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Clonal Mixing, Clonal Restriction, and Specification of Cell Types in the Developing Rat Olfactory Bulb

CHRISTOPHER B. REID,† IVAN LIANG,† AND CHRISTOPHER A. WALSH*
Division of Neurogenetics, Department of Neurology, Beth Israel Deaconess Medical Center, and Programs in Neuroscience, Biological/Biomedical Sciences, and Harvard-MIT Division of Health Sciences and Technology, Harvard Medical School, Boston, Massachusetts 02115

ABSTRACT

To understand the clonal relationship of various olfactory bulb (OB) cell types, OB progenitor cells were infected at embryonic day (E) 14, E15, and E17 with retroviral libraries encoding alkaline phosphatase or β-galactosidase. After survival to postnatal day 10–15, sibling relationships were identified by polymerase chain reaction DNA amplification of distinct sequences in the retroviral constructs. Within the OB, clonal progeny dispersed widely in all directions. In sharp contrast, however, clonal dispersion between the OB and neocortex was not observed, although occasional clonal dispersion between the OB and pyriform and hippocampal regions could not be excluded. Most clones (84%) contained a single cell type, especially after E17 injections, suggesting the existence of either restricted precursors, or multipotential progenitors instructed by a restricted cellular environment. Mixed OB clones (16%) contained multiple cell types in the OB, or occasionally glial or neuronal cells outside the OB, demonstrating the existence of multipotential OB progenitors, likely at a stage before formation of the olfactory rostral migratory stream. Surprisingly, OB glial cells were not labeled, suggesting distinct lineages or perhaps distinct migratory paths for glia and neurons into the OB. A hierarchical cell lineage is proposed that involves a multipotential progenitor that gives rise to potentially more limited progenitors. J. Comp. Neurol. 403:106–118, 1999.

Indexing terms: cell lineage; development; subventricular zone; neuronal migration; stem cell; progenitor cell

The olfactory bulbs (OBs) represent the most anterior extensions of the telencephalon and are located on the ventral surface of the forebrain. The bulb has two types of output neurons, i.e., tufted and mitral cells, and two types of local neurons or interneurons, i.e., periglomerular and granule cells (Cajal, 1955; Brunjes and Frazier, 1986). Neurons of the bulb are formed in two successive, overlapping waves of neurogenesis. The projection neurons are born first. Several days before the developing bulb protrudes from the forebrain, mitral cells are already being generated (Hinds, 1968a,b; Bayer, 1983). Eighty percent of mitral cells are generated between embryonic day (E)14 and E16, and all mitral cells are produced by E18. In contrast, interneurons (periglomerular and granule cells) are generated mostly postnatally. Seventy-six percent of periglomerular neurons are generated from postnatal day (P) 0 and P3, whereas a similar proportion of granule cells are generated over the same time period (Hinds, 1968a,b; Bayer, 1983).

OB interneurons are largely generated outside of the bulb (Altman, 1968; Luskin, 1993; Lois and Alvarez-Buylla, 1994), and migrate into the bulb through the subventricular zone (SVZ). Migration of OB neurons through the SVZ is predominantly perpendicular to radial glia. The widespread nonradial rostral migration of OB precursors is dependent upon their expression of polysialylated neural cell adhesion molecule (PSA-N-CAM) (Lois and Alvarez-Buylla, 1994; Rousselot et al., 1995). Microscopically, the migrating cells have the appearance of

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Dr. Liang's current address is Northwestern University Medical School, McGaw Hall, Chicago, Illinois.

†authors contributed equally

*Correspondence to: Christopher A. Walsh, Division of Neurogenetics, Beth Israel Deaconess Medical Center, Harvard Institutes of Medicine, 77 Avenue Louis Pasteur, Boston, MA 02115.
E-mail: cwalsh@bidmc.harvard.edu

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neuronal chains coalescing to form a rostral migratory stream (RMS; Lois et al., 1996). Interestingly, OB migration appears to depend on a repulsive activity produced near the base of the RMS (Hu and Rutishauser, 1996), which inhibits migration of OB cells to regions caudal to the bulb.

Postnatal injection of a βgalactosidase (βgal)-encoding retrovirus into the anterior region of the SVZ-labeled granule cells, periglomerular cells, and their undifferentiated precursors within the RMS en route to the OB, suggesting that the subventricular zone represented a specialized source of OB neurons postnatally (Luskin, 1993). Spatial analysis of retrovirally labeled cells from P2 to P28 demonstrated that neurons continued to migrate within the RMS for several millimeters, over the course of weeks, before executing 90° turns and exiting the SVZ in all directions (Luskin, 1993). Migrating cells in the RMS stain for neuronal markers, but continue to divide (Menezes et al., 1995). No identifiable glia were labeled by retroviral injections into the anterior SVZ , although all bulb glia appear to be generated postnatally in the mouse (Hinds, 1968a,b).

Although patterns of migration of OB neurons has been a subject of considerable recent interest, lineage relationships of OB neurons have not been elucidated. In this study, we show that OB clones show widespread dispersion within the bulb, although clonal dispersion between OB and neocortex is rare or nonexistent. We also find that clones labeled later in embryogenesis show progressive restrictions of cell fates. Furthermore, our data suggest that multipotential OB precursors are likely to reside outside the OB proper, and that some precursors in the RMS may be restricted to producing single cell types.

TABLE 1. Polymerase Chain Reaction–Defined Clones Labeled With an Alkaline Phosphatase–Encoding Retroviral Library at Embryonic Day 14[1]

Experiment 1
1
SVZ/SVZ (2600)
SVZ/SVZ (2600)

Experiment 2
2
IGL/GC (2600, 190°)
IGL/GC (3400, 110°)
IGL/GC (3900, 195°)
IGL/GC (4400, 200°)

3
IGL/GC (3200, 60°)
IGL/U (4000, 280°)

Experiment 3
4
IGL/GC (500, 90°)
IGL/GC (1000, 265°)
IGL/GC (1300, 180°)
IGL/GC (1400, 310°)
IGL/GC (1700, 50°)
SVZ/SVZ (2800)
IGL/U (3300, 210°)
SVZ/SVZ (3500)
IGL/U (4500, 350°)
IGL/U (4500, 150°)

5
IGL/U (900, 180°)

6
IGL/GC (2000, 200°)
SVZ/SVZ (2100)

7
IGL/GC (2100, 175°)
IGL/U (3200, 345°)

8
SVZ/SVZ (2600)

9
IGL/U (4300, 180°)

10
IGL/U (400)

Experiment 4
11
IGL/GC (1600, 340°)
MCL/U (1800, 10°)
IGL/GC (3200, 255°)

12
IGL/U (1700, 345°)

13
MCL/U (1800, 95°)
EPL/U (3600, 155°)

14
IGL/U (2100, 130°)
Glom/U (2300, 320°)

15
IGL/GC (3200, 0°)
IGL/U (4700, 100°)
IGL/GC (5000, 100°)

16
Glom/PG (3100, 125°)
IGL/GC (3400, 160°)

17
Glom/PG (3200, 285°)

18
Glom/PG (3000, 125°)
IGL/GC (3700, 160°)

19
SVZ/SVZ (2600)
IGL/GC (2300, 150°)
IGL/GC (2800, 310°)
IGL/GC (3100, 155°)
IGL/GC (3100, 20°)

20
SVZ/SVZ (2400)

21
SVZ/SVZ (4400)
SVZ/SVZ (4400)

22
GCL/GC (3800, 10°)

23
Glom/PG (3700, 45°)

[1]Topographic and morphologic analysis of 23 olfactory bulb (OB) clones from four experiments labeled with an alkaline phosphatase (AP)–encoding retroviral library at embryonic day 14 (E14), and analyzed at postnatal day 15 (P15). Each experiment represents a single OB. Sibling cells of each clone are listed vertically in order of proximity to the rostral tip of the olfactory bulb. The layer in which the cell was found as well as its morphology are listed. Likewise the position of the cell within the bulb is localized according to the parameters d (rostrocaudal distance from the tip of the bulb) and θ (position of the cell with the 360° plane perpendicular to the rostrocaudal axis). Labeled cells for which polymerase chain reaction (PCR) was not successful are not shown, so that each entry represents a single PCR-positive cell in the following form: laminar location/cell morphology of cell (d, θ). Cells in the subventricular zone were generally too close to the origin to get an accurate measure of θ, and so θ is not indicated. Glom, glomerular layer; EPL, external plexiform layer; MCL, mitral cell layer; IGL, internal granule cell layer; SVZ, subventricular zone/cell; PG, periglomerular cell; T, tufted cell; MC, mitral cell; GC, granule cell; U, only a cell body could be visualized, and cell morphology was hence uncertain. Morphology of cells was determined by AP staining, which allowed definition of neuronal vs. glial identity in >90% of labeled cells, and identification of broad neuronal classes in ≈85% of labeled neurons. Because neurogenesis continues into adulthood, migrating cells are commonly seen in the SVZ.

MATERIALS AND METHODS

Retroviral vector production

Preparation of the alkaline phosphatase (AP) and β-galactosidase retroviral libraries has been previously presented (Walsh and Cepko, 1992; Walsh, 1995). The βgal library consists of 85–100 constructs distinguishable by containing different short inserts of genomic DNA in the *Xho*I site (Walsh and Cepko, 1992) of the BAG vector (Turner and Cepko, 1987). The AP library, with which most of the present data were generated, was derived from ≈3,400 clones containing genomic DNA fragments inserted into the *Xho*I site (Reid et al., 1995) of the DAP vector (Fields-Berry et al., 1992). The AP library contains ≥100–400 distinct retroviral constructs at roughly equal titer (Reid et al., 1995).

Animal surgery

Timed-pregnant Long-Evans hooded rats were purchased from Charles River Laboratories, and were cared for and handled according to animal protocols approved by the Institutional Animal Care and Use Committees (IACUCs) of Harvard Medical School and Beth Israel Deaconess Medical Center. Pregnancies were timed from the day of vaginal plug (E0). Birth usually occurred on E21. Surgical procedures and injection of the retroviral supernatant into the lateral ventricles of fetal rat brains are described in detail elsewhere (Walsh and Cepko, 1992; Cepko et al., 1993).

To label the early-born OB projection neurons, as well as the later-born granule cells and periglomerular cells, we made retroviral injections at the earliest feasible stages of development. Progenitors for the various neuronal types were infected by injection of 2–4 μl of AP- or βgal-encoding retroviral libraries into the lateral ventricles of E14, E15, and E17 fetal rats (see Tables 1–4). Because the titer of the retroviral supernatants varied from 1 to 20 $\times$ 10^6 colony forming units (cfu)/ml, the amount injected corresponds to 2–80 $\times$ 10^3 cfu per injection. However, some virus is usually lost because of leakage at the injection site, and the rate of infection in vivo is typically 50–100 times lower than the rate of infection of cultured fibroblasts in vitro. The half-life of the virus at 37°C was also about 4 hours, so that the injections are reasonably regarded as delivered during brief time windows. Additional injections were

TABLE 2. Polymerase Chain Reaction–Defined Clones Labeled With an Alkaline Phosphatase–Encoding Retroviral Library at Embryonic Day 15[1]

Experiment 1	Experiment 2
1	**3**
MCL/U (500, 60°)	MCL/MC (400, 125°)
MCL/MC (600, 310°)	MCL/MC (700, 90°)
Glom/PG (700, 280°)	MCL/MC (1400, 165°)
Glom/PG (1000, 345°)	MCL/MC (1600, 15°)
IGL/U (1400, 355°)	MCL/MC (1600, 305°)
IGL/U (1200, 325°)	MCL/MC (1700, 35°)
MCL/MC (1600, 350°)	MCL/MC (1900, 140°)
IGL/U (1600, 10°)	MCL/MC (1900, 330°)
IGL/GC (1500, 0°)	Hippocampus? (12100)
Glom/PG (1500, 210°)	
MCL/MC (1700, 130°)	**Experiment 3**
MCL/MC (1700, 65°)	**4**
MCL/MC (1900, 0°)	Glom/PG (1100, 265°)
Glom/PG (1900, 20°)	
IGL/U (2000, 350°)	**5**
IGL/GC (2000, 60°)	IGL/GC (1300, 180°)
IGL/GC (2200, 160°)	IGL/GC (1400, 0°)
IGL/GC (2200, 55°)	IGL/GC (1600, 340°)
Glom/PG (2200, 80°)	SVZ/SVZ (4800)
Glom/PG (2500, 105°)	SVZ/SVZ (5400)
Glom/PG (2500, 15°)	SVZ/SVZ (5400)
IGL/U (2600, 345°)	SVZ/SVZ (5700)
Glom/PG (2700)	
IGL/GC (3100)	
SVZ/SVZ (4100)	
2	
IGL/U (400)	

[1]Topographic and morphologic analysis of five OB clones from three experiments labeled with the AP retroviral library at E15. Each experiment represents one hemisphere of three brains analyzed at P15. Other conventions are as in Table 1.

TABLE 3. Polymerase Chain Reaction–Defined Clones Labeled With a βgal-Encoding Retroviral Library at Embryonic Day 15[1]

Experiment 1		
1	**2**	**7**
IGL (600)	IGL (1700)	IGL (2400)
IGL (800)	IGL (2400)	IGL (2600)
IGL (900)	SVZ (3800)	
IGL (900)	SVZ (5200)	**8**
IGL (900)		IGL (2900)
IGL (1500)	**3**	
SVZ (2000)	PG (1000)	**9**
IGL (2200)	IGL (1500)	IGL (3100)
SVZ (2300)	IGL (2000)	
IGL (2600)	SVZ (2700)	**10**
IGL (2600)	SVZ (6200)	MCL (600)
IGL (2800)	SVZ (6400)	
IGL (2900)	SVZ (6700)	**11**
IGL (2900)		SVZ (4600)
IGL (3000)	**4**	
SVZ (3000)	IGL (1900)	**Experiment 2**
IGL (3100)	IGL (2500)	**12**
IGL (3200)	IGL (2700)	IGL (1000)
IGL (3200)	IGL (2800)	IGL (1300)
SVZ (3400)	IGL (4100)	IGL (2300)
SVZ (3700)	post.-lat. forebrain (12900)	SVZ (2300)
SVZ (3700)	post.-lat. forebrain (13000)	SVZ (2800)
SVZ (3900)	post.-lat. forebrain (13000)	SVZ (3100)
SVZ (4100)	post.-lat. forebrain (13100)	IGL (3100)
IGL (4100)	post.-lat. forebrain (13200)	SVZ (3300)
SVZ (4300)		SVZ (3800)
SVZ (4400)	**5**	SVZ (3900)
IGL (4400)	IGL (2000)	SVZ (4000)
SVZ (6700)	IGL (2200)	SVZ (4200)
SVZ (7000)	SVZ (4300)	
AOB (6700)	SVZ (4500)	**13**
AOB (6700)	SVZ (5900)	SVZ (2300)
AOB (7200)		SVZ (2800)
AOB (7300)	**6**	SVZ (3100)
AOB (7400)	Glom (1700)	SVZ (3600)
AOB (7500)	IGL (2600)	SVZ (3900)
	pyriform cortex (7800)	

[1]Topographic and morphologic analysis of 12 OB clones from three experiments labeled with the βgal retroviral library at E15. Each experiment represents one hemisphere of brains analyzed at P15. Since βgal-labeled brains were sectioned tangentially from whole-mounts, θ was not determined. Similarly, βgal staining did not allow cell morphology to be determined independent of laminar position for most neurons. Therefore, only layer and d (distance caudal from the rostral tip of OB) are listed for each cell. post.-lat.; posterior-lateral; other conventions are as in Table 1.

made with single retroviral constructs on P0. The brains of injected animals were harvested for analysis at P10 or P15. Progeny of infected cells expressed the appropriate retrovirally encoded histochemical marker revealing cell bodies and stained cellular processes.

Histology and analysis of labeled cells

Animals were killed 15 days after birth by an overdose of Nembutal and perfused with 2–4% paraformaldehyde in 2 mM $MgCl_2$ and 1.25 mM EGTA. The brains were removed and submerged in fixative overnight at 4°C. The brains were then transferred to 30% sucrose in phosphate-buffered saline (PBS, pH 7.4) with 2 mM $MgCl_2$ at 4°C until they sank. Brains were sectioned coronally (AP library) or the cortex and OBs were removed from the brainstem (βgal library), flattened, and sectioned tangentially at 100-μm thickness by using a Bright cryostat. The sections were mounted onto gel-coated glass slides and processed for AP or βgal activity according to protocols presented elsewhere (Cepko et al., 1993; Reid et al., 1995). Cell morphologies and positioning within the bulb were recorded by photography and camera lucida drawings.

In AP-labeled brains, the locations of labeled neurons was recorded by the parameters d and θ. d represents the distance of the cell caudal from the rostral tip of the OB. The distance (d) was calculated as was done previously (Reid et al., 1995, 1997) by multiplying the cell's coronal section number by the thickness of each section (100 μm). θ is an angular measure from an arbitrary origin determined by establishing an axis perpendicular to a tangent to the most ventral portion of each bulb section. The origin was fixed at the midpoint of the distance that the mitral cell layer spanned along the axis and was almost always located within the subventricular zone. Ventral along the axis was designated as 0° and measurements were made clockwise, facing the bulb. The measurements, d and θ, are not enough to locate a cell to a point in three-dimensional space, but to a line in space, which was sufficient for analysis. For βgal-labeled cells, distances (d) were measured directly from camera lucida drawings of tangential sections, whereas θ was not determined because sections were taken roughly parallel to the long axis of the OB.

Clonal analysis with polymerase chain reaction

Tissue analysis was performed by polymerase chain reaction (PCR) amplification of viral-encoded DNA tags from samples containing individual labeled cells and surrounding unlabeled cells, for amplification by PCR, as has been presented in detail elsewhere (Walsh and Cepko, 1992, 1993; Walsh et al., 1992). Small fragments of tissue (approximately 100 μm × 200 μm × 200 μm) containing the nucleus of each labeled cell were dissected into 200 μg/μl proteinase K in 1× PCR buffer, covered with 30 μl of mineral oil, and incubated at 65°C for ≥4 hours. The samples were heated to 85°C to inactivate proteinase K and then to 95°C for 5 minutes to denature the genomic DNA. A nested PCR protocol was used to increase the sensitivity and specificity of amplification and is described elsewhere (Walsh and Cepko, 1992, 1993; Walsh et al., 1992).

Retroviral sequences could be amplified by PCR from a significant minority of tissue fragments that did not appear to contain a histochemically labeled OB cell. About 10–20% of samples containing OB tissue without labeled

TABLE 4. Polymerase Chain Reaction–Defined Clones Labeled With an Alkaline Phosphatase–Encoding Retroviral Library at Embryonic Day 17[1]

Experiment 1

1
IGL/GC (2400, 155°)
IGL/GC (3200, 275°)
IGL/GC (4000, 340°)

2
Glom/PG (1800, 310°)

3
Glom/PG (1900, 350°)

4
Glom/PG (1800, 250°)

5
SVZ/SVZ (500, 10)
IGL/GC (1700, 355°)
IGL/U (3300, 40°)

6
IGL/U (300, 345°)
IGL/U (3100, 230°)
MCL/GC (4100, 330°)

7
IGL/GC (900, 170°)
IGL/GC (2300, 205°)

8
Glom/PG (3100, 55°)

9
Glom/PG (2900, 65°)

10
IGL/GC (1800, 75°)
IGL/GC (2500, 15°)

11
SVZ/SVZ (2500)
IGL/GC (3200, 70)

12
IGL/U (1200, 350°)

13
EPL/PG (500, 15°)

14
Glom/PG (2100, 45°)

15
Glom/PG (300, 10°)
EPL/PG (500, 170°)
EPL/PG (500, 170°)
Glom/PG (600, 20°)
EPL/U (600, 170°)
Glom/PG (1900, 95°)

16
IGL/GC (2500, 345°)
IGL/GC (2700, 225°)
SVZ/SVZ (2800)

17
MCL/MC (2000, 0°)

18
IGL/U (1800, 55°)

19
Glom/PG (2000, 45°)

20
IGL/GC (1800, 55°)
IGL/GC (2600, 150°)
IGL/GC (3400, 350°)

21
Glom/PG (500, 25°)
Glom/PG (2600, 245°)
AOB/Glia (6400)

22
IGL/GC (1900, 320°)

23
Glom/PG (2100, 105°)

24
IGL/GC (1600, 345°)

Experiment 2

25
IGL/U (400, 110°)
IGL/GC (500, 55°)
IGL/U (500, 330°)
IGL/GC (700, 55°)
IGL/GC (1300, 325°)
IGL/GC (1300, 325°)
IGL/GC (1400, 115°)
IGL/GC (1800, 285°)
IGL/GC (2100, 65°)

26
MCL/GC (400, 205°)
IGL/GC (2200, 100°)

Experiment 3

27
IGL/GC (100, 195°)
IGL/GC (200, ?)
IGL/GC (400, 0°)
IGL/GC (1300, 345°)
IGL/GC (1900, 55°)
IGL/GC (2800, 115°)

Experiment 4

28
IGL/GC (2400, 140°)
SVZ/SVZ (3900)

[1]Topographic and morphologic analysis of 28 OB clones from four experiments labeled with the AP-encoding retroviral library at E17 and analysis at P15. Each experiment represents one OB. 100% of clones labeled at E17 were single cell type clones. Other conventions are as in Table 1.

cells showed PCR products, whereas OB tissue from unlabeled brains, or other unlabeled regions of a labeled brain, did not show positive PCR products. Moreover, the PCR product sizes observed usually matched a DNA tag amplified from other cells within the same OB, strongly suggesting that some OB precursors failed to express histochemical markers encoded by integrated retroviruses. Retroviral inactivation occurred more commonly in OB samples than in the cerebral cortex (Walsh and Cepko, 1992). At least 10% of all PCR reactions were negative controls involving unlabeled tissue samples from the same brains or reagents alone. Any experiments in which reagent-only controls produced a PCR product were considered contaminated and were discarded.

Analysis of PCR products

The PCR products from the second PCR reaction were separated on a 3%/1% NuSieve/Seakem agarose (FMC Agarose) gels to determine tag sizes. Each tag was then digested with *Cfo*I, *Rsa*I, *Alu*I, *Mse*I, and *Msp*I. Finally, we ran samples of similar initial size side-by-side on agarose gels to allow direct comparison of restriction fragment sizes.

Quantitation of sibling and nonsibling cell spacings

To test for systematic spacings between sibling cells, we measured rostrocaudal cell spacings between sibling cells by a technique used previously (Reid et al., 1995, 1997) by calculating $|_{\text{cell a}} - d_{\text{cell b}}|$ for each pair of sibling cells for each clone, and generated a frequency histogram of intersibling cell spacings. Because no consistent differences were seen between cells labeled at E14, E15, and E17, data were pooled. For comparison, cell spacings were calculated by the same method for cells in different clones as well to create a frequency histogram of nonsibling cell spacings. Similar analysis was performed for the angles (θ) between pairs of sibling cells ($\Delta\theta = |\theta_{\text{cell a}} - \theta_{\text{cell b}}|$), and between pairs of nonsibling cells, with the smaller Δθ between each pair of cells being measured, so that Δθ was always ≤180°. Again, data were presented as frequency histograms.

Three-dimensional computer reconstruction

Computer reconstructions of a few clones were performed by using the Computer Assisted Reconstruction Program (CARP) running on a Silicon Graphics workstation (generously supplied by C.L. Cepko). The program prepares reconstructed images from serial coronal sections and generates an outline. The location of retrovirally labeled cells are indicated in the sections by using arbitrarily chosen colors (different colors indicating different DNA tags), with each PCR-positive cell shown as a 100-μm-diameter dot for clarity. Reconstructions can then be viewed from several angles.

RESULTS

Patterns of cell labeling

In total, >500 OB neurons were labeled by prenatal retroviral injections. Labeled OB neurons were invariably scattered widely as solitary cells. For example, labeled neurons occurring within the same section were frequently separated mediolaterally and dorsoventrally (Tables 1–4) and did not show apparent clustering. Labeled neurons were present throughout the bulb often involving regions in or near the anterior olfactory nuclei. This overall distribution of labeled neurons was similar to that seen after postnatal labeling of SVZ precursors and granule cells (Luskin, 1993).

The differentiation of AP-labeled and βgal-labeled neurons did not appear to be altered by expression of the retrovirally encoded markers. Greater than 85% of AP-labeled OB neurons could be classified as granule, periglomerular, or mitral cells based on their morphology and laminar position within the bulb (Figs. 1–6). Because labeling of neurons with βgal provided considerably less morphologic information than AP, cell type identification of βgal-labeled neurons was less precise and usually only inferred from laminar position. Consistent with tritiated thymidine birthdating studies suggesting that the projection neurons are generated first (Hinds, 1968a,b; Kaplan and Hinds, 1977; Bayer, 1983; Corotto et al., 1993), the early-born mitral cells were labeled in higher proportion after early (E14/E15) injections (see Tables 1–4). The relatively large mitral cells usually showed a clearly discernible primary dendrite and other dendrites in the external plexiform layer, although labeling was variable (Figure 3). E17 injections, on the other hand, labeled

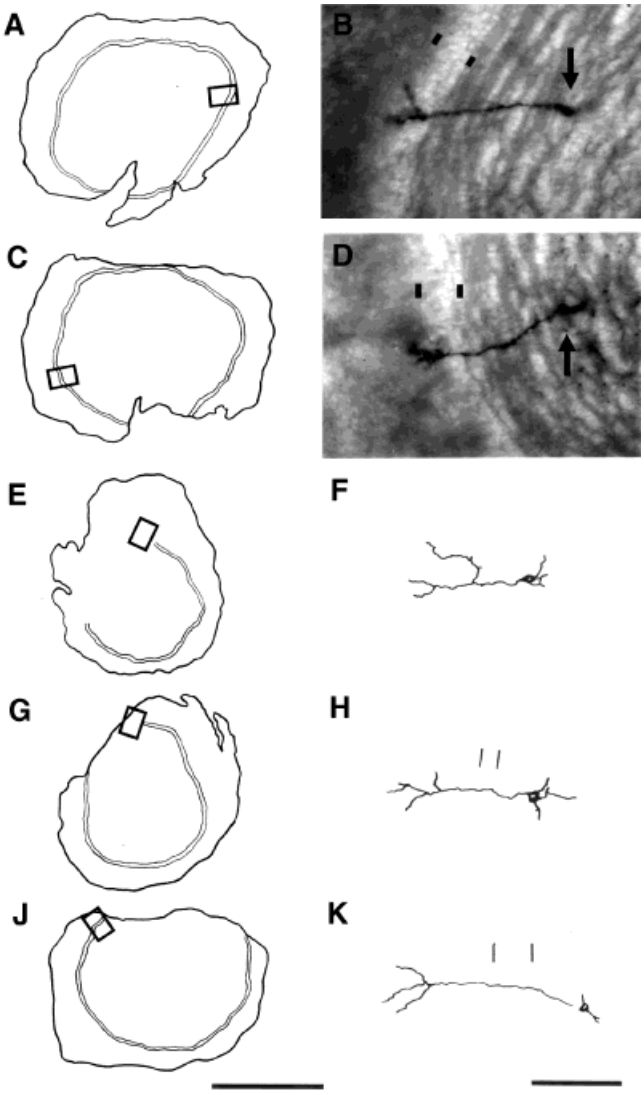

Fig. 1. A clone labeled with alkaline phosphatase-encoding retrovirus at embryonic day 17 that includes multiple granule neurons. **A**, **C**, **E**, **G**, and **J:** Low-power camera lucida drawings of one olfactory bulb (OB) that contained the individual retrovirally labeled cells illustrated by matching photomicrographs (**B**, **D**) or higher power camera lucida drawings (**F**, **H**, **K**). The clone illustrated corresponds to Table 4, clone 25. In the low-power drawings, the dark rectangle represents the field shown at higher power in the corresponding photomicrograph or drawing. The double lines indicate the approximate borders of the mitral cell layer, and the darker outlines indicate the border of the OB. Each drawing is oriented with dorsal up. In the photomicrographs and higher power photos, the two parallel tick lines indicate the approximate border of the mitral cell layer. Arrows in B and D indicate the cell soma. Scale bar = 200 μm in J (applies to A,C,E,G,J), 100 μm in K (applies to B,D,F,H,K).

relatively large numbers of the later-born interneurons, granule cells, and periglomerular cells, which are produced well into adult life (Table 4; Figs. 1, 2, 4, 5, 6). Granule cells had small cell bodies (>10 μm) and lacked axons but were usually seen to have a single unbranched cell process projecting into the external plexiform layer (Figs. 1, 5, 6). Periglomerular neurons had somata near the glomeruli and dendrites that usually branched many times within the confines of a single glomerulus or two neighboring glomeruli (Figs. 2, 4, 6).

Although most AP-labeled cells showed intense staining of soma and cellular processes and were therefore easily identifiable in coronal sections of the OB, not all retrovirally labeled cells were fully differentiated. Because OB neuronal production and migration continue into adulthood, many labeled cells were immature when brains were harvested and analyzed at P10 and P14. Virtually all labeled cells in the SVZ, for instance, resembled migratory cells described in previous experiments (Luskin, 1993; Lois and Alvarez-Buylla, 1994; Lois et al., 1996). They were spindle-shaped cells with thick leading and thin trailing processes (Figs. 5, 7). Tangential sections of βgal-injected brains facilitated visualization of these cells, which constitute the "rostral migratory stream" (Lois and Alvarez-Buylla, 1994; Lois et al., 1996) and their continuity with the cells in the telencephalic SVZ (Fig. 7).

Although all major neuronal cell types were labeled, remarkably no labeled glial cells were observed in the OB after retroviral injection at E14–E17. Glia were labeled throughout other regions of the telencephalon after these injections. Glial labeling typically consisted of dark, cloudy regions of precipitate in which individual cells could frequently not be resolved (Fig. 4C). This pattern of labeling invariably corresponds to glial cells in previous studies in which retroviral labeling has been combined with electron microscopy and appears to represent the dense filling of vellate astrocytic processes (Fields-Berry et al., 1992; Snyder et al., 1992; Luskin, 1993; Luskin et al., 1993; Zhang and Goldman, 1996; Goldman et al., 1997). A small number of glial clusters were observed in the region of the anterior olfactory nuclei, but none of these continued rostral into the OB proper. Labeled glial cells were easily recognized in the cerebral cortex and subcortical white matter of these same brains (Walsh and Cepko, 1992; Reid et al., 1995). Labeled glia were likewise absent after P0 retroviral injections into the SVZ that forms OB neurons (Luskin, 1993). Because labeled glial cells have never been observed within the OB after retroviral injections, it is likely that virtually all retrovirally labeled cells observed in the SVZ were limited to a neuronal fate. This finding is consistent with previous reports showing that cells in the RMS already express neuronal markers (Menezes et al., 1995). We also noted, however, that a minority (≈10%) of unlabeled OB tissue fragments contained retroviral sequences that could be amplified by PCR indicating significant inactivation of the histochemical marker gene. We cannot rule out, therefore, the possibility that OB glia, or any other cell type, preferentially inactivate the retroviral construct.

Clonal analysis by PCR

PCR amplification was successfully performed on four OBs labeled with the AP-encoding virus at E14 (Table 1), three labeled at E15 (Table 2), and four labeled at E17 (Table 4), yielding 23, 5, and 28 clones, respectively. Two bulbs labeled with a βgal-encoding retrovirus at E15 were also analyzed (Table 3) for an additional 12 E15-labeled clones. Overall, 69% of retrovirally labeled tissue samples were successfully amplified. Because the failure rate of PCR is unknown for any given clone, no mathematical correction was made for the amplification of less then 100% of histochemically marked cells. Given the complexity of the βgal and AP libraries (see Materials and Methods

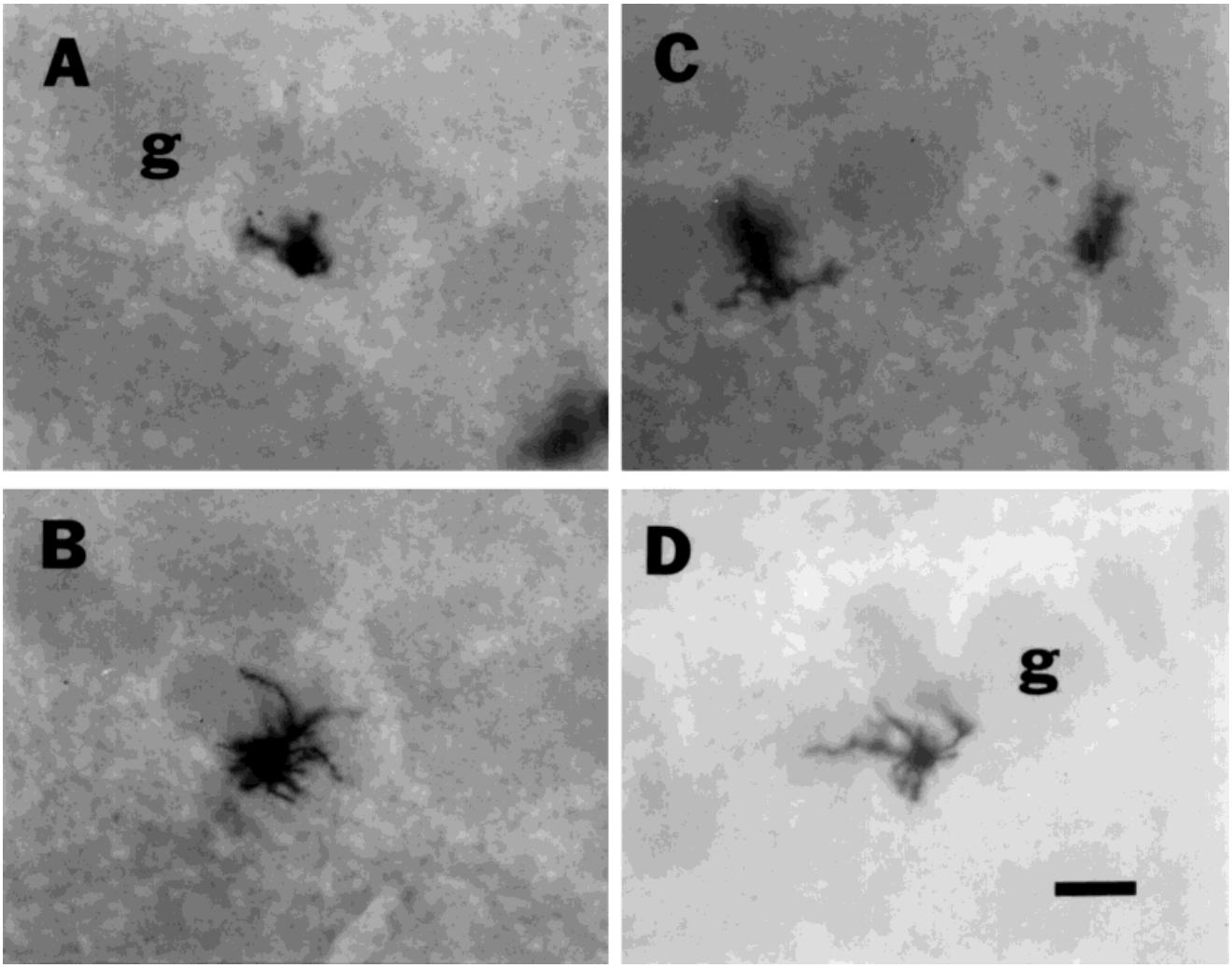

Fig. 2. **A–D:** A clone labeled with alkaline phosphatase-encoding retrovirus at embryonic day 17 that includes multiple periglomerular neurons. High-power photomicrographs of the periglomerular layer of an olfactory bulb that contains a clone (Table 4, clone 15) with multiple cells with strikingly similar morphology. Each of the cells is well stained so that dendrites can be seen. "g" indicates an individual glomerulus. Several fields (A,C) contain multiple labeled cells, of which at least one (the cell nearer the center of the photo in each case) was demonstrated by polymerase chain reaction to be a sibling cell of the other cells in other plates of this figure. Scale bar = 20 μm in D (applies to A–D).

section), the likelihood that retroviruses with the same DNA tag will coincidentally infect more than one progenitor cell and produce spurious clonal relationship is certainly <5% for experiments with fewer than four clones. On the other hand, it is possible that some experiments with more than five clones might have spurious clones (Walsh et al., 1992). However, clonal patterns did not obviously differ between more heavily and more lightly infected OBs, and in fact most larger clones happened to occur in experiments with fewer than four clones. Therefore, we did not attempt to correct for the possibility of coincidental infections, and it is unlikely to confound the data.

PCR-defined clones varied widely in size and composition, containing as few as 1 neuron or as many as 30 neurons. Average clone size varied little between E14 and E17, although the largest clones (clone 1, Table 2; clone 1, Table 3) were labeled at E15. The average size of all clones for E14 was 2.3 neurons compared with 2.2 neurons for E17 (Table 5). A high proportion of E17 clones (50%) consisted of a single cell. The average clone size at E15 was 8.9 neurons (Table 3). The reason for the much larger clone sizes after E15 labeling versus E14 or E17 is not obvious. The larger clones do not appear to reflect errors in staging of the fetuses, because the effect holds for multiple litters (Tables 2, 3). The differences reflect the labeling of a few large clones at E15, perhaps suggesting that multipotential progenitors are especially common or accessible to the virus injection at that age.

Clone size and distribution

Clones in which all neurons could be identified as the same morphologic cell type appeared more commonly after E17 injections than after E14/E15 injections (Table 4; Figs. 1, 2, 3). Of eight clones labeled at E14 that contained multiple, fully differentiated neurons, five of these were

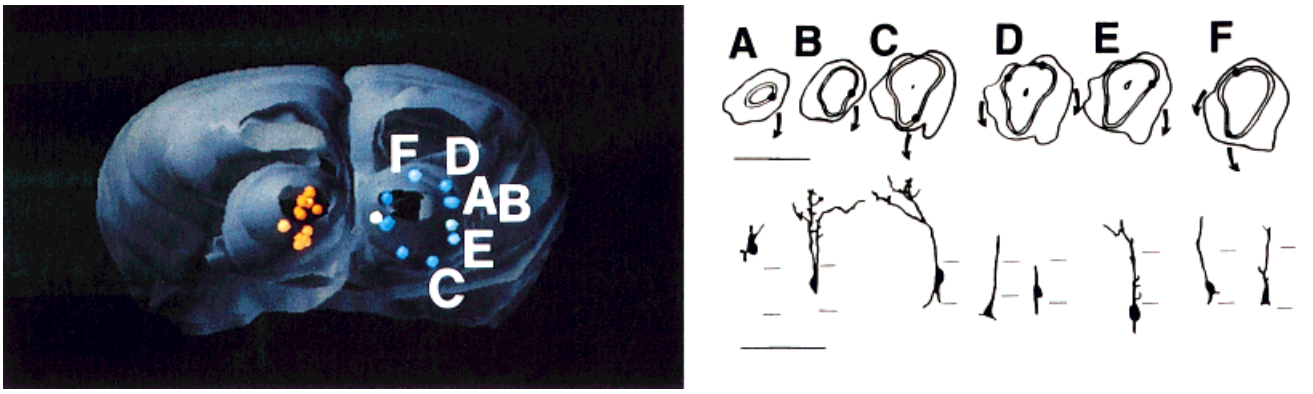

Fig. 3. A three-dimensional reconstruction of a clone containing several probable mitral cells (left panel, blue), labeled at embryonic day 15, along with camera lucida drawings of each individual cell (A–F). The left panel shows a rat brain viewed from the rostral end, with cells of the clone (Table 2, clone 3) shown in blue and labeled. Each cell is then represented in the right panel by a low-power camera lucida drawing of the corresponding olfactory bulb (top) and a higher power drawing of the cell (bottom), with the two drawings connected by curved arrows. The double lines in the lower power drawing indicate the borders of the mitral cell layer, also indicated by the dashes in the higher power drawings. The scale bar for the low magnification drawings shows 2 mm; the scale bar for the higher magnification drawings shows 100 μm. Although the level of morphologic detail of the cells is not always sufficient to definitively identify them all as mitral cells, their location in or around the mitral cell layer is striking (see the left panel) and the morphologies are consistent with mitral cell identity. The cells in the right hemisphere depicted in orange represent the clone illustrated in detail in Figure 5. Scale bars = 2 mm in the low magnification drawings, 100 μm in the higher magnification drawings.

composed of a single class of neuron. All five clones contained granule cells only. Four other clones labeled at E14 were composed of undifferentiated cells migrating within the bulb SVZ. At E15, 6 of 11 clones containing multiple fully differentiated neurons involved only a single neuronal cell type. The percentage of clones containing a single cell type rose sharply to 100% (11 of 11 clones containing multiple fully differentiated neurons) after injections at E17. Of 28 total E17 clones, 23 clones contained only periglomerular cells or granule cells.

In contrast to clones containing a single cell type, 11 of 68 clones labeled at E14–E17 clearly contained cells of divergent neuronal cell types within the OB or included divergent cell types in various other portions of the telencephalon. Within clones restricted to the OBs, the most common combination of mixed cell types (six clones) involved granule/periglomerular cell clones (G/P clones) (Fig. 6). All six G/P clones were labeled by early injections made at E14 or E15. No G/P clones were seen after E17 injections, although 11 periglomerular cell and 14 granule cell clones were labeled by E17 injection. In contrast, 48% of periglomerular neurons and 8.9% of granule neurons labeled at E14 and E15 were members of G/P clones. This result strongly indicated a common progenitor for the granule and periglomerular neurons within the ventricular zone at E14 and E15. The absence of G/P clones after later injections suggests the possibility that the lineages have largely diverged by E17. A single mixed clone involving mitral cells and SVZ cells in addition to granule and periglomerular cells was also observed after E15 injection (clone 1, Table 2). This clone suggested the possibility of a rare progenitor for both projection neurons and interneurons in the E15 ventricular zone.

Our data confirmed a gradient of interneuron production from granule cell to periglomerular cell over the course of bulb development (Bayer, 1983). At E14, without regard to clonal relationships, seven granule neurons were labeled for each labeled periglomerular neuron. This ratio fell, however, to 3:1 when injections were made at E15, and 2.5:1 after E17 injections. This trend is consistent with tritiated thymidine studies that also indicated a shift toward periglomerular cell production in the bulb over time (Bayer, 1983). With regard to PCR-defined clones, the ratio of G clones to G/P clones to P clones at E14 was 6.5:1:1 (Table 6), suggesting that granule cell progenitors strongly predominated at this stage of bulb development. This ratio, however, fell dramatically to 3.5:1.5:1 after E15 injection and to 1.7:0:1 after E17 injection (Table 6). These results demonstrate that the proportion of P progenitors increased greater than threefold from E14 to E17. Likewise, the proportion of G/P progenitors increased approximately twofold from E14 to E15, indicating a progressive transition from G clones toward G/P clones and P clones. The absence of any G/P clones after E17 injection and the further increase in P clones from E15 to E17 suggests a completed transition to separate lineages for granule cells and periglomerular cells by E17. However, any suggestion about restrictions to cell fates can only be tested by future studies that challenge cell fates (e.g., transplantation).

Clonal dispersion within the OB

The majority of neurons labeled by injections at all ages (>89%) were members of multicell clones, which showed substantial topographic dispersion within the OB. Multicell clones contained 2–30 neurons for an average of 6 neurons per multicell clone. With respect to the rostrocaudal axis, the most widely dispersed fully differentiated siblings found within the bulb were separated by 3.8 mm (Table 4, clone 6), over half the length of the OB at P14 (approximately 7 mm). Clone 1 of Table 3 included neurons dispersed over 3.2 mm in the OB as well as glial cells in the region of the anterior olfactory nuclei for a total dispersion of 6.9 mm. On average, multicell clones labeled by E14 injections were dispersed 1.3 mm anteroposteriorly, whereas E17-labeled clones were dispersed an average of 1.8 mm. SVZ cells were ignored in this analysis because their final position in the bulb had not been achieved when the brains were analyzed at P14.

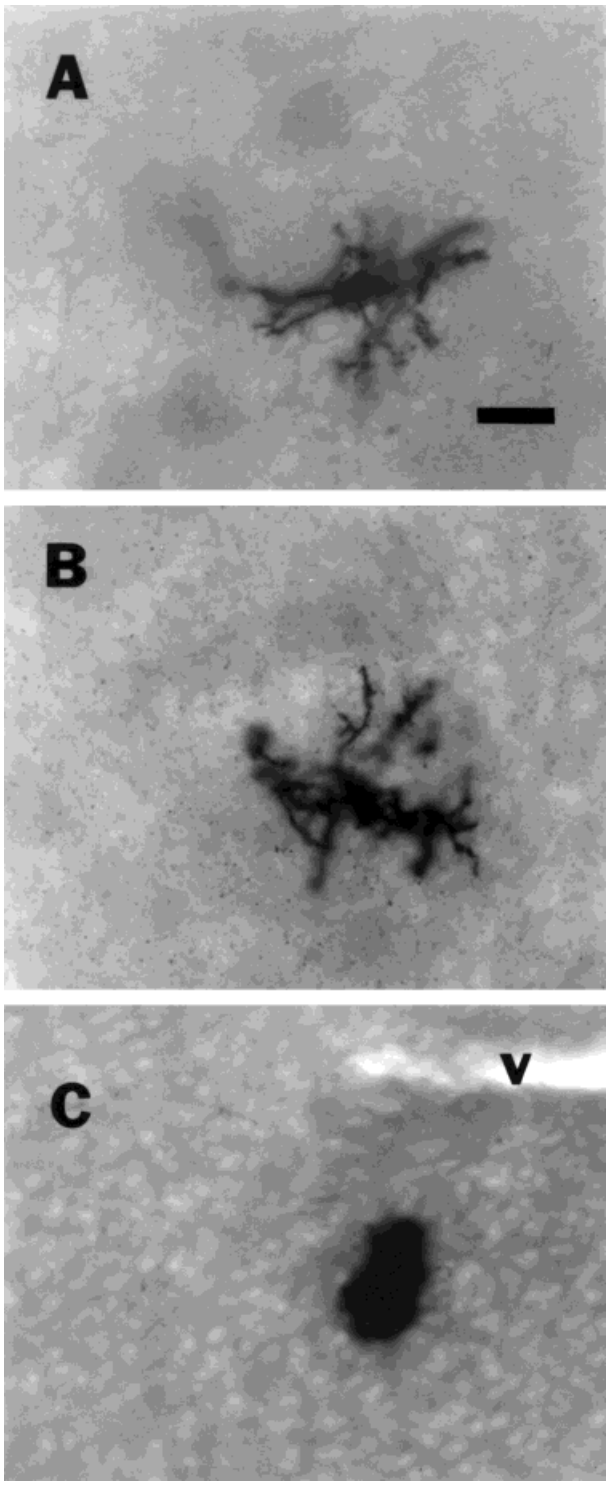

Fig. 4. Photomicrographs of a clone labeled at embryonic day 17 that contained both periglomerular cells (**A**,**B**) and cells in the region of the anterior olfactory nuclei (**C**). The photomicrographs are taken from coronal sections. The cells in the region of the anterior olfactory nuclei are difficult to classify morphologically because of the dense staining, although this dense, dark clustering is generally characteristic of alkaline phosphatase-labeled glial cells (Goldman and Vaysse, 1991; Snyder et al., 1992; Luskin et al., 1993). The clone corresponds to Table 4, clone 21. v, ventricle. Scale bar = 20 μm in A (applies to A–C).

To determine whether there was a systematic spacing of sibling cells within the OB, we examined the rostrocaudal distances between sibling cells. Histograms representing the distribution of sibling spacings did not show any obvious systematic pattern (Fig. 8). Moreover, the distances between sibling cells and between nonsibling cells showed very similar distributions, suggesting that the final position of a labeled neuron along the anteroposterior axis within the OB is apparently random with respect to the positioning of its siblings.

In a similar manner, we investigated whether or not sibling cells tended to migrate along a preferred axis from the SVZ at the bulb core to their final position in the bulb laminae. By measuring the angle (θ) within the plane of the bulb formed by any two sibling neurons (with respect to an origin in the bulb SVZ), we were able to quantify clonal dispersion within a coronal plane. The distribution of angles formed by sibling pairs did not differ significantly from the distribution of angles formed by nonsibling pairs (Fig. 9). In histograms representing the sibling as well as nonsibling distributions of θ, all angles from 0 to 180° were represented in roughly equal proportions (not shown). Thus, it appeared that sibling cells disperse from the bulb SVZ to the bulb layers with noncorrelated trajectories.

Clones dispersing into other forebrain regions

Retroviral injections into the lateral ventricles resulted in labeled cells not only in the OBs, but throughout the developing forebrain. Labeled cerebral cortical cells in these same brains were previously analyzed in separate studies (Walsh and Cepko, 1992; Reid et al., 1995). However, despite one previously observed clone that contained cells in both cerebral cortex and OB (Walsh and Cepko, 1993, Fig. 2f), in the present larger sample we failed to observe clones dispersing between cerebral neocortex and OB, suggesting that clonal dispersion between neocortex and OB is rare or nonexistent (affecting $\leq$1/100 clones in the present study plus Walsh and Cepko, 1993). To determine whether or not OB precursors were related to cells in other regions of the telencephalon, we performed PCR on cells labeled throughout the telencephalon. After E14–E17 injections, 5 of 68 clones in the OB also contained cells in other regions of the telencephalon. Two clones (clone 1, Table 3; clone 21, Table 4) contained OB neurons in addition to glia within the region of the anterior olfactory nuclei (Fig. 4). One other clone (clone 3, Table 2) contained eight apparent mitral neurons in the OB and another cell over 10 mm posterior, in a location deep to the cortex near or within the hippocampus. Another clone (clone 4, Table 3) contained OB granule cells and additional cells, probably glia, far posterior in the lateral forebrain in or near the hippocampus (although the exact location of these cells was impossible to determine because the brain was sectioned tangentially). A third clone contained labeled cells in the glomerular layer, the internal granule cell layer, and another neuron in the pyriform cortex. These clones suggest that bulb precursor cells might share a rare common progenitor with cells in various other regions of the forebrain such as the hippocampus, parahippocampal regions, and pyriform cortex. However, if such widespread dispersion occurs it would be a rare event ($\leq$3 of 68 clones). Furthermore, these few, widely dispersed patterns could also be potentially explainable as spurious "clones" produced by coincidental infection of two separate progenitors

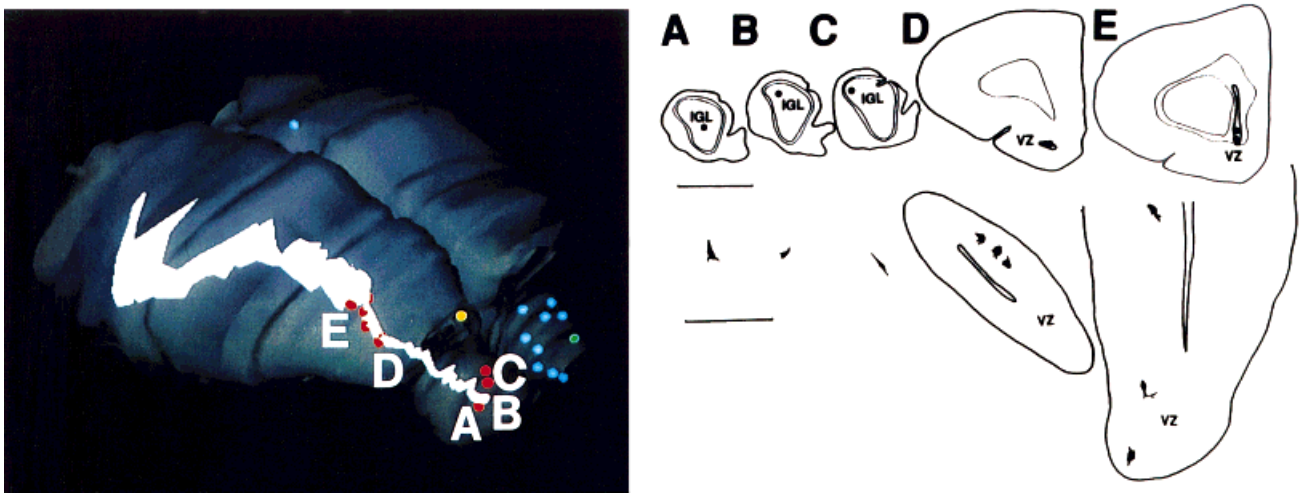

Fig. 5. Three-dimensional reconstruction (left) and camera lucida drawings (right) of a clone containing probably granule cells and cells of the subventricular zone and rostral migratory stream, labeled after injection on embryonic day 15. The figures represents Table 2, clone 5. The left panel shows a three-dimensional reconstruction of a rat's forebrain, viewed from above, rostrally, and from the right. The ventricle is indicated by white shading. Members of the illustrated clone are shown in red-orange and labeled A–E. Camera lucida drawings of the cells are shown in A–E, with corresponding lower power and higher power drawings. A–C indicate that cells are in the internal granule cell layer of the olfactory bulb (OB), but the cells are not highly developed morphologically, suggesting that some of them may still be migratory. D,E show that cells are in the ventricular zone (VZ)/subventricular zone (SVZ) of the OB and lateral ventricle region, indicating the continuity of clones from the SVZ into the olfactory bulb. The blue cells in the left panel represent the clone illustrated in detail in Figure 3, and the yellow and green dots represent two clones, each containing single cells, also present in the same olfactory bulb (see Table 2, Experiments 2 and 3). IGL, internal granule cell layer. Scale bars are as for Figure 3.

by retroviruses carrying the same DNA tag. Analysis of the entire telencephalon (i.e., cortex, striatum, hippocampus, OB, septum) typically entails an experiment with >10 clones and, thus, a significant possibility that two progenitor cells might be coincidentally infected by retroviruses carrying the same DNA tag.

DISCUSSION

In this report, we have used two retroviral libraries to analyze cell lineage in the rat's OB during the period of major prenatal neurogenesis (E14–E17). Our results show that many OB neurons are derived from progenitors that produce multiple cells of a single type; on the other hand, a significant minority of progenitors, especially at early ages, produce neurons of multiple types as well as occasional glia in the region of the anterior olfactory nuclei, suggesting that there may be a progressive restriction of cell fate as development proceeds. In contrast to the frequent phenotypic restriction of clones to a single cell type, clones showed no obvious spatial restrictions within the OB. Progeny of individual precursor cells appear to be scattered across the OB in a pattern not clearly different from random. Finally, despite the widespread clonal dispersion within the OB, we do not find evidence of frequent clonal dispersion between OB and neocortex at the ages examined. Although we cannot completely rule out widespread dispersion at earlier stages, or occasional clonal dispersion between OB and certain other cortical areas such as pyriform or hippocampal cortex, the bulk of neocortex is not involved by clones that generate OB neurons.

Cell types and progressive restriction of cell fate

Comparison of clonal patterns resulting from early infection and late infection strongly suggests a progressive restriction of cell fate as development proceeds. Within the OB, the most common combination of mixed cell types involved granule/periglomerular cell clones. In fact, half of all periglomerular neurons labeled at E14 and E15 shared a common progenitor with granule neurons. Other mixed cell type combinations were observed including a mixed interneuron/projection neuron clone at E15. Clones containing a single cell type were commonly seen in all experiments but were increasingly common as development proceeded from E14–E17. Fifty-eight percent of clones labeled at E14 and E15 were single cell type clones as opposed to 100% of E17 labeled clones. The absence of mixed neuronal cell type clones after E17 injection demonstrates a trend consistent with a transition from multipotential lineages to increasingly limited cell lineages over the course of development. Because cell lineage analysis does not directly test the commitment of precursor cells, we cannot rule out that precursors retain potential to produce a broader array of cell types than that observed after retroviral labeling: precursor cells that normally produce only single cell types might retain the potential, under different environmental conditions, to produce multiple cell types. In fact, because a number of studies (Reynolds and Weiss, 1992; Lois and Alvarez-Buylla, 1994; Craig et al., 1996) suggest that SVZ cells remain multipotential late in life, the apparent progenitor restrictions to cell fate are more likely to be the result of a defined microenvironment acting on multipotential progenitor cells to produce restricted offspring.

Formation of glia in the OB appears to show some differences compared with the adjacent cerebral cortex. In the rat cortex at E15, one of four glial clusters were members of clones that also generated neurons (Reid et al., 1995) but at E14 the proportion is considerably higher (Reid and Walsh, unpublished observations) and in the

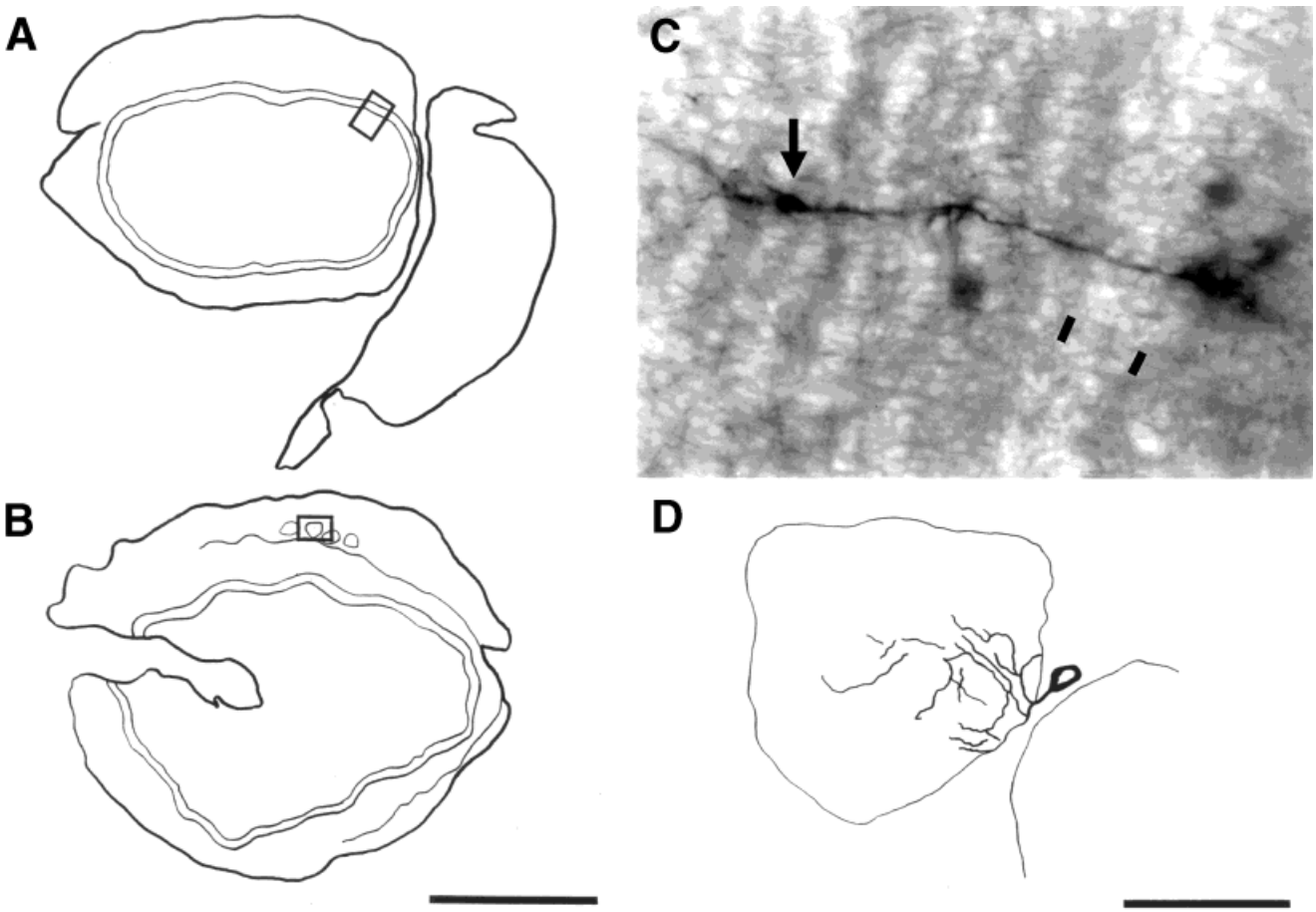

Fig. 6. A clone containing a well-labeled granule cell and periglomerular cell labeled at embryonic day 14 (clone 18, Table 1). **A:** The rectangle indicates the position of a labeled granule cell in a section of the olfactory bulb. The same area is shown at higher magnification in **C**. **B:** The rectangle indicates the position of a periglomerular cell seen at higher magnification in **D**. The thin lines in A and the lines in C indicate the mitral cell layer; the arrow in C shows the granule cell body. Scale bar = 200 μm in B (applies to A,B), 50 μm in D (applies to C,D).

ferret at E33–35, all labeled glia were part of neuronal clones (Reid et al, 1997). In contrast, OB glial cells were only rarely labeled in the present experiments (less than five glial clusters total), even when injections were made early in bulb development, and the only glia that were labeled were located outside of the OB proper. This observation confirms Luskin's finding (1993) and has several possible interpretations. The first possibility is technical, that OB glial cells selectively inactivate the retrovirally encoded marker gene, as some inactivation was detected in the bulb in these experiments by PCR. However, a second possibility is that OB neurons and glia arise from distinct sets of progenitors, and that OB glia arrive in the OB from a distinct source that has not yet been identified. For example, a small and distinctive source for most of the oligodendrocytes in the optic nerve has recently been identified (Ono et al., 1997), and an analogous point source for glia may exist for the OB and be labeled with retroviruses only under specific circumstances not used in these experiments. SVZ progenitors may produce glial cells as well as neurons, but those labeled glia may migrate to sites other than the OB.

Clonal dispersion and clonal restriction in the OB

Although most OB clones were restricted as to the cell types formed by a single progenitor, there was no clear topographic relationship between sibling neurons in the bulb. Cells within individual clones appeared to disperse randomly with respect to their siblings. This observation is consistent with previous retroviral studies (Luskin, 1993), and with observations of the migrating neurons in the SVZ and rostral migratory stream, because neurons appear to move along each other and do not maintain topographic relationships (Doetsch and Alvarez-Buylla, 1996; Lois et al., 1996; Doetsch et al., 1997; Wichterle et al., 1997). In contrast, no evidence was seen for clonal dispersion between OB and neocortex, because no clones contained neurons in both structures. Recently, there have been several suggestions of such widespread dispersion between cortex and OB-related structures, OB and pyriform regions (de Carlos et al., 1996), or the lateral ganglionic eminence SVZ (from which OB precursors presumably originate) and neocortex (Anderson et al., 1997; Brunstrom et al., 1997). We cannot rule out dispersion between

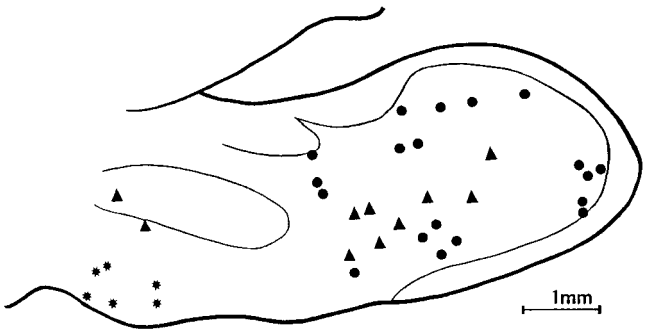

Fig. 7. A large clone labeled at embryonic day 15 containing apparently multiple cell types. The cells shown are all part of one polymerase chain reaction-defined clone labeled with the βgal library. The clone contains glia in the region of the anterior olfactory nuclei (asterisks), small cells in the subventricular zone (SVZ) and rostral migratory stream (triangles), and small cells in the internal granule cell layer representing presumptive granule cells (circles). The clone is viewed from a series of tangential sections taken through the olfactory bulb more or less parasagittally, and the cells are represented on a single section taken near the middle of the series; therefore, the architectural relationship are not preserved completely in three dimensions. Dorsal is up. Thin lines indicate the approximate location (in the section on which the cells were plotted) of the SVZ and the glomerular layer.

TABLE 5. Average Clone Size[1]

	Embryonic day		
Parameter	14	15	17
All clones (average)	2.3	8.9	2.2
All clones (median)	1.5	4	2
Multicell clones (average)	3.1	9.8	3.4
Multicell clones (median)	3	6	2
Maximum size	10	36	8

[1]Comparison of clone size resulting from AP and β-gal clones at embryonic day 14 (E14), 15, and 17. The average number of labeled neurons in E15-labeled clones was approximately fourfold greater than the average number of neurons in clones labeled at E14 or E17, although the median number of neurons labeled at E15 was only about twofold higher than the median number of neurons within a clone labeled at either E14 or E17 suggesting that E15 clones varied more widely in size.

TABLE 6. Clonal Composition After Labeling at Embryonic Days 14–17[1]

Embryonic day	% Granule cell (G) ratio of G to P clones	% Granule/ Periglomerular (G/P) ratio of G/P to P	% Periglomerular (P)
14	68.4	10.5	10.5
	6.5	1	
15	46.6	20	13.3
	3.5	1.5	
17	60.7	0	35.7
	1.7	0	

[1]Comparison of clonal composition at embryonic day (E)14, E15, and E17. The percentage of clones represented by each interneuron clone type, granule cell (G), periglomerular (P), or mixed (P/G), is indicated along with its ratio to the number of P clones. G/P clones were observed more commonly after injections at E15 (20% of clones) than at E14 (10.5%) or E17 (0%). Similarly, there is a dramatic shift in clonal composition from 6.5 granule cell clones per periglomerular clone to 3.5 to 1 at E15 and to 1.7 to 1 at E17, suggesting a gradual transition away from predominantly strict granule cell clones early in bulb development to more mixed and periglomerular cell clones at E15. Later, at E17, a variety of single cell type clones are apparent.

OB and neocortex, or between OB and other telencephalic regions, particularly given the occasional occurrence of cells with the same DNA tag in OB and pyriform or hippocampal regions. However, widespread dispersion is too rarely seen to establish definitively without using a more complex retroviral library (Golden et al., 1995).

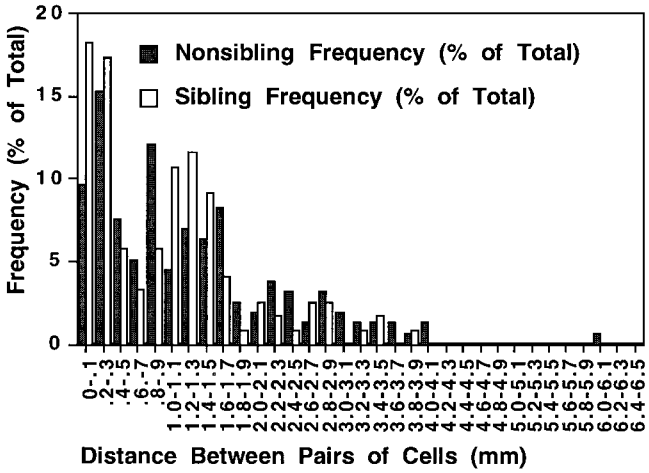

Fig. 8. Histogram of rostrocaudal spacings between sibling cells and between nonsibling cells (i.e., cells in different clones). Intersibling distances were measured as described in the text and previously (Reid et al., 1995, 1997). There were no obvious differences in the spacings between sibling cells and nonsibling cells, suggesting that rostrocaudal distribution of sibling cells with respect to one another is largely random.

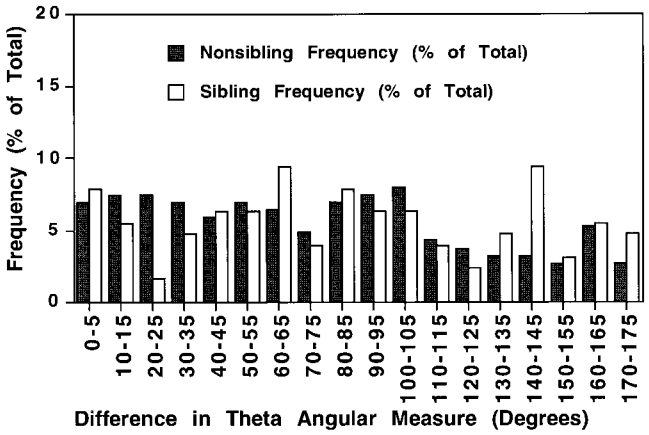

Fig. 9. Histogram of differences in θ(Δθ) between sibling cells and nonsibling cells, where θ represents the angle that the cell was located away from a line oriented directly ventral. The distribution of Δθ between sibling cells and between nonsibling cells was not obviously distinguishable, suggesting that sibling cells distribute essentially random around angle from 0 to 360° as they exit the subventricular zone.

OB progenitors most likely reside in the SVZ outside of the bulb

Although injection of retrovirus into the lateral ventricle, as done in these experiments, does not identify the precise locus of the progenitor cell labeled by the retrovirus, our data nonetheless confirm the earlier suggestion that the stem cells for the OB lie outside of the OB and that the cells in the rostral migratory stream may already be limited to a neuronal fate (Luskin, 1993; Lois and Alvarez-Buylla, 1994; Menezes et al., 1995; Doetsch et al., 1997; Luskin et al., 1997). One line of evidence supporting this is that lateral ventricle injections did not label OB glia at all, suggesting that OB progenitors were already limited to a

neuronal fate. Second, the small minority of clones that included multiple cells types (e.g., neurons in the OB and glia in the region of the anterior olfactory nuclei, or neurons elsewhere) also labeled cells outside of the OB proper. These data suggest a simple model in which the cells that generate OB neurons lie outside of the OB, presumably in the SVZ near the striatum where stem cells have previously been observed (Craig et al., 1996). Cells of the rostral migratory stream would be expected to be then limited to neuronal fate and appear even to become progressively limited to either granule cell or periglomerular fate as development proceeds. This model is quite consistent with data from electron microscopy reconstruction of the SVZ, which suggests the existence of a morphologically distinct cell type, thought to correspond to a potential stem cell, that is present in the SVZ but not in the rostral migratory stream (Doetsch et al., 1997). The SVZ near the striatum also appears to be the best source for bipotential cells that can make neurons and glial cells (Reynolds and Weiss, 1992; Lois and Alvarez-Buylla, 1993).

Retroviral injections at E15 labeled unusually large clones, and a surprisingly higher proportion of clones containing multiple cell types, compared with labeling at E14 or E17. The reasons for this finding are not clear. There is no obvious technical explanation, because larger E15 clones were observed with two different retroviral libraries and two different litters. However, it may also be that at E15 (as the bulb is extending outward from the rostral telencephalon) there is an unusual accessibility of the precursors that generate large clones, so that for whatever reason the precursors of large clones are easy to label at E15, but more difficult to label at E14 or E17. Thus, the failure of labeling of large clones after E17 injection may not necessarily suggest that these precursors are not present (indeed evidence from other labs suggests that "stem cells" persist throughout life in the SVZ) but instead may just mean that the precursors of large clones are either not accessible to the ventricle where the virus is placed, or not dividing rapidly so as to be easily infected by retroviruses.

The extent of progenitor dispersion within the SVZ is not certain. Originally, the SVZ was conceived of as a specialized source for neurons of the OB, hence the term anterior SVZ or aSVZ (Luskin, 1993; Menezes et al., 1995; Zigova et al., 1996; Luskin et al., 1997). However, recent histologic and ultrastructural studies have suggested that the characteristic SVZ structure extends over a much larger rostrocaudal extent in relationship to the striatum, though not the cerebral cortex (Lois and Alvarez-Buylla, 1994; Rousselot et al., 1995; Doetsch and Alvarez-Buylla, 1996; Lois et al., 1996; Doetsch et al., 1997). Given the persistent neurogenesis in the OB and in the hippocampus, it is possible that the rostrocaudally extended SVZ may feed both structures. The rare clones that appear to contain neurons in relationship to the OB and near the hippocampus support this intriguing suggestion.

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