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Postmitotic neurons migrate tangentially in the cortical ventricular zone

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SUMMARY

Patterns of cell movement play a key role in the establishment of the brain's functional architecture during development. The migration of neuronal progenitor cells has been hypothesized to disperse clonally related cells among different areas of the developing cerebral cortex. To test this model, we explored the migratory patterns of cells in the proliferative zone of the intact cortex of the ferret. After focal injections of DiI, labeled cells migrated in all directions and over long distances within the ventricular and subventricular zones. These cells expressed the neuron-specific marker TuJ1 and did not incorporate BrdU after cumulative labeling. Our results reveal an extensive tangential dispersion of cortical cells mediated predominantly or exclusively by the non-radial migration of postmitotic neurons.

Key words: migration, cerebral cortex, neurogenesis, pattern formation, ferret

INTRODUCTION

During development, many young neurons migrate from their site of origin to the final position in which they differentiate. In the cerebral cortex, neurons are generated in the ventricular zone (VZ) adjacent to the ventricle. After their final cell division, they assume a bipolar morphology and migrate out through the intermediate zone and into the cortical plate, where they differentiate and form the six layers of the adult cortex. This outward or radial migration was demonstrated directly with thymidine birthdating studies (Angevine and Sidman, 1961; Rakic, 1974). Both electron microscopy (Rakic, 1972) and in vitro studies (Edmondson and Hatten, 1987) revealed that radial glia provide a substrate for radially migrating neurons. For many years, radial migration along glia was the only migratory pattern recognized in the cortex and appeared sufficient to explain the neuronal movements known to occur during early cortical development.

Evidence that cortical neurons do not follow strict radial pathways was provided initially by retroviral lineage studies. Clones of cells derived from single retrovirally infected progenitors became dispersed tangentially over wide distances (Walsh and Cepko, 1992). In another study, X-inactivation was used to randomly label half of all cortical progenitors with β-galactosidase prior to migration and revealed that about a third of cortical cells moved tangentially from their sites of origin during migration (Tan and Breen, 1993; Tan et al., 1995). Time-lapse imaging of living slices through the developing cortex revealed at least one mode of migration that contributes to tangential dispersion (O'Rourke et al., 1992). Bipolar cells, resembling young neurons, migrated tangentially through the cortical intermediate zone. Immunohistochemical studies confirmed the neuronal identity of these cells (O'Rourke et al., 1995), demonstrating that cortical neurons have a more diverse repertoire of migratory pathways than suspected previously.

Recent studies have generated the intriguing notion that tangential migration may not be limited to postmitotic neurons. Retroviral lineage studies in rat suggest the existence of multipotent progenitor cells that migrate through the VZ and pause periodically to generate subclones of neural or glial progenitors (Reid et al., 1995). This hypothesis has received support from lineage studies employing short survival times, which indicate that clonally related cells can disperse tangentially within the VZ (Walsh and Cepko, 1993). In addition, Fishell et al. (1993) imaged cells moving randomly within the tangential plane of the mouse VZ and found that some of these cells divided as they migrated. While the notion of a migratory, multipotent progenitor is both intriguing and appealing, observations of ferret cortical progenitors (Chenn and McConnell, 1995) have suggested that these cells remain stationary in the tangential plane during the radially oriented basal and apical nuclear movements known as interkinetic nuclear migration (Sidman et al., 1959). Thus the exact nature of progenitor cell movements remains uncertain.

Another form of tangential dispersion in the VZ was suggested by studies using the neuron-specific antibody TuJ1 (Lee et al., 1990). A subset of TuJ1-positive cells with the elongated bipolar morphology of migrating neurons are present in the VZ of both rodents (Menezes and Luskin, 1994) and ferrets (O'Rourke et al., 1995). Most of these cells are oriented parallel to the ventricular surface, suggesting they may be migrating tangentially (O'Rourke et al., 1995). Thus it is possible that migrating neurons could underlie a portion or possibly all of the tangential dispersion that occurs in the cortical proliferative zone. The immunohistochemical studies, however, do not provide direct evidence that these cells are actually migrating.

Current studies thus suggest two possible means for the tangential dispersion of clonally related cells in the VZ. One possibility is that multipotent progenitor cells may migrate and divide at intervals within the VZ (Reid et al., 1995). Another is that elongated neurons, presumably postmitotic, may migrate tangentially in the VZ before entering the intermediate zone. To determine whether either or both of these mechanisms are active in vivo, we traced the migration of cells labeled in localized regions of the proliferative zone in intact ferret cortex. Our results demonstrate that postmitotic neurons migrate tangentially in the cortical proliferative zone, and suggest that the migration of young neurons, and not their progenitors, provides the major and possibly the sole means by which clonally related cells become dispersed in the developing cerebral cortex of the ferret.

MATERIALS AND METHODS

In vivo DiI injections

P1 ferrets (Marshall Farms) were anesthetized with Halothane. Two small holes were made in the skull and dura to expose the dorsal surface of occipital cortex. A glass micropipette (WPI) containing DiI (1,1′-dioctadecyl-3,3,3,3′-tetramethylindocarbocyanine perchlorate, Molecular Probes; 0.05% in dimethyl formamide) was lowered vertically to a position approximately 1.2 mm below the cortical surface using a hydraulic microdrive (Kopf). Approx. 20 nl of DiI solution was pressure injected into the VZ/SVZ using a Picospritzer (General Valve). The skin was sutured and ferrets were revived. After 1, 24, or 48 hours, ferrets were anesthetized with Nembutol and perfused with 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4. Brains were removed and stored in fixative at 4°C. To visualize DiI-labeled cells, brains were embedded in gelatin-albumin and sectioned at 75 or 150 μm (Vibratome). Sections were stored in fixative at 4°C, then mounted onto slides and examined using epifluorescence microscopy. Positions of DiI-labeled cells were recorded with a SIT camera (Hamamatsu). Images were acquired and digitized (Data Translation), analyzed (NIH Image), and assembled into figures (Adobe Photoshop). When brains were sectioned immediately following a 1 hour survival, DiI injection sites spanned the VZ and SVZ; no labeled cells were visible outside of injection sites. When similar brains were stored at room temperature for 1 week then sectioned, DiI diffused into radial glial and retrogradely into neurons in the cortical plate (c.f. Fig. 5 of Roberts et al., 1993), but did not label cells with a migratory morphology in the VZ/SVZ tangential to the injection site.

Cumulative BrdU labeling

Beginning at either 8 or 23 hours after DiI injection, ferrets received a total of 5 intraperitoneal injections of BrdU (Boehringer Mannheim) spaced 3 hours apart (15 hours cumulative labeling). Each BrdU injection was based on 5 mg/kg body weight (10 mg/ml in sterile saline). Kits were perfused with 4% paraformaldehyde 3 hours after the final injection.

Immunohistochemistry

Brains containing DiI-labeled cells were sectioned at 75 μm and immunostained with the mouse monoclonal antibody TuJ1 (Lee et al., 1990). Sections were blocked with serum, then incubated with TuJ1 (1:1000) followed by a fluoresceinated goat anti-mouse secondary antibody (Jackson; 1:200). To facilitate penetration, antibody incubations were carried out overnight at room temperature. All stained sections were imaged on a BioRad MRC-600 confocal microscope.

For BrdU immunostaining, 75 μm sections containing DiI-labeled cells were rinsed for 1 hour with phosphate buffer, treated with 2 N HCl (10 minutes), then incubated in 0.1 M borate buffer, pH 8.5 (15 minutes). Sections were incubated overnight at room temperature with a mouse monoclonal antibody against BrdU (Becton Dickinson; 1:100), followed by a fluoresceinated goat anti-mouse secondary antibody (Jackson; 1:200). Sections double-labeled for BrdU and TuJ1 were treated as above, except that treatment with 2 N HCl was for 1 hour. BrdU was visualized with a rat monoclonal antibody (Accurate; 1:500) and a Texas Red-conjugated goat anti-rat secondary antibody (mouse adsorbed, Jackson; 1:200). After rinsing, sections were stained with TuJ1 followed by fluorescein-conjugated goat anti-mouse (rat adsorbed, Jackson; 1:200).

Lectin staining

Sections were rinsed (0.1 M phosphate buffer, 30 minutes), then incubated in fluorescein-labeled lectin [*Griffonia (Bandeiraea) simplicifolia* seeds; Vector; 20 μg/ml] overnight at 4°C (Chugani et al., 1991; Streit and Kreutzberg, 1987; Wu et al., 1993). After rinsing, sections were mounted in 1:1 glycerol/phosphate buffer.

Syto-11 staining

Coronal cortical slices (400 μm) from E29, E36, and P0-3 ferrets were incubated in 5 mM Syto-11 (Molecular Probes) as described previously (Chenn and McConnell, 1995). Slices were imaged on a Bio-Rad MRC-600 confocal microscope every 2 minutes; images were obtained at least 50 μm below the slice surface. 344 cells from 188 divisions were imaged in 34 slices from 34 animals. Cells were imaged at all dorsoventral levels of the cortex and from several different functional areas. Their cleavage orientations were analyzed in a previous study (Chenn and McConnell, 1995). In these experiments, the imaging period begins when sister chromatids separate in anaphase, and ends when cells exit the focal plane, are obscured by other cells, or become indistinguishable from neighbors.

RESULTS

To assess the migratory patterns of cells in the proliferative regions of the developing cerebral wall, focal injections of DiI were made into the ventricular and subventricular zones of postnatal day (P) 1 ferrets, when layer 2/3 neurons are being generated (Jackson et al., 1989; McConnell, 1988). Ferrets provide an accessible model system in which to study cell movements in vivo because neurogenesis continues after birth, until roughly P14 (Jackson et al., 1989). DiI injected at a depth of 1.2 mm in dorsal regions of posterior cortex reliably targeted the VZ and subventricular zone (SVZ) (Figs 1, 2). Animals were perfused after survival times of 1, 24, or 48 hours, and brains were sectioned in the coronal or sagittal plane to visualize the positions and morphologies of DiI-labeled cells. Brains sectioned following a 1 hour survival revealed DiI injection sites that spanned the VZ and SVZ, but no labeling of migrating cells (see Methods).

Longer survivals revealed large numbers of DiI-labeled cells emanating from each injection site (Fig. 1). In the radial direction, cells with the elongated morphology of migrating neurons were found both in the intermediate zone directly above the injection site and in the cortical plate. Laterally, large numbers of DiI-labeled cells with migratory morphology were found in the VZ and SVZ. In addition, some labeled cells were displaced both laterally and radially at a distance from the injection site in the intermediate zone or cortical plate. Many cells dispersed over large distances following DiI labeling,

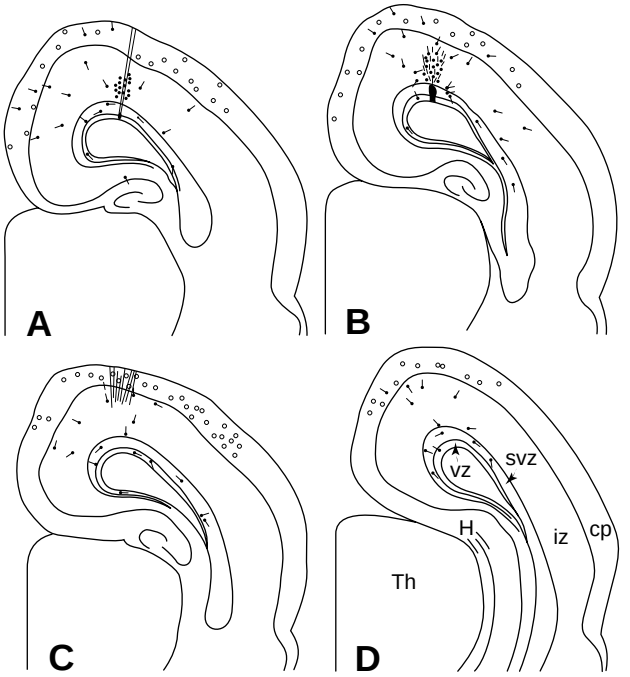

Fig. 1. Positions of DiI-labeled cells from a common origin in the VZ. Tracings of 4 alternate 150 μm coronal sections (A-D, anterior to posterior) through the cerebral cortex 48 hours after DiI injection. Elongated DiI-labeled cells (filled circles with lines) have migrated in all directions from the injection site (black area in A and B). Cells emanate into the cortical plate (CP) and move tangentially into lateral and medial neocortex. One cell has migrated into the hippocampus (H) in A. The orientation of each cell is indicated by the line extending from the filled circle. Open circles (A-D) in the cortical plate represent retrogradely labeled neurons. Long lines extending through the intermediate zone (IZ) and cortical plate (in A and C) indicate labeled radial glia. Filled circles above the injection site are astroglial cells. Short lines above the injection in B are densely packed radially migrating cells in the intermediate zone. Th, thalamus. Scale bar, 1 mm.

with a maximal dispersion of about 2.5 mm from the injection site after 48 hours (Fig. 1; Table 1). These distances translate into large displacements relative to the total dimension of the neocortex: in the medial direction, cells exited from neocortex into the hippocampus (Fig. 1A); lateral displacements placed cells in extreme temporal regions (Fig. 1B,C). In the sagittal plane, labeled cells reached the anterior and posterior boundaries of the proliferative zone, although no cells entered the anterior SVZ pathway into the olfactory bulb (Luskin, 1993).

Fig. 2. DiI-labeled cells in the VZ/SVZ have an elongated morphology. Photomontage of a DiI injection site in the VZ/SVZ (coronal section). A DiI-labeled cell in the SVZ with an elongated, bipolar morphology (arrow) has migrated 387 μm from the injection site. A cell adjacent to the injection site (arrowhead) resembling a radial glial cell has been labeled retrogradely via a process in the intermediate zone. Scale bar, 100 μm.

Morphology and distribution of cells in the VZ and SVZ

We focused our analysis on the distribution and morphologies of cells in the VZ and SVZ, where much of the widespread migration appears to have occurred. Fig. 2 shows an injection site spanning the VZ/SVZ; a single cell in the SVZ is located 387 μm medial from the injection site after a 24-hour survival. The cell has an elongated, bipolar morphology with a thick, long (70 μm) process similar to the leading process of a migrating neuron. Commonly extending from the opposite ends of such cells were short, thin processes resembling the trailing processes of migrating neurons (Fig. 3). This morphology typifies that seen in DiI-labeled cells distributed tangentially within the VZ/SVZ. Three arguments suggest that these cells have migrated away from the DiI injection sites. First, the cells have morphological features characteristic of both migrating neurons (Edmondson and Hatten, 1987; O'Rourke et al., 1992) and glia (Levison and Goldman, 1993). Second, it is unlikely that these cells were labeled retrogradely from injection sites because their trailing processes were only 10-20 μm in length and never extended into an injection site. These cells were typically brightly labeled with DiI; in contrast, retrogradely labeled neurons, located primarily in the cortical plate (Fig. 1), were more faintly labeled and extended clearly visible axons into injection sites. Finally, the average distance between labeled cells and injection sites increased over time. Labeled cells were found at average distances of 370

Table 1. Rates and distances of cell dispersion in the VZ/SVZ

	24 hour survival		48 hour survival	
Direction of dispersion	Average distance from injection site (μm)	Average rate of migration (μm/hr)	Average distance from injection site (μm)	Average rate of migration (μm/hr)
Mediolateral	367±269	15	731±676	15
Anteroposterior	510±410	21	620±401	13

Distances of DiI-labeled VZ/SVZ cells from injection sites were calculated after 24 hours (mediolateral plane from coronal sections, 75 cells, 7 animals; anteroposterior plane from sagittal sections, 99 cells, 5 animals) or 48 hour (mediolateral/coronal, 96 cells, 3 animals; anteroposterior/sagittal, 50 cells, 3 animals) survivals. Because DiI-labeled cells may exit injection sites at any time during the survival period, these calculations represent minimum estimates of the actual rates of movement and are quite variable. Mean ± s.d.

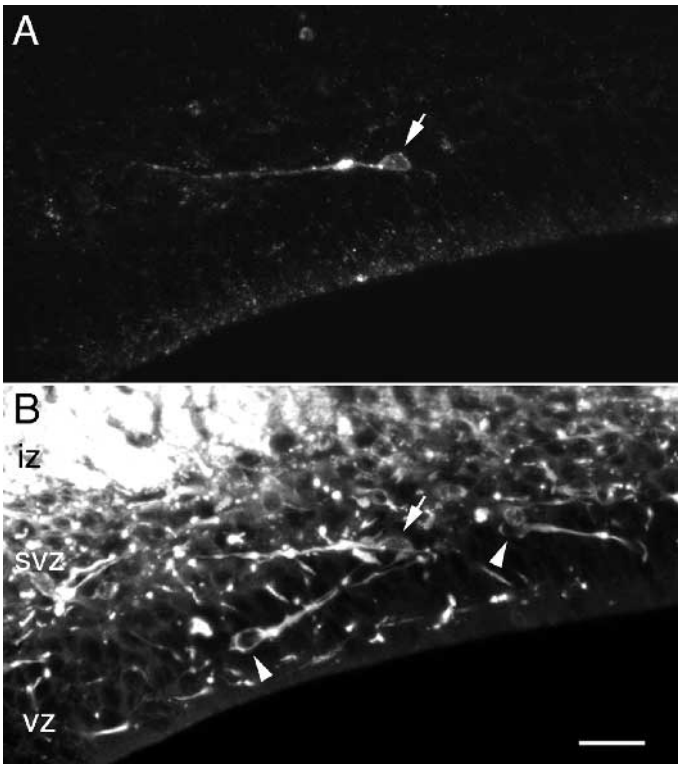

Fig. 3. DiI-labeled migrating cells in the VZ/SVZ are TuJ1-positive. **(**A) DiI-labeled cell (arrow) at the top of the VZ has migrated tangentially from an injection site. Immunostaining (B) demonstrates that the same cell (arrow) expresses TuJ1 immunoreactivity. Two other TuJ1-positive cells with migratory morphologies are present in the VZ (arrowheads). Scale bar, 20 μm.

μm (mediolateral) or 510 μm (anteroposterior) from these sites after 24 hr, and 620-730 μm after 48 hours (Table 1), consistent with their active and continued migration.

Migrating cells were distributed throughout the proliferative zone. Cell positions were examined in either coronal or sagittal sections after 24 or 48 hours (these cells are the same as those analyzed in Table 1; see Table 1 for numbers of cells and animals). In coronal sections at 24 or 48 hours, DiI-labeled cells showed similar distributions: 28% were in the VZ, 61% in the SVZ, and 11% were at the border between these two zones. Similarly, in sagittal sections after 24 or 48 hours, 30% were in the VZ, 57% in the SVZ, and 13% were at the VZ/SVZ border. Thus, roughly a third of cells migrating within the proliferative zone are located in the VZ, a region composed primarily of neuronal progenitor cells. Cells migrated away from injection sites in all directions (Fig. 1). Of labeled cells moving mediolaterally in the VZ/SVZ (assessed in coronal sections), 47% were displaced laterally and 52% medially. This contrasts with migratory patterns in ventrolateral cortex, where the bulk of tangential migration in the intermediate zone is directed laterally along the curved processes of glia (Bayer et al., 1991; Misson et al., 1991). In the sagittal plane, in which anteroposterior dispersion was assessed, comparable numbers of labeled cells in the VZ/SVZ migrated anteriorly (54%) vs posteriorly (46%) from injection sites.

The distances over which labeled cells migrated in the sagittal and coronal planes were measured at 24 and 48 hours (Table 1). These distances translate into average rates of cell movement in the VZ/SVZ of 13-21 μm/hour, rates similar to those of radially migrating neurons in the cortical intermediate zone (Angevine and Sidman, 1961; Rakic, 1974; McConnell, 1988; Jackson et al., 1989; O'Rourke et al., 1992). Because migratory distances were calculated only within the plane of section, and because DiI-labeled cells may exit injection sites at any time during the survival period, our calculations represent minimum estimates of the actual rates of movement and are quite variable. Calculations based on the maximal spread of labeled cells over time predict migration rates of 40-50 μm/hour. Indeed, tangentially migrating cells imaged in cortical slices move at comparable rates, averaging 30 μm/hour and reaching 50 μm/hour (O'Rourke et al., 1992). Thus our estimates of the maximal rate of migration are consistent with directly observed rates of tangential movement in the intermediate zone.

Orientations of migrating cells

Both their polarized morphology and migration rates suggest that the DiI-labeled cells migrate in a manner similar to that of young neurons in the intermediate zone. Imaging of the latter cells revealed that they move in a highly directional manner, extending a leading process in the direction through which the cell soma is subsequently translocated. To compare these populations further, we measured the orientations of DiI-labeled cells in the proliferative zone (same cells as those in Table 1). Overall, 78% were oriented tangentially (roughly parallel to the ventricular surface), and 16% were oriented radially. The remaining 6% were oriented at intermediate angles (30-60°) between radial and tangential. In contrast, only 13% of migrating cells in the intermediate zone were oriented tangentially and 17% were oriented at intermediate angles (O'Rourke et al., 1992). In the VZ/SVZ, radially migrating cells may be underrepresented because any cell spending a significant time moving radially is likely to enter the intermediate zone. Indeed, many radially oriented cells in the intermediate zone were displaced tangentially from injection sites (Fig. 1); however, we cannot determine whether these cells arrived at these positions through tangential migration in the VZ/SVZ, the intermediate zone, or both.

In general, labeled cells in the VZ/SVZ were oriented with their leading processes directed away from injection sites (70%); only 13% had leading processes oriented toward their point of origin, and the remainder were oriented radially. This bias in cell polarity, facing away from injection sites, is inconsistent with a random pattern of movement as described for some VZ progenitors (Fishell et al., 1993). Although their dynamic behavior has not been observed directly, the orientations and polarized morphologies of migrating cells in the present study suggest that they employ a directional mechanism for migration similar to that of neurons in the intermediate zone (O'Rourke et al., 1992). In the latter region, cells can migrate radially or tangentially, making sharp turns from one direction to another and reversing polarity in a matter of minutes. That only 70% of cells in the present study are oriented away from injection sites suggests that migrating cells in the VZ/SVZ are also capable of changing direction.

Neuronal identity of migrating cells

A key question is whether cells that disperse tangentially in the

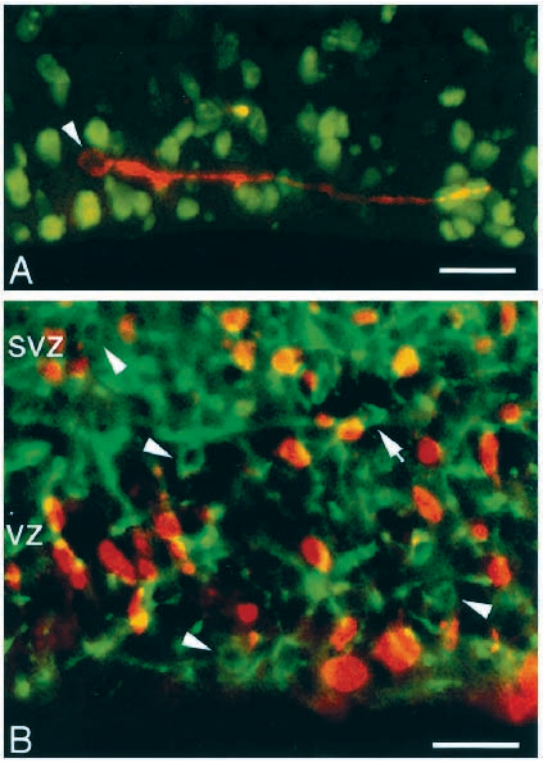

Fig. 4. DiI-labeled migrating cells and TuJ1-positive cells in the VZ/SVZ fail to incorporate BrdU. **(**A) A DiI-labeled cell (red, arrowhead) has migrated away from an injection site and extends its leading process parallel to the ventricular surface. The cell failed to incorporate BrdU after 15 hours cumulative labeling, although neighboring cells have, as indicated by immunostaining of their nuclei (green). (B) An elongated TuJ1-positive cell (green, arrow) at the top of the VZ has not incorporated BrdU after 15 hours cumulative labeling (red stained nuclei). Several other TuJ1-positive cell bodies (arrowheads) in the VZ/SVZ are also BrdU negative. Scale bars, 20 μm.

VZ/SVZ are dividing progenitor cells, as hypothesized to explain the tangential distribution of clonally related cortical cells in rat (Reid et al., 1995). Alternatively, tangentially migrating cells could be young postmitotic neurons. To distinguish between these possibilities, we asked whether DiI-labeled cells in the VZ/SVZ express a neuron-specific protein, class III β-tubulin, which is recognized by the TuJ1 monoclonal antibody (Lee et al., 1990). DiI was injected into the VZ/SVZ of P1 ferrets as above, then coronal sections were prepared after a 24-hour survival and immunostained with TuJ1 antibody. Fig. 3 shows an elongated, migratory DiI-labeled cell (Fig. 3A) in the upper VZ that expresses the TuJ1 antigen (Fig. 3B). Of 42 DiI-labeled cells (from 3 animals) that had dispersed tangentially in the VZ/SVZ, 38 were clearly TuJ1-positive; the labeling of the remaining 4 cells was ambiguous. This result is consistent with the possibility that migrating cells in the VZ/SVZ are postmitotic neurons. Indeed, the TuJ1 antibody labels only a subset of VZ cells, which do not include neuroepithelial progenitors. Previous studies of TuJ1-positive cells in the cortical VZ revealed that these cells have a migratory morphology (Menezes and Luskin, 1994; O'Rourke et al., 1995) and that at least 75% are tangentially

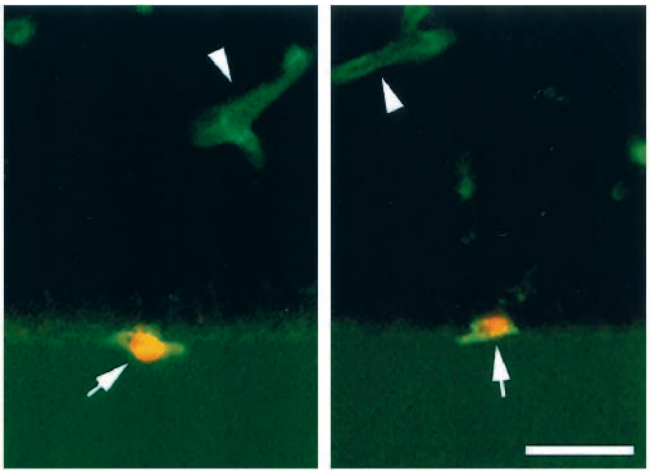

Fig. 5. Round cells at the ventricular surface bind GSA I-B_4 lectin. Injections of DiI into the lateral ventricle label a population of round cells at the ventricular surface (red, arrows). These cells bind GSA I-B_4 (green), which recognizes endothelial cells (arrowheads) and microglia, and may bind to other, as yet uncharacterized, cell types in the VZ. These cells resemble a rounded cell at the lumenal surface in Fig. 2A of Fishell et al. (1993). Scale bar, 20 μm.

oriented (O'Rourke et al., 1995). We have shown that similar numbers of DiI-labeled migratory cells (78%) are tangentially oriented. Together, these results link the identities of the TuJ1-positive and DiI-labeled migrating cells within the VZ/SVZ, and suggest that the DiI-labeled cells may be young neurons.

Migrating cells are postmitotic

TuJ1 staining does not demonstrate definitively that neuronal cells have exited the cell cycle: cells migrating from the anterior SVZ into the olfactory bulb both express TuJ1 and undergo cell division, subsequently generating olfactory neurons (Menezes et al., 1995). Thus it is possible that the TuJ1-positive cells in the cerebral proliferative zone might represent a similar subpopulation of migrating neuronal progenitor cells. To test this hypothesis, DiI was injected into the VZ/SVZ and labeled cells were allowed to migrate away from injection sites for either 8 or 23 hours. Beginning at these times, animals were exposed to 5-bromo-2′ deoxyuridine (BrdU) for 15 hours of cumulative labeling (5 injections spaced 3 hours apart), to label the nuclei of any cells in S-phase of the cell cycle. Brains of animals labeled with DiI and BrdU were fixed, sectioned in the coronal plane, and immunostained to reveal BrdU incorporation. Figure 4A illustrates a DiI-labeled cell extending tangentially above the ventricular surface; while this cell is surrounded by BrdU-positive nuclei, its nucleus is unlabeled. Of 65 DiI-labeled migratory cells in the VZ/SVZ, none incorporated BrdU during the 8- to 38-hour period following DiI injection (8- to 23-hour labeling, 31 cells, 3 animals; 23- to 38-hour labeling, 34 cells, 3 animals). Roughly 30% of all VZ cells were labeled with BrdU; thus, if DiI-labeled cells were mitotically active, a similar number should have incorporated BrdU. These results suggest strongly that the migrating cells in the proliferative zone have exited the cell cycle.

To sample a larger number of cells, we took advantage of the fact that nearly all DiI-labeled migratory cells were immunoreactive with the TuJ1 antibody, and asked whether

any TuJ1-positive cells incorporate BrdU. Sections from cumulative labeling experiments were immunostained with TuJ1 antibody. The vast majority (over 99%) of the 218 TuJ1-labeled cells from 4 animals failed to incorporate BrdU (Fig. 4B). Of 93 cells in the VZ and 46 cells at the VZ/SVZ border, none were BrdU positive; of 79 cells in the SVZ, only one appeared to be labeled with BrdU. These results provide strong support for the conclusions that migrating cells in the VZ/SVZ are postmitotic and that they are young neurons. Previous studies in mouse of TuJ1-immunoreactive VZ cells showed that these cells do not incorporate BrdU after single injections between E14 and E17 (Menezes and Luskin, 1994), further evidence that TuJ1-positive cells are postmitotic neurons.

Other cell populations in the cortical VZ

Our studies in ferret have revealed a population of elongated, postmitotic neurons that migrate tangentially within the VZ. In mouse VZ, round neural progenitors have been observed to disperse tangentially through random cell movements (Fishell et al., 1993). Because these cells in rodent appear dissimilar to the cells observed in our experiments, we sought to determine whether the DiI labeling techniques used in the two studies labeled distinct subpopulations of cells. The present experiments employed focal injections of DiI into the VZ/SVZ and followed cells over 1-2 days, whereas Fishell and colleagues (1993) bathed the ventricular surface with a dilute solution of DiI then immediately imaged the labeled cells. To ascertain whether different methods of DiI application result in the labeling of distinct cell populations, we injected DiI directly into the lateral ventricles of P1 ferrets. The ventricular delivery of DiI was verified by the presence of labeled choroid plexus cells. Such injections labeled radially oriented VZ cells (not shown; see Chenn and McConnell, 1995: Fig. 3), which resemble the neuroepithelial cells labeled by Fishell et al. (1993: Fig. 2A). Importantly, in both this and our previous studies (Chenn and McConnell, 1995), DiI injections into the ventricle failed to label tangentially oriented cells in the VZ/SVZ. This is not surprising because the tangentially migrating cells labeled from focal injections into the VZ/SVZ do not extend processes to the lumenal surface (Figs 2-4).

In addition to neuroepithelial cells, DiI injections into the ventricle labeled a population of round cells at the lumenal surface of the VZ (Fig. 5). These cells were not seen when injections were confined to the VZ/SVZ, and they represent an unusual and to our knowledge uncharacterized ventricular population. To explore their identity further, sections were incubated with a fluoresceinated lectin (GSA I-B_4) that binds microglia and endothelial cells in the mature and developing brain (Chugani et al., 1991; Wu et al., 1993), although this lectin may not mark these cells exclusively. As illustrated in Fig. 5, all rounded cells at the VZ surface (n = 24 cells, 3 animals) bound the GSA I-B_4 lectin. Neither the migratory nor mitotic behavior of these lectin-binding cells has been observed.

Behavior of dividing cells in brain slices

Neuronal progenitor cells are radially oriented with processes that span the VZ; during the cell cycle their nuclei undergo radial movements known as interkinetic nuclear migration (Sidman et al., 1959). In previous time-lapse studies, DiI-labeled VZ cells in E29 ferrets remained constrained at the same tangential position as they underwent interkinetic nuclear migration (Chenn and McConnell, 1995). To examine further the behavior of daughter cells after mitosis, cells in living cortical slices from E29, E36, and P0-P3 ferrets were labeled with the nucleic acid-binding dye Syto-11 and observed by time-lapse confocal microscopy (Chenn and McConnell, 1995) for an average of 40-70 minutes after mitosis (Table 2). The two daughters produced from a division typically elongated radially and moved outward in the radial direction after mitosis (Fig. 6). In only one division (out of 344 Syto-11-labeled cells; Table 2) did daughter cells move tangentially after mitosis. Radial movements were also observed when E29 VZ cells labeled with DiI were followed for ~8 hours after mitosis (Chenn and McConnell, 1995). Together these results suggest that the movements of ferret progenitor cells are radially constrained, and that the immediate behavior of daughters is to elongate or move radially within the VZ. We speculate that young neurons may subsequently turn and migrate tangentially within the VZ/SVZ, as demonstrated in our experiments, or in other regions of the cerebral wall, as shown previously (O'Rourke et al., 1992, 1995). The interval between cell birth in the radial domain and subsequent tangential migration is not

Table 2. Patterns of movement of Syto-11-labeled cells in the VZ after mitosis

	E29	E36	P0-P3
No. of cells imaged for at least 15 minutes after mitosis	172	91	81
Average time imaged after mitosis (minutes ± s.d.)	38.4±53.4	51.6±43.7	69.9±70.4
% moving radially*,‡	41.9	40.6	34.6
% elongating radially†,‡	17.3	13.2	20.3
% moving tangentially*,‡	1.1	0.0	0.0
% elongating tangentially†,‡	0.0	0.0	0.0

*Movement was defined as the translocation of a cell soma over a distance of at least one cell diameter away from the site of mitosis.

†Elongation was defined as a change in cell shape from a round to an elongated morphology.

‡The remaining cells (roughly 40-45% of the total at each age) neither moved nor elongated after mitosis.

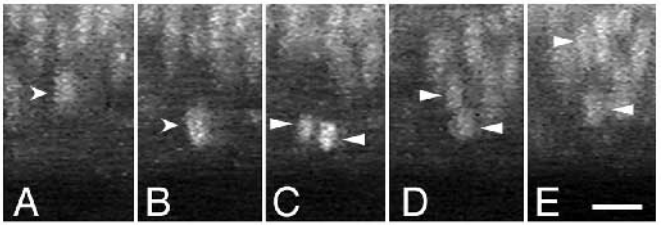

Fig. 6. Time-lapse imaging of Syto-11-labeled progenitor cells. Each frame represents a single optical section from a time-lapse series of confocal images. Syto-11 labeling was diffuse until chromatin condensation in metaphase, when a bright fluorescent bar demarcates the metaphase plate. (A-C) A progenitor cell (arrowhead, A) descends radially to the ventricular surface; sister chromatids separate at anaphase (C). (D,E) Following cleavage and chromosome decondensation, both daughter cells (arrowheads) move radially out from the lumenal surface. Times in each panel: (A), 0 minutes; (B), 15 minutes; (C), 26 minutes; (D), 80 minutes; (E), 151 minutes. Scale bar, 10 μm.

known; however, our double-labeling studies with TuJ1 and BrdU suggest that at least 15 hours elapse between a cell's final S-phase and TuJ1 expression, at which point many cells are oriented tangentially.

DISCUSSION

We have examined the patterns of tangential dispersion of cells emigrating from a common origin in the proliferative zone of the developing cerebral cortex. We found that postmitotic neurons can migrate over long distances within the VZ and SVZ, dispersing from dorsal cortex in all directions. In some cases, cells from neocortex migrated into sites as distant as the hippocampus. Although the hypothesis that neuronal progenitor cells may migrate tangentially within the VZ or SVZ has been presented (Reid et al., 1995), our data indicate that tangentially migrating cells in the ferret proliferative zone are terminally postmitotic and express a neuron-specific marker. These results, in conjunction with previous studies (O'Rourke et al., 1992), suggest that most if not all tangential dispersion of cortical neurons in developing ferrets is attributable to the tangential migration of postmitotic neurons. Because TuJ1-positive cells are also present in the mouse VZ/SVZ (Menezes and Luskin, 1994), we postulate that the tangential migration of postmitotic neurons in this zone contributes to the dispersion of clonally related cells in other species as well.

Tangential migration in cortical development

Several lines of evidence indicate that cortical neurons disperse tangentially during development. These include retroviral lineage studies (Walsh and Cepko, 1992, 1993; Reid et al., 1995; Kornack and Rakic, 1995), imaging of migrating cells in cortical slices (O'Rourke et al., 1992), studies of the orientations of migrating cells in fixed sections (O'Rourke et al., 1995), analysis of transgenic mice carrying an X-linked *lacZ* marker (Tan et al., 1995), and the present study. The question remaining is not whether tangential movements occur, but what fraction of neurons migrate nonradially and over what distances they become dispersed during development. Estimates of the numbers of tangentially migrating neurons range widely from 10% (Rakic, 1995; Soriano et al., 1995) up to 70% (Reid et al., 1995). Our studies in the ferret intermediate zone suggest that the fraction of nonradially migrating cells increases from 20% at E31 to over 35% at P10 (O'Rourke et al., 1992, 1995). Because tangential migration can occur at all levels of the developing cerebral wall, the actual numbers of cells employing nonradial pathways for migration may be even larger. Unfortunately the methods used in our experiments are not appropriate to resolve this issue. Although every injection labeled tangentially displaced cells, small variations in injection size and placement resulted in wide variations in the extent of DiI labeling radial to these sites. Thus we could not quantify reliably the fractions of radially vs tangentially migrating cells.

Nevertheless, the data presented here both confirm and broaden previous studies by providing a direct demonstration that cells in intact cortex migrate in all directions and over long distances from a single point at the ventricular surface. Our results are consistent with recent retroviral lineage studies in ferret, which indicate that cortical clones become widely dispersed during development (Reid, Tavazoie and Walsh, unpublished data). In our present study, cells originating in dorsal occipital cortex migrated into the hippocampus and the far reaches of the neocortex in only 2 days. A similar spread of clonally related cells between cortex and hippocampus was observed in rat (Reid et al., 1995). Although our studies involved only short survivals, the ferret retroviral lineage studies suggest that tangentially migrating neurons eventually enter and differentiate within the cortical plate. Thus it seems likely that tangentially migrating neurons survive and incorporate functionally into the cortex.

Mechanisms of tangential dispersion of cortical neurons

Our data suggest that this dispersion in the ferret cortex is achieved predominantly, and possibly exclusively, by the nonradial migration of postmitotic neurons. At least 90% of tangentially migrating cells in the VZ/SVZ express the neuron-specific antigen, TuJ1, and in no case did any DiI-labeled migrating cell incorporate BrdU during the 8-38 hours following DiI injection. Examination of a larger population of TuJ1-positive cells in the VZ/SVZ revealed that over 99% were BrdU-negative after 15 hours of cumulative labeling. TuJ1-positive cells in the mouse proliferative zone similarly fail to incorporate BrdU after single injections at several stages during cortical neurogenesis (Menezes and Luskin, 1994). These studies show definitively that postmitotic neurons migrate over long distances within the VZ/SVZ. Although the final resolution of this issue will come from observing directly the patterns of neuronal migration at earlier ages, tangentially oriented TuJ1-positive cells are common in the VZ/SVZ throughout cortical plate neurogenesis in both ferret (O'Rourke et al., 1995) and mouse (Menezes and Luskin, 1994). Studies of migration in vitro have suggested, however, that tangential migration may not occur during the establishment of the subplate (O'Leary and Koester, 1993).

Our results contrast with imaging studies of the mouse VZ, in which DiI-labeled progenitor cells dispersed tangentially over time (Fishell et al., 1993). The cell populations described in these two studies displayed several distinct features; indeed, we believe that they represent different populations. The cells imaged by Fishell et al. (1993) were described as rounded cells that extended short lamellar processes and moved in a random walk fashion. A fraction of these cells were observed to divide and thus may represent neural progenitors, although it is unclear what percentage of cells were actually cycling because mitosis represents only a brief period during the cell cycle. In contrast, migrating cells labeled by focal DiI injections in the present study were highly polarized, extending a long, thick process from one pole of the cell. Although the migration of the cells was not observed directly, their leading processes were directed predominantly away from injection sites, an observation suggestive of a directed migration and inconsistent with random movements. Fishell et al. (1993) labeled cells by bath application of DiI onto the ventricular surface. We found that DiI injections into the lateral ventricle fail to label the tangentially oriented migratory cells in our experiments, presumably because they do not contact the ventricular surface. Although we hypothesize that tangentially migrating neurons in the VZ/SVZ must be derived from neuroepithelial cells, at least 15 hours pass between a cell's final S-phase and the

expression of the TuJ1 antigen, a marker expressed by nearly all tangentially migrating cells in the VZ/SVZ.

Do our results rule out the possibility that dividing progenitors migrate tangentially? As shown here, no tangentially migrating, DiI-labeled cells incorporated BrdU, nor were DiI-labeled cells with a neuroepithelial morphology found at any distance from the injection site. Progenitor cells visualized throughout cortical neurogenesis with Syto-11 (this work) or with DiI (Chenn and McConnell, 1995) produced daughters that elongated or moved radially during the 1-8 hours following mitosis. Collectively these observations suggest that progenitor cell movements in the ferret VZ are radially constrained. We do not know why our results differ from those of Fishell et al. (1993), who found that DiI-labeled VZ cells disperse tangentially from one another over an 8-hour period. It is conceivable that there are species differences in the behavior of cells in the VZ. Until more is known about the ultimate fate of the tangentially migrating neural progenitors in the rodent VZ, this issue will remain unresolved.

Models for the tangential dispersion of cortical neurons

Retroviral lineage studies in rat suggest that many cortical clones are composed of single cells and/or small clusters of cells spaced periodically at 2-3 mm intervals; cells within each cluster have similar morphogical features and laminar positions, yet distant but related cells could have disparate phenotypes (Reid et al., 1995). To explain these patterns, two types of cortical progenitors were postulated: a migratory, multipotential progenitor that moves tangentially through the proliferative zone, and a nonmigratory progenitor with a more limited potential. The migratory progenitor would divide to produce two daughters. One daughter is a nonmigratory progenitor that produces one or more postmitotic cells that move radially from that site; the second is another multipotential cell that migrates tangentially within the VZ/SVZ, dividing later in a new position. This process could be reiterated to form several clusters of clonally related cells. The finding in the present paper that tangentially migrating cells in the ferret proliferative zone are postmitotic casts doubt on the generality of this proposal.

It is possible that the mechanisms of neurogenesis and migration differ between species. It appears that there are striking similarities in the tangential distribution of neurons in ferret and rodent; both show widespread neuronal dispersion (Walsh and Cepko, 1992, 1993; this study) and postmitotic TuJ1-positive cells in the VZ/SVZ (Menezes and Luskin, 1994; O'Rourke et al., 1995). However, recent lineage tracing studies in the ferret cortex reveal that clonally related neurons are not distributed periodically at regular intervals, as in rat, but are instead scattered randomly in the tangential plane (Reid, Tavazoie and Walsh, unpublished data). The absence of periodic clustering suggests that the ferret does not employ migratory progenitor cells that divide at regular intervals to create clusters of related cells. These results seem consistent with a mode of cell dispersion based on the migration of postmitotic neurons. Thus, it is plausible that the rat and ferret employ distinct mechanisms to disperse neurons tangentially.

Nevertheless, one may wonder whether the distributions of clonally related cells in rat require the existence of a migratory progenitor. A compelling argument for such a cell would be the presence of clones composed of multiple cell clusters, each containing more than one neuron. The existence of clustered glia would not support this argument because glial progenitors divide within the cortical plate (Levison and Goldman, 1993). Only one of the 74 clones analyzed by Reid et al. (1995; Table 1: clone 27) contained more than one cluster of neurons; all other multi-neuron clones were composed of one cluster of neurons flanked by one or more isolated clonally related neurons. These patterns appear not to require the existence of a migratory progenitor, and could be generated by the tangential migration of postmitotic neurons. Indeed, TuJ1-positive, BrdU-negative cells with migratory morphology do exist in the mouse VZ/SVZ (Menezes and Luskin, 1994), suggesting that the migration of postmitotic neurons through these regions must at least contribute to tangential dispersion in the rodent cortex. A direct test of whether neuronal progenitor cells migrate in rodents awaits studies similar to those performed in our experiments.

Fig. 7 illustrates a model for the delivery of neurons into their final positions in the ferret cortical plate. In this model, cortical progenitors remain stationary within the proliferative zone, giving rise to young neurons that can use various migratory pathways. Migration along radial glia delivers subsets of related neurons into clusters located radial to their site of origin. We postulate that remaining neurons become dispersed from their siblings by migrating tangentially within the VZ/SVZ, intermediate zone, or cortical plate (O'Rourke et al., 1992, 1995). The existence of cells with a common phenotype within clusters in rat could be explained by cell type-specific migration pathways, a possibility that remains to be explored experimentally. The question of what mechanism might generate the periodic distribution of clonally related cells in rat is unresolved; indeed, our data cannot rule out the existence of migratory neuronal progenitors in the rodent. Nevertheless, we have demonstrated that the tangential migration of postmitotic neurons can account for most if not all tangential dispersion in the ferret and is likely to contribute, at least in part, to clonal dispersion in a wide variety of species.

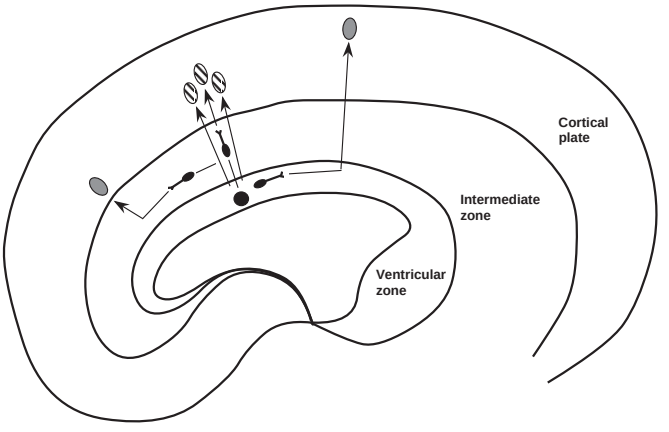

Fig. 7. A model for neuronal migration patterns in developing cerebral cortex. Our data are consistent with the model that cortical neurons arise from a stationary progenitor cell (black circle) that gives rise to neurons that may either migrate radially to form a cluster (striped cells), or tangentially (gray cells). Neurons can migrate tangentially at all depths of the developing cerebral wall, including the VZ, SVZ, intermediate zone and cortical plate.

Significance of tangential migration

Many CNS regions display tangential migration during development. Mitotic progenitors travel long distances to create the cerebellar external granular layer (EGL) (Sidman and Rakic, 1973) and olfactory bulb interneurons (Lois and Alvarez-Buylla, 1994; Menezes et al., 1995). Tangential dispersion is constrained at rhombomere boundaries in developing hindbrain, which may aid the compartmentalization of gene expression (Fraser et al., 1990). Cells in developing spinal cord migrate nonradially in the intermediate zone to disperse interneurons from ventral motor neurons (Leber and Sanes, 1995), and tectobulbar neurons disperse tangentially after arriving in the tectal plate (Gray and Sanes, 1991). Some of these movements have an obvious purpose, such as the creation of a new proliferative region like the EGL. The function of others is more obscure: for example, what function is subserved by cell movements within hindbrain rhomobomeres? One possibility is that if progenitors are specified to form a distinct class of neurons, as in the early hindbrain (Lumsden et al., 1994), their progeny must spread to fulfill their function throughout each compartment. In cortex, it is plausible that subclasses of neurons, such as interneurons vs projection neurons, may employ different pathways for migration. Until the nature and numbers of nonradially migrating cells in cortex are determined definitively, the functional significance of tangential migration remains elusive.

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