Coexistence of Widespread Clones and Large Radial Clones in Early Embryonic Ferret Cortex

Marcus L. Ware[1,2], Sohail F. Tavazoie[2], Christopher B. Reid[1,2] and Christopher A. Walsh[1,2,3]

[1]Division of Neurogenetics, Department of Neurology, Beth Israel Deaconess Medical Center, Harvard Institutes of Medicine, 77 Avenue Louis Pasteur, Boston, MA, [2]Program in Neuroscience and [3]Program in Biological and Biomedical Sciences, Harvard Medical School, Boston, MA, USA

Cell lineage analysis in rodents has shown that the cerebral cortex is formed from both widespread and large radial clustered clones representing partly distinct lineages and producing differing cell types. Since previous cell lineage analysis of the ferret cortex using retroviral libraries showed that most neurons labeled at E33–E35 formed widespread clones, we determined whether clones labeled earlier in neurogenesis showed a greater tendency to form coherent radial clones. Clones labeled at E27–E29 occasionally consisted of widespread multineuron clones (13% of PCR-defined clones), but commonly consisted of small clusters of two to four neurons (65%). Moreover, 6/21 hemispheres contained a single, much larger (6–150 cells) radial cluster. Although large clusters were observed in 28% of experiments, they contained many neurons, accounting for 38% of retrovirally labeled cells. The large clusters showed at most few widely scattered sibling cells, either by histological analysis or by PCR analysis, suggesting that radial and widespread clones coexist but are lineally separate at early stages of corticogenesis. Coexistence of large radial and widespread neuronal clones appears to be an evolutionarily conserved mechanism for cortical neurogenesis.

Introduction

Studies of cell lineage and migration in cortical neurons have demonstrated a striking diversity of clonal and migratory patterns. Morphological studies (Rakic, 1971), electron microscopic studies (Rakic, 1972; Rakic *et al*., 1974), and *in vitro* analysis of cerebellar cultures (Edmondson and Hatten, 1987) have long shown that radial glia provide a substrate for radially migrating neurons. However, studies of cell lineage using retroviral vectors (Price and Thurlow, 1988; Walsh and Cepko, 1988, 1992) or X-inactivation mosaic analysis (Tan and Breen, 1993; Tan *et al*., 1995) have provided a picture of diverse migratory routes, including evidence for both directly radial and less direct patterns. Direct studies of cortical cell migration in explant or slice cultures (O'Rourke *et al*., 1992; Fishell *et al*., 1993) have also shown the predominantly radial routes of some cells, but the strikingly non-radial routes of other cells. What is the mechanistic basis for these two widely differing clonal and migratory patterns?

Several lines of evidence suggest that cortical neurons derive from a heterogeneous population of progenitor cells with very different migratory properties and progeny. Retroviral studies suggested that distinct progenitors give rise to pyramidal and non-pyramidal neurons (Parnavelas *et al*., 1991), but this idea was extended with the observation that clones of pyramidal and non-pyramidal neurons showed distinct patterns of clonal organization (Mione *et al*., 1994). Although some clones expressed both pyramidal and non-pyramidal features, especially shortly following retroviral labeling (Lavdas *et al*., 1996), pyramidal neurons (defined either by electron microscopy, or by the expression of glutamate as a neurotransmitter) tended to be found in large radial clusters, whereas non-pyramidal neurons tended to be found in non-radial, widespread patterns (Mione *et al*., 1994, 1997). The notion that distinct progenitors preferentially produce widespread clones of mainly GABAergic neurons, or radial clones of mostly glutamatergic neurons, has recently been very elegantly extended and refined using highly unbalanced chimeras and X-inactivation mosaics (Tan *et al*., 1998). Large radial clones consisted of >90% glutamatergic neurons, whereas widespread 'horizontal' clones contained 51–92% GABAergic (average = 75%) neurons, with the remaining cells glutamatergic (Tan *et al*., 1998). The general notion that cortical clones consist of two fundamentally distinct types both topographically and physiologically appears able to reconcile most of the existing data from retroviral lineage studies, retroviral library analysis, and *in vitro* time-lapse studies (see Discussion). Although large radial clones only form up to 70% of cortical neurons and perhaps less and are apparently absent from far lateral neocortex (Tan *et al*., 1995), they include most pyramidal neurons and form a potential source of positional information as first proposed by Rakic (Rakic, 1978, 1988).

One of the most remarkable recent discoveries regarding the origins of cortical neurons is the observation that a substantial fraction of GABAergic, inhibitory cortical interneurons actually originates outside of the cortex altogether, in the proliferative zone that gives rise to the striatum. This area is called the lateral ganglionic eminence (LGE) (Anderson *et al*., 1997; Tamamaki *et al*., 1997). Neurons that express *Dlx-1* and *Dlx-2* arise here, and migrate through the striatum and into the overlying cerebral cortex (Anderson *et al*., 1997). Interestingly, transplantation studies of striatal precursors into the cortex had previously shown that the striatal cells could take on cortical fates (Fishell, 1995). The formation of inhibitory neurons outside the cortex is especially intriguing because the same proliferative region, the SVZ of the striatum, is a persistent source of inhibitory interneurons for the olfactory bulb (Alvarez-Buylla, 1990; Luskin, 1993; Reid *et al*., 1999). This suggests that inhibitory interneurons destined for large portions of the forebrain could arise from a common progenitor pool. The origin of some cortical neurons from the LGE begs the question whether all widespread/inhibitory cortical clones derive from the LGE, or whether widespread clones have more than one origin (e.g. from both the VZ of the LGE and the VZ of the cortex proper). In chimera experiments (Tan *et al*., 1998) most, though not all, experiments with widespread clonal patterns also showed labeled cells in the underlying striatum, suggesting that the striatum is the major, if not only, source of widespread clones. This question is difficult to address with current techniques, since there is no simple *in vivo* method that selectively labels just the striatal VZ or cortical VZ progenitors in clonal fashion.

Since evolutionary models (Rakic, 1988) have suggested potential phylogenetic expansion of the cortex by addition of radial modules, the question also arises whether similar patterns

of widespread and radial clones are conserved among species with larger cerebral cortices. Cell lineage studies in monkey (Kornack and Rakic, 1995) have demonstrated both radial and horizontal patterns of retrovirally labeled neurons. However, very large radial clusters have not been reported in primates, and in ferrets (members of the order Carnivora), retroviral library analysis of cortical clones at mid- to late corticogenesis demonstrated striking numbers of widespread clones that covered large portions of the cortical mantle with little evidence for radial clustering (Reid *et al.*, 1997). The *average* multineuron clone labeled at E33–E35 in ferret covered 40% of the cortex, while the radial clusters of labeled neurons were seen quite rarely. This may, however, be a reflection of the relatively later stage of development at which the labeling was performed.

The present study was undertaken to determine clonal relationships at earlier stages of ferret neurogenesis using the retroviral library technique. Rodents are very difficult to approach surgically at early stages, although this may soon change with improved technology (Olsson *et al.*, 1997). The larger ferret embryo is somewhat easier to mark with retroviruses at early stages. We have successfully labeled the ferret cortex with retroviral libraries at E27–E29, when some of the prelate and subplate cells are still being generated, and before most cortical plate cells are generated. We find that clonal patterns at E27–E29 are markedly different compared to E33–E35 injections. Occasional widespread clones are still seen, but are relatively less common, and instead small (two to four neurons), tightly clustered clones are frequent. Most strikingly, we occasionally observed very large, radial clusters that contained up to 150 cells which are similar in their overall appearance to the radial clones described by Tan (Tan *et al.*, 1998). The large clusters account for a small portion of all clones, but a significant fraction (38%) of all retrovirally labeled cells. Interestingly, we did not see evidence that large radial clones and widespread clones were derived from a common progenitor, suggesting that theses two clonal types may diverge at a very early stage of development, consistent with the possibility that they emerge from distinct geographic regions of the neuraxis. Some of these data have been previously presented in abstract form (Ware *et al.*, 1997a).

Materials and Methods

Alkaline Phosphatase (AP)-encoding Amphotropic Retroviral Library

The preparation and composition of the amphotropic retroviral library used for these experiments is described elsewhere (Reid *et al.*, 1997). In brief, an ecotropic retroviral library that infects only rodent cells (Reid *et al.*, 1995; Walsh, 1995), and that contains 400–2000 retroviral constructs, was repackaged to form an amphotropic virus by infection of the amphotropic producer cell line CR7, a subclone of the ΨCRIP producer line (Danos and Mulligan, 1988) with the ecotropic library. Producer cells (>20 000 colonies) were then grown in G418 to select for neomycin-resistant producer colonies. The amphotropic library then was isolated as the supernatant removed from confluent plates of producer cells, showed a titer of 2×10^6 cfu/ml, and was concentrated ×50 before use.

Animal Surgery

All animal housing and experimentation were performed according to protocols approved by the IACUC of Beth Israel Deaconess Medical Center and Harvard Medical School. Timed-pregnant ferrets were purchased from Marshall Farms (North Rose, NY, USA). 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 at embryonic days 27 (E27), E28 or E29 by a midline incision and transilluminated to facilitate identification of fetal skull landmarks. Concentrated amphotropic AP-encoding virus (2–8 μl) containing 80 mg/ml polybrene (Sigma, St Louis, MO, USA) 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 caused the ventricular system to appear blue. Incisions were closed with sutures and staples and animals were returned to their cages to allow development to continue normally. Kits were born vaginally, and nursed by the operated mother. On postnatal day 0 (P0), P8, or P24 the kits were killed by an overdose of Nembutal and perfused with 2–4% paraformaldehyde in 2 mM $MgCl_2$ and 1.25 mM EGTA in 0.1 M PIPES buffer (pH = 7.2).

Histology and Analysis of Clones

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 according to protocols presented elsewhere (Cepko *et al.*, 1995). Labeled cells were detected by microscopic examination of tissue sections, and cell morphology and location were recorded by photography and/or camera lucida drawings. AP staining of cell bodies and processes allowed identification of most labeled cells as presumptive neurons or glia by morphological criteria (Fig. 1). Rostral–caudal location was determined by counting frontal section number, using the rostral extreme of the forebrain as 0, and multiplying by the section thickness (generally 100 μm).

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, 1992; Walsh, 1995). Briefly, coverslips were removed in a 50 ml centrifuge tube filled with sterile water. Small tissue fragments (~100 μm

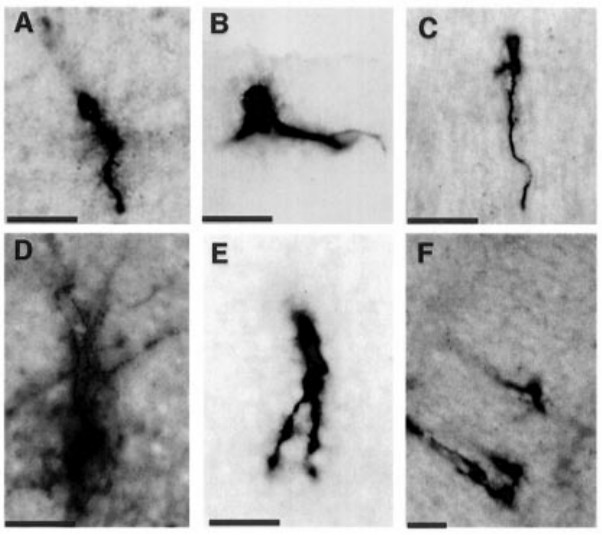

Figure 1. Morphology of retrovirally infected cells. Retrovirally encoded AP allowed for the morphological identification of many labeled neurons. Labeled cells were present in all layers of the cortex and included cell types produced during both earlier and later stages of development. Many of the cells observed were singly labeled cells (as in *A–E*) or in small cell clusters (as in *F*). *A–C* Cerebral cortical neurons labeled at E27 and analysed at P0 (experiment 6, Table 1). *D* and *F* Cells labeled at E29 and analysed at P24 (experiment 20, Table 1). *E* Cell labeled at E28 and analysed at P0 (experiment 9, Table 1). *A* Radially migrating cell (presumably a neuron) crossing the VZ/IZ border. *B* An embryonic Cajal–Retzius cell in layer I. *C* Young fusiform neuron with a long descending axon. *D* Layer II/III basket cell. *E* Immature neuron in layer VI with descending processes. *F* Cluster of four bipolar neurons in layer III of the medial cortex. Scale bar is 50 μm.

× 200 μm × 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 $MgCl_2$, 50 mM Tris buffer, pH = 8.3, 25 mM KCl, 0.5% Tween-20) at 65°C for 4–24 h. Each well was covered with 30 μl of mineral oil to prevent evaporation. Samples were then heated to 85°C for 20 min to inactivate proteinase K and then 95°C for 5 min to denature the DNA. A nested PCR protocol was employed 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, consisting either of unlabeled tissue, or reagents alone. No experiments contained false PCR-positives.

Analysis of PCR Products

The PCR products from the second PCR reaction were separated on a 3%/1% NuSieve/Seakem agarose gels to determine tag sizes. Each tag was then digested with *Cfo*I, *Rsa*I, *Alu*I, *Mse*I, and *Msp*I. Finally, digested samples of similar predigest size were run side by side on agarose gels to allow direct comparison of restriction fragment sizes. Cells from which a PCR product of indistinguishable size and restriction enzyme digestion pattern was amplified were interpreted as arising from a common progenitor; cells with distinguishable tags were interpreted as having arisen from separate progenitors.

Results

The ferret's short gestation period (41–42 days), coupled with a protracted period of cortical neurogenesis (35 days), makes this species ideal for the study of early cortical development. Neurogenesis in the ferret cerebral cortex begins at E19 and continues until P14 (where P0 = E42) (Jackson *et al.*, 1989). The preplate is formed from E19–E29, and the first cortical plate neurons, which reside in layer VI, appear on E25 (Jackson *et al.*, 1989). In this study, retrovirus was injected when preplate neurons and early cortical plate neurons are born, and labeled hemispheres were analysed before neurogenesis was complete. Because the postnatal survival of injected animals was low, many ferrets were analysed at P0–P8, while labeled progenitors are still proliferating and their progeny are still migrating. The age at injection and analysis also biases labeling toward neurons as glial production in the ferret occurs later in development (Jackson *et al.*, 1989). The active proliferation and migration at the time of analysis produced a consistent underestimation of the number of cells generated and of the final distance between migrating sibling cells. To correct for distances between sibling cells caused by analysis at different ages, we have converted all distances to percentages of the rostral–caudal length of the cortex at the time of analysis.

Spatial Distribution of Retrovirally Labeled Cells

In each of the 21 hemispheres successfully injected and AP-stained, retrovirally labeled cells most commonly consisted of dispersed single labeled cells and/or small clusters of two to four labeled cells (Table 1). Single labeled cells refer to labeled cells with a nearest neighbor of greater than 3% of the rostral–caudal cortex, and small cell clusters are labeled cells within 3% of the rostral–caudal cortex (Fig. 1). As in previous studies (Austin and Cepko, 1990; Parnavelas *et al.*, 1991; Walsh and Cepko, 1992; Mione *et al.*, 1994; Reid *et al.*, 1995), cells within small clusters in this study contained two to four phenotypically similar cells usually within the same laminae of the cortex, suggesting similar birthdates. The single cells and small clusters generally resembled the pattern of cell labeling described previously in the mouse (Luskin *et al.*, 1988; Austin and Cepko, 1990), the rat (Price and Thurlow, 1988; Walsh and Cepko, 1988; Parnavelas *et al.*, 1991; Luskin *et al.*, 1993) and ferret after E29 labeling (Reid *et al.*, 1997). However, compared to retroviral labeling at E33–35 ages in ferret, small clusters of retrovirally labeled neurons were notably more common in the present material.

Table 1
Overview of experiments

Experiment	Age at injection	Age at analysis	Small clusters or single cells	Large radial clusters
1	E27	P0	13	20
2	E27	P0	7	0
3	E27	P0	17	0
4	E27	P0	5	0
5	E27	P0	34	0
6	E27	P0	30	0
7	E27	P0	17	0
8	E28	P0	9	30
9	E28	P0	21	0
10	E28	P0	30	0
11	E28	P8	9	25
12	E28	P8	18	150
13	E28	P8	14	0
14	E28	P8	19	10
15	E28	P8	12	0
16	E28	P8	4	0
17	E29	P24	2	0
18	E29	P24	60	6
19	E29	P24	15	0
20	E29	P24	53	0
21	E29	P24	7	0
			396	241

The table lists the injection ages, analysis ages, number of neurons that were solitary or in small clusters of two to four neurons, and the number of neurons in large radial clusters in each experiment. Twenty-one hemispheres were infected with amphotrophic AP library at E27, E28, and E29, and analysed at P0, P8, or P24. All 21 hemispheres contained single labeled cells and small clusters of 2–4 cells. However, 6/21 labeled brains contained an additional pattern of large radial clusters of labeled neurons.

Besides the pattern of single cells and small cell clusters, we also occasionally observed strikingly large radial clusters of closely associated cells in considerably larger numbers than previously observed in retroviral studies in the rat, mouse, ferret, or primate. Based on the morphology of cells in these clusters as well as the early age of injection and analysis, we have identified many of the cells within these radial clusters as neurons (Fig. 2), although morphological criteria cannot rule out the inclusion of a few glia in these clusters. The radial pattern was markedly different from the more scattered pattern of AP labeling, since radial clusters contained many more cells than the small cell clusters and covered multiple layers of the cortex (Figs 2 and 3). Although the large radial clusters were somewhat uncommon (occurring in only 6/21 experiments as a single cluster in each experiment) given their striking nature and resemblance to the large radial clusters described previously (Tan *et al.*, 1998), we have analysed them in some detail.

Analysis of Radial Clusters

Large radial clusters are most clearly distinguished from the smaller clusters of retrovirally labeled cells observed here and in previous studies by the number of cells per cluster (Table 2). Radial clusters identified in this study showed 6–150 cells per cluster with an average of 40 cells per cluster. The large numbers of labeled cells in these clusters gave them a density and radial coverage that was not so clearly seen in the smaller clusters seen in other retroviral studies. Moreover, the large numbers of labeled neurons in each cluster caused them to account for a substantial fraction of all retrovirally labeled cells observed in

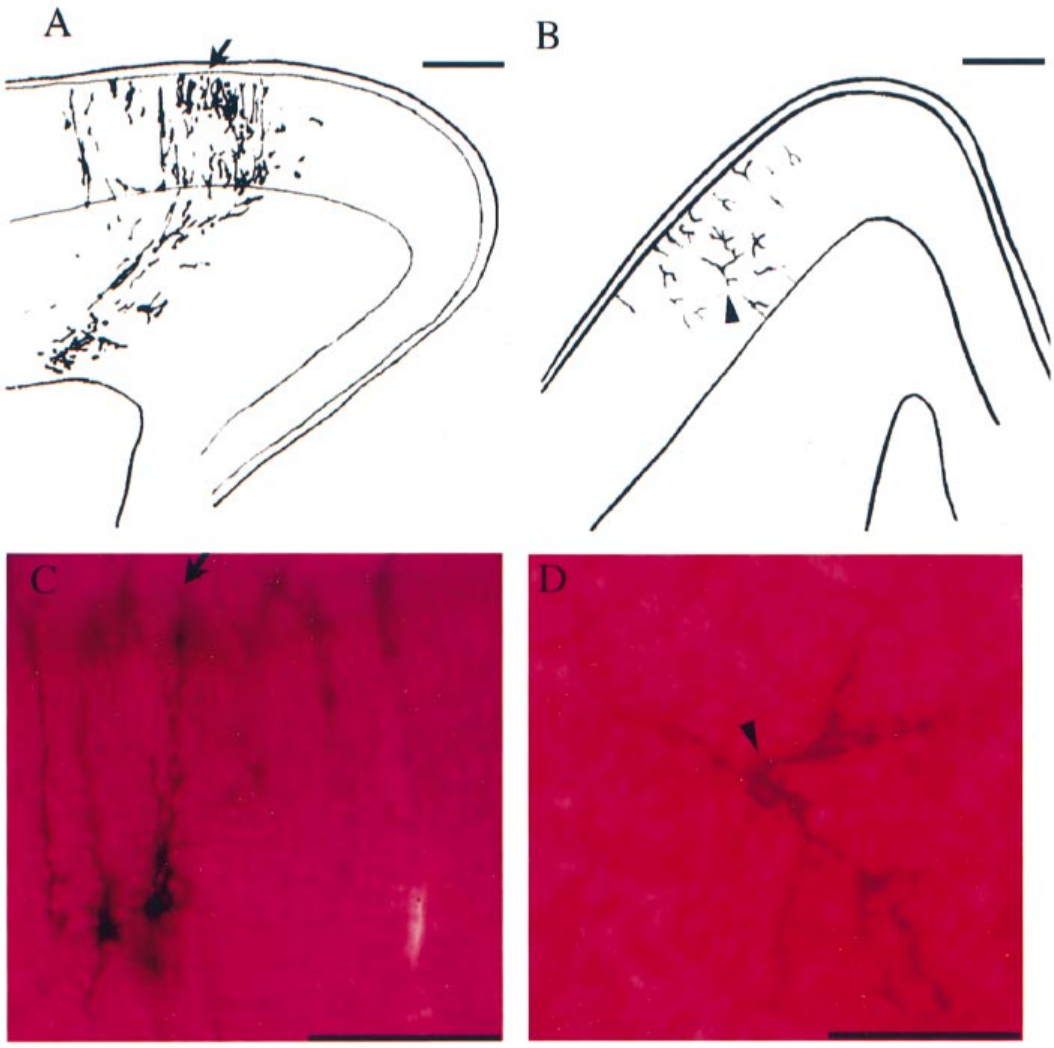

Figure 2. Hemispheres contained labeled neurons in large radial clusters. *A* Camera lucida drawing of a radial cluster labeled at E29 and analysed at P8 (experiment 12, Table 2). The large radial cluster consists of 150 cells and has a diameter of 9% of the rostral–caudal cortex. Radial cluster includes mature neurons in the subplate and all layers of the cortical plate as well as migrating neurons. *B* Camera lucida drawing of a radial cluster labeled at E29 and analysed at P8 (experiment 11, Table 2). The radial cluster contains 25 mature neurons and has a diameter of 7% of the rostral–caudal length of the cortex (measured at approximately layer III/IV). *C* Picture of layer V pyramidal cell with long apical dendrites ascending towards the pial surface, located within the radial cluster in *A*. *D* Picture of a layer III pyramidal cell with an ascending dendrite. Arrows in *A* and *C* indicate the same cell; arrowheads in *B* and *D* indicate same cell. Radial cluster diameters measured at approximately layer III/IV of the cortex. Scale bar in *A* and *B* is 500 μm, scale bar in *C* and *D* is 50 μm.

this study: the six radial clusters accounted for 241 of 637 (38%) of the labeled cells in this study (Table 1).

Despite the large numbers of neurons contained in the radial clusters, they were mainly confined to small cortical regions. Large clusters showed diameters of 1–9% of the rostral–caudal cortex (measured at approximately layer III/IV, which represented the radial midpoint of radial clusters in the cortex), with an average diameter of 5% of the rostral–caudal length of the cortex. Thus the diameter of the larger clusters is similar to the diameter of smaller neuronal clusters observed in this study and previous retroviral studies (Figs 2 and 3), but the large clusters contain a much denser population of labeled cells in the same region. Moreover, the large radial clusters were quite coherent with sharp borders, and did not show an obvious tendency towards containing widespread sibling cells in the near vicinity: outside of the border of the large clusters, the density of scattered labeled cells dropped off rapidly.

Another feature that distinguished the large radial clusters observed here from the smaller clusters observed in most earlier retroviral studies is that the large radial clusters contained cells in many different layers of the cortex (Figs 2 and 3), indicating that progenitors producing radial clusters are mitotic throughout most of cortical neurogenesis. Moreover, migrating cells were present in three of six radial clusters (experiments 1, 8, and 12). Because these radial clusters were analysed while the later-born upper layer neurons were being generated, the presence of young migrating presumptive neurons in these radial clusters suggests that radial cluster progenitors were mitotic shortly before analysis, as late as P8. Thus, despite the absence of the radial pattern of labeling after later injections (E33–E35),

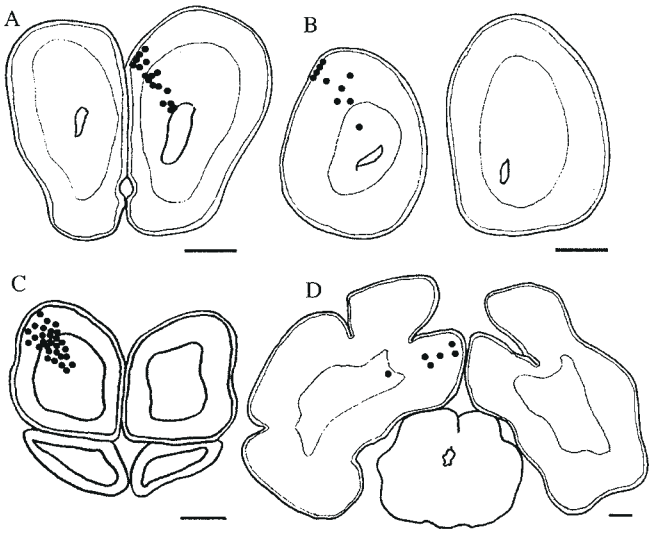

Figure 3. Camera lucida plots of large radial clusters. The location of cells within each radial cluster is indicated by filled circles. *A* Drawing of radial cluster in experiment 1. This radial cluster is located in the medial frontal cortex and contains 20 labeled cells. Cells drawn are within a rostral–caudal distance of 300 μm. *B* Drawing of radial cluster in experiment 14. This radial cluster is located in the frontal cortex and contains 10 labeled cells. All cells drawn are within a rostral–caudal distance of 400 μm. *C* Drawing of radial cluster in experiment 8. This radial cluster is located in the frontal cortex and contains 30 cells. All cells drawn are within a rostral–caudal distance of 200 μm. *D* Drawing of radial cluster from experiment 18. This radial cluster is located in the medial occipital cortex and contains six labeled neurons. All cells drawn are within a rostral–caudal distance of 200 μm. The clonal relationship of these neurons was confirmed by PCR analysis (see Table 3, clone 15). Scale bars are 1 mm.

progenitors producing radial clusters are apparently actively mitotic during the later stages of development, as late as P8.

The large radial clusters were observed in several different areas of the cortex (Figs 2 and 3), though the small number of clusters observed does not allow a comprehensive analysis of their topographic distribution. Three of six radial clusters identified were in the frontal cortex, two of six were in the parietal cortex, and one of six was in the occipital cortex (Table 2). Additionally, three of six radial clusters were in the medial cortex, while three of six were more laterally, though none was in the extreme lateral-most portion of the neocortex. Although the small number of radial clusters labeled here did not allow us to determine if the radial clusters occurred preferentially in different areas of the cortex, it did not appear that radial clusters were restricted to a single region of the cortex.

Clonal Analysis by PCR

To determine the clonal relationship of the AP-labeled cells, we performed PCR amplification of retrovirally encoded DNA tags. Cells containing the same tag were interpreted as siblings derived from a single retroviral infection. Given the complexity of the library [100–400 tags represented roughly equally, with as many as 2000 potential tags (Reid *et al*., 1997)], the possibility of clones derived from two different progenitors coincidentally infected with the same tag is <5% for experiments with fewer than four clones and <40% for experiments with fewer than eight clones (Walsh and Cepko, 1992). PCR amplification was successfully performed on four cerebral hemispheres infected with retrovirus at E27 and E29 representing 23 clones (Table 3, Fig. 4). The PCR efficiency of experiments included in this study ranged from 45 to 86%. Experiments with PCR efficiency of less than 40% were excluded from further analysis, since they showed a large number of tags seen in single cells only. Because PCR analysis is less than 100% successful, the number of sibling cells per clone is generally underestimated. Since the precise degree of underestimation could not be determined for each clone, we did not apply a numerical correction. However, on average the clone size is expected to be between 1.2 and 2.2 times higher than the number indicated.

Table 2
Analysis of radial clusters

Experiment	Analysis age	Labeled cells	Radial cluster diameter (mm)	% rostral–caudal cortex	Location
1	P0	20	300	5	Medial frontal
8	P0	30	400	6	Frontal
11	P8	25	750	7	Parietal
12	P8	150	1000	9	Medial parietal
14	P8	10	300	3	Frontal
18	P24	6	200	1	Medial occipital
Total		241			
Average		40		5	

The table lists the number of cells, radial cluster diameters, and locations of radial clusters. Radial cluster diameters were measured directly and converted to % rostral–caudal length of the cortex in order to compare diameters from animals analysed at different ages. Radial clusters are observed at every injection time in this study (E27, E28 and E29) and at every age of analysis (P0, P8 and P24). The number of cells/cluster, radial cluster diameters, and location within the cortex are quite variable and show no clear relationship with injection age or age of analysis. All radial cluster diameters were determined by measuring at approximately layer III/IV, which represented the radial midpoint of radial clusters in the cortex. The rostral–caudal lengths of the cortex used to calculate percentages are 6500 μm at P0, 10 500 μm at P8, and 18 500 μm at P24.

PCR analysis of four experiments (one injected at E27 and analysed at P0, and three injected at E29 and analysed at P24) showed a predominance of small clustered clones. Three of 23 clones (13%) consisted of single cells, 15 (65%) were small clusters containing two or three PCR-positive cells within a single cluster. Three clones (13%) were widespread, defined as having siblings separated by at least 10% of the rostral–caudal length of the cortex. Finally one clone (4%) was a large radial cluster, with six PCR-positive neurons in a single radial cluster (Table 3). Of the PCR-positive cells, three of 54 cells (6%) were from single-cell clones, 29 (54%) were from small clustered clones, 16 (30%) were from widespread clones, and six (11%) were from the large radial clone (Table 3, Fig. 4). Although proportionately far fewer progenitors at E27–E29 produce widespread clones than at E33–E35, widespread clones still account for about one third of all retrovirally labeled cells even after early injections, and still show dispersion over wide cortical areas. In contrast, small clustered clones and large radial clusters account for a much higher proportion of retrovirally labeled neurons than after later injections. PCR analysis of the large radial cluster in experiment 18 (Fig. 3*D*) verified that cells in this cluster were all clonally related, and that this cluster was not part of a widespread clone. Since PCR analysis is based on a small number of cells, it does not exclude the possibility that other large radial clones may have widely dispersed siblings.

PCR analysis confirmed that sibling cells in small clusters usually occurred within the same layer or in adjacent layers, as observed in previous retroviral studies. Of the mature two-cell clusters analysed by PCR (Table 3), nine of 14 (64%) showed identical laminar location. Although morphology could only be studied incompletely, small clusters appeared to contain cells with similar morphology as well. This observation is consistent with previous findings in later injections in the rat (Walsh and Cepko, 1992; Reid *et al*., 1995). Clusters of deep-layer neurons

Table 3
PCR analysis of labeled hemispheres

Experiment	Clone number	Cell type	Rostral–caudal distance	% rostral–caudal cortex	Clone type
5	1	V	900	14	SS
	2	III	3100	48	SS
	3	mIZ	5700	88	SS
	4	SP,RG	2000, 3300	31, 51	WS
	5	III, mCP, III	2300, 2500, 3700	35, 38, 57	WS
	6	mCP,CP,V, mIZ, mCP,mIZ, mSVZ, HI, mIZ, SVZ, SVZ	1800, 2500, 3900, 4100, 4400, 5000, 5000, 5400, 5800, 5900, 6000	28, 38, 60, 63, 68, 77, 77, 83,89, 91, 92	WS
18	7	III, III	3200, 3300	17, 18	SC
	8	III, III	4400, 4500	24, 25	SC
	9	II, II	5900, 6200	32, 34	SC
	10	IV, IV	9900, 9900	54, 54	SC
	11	II, VI	11 700, 12 000	63, 65	SC
	12	II, mIZ	11 500, 12 000	62, 65	SC
	13	HI, HI, HI, HI	11 600, 12 000, 12 000, 12 100	63, 65, 65, 65	HC
	14	II, II	12 900, 12 900	70, 70	SC
	15	IV, WM, V, V, II, II	14 700, 14 700, 14 800, 14 800, 14 800, 14 800	79, 79, 80, 80, 80, 80	RC
	16	III, III	14 900, 14 900	81, 81	SC
	17	VIb, SP	14 900, 15 000	81, 81	SC
	18	II, II	16 300, 16 600	88, 90	SC
	19	IV, IV, IV	16 800, 16 900, 17 500	91, 91, 95	SC
20	20	VI, V	14 600, 15 100	79, 82	SC
21	21	II, II	5300, 5400	29, 29	SC
	22	III, III	7500, 7600	41, 41	SC
	23	II, IV	8000, 8000	43, 43	SC
SS clones	3				
SC clones	15				
WS clones	3				
RC clones	1				

The table lists results of clonal analysis of presumptive neurons from four experiments. Clonally related cells are grouped together and classified as single cell clones (SS), widespread clones (WS), small clustered clones (SC), radial clones (RC) or hippocampal clones (HC). Neuronal identity was determined based on cell morphology. Cells were classified by their location. Roman numerals (II–VI) represent neurons within layers II–VI in the cortex. Cells were also identified in the ventricular zone (VZ), subventricular zone (SVZ), subplate (SP), and hippocampus (HI). Cells in each of these locations are presumptive neurons except for one radial glial (RG) cell. All cells whose morphology suggested migratory profiles are indicated by 'm'. The rostral–caudal location of each cell was measured and converted to % rostral–caudal cortex to allow comparison of clones from animals analysed at different ages. The rostral–caudal lengths of the cortex used to calculate percentages are 6500 μm at P0, 10 500 μm at P8, and 18 500 μm at P24.

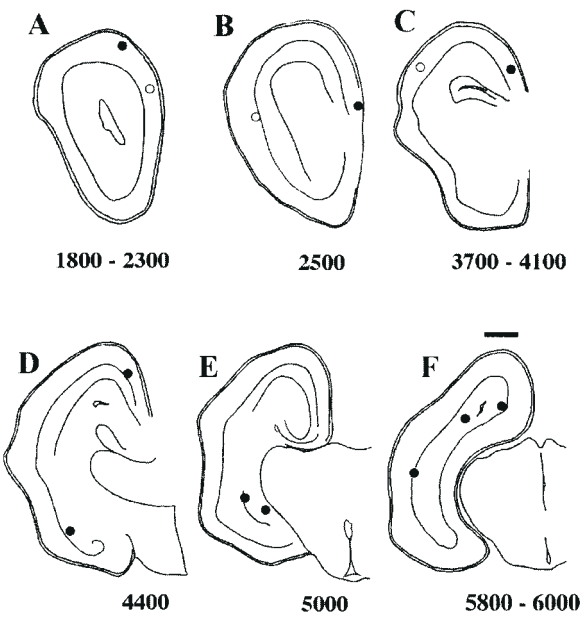

Figure 4. Camera lucida plots of widespread multineuron clones. Widespread clones appear to consist of neurons in different functional areas of the brain. *A–F* Camera lucida drawings of coronal slices of a hemisphere containing two widespread clones. The open circles represent clone 5 (Table 3, experiment 5), and the filled circles represent clone 6 (Table 3, experiment 5), although one cell (5400 μm) is not illustrated. Distances indicated below each drawing represent the rostral–caudal distances of cells from the rostral tip of the olfactory bulb. Scale bar is 1 mm.

also showed similar morphological and laminar fate, with one clone composed of a layer VI neuron and a subplate neuron (Table 3, clone 17), and another composed of a layer V neuron and a layer VI neuron (Table 3, clone 20). The similarities between small clusters generated early in development with those generated later suggests that the processes that produce small clusters in earlier development persist to produce similar patterns later in development. Interestingly, however, these deeper-layer clusters did not obviously contain sibling cells in upper layers of the cortex in the same or different cortical regions, suggesting that the clusters may arise from a progenitor that does not continue dividing throughout neurogenesis.

Discussion

In this report, we used an amphotropic retroviral library to determine whether cortical lineage patterns observed with retroviral injections during the late preplate stage and early cortical plate stage are similar or different to patterns previously observed after later injections. Consistent with findings with later injections (Reid *et al.*, 1997), we observed that clones labeled at E27–E29 commonly consisted of small clusters of neurons, single neurons and widely dispersed multineuron clones. In addition, however, we observed a striking radial pattern of clonal dispersion in a small but significant number of experiments. The radial pattern is seen only after early progenitor labeling, and radial progenitors appear to coexist with progenitors of single cell clones, small clustered clones, and widespread clones.

Radial and Non-radial Clones

Analysis of highly unbalanced chimeras (Tan *et al.*, 1998) and previous retroviral experiments (Parnavelas *et al.*, 1991; Mione *et al.*, 1994, 1997) has suggested that large radial clusters and widespread clones are lineally distinct, and several lines of evidence in this study confirm that. First, in several experiments (experiments 8, 11, 12) the large radial cluster accounts for the majority of the labeled cells in the entire experiment, suggesting that the progenitor(s) that produce the large radial cluster give rise to few if any widely scattered cells. Second, large radial clusters were only labeled in a minority (6/21) of experiments, with most experiments showing only labeled single neurons or small clusters of neurons; since progenitors of large radial clusters are rarely labeled, the progenitors of scattered neurons or small clusters usually seem to be distinct from progenitors of large clusters. Third, large radial clusters, when labeled, generally had sharp borders without obvious widely scattered neurons near the edges. Fourth, limited PCR analysis also showed that a large cluster found in an experiment with many scattered labeled cells and small clusters of cells (experiment 18) nonetheless was clonally distinct from other labeled cells.

While large radial clones and widespread clones may be two distinct extreme patterns, the small clonal clusters appear to be less distinct in their identity, and may relate both to widespread clones or to radial clones (Fig. 5). For example, small clusters have been commonly seen as 'subunits' of widespread clones in rat (Reid *et al.*, 1995), and widespread clones in ferrets also can contain some neurons that are individually clustered (Table 3, and see Reid *et al.*, 1997). On the other hand, some small clustered clones seem likely to represent products of the same progenitor types that produce large labeled clusters after earlier labeling. Birthdating studies have suggested that, once cortical plate formation begins, a large number of cortical progenitors may become extinguished after relatively few cell cycles; these observations suggest that cortical clone size may change radically when labeling is performed at slightly different ages (Takahashi *et al.*, 1997). Therefore, smaller clusters of retrovirally labeled neurons may be heterogeneous with regard to their derivation and progeny, representing portions of clones that, if labeled earlier, would form either large radial or widespread clones (Fig. 5*A*, *B*). This interpretation appears to fit nicely with the observation of small or large clusters of retrovirally labeled cells that are often homogeneous anatomically or neurochemically (Mione *et al.*, 1994, 1997).

The overall appearance of the large radial clusters and widespread clones in the ferret suggest that they may represent the same fundamental biological mechanism in Carnivora as previously observed in rodents. Although the radial columns described by Tan (Tan *et al.*, 1998) contain larger numbers of neurons than the radial clusters described here, the gross appearance of radial columns labeled by the two techniques is quite similar. The radial columns observed by Tan (Tan *et al.*, 1998) contained neurons in quantal multiples of 600, suggesting that a single precursor in the cerebral vesicle produces 600 labeled neurons, or the result of 10 cell cycles, in the mature mouse cortex. The radial patterns that we observed, with an average of 40 cells per radial cluster, consisted of far fewer cells than the radial clones observed in chimera experiments. Our largest radial cluster contained 150 cells, the result of at least eight cell cycles. Our injections were made into the ferret at E27–E29, and since preplate development begins at E20, it is possible that we labeled single progenitors several cell cycles after the onset of preplate development. Moreover, most of the injected ferrets were harvested from P0 to P8, while the cortical plate cells were still being generated. Thus, in most experiments, we harvested animals before labeled progenitors had completed proliferation.

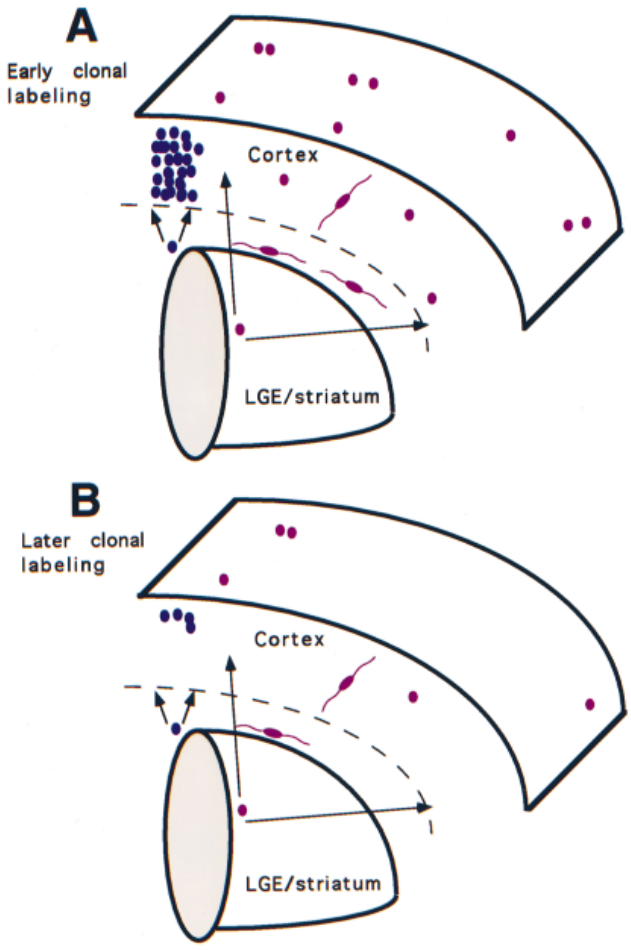

Figure 5. Schematic summary of a model of cortical neurogenesis based on lineage data from many sources. Two basic clonal types are shown, though there may be more. Large radial clustered clones (blue) form neurons, predominantly pyramidal, in most or all cortical layers. After early retroviral labeling (or using chimera analysis or X-inactivation mosaics) the large radial clusters can be seen (*A*), but labeling late in neurogenesis produces only small clusters of labeled cells (*B*). Widespread progenitors (purple) are formed in part in the LGE (*A*), though additional sources of widespread clones are not ruled out. They form mainly inhibitory interneurons that seem to take very complicated migratory trajectories to the cortex. After later labeling (*B*), these widespread clones are still clearly seen.

The retrovirally labeled clusters do not form simple quantal multiples in terms of cell number. However, the integration of retroviruses into daughter cells of infected cells makes retrovirally labeled clones subject to large variability, and tends to be subject to 'lineage tree' extinction (Takahashi *et al.*, 1997) effects that limit the size of labeled clones as well. Therefore, we believe that the differences in the number of cells seen in these experiments may be primarily due to the timing of injections and analysis, in addition to differences between ferret and mouse cortical development. The radial clusters here may represent a subset of clones described in unbalanced chimera experiments. Given the differences in these two techniques, it is particularly appealing that we have identified radial clusters that may be

the same patterns as the radial clusters observed in chimera experiments.

Although the very large radial clusters have not been observed previously with retroviral labeling, smaller radially aligned clones or up to 23 neurons have been labeled at later stages of corticogenesis the rat (Walsh and Cepko, 1988; Luskin *et al*., 1993; Mione *et al*., 1997) and in the monkey (Kornack and Rakic, 1995). In monkeys, hemispheres labeled between E63 and E68, when 40% of cortical neurogenesis is complete, contained arrays of two to six cells with 54% of the total arrays composed of only two cells. Similarly, E16 injection of retrovirus into the developing rat cortex produced clones showing radial migration in up to seven neurons in different layers of the cortex (Mione *et al*., 1997). Interestingly, only pyramidal neurons were seen in these radial clones, whereas non-pyramidal neurons were found in pairs or as single neurons. The radial clones observed in the rat and monkey are similar to parts of the larger radial clusters observed here. Taken together, these findings suggest that radial clones are present in the cortex of a number of species and that the pattern of cell proliferation and migration involves biases at different times in development towards symmetric and asymmetric division.

Given that a substantial proportion of inhibitory interneurons of the cerebral cortex are formed in the lateral ganglionic eminence (Anderson *et al*., 1997; Tamamaki *et al*., 1997), and given that inhibitory interneurons are more commonly found in the widespread clones, the most parsimonious model (though probably too simple) is that widespread clones are preferentially if not exclusively derived from the LGE and/or other sources outside of the cortical VZ/SVZ. The observation that widespread and large radial clones are mainly if not exclusively lineally distinct, even at the earliest stages of cortical neurogenesis, is most consistent with the derivation of widespread and radial clones from two different, non-overlapping sources. However, this remains uncertain and it is still possible that some widespread cortical clones derive from the cortical ventricular zone as well.

Radial and Non-radial Migration

The observation that there are distinct lineages for widespread and radial clones appears to resolve a number of studies of migration into the cortex. Time-lapse imaging has shown a variety of radial and non-radial migratory routes between the VZ and the cortex. For example, there is rapid dispersion of cortical cells in the ventricular zone of the cerebral cortex (Fishell *et al*., 1993; Walsh and Cepko, 1993; Neyt *et al*., 1997; O'Rourke *et al*., 1997), and there is radial and non-radial migration in the intermediate zone (O'Rourke *et al*., 1992, 1995, 1997). However, virtually all non-radially migrating cells in the layers beneath the cortex are postmitotic (Neyt *et al*., 1997; O'Rourke *et al*., 1997). The derivation of widespread clones from the LGE would produce a population of non-radially migrating cells in the layers beneath the cortex that might be expected to be postmitotic, since they are far from the respective proliferative zone. Taken together, these studies show that patterns of cell migration change markedly over time, that there are many migratory pathways into the developing cortex, and that cortical precursors sometimes derive from distant sites.

Relationship to Genetic Defects in Neuronal Migration

It remains a remarkable fact that two sets of progenitor cells give off similar-appearing neurons that appear to migrate through the same terrain of the subcortical intermediate zone according to completely different rules. These differences may arise simply from the location of the progenitors (i.e. in the cortical VZ versus the LGE), or from the extensive interconnection of cortical VZ cells by gap junctions (Bittman *et al*., 1997), but more likely reflects two distinct genetic programs. For example, LGE progenitors are characterized by expression of *Dlx-1*, *2*, and other family members, whereas the cortical VZ cells express *Emx-1*, *2*, and other distinct transcription factors (Rubenstein and Lai, 1999). These transcription factors may control very distinct sets of downstream targets in the form of adhesion molecules that control differential adhesion to radial glial cells. For example, neuronal outgrowth in distinct neuronal populations shows opposite effects in response to applied netrins (Shirasaki *et al*. 1996), and neuronal migration in *C. elegans* also shows cell-specific effects of netrin homologues (Hedgecock *et al*., 1990). Similar guidance cues in vertebrates may have differential effects on distinct populations that ultimately reach the cerebral cortex.

A number of single gene mutations disrupt the migration of neurons into the cerebral cortex and some may ultimately shed light on the differing genetic programs underlying the distinct migrational patterns of widespread and clustered clones. For example, in mice that carry mutations in the *reelin* gene (Caviness, 1982) or *mdab1* (Gonzalez *et al*., 1997; Howell *et al*., 1997b; Sheldon *et al*., 1997; Ware *et al*., 1997b), the cortical preplate fails to split normally, with a consequent secondary disruption of the layers of the cortical plate. *Reelin* encodes a large secreted protein expressed only in the marginal zone of the cortex (D'Arcangelo *et al*., 1995); *mdab1* encodes a protein that was first isolated via its binding to non-receptor tyrosine kinases such as Src and Abl (Howell *et al*., 1997a), and is homologous to the fly *disabled* gene that is so named because it interacts genetically with fly *Abl* in developing neurons (Gertler *et al*., 1993). mDab1 protein contains a phosphotyrosine binding (PTB) domain similar to domains in Shc and Numb that interact with transmembrane receptors. *mdab1* is expressed in a complementary pattern to *Reelin* [in the migrating cortical plate neurons and not in the marginal zone (Howell *et al*., 1997b; Sheldon *et al*., 1997; Rice *et al*., 1998)] and Reelin expression is normal in *mdab1* mutant mice (Gonzalez *et al*., 1997). Therefore, Reelin and mDab1 are thought to represent an extracellular ligand and intracellular adapter protein that may each bind to a common receptor, though that receptor is unknown at present. The Reelin-mDab1 signaling pathway appears to be critical to allowing cortical plate neurons to divide the preplate, and to allow later-born cortical plate neurons to migrate past earlier-born cortical plate neurons, although how this happens is also not clear. Mutations in the *cdk-5* kinase gene (Ohshima *et al*., 1996; Gilmore *et al*., 1998), or its regulator, *p35* (Chae *et al*., 1997), also cause inversion of the layers of the cortical plate, though some splitting of the preplate occurs in these mutants. Therefore, these proteins may represent part of a biochemical effector pathway for Reelin/mDab1, or may form a separate pathway.

It is unknown whether the several mouse mutations that alter cortical neuronal migration affect widespread clones or radial clones equally, or whether the mutations affect the two migratory patterns differentially. Since all of the known mutations affect the vertical or radial organization of the layers of the cortex, it seems unavoidable that the radial type of migration, i.e. that which occurs in relation to radial glial fibers, is affected. However, migration from the LGE occurs largely or completely independently of radial glial fibers. Hence, one possibility is that

some of these genes may be required for normal migration of radial clones but expendable for non-radial migration from the LGE. These possibilities can be evaluated by direct analysis of clonal patterns in mutant mice.

A number of single gene mutations affect neuronal migration to the cortex in humans as well, though their modes of action are even less well understood. Some neuronal migration defects in humans are associated with lissencephaly ('smooth brain') and can be caused by genes such as *LIS1* (*PAFAH1β1*) (Reiner *et al*., 1993) and *doublecortin (*des Portes *et al*., 1998; Gleeson *et al*., 1998). Although doublecortin is a likely substrate for c-Abl (des Portes *et al*., 1998; Gleeson *et al*., 1998), implying potential biochemical links to mDab1, there is accumulating evidence that PAFAH1β1 and DCX proteins may regulate migration via actions on microtubules. *PAFAH1β1* has a strong homologue in *Aspergillus nidulans* (*nudF*), which is required for the translocation of nuclei along the fungal mycelium (Xiang *et al*., 1995), and which interacts genetically with other genes (*nudA*, *nudC*, *nudE*, etc.). These genes encode, among other things, the heavy chain of dynein, which in neurons represents a microtubule-based motor (Xiang *et al*., 1994; Morris *et al*., 1998). Furthermore, PAFAH1β1 itself binds in part to microtubules and regulates microtubule dynamics (Sapir *et al*., 1997). Preliminary evidence (Gleeson, Lin, Flanagan, and Walsh, unpublished observations) also implicates DCX protein in regulating microtubule polymerization. Moreover, the phenotype of mouse mutations in *PAFAH1β1* is quite distinct from that of *Reelin*, *mdab1*, *p35*, or *cdk5*, consistent with the involvement of *PAFAH1β1* in different cellular mechanisms (Hirotsune *et al*., 1998). Given the dramatic changes that these mutations induce in the patterns of radial organization of cortical neurons, it will be interesting to determine whether these mutants show differential effects on the radially clustered versus widespread clonal patterns.

One neuronal migration disorder that seems to show an intriguing differential effect on different migratory patterns is the human syndrome of periventricular heterotopia, due to mutations in the X-linked gene encoding a large cytoplasmic actin-binding protein called filamin 1 (Fox *et al*., 1998). In PH, 'heterotopic' neurons collect in the ventricular zone beneath the cerebral cortex (Eksioglu *et al.*, 1996), and generally form large nodules. These neurons are highly developed and most of them are pyramidal in morphology (Eksioglu *et al*., 1996). Interestingly, the basal ganglia in PH have never been noted to show heterotopic neurons as would be expected if the migration of cortical neurons that are formed in the LGE were also arrested. This may imply that filamin 1 (*FLN1*) is selectively required for the highly radial migration that takes place between the cortical ventricular zone and the cortex, but may be expendable for the migration of cells from the LGE to the cortex that requires *Dlx-1* or *2*, and perhaps other downstream genes. However, careful review of human specimens, and analysis of an animal model with *FLN1* mutations, will be required to determine whether there are specific defects in migration of specific clonal types, and whether that implicates interaction of filamin with receptor systems specific to radial versus widespread migration.

Notes

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Address correspondence to C.A. Walsh, Division of Neurogenetics, Beth Israel Deaconess Medical Center/Harvard Medical School, Harvard Institutes of Medicine, 77 Avenue Louis Pasteur, Boston, MA 02115, USA. Email: cwalsh@caregroup.harvard.edu.

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