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# Clonal dispersion and evidence for asymmetric cell division in ferret cortex

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## SUMMARY

**Cell lineage analysis with retroviral libraries suggests that clonal progeny disperse widely in rodent cortex. To determine whether widespread dispersion is a general mammalian plan and to investigate phylogenetic differences in cortical development, we analyzed cell lineage in the ferret, a carnivore and near relative of the cat. The ferret possesses a highly developed, folded cerebral cortex, characteristic of higher mammalian species. Progenitor cells of the ferret cerebral cortex were tagged with an amphotropic retroviral library encoding alkaline phosphatase, and sibling relationships were determined using the polymerase chain reaction. Neuronal clones were single neurons (52%) or large clones (48%; average, 7 neurons) containing neurons and glia in widespread cortical locations. Neuronal clones in the ferret labeled at middle to late neurogenesis (embryonic day 33-35) contained large numbers of neurons and showed little tendency to cluster. The large proportion of single neuron clones, contrasted with the large size of multicell clones, suggests that some progenitors divide asymmetrically, producing a postmitotic neuron and regenerating a multipotential cell.**

Key words: cell lineage, asymmetric, cerebral cortex, protomap, protocortex

## INTRODUCTION

The mammalian cerebral cortex shows striking cellular diversity, precise laminar stratification and a functional organization that reflects species-specific behavior. While anatomical studies have uncovered the gross morphological changes associated with forebrain development (Bayer and Altman, 1991), they have left many unanswered questions about the cellular and molecular processes that generate the structure most responsible for cognition and complex behavior. The cerebral cortex varies tremendously in size, shape and function among mammalian species. Not only do the absolute and relative sizes of cortical regions vary between species, but local cortical architecture – cell number and density, cell type and intrinsic synaptic connectivity – varies as well.

In all mammals, cortical neurons and macroglia derive from an apparently homogenous pseudostratified neuroepithelium (Boulder-Committee, 1970). Early in cortical development, progenitor cells in all species appear to undergo mainly symmetric cell divisions leading to extensive cellular proliferation (Chenn and McConnell, 1995; Takahashi et al., 1993, 1994) before progenitors divide to form postmitotic neurons of the cerebral cortex in an orderly 'inside-out' sequence (Angevine and Sidman, 1961). While all mammalian species appear to show similar overall neurogenetic patterns, one grossly apparent difference between species is the length of neurogenesis, which increases from approximately 8 days in the rat (E13 to E21) (Bayer and Altman, 1991) and 34 days in ferret (E20-P14, where P0=E42) (Jackson et al., 1989) to 60 days in the macaque monkey (Rakic, 1974).

In an attempt to understand the relationship between the activity of progenitor cells in the germinal zone and the final organization of the adult cortex, the behavior of single cortical progenitors has been investigated using replication-incompetent retroviruses (Sanes et al., 1986; Turner and Cepko, 1987), which mark cortical cells in clonal fashion. In the rodent cortex, retroviral lineage analyses revealed loose cell clusters scattered over approximately 500 μm, containing morphologically similar neurons (Luskin et al., 1993; Mione et al., 1994; Parnavelas et al., 1991) or glia (Grove et al., 1993; Luskin et al., 1993). However, when cortical clones were analyzed using libraries of retroviral vectors, so that sibling relationships could be determined by PCR, the clusters of labeled cells frequently were seen to represent subunits of widespread clones dispersed throughout large parts of the cerebral cortex (Walsh and Cepko, 1992; Reid et al., 1995).

In contrast to rodents, clonal studies in primates, which display a more precise physiological organization in the cortex, have suggested a more strict pattern of radially oriented clonal migration from the ventricular zone to the cortex, without the widespread clonal dispersion seen in rodents (Kornack and Rakic, 1995; Rakic, 1972). On the other hand, video microscopy (O'Rourke et al., 1992) and histological studies (O'Rourke et al., 1995, 1997) have shown that large numbers of cells take nonradial paths from the germinal zone to cortical plate (O'Rourke et al., 1992) in the developing ferret cortex,

although the ultimate fate of the nonradially migrating cells is not known. The ferret, a member of the order Carnivora, is widely used in studies of cortical neurogenesis (McConnell, 1988; McConnell and Kaznowski, 1991). Although ferrets are most closely related to dogs and bears, ferret and cat brains develop, over a very similar time course, to a similar size and morphology.

In order to test whether widespread clonal dispersion is a general feature of mammalian development, we studied cell lineage in the ferret. The present study demonstrates that the cerebral cortex of the ferret is produced at least in part by multipotential progenitor cells that give rise to clones spanning multiple functional domains. Surprisingly, single neuron clones constitute a large proportion of clones, suggesting that many cortical neurons labeled at E35 and E35 are formed by the asymmetric division of multipotential progenitor cells.

## MATERIALS AND METHODS

### Preparation and characterization of an amphotropic retroviral library

A new amphotrophic retroviral library encoding alkaline phosphatase (AP) was prepared from an ecotropic AP-encoding library previously described (Reid et al., 1995; Walsh, 1995). CR7 amphotrophic producer cells were infected with the ecotropic DAP library. CR7 is a subclone of the ψCRIP amphotropic producer cell line (Danos and Mulligan, 1988), engineered to preclude formation of recombinant helper virus. After infection, >20,000 colonies of retroviral producer cells were selected by growth in G418-containing medium. The supernatant from confluent cells was harvested and showed a titer of $2\times10^6$ cfu/ml on NIH 3T3 cells. The supernatant was concentrated 50- to 100-fold by centrifugation (Cepko, 1992) and stored at −80°C.

Accurate determination of sibling relationships with retroviral libraries requires that the library contain large numbers of retroviruses with different DNA tags present at relatively equal titers (Walsh et al., 1992). The rodent ecotropic library was prepared from approx. 3400 plasmid constructs, and contained 250-400 tags present at equal titers, so that experiments with $\leqslant 4$ clones provide a $P<0.05$ probability that two clones coincidentally contain the same tag (Reid et al., 1995). The composition of the amphotropic library was assayed by comparing tags amplified from 29 labeled clones in five different experiments. No repetitions of any tag were seen between different experiments, suggesting that the complexity of the retroviral library was maintained during the repackaging.

### Animal surgery

Timed-pregnant ferrets, purchased from Marshall Farms (North Rose, NY), were housed and handled according to approved protocols. Pregnancies were timed from the day after breeding (E0). Birth usually occurred on E42. Pregnant mothers were anesthetized with Nembutal or inspired halothane. The uterus was exposed by a midline incision and transilluminated to facilitate identification of fetal skull landmarks. Concentrated AP virus (2-8 μl) containing 80 μg/ml Polybrene (Sigma) and 0.05% trypan blue was injected through the uterine wall and fetal membranes into the lateral ventricles of each fetus using pulled glass capillary pipettes (Drummond Instruments). The accuracy of the injection was monitored by direct observation, since the trypan blue causes the ventricular system to appear blue. Incisions were closed with suture and staples.

### Histology and analysis of clones

Animals were killed 14-26 days after birth by an overdose of Nembutal and perfused with 2-4% paraformaldehyde in 2 mM $MgCl_2$, 1.25 mM EGTA in 0.1 M PIPES buffer (pH 7.2). The brains were removed and submerged in fixative overnight at 4°C, then transferred to 30% sucrose in phosphate-buffered saline (PBS) at 4°C until they sank. Brains were sectioned at 100 μm thickness using a Bright cryostat. Sections were mounted onto gel-coated glass slides and processed for AP activity as described (Cepko et al., 1993). Labeled cells were detected by microscopic examination of tissue sections, and cell morphologies and locations were recorded by photography and/or camera lucida drawings.

### Corrected cell counts

Average numbers of cells in layers I-VI in several cortical regions were taken from studies in the cat (Beaulieu and Colonnier, 1989). A weighted average of area-specific counts was determined. Relative sizes of major cortical areas were determined from a two-dimensional map of the cat cortex showing visual, somatosensory and motor areas (Van Essen and Maunsell, 1980, Fig. 12) by cutting out each cortical area and weighing it on a balance. For cortical areas in which specific counts were not available, counts of PMLS (association cortex) were used. Varying relative contributions of cortical areas had little effect on the numbers, since layer-specific counts do not vary greatly between cortical areas, or between different species (Rockel et al., 1980). Final correction factors were 1.4, 26, 10, 5 and 13 for layers I-VI, respectively. Cell counts in subplate were not corrected because appropriate numbers were not available.

### Clonal analysis with PCR

Tissue analysis was performed by preparing DNA samples from labeled cells for amplification by PCR, as presented elsewhere (Walsh and Cepko, 1993, 1992). Coverslips were removed in a 50 ml centrifuge tube filled with sterile water. Small tissue fragments (approximately 100×200×200 μm) containing the nucleus of each labeled cell were dissected using a fresh razor blade edge for each cell. Tissue fragments were digested in 10 μl of proteinase K (0.2 mg/ml) in 1×PCR buffer (2.5 mM MgCl2, 50 mM Tris buffer, pH 8.3, 25 mM KCl) with 0.5% Tween-20 at 65°C for 4-24 hours. Each well was covered with 30 μl of mineral oil to prevent evaporation. Samples were then heated to 85°C (20 minutes) to inactivate proteinase K and then to 95°C for 5 minutes to denature the DNA. A nested PCR protocol was employed in order to increase the sensitivity and specificity of amplification and is described elsewhere (Walsh and Cepko, 1992; Walsh, 1995). At least 10% of all PCR reactions were negative controls. No experiments showed false PCR-positives.

### Analysis of PCR products

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

## RESULTS

Ventricular zone progenitors were infected by injection of 2-8 μl of the AP retroviral library into the lateral ventricles of fetal ferrets at embryonic day 29 (E29), E33, E35 and P0 (postnatal day 0). Injected animals were allowed to develop normally and their brains were subsequently harvested for analysis at postnatal day 14 (P14) and P16, after cortical neurons are postmitotic but while some neurons are still migrating to the cortex (Jackson et al., 1989), or at P26, when neuronal migration is complete. AP-staining of cell bodies and processes allowed morphological identification of >85% of labeled cells as neuronal or glial by standard morphological criteria (Fig. 1).

Neurons and astrocytes were labeled in large numbers, while most oligodendrocytes are formed late, and therefore were labeled only after late injections (P0). The morphologies of retrovirally labeled neurons did not appear to be altered by AP expression since those neurons displayed morphologies similar to those described in Golgi studies (see Fig. 1).

### Pattern of cortical labeling

While virtually all cells demonstrated intense staining of the soma and cellular processes, labeled cells did vary in their degree of differentiation. Some labeled cells in the subcortical white matter (WM) matched the profile of the classic migratory neuron (Fig. 1D). These cells were well stained and possessed large cell bodies with what appeared to be leading and lagging cell processes (Rakic, 1972). Most of these cells were probably destined for the superficial layers of the cerebral cortex (Jackson et al., 1989). Astrocytes occurred singly or as densely packed clusters with intensely stained and overlapping processes, just as they did in the rodent (Reid et al., 1995). The dense staining often precluded precise determination of glial cell number.

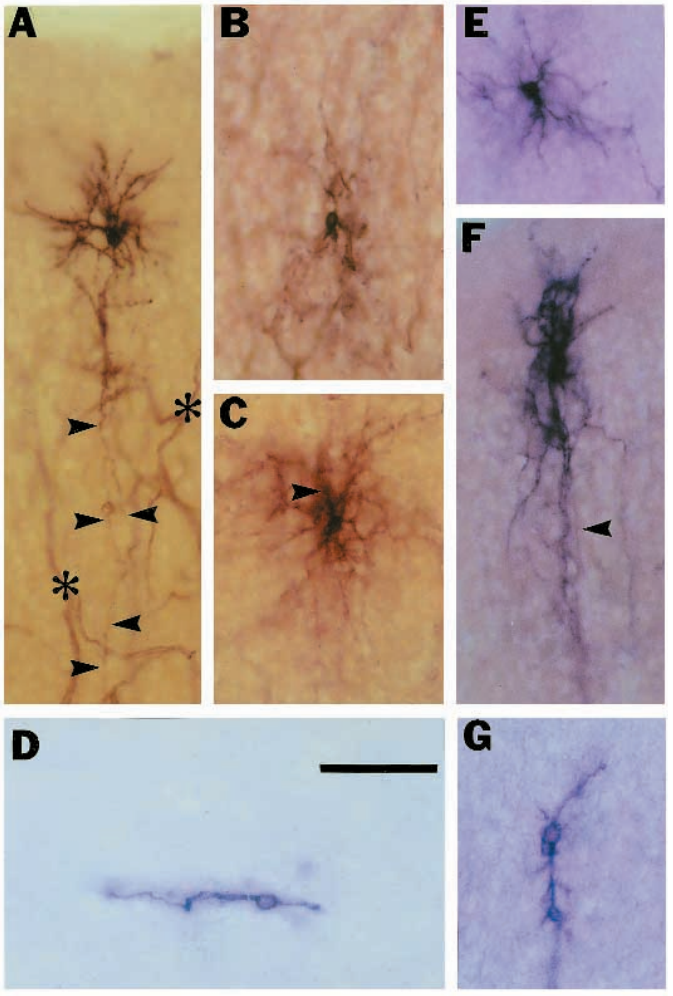



**Fig. 1.** Retrovirally encoded AP labeled many morphological types of neurons, suggesting that retroviral infection did not alter normal cellular development. (A-C) Cerebral cortical neurons labeled at E29 and analyzed at P21; (D-G) cells labeled at E33 and analyzed at P16. (A) A neuron at the top of layer II, with its axon descending to deeper cortical layers (arrowheads). Background staining of blood vessels (asterisks) was eliminated in later experiments by heat treatment. (B) A stellate neuron in layer IV. (C) A large pyramidal neuron in layer V with its apical dendrite severed at the edge of the section (arrowhead). (D) A tangentially migrating cell (presumably a neuron) within the cortical white matter, which displays leading and lagging cell processes. (E) A stellate cell in layer II. (F) A Martinotti neuron in layer II, with its axon descending to deeper cortical layers (arrowhead). (G) A bipolar cell in layer III. Its ascending and descending processes are clearly visible in lower power photographs. The scale bar, 100 μm.

Histological analysis of labeled neurons showed an expected distribution across cortical layers consistent with the 'inside-out' pattern of cortical neurogenesis (Jackson et al., 1989). Injections at all prenatal ages labeled relatively large numbers of the youngest neurons (layer II), while earlier injections labeled progressively larger proportions of early born, deep cortical plate and subplate neurons (Fig. 2A).

To assess whether the retroviral library preferentially labeled certain cell types or certain progenitor types, the counts of labeled cells were corrected for the fact that cortical laminae do not contain equal cell numbers. Although no quantitative data were available specifically for the ferret, many mammalian species (e.g. rat, cat, monkey) show similar cell ratios between cortical layers (Rockel et al., 1980). Therefore, the neuronal cell counts were adjusted using numbers from the cat since careful counts have been obtained in this species (Beaulieu and Colonnier, 1989). We derived a weighted average of cells in each layer per cortical column by averaging area-specific counts according to published estimates of the relative sizes of cortical areas (Van Essen and Maunsell, 1980). After correction (Fig. 2B), a remarkably similar proportion of cells in most cortical laminae were labeled by injection at E29. The corrected counts of labeling in different layers imply that the AP retrovirus labeled cortical progenitors that produce cells in most cortical layers in an unbiased manner.

### Spatial distribution of retrovirally labeled cells

Although PCR analysis of DNA tags provides more definitive information about clonal relationships, the distribution of retrovirally labeled neurons gives general information about whether labeled cells cluster, and has been widely used in the past to infer clonal relationships. We observed clusters composed typically of two neurons within the same cortical layer following injections at E29 and morphological analysis at P26. 51% of cortical neurons in E29-labeled brains were members of discrete (usually two-cell) clusters. In contrast, neurons labeled at somewhat later stages of development (E33 and E35) rarely (<5%) occurred within 500 μm of one another in the same histological section. In addition, E33- and E35-labeled neurons occurring within the same section were typically widely separated mediolaterally, and often in different gyri. One cluster in the ferret (1/17 clusters in three experiments) formed a radial array after E29 (Kornack and Rakic, 1995). No such radial clustering occurred after E33 or later injections.

### Clonal analysis by PCR

PCR amplification was successfully performed on five cerebral hemispheres labeled at E33 (4) or E35 (1), representing 25 neocortical clones. In each experiment, PCR was successful for

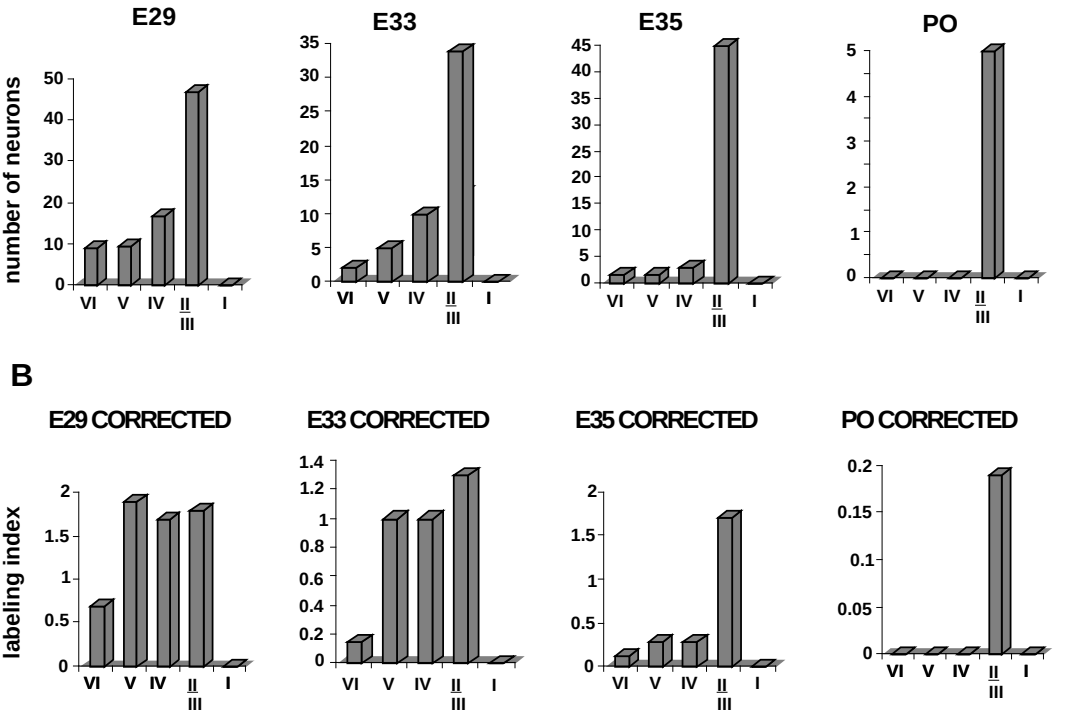



**Fig. 2.** (A) Histograms of labeled neurons in each cortical layer of adult ferret brains following injections at E29, E33, E35 and P0. Retroviruses label the older and newer progeny of infected cells. Thus earlier injections labeled neurons in all cortical layers, while progressively later injections labeled an increasingly restricted population of neurons in superficial layers. The deeper cortical layers also contain fewer neurons than the upper layers, exaggerating this effect. The laminar patterns of retrovirally labeled neurons are consistent with the inside-out pattern of neurogenesis, suggesting that the behavior of infected cortical progenitors is not obviously different from the behavior of unlabeled cortical progenitors.
(B) Corrected histograms of retrovirally labeled cells show remarkably equivalent labeling of neurons formed after the time of the injection. Corrected 'labeling index' (arbitrary units) represents the number of retrovirally labeled cells in each layer divided by the average number of cells in that layer per cortical column. The efficiency of labeling was remarkably similar in layers formed after viral infection (Jackson et al., 1989), suggesting that retrovirally labeled progenitor cells accurately reflect the behavior of cortical progenitor cells as a whole. Subplate neurons were also labeled by E29 injections, but are not illustrated because appropriate corrections are not available.

45-66% of retrovirally labeled tissue samples. Since amplification was less than 100% successful, the number of sibling cells per clone, the dispersion of sibling cells and the diversity of cell types within clones are likely to be underestimated.

52% of the neuron-containing clones labeled at E33 and E35 contained only a single cell. These clones represented 13/97 PCR-positive neurons. None of the single cell clones labeled at E33 and E35 were members of obvious cell clusters in which PCR-positive cells adjoined PCR-negative neurons, suggesting that many of these clones represent true 'single cell clones.' Nine of the 13 neurons in single cell clones resided in layers II or III and four of the nine were clearly pyramidal. Three more clones consisted of lone migrating cells (presumably neurons) still within the cortical WM. The remaining single cell clone involved a layer IV neuron. Single cell clones were located throughout the AP axis with no obvious preference for any cortical region.

## Clones containing multiple neurons

The vast majority of neurons labeled by injections at E33 and E35 (>85%) were members of multicell clones. These clones contained 2-17 neurons for an average of seven neurons per multicell clone, with 4/12 clones containing glial cells as well. The least dispersed cortical clone, clone 2, measured 1.6 mm in the anterioposterior (A-P) dimension, while the most widely dispersed clones (17 and 22) attained dimensions of 15.2 and 16.7 mm, close to the entire A-P length of the ferret cortex at P16 (approximately 19 mm). On average, multicell clones were dispersed over more than 40% of the rostrocaudal dimension of the cortex (Figs 3, 4). In addition, 4/12 multicell clones were dispersed over more than 85% of the mediolateral extent of the cortex (Fig. 5).

Given the large dispersion of multicell clones labeled by E33 and E35 injections, it is not surprising that clones freely spanned anatomical and functional boundaries, contributing cells to cortical areas devoted to very different functions. This was true of all clones containing multiple neurons. The unpredictable pattern of dispersion is represented graphically in Fig. 4A. The clone shown in green, clone 17 (Fig. 4A), for instance, contributed cells to prefrontal, motor, somatosensory and visual cortices. It is dispersed 15.2 mm along the A-P axis, 5.3 mm mediolaterally and 7 mm in the superior-inferior dimension. Clone 20 (Fig. 4A, blue), was represented in five functional regions: prefrontal, motor, somatosensory, auditory and visual, and spanned 10.3 mm in the A-P dimension and 3.5 mm in both the mediolateral and superior-inferior dimensions. A third clone, clone 15 (Fig. 4B, red), contributed cells to somatosensory and auditory cortices. Clone 15 dispersed 5.1 mm rostrocaudally, and spread 4.4 mm mediolaterally and 3.1 mm in the superior-inferior plane. Finally, clone 22 (Fig. 4B, yellow) encompassed cells that were present in several cortical regions including prefrontal, motor, somatosensory, auditory and visual cortex. Clone 22 measured 16.7 mm (A-P), 5.3 mm (M-L) and 4.4 mm (S-I). The A-P dispersion of all multicell clones is summarized in Fig. 3.

Four multicell clones extended less than 4 mm rostrocaudally (clones 2, 3, 12 and 14) (see Fig. 3), but dispersed more widely in the mediolateral plane than along the anterio-

posterior axis. Clone 12, for instance, dispersed 6 mm mediolaterally compared to 3.7 mm along the A-P axis, but nevertheless contained cells in frontal, motor and olfactory cortex (Fig. 5). Clone 19 (neocortex and hippocampus), as well as clone 1 (neocortex and olfactory cortex), also contained neurons in both neocortex and allocortex, suggesting that the border between these regions does not restrict clonal mixing. In summary, we find no evidence for confinement of any multineuron clones within even broadly defined functional boundaries.

In order to analyze clonal dispersion more quantitatively, we prepared a frequency histogram of the distances measured between related cells (i.e. those that contained the same DNA tag). We compared the distances between sibling cells to the distances measured between unrelated, retrovirally labeled cells (i.e. those containing different tags). In previous rat studies, we noted that sibling cells were seen to be distributed nonrandomly within the cerebral cortex. An analogous histogram generated from all ferret multicell clones (Fig. 6A) appeared nonrandom in only one respect: there was an almost twofold increase in A-P 'clustering' among related cells compared to unrelated retrovirally labeled cells. More than 15% of all intersibling spacings occurred at <500 μm versus 8% of nonsibling spacings. Except for the A-P 'clustering' effect (which, as noted above, often reflected cells in nearby sections at widely different M-L locations), the distribution of intersibling cell spacings in the ferret at E33-35 did not differ significantly from the distribution of nonsibling intercell spacings (Fig. 6B) or from a theoretical distribution based on a model of simple diffusion (not shown).

Although the pooled set of intersibling measurements of all clones did not show evidence for preferred spacings of sibling cells, four individual clone-specific histograms showed distributions of intersibling distances that hinted at nonrandom spacings similar to those seen in the rat. Two clone-specific histograms differed from a uniform distribution at $P$=0.0001 (clone 17) and $P$=0.003 (clone 24) according to a chi-square analysis (Fig. 6C,D). Clone 17 is also illustrated in the three-dimensional reconstruction in Fig. 4. The absence of nonrandom intersibling distances derived from pooled measurements of all clones may reflect different periodicities amongst individual clones. Alternatively, individual clones may only show apparent periodic spacings because PCR was successful for less than 100% of labeled cells.

### Cell phenotypes in multicell clones

PCR-defined clones labeled by E33 and E35 injections contained multiple cells displaying a variety of neuronal morphologies, often located in different layers. Most of the PCR-positive neurons were found in layers 2 and 3, since these are the last layers generated during development and they also contain relatively more cells (Fig. 5). On the other hand, sufficient neurons were labeled in deeper layers, and enough glial cells were also labeled, to demonstrate clearly that the progenitors infected at E33-E35 were multipotential (Fig. 3). However, we noted no consistent pattern of clonally related superficial and deep layer cells with respect to their position along the anterioposterior or mediolateral axis (Moore and Price, 1992). In the same manner, glial cells did not distribute preferentially with respect to neurons.

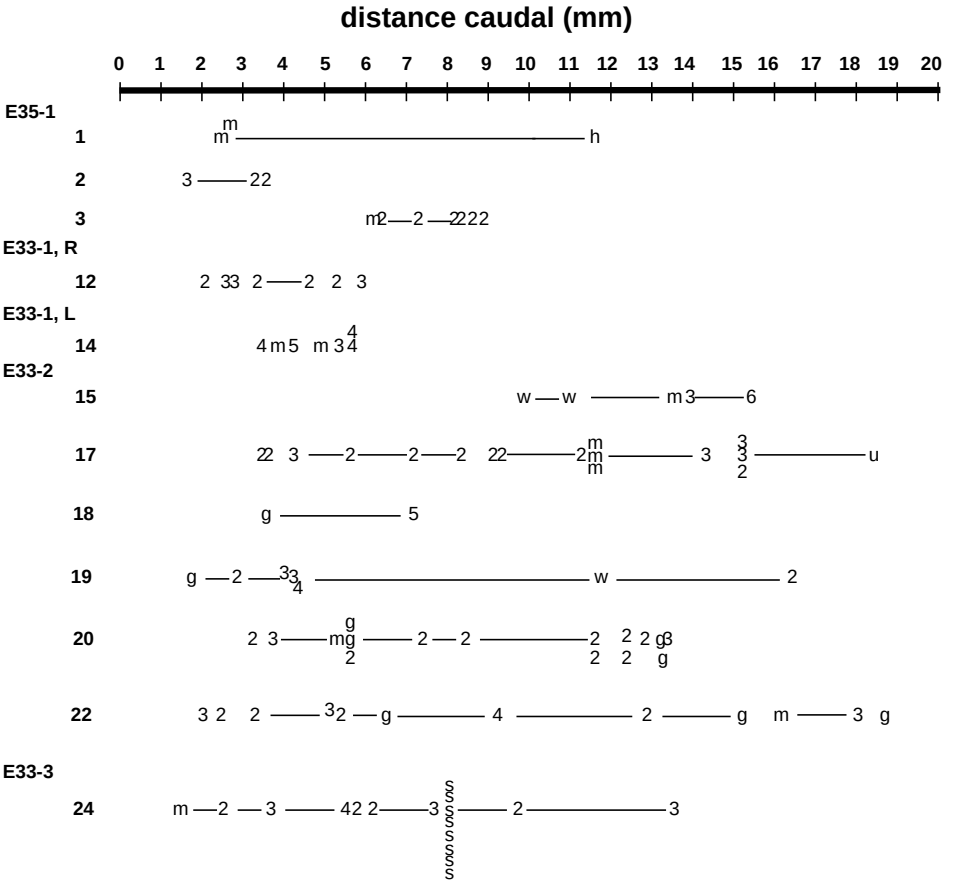



**Fig. 3.** Topographic and morphologic analysis of 12 multicell cortical clones labeled with the AP retroviral library at E33 and E35. Clones are from five experiments, but only four brains, since separate hemispheres of the same brain were considered separate experiments. Each cell or glial cluster has been plotted along the anterioposterior axis shown (in mm) at the top of the figure according to its location in the adult cortex. The origin represents the rostral tip of the olfactory bulb. Morphology of cells was determined by alkaline phosphatase staining alone, allowing definition of neuronal versus glial identity in >80% of labeled cells. The numbers indicate the cortical layer (2-6) in which a given neuron resides. g, glial clusters (almost exclusively astrocytes); h, hippocampal neurons; u, cells of unknown identity in the cortical plate; m, migrating cells that are probably neurons; w, unidentified white matter cells; s, subventricular zone cell.

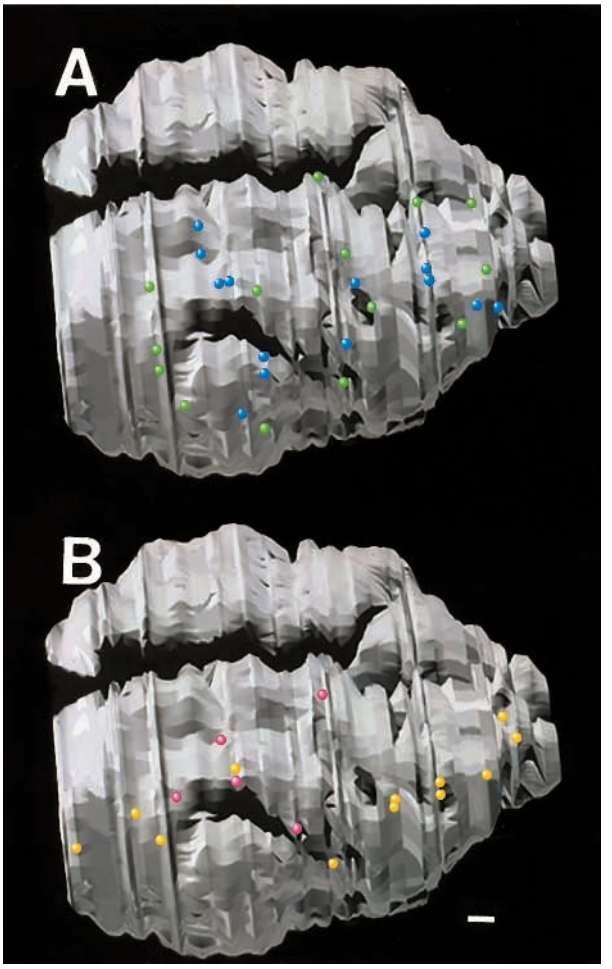



**Fig. 4.** (A) Three-dimensional reconstructions of two large clones from experiment E33-2. The computer-generated reconstruction of the ferret cerebral cortex is shown in gray. It is viewed from the right side and slightly from behind, and is oriented with the rostral end, including olfactory bulbs, pointing towards the right. Clone 17 (green) contributes cells to the prefrontal, motor, somatosensory and visual areas. This clone is dispersed 15.2 mm along the A-P axis and 5.3 mm mediolaterally. Clone 20 (blue) contributes cells to the prefrontal, motor, somatosensory, auditory and visual cortices. It is dispersed 10.3 mm along the A-P axis and 3.5 mm mediolaterally. Major sulci can be seen by shadowing (compare Fig. 5 A,B). (B) Three-dimensional reconstructions of two additional multicell clones from experiment E33-2. Clone 22 (yellow) contributes cells to motor, prefrontal, somatosensory, auditory and visual areas. This clone is dispersed 16.7 mm in the A-P axis and 5.3 mm mediolaterally. Clone 15 (red) contributes cells to auditory and somatosensory cortices. The cells of this clone disperse 5.1 mm along the A-P axis and 4.4 mm mediolaterally. Scale bar, 1 mm.

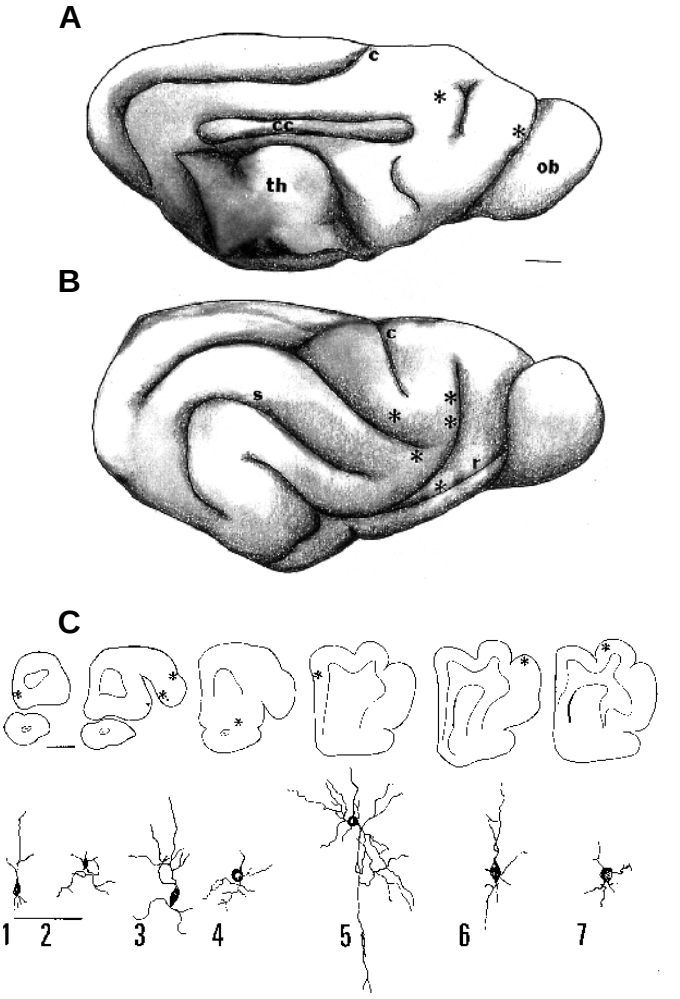
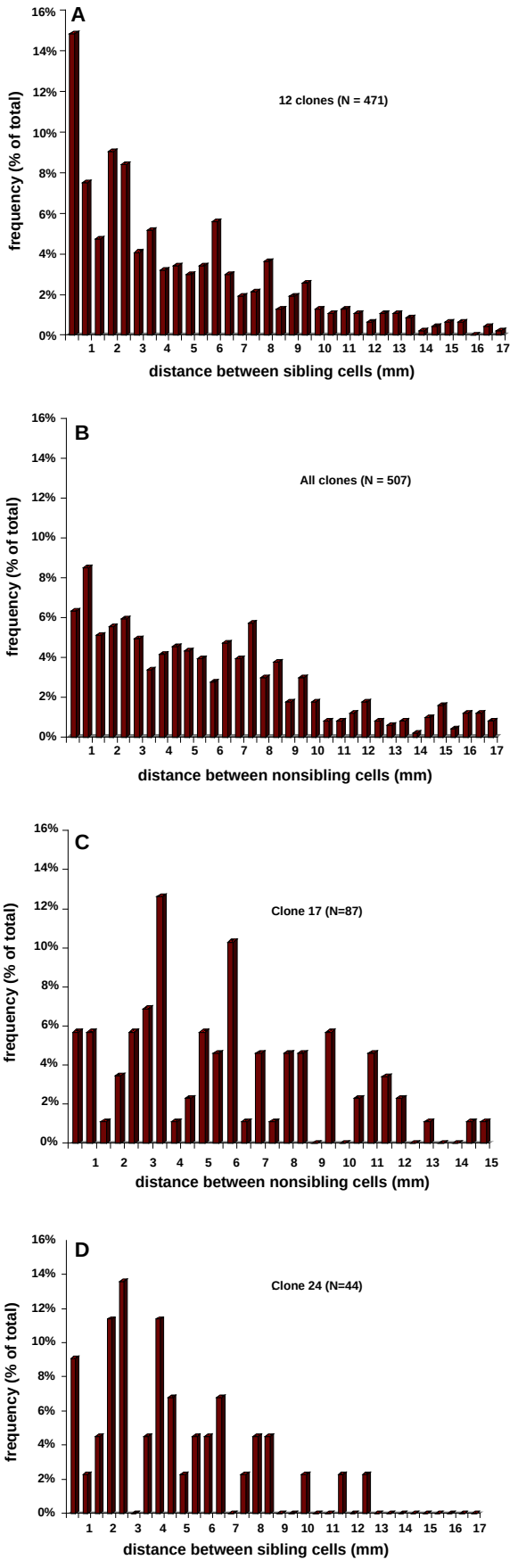



**Fig. 5.** Simplified graphical reconstruction and camera lucida drawings of PCR-positive members of clone 12 and the sections in which they were identified. The tracings in A and B represent medial and lateral views (respectively) of the adult ferret cerebral cortex (Lockard, 1980). Asterisks represent PCR-positive neurons that all contained the same tag (clone 12, Fig. 3). This hemisphere contained eight PCR-positive cells, seven in the clone illustrated and the last cell representing a single cell clone (not illustrated). Locations of the cells were determined based on (1) computerized 3-dimensional reconstruction of the brain, (2) locations of the cells relative to major gyri and (3) rostral-caudal locations of cells (Fig. 3). Major sulci are illustrated, including the cruciate (c) and suprasylvian (s), and can also be seen on the reconstructions in Fig. 4. The olfactory bulb (ob), thalamus (th) and corpus callosum (cc) are also indicated. In C, the outline of each section containing a neuron from this clone is illustrated: the sections are shown from rostral (left) to caudal. Each cell is represented below by camera lucida drawings (1-7) and each demonstrated neuronal morphology. Neurons 2 and 3 were actually in adjacent sections but are represented in a single section for the sake of space. Histochemically labeled cells that were not amplified successfully with PCR are indicated by small inverted triangles. Scale bars, 1mm (A and B), 100 μm (C).

Most labeled glia, 7/10 astrocyte clusters in the cerebral cortex, were found to be part of widespread clones that also included neuronal clusters. Of the clones labeled at E33, 4/12 contained both astrocytes and neurons, indicating the existence of a common progenitor for astrocytes and neurons in the germinal zone of the E33 ferret embryo. Single astrocyte clusters represented 12% of neocortical clones at E33. Mixed glia-neuron clones have also been reported in the rat in vitro (Davis and Temple, 1994; Grove et al., 1993; Temple and Davis, 1994; Williams and Price, 1995) though rarely in vivo (Price and Thurlow, 1988; Reid et al., 1995).




## DISCUSSION

In this report, we have developed a new amphotropic retroviral library that allows comparison of cortical cell lineage patterns between species. We applied this library to the ferret, a carnivore with a large, gyrencephalic and highly organized cortex, and found no evidence for segregation of clones within even broad functional subdivisions of the cortex. In fact, all PCR-determined clones containing two or more neurons appeared to span multiple functional cortical areas, even crossing neocortical and allocortical boundaries. In contrast, no PCR-defined clones (labeled at E33-35) contained neurons that spread beyond the limits of the cerebral cortex. Most clones contained neurons that were dispersed widely in the mediolateral plane as well as rostrocaudally. These data have important implications for the ontogeny and phylogeny of the mammalian cerebral cortex.

### Clonal dispersion and regional specification

Two contrasting views of how functional areas of the cortex become distinct have been referred to as the 'protomap' and the 'protocortex' hypotheses. The protomap theory proposes that neurons within each cortical area are inherently different, their fate specified in the germinal zone according to the cortical area they will eventually occupy (Rakic, 1978, 1988). According to this view, neurons migrate predominantly in a radial fashion from the germinal zone to the overlying cortex, although there may be occasional exceptions (Kornack and Rakic, 1995). In contrast to the protomap hypothesis, however, a series of heterotopic cortical transplants have suggested that various regions of developing cerebral cortex retain a similar capacity to produce the area-specific features that distinguish functional areas (O'Leary, 1989), suggesting that developing cerebral cortex adopts a structure and function appropriate to its environment (Schlaggar and O'Leary, 1991; see also Barbe and Levitt, 1991; Levitt et al., 1995). According to this view, the developing cortex might be seen as a 'protocortex'

**Fig. 6.** (A) The distribution of 471 intersibling cell spacings of E33 and E35 injected brains. Only rostral-caudal spacing was measured. Measurements were made by counting the number of sections separating cells and multiplying that number by the section thickness (usually 100 μm), although medial-lateral spacings did not show any obvious pattern. For each clone, all possible segments connecting sibling cells were measured. Note that intersibling spacings are commonly ≤0.5 mm (15% of all sibling spacings, compared to 6% of nonsibling spacings and 7.5% of spacings predicted by the diffusion model). Other distances between sibling cells were not obviously nonrandom, or significantly different from the distribution of nonsibling spacings (Fig. 4B). (B) Distances between retrovirally labeled nonsibling cells do not show preferred spacings. The histogram shows the distribution of 507 pairwise rostral-caudal distances (determined as the number of sections between cells multiplied by the section thickness) between retrovirally labeled cells that contained *different* PCR tags. Distances were computed between AP nonsibling cells within the same brains and between brains injected and processed identically. The distribution of pairwise distances appears to approximate a uniform distribution in the A-P dimension. (C,D) Intersibling distances from two individual clones that showed a suggestion of preferred spacings between sibling cells. Intersibling spacings appear to be somewhat more commonly 3-3.5 mm, 5-5.5 mm in clone 17 (C) and 1.5-2.5 and 4-4.5 mm in clone 24 (D), although this could be due to sampling phenomena.

composed of comparable populations of neurons across its extent, with area-specific patterns of neuronal differentiation being primarily a consequence of region-specific, extrinsic cues (O'Leary, 1989; but see Ebrahim-Gaillard and Roger, 1996).

The patterns of clonal dispersion observed in the ferret suggest that neurons originating from a single progenitor are capable of acquiring divergent fates in functionally distinct regions of the cortex. Recent experiments in monkeys suggesting some clustering of retrovirally labeled cells (Kornack and Rakic, 1995) relied only on histological examination of labeled sections. Therefore, the widespread dispersion seen after E33 and E35 ferret injections could not have been seen if it occurred in the monkey. It is impossible to determine whether the primate cortex shows a contrasting pattern of clonal dispersion until the retroviral library is applied to primates. Nevertheless, the finding that the widely separated rodent and carnivore orders show similar widespread clonal dispersion following injections at mid-neurogenesis makes it highly likely that such dispersion is a plan general to mammals.

Intermediates between the protomap and protocortex models are most consistent with data from several sources. For example, patterns of retroviral labeling produced by earlier injections with the ferret retroviral library (E29) suggest that sibling cells may show a somewhat greater tendency to cluster at earlier stages of development. Additional evidence that early developmental stages may show relatively more precise maintenance of spatial relationships between ventricular zone and cortex comes from time-lapse imaging of the ventricular zone (O' Leary and Borngasser, 1992), and from the analysis of mosaic expression of a transgene inserted into the X chromosome (Tan and Breen, 1993; Tan et al., 1995; see also Cohen-Tannoudji et al., 1994; Soriano et al., 1995). Analysis of retroviral labeling carried out at earlier stages of neurogenesis (E29 and earlier in ferret) may help to clarify the relative roles of clonal dispersion and radial migration in regional specification of the cortex.

Whereas ferret clones labeled at E33 and 35 dispersed without regard to functional and anatomical boundaries of the mature cerebral cortex, the limits of clonal dispersion did correlate with area domains specified by the regional expression of several genes recently cloned from the embryonic forebrain. For example, *Emx1* and *Emx2* are two homologues of the *Drosophila empty spiracles* gene, with sharp expression boundaries at the border between developing cerebral cortex and striatum (Simeone et al., 1992a,b). Mice mutant for *Emx1* show dramatically reduced corpus callosi, suggesting a role for *Emx1* in the generation of interhemispheric projections by neurons in layers 2 and 3 of the cerebral cortex (Qiu et al., 1996). Mice mutant for *Emx2* lack dentate gyri and show arrested development of the hippocampus and medial limbic cortex (Yoshida et al., 1997). *Emx2* expression, therefore, appears to play a role in defining the medial pallium and limbic region of the telencephalon. Widespread clones disperse across neocortical and hippocampal regions, mirroring in many ways the major domains of *Emx2* expression. In contrast, the widespread clones of the ferret, as well as those of the rat (Fishell et al., 1993; Reid et al., 1995), appear to consistently respect the striato-cortical boundary, not crossing it. The expression of several other homeodomain-encoding gene families such as *Nkx* and *Dlx* occur in patterns complementary to those of *Emx2*, suggesting that the cortico-stiatal boundary represents a major developmental border in forebrain development (for a review see Alvarez-Bonaldo et al., 1995).

An increasing number of other genes have been found to be expressed differentially within the cortex. Limbic associated membrane glycoprotein (LAMP), for example, is a member of the immunoglobulin superfamily and is expressed specifically in developing limbic cortices such as prefrontal, perirhinal and entorhinal cortex. Migrating postmitotic neurons destined for limbic regions and differentiating limbic neurons express LAMP, although progenitors within the ventricular zone do not (Barbe and Levitt, 1991; Levitt et al., 1995). A promoter for a yet-unidentified gene, has been shown to drive a similarly restricted pattern of cortical expression in the somatosensory region of the neocortex during middle and late stages of mouse cortical development (Cohen-Tannoudji et al., 1994). Likewise, discontinuous expression of the *T-brain-1* gene may help to delimit paleo-, limbic and neocortical domains (Bulfone et al., 1995). The expression patterns of these later-acting genes, in contrast to the expression domains defined by the *Emx* genes, did not correspond in any way to the pattern of clonal dispersion observed in this study. However, *LAMP* and *T-brain-1* expression may be part of a later stage of area and laminar differentiation where cortical neurons adopt their final cell fate according to other than lineage-based mechanisms.

## Nonradial migration

The clonal dispersion observed in the ferret cortex presumably reflects substantial nonradial movements by most cortical neurons formed late in neurogenesis (or their progenitors) during radial ascent to the cortical plate. In vitro imaging and retroviral labeling indicated that substantial nonradial migration occurred in the VZ (Fishell et al., 1993; Walsh and Cepko, 1993). Labeling of ferret cells with DiI has shown that most nonradial dispersion occurs in the VZ and SVZ and involves almost exclusively postmitotic neurons. 24 hours after labeling with intracortical injection of DiI, postmitotic neurons were shown to have migrated up to 500 μm mediolaterally or anterioposteriorly within the VZ and SVZ, suggesting a surprisingly rapid tangential migration (O'Rourke et al., 1997). Less extensive nonradial dispersion of DiI-labeled cells was seen at other levels of the developing cortex as well (O'Rourke et al., 1995, 1997). Therefore, multiple mechanisms are available to explain the widespread clonal dispersion we observed, though the retroviral technique is not well-suited to determining the predominant mode of nonradial migration.

## Evidence for asymmetric cell divisions

The patterns of cortical cell lineage that we have observed in the ferret suggest a prominent role for asymmetric cell divisions in the generation of cerebral cortical neurons. Asymmetrical cell divisions are defined as any division that generates two daughter cells with different fates. Many ferret progenitors produce multiple progeny with different fates: multiple cortical layers, neurons, glia, etc., perhaps by dividing to yield a postmitotic cell and a mitotic cell that goes on to form multiple other cell types. While we cannot determine the precise patterns of division that produce multicell clones, those clones containing multiple cell types certainly imply some sort of asymmetric fate decision. Moreover, a large number of retrovirally labeled clones observed in the ferret consisted of only

a single neuron. Since retroviruses label only one of two daughter cells of the infected cell, single cell clones probably reflect progenitors that divide to produce one postmitotic neuron and a mitotic daughter cell. Although cell death in the developing cortex may be substantial (Blaschke et al., 1995), preliminary data (S. F. Tavazoie, C.B. Reid, L. Zhang and C.A. Walsh, unpublished observations) suggest that earlier retroviral injections (E27-E29) and shorter survival times also label approx. 50% single-cell clones, so that single-cell clones are unlikely to result exclusively from cell death. Moreover, the large number of single-cell clones, and the large size of clones with more than one cell (average 7 neurons), make it unlikely that all single-cell clones can be accounted for by technical reasons such as inability to amplify multiple members of a larger clone successfully.

Direct visualization of the ferret ventricular zone with time-lapse video microscopy has previously suggested an important role for asymmetric cell divisions in ferret cortex. DiI-labeled progenitors divided along both horizontal and vertical cleavage planes (Chenn and McConnell, 1995). At an early stage, prior to the onset of neurogenesis, most progenitor cells divided vertically with cleavage planes perpendicular to the ventricle. As a result, both daughter cells maintained contact with the ventricular surface during cell division (Chenn and McConnell, 1995). Early progenitor cell mitoses probably represent proliferative divisions that serve to expand the number of proliferating cells within the epithelium (Takahashi et al., 1994). Such symmetric divisions appear to produce daughter cells with similar precursor cell morphologies and behavior. Horizontal cleavage planes, on the other hand, may represent asymmetric divisions resulting in a mitotic daughter at the ventricular surface, and a second basally oriented cell which appears to have retained its pial contact (Chenn and McConnell, 1995). Additional evidence suggests that some asymmetric mitoses are accompanied by asymmetrical inheritance of proteins such as Notch1 (Chenn and McConnell, 1995) and m-numb (Zhong et al., 1996) that may bias cell fate selections. It will be interesting to determine whether alterations in expression of such proteins can induce changes in cell lineage patterns.

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