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Cortical Ventricular Zone Progenitors and Their Progeny Maintain Spatial Relationships and Radial Patterning during Preplate Development Indicating an Early Protomap

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The graded expression of transcription factors by progenitors in the ventricular zone (VZ) confers positional or area identity that is inherited by subplate (SP) neurons and governs their expression of guidance molecules for thalamocortical axons and other properties required for cortical area specification. This mechanism would be most efficient if VZ progenitors and their SP neuronal progeny maintain neighbor relationships during the generation of the preplate (PP), the precursor of the SP. Therefore, a major goal of this study is to determine whether progenitors in the cortical VZ and their progeny maintain neighbor relationships during the genesis of the neocortical PP. We used time-lapse video microscopy to follow the movements of VZ progenitors and the radial movement of their progeny and distribution in the PP in whole-mount or slice cortical explants from embryonic rats at stages when PP neurons are generated. We show that labeled VZ cells proliferate and have a strong tendency to retain neighbor relationships within the VZ and that their neuronal progeny move superficially along a radial column to form the overlying PP; during this process, their neuronal progeny also retain neighbor relationships and thereby form the PP in spatial register with the VZ progenitors that generate them. This behavior differs from that reported at later stages of cortical development, when cortical plate (CP) neurons are generated, and considerable dispersion is evident among both cells within the VZ and neuronal progeny as they migrate from the VZ to the CP. However, our findings show that at the early stage of cortical development, when PP/SP neurons are generated, the VZ is, at a cellular level, a "protomap" of the PP/SP.

Keywords: area specification, clonal dispersion, cortical development, subplate, time-lapse imaging

Introduction

The neocortex, a dorsal telencephalic structure unique to mammals, is organized in its radial dimension into 6 major layers, distinguished by differences in the morphology and density of their constituent neurons, connectivity, and gene expression. These features also differ along the tangential dimension of the neocortex, dividing it into anatomically, genetically, and functionally distinct "areas" (O'Leary and Nakagawa 2002). Neurons that populate the neocortex are of 2 general types, γ-aminobutyric acidergic (GABAergic) interneurons, which are generated in the ganglionic eminences and migrate into the cortex, and glutamatergic neurons, including all projection neurons, which are generated by progenitors within the ventricular zone (VZ) and subsequently in the subventricular zone (SVZ) of the dorsal telencephalon (dTel) (Parnavelas 2000; Marin and Rubenstein 2003; Kriegstein and Noctor 2004).

The earliest population of neurons generated in the neocortical VZ of the dTel accumulates above the VZ, forming the preplate (PP) (Bayer and Altman 1991). A smaller population of cells, mainly reelin-positive Cajal–Retzius (C–R) neurons, are generated in the cortical hem and subcortically, migrate tangentially into the cortex, and reside just beneath the pial surface on top of the PP (Bielle and others 2005). Neurons generated later in the VZ and SVZ accumulate within the PP and form the cortical plate (CP), which displaces most of the original PP neurons deep to the CP, thereby forming the subplate (SP) layer, leaving behind a superficial marginal zone (future layer 1) containing the reelin-positive C–R neurons (McConnell 1995). The CP is progressively populated by GABAergic interneurons and differentiates into layers 2 through 6 in a deep to superficial pattern (Marin and Rubenstein 2003; Kriegstein and Noctor 2004).

Thalamocortical axons (TCAs) originating from nuclei in the dorsal thalamus are the primary afferent input to the neocortex and are the major source of modality-specific sensory information to neocortical areas. Therefore, the functional specializations of sensory areas are defined by, and dependent upon, the TCA input. In adult mammals, TCAs project to the neocortex in an area-specific pattern and primarily terminate within layer 4, but to a lesser degree in other layers (Hohl-Abrahao and Creutzfeldt 1991). In contrast to the initially broad distribution of projection neurons that form the major efferent output projections of the cortex, TCAs target their appropriate cortical areas in a highly specific manner from the outset (O'Leary and Koester 1993; Chenn and others 1997). During embryonic development, TCAs extend through the internal capsule to reach the neocortex and then grow tangentially on an intracortical pathway centered on the SP to reach their specific cortical target area (Ghosh and Shatz 1993; Miller and others 1993; Bicknese and others 1994). Few TCAs extend beyond their appropriate cortical target areas or make gross directional errors. After reaching their target area, TCAs extend collateral branches from the SP superficially into the overlying CP, showing a strong preference for their appropriate target areas (Crandall and Caviness 1984; Ghosh and Shatz 1993; Miller and others 1993).

This precision in both the extension and collateral branching phases of developing TCAs suggests the presence of an efficient system of position-dependent guidance information within the developing cortex that controls their area-specific targeting. Several lines of evidence suggest that this guidance information is expressed within the SP: for example, TCAs take an intracortical path centered on the SP and apparently make their targeting decisions within it (De Carlos and O'Leary 1992; Bicknese and others 1994), and experimental ablation of the SP leads to defects in area-specific TCA targeting (Ghosh and others 1990; Ghosh and Shatz, 1993).

Recent studies show that the homeodomain protein EMX2 confers positional or area identity to cortical neurons, in both

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CP and SP, and regulates in the SP the expression of unidentified axon guidance molecules that control the area-specific targeting of TCAs (Leingartner and others 2003; Hamasaki and others 2004). Alterations in the organization of area-specific TCA projections in EMX2 mutants are also consistent with this interpretation (Bishop and others 2000; Mallamaci and others 2000), although subject to caveats (Lopez-Bendito and others 2002). However, EMX2 is expressed in a graded pattern by progenitor cells of the dTel VZ but is not expressed by their neuronal progeny that form either the SP or the CP. Taken together, though, these observations suggest a mechanism to establish TCA guidance information within SP neurons, not unlike the mechanism shown to control development of other neural maps, such as retinotopic maps (McLaughlin and others 2003; McLaughlin and O'Leary 2005). Specifically, the graded levels of EMX2 and likely other regulatory genes that cooperate with it expressed in VZ progenitors specify positional or areal identity that is inherited by SP neurons and governs their expression of TCA guidance molecules.

This mechanism would be most efficient if VZ progenitors and their SP neuronal progeny maintain neighbor relationships during the time the PP is established. However, studies of the movements of progenitors and their progeny in rodents and other mammals have reported considerable tangential dispersion of cells within the VZ as well as migrating cortical neurons during the establishment of the CP. Therefore, a major goal of this study is to determine whether progenitors in the dTel VZ and their progeny maintain neighbor relationships during the genesis of the neocortical PP. Toward this end, we have used time-lapse video microscopy to follow within dTel explants from embryonic rats at stages when PP neurons are generated, the relative movements of VZ progenitors and the radial movement of their progeny and distribution in the PP. We find that focally-labeled VZ cells proliferate and remained clustered within the VZ, that their neuronal progeny move superficially along a radial column to form the overlying PP, and that during this process the neuronal progeny retain neighbor relationships and stay in spatial register with the VZ progenitors from which they arise.

Materials and Methods

Animals

Embryonic day (E) 12 to E13 fetuses were obtained from timed-pregnant Sprague-Dawley rats (Harlan Indianapolis, IN). The day of insemination is designated as E0.

Explant Cultures

Pregnant rats were anesthetized with an intraperitoneal injection of Nembutal (50 mg/kg body weight) before removal of embryos by cesarean section. The dTel was removed in cold L15 media under a dissecting microscope and then cultured on Millicell membranes (Millipore CMF) for 24–48 h. Explants were placed with the pial surface apposed to the Millicell membrane and covered with a collagen matrix and a thin film of serum-free EOL-1 medium (Annis and others, 1991) with penicillin–streptomycin (Fig. 1*A–C*). For coronal and sagittal sections, the telencephalon was removed from the embryo and embedded in 3% agar dissolved in L15 media. Blocks were then mounted on the vibratome and sectioned at 200 μm in cold L15 media. These sections were then placed on the Millicell membrane and kept under a thin film of EOL-1 media without a collagen matrix (Fig. 1*D–F*)

DiI Labeling

The fluorescent carbocyanine dye 1,1′-dioctodecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (DiI) was used to label small clusters of cells in the apical aspect of the VZ (Fig. 1). Three different methods were used for labeling: 1) DiI was dissolved in dimethylformamide at 0.05–0.02% and injected through a fine-tipped glass micropipet using a short pressure pulse from a picospritzer into the ventricular surface, 2) a 0.001% suspension of DiI was prepared in L15 by sonication and then injected as in (1), or 3) a saturated solution of DiI in 100% ethanol in a fine-tipped glass micropipet was inserted into the ventricular surface of the explant allowing a small amount of DiI to diffuse from the tip. Ethanol diffusion consistently gave small discrete injections. In a small number of explants, 2 DiI labelings were done. Injections were monitored using green-light bright-field and/or low-light–level fluorescence microscopy with a silicon-intensified target (SIT) camera (Hamamatsu Photonics, Inc., Shizuoka, Japan).

Imaging

Labeled cells in the explants were imaged in a 37 °C humidified, CO_2 equilibrated chamber on a upright microscope (Nikon Microphot-FX) using ×20 and ×40 super long working distance lenses (Nikon, Melville, NY). Approximately 1 h after the injections, images were captured with an SIT camera controlled by Image-1 system (Fig. 1*C,F*). A selection of appropriately labeled explants were imaged every 15 min for approximately 24–48 h. Others were imaged at irregular intervals. After imaging, the explants were fixed with 4% paraformaldehyde.

Sectioning

After fixation, explants were sectioned on a vibratome or cryostat. For vibratome sections, explants were removed from the collagen matrix and embedded in 3.5% agar and 20% sucrose. Transverse sections were cut at 50–100 μm and transferred to 0.1 M $NaPO_4$ buffer. Sections containing labeled cells were photographed using rhodamine optics. For cryostat sectioning, explants were cryoprotected with 20% sucrose in phosphate buffer, removed from collagen, placed in tissue-tek, and frozen. Transverse sections were cut at 10–20 μm and mounted on gelatin-subbed slides for 5-bromodeoxyuridine (BrdU) immunohistochemistry.

BrdU Labeling

A subset of imaged explants and nonimaged explants prepared in the same manner were assessed for cell proliferation and movement by exposure to EOL-1 medium containing 10 μM of BrdU and 1 μM of fluorodeoxyuridine for 2 h at various times during the culture period. The BrdU was then washed away, and the explants were fixed or returned to the incubator and/or imaged and fixed at a later time point. BrdU labeling was examined in 10- to 20-μm cryostat sections mounted on gelatin-subbed slides. Sections were covered with 1 N HCl for 30 min at 60 °C, rinsed off with phosphate-buffered saline (PBS), and then covered with anti-BrdU at 1:100 in PBS with 5% rat serum for 1–2 h at room temperature. Goat anti-mouse antibody with fluorescein isothiocyanate (FITC) was diluted at 1:100 and left at room temperature for 2–3 h before rinsing with PBS and coverslipping the sections with *n*-propylgallate in glycerol and 0.01 M $NaPO_4$ for photographing.

TuJ1 Immunobistochemistry

Floating vibratome sections were blocked with 0.25% triton X-100 and 5% goat serum in PBS for 30 min at room temperature. Sections were then transferred to TuJ1 antibody (Lee and others, 1990) diluted at 1:750 in the same solution and kept overnight at 4 °C. After rinsing the sections with PBS, FITC-labeled goat anti-mouse antibody was added at 1:100 for 2 h at room temperature. Sections were then rinsed before mounting and coverslipping with the fade retardant *n*-propylgallate for photographing.

Results

The major goal of this study is to determine whether progenitors in the neocortical VZ and their progeny maintain neighbor relationships during the genesis of the neocortical PP. Specifically, we have investigated the relative movements of progenitors within the embryonic rat VZ of the dTel; the movement of their neuronal progeny into the PP, the precursor of the SP; and the spatial registration of PP neurons with their VZ

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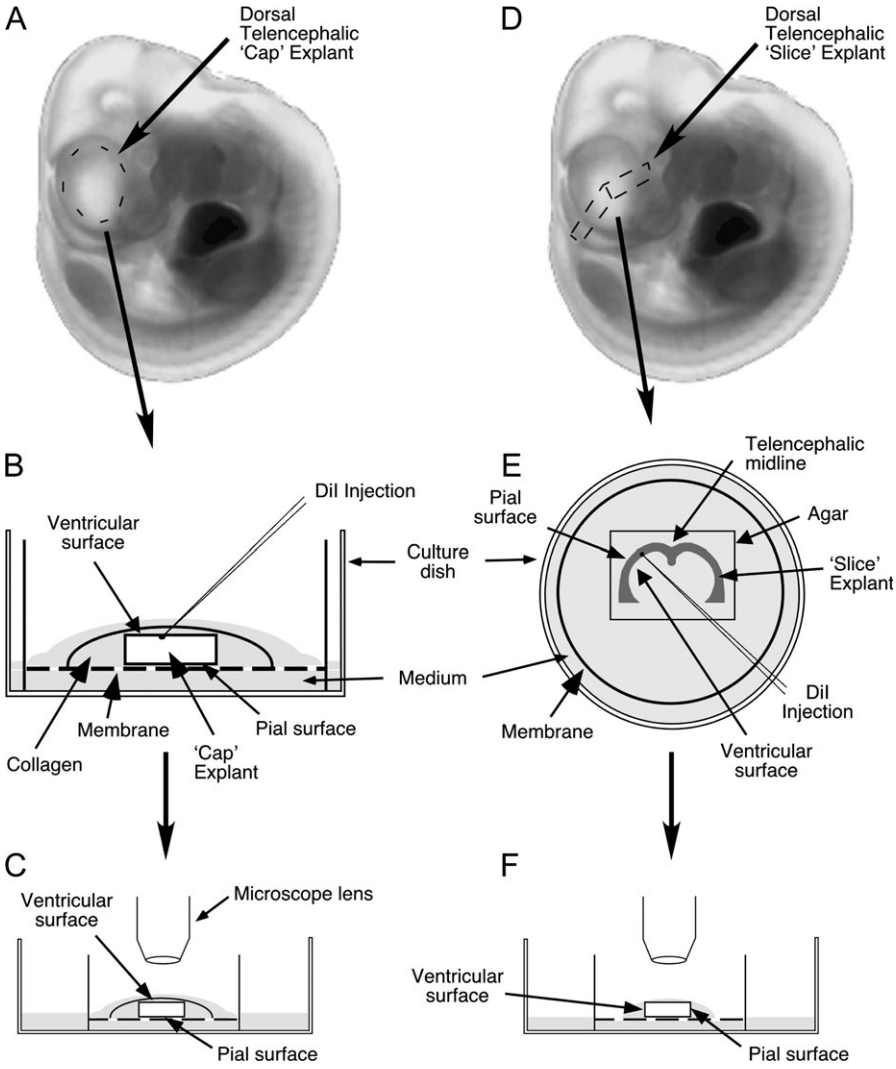

Figure 1. Preparation of dTel explants for time-lapse video imaging of labeled cells. dTel cap explants from E12 or E13 rats were prepared and analyzed as illustrated in (*A–C*); dTel slice explants were prepared and analyzed as illustrated in (*D–F*). (*A*) and (*D*) are photos of a side view of an E12 rat embryo; dashed oval or bent rectangle indicates the approximate piece of dTel used as an explant; (*B*) and (*E*) show culturing and injection of explants; (*C*) and (*F*) show imaging. The dTel cap explants were approximately 2 mm in diameter; dTel slice explants were cut 300 μm thick. See Materials and Methods for details.

progenitors. In rat, PP neurons are generated from E12 to E14 (Valverde and others 1989; Bayer and Altman 1991). Therefore, to accomplish our goal, we developed an in vitro system to culture explants of dTel from E12 or E13 rat embryos, as described in Figure 1. Our primary strategy involves labeling individual or very small groups of progenitors in the VZ with DiI in these explants and following the movements of the labeled cells using low-level fluorescence, time-lapse imaging, as well as performing a post hoc analysis of their distribution in the VZ and PP. We also carried out several control experiments to demonstrate the viability of the explants and the feasibility of this study.

PP Development and Cell Proliferation Appear to Be Normal in Cultured dTel Cap Explants

We assessed the viability and development of explants of dTel dissected from E12 or E13 rat embryos, focusing on the dTel "cap" explants, which were the main explant type used in this study (Fig. 1*A–C*). In addition to our findings from the imaging experiments to be described later, findings from these control experiments indicate that the explants remain viable and develop normally under these culture conditions. First, at the end of each experiment, each explant was stained with carboxyfluorescein diacetate succinimidyl ester, a vital dye that selectively stains living cells, and all are found to be brightly fluorescent, indicating that they remained viable (not shown). We also assessed the progressive development of a PP in the explants and used incorporation of the thymidine analog BrdU to show that the explants continued to exhibit proliferation and appropriate movement of BrdU-labeled cells from the VZ to the PP.

Neuronal differentiation and PP development was examined in the explants by immunostaining using the neuron-specific marker TuJ1 (Lee and others 1990). For this experiment, E12 dTel cap explants were fixed at progressively longer times in culture, ranging from 3 to 50 h and then transversely sectioned and immunostained (Fig. 2*A–D*). In vivo, the first PP neurons are generated on E12, and by E14, when generation of PP neurons comes to an end, a well-defined PP is evident. Consistent with in vivo development, when the dTel cap

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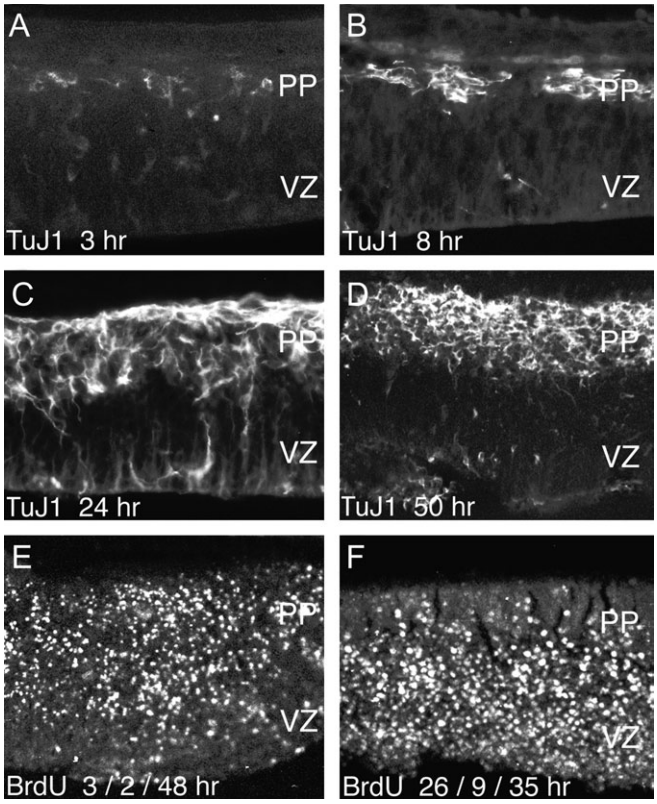

Figure 2. Dorsal telencephalic explants exhibit proliferation, cell movements, and PP development that closely resemble in vivo development. Sections shown are from dorsal telencephalic cap explants from E12 rats cultured for up to 50 h as described in Figure 1 and then fixed and sectioned before immunostaining. (*A–D*) Sections are from explants cultured for the hours indicated on each panel; explants in (*C*) and (*D*) had a small DiI injection in the VZ, and the DiI-labeled cells were time-lapsed imaged, with images collected every 15 min (as in Figs 3 and 4). Immunostaining with the neuron-specific marker TuJ1, an antibody for Beta III tubulin, reveals that a PP is not present when the explants are placed in culture but progressively develops over time in culture, similar to in vivo. These findings also imply that appropriate neurogenesis and cell movements occur in the dTel cap explant cultures. (*A*) After 3 h in culture, few TuJ1-labeled neurons are present, and all are located above just below the pial surface where the PP should form. (*B*) The density of TuJ1-positive neurons just below the pial surface increases by 8 h in culture. (*C*) At 24 h in culture, the density of TuJ1-positive neurons below the pial surface further increases, and their radial distribution is broader, taking on the appearance of a PP. (*D*) By 50 h, a well-defined, neuron-dense PP is established. BrdU labeling confirms proliferation and migration in explant cultures. (*E–F*) Cell proliferation and movement were examined in the explants by exposure to the thymidine analog BrdU for 2 h, and then the explants were fixed or returned to the incubator and/or imaged and fixed at a later time point. Explants were sectioned and processed to reveal BrdU labeling. (*E*) The explant was cultured for 3 h before exposing to BrdU for 2 h and then fixed at 48 h. BrdU-labeled cells are found in VZ, as well as in the PP layer, indicating that they were generated in culture and migrated from the VZ to the PP. (*F*) The explant was first cultured for 26 h, exposed to BrdU for 9 h, and then fixed (35 h total culture time). A high density of BrdU-labeled cells are seen in the VZ, and a proportion of cells in the PP are also BrdU labeled, indicating that proliferation and generation of PP neurons, as well as the movement of cells from the VZ to the PP, continue throughout the culture period.

explants are fixed after 3 h in culture, very few TuJ1-positive cells are found, and they are positioned just beneath the pial surface (Fig. 2*A*). The numbers and density of TuJ1-positive neurons present beneath the pial surface progressively increases with longer times in culture (Fig. 2*B*,*C*), such that by 50 h in culture a dense accumulation of TuJ1-positive cells indicative of a mature PP is evident (Fig. 2*D*). The TuJ1-staining patterning observed in these explants closely resembles the normal in vivo development at the equivalent time points (E12 plus the hours of culture time added on) (Menezes and Luskin 1994; Richards and others 1997; data not shown).

Cell proliferation in the explants was examined by incorporation of BrdU. E12 dTel cap explants were exposed to BrdU added to the medium for 2–9 h and washed out and then either immediately or after an additional period of time the cultures were fixed and transversely sectioned and processed for BrdU immunostaining. In the representative examples shown in Figure 2*E*,*F*, a high density of cells is heavily labeled by BrdU, and the labeled cells are found at the appropriate radial positions. With long culture times after a brief BrdU exposure (Fig. 2*E*), many labeled cells are found superficially, that is, in the PP. When an explant is exposed to BrdU after roughly a day in culture and fixed during the period of BrdU exposure (Fig. 2*F*), the majority of labeled cells are deep, that is, in the VZ, and a proportion of cells in the PP are well labeled. The distribution of labeled cells is very similar to that reported for comparable exposure and survival times at the same stages in vivo (Bayer and Altman 1991). These findings show that cell proliferation continues in the VZ over the period of culturing and that cells move from the VZ superficially in an apparently normal manner; both of which are consistent with the demonstration that a PP forms in these explants. Further, because cells in these explants had been coincidentally labeled with DiI and time-lapsed imaged, the findings also show that the imaging method that we used in this study does not alter normal development.

Time-Lapse Imaging of DiI-Labeled Explants Shows Minimal Dispersion of Neighboring Cells

To facilitate the clearest imaging of labeled cells in the VZ, dTel cap explants were placed with the pial surface apposed to the membrane of the Millicell insert, and individual or small clusters of neighboring cells in the VZ were labeled with DiI (Fig. 1*A–C*). Low-light–level fluorescence images of the labeled cells were taken with an SIT camera at 15 min or longer intervals over the culturing period of approximately 24–48 h.

Figure 3 illustrates an E12 dTel cap explant in which one or possibly 2 progenitors were initially labeled with DiI on the ventricular surface of the explant soon after it was placed in culture. The first image presented was taken 1 h after labeling and shows what appears to be a closely apposed pair of labeled cells. The next image, taken 15 min later, clearly shows at least 2 labeled cells, separated by a very small distance with a narrow labeled "bridge" apparently connecting them. These 2 images 15 min apart are suggestive of capturing a progenitor cell in the VZ undergoing mitosis at the ventricular surface. The appearance of labeled cell cluster changes somewhat over the next 75 min. The image collected 17 h later (19.5 h after initial labeling) shows that the labeled cell cluster in the VZ has expanded and contains appreciably more cells, presumably through cell division. We estimate that approximately 8–10 labeled cells are present in the cluster, which is roughly consistent with the number predicted by cell cycle estimates of approximately 8 h (Caviness and others 2003) and an initial population of 2 labeled cells in a proliferative mode. These observations are consistent with the findings obtained with TuJ1 immunostaining and BrdU labeling (Fig. 2) that progenitor cells in the VZ continue to proliferate in the explants in a manner that approximates that normally observed in vivo. Another important observation is that although the cluster of

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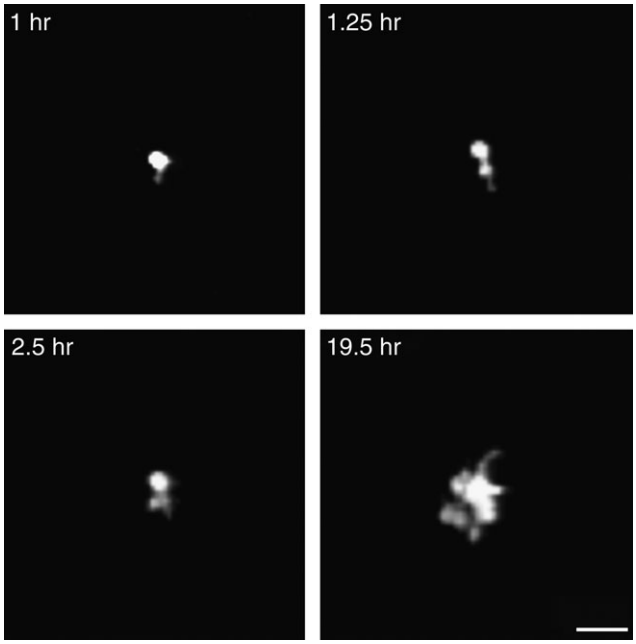

Figure 3. Time-lapse imaging of labeled cells in VZ of dorsal telencephalic cap explant shows that they increase in number but remain clustered. Time-lapse images of DiI-labeled cells in the VZ of an E12 dTel cap explant taken with low-light–level rhodamine fluorescence and an SIT camera as described in Figure 1*A–C*. The ventricular surface is toward the camera. One or two cells in the VZ were labeled on the explant's ventricular surface at 0 h, soon after the explant was put in culture. Images were taken at 15-min intervals (0.25 h) over 24 h. The number of labeled cells increases over the period shown, presumably through cell division, and they remain clustered. Scale bar: 20 μm.

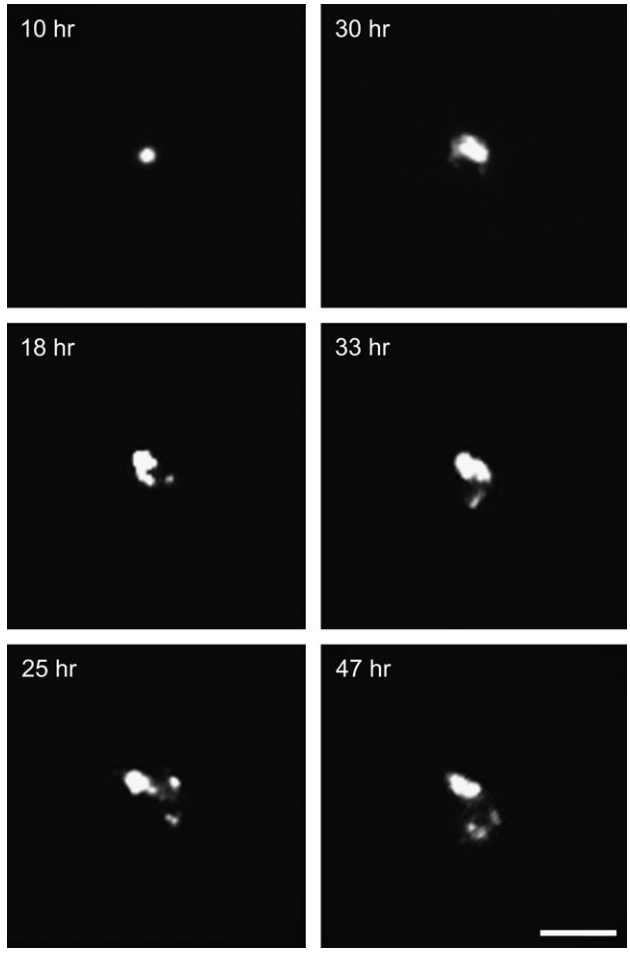

Figure 4. Time-lapse imaging of DiI-labeled cells in VZ of dorsal telencephalic cap explants suggests radial movement and minimal tangential dispersion. Time-lapse images of DiI-labeled cells in the VZ of an E12 dTel cap explant as described in Figures 1*A–C* and 3. Images were taken at 15-min intervals (0.25 h). The first image shown is at 10 h after labeling; the last image is at 47 h. Over this 37 h, an initially focal, dense cluster of labeled cells expands through addition of labeled cells. The prominent group of labeled cells remains clustered, but a few labeled cells disperse a short distance (20–30 μm) from it. Some of the labeled cells appear, disappear, and apparently reappear over the imaging period: these dynamics suggest that the cells move out of the focal plane in the radial dimension of the explant and subsequently some move back into the focal plane. Scale bar: 50 μm.

labeled cells expands and changes in shape, the labeled cells visible in the VZ remain clustered.

Figure 4 illustrates another example, an E12 dTel cap explant. In the first image presented, collected at 10 h after the explant was placed in culture, one or possibly 2 labeled cells are visible on the ventricular surface of the explant. Five additional images are shown of this cluster of labeled cells at times ranging from 18 to 47 h in culture. Over this period, the cluster of labeled cells changes in shape and size and appears to add additional cells, again likely through proliferation. Some of the cells move in and out of focus during imaging, which contributes to the shape changes and apparent disappearance and appearance of labeled cells (e.g., compare 25 h with 30 and 33 h); this behavior is consistent with the radial movement of the labeled VZ cells superficially, likely due to either normal interkinetic nuclear migration of progenitors away from the apical surface of the VZ and then back to it or the migration of newly postmitotic neurons superficially to the PP.

Similar to the case illustrated in Figure 3, the labeled cells in the explant shown in Figure 4 also tend to remain clustered. However, in contrast, in this case, a few of the labeled cells move tangentially a short distance away from the main group of labeled cells. We analyzed this behavior for all the dTel cap explants that were appropriately labeled and had adequate time-lapse imaging over a period ranging from approximately 24 to 48 h. In 38 E12 explants imaged for approximately 48 h, only 5 clusters had labeled cells move an appreciable distance tangentially away from the main cluster. The total number of cells was 13 (at a minimum), and as measured from the edge of the cluster, they moved away from the cluster by an average distance of 34.4 ± 12.2 μm. In 59 E12 explants imaged for approximately 24 h, only 3 cases had labeled cells move an appreciable distance tangentially away from the main cluster, with an average distance of 27.7 ± 13.1 μm. In 37 E13 explants imaged for approximately 24 h, only 5 cases had labeled cells move an appreciable distance tangentially away from the main cluster, with an average distance of 16.8 ± 4.5 μm. In summary, the groups of labeled cells visible from the apical VZ surface do exhibit changes in their shape and size but tend to remain clustered in the tangential plane of the explant.

Low-Light Fluorescence Imaging Does Not Affect Cell Movements: Labeled VZ Cells Remain Clustered without Frequent Imaging

Our findings show that the imaged explants exhibit cell proliferation in the VZ and that cells move from the VZ superficially to form a normal-appearing PP over the proper developmental period. It remains possible though that the

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clustering of labeled cells with minimal dispersion is due to deleterious effects of the low-light fluorescence imaging on movements of labeled cells in the VZ. We did additional experiments to rule out this possibility; 2 will be described here, and the third experiment will be described in the following section in which we imaged the movements of labeled cells in dTel "slice" explants.

In one set of experiments, we made small DiI injections into the dTel VZ in intact forebrains, dissected them free, and maintained them floating in oxygenated EOL-1 medium for up to 48 h (n = 11). At the end of the incubation period, the viability of the neural tissue was demonstrated by its strong staining with the vital dye carboxyfluorescein. The brains were then fixed and transversely sectioned. Small clusters of DiI-labeled cells, both in the VZ and in the overlying PP, were identified; although they tended to be larger (due to larger initial injections), the great majority of labeled cells whether in the VZ or PP remained clustered (data not shown).

In the other set of experiments (n = 12), we labeled and imaged cells in the VZ of E12 dTel cap explants, as described for the cases illustrated in Figures 3 and 4. However, rather than collecting time-lapse images at frequent intervals (e.g., every 15 min), we imaged each explant only twice, once at a relatively early time point after labeling and the other roughly 12–36 h later (Fig. 5 and data not shown). We find that in these cases, the appearance of the DiI-labeled cells and their tendency to remain clustered are indistinguishable from the cases imaged at frequent intervals. Three representative explants are shown in Figure 5. At the time of the initial image, the number of labeled cells and the size of the labeled clusters varied across cases, as did the separation between labeled cells. In each case, the number of labeled cells and the size of the clusters increased over the lengthy period between the first and second images, indicative of proliferation of the labeled cells. Importantly, though, the great majority of labeled cells remained clustered, and a small proportion of labeled cells moved short distances from the main cluster, behaviors similar to those observed in the frequently imaged explants.

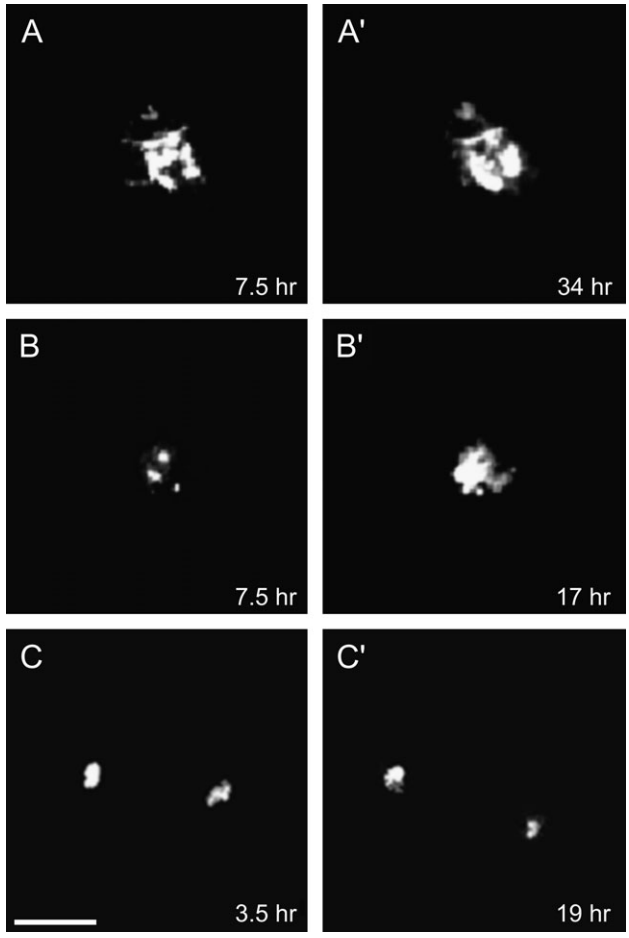

Figure 5. Techniques employed to time-lapse image DiI-labeled cells in VZ do not appear to affect their behavior. Paired images of 3 different E12 dTel cap explants cultured as in Figures 1*A–C*, 3, and 4. In each, a single injection of DiI was made at the ventricular surface at 0 h and verified. The explants were not time-lapse imaged; only 2 images were collected for each explant at the times indicated. The numbers of labeled cells and cluster size increased over time, and the labeled cells remained clustered with minimal dispersion. These findings are similar to those obtained from cases imaged every 15 min, indicating that the time-lapse imaging techniques that we have used does not noticeably influence the behavior of the labeled cells, including their proliferation or movements. (*A*) At 7.5 h in culture, a single small cluster of labeled cells is seen from the ventricular surface. By 34 h, these cells still retain their close neighbor relationships. (*B*) In a second example, 7.5 h after preparing and labeling the dTel cap explant, a small focus of label is visible on the ventricular surface. By 17 h, the labeling site has enlarged possibly due to cell division while maintaining the neighbor relationships. (*C*) Two small injections visualized at 3.5 h reveals 2 clusters of labeled cells on the ventricular surface. By 19 h, the clusters have different appearances, but their positions relative to each other have changed little. Scale bar in (*C*) equals 50 μm in (*A*) and (*B*), 20 μm in (*C*).

Distribution of DiI-Labeled Progenitors and Their PP Progeny: The PP Forms in Register with the Underlying VZ in a Manner That Retains Neighbor Relationships

A primary goal of these analyses was to assess whether the labeled PP progeny of progenitors initially labeled in the VZ remain in radial register with their progenitors in the underlying VZ. We performed this analysis in 2 ways, as described below. First, after imaging, the labeled dTel cap explants were fixed and transversely sectioned to examine the distribution of DiI-labeled cells in the VZ and the PP (Fig. 6). Second, we prepared an alternative type of explant distinct from the dTel cap explant that allowed us to label cells in the VZ and follow their movements in the VZ and from the VZ to the PP (Fig. 7).

Labeled PP Neurons and VZ Progenitors Are in Radial Register in Sections of Imaged dTel Cap Explants

The distribution of labeled cells was determined in transverse sections through the dTel cap explants that had been previously imaged. Only cases in which a single cluster of labeled cells was initially filled by the DiI injection and in which each section was collected, intact, and analyzed for labeled cells were used for this analysis; 12 cases met these criteria. Figure 6 illustrates a section through a representative example. Within the VZ, the labeled cells formed a fairly condensed cluster, very similar to the labeled cell clusters observed with time-lapse imaging of the ventricular side of these explants. Similarly, the labeled cells beneath the pial surface, within the PP, were also clustered. The cluster of labeled cells in the PP were positioned directly above the cluster of labeled cells in the VZ, and a few labeled cells were typically found between them; these were likely either progenitors moving superficially within the VZ to undergo "S" phase or newly generated neurons moving out to the developing PP. In summary, labeled cells in the PP are clustered as they are in the VZ, and the 2 clusters are

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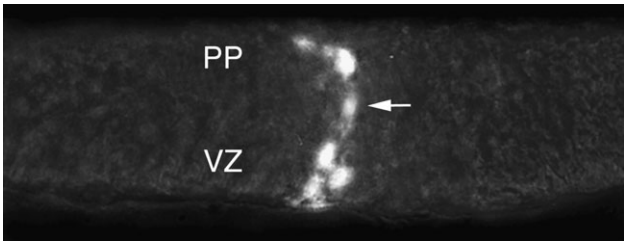

Figure 6. Labeled VZ progenitors and their PP progeny exhibit radial alignment in dorsal telencephalic cap explants. After time-lapse imaging of labeled VZ cells from the ventricular surface of dTel cap explants, explants were sectioned to determine the radial distribution of labeled cells. The image shown is representative of our findings. In this example, which is a section through the imaged explant presented in Figure 3, a small deposit of DiI was made into the ventricular surface at 0 h that focally labeled cells in the apical aspect of the VZ; after 24 h of culturing, the explant was fixed and sectioned. A radial column of labeled cells is seen spanning the extent of the cross section of the explant, with accumulations deep in the VZ and superficially in the PP, and some labeled cells are found in between (arrow). Little if any tangential spread of labeled cells is apparent in this and adjacent sections. Scale: distance between ventricular and pial (top) surfaces is 150μm.

spatially in register in the cortical wall along its radial (laminar) axis, with the labeled cluster in the PP positioned directly above that in the VZ.

Imaging in dTel Slice Explants to Observe Cell Movements between the VZ and the PP

To extend our findings obtained from analyzing the distribution of DiI-labeled cells in transverse sections of imaged dTel cap explants, we carried out time-lapse imaging of DiI-labeled cells in 300-μm-thick slices of E12 or E13 dTel (Fig. 1*D–F*). This approach allowed us to observe the movement of labeled cells from the VZ to the PP. As with the dTel cap explants, in the dTel slice explants, single cells or small groups of cells were labeled with DiI injected into the VZ and followed using low-light–level, time-lapse imaging. Because of the possibility that tangential cell movements might occur along either the coronal (lateral to medial; as in Fig. 1*D*,*E*) or sagittal (anterior to posterior) plane, we imaged 27 coronal and 8 sagittal sections for up to 48 h. The same patterns of cell movements were observed in both planes of slice explants and therefore are considered together.

Similar to our findings from imaging-labeled VZ cells from the ventricular surface of dTel cap explants, imaging of the sectioned explants reveals that the labeled cells remained clustered in the VZ and few migrated tangentially out of the cluster. In contrast, a significant number of labeled cells migrated radially from the VZ toward the pial surface, as expected from our findings described for the dTel cap explants (e.g., see Figs 2 and 6). Figure 7 illustrates 3 representative cases: early- and late-paired images of E13 explants are shown in Figure 7*A*,*A′*,*B*,*B′*; 3 temporally spaced images of an E12 explant are shown in Figure 7*C–C″*. Images of the 2 E13 explants show the distribution of the labeled cells at 4 h (Fig. 7*A*,*B*) and 18 h (Fig. 7*A′*,*B′*) after a DiI injection was placed near the apical surface of the VZ. Comparisons of the images collected at 4 h and at 18 h show that the clusters of labeled cells in both the VZ and the PP increased in size and in number of labeled cells. Examination of the series of time-lapse images collected between the 2 time points shown (not shown) indicates that the increase of cluster size of labeled cells within the VZ is due to proliferation and that the presence of labeled cells in the PP and their increase in number are due to the radial movement of labeled cells from the

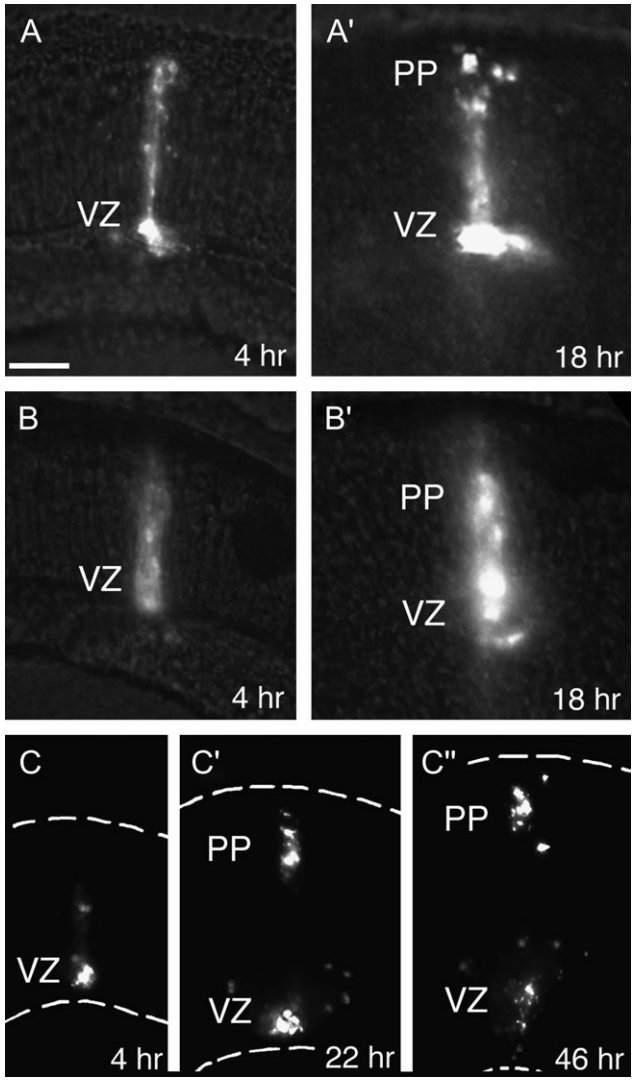

Figure 7. Progeny of labeled VZ progenitors exhibit radial movement along a columnar path to the PP in dorsal telencephalic slice explants. Time-lapse images of 3 different dTel slice explants as described in Figure 1(*D–F*). The paired images shown in (*A*, *A′*) and (*B*, *B′*) are from E13 explants and were collected at 4 h and 18 h after injection; the 3 images shown in (*C–C″*) are from an E12 explant and were collected at 4, 22, and 46 h after injection. In each case, a single small deposit of DiI was made into the apical aspect of the VZ and verified. Images were taken at 15-min intervals (0.25 h). The injection labeled a small, focal group of cells deep in the VZ, which over time expanded in size and number of labeled cells but remained focally clustered. Labeled cells migrated radially from deep to superficial, forming a columnar path with labeled cells distributed along it. The number of labeled cells beneath the pial surface in the PP progressively increased. Scale bar in (*A*) is 50 μm in (*A*) and (*B*), 70 μm in (*C*).

VZ to the developing PP. Within both the VZ and the PP, the labeled cells remain relatively clustered, with few labeled cells significantly outside the main column of labeled cells and their path of radial movement. In these and other such cases, a clear columnar pattern of labeling is evident, with labeling visible along the entire route between the cluster of labeled cells in the VZ and that directly above it in the PP. Labeled cell bodies are present within this column of labeling; these are likely a combination of progenitors undergoing interkinetic nuclear migration and postmitotic neurons migrating to the PP. In addition, some of the columnar labeling has the appearance of labeled processes; these are likely a combination of radial processes of

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progenitors within the VZ and processes of migrating PP neurons.

Panels *C*,*C′*, and *C″* in Figure 7 show images at 3 time points of an E12 coronal slice explant again selected from a full series of images collected every 15 min from 1 h after DiI was injected into the VZ to 48 h later. At 4 h, a discrete cluster of labeled cells is seen deep in the VZ, with one or more labeled cells outside of the main cluster but radially above it within the VZ. At 22 h, the number of cells in the VZ cluster has increased, and a radially distributed group of cells are positioned within the PP directly above the VZ cluster. At 46 h, the cluster of labeled cells in the VZ has spread radially, and the number of labeled cells in the PP has increased substantially. The majority of labeled cells in the PP form a radial cluster, with a few labeled cells tangentially positioned a short distance away. In summary, in these and other imaged dTel slices, the cells in the PP and the migratory route between them and the labeled cells in the VZ retain a "columnar organization" with only a small proportion of cells being displaced a small distance from the radial path (or column). These findings are similar to those observed in the transverse sections of dTel cap explants fixed after imaging (e.g., Fig. 6).

Discussion

Evidence for Maintenance of Neighbor Relationships by VZ Progenitors and Their Neuronal Progeny during PP Development

We show that during early stages of cortical development 1) progenitors maintain neighbor relationships in the VZ as they generate PP neurons, 2) PP neurons that arise from the same or neighboring progenitors retain neighbor relationships as they form the overlying PP layer, and 3) PP neurons are positioned above their progenitors in the VZ, thereby retaining a spatial, point-to-point, registration between the VZ and the PP. To accomplish this goal, we primarily used time-lapse video microscopy to study over a period of approximately 24–48 h the movements of small numbers of DiI-labeled cells in either whole-mount explants of essentially the entire cortex or a thick slice through it, from E12–E13 rats, when PP neurons are generated. We find that labeled cells in the VZ exhibit minimal tangential dispersion and a strong tendency to remain clustered, even as the clusters expand in both size and the number of labeled cells within them. We find that their neuronal progeny that form the PP also tend to remain clustered in a columnar pattern that retains radial point-to-point spatial relationships and effectively maps the VZ onto the PP.

Several observations indicate that our in vitro findings present an accurate picture of in vivo development. First, we show using a neuron-specific marker, TuJ1, that a PP develops in the explants over the same time frame that it develops in vivo and has a very similar appearance to the in vivo PP. Second, using BrdU incorporation to mark cell proliferation, we show that proliferation continues within the explants, that it is localized to the VZ, and that the BrdU-labeled cells move from the VZ to the PP as they do in vivo. These 2 sets of data, TuJ1 immunostaining and BrdU labeling, are consistent with each other. The progressive increase in the number of TuJ1-immunopositive neurons at the position where the PP would develop in vivo is consistent with the continued cell proliferation observed with BrdU labeling. In addition, the accumulation of a layer of TuJ1-marked neurons at a laminar position just above the VZ and beneath the pial surface is consistent with appropriate movements of early-generated neurons from the VZ to the PP, as also suggested by the distributions of BrdU-labeled cells.

These data on cell movements and proliferation are also consistent with our findings using time-lapse imaging of DiI-labeled cells, establish confidence in our findings using time-lapse imaging of DiI-labeled cells in the dTel explants, and provide a framework for interpreting our imaging data. Imaging of DiI-labeled cells in the VZ shows that they increase in number over time, again consistent with the BrdU labeling showing continued cell proliferation over the period of explant culturing. Our time-lapse imaging of DiI-labeled cells in the VZ shows that as the cluster of labeled cells increases in number, they exhibit a strong tendency to remain clustered in the tangential plane. Although the cells remain clustered, they do move, often in and out of the plane of focus, indicating movement in the radial axis. This observation and more importantly our control studies showing that exposure to the imaging itself does not affect movement or the tendency to cluster confirm that our observations reveal a real behavior of VZ cells at this stage of cortical development.

Implications of Maintenance of Neighbor Relationships for Mechanisms of Establishing and Maintaining Smooth Gradients of Transcription Factors That Specify Positional or Area Identities

Our findings have implications for the establishment and maintenance of graded distributions of transcription factors that are expressed by VZ progenitors and impart positional identity (or area identity) to their progeny, both additional progenitors and neuronal progeny. All transcription factors identified to date to be differentially expressed across one or both axes of the cortical VZ are expressed in graded patterns (Liu and others 2000; O'Leary and Nakagawa 2002). Considerable evidence shows that one of these graded transcription factors, EMX2, imparts area or positional identity by a mechanism that depends upon its absolute level of expression within a progenitor (Hamasaki and others 2004). Given the critical relationship between the absolute level of expression of a transcription factor in a progenitor cell and the area identity of its progeny (Hamasaki and others 2004), it is important that neighboring progenitors have similar levels of expression. However, multiple mechanisms likely cooperate to create and maintain this scenario in which an expanding population of progenitors have similar levels of a transcription factor, such as EMX2, at the level of near neighbors, yet that level changes in a progressive way across the cortical axes to generate a smooth gradient of expression .

Morphogens, such as fibroblast growth factor, bone morphogenic protein, and wingless-int, secreted by patterning centers positioned around the perimeter of the cortical VZ, including the anterior neural ridge/commissural plate and the cortical hem, establish the initial graded expression of EMX2 (Ragsdale and Grove 2001; O'Leary and Nakagawa 2002; Shimogori and others 2004). Although the expression of EMX2 and like transcription factors are presumably under tight transcriptional control, other mechanisms might act in the VZ to equilibrate differences among neighboring progenitors in their protein levels, including reciprocal activation/repression loops, as well as the potential release and local uptake of transcription factors among neighboring progenitors, as described in vitro for

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homeodomain proteins (Prochiantz and Joliot 2003). Although these same mechanisms could potentially operate to modify levels in a progenitor to match those of its neighbors should it move to a new position within the cortical VZ, our demonstration that progenitors in the VZ retain neighbor relationships would contribute to the stability and maintenance of the gradient.

Implications of the Maintenance of Spatial Relationships between VZ Progenitors and PP Progeny

We find DiI-labeled cells in the PP of cultured explants fixed and sectioned after imaging their DiI-labeled progenitors in the VZ. In addition, time-lapse imaging of DiI-labeled cells in thick dTel slice explants shows that DiI-labeled cells in the VZ increase in numbers, and a proportion of it moves away from the VZ in a radial column and accumulate beneath the pial surface. Both approaches show that labeled cells move along a radial, columnar path and accumulate beneath the pial surface in spatial register with the labeled cells in the VZ. The most straightforward interpretation of these observations, and strongly implied by the TuJ1 and BrdU data, is that DiI-labeled cells in the VZ are progenitors, and over time they generate neuronal progeny that migrate superficially along a radial path and form the overlying PP. Although the resolution of our imaging did not allow us to address the mode of movement, it is likely that it is accomplished by translocation, which has been proposed to be the major mode of migration during these early PP stages of cortical development (Nadarajah and Parnavelas 2002).

We conclude from our findings that during the time the PP is established, progenitors in the VZ that generate PP neurons maintain neighbor relationships, and through the radial columnar movement of their neuronal progeny to the overlying PP, PP neurons generated by neighboring progenitors also tend to maintain neighbor relationships as they accumulate in the PP. Our findings, then, show that spatially the VZ is mapped onto the PP, much like that proposed in the "protomap" hypothesis for cortical areal patterning in general (Rakic 1988) and in other models for establishing positional information in the SP to control development of area-specific TCAs (O'Leary 1989; O'Leary and others 1994). Once in the PP, the neuronal cells rapidly differentiate, extend long axons, and establish elaborate processes (Marin-Padilla 1971; De Carlos and O'Leary 1992); therefore, as the majority of PP neurons are displaced deep to the pial surface by the developing CP, forming the SP, it is likely that they would maintain in the SP the neighbor relationships that they had established with one another in the PP. In this way, positional information or specification passed onto PP progeny by their VZ progenitors, and mapped in the PP, would be faithfully remapped in the SP through the maintenance of this spatial ordering. This would be a very efficient mechanism to establish within the SP a framework of genetically specified, positional information that controls the expression of axon guidance molecules that direct the development of area-specific TCA projections.

This mechanism is consistent with the recent evidence that the homeodomain protein EMX2 confers positional identity to SP neurons and regulates in the SP the expression of axon guidance molecules that control the area-specific targeting of TCAs (Bishop and others 2000; Leingartner and others 2003; Hamasaki and others 2004). EMX2 also influences the differential expression of p75 in the SP (Bishop and others 2002), which has been implicated in the targeting of TCAs from the dorsal lateral geniculate nucleus to the primary visual area (McQuillen and others 2002), as well as ephrin-A5 in primary somatosensory area (Bishop and others 2002), which influences the patterned projection of TCAs from the ventroposterior nucleus to the barrelfield (Vanderhaeghen and others 2000).

Differences between Early and Late Stages of Cortical Development in Tendencies for Progenitors and Neurons to Retain Neighbor Relationships or Disperse

Our findings show a strong tendency for progenitors in the VZ and their progeny that form the PP to retain neighbor relationships and for neurons that form the PP to accumulate within the PP radially above their VZ progenitors (Fig. 8). This

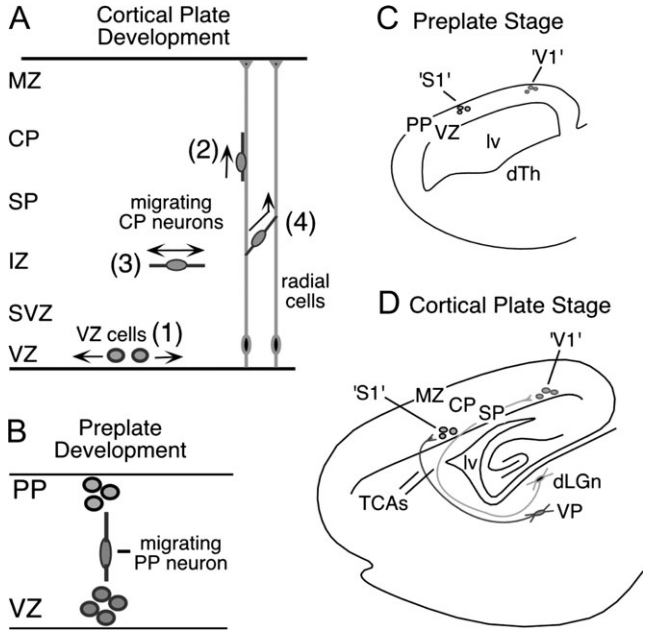

Figure 8. Cell movements in the VZ and degree of neuronal dispersion differ between early PP development and later CP development. (*A*) Previous studies examining cell movements using time-lapse imaging or static analyses of serial, progressive time points in different animals, during the period of CP development, have revealed several forms of cell movements or migration. Within the VZ, time-lapse imaging has shown movements and dispersion of once neighboring cells labeled within the VZ (Cell 1). Other studies have found that the most prominent form of migration of postmitotic neurons from the VZ and SVZ is by radially directed migration along radial processes (Cell 2). In addition, both tangential movements within the IZ (Cell 3) and the "jumping" of clonally related marked neurons from one radial cell process to another as they migrate from deep to superficial (Cell 4). (*B*) Our findings reported here show that earlier in cortical development, during the time PP neurons are being generated, neighboring cells within the VZ retain their neighbor relationships and that their neuronal progeny that form the PP maintain these neighbor relationships as they migrate superficially along a radial path and accumulate in a cluster beneath the pial surface in the PP directly above their progenitors in the VZ. This process results in a columnar arrangement of spatially related progenitors in the VZ, migrating cells, and neurons in the PP. (*C*, *D*) Our findings show that PP neurons cluster within the PP directly above their progenitors in the VZ. Thus, at a cellular level, the VZ is "remapped" in the PP through this process. We propose that when the majority of PP neurons are passively repositioned deep into the SP by the expanding CP, the neighbor relationships established in the PP are retained in the SP. Thus, positional information or "areal" identities that they inherit from their progenitors is faithfully maintained in a cellular map in the SP, providing appropriate guidance cues for the area-specific targeting of TCAs. See text for further discussion. dLGn, dorsal lateral geniculate nucleus; dTh, dorsal thalamus; lv, lateral ventricle; MZ, marginal zone; S1, SP neurons with positional identities appropriate for primary somatosensory area; V1, SP neurons with positional identities appropriate for primary visual area; VP, ventroposterior nucleus.

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is distinct from several studies of cell movements and dispersion carried out at later stages of cortical development, during the period that the CP is developed. Studies done at these later stages have shown that CP neurons migrate along processes of radial glia (Rakic 1972) and more recently have shown that CP neurons not only arise from radial glia but also often migrate along their parental radial process (Kriegstein and Noctor 2004). However, clonally related CP neurons or CP neurons arising from neighboring progenitors have been found to use different migratory routes and have been reported in rats and mice to exhibit considerable dispersion (e.g., Walsh and Cepko 1993; Reid and others 1995; Tan and others 1998). Using polymerase chain reaction identifiable retroviral tags, Walsh and Cepko (1992, 1993) have shown that clonally related cells in the neocortex can be distributed over large distances and have reported mean clonal dispersion of greater than 2 mm in adult rat cortex, with some clones spanning 10 mm, the full anterior to posterior extent of the neocortex. A single clone can therefore contain cells in many distinct cortical areas. Such dispersion can occur through multiple mechanisms. For example, clonally related CP neurons can migrate along parallel paths (Walsh and Cepko 1988; Noctor and others 2004) and have been reported to jump from one radial process to another enroute during migration (Misson and others 1991). Time-lapse and static imaging has also confirmed tangential dispersion of neurons apparently enroute to the CP (O'Rourke and others 1992, 1995, 1997; Tamamaki and others 1997). And, in contrast to our findings during early stages of cortical development, others have found using similar labeling and imaging techniques at later stages of development that VZ cells tend to disperse (Fishell and others 1993), a finding recently supported by genetic approaches (Wilkie and others 2004).

As we have shown, at early stages of cortical development, when the PP is generated, VZ progenitors and their progeny have a strong tendency to retain neighbor relationships that leads to PP neurons settling in a pattern that maintains a radial spatial registration with their progenitors in the VZ. This behavior differs with that reported at later stages of cortical development, when the CP is generated, and considerable dispersion is evident in both the VZ and along the migratory route to the CP. These distinctions are likely related to differences in 1) the mode of migration, translocation versus guidance by radial glial fibers; 2) molecular differences; and 3) the substantial increase in size and complexity of the cortical wall over the course of its development, which presents migrating CP neurons with multiple choice points and alternate paths distinct from a simple radial one (reviewed in Nadarajah and Parnavelas, 2002; Kriegstein and Noctor 2004).

Notes

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