In vivo Evidence for Radial Migration of Neurons by Long-Distance Somal Translocation in the Developing Ferret Visual Cortex

During the development of the cerebral cortex, neurons generated in the cortical ventricular zone migrate radially toward the marginal zone. Radially migrating neurons are thought to display 1 of 2 morphologies: cells with a long, pia-contacting, apical process utilized for somal translocation early in development, when the cortex is still relatively thin; or cells with a short leading process, abundant at late stages of corticogenesis when neurons need to travel for longer distances. In large convoluted brains, like those of many primates and carnivores, radially migrating neurons must travel distances up to several millimeters before reaching their final destination, often following curvilinear trajectories. Here we analyze modes and morphologies of radially migrating neurons in convoluted brains by studying the visual cortex of developing ferrets. We provide in vivo and in vitro evidence for the existence of late-born cortical neurons that migrate radially by long-distance somal translocation within a long apical process extended to the cortical plate, in contrast to the early somal translocation observed in rodents. Long-distance translocating neurons in the ferret show a discontinuous rhythm of migration, alternating periods of advance with periods of stall. Furthermore, by combining different labeling methods we find the simultaneous presence in the developing ferret cortex of long-distance translocating neurons and neurons migrating within a short leading process.

Keywords: adenovirus, cerebral cortex, DiI, electroporation, gyrus, neurogenesis, neuron tracing, radial glia, time lapse

Introduction
The normal development of the cerebral cortex requires the orchestrated migration of multiple generations of neurons from the germinative ventricular zone (VZ) to the superficial cortical plate (CP) following a radial trajectory (Rakic 1974, 1995). Early studies of brain development already proposed that neuronal migration involves the displacement of the cell nucleus and soma along a leading process, a mechanism termed nuclear translocation (Rakic 1971, 1972; Sidman and Rakic 1973). These observations were confirmed by real-time imaging studies (Edmondson and Hatten 1987; Komuro and Rakic 1995, 1998; Rivas and Hatten 1995; Nadarajah and Parnavelas 2002; Ang and others 2003; Moya and Valdeolmillos 2004), which also revealed that radially migrating cells in the cerebral cortex display 1 of 2 different morphologies: 1) cells with a short leading process tipped with a growth cone-like structure, which combine cycles of elongation–retraction of this process with nuclear translocation; and 2) cells with a pia-contacting apical process that is never detached from the pial surface during migration but that is gradually shortened as nuclear translocation progresses (Miyata and others 2001; Nadarajah and others 2001; Nadarajah and Parnavelas 2002; Noctor and others 2001, 2004).

Previous studies have referred to these morphologies as “locomoting” and “traslocating” cells, respectively (Nadarajah and others 2001; Nadarajah and Parnavelas 2002; Gupta and others 2003; Marin and Rubenstein 2003; Tabata and Nakajima 2003; Noctor and others 2004). Studies in rodents, carnivores, and humans have shown that at late stages of development radial glial cells undergo somal translocation from the VZ/subventricular zone (SVZ) to superficial positions without retraction of the pia-contacting process (Voigt 1989; deAzvedo and others 2003). The mechanistic significance of these morphological differences is highlighted by the fact that several mouse mutations produce deficits of radial migration in late-born but not early-born neurons, suggesting that the signaling mechanisms that orchestrate radial navigation and migration in the cerebral cortex may be different for neurons with a pia-contacting process versus neurons with a short leading process (Gilmore and others 1998; Ohshima and others 2001; Gupta and others 2002, 2003; Hatanaka and others 2004).

Previous studies in rodents, carnivores, and humans have shown that at late stages of development radial glial cells undergo somal translocation from the VZ/subventricular zone (SVZ) to superficial positions without retraction of the pia-contacting process (Voigt 1989; deAzvedo and others 2003; Noctor and others 2004). Other studies demonstrate that radial glial cells in the VZ/SVZ are mitotically active in both rodent and human cortex, giving off daughter cells that differentiate into neurons (Malatesta and others 2000, 2003; Miyata and others 2001, 2004; Noctor and others 2001, 2004; Weissman and others 2003; Anthony and others 2004). It has also been shown that neurons produced by dividing radial glia may inherit the long radial process of the mother cell (Miyata and others 2001), which spans hundreds of microns from its cell body to the pial surface. Taken together, these studies prompted us to...
hypothesize that migration using a pia-contacting apical process might not be exclusive to short-range migrations early in corticogenesis and may also be a mode used for long-distance radial migration. Here we have addressed this issue by studying the ferret (*Mustela putorius*), a carnivore whose gyrated cerebral cortex develops during a protracted period of time, with the late stages occurring postnatally (Jackson and others 1989; Welker 1990). By using a biased labeling method, we were able to identify a population of radially migrating neurons that contain an extraordinarily long apical process, which these cells maintain extended up to the pial surface during their long somal translocation through a thick intermediate zone (IZ). We also show that these neurons displace their soma in a salutatory fashion, alternating periods of advance with periods of stall. By using an alternative labeling method we also find cells with a short leading process undergoing radial migration, which coexist with long-distance translocating neurons in the developing ferret visual cortex. Our findings represent strong evidence for the existence of neuronal migration by long-distance somal translocation in gyrencephalic cerebral cortices.

### Materials and Methods

**Subjects and 1,1'-Dioctadecyl-3,3,3,3'-Tetramethylindocarbocyanine Perchlorate Injections**  
Thirty-nine pigmented ferret kits (16 litters) were obtained from Marshall Farms (North Rose, NY) and kept on a 12 h light/12 h dark cycle. All animals were treated in accordance with institutional and National Institutes of Health (NIH) guidelines for the Care and Use of Laboratory Animals. Animal protocols were approved by the Yale Institutional Animal Care and Use Committee.

Kits aged postnatal day (P) 16 were perfused with phosphate-buffered 4% paraformaldehyde, and small crystals of 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate (Dil, Molecular Probes, Invitrogen, Carlsbad, CA) were delivered to the occipital surface of the primary visual cortex (V1) of individual hemispheres (n = 4). Dil was allowed to diffuse and then these sections were sectioned and analyzed under rhodamine optics.

**Viral Stocks and Injections In Vivo**  
Two different viruses were used. Ad-WGA-myc is a replication-incompetent adenovirus (ΔE1,ΔE3) engineered using Adeno-X (Clontech, Mountain View, CA) to encode for myc-tagged wheat germ agglutinin (5 x 10^11 vp/mL). Ad-GFP is a Green Fluorescent Protein (GFP)-encoding ΔE1,ΔE3 adenovirus (QBioGene, Irvine, CA) (9.3 x 10^11 vp/mL). Virus injections were delivered stereotaxically by pressure through pulled glass micropipettes. Twenty-three ferret kits (16 litters) received 2 adjacent injections of Ad-WGA-myc or Ad-GFP (3-6 nL/injection site-2) and were sacrificed at P16 (n = 22) or P18 (n = 1).

**In Vivo Electroporation of Ferret V1**  
The DNA construct used to label transfected cells was pCAGGS-EGFP. Ferrets aged P1 (n = 6) were deeply anesthetized with isoflurane and placed in a stereotronic micromanipulator. Approximately 2 µL of DNA solution was injected using a pulled glass micropipette into the lateral ventricle in a region subjacent to the prospective V1. A tweezers-type electrode (CUY661-3X7; NEPA Gene, Chiba, Japan) was then used to electroporate the DNA into the VZ of the prospective V1. Square electric pulses (60 V, 50 ms) were passed 5 times at 1-s intervals using an electroporator (CUY21E; NEPA Gene). The skin was suture-secured, and the animals were allowed to recover from anesthesia before being placed back with the dam. Electroporated ferrets were transcardially perfused with 4% paraformaldehyde at P6 (n = 4) or P55 (n = 2), their brains were cryoprotected and sectioned, and sections were stained for the detection of GFP as described below.

**Slice Culture and Time-Lapse Imaging**  
V1 slices were prepared from ferrets aged P14 (n = 4)/P18 (n = 4) and maintained in culture as described previously (Borrell and Callaway 2002). Briefly, brains were dissected out in ice-cold N-2-hydroxyethylpiperazin-N’-2-ethane sulfonic acid (HEPES) buffered artificial cerebral spinal fluid (140 mM NaCl, 5 mM KCl, 1 mM MgCl2, 24 mM Na-glucose, 10 mM HEPES, 1 mM CaCl2, and pH 7.4), the occipital pole of each hemisphere was blocked, and tissue blocks were sliced at 400 µm thickness using a homemade “egg slicer.” Selected slices were placed onto tissue culture inserts (Millipore, 0.4 µm pore size) and were cultured in a 37 °C, 5% CO2 incubator with 1 mL of incubation medium (50% basal medium of eagle [BME], 25% flanks’ balanced salt solution, 25% normal horse serum, 2 mM glutamine, 0.45% Na-glucose, 10 mM HEPES, 1% Pen/Strep, all from GIBCO-Invitrogen). One hour later, slices received various injections of Ad-WGA-myc or Ad-GFP (3-6 nL/injection site-2) and were sacrificed at P16 (n = 22) or P18 (n = 1). The DNA construct used to label transfected cells was pCAGGS-EGFP. Ferrets aged P1 (n = 6) were deeply anesthetized with isoflurane and placed in a stereotronic micromanipulator. Approximately 2 µL of DNA solution was injected using a pulled glass micropipette into the lateral ventricle in a region subjacent to the prospective V1. A tweezers-type electrode (CUY661-3X7; NEPA Gene, Chiba, Japan) was then used to electroporate the DNA into the VZ of the prospective V1. Square electric pulses (60 V, 50 ms) were passed 5 times at 1-s intervals using an electroporator (CUY21E; NEPA Gene). The skin was suture-secured, and the animals were allowed to recover from anesthesia before being placed back with the dam. Electroporated ferrets were transcardially perfused with 4% paraformaldehyde at P6 (n = 4) or P55 (n = 2), their brains were cryoprotected and sectioned, and sections were stained for the detection of GFP as described below.

**Bromodeoxyuridine Injections and Immunohistochemistry**  
Bromodeoxyuridine (BrdU) (SIGMA, St. Louis, MO) was administered intraperitoneally at 50 mg/kg body weight. BrdU was injected at P0.5 and P1 (n = 4) or P6.5 and P7 (n = 2); all animals received injections of Ad in the CP at P14 and were sacrificed at P16 by perfusion with paraformaldehyde. Single and double immunostainings were performed on 50-µm-thick free-floating brain sections or whole-mount slice cultures. Tissue was blocked and incubated in primary antibodies overnight at 4 °C. Primary antibodies and dilutions were as follows: anti-myc (Santa Cruz [Santa Cruz, CA], 1:300); anti-TU-C4, anti-vimentin, and anti-NeuN (all from Chemicon [Temecula, CA], 1:300); TUJ-1 (Covance [Berkeley, CA], 1:500); anti-glial fibrillary acidic protein (GFAP) (DAKO [Carpinteria, CA], 1:500); anti-Hu (1:40) and anti-GFP (1:500, Molecular Probes); anti-BrdU (Accurate [Webstbury, NY], 1:100); anti-doublecortin (DCX) (1:100) and anti-PSA-NCAM (1:1000). Sections were then incubated with appropriate fluorescently conjugated secondary antibodies (Chemicon; Jackson IR, West Grove, PA) and counterstained with 4’-6-diamidino-2-phenylindole (DAPI, SIGMA) when appropriate.

**Definition of Layer Boundaries**  
Two different criteria were used in this study to determine the exact location of borders between layers: changes in cell density and distribution of cells in S-phase of the mitotic cycle (see Supplementary Material online). The IZ was defined as the cell-sparse layer contained between the cell-dense CP and the cell-dense SVZ (Bayer and Altman 1991; Brazel and others 2003). The border between the IZ and the SVZ was also defined by the characteristic accumulation of S-phase cells along this boundary (Supplementary Fig. 1 online).

**Cell Measurements and Quantification**  
The distance from the cell body of Ad-labeled cells to the pial surface was measured from all sections containing Ad-labeled cells in a single brain (1-in-7 series) using Neurolucida (Microbrightfield). Distances were measured taking into account the curvature of the cortex. Ad-labeled IZ cells in slice cultures were reconstructed using Neurolucida or by photomontage of overlapping confocal microscopy images using Adobe Photoshop. Quantification of cells containing for specific markers...
was performed by confocal microscopy (Bio-Rad, Hercules, CA) through a 40× objective, obtaining Z-series of 0.5- to 1-μm-thick optical sections. In each case, cells were sampled randomly from 3 to 4 sections from each of 2–3 different animals. For quantification of BrdU labeling, all Ad+ cells in the IZ of selected brains (1-in-7 series) were examined under a 65× objective and classified as follows: cells whose nuclei were completely filled with dense staining were designated strong labeling; cells clearly containing the BrdU signal in the nucleus above background, but with only a few corpuscles of staining, were designated weak labeling; intermediate levels of nuclear staining were designated medium labeling (see Fig. 7 for examples).

**Results**

**Cells with Migratory Morphology in the Cortical IZ Display a Long Apical Process**

Birth-dating analyses in ferret have shown that V1 neurons committed to layer 2/3 are born between P1 and P12 and are still migrating through the IZ between P14 and P21 (Jackson and others 1989). Therefore we decided to study late cortical migration using ferrets aged P14–P18. In order to identify putative migrating neurons with a pia-contacting apical process and distinguish them from neurons with a short leading process, we used methods of labeling that are selectively biased toward cells with processes extended up to the cortical surface. In previous studies involving small DiI injections on the surface of the ferret cerebral cortex, cells in the IZ have been retrogradely labeled through their long apical processes and have been shown to undergo migration to superficial aspects of the cortex (Voigt 1989). We started our study by performing similar injections of DiI crystals on the surface of V1 of P16 ferrets. In agreement with previous observations, we found that DiI injections retrogradely labeled a number of cells distributed throughout the IZ, SVZ, and VZ, which characteristically displayed long and fine apical processes (Fig. 1A,B). At this late stage of cortical development both the VZ and SVZ are mostly proliferative layers, whereas the IZ is a layer where cortical cells are believed to be exclusively migrating (Bayer and Altman 1991; Haubensak and others 2004; Noctor and others 2004). Thus, for subsequent studies we focused on cells whose somata were located in the IZ.

DiI labeling is a nonspecific tracing method that reveals the morphology of any cell type and process that is in contact with the injection site, including glia and neurons. Accordingly, all cells observed in the IZ and their apical process were immersed within thick bundles of intimately associated, DiI-labeled processes, likely including fibers of radial glia and axons (Fig. 1B). Therefore, we decided to design an alternative and more specific, but still biased, labeling method. Taking advantage of the fact that adenoviruses (Ad) have the ability to retrogradely infect differentiated neurons through their processes, which transport virions back to the cell nucleus (Terashima and others 1997), we decided to use Ad to retrogradely infect putative translocating neurons located in the IZ. By means of small Ad injections in the CP of V1, cells in the IZ would be infected through their apical processes. After in vivo injection of Ad in the CP of P14 ferret V1, a population of cells was revealed whose cell bodies were distributed continuously through the depth of the IZ, SVZ, and VZ, in most cases several millimeters away along a radial trajectory from the injection site (Fig. 1C,E). In contrast to the DiI labeling pattern, Ad labeling revealed only individualized cells, whose soma and apical process were unmistakably identifiable (Fig. 1D). In the IZ, cells had an oval

Figure 1. Retrograde labeling of cells in the IZ of ferret V1. (A, C) Patterns of labeling at P16 after injection of DiI (A, red) or Ad (C, red) in the CP (asterisk) of V1. Dashed lines indicate borders between layers. Samples were counterstained with DAPI (blue). Scale bar = 1 mm. (B, D) Enlarged images of the boxed areas in (A) and (C), respectively. Many labeled cells have typical migratory morphology, including elongated or oval cell bodies (B, arrows) and a single, long apical process. Only cells with this morphology are labeled by Ad (D), whereas DiI also labels many other processes (B), likely including radial glia and axons. Scale bar = 20 μm. (E) Cumulative plot showing the distribution of Ad+ cells with respect to the pial surface following an injection into the CP (n = 1140). Because the cell measurements plotted were obtained from multiple sections, the distance from the pial surface to the borders between layers varied between sections. The range of the position of layer borders is represented with gray bars.
or elongated cell body typical of migrating cells (Fig. 1D), which occasionally contained several fine processes (not shown). Each cell also typically displayed a single long apical process extended toward the injection site. These apical processes never displayed any lateral extensions or protrusions, like those typically observed in the fibers of radial glia (Rakic 1972; Voigt 1989; deAzevedo and others 2003). Occasionally, cells were seen displaying an apical process up to 500 μm in length within a single, 50-μm-thick section. In one of the specimens studied, we calculated that out of all the Ad-labeled cells found in the IZ, 65.7% (389 of 592 cells) were located at least 2000 μm away from the pial surface (Fig. 1E).

These observations strengthened the initial hypothesis that this population of radially oriented cells should have been labeled in deep layers after superficial injections of Ad through very long apical processes, extending from the cell body up to the cortical surface, in the vicinity of the injection site. If this was the case, it would mean that a large proportion of the Ad-labeled cells observed within the IZ would have an apical process 2000–3200 μm long (Fig. 1E). To confirm that Ad-labeled cells contain such processes, we prepared 400-μm-thick slice cultures from P14-P18 ferret V1 and injected Ad in the superficial aspects of the cortex (see Materials and Methods). These injections resulted again in labeling of cells in the IZ (Fig. 2A, boxed area; Fig. 2D) that were morphologically indistinguishable from the cells observed in the IZ after virus injection in vivo (Figs. 1D and 2B). Each of these cells displayed a simple apical process containing no lateral varicosities that was extraordinarily long (2000–2400 μm in many cases) and that could be followed into the injection site (Fig. 2). Moreover, the apical process of these cells was not simply extended straight away from the cell body to the closest point of the pial surface, or to the closest Ad injection site, but always followed the curvature of the cortical folding (Fig. 2A,C,D). Thus, Ad labels a population of cells distributed through the IZ that have a morphology of migrating cells, each with an apical process extended all the way to the CP and exceeding 2 mm in length in numerous cases.

**Ad-Labeled Cells with Migrating Morphology Are Neurons**

Next, we wanted to distinguish whether the Ad-labeled cells located in the IZ are migrating neurons or, as shown previously, radial glial cells translocating superficially to differentiate into astrocytes (Voigt 1989; Noctor and others 2004). Two different markers specific for radial glia in the ferret were tested, and their stains were analyzed by 3-dimensional confocal microscopic reconstructions: vimentin is specific for radial glial cells, and GFAP is specific for radial glia and differentiated astrocytes (Engel and Muller 1989; Voigt 1989). Staining with these markers revealed that less than 5% of Ad-labeled cells in the IZ were positive for vimentin or GFAP (Fig. 3, Table 1). Because it is possible that vimentin+ and GFAP+ cells could be 2 different glial cell populations, expressing only one of the glial-specific markers but not the other, we tested the overlap of these markers in unmanipulated ferrets of the same age. The majority of GFAP+ radial processes analyzed (90.1%, 128/142 processes) also contained vimentin (not shown), indicating that GFAP is mostly present in a subset of vimentin+ radial glial processes. Therefore, the vast majority of Ad-labeled cells (>95%) did not appear to be radial glial cells.

We next tested whether the remaining majority of Ad-labeled cells are migrating neurons. Several neuron-specific markers were tested, and stains were analyzed by 3-dimensional confocal reconstructions: TUC4, DCX, NeuN, Hu, class III β-tubulin, and PSA-NCAM. TUC4 is a protein expressed specifically by neurons beginning right after they become postmitotic (Minturn and others 1995). It is important to note that in mature ferret neurons, the TUC4 antibody staining appears reticulated in the
cytoplasm, around the cell nucleus and in the apical dendrite (Fig. 4). Correspondingly, in immature migrating cells of the IZ, which have a very small cytoplasm, the TUC4 staining appears consistently in all cases as a bright, small and elongated corpuscle at the base of the apical process, right above the cell nucleus (Fig. 3). Quantification of stains revealed that the vast majority of Ad-labeled cells in the IZ (96.5%) were positive for TUC4. Also, a majority of cells (74.5%) were positive for DCX, a microtubule-associated protein expressed by migrating neurons (Francis and others 1999; Gleson and others 1999; Fig. 3, Table 1). In contrast, cells in the IZ were rarely observed to be positive for either NeuN or Hu (Table 1). This is consistent with the notion that HuC (a member of the Hu family of genes) and NeuN are expressed in the neocortex by postmigratory/mature neurons (Okano and Darnell 1997; Seki 2002; van Praag and others 2002). Specific antibodies against class III β-tubulin and PSA-NCAM were also tested; however, these antibodies stained the IZ very strongly and homogeneously (as previously described; O’Rourke and others 1995), making it impossible to draw conclusions about the immunoreactivity of individual cells for either of the markers.

Next we asked whether Ad+ cells in the IZ may derive and inherit their long apical process from SVZ/VZ radial glial cells. The observation that CP injections of Ad produce infection of cells located not only in the IZ but also in the SVZ and VZ (Fig. 1) suggests that Ad+ IZ neurons may not grow the apical process on their own but rather they may inherit it from parental radial glial cells located deeper, in the proliferative layers. If this hypothesis is correct, we might expect to find SVZ/VZ Ad+ cells that are positive for radial glial markers. In order to address this question we extended the analysis of glial and neuronal marker expression to cell populations in the SVZ.

Table 1

<table>
<thead>
<tr>
<th>Marker</th>
<th>IZ cells</th>
<th>SVZ cells</th>
<th>VZ cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vimentin</td>
<td>3.6% (2/56)</td>
<td>9% (3/33)</td>
<td>9% (1/11)</td>
</tr>
<tr>
<td>GFAP</td>
<td>4.5% (3/66)</td>
<td>3% (1/33)</td>
<td>0% (0/15)</td>
</tr>
<tr>
<td>TUC4</td>
<td>96.5% (55/57)</td>
<td>89% (25/28)</td>
<td>77% (10/13)</td>
</tr>
<tr>
<td>DCX</td>
<td>74.5% (41/55)</td>
<td>34% (11/32)</td>
<td>56% (9/16)</td>
</tr>
<tr>
<td>NeuN</td>
<td>8.0% (5/62)</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Hu</td>
<td>1.6% (1/62)</td>
<td>—</td>
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Note: Percentage of Ad+ IZ, SVZ, and VZ cells, labeled by Ad injections in the CP; immunopositive for each of the markers. NeuN and Hu were not tested for SVZ and VZ because these layers are negative for those markers. Absolute numbers of positive cells versus cells analyzed are indicated in parentheses.
and VZ. Contrary to our predictions, less than 10% of Ad+ cells in the SVZ or VZ were positive for the radial glial markers vimentin or GFAP (Table 1), whereas a substantial proportion expressed DCX (34% of SVZ cells, and 56% of VZ cells) and the majority were positive for TUC4 (89% and 77%, respectively; Table 1). These results suggest that cells positive for neuronal markers and displaying migratory morphology may start their radial migration in the SVZ and VZ, where they already contain a long apical process reaching the CP. However, our stains are not informative about the origin of Ad+ cells because Ad-labeled cells across all layers seem to be part of the same population of immature neurons.

Previous studies have shown that infection of neurons in culture with replication-incompetent Ad can affect the expression levels of certain genes within a few days (Easton and others 1998). To directly test the possibility that infection with Ad might downregulate the expression of vimentin or GFAP in vivo, we analyzed the immunoreactivity for these 2 markers in cells heavily labeled with Ad and displaying typical glial morphology. To test for changes in vimentin immunoreactivity in radial glial cells, Ad was injected into the SVZ/VZ of V1 in P14 ferrets. Two days later numerous cells in the SVZ/VZ expressed the reporter gene. Each of these cells displayed a long apical process oriented radially toward the pial surface, resembling radial glial cells. Double staining analyses revealed that 97% of the Ad+ radial processes in the IZ were strongly immunoreactive for vimentin (Fig. 4; Table 2). Next we examined cells with astrocytic morphology that could be seen near the injection site in the CP: 96% of these cells were strongly immunoreactive for GFAP (Fig. 4; Table 2). Thus, antibody detection of neither GFAP nor vimentin is prevented in cortical glial cells by infection with our Ad. Interestingly, these experiments also revealed that radial glial cells appear to express the transgene delivered by Ad only when the virus is applied to the vicinity of the cell body (SVZ/VZ). This indicates that Ad is an excellent tool to deliver genes selectively to translocating neurons but not radial glia when applied by remote injections.

Figure 4. Ad infection does not alter expression of glial or neuronal markers. Confocal micrographs (single 1-μm-thick optical sections) showing examples of identified Ad+ radial glial fibers and astrocytes (red, 2 left columns) and pyramidal neurons in the CP (red, 2 right columns). Radial glial cells were labeled by Ad injections in the SVZ. Astrocytes and pyramids were labeled by injections in the CP. Immunostaining patterns for the different markers are shown in green. Bottom row shows merged images. Ad-infected radial glia and astrocytes are strongly immunoreactive for the glial markers vimentin (Vim) and GFAP, respectively. Infected neurons are immunoreactive for the neuronal markers TUC4 (corpuscles in the apical dendrite and cell body) and DCX. Scale bar = 10 μm.
Table 2
Marker expression in soma-labeled Ad+ cells

<table>
<thead>
<tr>
<th>Marker</th>
<th>Radial glial fibers</th>
<th>Astrocytes</th>
<th>Pyramidal neurons</th>
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<tbody>
<tr>
<td>Vimentin</td>
<td>97% (206/212)</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>GFAP</td>
<td>---</td>
<td>96% (77/80)</td>
<td>---</td>
</tr>
<tr>
<td>TUC4</td>
<td>---</td>
<td>---</td>
<td>100% (84/84)</td>
</tr>
<tr>
<td>DCX</td>
<td>---</td>
<td>---</td>
<td>87% (67/77)</td>
</tr>
<tr>
<td>NeuN</td>
<td>---</td>
<td>---</td>
<td>100% (87/87)</td>
</tr>
<tr>
<td>Hu</td>
<td>---</td>
<td>---</td>
<td>97% (72/74)</td>
</tr>
</tbody>
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Note: First column: percentage of Ad+ radial glial fibers, labeled by Ad injections in the SVZ, positive for the radial glial marker vimentin; second column: percentage of Ad+ astrocytes, labeled by Ad injections in the CP, immunopositive for the glial marker GFAP; third column: percentage of Ad+ pyramidal neurons, labeled by Ad injections in CP, immunopositive for neuronal markers (TUC4, DCX, NeuN, Hu). Absolute numbers of positive cells versus cells analyzed are indicated in parentheses.

Complementarily, we investigated whether infection with Ad could significantly modify the levels of expression of the neuron-specific markers tested. After in vivo injections of Ad in V1 of P14 ferrets, layer 2/3 and layer 5 cortical pyramidal cells were retrogradely labeled, expressing low levels of the reporter gene (Fig. 4). We tested whether these well-differentiated pyramidal neurons presented any change in the expression of neuronal markers after Ad infection. We found that all infected pyramidal cells in V1 stained for TUC4 and NeuN, 97% stained for Hu, and 87% could be identified as being immunoreactive for DCX (Fig. 4, Table 2). Also, the patterns of immunostaining with the different antibodies were identical to those observed in cells of the IZ (compare Figs. 3 and 4). We conclude that the immunoreactivity for neither the neuron-specific nor the glia-specific markers used in this study was altered by infection with Ad.

Thus, our results so far demonstrate the existence of a population of cells in the IZ of the developing ferret visual cortex that morphologically resemble migrating cells, have very long apical processes extended to the CP, and express neuron-specific but not glia-specific proteins. According to current views of cortical development, these traits strongly suggest the existence of neurons migrating radially for very long distances by somal translocation along a pia-contacting apical process. From here on we will refer to this type of cells as long-distance translocating neurons.

Neurons Migrate by Long-Distance Somal Translocation In Vitro
To confirm that these IZ neurons do in fact migrate, we performed time-lapse imaging experiments in slice cultures. Slices of ferret V1 were obtained, and injections of Ad were delivered into the CP to visualize putative long-distance translocating neurons in the IZ (see Fig. 2A). Injected slices were incubated for 24 h to allow for gene expression and onset of GFP fluorescence. Twenty-four hours after Ad injection the vast majority of Ad-labeled cells in the IZ displayed a very long apical process (92.6%; 486/525 cells, 15 slices, 5 ferrets; Fig. 2D). Time-lapse images were obtained from some of these cells out of 4 selected slices (4 ferrets; see Supplementary Movie online).

Twenty cells were recorded translocating the cell body radially along their apical process and toward the CP. The distance traveled in a single experiment varied between cells, ranging from 15 to 85 μm (38.6 ± 4.7 μm, mean ± SEM), and the cells also varied in their average migration speed from 1 to 16 μm/h (3.9 ± 0.8 μm/h). Migrating cells appeared to display 3 different behaviors (Fig. 5D): most cells (n = 10) were stationary at the beginning of the recording and then migrated for some time (2–3 h) before stopping for the remaining of the recording (Fig. 5A–C, cell 2; Fig. 5D). Other cells (n = 5) migrated from the beginning of the recording period but stopped after a few hours (11–28 h; Fig. 5A–C, cell 1; Fig. 5D). Finally, a few cells (n = 2) were stationary at the beginning of the recording and only later started migrating until the end of the recording (6 to 36 h; Fig. 5D). In 3 additional cases a combination of these patterns of migration was observed: cells were stationary at the beginning of the recording, migrated for a period of time (3–16 h), stopped for a few hours, and migrated again until coming to a halt (n = 2; Fig. 5D) or until the end of the experiment (n = 1; Fig. 5D). Taken together, our results demonstrate that Ad-labeled neurons in the IZ are in the process of radial migration. Furthermore, all these migration patterns can be reconciled in one unique pattern: cells translocate the soma within their long apical process in a discontinuous or saltatory fashion, alternating periods of advancement with periods of stall.

The technical requirements of the imaging experiments did not allow us to monitor the full extent and morphology of the cells during migration. This restriction prevented us from being able to discern between 2 possibilities: did these cells maintain the exceptionally long apical process fully extended during migration, or did these cells retract the apical process they were displaying at the beginning of the experiment (see discussion earlier) to proceed radial migration with a short leading process? To resolve this dichotomy we performed a full reconstruction of all the cells observed migrating during the imaging experiments by means of 3-dimensional confocal microscopy. In every case, cells that had been seen migrating radially in the IZ still exhibited an apical process of more than 800 μm in length (up to the injection site) after the 3-day imaging period (Fig. 5A′,A″). Therefore, our observations indicate that the Ad-labeled neurons migrate radially by translocating the cell soma within the long apical process while maintaining it fully extended to the CP at all times: they undergo long-distance somal translocation. This conclusion, based partially on our in vitro findings, is further supported by the aforementioned in vivo observation that cells retrogradely labeled after a single Ad injection in the CP are distributed continuously through the IZ (Fig. 1E). If migrating cells were to retract their apical process at any given point along their trajectory (or immediately after being born) and then migrate using a short leading process, the cells in the IZ labeled after a superficial injection of Ad would show a biphasic distribution: deep cells still containing a long apical process, and superficial cells infected through their short leading process, but no labeled cells would be found in intermediate positions. Therefore, the continuous distribution of Ad-labeled neurons through the IZ observed in vivo (Fig. 1E) is further indicative of radial migration by true long-distance somal translocation.

The Apical Process of Ad-Labeled Long-Distance Translocating Neurons Terminates in Multiple End-Feet at the Pial Surface
Rodent studies have proposed that migrating cortical neurons with a pia-contacting process do not grow such apical process de novo but rather inherit it from the parental radial glial cells (Miyata and others 2001). If this is the case, the apical process of
long-distance translocating neurons should terminate with multiple end-feet at the pial surface, which are the characteristic terminations of radial glia. Thus, we wondered if the long radial process of Ad-labeled cells makes contact with the pial surface, and what is its detailed morphology. Unfortunately and due to the labeling method used, the apical portion of Ad-labeled cells was immersed within a dense group of labeled cells right at the injection site, which prevented us from distinguishing the apical terminations (Figs. 1C, 2D). On a few occasions, however, the full morphology of individual Ad-labeled cells could be distinguished. In these cases, the long apical process of Ad-labeled cells branched near the CP-marginal zone border, and each of the branches terminated in an end-foot apposed to the pial surface (Fig. 6A). These cells were always positive for...

Figure 5. Retrogradely labeled cells in the IZ migrate radially in vitro by somal translocation. (A) Time-lapse image sequence from the IZ demonstrating pia-directed movement of 2 cells (1 and 2) compared with a stationary third cell (3). t = elapsed time in h:min. Arrowheads point at the apical process of cell 1. Scale bar = 20 μm. (A’, A”) Confocal microscopic reconstructions of a portion of cell 1 (A’) and cell 2 (A”) at the end of the imaging experiment (t = 66:30), demonstrating that migrating cells maintain their long apical process during migration (arrowheads). Arrows point at the cell soma. Dashed boxes indicate the corresponding area visible in A at t = 66:30. Scale bar = 100 μm. (B, C) Quantification of displacement (B) and speed (C) of identified cells in (A). Cell 1 (green trace) migrated slowly for the first 7 h of imaging, and then it accelerated toward the pia (upward) along its own apical process (arrowheads in A), translocating 70 μm from t = 7:00 to t = 28:00 at 3.3 μm/h on average, with a peak of 15 μm/h at t = 11:30. Cell 2 (red trace) was stationary at the beginning of the experiment and then translocated 23 μm from t = 5:00 to t = 9:00, at speeds of 5–10 μm/h. (D) Diagram illustrating the types of behavior of migrating IZ cells during time-lapse imaging experiments: cells either 1) migrated from the beginning of the experiment (green bar) and stopped after a few hours (red bar), 2) showed a period of migration between periods of stall, or 3) after being stationary for a few hours migrated until the end of the experiment. Additionally, 4) two cells started and stopped migration twice during the experiment, and 5) one cell exhibited a combination of behaviors 2) and 3). Cells 1 and 2 shown above in this figure represent typical examples of migratory behaviors type 1) and type 2), respectively.
TUC4 (Fig. 6B,B'), confirming their neuronal identity. Very similar morphologies were also observed in cortical cells labeled by in vivo electroporation of VZ progenitors (Fig. 6C; see discussion later). These observations suggest that the long apical process of Ad-labeled cells may derive from radial glial fibers (Miyata and others 2001).

Radially Migrating Cells with a Short Leading Process Are Also Present in the Developing Ferret Cortex

Studies in rodents, carnivores, primates, and humans have either demonstrated or strongly suggested the existence of radially migrating cortical neurons with a short leading process (Rakic 1972; Sidman and Rakic 1973; O'Rourke and others 1992; Nadarajah and others 2001; Tabata and Nakajima 2003; Noctor and others 2004). Once we had demonstrated the existence of cortical neurons undergoing long-distance somal translocation during late ferret corticogenesis, we wondered whether migrating cells with a short leading process also exist at this stage of development. Cortical progenitors were labeled by electroporation of GFP-encoding DNA into the VZ of newborn ferrets, as described previously in rodents (Tabata and Nakajima 2001; Borrell and others 2005). Seven days after electroporation (by P8) numerous GFP+ cells were seen dispersed along the entire depth of the CP and IZ (Fig. 6D). The majority of these cells displayed a short and thick leading process tipped with a small growth cone-like structure, and a very thin axon-like trailing process.

Figure 6. Long and short translocating cells coexist in the early postnatal ferret cortex. (A) Full morphology of a long translocating Ad-infected cell. The soma of this cell is in the CP, and the apical process branches at the border between CP and marginal zone (MZ), terminating at the pial surface in multiple end-feet (arrowhead). Scale bar = 10 μm. (B, B') Detail of the translocating cell in (A) (boxed area) double stained for the early neuronal marker TUC4 (green label). Note the presence of TUC4+ corpuscles at the base of the apical process of this Ad+ cell (arrowheads), besides other label belonging to nearby neurons. Scale bar = 10 μm. (C) Example of a GFP+ cell at P8 after electroporation of VZ at P1. The apical process of this cell is also branched and terminates in multiple end-feet (solid arrowheads), presumably contacting the pial surface. The cell body is indicated by an arrow and the trailing process by an open arrowhead. Scale bar = 10 μm. (D, E) Images showing the abundant presence of putative short translocating cells in the IZ and CP in the prospective V1 of a P8 ferret. The image in (E) is a detail of the upper IZ area boxed in (D). These cells labeled by electroporation of the VZ display distinctive morphologies (E), including a short leading process capped with a small growth cone-like structure (solid arrowhead) and a very thin trailing process (open arrowhead). The cell soma is indicated by an arrow and located in the IZ. Scale bars: D = 150 μm; E = 25 μm. (F) Layer 2/3 pyramidal neurons at P35, which were labeled by electroporation of the VZ at P1. At least some proportion of these neurons migrated radially through the IZ, as shown in (E). Scale bar = 50 μm.
process (Fig. 6E). When sibling ferrets treated identically were allowed to develop for another 4 weeks, GFP+ layer 2/3 pyramids were observed in the CP (Fig. 6F). These results strongly suggest the existence of radially migrating neurons with a short leading process late in the cortical development of the ferret.

**Ad-Labeled Long-Distance Translocating Neurons Are Committed to Layer 2/3**

To further characterize the population of long-distance translocating neurons, we next investigated their laminar fate. Contrary to previous analyses in rat brain slices (Noctor and others 2004), the cells imaged in our ferret slices could not be followed for the entire extent of their migration phase mainly because their movement is so slow that radial migration requires 2–3 weeks to be completed in vivo in most cases (Jackson and others 1989). However, previous birth-dating and cell-transplantation studies performed in ferret V1 indicate that by P14–P16 only neurons committed to layer 2/3 are still migrating, and these cells become postmitotic between P1 and P12 (Jackson and others 1989; Desai and McConnell 2000). Therefore, because the populations of cells we observe in the IZ at P14–P16 are migrating neurons, these cells should be committed to layer 2/3, and thus they must have become postmitotic between P1 and P12. We determined the birth date of Ad-labeled cells by injecting BrdU in ferret kits at P0.5–P1 or P6.5–P7, injecting Ad in the CP at P14 and sacrificing them at P16 (Fig. 7A). Ad+/BrdU+ cells in the IZ were counted and classified according to their level of BrdU labeling (Fig. 7B). Only a small minority of Ad-labeled cells were strongly labeled with BrdU at P0.5–P1 (0.37%), whereas a higher percentage contained medium or weak BrdU label (18.2%; Fig. 7C). In contrast, a larger proportion of Ad-labeled cells were medium or strongly labeled with BrdU at P6.5–P7 (16.1%), together with other cells being weakly labeled (Fig. 7C). These experiments therefore revealed that some Ad-labeled neurons in the IZ become postmitotic during the first postnatal week, suggestive of their commitment to migrate to and become part of layer 2/3 of the V1.

**Discussion**

The arrangement of cortical neurons into distinct layers is a central step in the development of a functional cerebral cortex. In humans, a number of genetic mutations are known to cause abnormal neuronal migration in the cerebral cortex, which will in turn be responsible for severe alterations in brain function, including mental retardation and epilepsy (Ross and Walsh 2001; Gaitanis and Walsh 2004). Real-time imaging studies performed in the lyssencephalic brain of rodents have profoundly changed our view of the cellular changes occurring during neuronal migration. These studies have identified an unpredicted variety of morphologies that cortical neurons can display during radial migration: neurons with a long pia-contacting process, with a short apical leading process, or with multiple short processes (Anton and others 1997; Nadarajah and others 2001, 2003; Nadarajah and Parnavelas 2002; Tabata and Nakajima 2003; Kriegstein and Noctor 2004; Noctor and others 2004).

Here we have studied neuronal migration in the developing gyrencephalic cortex of the ferret. We demonstrate that in the latest period of cortical development some neurons migrate radially by moving the cell nucleus along their own pia-contacting apical process, which we find can exceed 3 mm in length. We also demonstrate the coexistence of long-distance translocating neurons together with neurons migrating with a short leading process (previously also referred to as locomoting cells; Nadarajah and others 2001; Nadarajah and Parnavelas 2002; Gupta and others 2003; Marin and Rubenstein 2003; Tabata and Nakajima 2003; Hatanaka and others 2004; Noctor and others 2004). This result was not predicted based on classical developmental studies in primates or on recent work using time-lapse imaging in rodent cortex (Rakic 1972; Rakic and others 1974; Nadarajah and others 2001, 2003; Noctor and others 2004). It is possible that the use of a nonretractable

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**Figure 7.** Some Ad+ neurons in the IZ become postmitotic during the first postnatal week. (A) Experimental design for birth dating of Ad+ neurons. Two doses of BrdU (50 mg/kg) were administered 12 h apart starting at P0.5 or at P6.5. Ad was then injected into the CP of V1 at P14, and animals were sacrificed for analysis at P16. (B) Examples of Ad+/BrdU+ cells in the IZ at P16 after BrdU administration during the first postnatal week. The top row shows examples of nuclear BrdU stains (green), and the bottom row shows those same images merged with the corresponding cytoplasmic Ad stains (red). Ad-labeled IZ cells were classified according to the intensity of their BrdU staining, as containing weak, medium, or strong levels of BrdU, as indicated. Scale bar = 10 μm. (C) Histogram showing the percentage of Ad+ cells in the IZ displaying weak, medium, or strong levels of BrdU labeling at P16, after injection of BrdU at P0.5–P1 (n = 810) or P6.5–P7 (n = 760). In most cases weak levels of BrdU are likely to reflect multiple cell divisions after an initial strong BrdU intake.
pia-contacting process for long-distance migrations may be the result of the increased complexity of the process of migration for the formation of a gyrated cortex.

**Long-Distance Somal Translocation of Neurons in a Gyrencephalic Cortex**

Time-lapse imaging studies have shown that in rodents radial migration of neurons with a pia-contacting process occurs early in development, when the cerebral cortex is thin. Later in development, when the cerebral cortex is much thicker, neurons migrating with a short leading process seem to predominate (Miyata and others 2001; Nadarajah and others 2001, 2003; Tamamaki and others 2001; Noctor and others 2004). Indeed, the studies that have so far addressed the question of late radial migration in gyrencephalic cortices conclude that migrating neurons display a short leading process and depend on radial glia (Rakic 1972, 2003; Sidman and Rakic 1973; Rakic and others 1974). By using a labeling method designed to bias toward potential cells with long-processes we have found radially migrating neurons displaying a pia-directed process of up to 3 mm in length (Book and Morest 1990; Nadarajah and others 2001). Although it has been suggested that somal translocation within a pia-contacting process is a mechanism independent of the radial glial scaffold, our results cannot exclude the fact that long-distance translocating neurons may require radial glial fibers for their migration. Cortical migration following a long pia-contacting apical process was already proposed by Morest (1970), after his studies in the opossum pouch young using the Golgi staining technique. A similar mechanism has also been demonstrated for basal pontine neurons, which migrate tangentially from the dorsal rhombencephalic neuripithelium to the ventral midline using a previously extended long apical process (Yee and others 1999).

**Coexistence of Migrating Neurons with a Long Pia-Contacting Process and with a Short Leading Process**

Rodent studies have concluded that radial migration by somal translocation within a pia-contacting process is predominant in the early stages of cortical development, whereas migrating cells with a short leading process predominate later in development, when the radial distance to be traveled is much longer (Nadarajah and others 2001; Nadarajah and Parnavelas 2002; Tabata and Nakajima 2003; Noctor and others 2004). Here we show that in the ferret cerebral cortex neurons also migrate by translocation within a long apical process late in development. This brings up the question: Is this the only mode of migration in the ferret? The answer is probably no. By using a labeling technique different than Ad infection, we were also able to label numerous migrating neurons with a short leading process in the early postnatal cerebral cortex. Therefore, our data suggest that in the postnatal ferret, migrating neurons with long pia-contacting and short apical processes coexist and converge in the CP to constitute layer 2/3.

**Kinetics of Long-Distance Somal Translocation**

Studies in early embryonic rodent cortex, where the radial distance to migrate is short, have shown that somal translocation along a pia-contacting apical process is a continuous and fast event, with average speeds of 60 μm/h (Nadarajah and others 2001). In contrast, our time-lapse imaging analysis reveals that long-distance translocating neurons migrate in a saltatory fashion along their trajectory. Given the difference between our observations and previous analyses in rodents (Nadarajah and others 2001), it could be argued that cells in our slices stop migrating simply due to unhealthy in vitro conditions. However, we observed cells not only stopping but also starting their migration at virtually any point during the 2–3 day recording period, which is a good indication that slice health was not an issue for cell migration throughout the extent of these experiments. Therefore, saltatory movement seems to be a characteristic of long-distance somal translocation of ferret cortical neurons.

Another significant difference between mouse and ferret somal translocation is the average speed of displacement: 4 μm/h in ferret versus 60 μm/h in mouse. Our results are consistent with previous BrdU-labeling studies, which have shown that many of the neurons born in ferret visual cortex during the first postnatal week (such as those in our study) migrate extremely slowly, requiring 2–4 weeks to migrate from the VZ/SVZ to the CP (Jackson and others 1989). This is equivalent to an average rate of 4.5–9 μm/h of continuous displacement, which seems consistent with our observations. However, although our time-lapse analyses reveal that long-distance translocating neurons migrate in vitro at 4 μm/h, the resting periods are not included. The time required for long-distance translocating neurons to travel the full distance in vitro would thus be much longer than 2–3 weeks. We have not analyzed the origin of these differences in any depth, but it is likely that they are due to intrinsic differences between the biological conditions in vitro and in vivo, as has been proposed to occur in mouse studies (Tabata and Nakajima 2003). Another possibility is that these long-distance translocating neurons never really make it to the CP but rather become other types of neurons (i.e. subplate cells). However, our birth-dating analysis included here, together with previous birth-dating studies in ferret (Jackson and others 1989; Noctor and others 1997), unmistakably identify Ad-labeled translocating neurons as layer 2/3 neurons.

**Lineage Relationship between Long-Distance Translocating Neurons and Radial Glia**

Radial glial cells have been shown to be the source of a large percentage of the cortical neurons during development (Malatesta and others 2000, 2003; Noctor and others 2002; Anthony and others 2004). This appears to result in cells transiently displaying both neuronal and glial features. For example, the neuron-specific promoter Tα1 can drive GFP expression in cortical radial fibers (Hatanaka and Murakami 2002). Also, neuronal markers (Hu) and radial glial markers (RC2 antigen or nestin) can be found simultaneously expressed in translocating cells (Miyata and others 2001). Although this has not been observed in human fetuses (deAzevedo and others 2003), neither did our marker expression analysis reveal an overlap between TUC4+ and vimentin/GFAP+ in Ad-labeled cells, the previous reports strongly support the notion that the long-distance translocating neurons we observe in ferret may derive directly from mitotic radial glial cells, likely inheriting the pia-connected radial process after the last mitotic division (Britts and others 1995; Miyata and others 2001; Campbell and Gotz 2002). Unfortunately, our marker staining data of Ad+ cells in the SVZ/VZ neither confirm nor disprove this hypothesis. However, the identification of Ad+ cells with a long apical
process terminating in multiple end-feet at the pial surface does provide further support for the hypothesis of a role of radial glia as a provider of the long-spanning apical process of long-distance translocating neurons. If this is indeed the case, the prediction is that the mother radial glial cell would reextend a new radial process to the pial surface (Fishell and Kriegstein 2003). Several studies have reported the existence of radial glial growth cones, which may lead the extension of radial processes (Takahashi and others 1990; Gadisseux and others 1992; Miyata and others 2001). Also, in our samples of visual cortex of the postnatal ferret we have observed long radial glial fibers (vimentin+ / GFAP+) tipped with growth cones (V.B. and E.M.C., unpublished data). This hypothesis, however, will need to be confirmed in the ferret model by direct visualization of radial glia giving off long-distance translocating neurons.

**Supplementary Material**

Supplementary material can be found at http://www.cercor.oxfordjournals.org/

**Supplementary Movie.** Time-lapse movie of cells in the IZ labeled from Ad injections in the cortical plate (CP), demonstrating their translocating behavior along the apical process. Multiple cells are seen translocating. The direction toward the pia is up, and toward the ventricle is down. The movie shows only one frame every 50 min (1 in 5 frames that were imaged at 10-min intervals) from the first 30 h of this experiment. Elapsed time is indicated in days hours minutes.

**Supplementary Figure 1.** Criteria to determine the borders between cortical layers in the V1 of a P14 ferret. (A, B) Low magnification views through V1 of a P14 ferret showing the distribution of all cortical cells (A, DAPI staining in blue) and of cells in S-phase of the mitotic cycle (B, BrdU staining in red). DAPI staining highlights changes in cell densities (dashed lines), which are used to define the border between cortical plate (CP) and intermediate zone (IZ), between IZ and subventricular zone (SVZ), and between SVZ and ventricular zone (VZ). The border between IZ and SVZ also corresponds to the line along which cell divisions occur most densely at this stage of development (B, arrowheads). Scale bar = 1 mm. (C–C’) Detail of the SVZ and its neighboring layers, illustrating the anatomical features used in this study to define the border between SVZ and IZ (arrowheads and dashed line): sudden change in density and pattern of DAPI staining (C, blue; and C’) and accumulation of cells in S-phase (C, red; and C’). Scale bar = 500 μm.

**Supplementary Methods—Labeling of Cells in S-Phase.** BrdU (SIGMA) was administered intraperitoneally at 50 mg/kg body weight in 0.9% NaCl. Two BrdU doses were injected, with a lapse of 1 h between them. One hour after the second injection, ferrets were perfused transcardially with 4% paraformaldehyde in 0.1 M phosphate buffer. Brains were then cryoprotected, sectioned, and sections were stained for the detection of BrdU as described in Materials and Methods.

**Notes**

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