Tangential migration of neurons in the developing cerebral cortex

Nancy A. O'Rourke*, Daniel P. Sullivan, Christine E. Kaznowski, Alice A. Jacobs and Susan K. McConnell

Department of Biological Sciences, Stanford University, Stanford, CA. 94305, USA

*Author for correspondence

SUMMARY

The mammalian cerebral cortex is divided into functionally distinct areas. Although radial patterns of neuronal migration have been thought to be essential for patterning these areas, direct observation of migrating cells in cortical brain slices has revealed that cells follow both radial and nonradial pathways as they travel from their sites of origin in the ventricular zone out to their destinations in the cortical plate (O'Rourke, N. A., Dailey, M. E., Smith, S. J. and McConnell, S. K. (1992) *Science* 258, 299-302). These findings suggested that neurons may not be confined to radial migratory pathways in vivo. Here, we have examined the patterns of neuronal migration in the intact cortex. Analysis of the orientations of [^{3}H]thymidine-labeled migrating cells suggests that nonradial migration is equally common in brain slices and the intact cortex and that it increases during neurogenesis. Additionally, cells appear to follow nonradial trajectories at all levels of the developing cerebral wall, suggesting that tangential migration may be more prevalent than previously suspected from the imaging studies. Immunostaining with neuron-specific antibodies revealed that many tangentially

migrating cells are young neurons. These results suggest that tangential migration in the intact cortex plays a pivotal role in the tangential dispersion of clonally related cells revealed by retroviral lineage studies (Walsh, C. and Cepko, C. L. (1992) *Science* 255, 434-440). Finally, we examined possible substrata for nonradial migration in dorsal cortical regions where the majority of glia extend radially. Using confocal and electron microscopy, we found that nonradially oriented cells run perpendicular to glial processes and make glancing contacts with them along their leading processes. Thus, if nonradial cells utilize glia as a migratory substratum they must glide across one glial fiber to another. Examination of the relationships between migratory cells and axons revealed axonal contacts with both radial and nonradial cells. These results suggest that nonradial cells use strategies and substrata for migration that differ from those employed by radial cells.

INTRODUCTION

During embryogenesis, neurons of the cerebral cortex are generated in the ventricular zone adjacent to the lateral ventricles. They then migrate outward through the intermediate zone and into the cortical plate, where they form the six layers of the adult cortex. Cells labeled with [^{3}H]thymidine during their final mitosis can be traced using autoradiography as they move outward (Angevine and Sidman, 1961; Rakic, 1974). Migration toward the pial surface appears to occur along radial glia which extend long processes from the ventricular to the pial surfaces. Using electron microscopy, Rakic (1972) observed a close apposition between migrating neurons and radial glia. Subsequently, Edmondson and Hatten (1987) demonstrated that cerebellar neurons could migrate along glia in vitro. These data led many to believe that neurons in the cerebral cortex employed glia as their sole substratum and consequently followed strictly radial pathways into the cortical plate. Based on these concepts, Rakic proposed that cortical areas may be laid out in a 'proto-map' in the ventricular zone that is then transferred faithfully to the cortical plate by neurons that maintain their

neighbor-neighbor relationships as they migrate radially (Rakic, 1988).

Indications that migration in the cortex may not be strictly radial have come from retroviral cell lineage experiments. Retroviral infections in the cortex generated both clusters of labeled cells, assumed to be clones, and large numbers of dispersed cells whose relationship to other labeled cells was unclear (Austin and Cepko, 1990; Luskin et al., 1988; Price and Thurlow, 1988). To positively identify clonally related cells, Walsh and Cepko (1992) infected the rat telencephalon with a large library of retroviruses, each with its own genetic marker. They then used the polymerase chain reaction to genotype individual retrovirally infected cells and assign them to clones, regardless of their location. In contrast to other layered structures such as retina and tectum in which clones are primarily radially arranged (Gray and Sanes, 1991; Holt et al., 1988; Turner and Cepko, 1987; Wetts and Fraser, 1988), clones in the cerebral cortex spread tangentially over wide expanses, with some clonally related cells ending up in different cortical areas. These results suggested that cortical cells undergo significant tangential dispersion during development and have called into question previous hypotheses

that cortical areas are prepatterned within the ventricular zone.

One possible mode for the tangential dispersion of clonally related cells was revealed by direct observations of individual migrating cells in slices of developing cortex (O'Rourke et al., 1992). Fluorescently labeled cells with the bipolar morphology characteristic of migrating neurons were visualized migrating through the intermediate zone using time-lapse confocal microscopy. While a majority of the cells migrated radially, 12.6% migrated at a 90° angle to the radial direction and 17.2% migrated tangentially at intermediate angles. These results suggest that nonradial or tangential migration in the intact cortex contributes to the dispersion of clonally related cells. Here, we examined the extent to which cells, specifically neurons, follow tangential migratory routes in the developing cerebral cortex. Furthermore, in our time-lapse studies we observed some cells making sharp, right-angle turns from radial to orthogonal directions. These observations highlight the question of whether a non-glial substratum must be invoked to support tangential migration in the cerebral cortex. Thus, we have employed both confocal and electron microscopy to explore possible migratory substrata for tangential migration.

MATERIALS AND METHODS

Autoradiography

To identify migrating neurons in intact ferret brains, ventricular cells were labeled with [³H]thymidine by intrauterine injection (10 μCi/embryo, New England Nuclear) on embryonic day (E) 29 to label presumptive layer 6 neurons (Jackson et al., 1989; McConnell, 1988), or by intraperitoneal injection (10 μCi/animal) on postnatal day (P)1 to label neurons destined for layer 2/3. Ferrets labeled on P1 were perfused with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) on P2, P3, P4, P6, or P10, a time course spanning the duration of layer 2/3 migration. Animals labeled on E29 were perfused on E31, E32, E33, and E35. Brains were removed, embedded in paraffin, then sectioned at 10 μm in the coronal plane. Sections were deparaffinized, dipped in autoradiographic emulsion (Kodak NTB2), exposed for 4-6 weeks, developed in Kodak D19, and counterstained with cresyl violet.

Analysis of cell orientation

Because lateral cortical regions contain radial glia with complex trajectories, we focused our analysis on sections through the posterodorsal cortex in which radial arrays of cells extend straight toward the pial surface. These cellular arrays were used to define the radial direction, to which the orientation of each migrating cell was compared. Images of selected sections were collected using a Nikon Optiphot microscope connected to a Dage CCD camera, digitized, and analyzed on a Macintosh Quadra computer using Image1 (NIH) and Adobe Photoshop. The orientation of each cell was measured with respect to the radial direction. Cells oriented within 30° of radial were classified as radial; those from 60°-90° from radial were classified as orthogonal. Cells oriented in the intermediate range from 30°-60° were also considered to be nonradially oriented. Rounded cells without a clear orientation (between 25-40% of all cells) were excluded from the analysis. Over 100 cells in two animals were analyzed for each timepoint shown in Figs 2 and 3, except for Fig. 2B in which one animal was analyzed on P2 and one on P3. Data are presented as mean ± s.e.m.

Brain slices and fluorescent dye injections

Migrating cells in living slices were labeled with DiI (DiIC18(3),

Molecular Probes), using published procedures (O'Rourke et al., 1992). Briefly, brain slices from P0-P2 ferrets were prepared for organotypic culture (Roberts et al., 1993). After 24 hours, DiI (0.5% in dimethyl formamide; 0.1-0.2 nl) was injected into the ventricular zone of cortical slices using a Picospritzer (General Valve) and a glass micropipette (tip diameter <10 mm). Slices were incubated for 15-36 hours to allow migration of labeled cells away from injection sites, and then fixed in 4% paraformaldehyde (0.1 M phosphate buffer, pH 7.4). To permanently mark migrating cells in slices, the fluorescent label was photoconverted to a dark diaminobenzidine (DAB) reaction product using published procedures (McConnell et al., 1989). Radial glia were DiI-labeled in fixed slices using the same injection techniques.

Immunohistochemistry

Cortical neurons were identified in sections with two mouse monoclonal antibodies that stain neurons during early phases of differentiation, TuJ1 (Lee et al., 1990) and NeuN (Mullen et al., 1992). Tissue used for NeuN staining was prepared in low-temperature melting wax to preserve immunoreactivity (Mullen et al., 1992). Polyclonal antibodies against the NG2 antigen were used to identify O-2A progenitors (Levine and Stallcup, 1987) in sections of postnatal rat cortex, and mouse monoclonal antibodies directed against vimentin (Boehringer Mannheim) were used to identify radial glia in brain slices. To facilitate antibody penetration into slices, tissue was extracted with 1% Triton X-100 in 1 mg/ml BSA (0.1 M phosphate buffer, pH 7.4) for 1-2 days at room temperature. Either peroxidase staining or immunofluorescence (ABC kit, biotinylated secondary antibodies, fluorescein-Avidin D; Vector) were used to visualize antibody binding. Quantification of cell orientations was carried out as described above. Over 100 cells in two animals and over 50 cells in four animals were analyzed for each timepoint in the NeuN and TuJ1 studies, respectively.

Electron microscopy

Cellular contacts made by migrating cells in the intermediate zone were analyzed using electron microscopy. P1 ferrets were perfused with 4% paraformaldehyde-1% gluteraldehyde in 0.1 M cacodylate buffer, pH 7.3 at room temperature. Brains were sliced at 400 μm and fixed overnight (4°C). Slices were then osmicated (1% OsO₄, 0.8% K₃Fe(CN)₆, 0.1 M cacodylate (McDonald, 1984), stained with uranyl acetate, and flat embedded in Epon-Araldite between sheets of plastic. Thin sections were cut on a Reichert-Jung ultramicrotome and viewed in a Phillips 400 electron microscope.

RESULTS

Orientations of migrating cells in the developing cerebral cortex

Time-lapse imaging studies have revealed that cells can migrate along nonradial pathways in the intermediate zone of cortical brain slices (O'Rourke et al., 1992), thus nonradial migration provides a likely mechanism for the dispersion of clonally related cells during development. One possibility, however, is that the migratory pathways observed in slices do not accurately portray those that occur in vivo. Although brain slices appear to retain the normal three-dimensional environment of the cortex, it is conceivable that some critical feature is altered during the culture period, introducing artifactual patterns of migration. To determine whether the pathways of migrating cells in cortical slices reflect those that occur in vivo, we measured the orientations of migrating cells in fixed sections through the developing cortex.

Migrating cortical cells can be identified in fixed tissue using
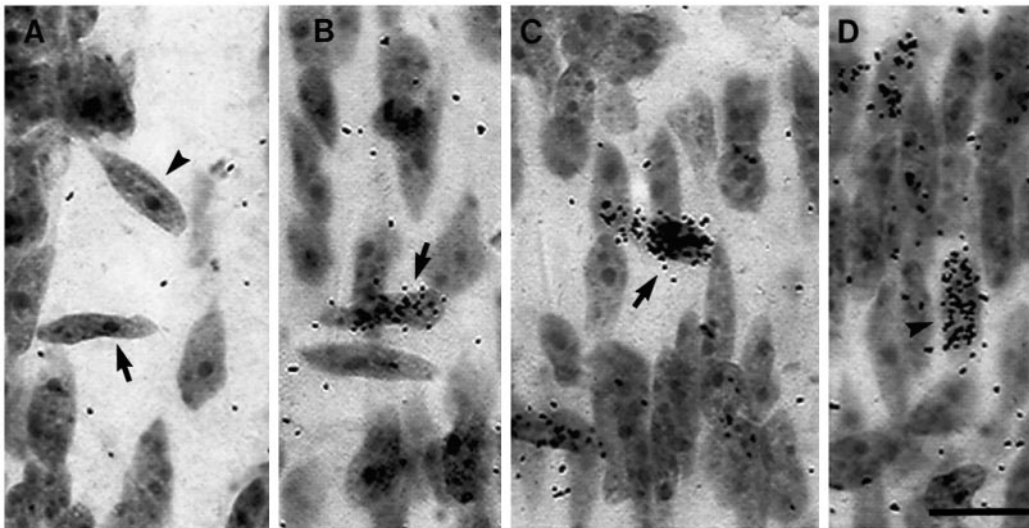
Fig. 1. Autoradiograghs reveal the orientations of cells in the intermediate zone of a P4 ferret cortex (radial, vertical). (A) One elongated cell oriented 90° from radial (arrow); another oriented 40° from radial (arrowhead). Among the [3H]thymidine-labeled cells, some are oriented orthogonally (arrows; B, C) and others are oriented radially (arrowhead; D). Scale bar, 10 μm.

[3H]thymidine labeling (Angevine and Sidman, 1961; Rakic, 1974). Precursor cells labeled with [3H]thymidine during S-phase generate postmitotic neurons that retain the thymidine label as they migrate out toward the cortical plate. Developing ferrets were pulse labeled with [3H]thymidine and perfused at various times during the main period of neuronal migration. Fixed cortical tissue was sectioned, processed for autoradiography and counterstained with cresyl violet to reveal the orientations of the migrating cells. Because radial glial fibers in lateral regions of cortex are commonly bent into 'S' shapes (Misson et al., 1991), we focused our analysis on dorsal regions of cortex in which the radial glia extend processes straight into the cortical plate. In such sections through the intermediate zone (Fig. 1), the majority of cells are oriented radially toward the pial surface, but nonradially oriented cells can also be seen scattered among them (Fig. 1A). Some of the cells have silver grains overlying their nuclei, identifying them as cells that have migrated out from the ventricular zone (Fig. 1B-D). Our previous time-lapse observations revealed that in all cases (87 cells) the orientation of the elongated cell soma predicts the direction in which the cell is migrating (O'Rourke et al., 1992). Thus, by quantifying the orientations of these migrating cells, we assessed the prevalence of nonradial migration in the intact cortex.

To compare the pathways of migration in the cortex with those observed in P1 cortical slices (O'Rourke et al., 1992), cells were labeled with [3H]thymidine on P1 and brains were analyzed in the coronal plane on P2, 3, 4, 6, and 10. Labeled cells in the intermediate zone were grouped into three categories according to their orientation: radial cells were oriented from 0-30° from radial, orthogonal cells were 60-90° from radial, and those oriented at intermediate angles were 30-60° from radial. On P2, P3 and P4 the percentage of nonradially oriented cells (oriented 30-90° from radial) ranged from 30 to 35% (Fig. 2B). At slightly older ages, P6 and P10, the percentage of nonradially oriented cells remained at about the same level, 35%. By comparison, in living cortical slices prepared on P1 and observed on days equivalent to P3 or 4, a total of about 30% of the cells migrated along nonradial pathways (12.6% at 60-90° and 17.2% at 30-60°). Thus, the results suggest that the migratory pathways observed in slices

accurately reflect those present in the intact brain. Radial migration in cortical slices also appears normal; cells travel radially into the cortical plate at roughly normal rates (Götz and Bolz, 1992; Roberts et al., 1993). Thus, brain slices appear to provide a reliable system for studying both tangential and radial migration in the developing cortex.

Neurons that will contribute to a particular layer of cortex are generated at reproducible stages of development and in an inside-first, outside-last sequence (Angevine and Sidman, 1961; Luskin and Shatz, 1985; Rakic, 1974). In the ferret, deep layer neurons (primarily layer 6) are born on E29 and superficial layer 2/3 cells are born on P1 (Jackson et al., 1989; McConnell, 1988). By analyzing tissue at different ages, one can ask whether neurons destined for different cortical laminae follow similar migratory pathways. To reveal the orientations of migrating deep layer cells, ferrets were labeled with [3H]thymidine on E29 then fixed on E31, 32, 33, or 35. In the intermediate zone, about 10% of labeled migrating cells were oriented orthogonally and 10% were oriented at intermediate angles on each of the days analyzed (Fig. 2A). Thus, a total of 20% of the [3H]thymidine labeled cells appeared to be migrating along nonradial pathways. In contrast, layer 2/3 cells generated on P1 appear to migrate along nonradial pathways about 30-35% of the time (Fig. 2B). These results suggest that migrating cells that are born during later stages of development and contribute to more superficial layers of the cortex are more likely to migrate tangentially.

Our observations have so far concentrated on the intermediate zone, which contains the highest relative proportion of migrating cells. To ask whether nonradial migration occurs at other levels along the migratory route, the orientations of cells throughout the depth of the cerebral wall were analyzed in coronal sections of P4 brains which had [3H]thymidine injections on P1 (Fig. 3). Cell orientations were measured in the subventricular zone, intermediate zone, subplate and cortical plate. Within the ventricular zone, the dense packing of cells made it impossible to determine the orientations of cells using these methods. Because [3H]thymidine labeled cells migrate as a cohort and are not found at all depths at a single age, the orientations of both labeled and unlabeled cells were tabulated. Unlabeled cells are more likely to be radially oriented than

A

B
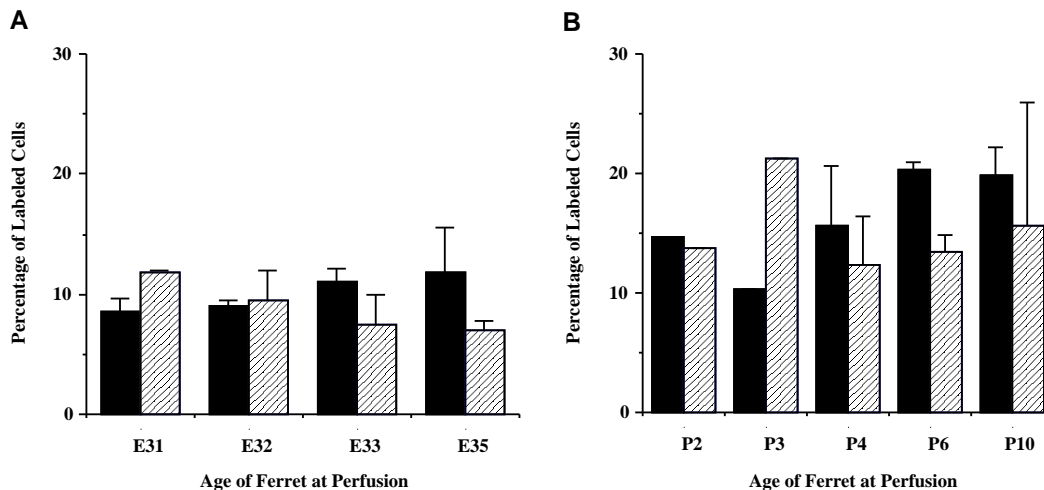
Fig. 2. The orientations of cells migrating through the intermediate zone during early and late phases of neuronal migration. The percentage of [³H]thymidine-labeled cells oriented in nonradial directions (30-60° from radial, black bars; 60-90° from radial, striped bars) is plotted according to the ferret's age. (A) Cells were labeled on E29 and animals perfused on E31, 32, 33 and 35. (B) Cells were labeled on P1 and fixed on P2, 3, 4, 6, and 10.

labeled cells (compare Figs 1B and 2); thus, these results are likely to provide an underestimate of the number of tangentially migrating cells. The prevalence of nonradially oriented cells decreased gradually from the ventricle out toward the pial surface. The highest percentage, 25%, was found in the subventricular zone. In the intermediate zone, 20% of the cells were oriented nonradially, in the subplate 17%, and in the cortical plate 10%. Thus, nonradially oriented cells were found at all the depths of the cortex, suggesting that nonradial migration can occur along the entire migratory route.

Phenotypes of nonradially migrating cells

In order to understand the functional significance of nonradial migration for cortical development, it is crucial to determine whether nonradially migrating cells ultimately differentiate into neurons. In the studies described above, cells pulse labeled with [³H]thymidine at both E29 and P1 had nonradial orientations during their migration. Previous studies have shown that
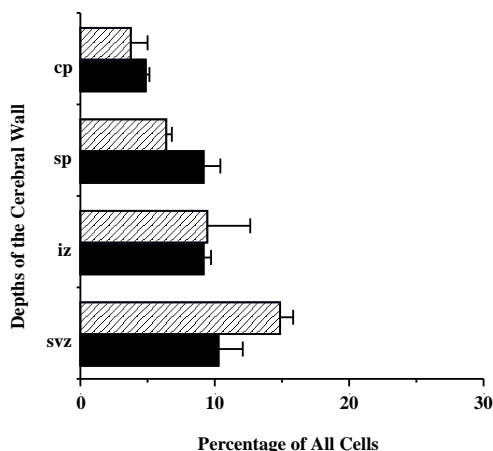

Fig. 3. The orientations of cells at different depths within the cerebral cortex of the P4 ferret. The percentages of all cells oriented in nonradial directions (30-60° from radial, black bars; 60-90° from radial, striped bars) were measured at different depths within the cerebral wall, including the subventricular zone (svz), intermediate zone (iz), subplate (sp), and cortical plate (cp).

the majority of cells generated on E29 and P1 in the ferret ultimately differentiate into the neurons of layers 6 and 2/3, respectively (Jackson et al., 1989; McConnell, 1988). These results strongly suggest that young neurons can follow nonradial pathways in the cortex. However, to confirm directly that nonradially migrating cells include young neurons, two antibodies that recognize neurons early during their differentiation were used to stain cortical sections and determine whether nonradially oriented cells with a migratory morphology express a neuronal phenotype.

The first antibody, NeuN, recognizes a neuron-specific nuclear epitope expressed in the vast majority of neurons in the brain; it does not stain glia as identified in vitro by the GFAP marker for astrocytes (Mullen et al., 1992). In addition, immunostaining with the NeuN antibody and an antibody to NG2, a marker of O-2A progenitor cells (Levine and Stallcup, 1987) reveal no co-localization in sections of P3 rat cortex (data not shown). Thus, NeuN does not label glial progenitors that appear to migrate tangentially during postnatal life (Levison et al., 1993). In sections through ferret cortex, NeuN nuclear staining was present in cells located above the ventricular zone (Fig. 4). Strong NeuN immunoreactivity is expressed in the cortical plate and subplate (not shown) within large nuclei characteristic of differentiated neurons. In the intermediate zone on E29, cells with the elongated nuclei characteristic of migrating cells stain with the NeuN antibody, but at a lower intensity, suggesting they are not fully differentiated. The elongated NeuN-positive neurons were not only found with radial orientations (Fig. 4, arrowheads), but they were also oriented along transverse pathways (Fig. 4, arrows). Nonradially oriented NeuN-positive nuclei were roughly as common as nonradially oriented [³H]thymidine-labeled cells at E29 (30-60°, 8.7%; 60-90°, 17.3%), E35 (30-60°, 13.7%; 60-90°, 16.4%), and P1 (30-60°, 10.1%; 60-90°, 12.8%), in strong support of the notion that most nonradially migrating cells at these stages are in fact young neurons.

A second neuron-specific antibody, TuJ1, recognizes class III β-tubulin, a cytoskeletal protein expressed exclusively by neurons (Lee et al., 1990). In a recent study, Menezes and Luskin (1994) demonstrated that TuJ1 labels a subset of cells that appear to be migrating in the ventricular zone of the mouse cortex. We observed a similar population of cells in ferret with
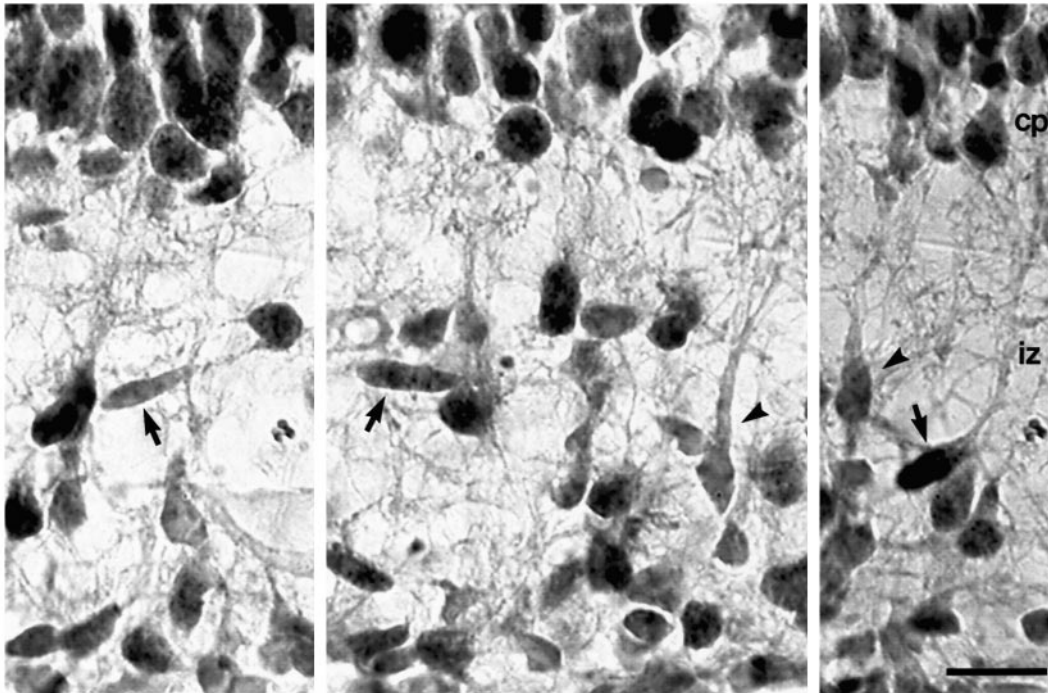
Fig. 4. NeuN-immunoreactive cells in the intermediate zone (iz) on E29 (radial, vertical). The NeuN-positive cells have the elongated nuclei characteristic of migrating neurons. Neurons are oriented in both radial (arrowheads) and nonradial (arrows) directions. The large NeuN-positive nuclei (top) belong to differentiating neurons in the cortical plate (cp). Scale bar, 10 μm.

elongated bipolar morphologies, thick leading processes and thin trailing processes typical of migrating neurons (Fig. 5). The orientations of TuJ1-positive neurons in both coronal and sagittal sections of the ferret ventricular zone were quantified. Heavily labeled axons obscure the outlines of individual cells in the cortical plate and intermediate zone; thus, these regions could not be analyzed. TuJ1-positive cells were present in the ventricular zone throughout the period of neurogenesis, including E29, E35, P1, and P4. A majority of the immunola-beled cells, 75% in the sagittal plane and 87% in the coronal plane, were oriented tangentially within the ventricular zone. In previous imaging studies (Fishell et al., 1993), rounded cells were observed migrating randomly along tangential pathways. If the TuJ1-positive cells are indeed migrating, as their mor-

phology suggests, their presence provides further evidence for tangential migration in the cortical ventricular zone.

Possible substrata for tangential migration

Cortical neurons migrating radially toward the cortical plate appear to travel along glial processes (Edmondson and Hatten, 1987; Rakic, 1972). The migrating neurons extend parallel to and in close contact with radially oriented glial fibers. The sub-stratum for tangential neuronal migration, on the other hand, is unknown. One possible substratum is nonradially oriented glial fibers. In areas of lateral cortex, radial glia turn in shallow S-shaped trajectories toward the ventrolateral cortex (Misson et al., 1991), and may provide cells with a tangentially oriented migratory substratum. Our studies, however, focus on dorsal
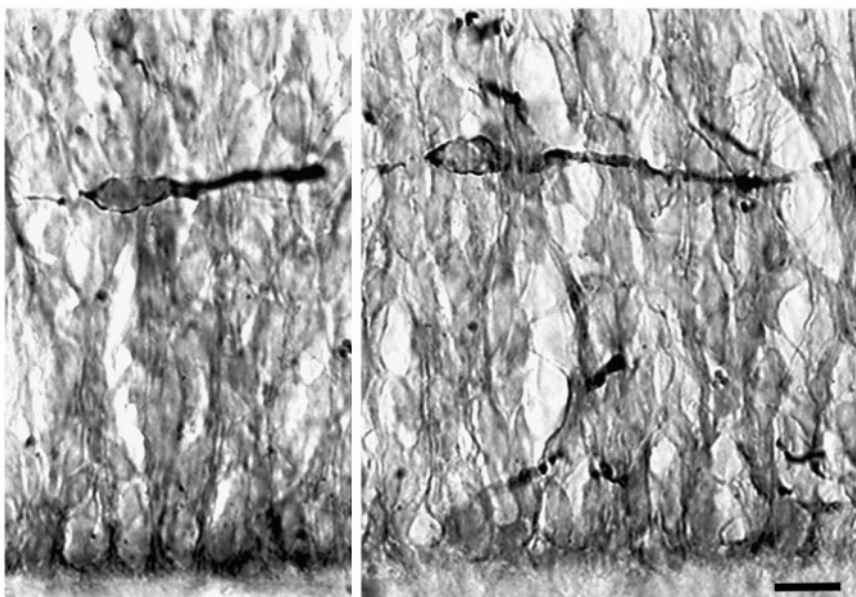
Fig. 5. TuJ1-immunoreactive cells in the ventricular zone on E29 have the elongated bipolar morphology of migrating neurons. The cells are oriented parallel to the ventricular surface (bottom) and are located in the lower portion of the ventricular zone (radial, vertical). Scale bar, 10 μm.

cortex where glial orientations are predominantly radial. A second possible cellular substratum for nonradial migration is the large number of axons that interconnect the thalamus and cortical plate, and project tangentially through the intermediate zone en route to their targets. While axons appear to provide a substratum for neuronal migration in other brain regions (Gray et al., 1990; Rakic, 1985), the possibility that cortical plate neurons migrate along axons is largely unexplored. Here, both light and electron microscopy were used to discern whether either glial or neuronal processes present plausible substrata for tangential migration.

Orientations of radial glia

We wondered whether nonradially oriented glial processes are prevalent enough to support the amount of nonradial migration that occurs in dorsal cortex. Radial glia in cortical slices were labeled with DiI (Voigt, 1989), and the pathways of glial processes were reconstructed with a confocal microscope. DiI injections at the pial surface retrogradely labeled glial processes through the intermediate zone out to their cell bodies in the ventricular zone. Of the 49 glial fibers (4 slices) traced, the vast majority, 90%, were oriented radially. Although many glial fibers meandered along their course, the majority did not venture farther than 30° from a radial orientation (Fig. 6A). Only 2 glial processes (4%) made 90° turns within the intermediate zone (Fig. 6B). The remainder of the nonradial processes (6% of the total) turned along angles from 30-60°. Injections in the ventricle labeled glial processes out to their endfeet at the pial surface. The orientations of these processes were analyzed within the upper intermediate zone and cortical plate. Except at the pial surface where they branch extensively, glial fibers were almost exclusively oriented radially (98%, n=179, 5 slices). Thus, overall only 3% of 228 labeled glia were nonradially oriented along their length. In contrast, 30% or more of migrating cells follow nonradial pathways. It is therefore unlikely that nonradially oriented glia provide a substratum sufficient to support all of the tangential migration in cortex.

Relationship between migrating cells and glia

Confocal microscopy was used to visualize the spatial relationships between glial processes and migrating cells in the 3-dimensional environment of cortical brain slices. Focal injections of DiI were made into living cortical slices (O'Rourke et al., 1992); cells with bipolar morphologies typical of migrating neurons migrated away from the injection sites. The slices were fixed and the fluorescent label converted to a brown DAB reaction product (see Methods). Radial glial processes in the intact slice were labeled with antibodies against vimentin. The juxtapositions between dark brown migrating cells and fluorescently labeled glial processes were visualized clearly in individual optical sections collected through the depth of each cell with the confocal microscope. While the confocal microscope can reveal whether a glial process and a cell are in the same plane of focus, it cannot, of course, reveal whether they are in direct contact. The advantage of this technique, however, is that it reveals the range of possible contacts between a migrating cell and the many glia in its close proximity. The spatial relationships between migrating cells and surrounding glia were classified into two groups. If the long axis of the leading process or soma of a migrating cell ran parallel to and
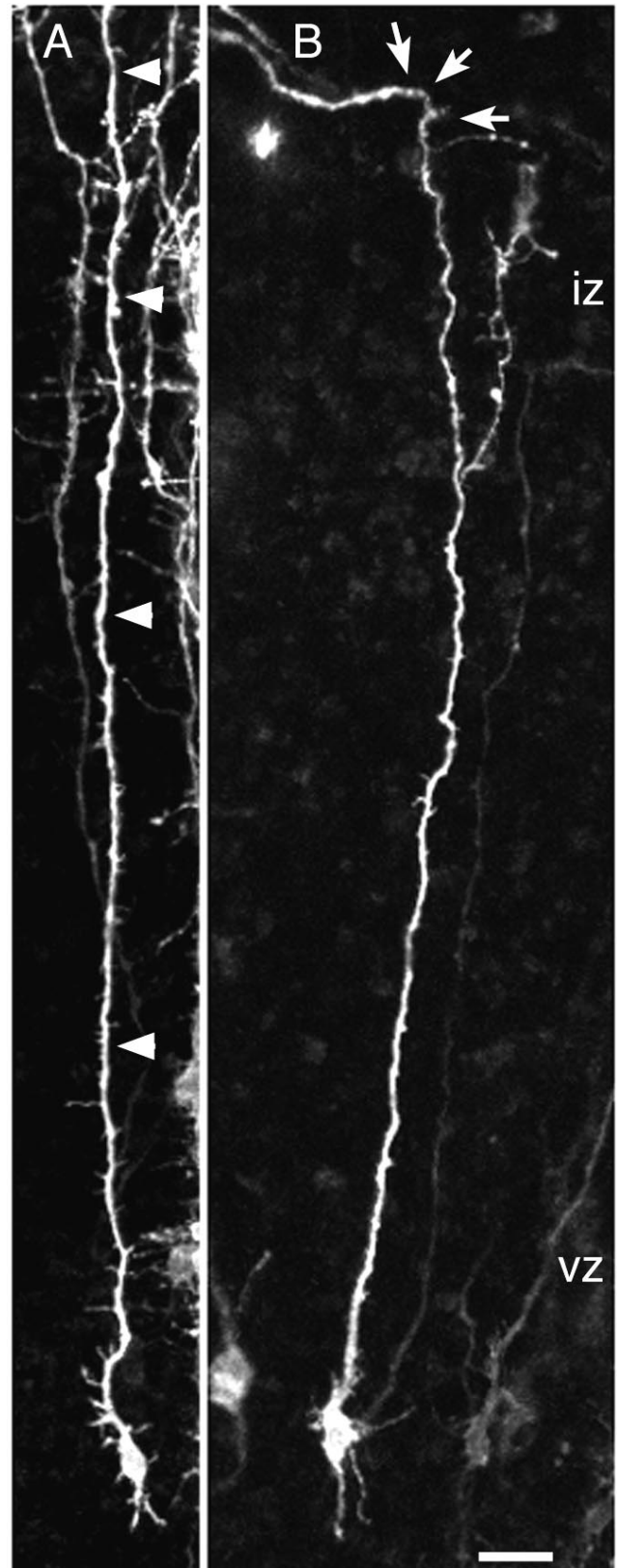
Fig. 6. The paths of DiI-labeled radial glial processes in P1 cortical slices were reconstructed using confocal microscopy. Glial fibers extend from their cell bodies in the ventricular zone (vz) out into the intermediate zone (iz). (A) A radial glial cell extends straight toward the pial surface (arrowheads). (B) Another glial fiber turns at roughly 90° within the intermediate zone (arrows). Scale bar, 10 μm.
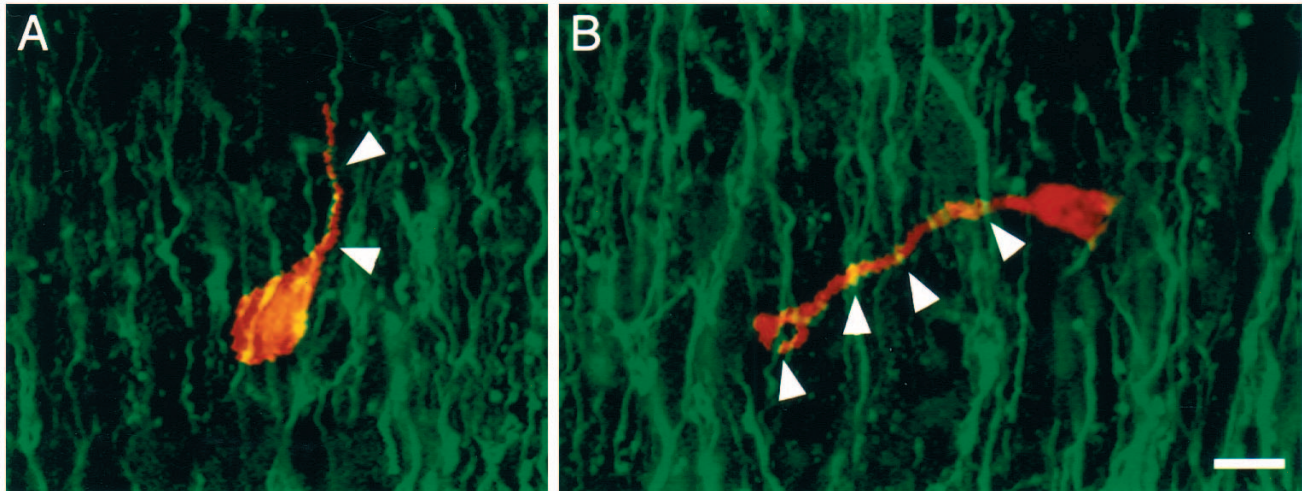
Fig. 7. The juxtaposition of a radially and a nonradially oriented cell with neighboring radial glial fibers in a P1 cortical slice (radial, vertical). Each image is a reconstruction of several optical sections through a DAB-labeled migrating cell (pseudocolored red) and vimentin-immunoreactive glial fibers (green). (A) The leading process of the radially oriented bipolar cell follows closely along the path of a radially oriented glial fiber (arrowheads). (B) An orthogonal migratory cell is oriented perpendicular to the radially oriented glial fibers and appears to contact them at several points (arrowheads). Scale bar, 10 μm.

coincident with the length of a glial process, this was classified as 'parallel contact'. If the long axis of a migrating cell crossed the length of the glial process at an angle, it was classified as a 'glancing or nonparallel contact'. The percentage of each type of contact was measured as the length of contact relative to the total perimeter of the migrating cell. Using this scheme, a theoretical neuron migrating along the length of a glial fiber and in contact with it along the entire length of one side would have 50% parallel contact.

We compared the parallel contacts made between both radially and tangentially oriented cells and glial processes, using cells that had migrated away from a focal DiI injection in a cortical slice. Many radially oriented cells appeared to extend their leading process along the length of a single glial process (Fig. 7A). Others made parallel contacts with several glial processes, as has been described in EM studies (Rakic et al., 1974). These data suggest that radially oriented neurons can migrate in close apposition to one glial process but then extend over to an adjacent process and migrate along its length. Direct observations of such behavior have been made in imaging experiments (Edmondson and Hatten, 1987; O'Rourke et al., 1992). When parallel contacts were assessed quantitatively, radially oriented cells (n=23 cells in 4 slices) were found in apparent parallel contact with glial fibers over an average of 37.6±4.4% of their perimeters (range, 15-82%). This nearly 40% average contact translates to 80% of one side of the cell in apparent close apposition to a radial glial process. These data are therefore consistent with the notion that glia provide a direct substratum for the radial migration of cells toward the pial surface (Edmondson and Hatten, 1987; Rakic, 1972). In contrast, tangentially migrating cells made few parallel contacts with glia. Indeed, one third of all orthogonally oriented cells made no parallel contacts whatsoever with glial fibers. The amount of parallel contact between nonradially oriented cells (n=13 cells from 5 slices) and glia ranged from 0 to 25%, with a mean of 8.5±2.5%, (i.e. on average 17% of the length of one side of the cell ran parallel to a glial fiber).

Because many cells did not appear to make any parallel contact with glial fibers, it seems unlikely that tangentially migrating neurons travel along the length of glial processes, as do radially migrating neurons.

The amount of glancing or nonparallel contact between migrating cells and glial fibers crossing their path at an angle was measured for the same set of cells. The percentage of nonparallel contacts between radial cells and glial process was very low, 0.7±0.3% (range, 0-1.8%). These results suggest that when a radially oriented cell contacts a glial process, its long axis nearly always runs parallel to the length of that process. However, nonradially oriented cells were much more likely to contact glial fibers at an angle. Many cells ran entirely perpendicular to the palisades of vimentin-positive radial glia that course through the intermediate zone (Fig. 7B). Tangentially oriented cells made an average of 11.1±3.0% nonparallel contact with radial glial fibers, apparently making direct but glancing contacts with several radial glial processes. While these observations suggest that tangentially migrating neurons do not migrate directly along radial glia, they do not rule out a role for glia in tangential migration. Radial glia could play a role if migrating neurons were to reach across at an angle from one glial palisade to another, like Tarzan swinging from vine to vine through the trees.

Electron microscopy

The electron microscope allows direct examination of the contacts made between migrating neurons and either glial or neuronal processes. Low magnification views of the intermediate zone reveal the cellular milieu in which neurons migrate (Fig. 8B,E). Alternating bundles of glial fibers and axons running at right angles to one another form a patchwork that fills the intermediate zone. Scattered among them are neurons that we presume to be migrating, based on their elongated morphology (Gregory et al., 1988; Rakic, 1972). Thick leading processes or thinner trailing processes can sometimes be seen extending from the soma; the elongated nuclei are notched at
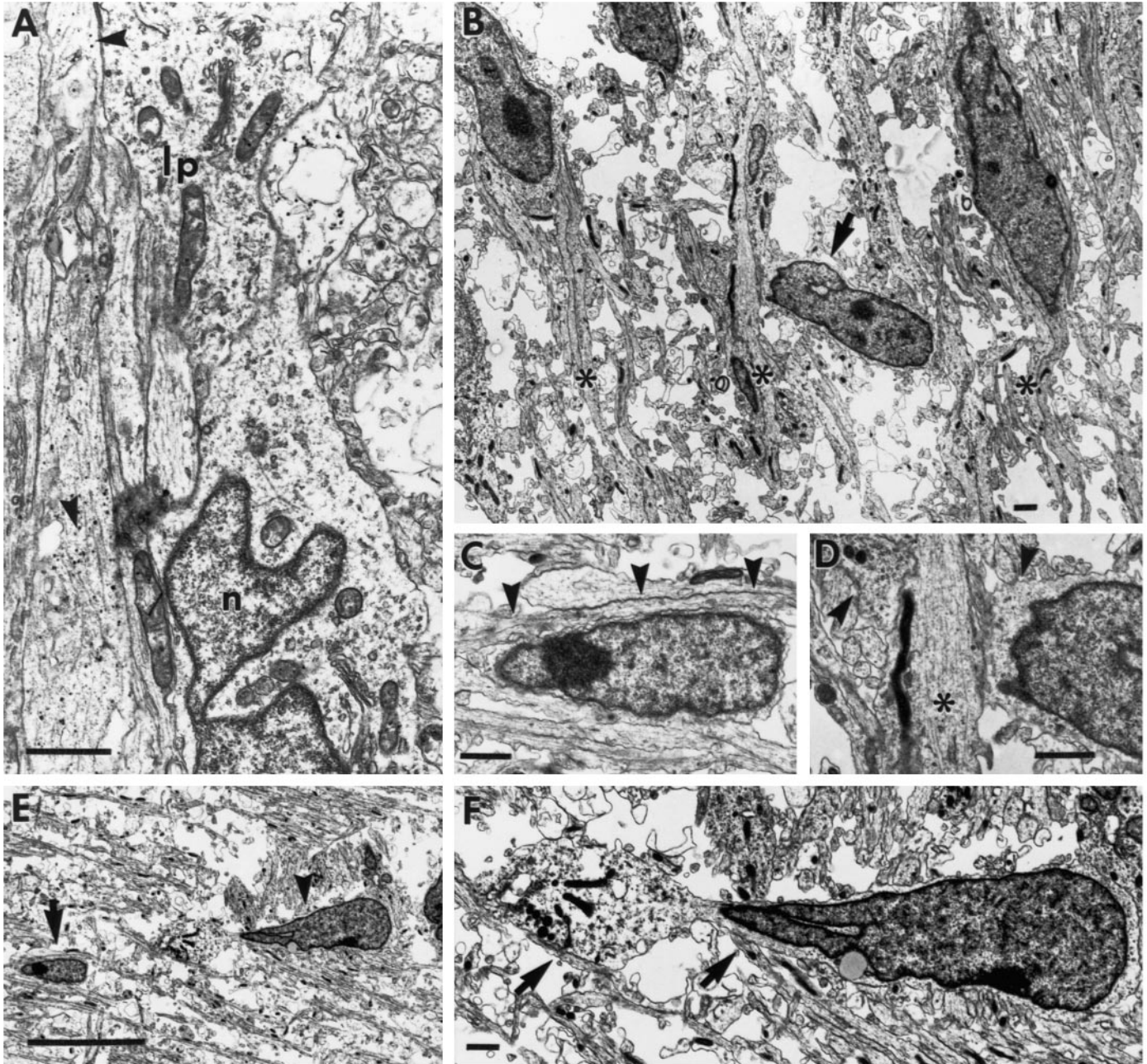
Fig. 8. Electron micrographs of migratory cells in the intermediate zone of a P1 ferret. (A) Radially oriented cell with the characteristics of a migrating neuron, an elongated nucleus (n) with a notch at its leading edge and organelles at the base of the leading process (lp). The cell is closely apposed to a radial glial process that can be identified by glycogen granules (arrowheads). (B) Low magnification view of several palisades of radial glia (asterisks) and migrating cells. The leading process of one nonradially oriented migratory cell (arrow) crosses a glial fiber (see D). (C) A nonradially migrating cell contacts axons extensively (arrowheads). This panel is a high magnification view of a cell in E (arrow). (D) High magnification view of the nonradially oriented cell in B. The leading process (arrowheads) crosses a glial process (asterisk). (E) Low magnification view of two nonradially oriented cells (also shown in C, arrow; and F, arrowhead) migrating through a region of tangentially oriented axons. (F) High magnification view of nonradially oriented cell from E (arrowhead). Both the cell body and leading process contact axons (arrows). Scale bars, (A-D, F) 1 μm; (E) 10 μm.

the cells' leading ends and organelles accumulate at the base of the leading processes. At the magnifications used here, we observed only hints of the specialized junctions made between migrating neurons and glia in vitro (Gregory et al., 1988). In our examination of the intermediate zone of the intact cortex, radially oriented cells were found frequently in parallel contact

with glial processes (Fig. 8A). In contrast, tangentially oriented cells made only glancing contacts with radial glia (Fig. 8B,D). Again, these observations suggest that the mechanism for orthogonal migration differs from that used by the radially migrating cells.

To further explore the possible substrata for orthogonal

migration, direct contacts made between cells with migratory morphology and glial processes, identified by punctate glycogen granules in their cytoplasm (Gadisseux et al., 1990), were quantified. Single sections of 9 radially oriented and 10 orthogonally oriented cells were analyzed from two animals. Radially oriented migrating cells made parallel contact with glial processes along an average of 15±2.4% of their perimeter. In sharp contrast, no parallel contacts were found between orthogonally oriented cells and glia, confirming the observation made using confocal microscopy that tangentially oriented cells tend not to be found in parallel alignment with glial fibers. The complementary result was revealed when the nonparallel contacts were measured. Radially oriented cells made nonparallel contact along only an average of 0.7±0.5% of their perimeter. Of the 9 cells analyzed, only two made any nonparallel contacts with glial processes. In contrast, orthogonally oriented cells made nonparallel contact with glial processes along 7.8±2.4% of their perimeters, again confirming observations made using confocal microscopy. While the absence of parallel contacts between tangentially oriented cells and glia suggests that these cells are not migrating along the length of glial fibers, migrating cells do make multiple glancing contacts with glia. Thus, we cannot exclude the possibility that glia play a role in tangential migration.

Relationship between migrating cells and axons

An alternative cellular substratum for tangential migration is also apparent in Fig. 8E: the intermediate zone (which becomes the white matter of the mature cortex) is rich with axons, many of which run at right angles to radial glia and could thus provide a substratum for nonradial migration in the cerebral cortex. To examine this possibility, we analyzed the contacts made between axons and cells with a migratory morphology at the EM level. Axons were distinguished from other processes by their small diameter, abundance of microtubules, and lack of glycogen granules in their cytoplasm. Several orthogonally oriented cells made striking contacts with tangentially oriented axons (Fig. 8D,F). However, when examined quantitatively, orthogonally and radially oriented cells with migratory morphologies made about the same amount of contact with axons, 39.8±6.6% and 32.6±4.7%, respectively. Contacts were broken down into two types, parallel and nonparallel, as was done for glia. Radial cells made 10.3±4.7% parallel contact and 22.3±4.7% nonparallel contact with axons. Similarly, nonradially oriented cells made 12.2±7.4% parallel and 27.6±5.8% nonparallel contact with axons. Thus, no differences were found between the types of contacts made by the two groups of cells. However, the presence of contacts between nonradial cells and axons is consistent with the possibility that axons may play a role in guiding the migration of young neurons.

DISCUSSION

In previous imaging studies, cells were observed migrating along nonradial pathways in slices of the developing cerebral cortex (O'Rourke et al., 1992). Here, we have investigated the extent to which nonradial migration occurs in the intact cortex. Migrating cells labeled with [3H]thymidine frequently displayed nonradial orientations, suggesting that they undergo tangential migration in vivo. Labeling with neuron-specific antibodies indicates that many of the cells that migrate tangentially will ultimately differentiate into neurons. Thus, our findings further support the notion that nonradial migration of young neurons presents a mechanism for the dispersion of clonally related cells during cortical neurogenesis. In addition, our observations suggest that tangentially migrating neurons in the dorsal cortex migrate at angles to radial glia rather than along their lengths. These results raise the possibility that tangentially migrating neurons utilize migratory mechanisms that differ from those used by radially migrating cells.

Nonradial pathways are used by migrating cortical neurons in vivo and in vitro

One concern in our studies is whether the cells we analyzed were actually migrating. In live cortical slices, cells were observed directly migrating along tangential pathways (O'Rourke et al., 1992). In the present studies, two approaches were employed to identify migrating cells. The first was to label cells with [3H]thymidine; any labeled cells located above the ventricular zone were certain to have migrated out to that point because [3H]thymidine is incorporated only by dividing ventricular precursors. Because we only analyzed the orientations of cells with elongated nuclei, we selected for cells that were in the process of migration. In our analysis of migratory substrata, migrating cells were labeled in living brain slices with DiI. After focal injections of the dye into the upper ventricular zone, fluorescently labeled cells with the elongated, bipolar morphology of migrating neurons had clearly migrated away from the injection sites. Thus, both thymidine-tagging and vital labeling were employed to positively identify cells that were likely to be migrating when brain tissue was fixed for analysis.

A critical issue for evaluating models for the specification of cortical areas is whether young neurons migrate tangentially as they travel toward the cortical plate. If neurons mix extensively with their neighbors en route to their final destinations, it is unlikely that they are specified to become part of a functional area while still in the ventricular zone. We have obtained evidence using immunohistochemistry that neurons can migrate tangentially. Young neurons were identified definitively with the NeuN antibody; the cells do not express the glial markers GFAP (Mullen et al., 1992) and NG2 (data not shown). The NeuN-positive neurons we analyzed in the intermediate zone had the elongated nuclei characteristic of migrating neurons and were not as darkly stained as neurons of the subplate and cortical plate, suggesting they had not fully differentiated and were destined to inhabit the six layers of the mature cortex. Additional evidence for tangential neuronal migration comes from the thymidine-labeling studies. Elongated cells that were labeled with [3H]thymidine on E29 and P1 and had migrated out of the ventricular zone displayed nonradial orientations. The results from previous birthdating studies indicate the majority of cells generated on E29 and P1 in ferret will become neurons (Jackson et al., 1989; McConnell, 1988). Finally, in retroviral lineage studies in rodents, clones of cells infected at early stages, comparable to those studied here in the ferret, are composed primarily of neurons (Parnavelas et al., 1993; Walsh and Cepko, 1992). In contrast, clones from infections at later stages are composed exclusively of glia (Levison et al., 1993; Levison and

Goldman, 1993). Thus, many of the glia arising from early retroviral infections are probably generated during the later period of gliogenesis. Again, these results suggest that cortical neurogenesis in the ferret occurs between E29 and P10.

The TuJ1 antibody appears to stain another subpopulation of tangentially migrating neurons in the ventricular zones of both mouse (Menezes and Luskin, 1994) and ferret. Examination of retrovirally labeled clones shortly after infection revealed that some tangential dispersion occurs before the cells have entered the intermediate zone (Walsh and Cepko, 1993). Imaging studies provided evidence for random tangential movements of rounded cells within the ventricular zone (Fishell et al., 1993). The TuJ1-positive neurons have an elongated bipolar morphology which resembles that of migrating neurons in the intermediate zone, suggesting they may migrate along more linear trajectories than the randomly moving rounded cells observed by Fishell and colleagues. While the morphology of the TuJ1-positive cells provides a tantalizing suggestion that these cells are migrating, no direct evidence for such movements exists. If indeed they are migrating tangentially, however, these cells may contribute to the dispersion of clonally related cells in the proliferative zone of the cortex.

While most glia are generated at later stages relative to neurons (Levison et al., 1993; Levison and Goldman, 1993), the pathways for glial migration appear to bear many similarities to those for migrating neurons. Some cortical glia are generated after periods of neuronal migration from radial glia that translocate into the cortical plate and transform into astroglia (Voigt, 1989). It appears that many of these cells maintain their radial orientation as their cell bodies move outward into the cortical plate. Retroviral lineage studies reveal that oligodendrocytes and some astrocytes are generated in the subventricular zone, largely following neurogenesis (Levison et al., 1993; Levison and Goldman, 1993). These glia migrate out into the white matter and cortical plate, perhaps using mechanisms similar to those employed by neurons. Clones of glia spread in the coronal plane suggestive of tangential modes of migration within the subventricular and intermediate zones (Levison et al., 1993). These findings suggest that migrating glia, like neurons, may follow both radial and tangential pathways.

Migratory pathways in vivo

Analysis of the orientations of [3H]thymidine-labeled migrating cells in the intermediate zone over time suggests that nonradial migration pathways are employed increasingly as development proceeds. The percentage of cells with nonradial orientations increased from 20% when they had been thymidine-labeled on E29, when deep-layer neurons are generated, to 30-35% after presumptive layer 2/3 cells had been labeled on P1. These data are consistent with retroviral lineage studies suggesting that labeled clones become progressively more dispersed over time (Austin and Cepko, 1990; Walsh and Cepko, 1992). In addition, they suggest the possibility that cells from different layers of the cortex may have different propensities to migrate tangentially. A dramatic example of this is seen in retroviral lineage studies in the tectum where most clonally related neurons are radially arranged in the tectal plate, but one subclass becomes tangentially dispersed from the others and appears to migrate tangentially along axons (Gray and Sanes, 1991). At this time it is unclear in either system whether the pathway influences the phenotypic choice or the phenotype dictates the choice of pathway.

Our analysis of the orientations of [3H]thymidine-labeled cells in the cortex suggests that tangential migration occurs at all depths of the developing cerebral wall. Slightly higher percentages of cells appear to migrate tangentially in the subventricular and intermediate zones than in the subplate and cortical plate. The TuJ1 antibody stained another apparently small population of cells that may migrate tangentially in the ventricular zone. These data raise the possibility that the number of cells that migrate tangentially at some point along their journey out to the cortical plate may be much higher than the original number (30%) that were originally observed in the cortical slices. To assess this possibility, we must ultimately know how to 'sum' the fractions of nonradially oriented cells described here – do only 30% of all cells move tangentially, or does every cell spend on average 30% of its time following nonradial pathways?

In retroviral lineage studies in rat, Walsh and Cepko (1992, 1993) observed that 40-50% of labeled clones showed evidence for tangential dispersion. Can the tangential migration described here account for all the dispersion demonstrated in the clonal analysis? While the percentage of cells migrating tangentially may be similar, several variables remain unknown. First, we are unsure how far cells can move in the tangential direction. The minimum criterion for a 'dispersed' clone is 500 μm (Walsh and Cepko, 1993), while the longest distance a cell has been observed migrating tangentially is 350 μm (O'Rourke et al., 1992). Second, the amount of tangential dispersion could vary among species. In mice, analysis of cell orientations in dorsal cortex revealed that about 50% of cells were nonradially oriented (Bayer et al., 1991). Although the migrating cells were not identified definitively with [3H]thymidine, the results suggest that tangential migration may be more common in rodents than in ferrets. Finally, in lineage studies as many as 30% of clones appeared to undergo tangential dispersion in proliferative zones (Walsh and Cepko, 1993). While some of this dispersion could be accounted for by tangential migration in the subventricular zone, as described above, tangential dispersion of dividing precursor cells in the ventricular zone also appears to contribute to the total dispersion (Fishell et al., 1993). Thus, while a final resolution of these issues will require further study, our current results suggest that a significant proportion of clonal dispersion occurs through tangential migration of cells that have left the ventricular zone and started to differentiate into neurons.

What are the substrata for radial and nonradial migration?

Our examination of the migratory substrata of radially migrating cells showed that radially oriented cells made extensive parallel contacts with one or more radial glial processes, consistent with the results of EM studies (Gregory et al., 1988; Rakic, 1972; Rakic et al., 1974) and in vitro observations (Edmondson and Hatten, 1987). The substratum for nonradial migration, however, has been unclear. In the lateral cortex, glial fibers turn to extend tangentially in a pattern that could support nonradial migration (Misson et al., 1991). In the dorsal cortex, however, only 3% of glia labeled with DiI extend in nonradial directions, a level that seems unlikely to support the 30% of migrating cells that appear to move along nonradial

trajectories. Analysis at both the light and EM levels supports this notion. While the leading process of radially oriented cells typically extended in close apposition to a glial process, non-radially oriented cells generally crossed over glial fibers and appeared to make numerous glancing contacts with them along their leading processes. Thus, if nonradially migrating cells require radial glia for their migration, they must do so essentially by gliding across at an angle from one glial palisade to the next.

Axons have also been promoted in a variety of other brain regions as a substratum for neuronal migration (Gray et al., 1990; Rakic, 1985). We used electron microscopy to examine the contacts made between axons and cells with migratory morphologies. Both radially and nonradially oriented cells in the intermediate zone make roughly the same proportion of parallel and nonparallel contacts with axons. These results suggest that axons may provide a substratum for migration, but there is no bias toward nonradially oriented cells making more extensive contacts with axons. Thus, the hypothesis that axons provide a substratum for nonradial neuronal migration remains to be tested directly. A final possibility that we have not addressed in this study is that the tangentially migrating cells may not employ a cellular substratum for migration, but may use a substratum such as one of the molecular components of the extracellular matrix. Again, this possibility must be examined directly in perturbation studies.

Compartmental models of cortical specification

Our results bolster the idea that tangential migration plays a significant role in the movements of neurons during cortico-genesis. These movements could lead, in part, to a widespread dispersion of clonally related cortical cells during development (Walsh and Cepko, 1992). Rakic (1988) proposed that cortical areas are specified in the ventricular zone and that strict radial migration preserves the integrity of the map as it is transferred out to the cortical plate. The fact that many cortical plate neurons appear to travel tangentially over relatively long distances and mix randomly with neighboring cells suggests that neurons are not specified to a functional area until they reach the cortical plate. Preliminary evidence suggests, however, that migration of preplate cells may be strictly radial (O'Leary and Borngasser, 1992); the subplate or marginal zone cells, that are generated from the preplate, may then specify the areal fate of later-generated neurons as they migrate into the cortical plate (Ghosh et al., 1990). The reason for the mixing of cortical cells during their migration remains unclear. Future studies must discern whether there are consistent patterns of tangential migration within different regions or among specific cell types in the cortex. Clearly, much remains to be learned about movements of young neurons during development and how they contribute to the overall patterning of the central nervous system.

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Note added in proof

Tan and colleagues (1995) have employed inactivation of an X-linked *lacX* transgene to assess patterns of dispersion in the developing mouse cerebral cortex and found that roughly 33% of neurons appear to undergo tangential movements sometime during development. The levels correspond closely with the levels of tangential migration we have observed during later stages of development in the ferret.

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