricle. LV, lateral ventricle. Scale bars equal 5 μm, main image; 0.5 μm, inset.

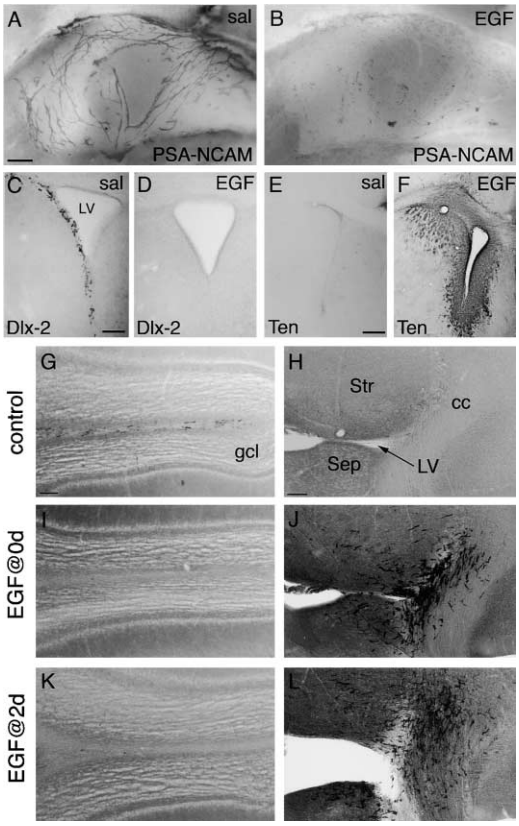


Figure 4. EGF Induces Invasion of SVZ Cells and Arrests Neuroblast Formation

(A and B) Whole-mount immunostaining of the PSA-NCAM$^+$ SVZ network of chains of vehicle (A) and EGF-infused (B) mice. After infusion of EGF for 6 days, the network of chains is completely disrupted. Very few PSA-NCAM$^+$ cells are present. Scale bar equals 200 μm.

(C and D) Frontal sections of control and EGF-infused mice immunostained for Dlx2. Note the absence of Dlx2 staining in EGF-infused mice.

(E and F) In control mice, tenascin is confined to the SVZ. After EGF infusion there is a dramatic expansion of tenascin immunostaining. Note the extensive staining along fiber tracts and blood vessels and staining present in the corpus callosum and cortex.

(G–L) SVZ cells arrest neuron production and migrate away from the SVZ upon EGF infusion. All sections are horizontal: anterior is to the right, lateral is to the top.

(G and H) In control GFAP-tva mice 6 days after infection with RISAP, many AP$^+$ cells generated from infected SVZ astrocytes are in the core of the olfactory bulb (G). Some have already migrated into the granule cell layer and begun differentiation into granule neurons. AP$^+$ cells are confined to the SVZ, adjacent to the lateral ventricle (H).

(I and J) In mice infused with EGF at the time of RISAP injection (EGF at 0 day), no AP$^+$ cells are present in the olfactory bulb (I). In contrast, AP$^+$ cells disperse into the striatum and septum upon EGF infusion (J). Many are associated with blood vessels and some migrate into the corpus callosum.

(K and L) In mice infused with EGF 2 days after RISAP injection (EGF at 2 days), no AP$^+$ cells are present in the olfactory bulb of mice (K). In contrast, cells undergo an invasive migration into the striatum, corpus callosum, and prefrontal cortex upon infusion of EGF (L). They are aligned along blood vessels and white matter fiber tracts. Scale bars equal 90 μm in (G), (I), and (K); 110 μm in (H), (J), and (L). Abbreviations: cc, corpus callosum; LV, lateral ventricle; Sep, septum; Str, striatum; gcl, granule cell layer.

SVZ astrocytes prior to EGF infusion. SVZ astrocytes infected with an avian leukosis virus encoding alkaline phosphatase (AP) in GFAP-tva mice, which express the receptor for this virus under the GFAP promoter (Holland and Varmus, 1998), generate the entire lineage that leads to olfactory bulb neurons (Doetsch et al., 1999b). In control GFAP-tva mice, many AP$^+$ neuroblasts had migrated to the olfactory bulb and begun differentiation into neurons 6 days later (Figure 4G); very few AP$^+$ cells were left in the SVZ (Figure 4H), and cells were not observed outside the SVZ, RMS, or olfactory bulb. In contrast, in animals infused with EGF for 6 days beginning 0 or 2 days after viral labeling, very few cells reached the olfactory bulb (Figures 4I and 4K). Instead, large numbers of AP$^+$ cells escaped from the SVZ and migrated into the striatum, septum, corpus callosum, and cortex (Figures 4J and 4L), where they were found primarily aligned along white matter tracts and blood vessels. Similar results were obtained using both RCAS-AP (replication competent avian leukosis virus) and RI-SAP, a replication incompetent avian virus (Chen et al., 1999), supporting the notion that in GFAP-tva mice RCAS-AP is replication incompetent in vivo (Fisher et al., 1999). The number of AP$^+$ cells that invaded the brain was greater when EGF infusion began 2 days after infection, a time when most labeled SVZ astrocytes have converted into secondary precursors, suggesting that many EGF respondents correspond to secondary precursors derived from GFAP$^+$ SVZ astrocytes. Thus, EGF infusion causes the arrest of neuroblast production and induces SVZ cells to become highly proliferative and invasive.

### In Vivo Response to EGF Is Greatly Reduced by Selective Killing of C Cells

In order to directly test whether C cells respond to EGF infusion, we used mice in which the Dlx2 locus is targeted with IRES-herpes simplex virus thymidine kinase (HSVTK)-IRES-tau-LacZ (L.P. and A.A-B., unpublished). Ganciclovir (GCV) is metabolized by HSV-TK to form toxic intermediates, resulting in the selective killing of dividing Dlx2$^+$ cells. Since C cells, but not SVZ astrocytes, express Dlx2, GCV in Dlx2-IRES-HSVTK-IRES-tau-LacZ mice should result in the elimination of C cells and neuroblasts. EM analysis after infusion of GCV for 6 days in Dlx2-IRES-HSVTK-IRES-tau-LacZ mice showed that C cells and neuroblasts were selectively depleted by more than one-half while sparing the astrocytes. In order to test if the population of EGF-responsive cells is affected by GCV treatment in these mice, we infused GCV into the lateral ventricles of wild-type and Dlx2-IRES-HSVTK-IRES-tau-LacZ mice with a miniosmotic pump for 6 days, followed by EGF infusion for another 6 days. On the last day of EGF infusion, BrdU was injected four times to label dividing cells. In the Dlx2-IRES-HSVTK-IRES-tau-LacZ mice, there was a 68% reduction ($p < 0.0001$) in the number of BrdU-labeled cells compared to wild-type mice infused with GCV and EGF (Figure 5). This experiment strongly suggests that the majority of EGF-responsive cells in vivo correspond to C cells.

Taken together, the above results suggest that, in vivo, exogenous EGF first causes C cells to undergo self-

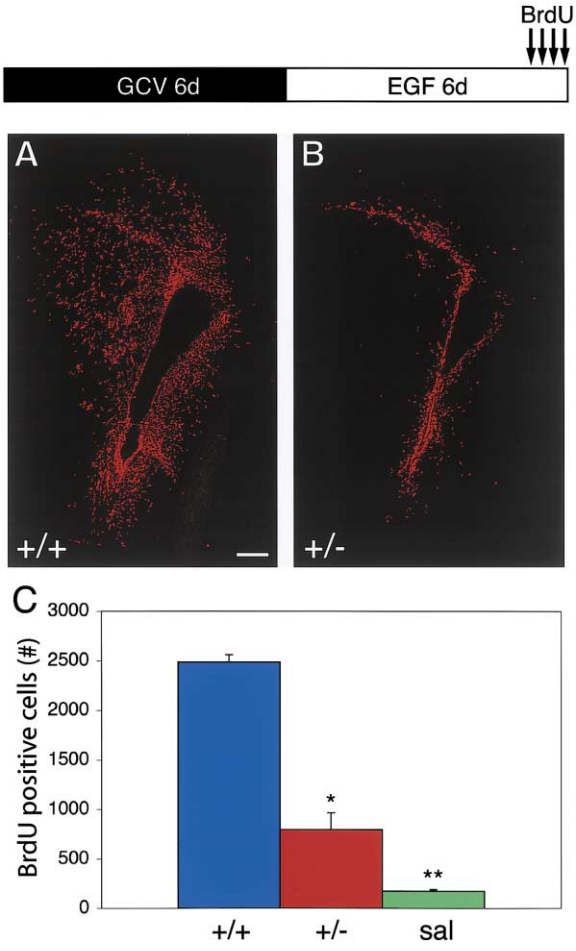


Figure 5. Selective Killing of Dlx2$^+$ Cells Dramatically Reduces In Vivo Response to EGF

Schematic timeline shows the experimental design. Ganciclovir was infused for 6 days into the lateral ventricles of wild-type or Dlx2-IRES-HSVTK-IRES-tau-LacZ mice to selectively kill dividing Dlx2$^+$ cells. EGF was then infused for 6 days and BrdU injected throughout the last day of EGF infusion to label dividing SVZ cells.

(A) Photomicrograph of a frontal section of a wild-type mouse showing the extent of BrdU labeling (red) around the lateral ventricle.

(B) Photomicrograph of a mutant mouse showing dramatic decrease in the number of BrdU$^+$ cells after selective killing of Dlx2$^+$ cells prior to EGF infusion.

(C) Histogram showing that the proliferative response in mutant mice is reduced to 32% of that in wild-type mice. The number of BrdU$^+$ cells in mutant mice is statistically higher than in saline-infused mice.

renewing divisions, thereby arresting the production of neuroblasts. With prolonged exposure to EGF, C cells downregulate Dlx2, continue to proliferate, and become migratory and more astrocyte-like.

### Dividing Cells Give Rise to Neurospheres

EGF-responsive cells from the SVZ can be cultured in vitro in the presence of EGF to generate neurospheres, which exhibit features of stem cells (Weiss et al., 1996a). Previous work has suggested that SVZ neural stem cells that can be cultured as neurospheres correspond to a rare population of relatively quiescent cells (Morshead et al., 1994). We reevaluated this hypothesis, as our present data indicate that C cells, which divide actively (Doetsch et al., 1997), express the EGF-R and are EGF responsive in vivo.

To label a cohort of dividing SVZ cells, adult mice were given a single injection of [$^3$H]-thymidine, which labels one-third of actively dividing SVZ cells in the proliferative pool (Morshead and van der Kooy, 1992). In control animals that were killed 4 hr after [$^3$H]-thymidine injection, heavily labeled cells were observed in the SVZ (Figure 6A). Neurospheres were prepared from [$^3$H]-thymidine-injected mice. Half of the neurospheres (535) were processed for [$^3$H]-thymidine autoradiography after 5 days. Almost half (43%) of these neurospheres were [$^3$H]-thymidine labeled (Figure 6B). To rule out that these small neurospheres were abortive growths of non-stem cells, we cultured the remaining neurospheres for 10 additional days and plated them to test their differentiation potential. Of 512 neurospheres analyzed, 84% gave rise to progeny that expressed glial (GFAP) and neuronal (TuJ1) markers (Figure 6C). These results indicate that almost half of the neurospheres arise from dividing SVZ cells after a single pulse of [$^3$H]-thymidine in vivo, but this number is likely an underestimation as only a fraction of the proliferative pool is labeled.

We next used a pseudotyped replication incompetent retrovirus encoding green fluorescent protein (GFP) to label dividing SVZ cells and prepared neurospheres 24 hr later. Although the efficiency of infection with the retrovirus was much lower than the labeling obtained with [$^3$H]-thymidine (100–300 cells per hemisphere), GFP-labeled neurospheres could be generated and their growth followed without loss of label due to dilution. From SVZs dissected 24 hr after viral infection in vivo, 25 fluorescent GFP$^+$ neurospheres grew (Figures 6D and 6E). Of these, 15 were cultured for 10 days and generated neurons and glia after plating for differentiation. The remaining 10 labeled neurospheres were dissociated into single cells, which gave rise to secondary neurospheres that retained their capacity to generate glial cells and neurons. This experiment further confirms that actively dividing SVZ cells in vivo can give rise to neurospheres that are self-renewing and multipotent.

### Neurosphere Number Increases as C Cells Reappear during SVZ Regeneration

The adult SVZ regenerates after elimination of all migrating neuroblasts and C cells with the antimitotic drug cytosine-β-D-arabinofuranoside (Ara-C) (Doetsch et al., 1999a). Following this antimitotic treatment, SVZ astrocytes but not ependymal cells divide to generate C cells followed by neuroblasts (Doetsch et al., 1999b). We used this method to determine whether the number of neurospheres that could be generated from the SVZ changed at different stages of regeneration. Figure 6G shows that a small number of cells capable of generating neurospheres are present in the SVZ at 0 and 12 hr after Ara-C treatment, when no C cells are present but the first dividing SVZ astrocytes appear (Doetsch et al., 1999b). However, between 1 and 3 days after Ara-C treatment, when C cells are present but before neuroblasts have reappeared, the number of neurospheres formed increased dramatically to approximately the levels ob-

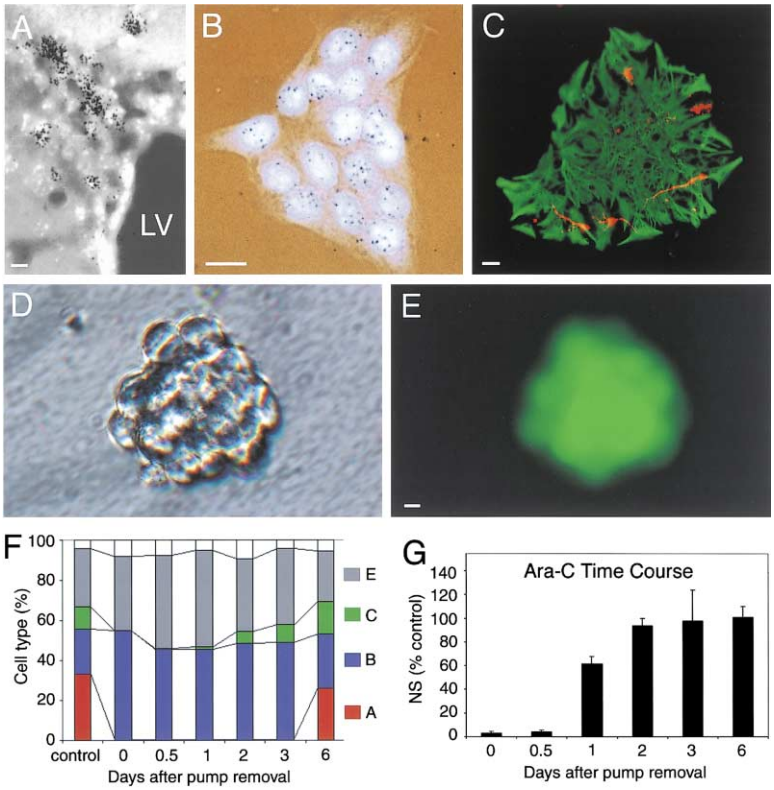


Figure 6. Neurospheres Arise from Dividing Cells and Their Number Increase with SVZ Regeneration

(A) Autoradiogram of a frontal section through the SVZ showing heavy labeling of SVZ cells 4 hr after a single [$^3$H]-thymidine injection. LV, lateral ventricle.

(B) Autoradiogram showing [$^3$H]-thymidine-labeled neurospheres cells cultured for 5 DIV from mice that received a single [$^3$H]-thymidine injection as in (A). Cell nuclei in (A) and (B) have been counterstained with Hoechst 33258.

(C) Double labeling of a differentiated neurosphere from a parallel culture to that shown in (B) (see text for details) showing immunoreactivity for astrocytic (GFAP in green) and neuron-specific (TuJ1 in red) markers.

(D and E) Dividing cells in the SVZ infected with a GFP retrovirus give rise to fluorescent neurospheres. Phase contrast (D) and epifluorescent (E) images of the same neurosphere prepared from the SVZ of mice infected by a replication defective retrovirus expressing GFP. Scale bars equal 25 $\mu$m in (A), (D), and (E); 50 $\mu$m in (C).

(F and G) The number of neurospheres increases with SVZ regeneration after Ara-C treatment.

(F) Sequence of appearance of SVZ cell types at different survivals after Ara-C treatment (modified from Doetsch et al., 1999a).

(G) Histogram showing the number of neurospheres (as percentage of control untreated mice) generated at six time points during SVZ regeneration following Ara-C treatment. Increased numbers of neurospheres are formed beginning on day 1 when C cells reappear in the SVZ. Very few neurospheres (but some) are generated immediately and at 12 hr after pump removal when only SVZ astrocytes are present in the SVZ. Error bars show SD of two independent experiments.

served in control animals. Neurospheres prepared 0 and 12 hr and 1, 2, 3, and 6 days after Ara-C treatment had the capacity to form GFAP$^+$ astrocytes and TuJ1$^+$ neurons when plated for differentiation. Furthermore, secondary neurospheres could be cultured, which upon plating gave rise to differentiated neurons and glia. In all respects, the neurospheres generated at each time point after Ara-C had the same properties as neurospheres produced from nontreated animals.

### Selective Killing of C Cells Results in Decreased Neurosphere Formation

The above experiments suggest that EGF-responsive neurospheres arise from actively dividing secondary precursors, likely C cells. In order to test this hypothesis more directly, we selectively eliminated C cells in vitro using Dlx2-IRES-HSV-TK-IRES-tau-LacZ mice and determined the number of neurospheres that could be generated. Dissociated SVZ cells from adult Dlx2-IRES-HSV-TK-IRES-tau-LacZ mice were cultured in the presence of EGF and GCV for 6 days. These cells gave rise to less than one-third (31.58% $\pm$ 3.98%, $p < 0.001$, t test) the number of neurospheres that grew from control wild-type cells treated with GCV (Figure 7C). GCV did not kill cells nonselectively, as control experiments in which wild-type SVZ cells were cultured either in the presence or absence of GCV yielded similar numbers of neurospheres (Figure 7C). However, it was possible that the neuroblasts, which also express Dlx2 and divide, were the cells that were eliminated and responsible for neurosphere formation. This was not the case, as neuroblasts purified to 99% purity (Lim and Alvarez-Buylla, 1999) failed to generate neurospheres when exposed to EGF (not shown). Together the above results suggest that ~70% of neurospheres arise from C cells. To rule out that C cells are not the precursors for neurospheres but are required to support the formation of neurospheres by another SVZ cell type in a nonautonomous manner, such as by the secretion of a factor into the medium, we performed a rescue experiment: cells from the SVZ of mice in which all cells express GFP (Okabe et al., 1997) were cocultured with Dlx2-IRES-HSV-TK-IRES-tau-LacZ cells in the presence of GCV (Figure 7). The number of neurospheres that grew from GCV-treated Dlx2-IRES-HSV-TK-IRES-tau-LacZ cells in the rescue conditions (36.99% $\pm$ 8.2%) did not increase significantly compared to those in the nonrescue conditions ($p = 0.26$, t test), indicating that the reduction of neurospheres formed is cell autonomous and is the result of killing C cells. These findings strongly suggest that C cells give rise to the majority of EGF-responsive neurospheres.

### Purified C Cells Generate Neurospheres

To directly evaluate the potential of C cells to act as stem cells in vitro, we purified C cells from the adult SVZ by fluorescence-activated cell sorting (FACS) and cultured them at low density in the presence of EGF. C

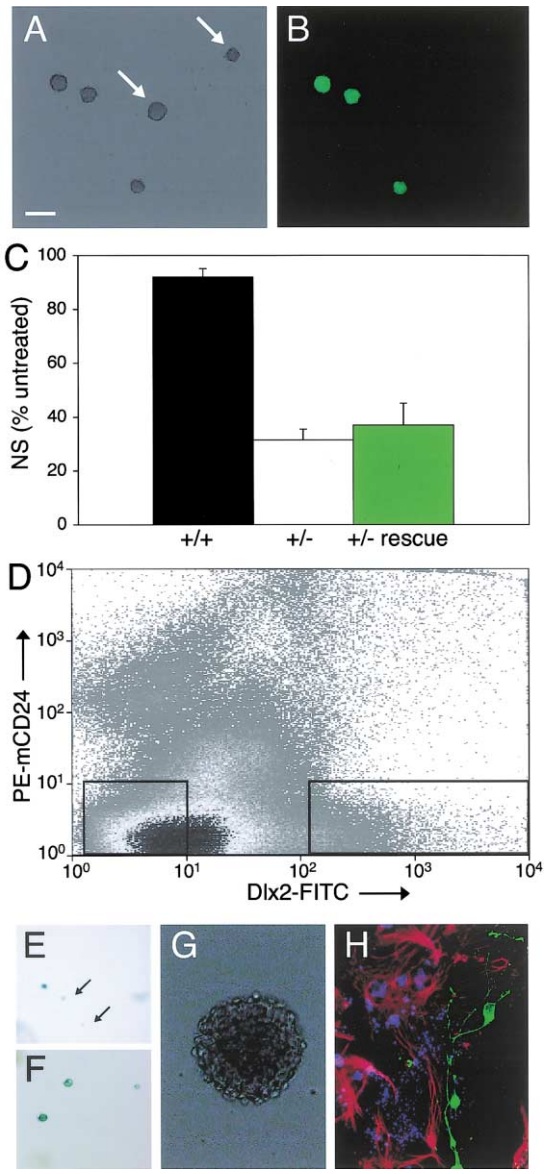


Figure 7. Dlx2$^+$ Cells Give Rise to Neurospheres

(A–C) The number of neurospheres that grow after selective killing of dividing Dlx2$^+$ cells is dramatically reduced; this effect is cell autonomous. SVZ cells from Dlx2-IRES-HSVTK-IRES-tau-LacZ mice were cultured in the presence of ganciclovir and EGF.

(A) Bright field photograph of five neurospheres that grew in the presence of ganciclovir and EGF from the rescue experiment. Arrows show two neurospheres derived from Dlx2-IRES-HSVTK-IRES-tau-LacZ cells (see B).

(B) Fluorescent image of field in (A), showing that two neurospheres are derived from Dlx2-IRES-HSVTK-IRES-tau-LacZ mice and three from GFP$^+$ mice.

(C) Histogram of the number of neurospheres (as percent of the number of untreated neurospheres) that grow from Dlx2-IRES-HSVTK-IRES-tau-LacZ wild-type (+/+, black) and mutant (+/−, white) mice in the presence of ganciclovir and EGF. The number of neurospheres that grow is reduced to 31%. The number of neurospheres that grow from mutant Dlx2-IRES-HSVTK-IRES-tau-LacZ mice is not rescued by coculturing with GFP$^+$ cells (+/− rescue, green).

(D–H) Purified C cells are highly enriched in neurosphere-forming ability. C cells were purified by FACS sorting from Dlx2-tau-LacZ mice using a fluorescent β-galactosidase reporter substrate and immunostaining for mCD24.

cells were purified from Dlx2-IRES-tau-LacZ mice (Corbin et al., 2000) using the vital fluorogenic substrate for β-galactosidase, fluorescein digalactopyranoside (FDG), in combination with immunostaining for mCD24 (heat stable antigen). This combination allowed us to separate C cells (Dlx2$^+$/mCD24$^-$) from neuroblasts (Dlx2$^+$/mCD24$^+$) (Figure 7D). Dlx2$^+$/mCD24$^-$ cells were plated at clonal density in serum-free medium containing EGF for 7 days. Additionally, single cells were plated in single wells of a 96-well plate using a FACS automated deposition unit. C cells were highly enriched in the ability to form neurospheres as compared to the unsorted fraction. In an unsorted fraction, 1 in 370 ± 87.2 cells gave rise to a neurosphere. In contrast, 1 in 6.93 ± 1.1 sorted cells was able to generate neurospheres. This represents a 53-fold ($p < 0.001$) enrichment over unsorted cells. Neurospheres from the sorted Dlx2$^+$/mCD24$^-$ fraction were capable of forming secondary neurospheres (Figure 7G) and were multipotent, differentiating into neurons, astrocytes, and oligodendrocytes upon plating (Figure 7H).

## Discussion

It has largely been assumed that the primary stem cells of new neurons and glia in vivo are the source of neurospheres, which function as stem cells in vitro. Here, we have identified which cells in the adult mouse SVZ respond to exogenous EGF in vivo and in vitro. Surprisingly, our results suggest that the majority of EGF-responsive cells correspond to the rapidly cycling transit-amplifying cells rather than to the primary and less proliferative in vivo stem cells.

The transcription factor Dlx2 is involved in the generation of GABA-ergic neurons and oligodendrocytes during development (He et al., 2001; Panganiban and Rubenstein, 2002). We show here that in the adult mouse SVZ, Dlx2 is highly expressed by C cells, the immediate precursors of neuroblasts destined for the olfactory bulb, and by the neuroblasts themselves. The expression of Dlx2 by C cells suggests that in vivo these cells have begun their differentiation. The expression of Dlx2 in C cells allowed us to investigate their responsiveness to EGF. Despite their apparent in vivo commitment, our results show that C cells are EGF responsive in vivo and behave as multipotent stem cells in vitro when exposed to EGF.

This conclusion is based on several lines of supporting evidence: (1) the majority of EGF-R-expressing cells in the SVZ are C cells; (2) short infusions of EGF result in increased proliferation of C cells and decreased neuroblast production; (3) with longer infusions of EGF, C cells downregulate Dlx2 expression and become highly migratory and proliferative glial-like cells with invasive

(D) Density plot showing gates for selecting C cells (Dlx2$^+$ and mCD24$^-$).

(E) X-gal stained unsorted cells from a Dlx2-tau-LacZ mouse after plating for 1 hr.

(F) X-gal stained sorted C cells after plating for 1 hr. Single purified C cells generate neurospheres that can be passaged to make secondary neurospheres (G) and are multipotent, generating neurons (green), astrocytes (red), and oligodendrocytes (blue) (H).

characteristics; (4) selective killing of C cells dramatically reduces the in vivo response to EGF; (5) actively dividing cells give rise to multipotent self-renewing neurospheres; (6) neurosphere formation coincides with the appearance of dividing cells during regeneration of the SVZ; (7) selective killing of Dlx2$^+$ cells in vitro results in a ~70% reduction in neurosphere formation; and (8) purified C cells are highly enriched in neurosphere-forming ability.

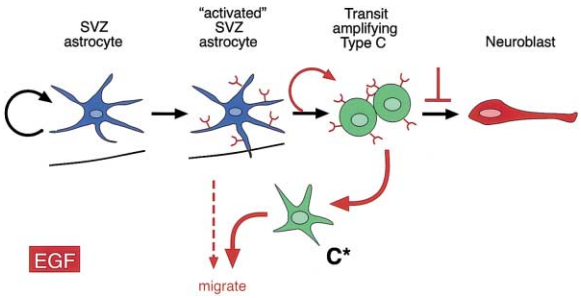


Figure 8. Model of EGF-R Stimulation in the SVZ Lineage of Olfactory Bulb Interneurons

The effects of exogenous EGF are indicated by red arrows and symbols in the figure. SVZ astrocytes are neural stem cells that divide to generate neuroblasts destined for the olfactory bulb via rapidly dividing transit-amplifying precursors (C cells). We propose that under normal conditions the in vivo stem cells (SVZ astrocytes) do not express the EGF receptor, but as they are "activated" they begin to express the EGF-R and touch the lateral ventricle. C cells maintain high levels of EGF-R expression, perhaps related to their rapid division. Neuroblasts downregulate expression of the EGF-R. The major effect of EGF on SVZ cells is on C cells. Under EGF, these cells stop generating neuroblasts that migrate to the olfactory bulb and instead undergo extensive proliferation. Prolonged exposure to high doses of EGF causes C cells to become reprogrammed to a more glial cell-type (C*) and induces an invasion into the neighboring brain. Some SVZ astrocytes also respond (dashed lines) to exogenous EGF and become migratory cells. Neurospheres arise from cells encompassing the proliferative stages in the SVZ lineage from activated SVZ stem cells to C cells, with the majority arising from C cells.

### Identity of Stem Cells: In Vivo versus In Vitro Stem Cells

GFAP$^+$ SVZ astrocytes are the primary stem cells that generate olfactory bulb interneurons (Doetsch et al., 1999b). In the hippocampus, the other major region of neurogenesis in adult mammals, subgranular layer astrocytes give rise to neurons in the dentate gyrus (Seri et al., 2001). Interestingly, astrocytes from multiple brain regions are able to generate neurospheres if isolated before postnatal day 11, when this capacity becomes restricted to SVZ astrocytes (Laywell et al., 2000). The finding that SVZ astrocytes are the primary neural stem cells in the adult mammalian brain fits well with the emerging view that radial glia in the embryonic brain and adult avian brain also act as neuronal precursors (Alvarez-Buylla et al., 1990; Noctor et al., 2001; Malatesta et al., 2000; Miyata et al., 2001). Stem cells are likely contained within the neuroepithelial-radial glia-astrocyte lineage (see Alvarez-Buylla et al., 2001, for review).

The in vivo identity of the stem cells that reside in the SVZ has been controversial. Besides astrocytes, it has been suggested that ependymal cells (Johansson et al., 1999) can function as stem cells. This conclusion is, however, not supported by other studies (Doetsch et al., 1999b; Chiasson et al., 1999; Laywell et al., 2000; Rietze et al., 2001; Capela and Temple, 2002). Purified ependymal cells fail to generate neurospheres; instead, neurosphere-forming activity is associated with SVZ cells expressing the carbohydrate LeX/SSEA-1 (Capela and Temple, 2002). A nestin$^+$ cell that is negative for both glial and ependymal markers has also been shown to form neurospheres in vitro (Rietze et al., 2001) and represents about 63% of neurosphere-forming activity in the SVZ. Based on our present results, we hypothesize that these cells, and much of the LeX/SSEA-1-expressing population, probably correspond to C cells. C cells, which we purify here as Dlx2-high/mCD24-low cells, represent about 70% of neurosphere-forming activity in the SVZ. Consistent with the hypothesis that C cells make neurospheres, mice in which the cell cycle inhibitor p27Kip1 is knocked out have increased numbers of C cells and a concomitant increase in the number of neurospheres that can be cultured from the SVZ of these mice (Doetsch et al., 2002). We do not know, however, whether all C cells have the potential to form neurospheres or if this is a property of a subpopulation of these cells, such as the first generations of C cells.

Our current findings clarify the relationship between the in vivo stem cells and neurosphere-forming cells. The ability to generate neurospheres and respond to EGF appears to reflect an in vivo biological state of precursor cells within a lineage, rather than the presence of a specific rare cell type (Figure 8). Quiescent SVZ astrocytes become activated and generate neuroblasts via Dlx2$^+$ transit-amplifying C cells. The EGF-responsive cells in the SVZ likely encompass the proliferative stages in the SVZ lineage from activated SVZ stem cells to C cells (Figure 8). Interestingly, LeX/SSEA-1$^+$ cells comprise all neurosphere-forming cells in the adult SVZ and include both a subpopulation of GFAP$^+$ SVZ astrocytes and a large fraction of cells that are negative for GFAP and TuJ1 (Capela and Temple, 2002). The majority of the LeX$^+$ GFAP$^-$ population probably corresponds to Dlx2$^+$ C cells. We show here that a few neurospheres can be cultured soon after termination of Ara-C treatment, when no C cells are present in the SVZ, but when some SVZ astrocytes undergo division (Doetsch et al., 1999b). Some SVZ astrocytes vitally labeled in vivo also generate neurospheres (Doetsch et al., 1999b). It is likely that a small fraction of neurospheres arise from "activated" astrocytes, perhaps those that contact the lateral ventricle, and/or from cells in transition from SVZ astrocytes to C cells before they express Dlx2. The greatest proportion of EGF-responsive neurospheres are, however, derived from C cells. It will be interesting to compare the stem cell properties of SVZ astrocytes and C cells. Neurospheres derived from these two populations may have different potentials.

Our findings contradict previous work suggesting that neurospheres are derived from relatively quiescent neural stem cells in the SVZ (Morshead et al., 1994). We show here, using [$^3$H]-thymidine labeling or infection with a pseudotyped replication-deficient retrovirus, that labeled neurospheres are derived from actively dividing

cells. Morshead et al. suggested that the neurosphere precursors are normally quiescent, and therefore not depleted by a first kill using high doses of [$^3$H]-thymidine. Morshead et al. argue that this first kill induces stem cells to divide, making them susceptible to a second kill with [$^3$H]-thymidine. However, the composition of the SVZ after the first or second [$^3$H]-thymidine kill was not studied and it is difficult to precisely define which cell population was affected. In a similar study, a single kill using high doses of [$^3$H]-thymidine reduced neurosphere formation by 50% (Gritti et al., 1999). Together with our experiments above, this observation indicates that neurospheres are derived from actively dividing cells.

Neurospheres can be isolated from the third ventricle and spinal cord, but these precursors also require the addition of basic FGF (bFGF) to proliferate (Weiss et al., 1996b). EGF-responsive neurospheres, however, can only be isolated from the SVZ (Weiss et al., 1996b). This could be due to the presence in this region, but not in others, of C cells and their immediate precursors. Preliminary EM observations indicate that C cells are not present in the third ventricle (unpublished observations). Neurosphere-forming cells along the periventricular axis and multipotent precursors that can be isolated from nonneurogenic regions in the adult brain with bFGF (Palmer et al., 1995, 1999) may correspond to astrocyte-like cells, similar to SVZ astrocytes. In the embryonic brain, bFGF-responsive stem cells precede the appearance of EGF-responsive stem cells (Santa-Olalla and Covarrubias, 1999; Tropepe et al., 1999). bFGF signaling may be involved in the activation of early quiescent precursors in the SVZ and other brain regions into EGF-responsive amplifying cells. Further work is required to identify the bFGF-responsive neural stem cells of the adult brain.

Many groups are currently using the neurosphere assay as a means to study neural stem cells. As we show here, cultured neurospheres do not necessarily reflect the properties of the in vivo primary stem cells. It will be important to take this into consideration when using neurospheres as a source of neural stem cells for comparison with other tissues and embryonic stem cells (Geschwind et al., 2001; Terskikh et al., 2001; Ivanova et al., 2002; Ramalho-Santos et al., 2002).

### Normal and Abnormal EGF-R Signaling in the SVZ

Signaling through the EGF-R could provide a means of amplifying activated stem cells and the transit-amplifying cells without affecting the primary stem cells that are being maintained in a more quiescent state. The signals underlying regulation of the primary stem cells and their activation remain unknown. These primary precursors divide less frequently and may be more resilient to expansion. In contrast, the transit-amplifying cells divide actively in vivo; their expression of the EGF receptor is probably associated with this proliferative state. Tightly regulated activation of EGF receptors may be part of the mechanism that allows the controlled amplification of C cells in vivo. The source of the endogenous ligand for signaling through the EGF-R is likely TGF-$\alpha$, which is present in the choroid plexus (Seroogy et al., 1993; Tropepe et al., 1997). Signaling through the EGF receptor, however, may also be involved in cell fate choice in the SVZ. In the developing retina and embryonic cortical ventricular zone (VZ), altering the concentration of ligand and number of copies of EGF-R results in precursors adopting a glial rather than a neuronal fate (Lillien, 1995; Burrows et al., 1997). EGF-R also mediates chemotactic migration in the developing cortex and radial migration in the olfactory bulb (Caric et al., 2001).

Exogenous EGF derails the normal differentiation program in the SVZ. C cells respond to EGF stimulation by undergoing division at the expense of neuroblast production, although an additional direct effect on neuroblasts cannot be ruled out. Interestingly, prolonged exposure of C cells to high doses of EGF results in the conversion of C cells into a highly invasive proliferative cell with glial characteristics, including upregulation of tenascin (Figure 8). Dlx2 is no longer expressed after 6 days of continuous EGF infusion, consistent with C cells being diverted away from a GABA-ergic neuronal lineage. Interestingly, infusion of lower concentrations of EGF also stimulates C cells to divide but does not result in downregulation of Dlx2 (F.D. and A.A.-B., unpublished data). It may thus be possible to reprogram C cells, stop their normal differentiation, and allow them to function as multipotent precursors when exposing them to growth factors. This may be a common property of intermediate precursors. Committed O2A progenitors can be reprogrammed into multipotent stem cells via an intermediate astrocytic state when they are sequentially exposed to extracellular signals in vitro (Kondo and Raff, 2000). Oligodendrocyte progenitors and cells with stem cell properties have been isolated from the adult human brain (Roy et al., 1999; Palmer et al., 2001; Suslov et al., 2002). Thus, the adult human brain probably contains a population of secondary precursors that could be reprogrammed for therapeutic goals. The notion that neurospheres are derived from secondary rather than from the primary in vivo precursors fits well with the observation that neurospheres are heterogeneous (Suslov et al., 2002) and that neurospheres isolated from different brain regions express different lineage markers (Hitoshi et al., 2002; Ostenfeld et al., 2002).

Transit-amplifying precursors may also contribute to brain pathology. SVZ cells may be a source of tumors (Globus and Kuhlenbeck, 1944) such as gliomas, which are characterized by high motility, association with blood vessels and white matter tracts, and expression of the EGF-R and tenascin (reviewed in Holland, 2001). Interestingly, these properties are similar to those described above for C cells exposed to EGF for 6 days in vivo. Deregulation of EGF receptor signaling in C cells may result in the widespread dispersion of proliferating cells through the adult brain. Additional mutations, for example in the cell cycle regulators p19$^{ARF}$ and p16$^{Ink4}$, may be required for the formation of tumors (Holland et al., 1998; Bachoo et al., 2002). It will be interesting to specifically introduce genes that are frequently mutated in brain cancers into SVZ astrocytes and C cells in the adult brain to determine if they result in the migration of SVZ cells into the parenchyma and subsequent generation of gliomas or other brain tumors. The response of C cells presented here may serve as a model system to study tumor origin and invasion in the adult brain.

## Stem Cell Potential of Transit-Amplifying Cells

Our findings suggest that transit-amplifying cells in the adult brain retain stem cell properties when exposed to EGF. C cells divide a few times in vivo, but do not function as long-term stem cells, as they can be eliminated with antimitotics without affecting SVZ regeneration (Doetsch et al., 1999a). When C cells are exposed to high concentrations of EGF, they act as stem cells, revealing that they are not irreversibly committed to a particular differentiation pathway. Differentiation of cells along a specific lineage may be gradual and may depend on accumulation of critical levels of transcription factors. Our data shows that the profound morphological and molecular transformation that occurs as cells go from SVZ astrocytes to C cells is not accompanied by irreversible commitment. Competence for self-renewal and multipotency may be a property of multiple cells derived from primary stem cells. As such, the stem cell concept may be better applied to the potency of cells within the roots of a lineage rather than to a specific "rare" cell type.

## Experimental Procedures

### Animals Used

For all experiments we used 2- to 4-month-old CD-1 or GFAP-tva mice in accordance with institutional guidelines.

### Immunohistochemistry

Adult mice were perfused with 3% parafomaldehyde (PF) and the brains were postfixed overnight. 40 μm sections were cut on a Vibratome, blocked in 10% goat serum in PBS/0.5% Triton-X 100 (except where noted), and incubated at 4°C for 48 hr with PSA-NCAM (1:1000; G. Rougon, Univ. of Marseilles), monoclonal GFAP (1:200; Boehringer Mannheim), polyclonal GFAP (1:1000; DAKO), S100 (1:1000; DAKO), Dlx2 (1:60; John Rubenstein, UCSF), Dll (1:50; G. Panganiban, Univ. of Wisconsin), EGF-R (1:50; Upstate Biotechnology, in 1% horse serum/0.5% TX), tva (1:200; A. Leavitt, UCSF), mCD24 (heat stable antigen) (1:1000 without TX; G. Rougon, Univ. of Marseilles), or Tenascin (1:7500; Sigma). Sections were revealed using species-specific secondary antibodies directly conjugated to fluorophores (Jackson Immuno) or to biotin (Vector). Biotinylated antibodies were revealed using avidin-Texas red or the ABC Elite kit (Vector) followed by DAB. Controls in which the primary antibody was omitted resulted in no immunostaining for all antibodies. Fluorescent sections were examined with under a Zeiss LSM510 confocal microscope. Whole mounts were dissected after EGF infusion and immunostained for PSA-NCAM as described (Doetsch and Alvarez-Buylla, 1996). Mice (n = 4–6/group) were injected with BrdU (100 μl of 10 mg/ml) 3 hr prior to sacrifice or four times every 4 hr on the last day of infusion and immunostaining was performed as described (Doetsch et al., 1999b).

#### *Pre-Embedding Immunohistochemistry*

Brains were perfused with 3% PF/0.5% glutaraldehyde and processed for pre-embedding immunostaining for Dlx2 (1:100) and EM analysis as described in Doetsch et al. (1997).

### EGF and Ganciclovir Infusion

EGF (Upstate Biotechnology) (400 ng/day) in vehicle (BSA/0.9% saline) or vehicle alone was infused into the lateral ventricle for 7 hr or 6 days with a miniosmotic pump (Alzet Model 1007D flow rate 0.5 μl/hr, 7 days) (n = 4 per group). Ganciclovir sodium (Cytovene-IV) (Roche) (8 mg/day in PBS) was infused for 6 days using miniosmotic pumps (Alzet Model 1002 flow rate 0.25 μl/hr, 14 days). Cannulas were implanted at 0 mm relative to bregma, 1.1 mm lateral and 2.3 mm deep.

### [$^3$H]-Thymidine Autoradiography and EM Analysis

Mice were injected with [$^3$H]-thymidine 3 hr prior to sacrifice and processed for EM and [$^3$H]-thymidine autoradiography as described (Doetsch et al., 1997). EM quantification was performed as described (Doetsch et al., 1997).

### RCAS/RISAP Injections and AP Histochemistry

0.2 μl of RCAS-AP or RISAP was injected into the SVZ of GFAP-tva mice (Holland and Varmus, 1998) at three coordinates (total of 0.6 μl): anterior, lateral, depth (mm) relative to bregma (0, 1.4, 1.6; 0.5, 1.1, 1.7; and 1, 1, 2.3). EGF was infused either at the time of retrovirus injection or 2 days later (n = 3/time point). Brains were processed for AP histochemistry as described (Doetsch et al., 1999b).

### Neurosphere Preparation, Differentiation, and Immunostaining

Neurospheres were prepared from the SVZ of the lateral wall of the lateral ventricle of adult mice (5 mice per experiment), passaged, differentiated, and immunostained as described (Doetsch et al., 1999b).

### [$^3$H]-Thymidine Injections for Neurosphere Studies

Mice (5/expt) were injected intraperitoneally with 200 μCi of [$^3$H]-thymidine and neurospheres prepared 4 hr later (n = 2). After 5 DIV, neurospheres were plated onto poly-L-ornithine-coated slides, fixed 5 hr later with 3% PF, and processed for autoradiography. Two control brains were perfused, sectioned, and processed for autoradiography as described before (Alvarez-Buylla et al., 1990)

### Retroviral Injection into the SVZ and Neurosphere Preparation

200 nl of pseudotyped replication defective retrovirus encoding GFP (pLIA-IRES-GFP, $1 \times 10^8$ cfu/ml, N. Gaiano and G. Fishell, Skirball) supplemented with 8 μg/μl of polybrene was injected stereotaxically into the SVZ bilaterally at the following coordinates: anterior, lateral, depth (mm) relative to bregma: 1, 1, 2.2; 0.5, 1.1, 1.7; 0.5, 1, 2.1; 0, 1.4, 1.6; and 0, 1.3, 1.8 (5 mice/expt). 24 hr later, cells were dissociated and plated in clonal isolation dishes as described (Doetsch et al., 1999b).

### Ara-C Infusion and Neurosphere Preparation

2% Ara-C (Sigma) or vehicle alone was infused as described (Doetsch et al., 1999b). Neurospheres were prepared at the indicated survivals after pump removal (5 mice/group, n = 2).

### Neurosphere Cultures in the Presence of Ganciclovir

Neurospheres were prepared from the SVZ of wild-type and heterozygous (n = 3x) Dlx2-IRES-HSVTK-IRES-tau-LacZ mice (L.P. and A.A.-B., unpublished) with the following modifications. Dissociated cells were passed over a 22% Percoll gradient, washed three times by centrifugation at 300 g for 10 min, and plated at 1000 cells/cm$^2$ in the presence of EGF (20 ng/ml) with or without 20 μM ganciclovir. For the rescue experiment, equal numbers (for a total of 1000 cells per 35 mm dish) of wild-type or Dlx2-IRES-HSVTK-IRES-tau-LacZ cells and β-actin-eGFP cells (Okabe et al., 1997) (Jackson Laboratory) were plated in the same well.

### FACS Sorting

Dissociated cells from adult Dlx2-IRES-tau-LacZ heterozygous mice (Corbin et al., 2000) were passed through a 40 μm cell strainer, centrifuged for 10 min at 1300 rpm, resuspended in 1:100 PE-conjugated CD24 (Pharmingen) in PBS, incubated on ice for 20 min, washed three times in PBS, and loaded by hypotonic shock with FDG substrate (Molecular Probes) at 37°C for 1 min as described in Nieto et al. (2001). After centrifugation, cells were resuspended in PBS and stored on ice until sorting on a MoFlo machine. Electronic gates were defined using wild-type cells loaded with FDG substrate. Cells were plated at 1000 cells/cm$^2$ or at densities from 1 to 100 cells per well in 96-well plates using an automated FACS deposition unit. Single neurospheres were passaged and plated for differentiation. Cells were live-stained for 30 min in 1:100 O4 antibody (Chemicon) at 37°C, washed, fixed for 30 min in 3% PF, and immunostained for GFAP and TuJ1.

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**References**

Alvarez-Buylla, A., and Garcia-Verdugo, J.M. (2002). Neurogenesis in adult subventricular zone. J. Neurosci. *22*, 629–634.

Alvarez-Buylla, A., Theelen, M., and Nottebohm, F. (1990). Proliferation "hot spots" in adult avian ventricular zone reveal radial cell division. Neuron *5*, 101–109.

Alvarez-Buylla, A., García-Verdugo, J.M., Mateo, A., and Merchant-Larios, H. (1998). Primary neural precursors and intermitotic nuclear migration in the ventricular zone of adult canaries. J. Neurosci. *18*, 1020–1037.

Alvarez-Buylla, A., Garcia-Verdugo, J.M., and Tramontin, A.D. (2001). A unified hypothesis on the lineage of neural stem cells. Nat. Rev. Neurosci. *2*, 287–293.

Bachoo, R.M., Maher, E.A., Ligon, K.L., Sharpless, N.E., Chan, S.S., You, M.J., Tang, Y., DeFrances, J., Stover, E., Weissleder, R., et al. (2002). Epidermal growth factor receptor and Ink4a/Arf. Convergent mechanisms governing terminal differentiation and transformation along the neural stem cell to astrocyte axis. Cancer Cell *1*, 269–277.

Burrows, R.C., Wancio, D., Levitt, P., and Lillien, L. (1997). Response diversity and the timing of progenitor cell maturation are regulated by developmental changes in EGFR expression in the cortex. Neuron *19*, 251–267.

Calaora, V., Chazal, G., Nielsen, P.J., Rougon, G., and Moreau, H. (1996). mCD24 expression in the developing mouse brain and in zones of secondary neurogenesis in the adult. Neuroscience *73*, 581–594.

Capela, A., and Temple, S. (2002). LeX/SSEA-1 is expressed by adult mouse CNS stem cells, identifying them as non-ependymal. Neuron *35*, 865–875.

Caric, D., Raphael, H., Viti, J., Feathers, A., Wancio, D., and Lillien, L. (2001). EGFRs mediate chemotactic migration in the developing telencephalon. Development *128*, 4203–4216.

Chen, C.-M.A., Smith, D.M., Peters, M.A., Samson, M.E.S., Zitz, J., Tabin, C.J., and Cepko, C.L. (1999). Production and design of more effective avian replication-incompetent retroviral vectors. Dev. Biol. *214*, 370–384.

Chiasson, B.J., Tropepe, V., Morshead, C.M., and van der Kooy, D. (1999). Adult mammalian forebrain ependymal and subependymal cells demonstrate proliferative potential, but only subependymal cells have neural stem cell characteristics. J. Neurosci. *19*, 4462–4471.

Corbin, J.G., Gaiano, N., Machold, R.P., Langston, A., and Fishell, G. (2000). The Gsh2 homeodomain gene controls multiple aspects of telencephalic development. Development *127*, 5007–5020.

Craig, C.G., Tropepe, V., Morshead, C.M., Reynolds, B.A., Weiss, S., and Van der Kooy, D. (1996). *In vivo* growth factor expansion of endogenous subependymal neural precursor cell populations in the adult mouse brain. J. Neurosci. *16*, 2649–2658.

Doetsch, F., and Alvarez-Buylla, A. (1996). Network of tangential pathways for neuronal migration in adult mammalian brain. Proc. Natl. Acad. Sci. USA *93*, 14895–14900.

Doetsch, F., Garcia-Verdugo, J.M., and Alvarez-Buylla, A. (1997). Cellular composition and three-dimensional organization of the subventricular germinal zone in the adult mammalian brain. J. Neurosci. *17*, 5046–5061.

Doetsch, F., Garcia-Verdugo, J.M., and Alvarez-Buylla, A. (1999a). Regeneration of a germinal layer in the adult mammalian brain. Proc. Natl. Acad. Sci. USA *96*, 11619–11624.

Doetsch, F., Caille, I., Lim, D.A., Garcia-Verdugo, J.M., and Alvarez-Buylla, A. (1999b). SVZ astrocytes are neural stem cells in the adult mammalian brain. Cell *97*, 703–716.

Doetsch, F., Verdugo, J.M., Caille, I., Alvarez-Buylla, A., Chao, M.V., and Casaccia-Bonnefil, P. (2002). Lack of the cell-cycle inhibitor p27Kip1 results in selective increase of transit-amplifying cells for adult neurogenesis. J. Neurosci. *22*, 2255–2264.

Eisenstat, D.D., Liu, J.K., Mione, M., Zhong, W., Yu, G., Anderson, S.A., Ghattas, I., Puelles, L., and Rubenstein, J.L. (1999). DLX-1, DLX-2, and DLX-5 expression define distinct stages of basal forebrain differentiation. J. Comp. Neurol. *414*, 217–237.

Fallon, J., Reid, S., Kinyamu, R., Opole, I., Opole, R., Baratta, J., Korc, M., Endo, T.L., Duong, A., Nguyen, G., et al. (2000). In vivo induction of massive proliferation, directed migration, and differentiation of neural cells in the adult mammalian brain. Proc. Natl. Acad. Sci. USA *97*, 14686–14691.

Fisher, G.H., Orsulic, S., Holland, E., Hively, W.P., Li, Y., Lewis, B.C., Williams, B.O., and Varmus, H.E. (1999). Development of a flexible and specific gene delivery system for production of murine tumor models. Oncogene *18*, 5253–5260.

Gage, F.H. (2000). Mammalian neural stem cells. Science *287*, 1433–1438.

Geschwind, D.H., Ou, J., Easterday, M.C., Dougherty, J.D., Jackson, R.L., Chen, Z., Antoine, H., Terskikh, A., Weissman, I.L., Nelson, S.F., and Kornblum, H.I. (2001). A genetic analysis of neural progenitor differentiation. Neuron *29*, 325–339.

Globus, J.H., and Kuhlenbeck, H. (1944). The subependymal cell plate (matrix) and its relationship to brain tumors of the ependymal type. J. Neuropathol. Exp. Neurol. *3*, 1–35.

Gritti, A., Frolichsthal-Schoeller, P., Galli, R., Parati, E.A., Cova, L., Pagano, S.F., Bjornson, R., and Vescovi, A.L. (1999). Epidermal and fibroblast growth factor behave as mitogenic regulators for a single multipotent stem cell-like population from the subventricular region of the adult mouse forebrain. J. Neurosci. *19*, 3287–3297.

He, W., Ingraham, C., Rising, L., Goderie, S., and Temple, S. (2001). Multipotent stem cells from the mouse basal forebrain contribute GABAergic neurons and oligodendrocytes to the cerebral cortex during embryogenesis. J. Neurosci. *21*, 8854–8862.

Hitoshi, S., Tropepe, V., Ekker, M., and van der Kooy, D. (2002). Neural stem cell lineages are regionally specified, but not committed, within distinct compartments of the developing brain. Development *129*, 233–244.

Holland, E.C. (2001). Gliomagenesis: genetic alterations and mouse models. Nat. Rev. Genet. *2*, 120–129.

Holland, E.C., and Varmus, H.E. (1998). Basic fibroblast growth factor induces cell migration and proliferation after glia-specific gene transfer in mice. Proc. Natl. Acad. Sci. USA *95*, 1218–1223.

Holland, E.C., Hively, W.P., DePinho, R.A., and Varmus, H.E. (1998). A constitutively active epidermal growth factor receptor cooperates with disruption of G1 cell-cycle arrest pathways to induce glioma-like lesions in mice. Genes Dev. *12*, 3675–3685.

Ivanova, N.B., Dimos, J.T., Schaniel, C., Hackney, J.A., Moore, K.A., and Lemischka, I.R. (2002). A stem cell molecular signature. Science *298*, 601–604.

Jankovski, A., and Sotelo, C. (1996). Subventricular zone-olfactory bulb migratory pathway in the adult mouse: cellular composition

and specificity as determined by heterochronic and heterotopic transplantation. J. Comp. Neurol. *371*, 376–396.

Johansson, C.B., Momma, S., Clarke, D.L., Risling, M., Lendahl, U., and Frisén, J. (1999). Identification of a neural stem cell in the adult mammalian central nervous system. Cell *96*, 25–34.

Kondo, T., and Raff, M. (2000). Oligodendrocyte precursor cells reprogrammed to become multipotential CNS stem cells. Science *289*, 1754–1757.

Kuhn, H.G., Winkler, J., Kempermann, G., Thal, L.J., and Gage, F.H. (1997). Epidermal growth factor and fibroblast growth factor-2 have different effects on neural progenitors in the adult rat brain. J. Neurosci. *17*, 5820–5829.

Laywell, E.D., Rakic, P., Kukekov, V.G., Holland, E.C., and Steindler, D.A. (2000). Identification of a multipotent astrocytic stem cell in the immature and adult mouse brain. Proc. Natl. Acad. Sci. USA *97*, 13883–13888.

Lillien, L. (1995). Changes in retinal cell fate induced by overexpression of EGF receptor. Nature *377*, 158–162.

Lim, D.A., and Alvarez-Buylla, A. (1999). Interaction between astrocytes and adult subventricular zone precursors stimulates neurogenesis. Proc. Natl. Acad. Sci. USA *96*, 7526–7531.

Lois, C., and Alvarez-Buylla, A. (1994). Long-distance neuronal migration in the adult mammalian brain. Science *264*, 1145–1148.

Lois, C., Garcia-Verdugo, J.M., and Alvarez-Buylla, A. (1996). Chain migration of neuronal precursors. Science *271*, 978–981.

Luskin, M.B. (1993). Restricted proliferation and migration of postnatally generated neurons derived from the forebrain subventricular zone. Neuron *11*, 173–189.

Malatesta, P., Hartfuss, E., and Gotz, M. (2000). Isolation of radial glial cells by fluorescent-activated cell sorting reveals a neuronal lineage. Development *127*, 5253–5263.

McKay, R. (1997). Stem cells in the central nervous system. Science *276*, 66–71.

Miyata, T., Kawaguchi, A., Okano, H., and Ogawa, M. (2001). Asymmetric inheritance of radial glial fibers by cortical neurons. Neuron *31*, 727–741.

Morshead, C.M., and van der Kooy, D. (1992). Postmitotic death is the fate of constitutively proliferating cells in the subependymal layer of the adult mouse brain. J. Neurosci. *12*, 249–256.

Morshead, C.M., Reynolds, B.A., Craig, C.G., McBurney, M.W., Staines, W.A., Morassutti, D., Weiss, S., and Van der Kooy, D. (1994). Neural stem cells in the adult mammalian forebrain: a relatively quiescent subpopulation of subependymal cells. Neuron *13*, 1071–1082.

Nieto, M., Schuurmans, C., Britz, O., and Guillemot, F. (2001). Neural bHLH genes control the neuronal versus glial fate decision in cortical progenitors. Neuron *29*, 401–413.

Noctor, S.C., Flint, A.C., Weissman, T.A., Dammerman, R.S., and Kriegstein, A.R. (2001). Neurons derived from radial glial cells establish radial units in neocortex. Nature *409*, 714–720.

Okabe, M., Ikawa, M., Kominami, K., Nakanishi, T., and Nishimune, Y. (1997). "Green mice" as a source of ubiquitous green cells. FEBS Lett. *407*, 313–319.

Ostenfeld, T., Joly, E., Tai, Y.T., Peters, A., Caldwell, M., Jauniaux, E., and Svendsen, C.N. (2002). Regional specification of rodent and human neurospheres. Brain Res. Dev. Brain Res. *134*, 43–55.

Palmer, T.D., Ray, J., and Gage, F.H. (1995). FGF-2 responsive neuronal progenitors reside in proliferative and quiescent regions of the adult rodent brain. Mol. Cell. Neurosci. *6*, 474–486.

Palmer, T.D., Markakis, E.A., Willhoite, A.R., Safar, F., and Gage, F.H. (1999). Fibroblast growth factor-2 activates a latent neurogenic program in neural stem cells from diverse regions of the adult CNS. J. Neurosci. *19*, 8487–8497.

Palmer, T.D., Schwartz, P.H., Taupin, P., Kaspar, B., Stein, S.A., and Gage, F.H. (2001). Progenitor cells from human brain after death. Nature *411*, 42–43.

Panganiban, G., and Rubenstein, J.L.R. (2002). Developmental functions of the Distal-less/Dlx homeobox genes. Development *129*, 4371–4386.

Peretto, P., Merighi, A., Fasolo, A., and Bonfanti, L. (1997). Glial tubes in the rostral migratory stream of the adult rat. Brain Res. Bull. *42*, 9–21.

Petreanu, L., and Alvarez-Buylla, A. (2002). Maturation and death of adult-born olfactory bulb granule neurons: role of olfaction. J. Neurosci. *22*, 6106–6113.

Porteus, M.H., Bulfone, A., Liu, J.-K., Puelles, L., Lo, L.-C., and Rubenstein, J.L.R. (1994). DLX-2, MASH-1, and MAP-2 expression and bromodeoxyuridine incorporation define molecularly distinct cell populations in the embryonic mouse forebrain. J. Neurosci. *14*, 6370–6383.

Potten, C.S., and Loeffler, M. (1990). Stem cells: attributes, cycles, spirals, pitfalls and uncertainties lessons for and from the crypt. Development *110*, 1001–1020.

Ramalho-Santos, M., Yoon, S., Matsuzaki, Y., Mulligan, R.C., and Melton, D.A. (2002). "Stemness": transcriptional profiling of embryonic and adult stem cells. Science *298*, 597–600.

Reynolds, B., and Weiss, S. (1992). Generation of neurons and astrocytes from isolated cells of the adult mammalian central nervous system. Science *255*, 1707–1710.

Rietze, R.L., Valcanis, H., Brooker, G.F., Thomas, T., Voss, A.K., and Bartlett, P.F. (2001). Purification of a pluripotent neural stem cell from the adult mouse brain. Nature *412*, 736–739.

Roy, N.S., Wang, S., Harrison-Restelli, C., Benraiss, A., Fraser, R.A., Gravel, M., Braun, P.E., and Goldman, S.A. (1999). Identification, isolation, and promoter-defined separation of mitotic oligodendrocyte progenitor cells from the adult human subcortical white matter. J. Neurosci. *19*, 9986–9995.

Santa-Olalla, J., and Covarrubias, L. (1999). Basic fibroblast growth factor promotes epidermal growth factor responsiveness and survival of mesencephalic neural precursor cells. J. Neurobiol. *40*, 14–27.

Seri, B., Garcia-Verdugo, J.M., McEwen, B.S., and Alvarez-Buylla, A. (2001). Astrocytes give rise to new neurons in the adult mammalian hippocampus. J. Neurosci. *21*, 7153–7160.

Seroogy, K.B., Lundgren, K.H., Lee, D.C., Guthrie, K.M., and Gall, C.M. (1993). Cellular localization of transforming growth factor-alpha mRNA in rat forebrain. J. Neurochem. *60*, 1777–1782.

Seroogy, K.B., Gall, C.M., Lee, D.C., and Kornblum, H.I. (1995). Proliferative zones of postnatal rat brain express epidermal growth factor receptor mRNA. Brain Res. *670*, 157–164.

Sotelo, J.R., and Trujillo-Cenóz, O. (1958). Electron microscope study on the development of ciliary components of the neural epithelium of the chick embryo. Z. Zellforsch. *49*, 1–12.

Suslov, O.N., Kukekov, V.G., Ignatova, T.N., and Steindler, D.A. (2002). Neural stem cell heterogeneity demonstrated by molecular phenotyping of clonal neurospheres. Proc. Natl. Acad. Sci. USA *99*, 14506–14511.

Temple, S. (2001). The development of neural stem cells. Nature *414*, 112–117.

Terskikh, A.V., Easterday, M.C., Li, L., Hood, L., Kornblum, H.I., Geschwind, D.H., and Weissman, I.L. (2001). From hematopoiesis to neuropoiesis: evidence of overlapping genetic programs. Proc. Natl. Acad. Sci. USA *98*, 7934–7939.

Thomas, L.B., Gates, M.A., and Steindler, D.A. (1996). Young neurons from the adult subependymal zone proliferate and migrate along an astrocyte, extracellular matrix-rich pathway. Glia *17*, 1–14.

Tropepe, V., Craig, C.G., Morshead, C.M., and Van der Kooy, D. (1997). Transforming growth factor-$\alpha$ null and senescent mice show decreased neural progenitor cell proliferation in the forebrain subependyma. J. Neurosci. *17*, 7850–7859.

Tropepe, V., Sibilia, M., Ciruna, B.G., Rossant, J., Wagner, E.F., and van der Kooy, D. (1999). Distinct neural stem cells proliferate in response to EGF and FGF in the developing mouse telencephalon. Dev. Biol. *208*, 166–188.

Weickert, C.S., Webster, M.J., Colvin, S.M., Herman, M.M., Hyde, T.M., Weinberger, D.R., and Kleinman, J.E. (2000). Localization of

epidermal growth factor receptors and putative neuroblasts in human subependymal zone. J. Comp. Neurol. *423*, 359–372.

Weiss, S., Reynolds, B.A., Vescovi, A.L., Morshead, C., Craig, C.G., and Van der Kooy, D. (1996a). Is there a neural stem cell in the mammalian forebrain? Trends Neurosci. *19*, 387–393.

Weiss, S., Dunne, C., Hewson, J., Wohl, C., Wheatley, M., Peterson, A.C., and Reynolds, B.A. (1996b). Multipotent CNS stem cells are present in the adult mammalian spinal cord and ventricular neuroaxis. J. Neurosci. *16*, 7599–7609.

Wichterle, H., Garcia-Verdugo, J.M., and Alvarez-Buylla, A. (1997). Direct evidence for homotypic, glia-independent neuronal migration. Neuron *18*, 779–791.