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POSTNATAL SHIFTS OF INTERNEURON POSITION IN THE NEOCORTEX OF NORMAL AND *REELER* MICE: EVIDENCE FOR INWARD RADIAL MIGRATION

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Abstract—During development, interneurons migrate to precise positions in the cortex by tangential and radial migration. The objectives of this study were to characterize the net radial migrations of interneurons during the first postnatal week, and to investigate the role of reelin signaling in regulating those migrations. To observe radial migrations, we compared the laminar positions of interneurons (immunoreactive for GABA or Dlx) in mouse neocortex on postnatal days (P) 0.5 and P7.5. In addition, we used bromodeoxyuridine birthdating to reveal the migrations of different interneuron cohorts. To study the effects of reelin deficiency, experiments were performed in *reeler* mutant mice.

In normal P0.5 cortex, interneurons were most abundant in the marginal zone and layer 5. By P7.5, interneurons were least abundant in the marginal zone, and were distributed more evenly in the cortical plate. This change was attributed mainly to inward migration of middle- to late-born interneurons (produced on embryonic days (E) 13.5 to E16.5) from the marginal zone to layers 2–5. During the same interval, late-born projection neurons (non-immunoreactive for GABA or Dlx) migrated mainly outward, from the intermediate zone to upper cortical layers. In *reeler* cortex, middle- and late-born interneurons migrated from the superplate on P0.5, to the deep cortical plate on P7.5. Late-born projection neurons in *reeler* migrated in the opposite direction, from the intermediate zone to the deep cortical plate.

We conclude that many middle- and late-born interneurons migrate radially inward, from the marginal zone (or superplate) to the cortical plate, during the first postnatal week in normal and *reeler* mice. We propose that within the cortical plate, interneuron laminar positions may be determined in part by interactions with projection neurons born on the same day in neurogenesis. **© 2004 IBRO. Published by Elsevier Ltd. All rights reserved.**

Key words: GABA, Dlx, bromodeoxyuridine, marginal zone, superplate.

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Abbreviations: BrdU, bromodeoxyuridine; E, embryonic day; IZ, intermediate zone; mz, marginal zone; P, postnatal day; SDF-1, stromal cell derived factor-1; svz, subventricular zone; vz, ventricular zone.

The cerebral cortex contains two major classes of neurons, *projection neurons* and *interneurons*, which have fundamentally different cellular properties and developmental origins (reviewed by Marín and Rubenstein, 2001). Projection neurons are glutamatergic; express transcription factors Emx1 and Tbr1; and are generated in the cortical ventricular zone (Chan et al., 2001; Hevner et al., 2001; Miyata et al., 2001; Noctor et al., 2001; Hatanaka and Murakami, 2002; Weissman et al., 2003). In contrast, interneurons are GABAergic; express Dlx, Nkx2.1, and Lhx6 transcription factors; and are produced in the medial ganglionic eminence, caudal ganglionic eminence, and lateral ganglionic eminence of the basal forebrain (Anderson et al., 1997, 2001; Tamamaki et al., 1997; Tan et al., 1998; Lavdas et al., 1999; Sussel et al., 1999; Wichterle et al., 2001; Ang et al., 2003). Interneurons enter the cortex postmitotically, by tangential migration through the marginal zone, subplate, intermediate zone, and subventricular zone (Anderson et al., 1997; Lavdas et al., 1999; Polleux et al., 2002; Ang et al., 2003; Tanaka et al., 2003).

Within the cortex, interneurons also migrate radially, to settle in different layers. Despite the temporal delay and dispersion associated with tangential migration, the laminar fates of interneurons are highly regulated, and by adulthood are highly correlated with the laminar fates of projection neurons with the same birthday (Fairén et al., 1986; Peduzzi, 1988). Interneurons and projection neurons demonstrate very similar "inside-out" relations between cell birthday and laminar fate, in which early-born neurons settle in deep layers, and late-born neurons settle in superficial layers (Angevine and Sidman, 1961; Fairén et al., 1986; Peduzzi, 1988). This raises the question of how the laminar fates of interneurons and projection neurons are regulated and coordinated.

One proposed explanation is that interneurons and projection neurons use the same mechanisms of radial migration (Fairén et al., 1986; Peduzzi, 1988; Valcanis and Tan, 2003). However, recent evidence from morphological and molecular studies suggests that this may not be the case. Direct observation of migrating cells in slice cultures has shown that projection neurons and interneurons undergo different morphological changes during radial migration. Projection neurons migrate by "somal translocation" and "glia-guided locomotion," and move exclusively away from the ventricular surface (Miyata et al., 2001; Nadarajah et al., 2001; Noctor et al., 2001; Hatanaka et al., 2002). Interneurons migrate by "branching cell" locomotion, and move both toward and away from the ventricle (Nadarajah et al., 2002, 2003; Polleux et al., 2002; Ang et al., 2003;

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Tanaka et al., 2003). Molecularly, chemokine SDF-1 is a potent chemoattractant for migrating interneurons, but not for projection neurons (Stumm et al., 2003). Mutant mice with deficiency of SDF-1 or its receptor, CXCR4, have a migrational defect that affects upper-layer interneurons but not projection neurons (Stumm et al., 2003). These morphological and molecular differences suggest that interneurons and projection neurons may use distinct mechanisms of radial migration.

Another molecule that regulates radial migration in the cortex is reelin (D'Arcangelo et al., 1995; Ogawa et al., 1995). Mice that lack reelin, its receptors (VLDLR/ApoER2), or a downstream molecule (Dab1) have identical cortical malformations, characterized by overall "inversion" of the cortical layers (Caviness, 1982; Rice et al., 1998; Trommsdorff et al., 1999). The effects of reelin deficiency on some classes of projection neurons have been studied (Polleux et al., 1998, 2001), but it is unknown how interneuron laminar fates are affected. Interneurons may respond to reelin signaling differently from projection neurons. Reelin prevents projection neurons from entering the marginal zone (Hammond et al., 2001), but interneurons migrate into, through, and out of the marginal zone (Polleux et al., 2002; Ang et al., 2003; Stumm et al., 2003; Tanaka et al., 2003). Reelin is never expressed by projection neurons, but is expressed by many interneurons postnatally (Pesold et al., 1998; Alcántara et al., 1998; Hevner et al., 2003a). In chimeric *Dab1*$^{+/+}$/*Dab1*$^{-/-}$ cortex, interneurons migrate into layers that contain *Dab1*$^{-/-}$ projection neurons, but not into layers that contain *Dab1*$^{+/+}$ projection neurons (Hammond et al., 2001). This last observation suggests that interneuron positions may be regulated not by reelin directly, but instead by projection neurons and their responses to reelin.

The present study was designed to evaluate the radial migrations of interneurons in the early postnatal cortex, by bromodeoxyuridine (BrdU) birthdating and laminar fate analysis of GABA$^+$ cells and Dlx$^+$ cells on P0.5 and P7.5. GABA is a neurotransmitter present in migrating and mature interneurons, and Dlx is a transcription factor that is specifically expressed in interneurons and their precursors (Anderson et al., 1997; Eisenstat et al., 1999). To examine the role of reelin signaling, we also studied interneuron migration in reelin-deficient (*reeler*) mutant mice. Our results suggest that during the first postnatal week, many late-born interneurons migrate "inward," from the marginal zone to the cortical plate. At the same time, other late-born interneurons and projection neurons appear to migrate "outward" from the intermediate zone, subventricular zone (svz), and ventricular zone (vz) to the cortical plate. Our results support the hypothesis that the laminar fates of interneurons may be regulated by signals from projection neurons in the cortical plate, independently of reelin.

EXPERIMENTAL PROCEDURES

Animals and genotyping

Mice were used according to a protocol approved by the Institutional Animal Care and Use Committee at the University of Washington, and in accordance with NIH guidelines. All efforts were made to minimize the number of mice used, as well as any pain or suffering. B6 *Reln* heterozygous mutant mice were purchased from Jackson Laboratories (Bar Harbor, ME, USA) and bred in a colony. Homozygous (−/−) mice were recognized phenotypically by ataxic behavior, abnormal cerebellar anatomy, and/or cortical malformation. Animals were genotyped to detect the mutant *Reln* allele, using PCR according to instructions from the supplier (Jackson Laboratories). Insemination was assessed by the presence of a vaginal plug. Noon of the vaginal plug day was designated embryonic day (E) 0.5. Wild-type (+/+) and heterozygous (+/−) mice were used as controls. The animal procedures were described in detail previously (Hevner et al., 2003a,b), and are only briefly outlined here.

BrdU injections and animal perfusion

Of 42 pups studied, 28 were postnatal day (P) 0.5, and 14 were P7.5. Control and *reeler* mutant littermate embryos were labeled with BrdU in utero by a single i.p. injection (40 mg/kg) on E10.5, E11.5, E12.5, E13.5, E14.5, E15.5, or E16.5. For P0.5 experiments, each birthday was studied in two control and two *reeler* brains. For P7.5 experiments, each birthday was studied in one control and one *reeler* brain. Pups were anesthetized by hypothermia on P0.5, or with Avertin (0.02 ml/g body weight, i.p.) on P7.5, and were perfused with cold 4% paraformaldehyde in 0.1 M sodium phosphate buffer, pH 7.2. After postfixation overnight in the same fixative, brains were removed, cryoprotected through increasing concentrations of sucrose (10%, 20%, 30%), embedded in OCT, and sectioned on a cryostat (12 μm). Sections were stored at −80 °C.

Antibodies and immunohistochemistry

Primary antibodies included the following: mouse monoclonal anti-BrdU (1:200; Chemicon, Temecula, CA, USA), rabbit polyclonal anti-pan-Dlx (1:75; J.K.), and rabbit polyclonal anti-GABA (1:5000; Sigma, St. Louis, MO, USA). Fluorophore-conjugated (Alexa 488, Alexa 594) secondary goat antibodies against mouse and rabbit IgG were purchased from Molecular Probes (Eugene, OR, USA), and were used at 1:200 dilution. For double-label immunofluorescence, cryostat sections through the parietal cortex were briefly microwaved to boiling in 10 mM sodium citrate (pH 6.0) for antigen enhancement three times, cooled, treated with 2N HCl for 60 min at 37 °C, rinsed with PBS, incubated in primary antibodies overnight at 4 °C, rinsed, and incubated in secondary antibodies for 2 h at room temperature. Sections were counterstained for 10 min with filtered 0.1% DAPI (Sigma) in phosphate-buffered saline (pH 7.4).

TUNEL labeling

Slides were reacted using the *in situ* cell death detection kit (fluorescein) from Roche (Indianapolis, IN, USA) on 12 μm cryosections. The manufacturer's labeling protocol for tissue was followed except that the permeabilization step was omitted; pilot studies showed that this step did not affect sensitivity. TUNEL-reacted tissues were then processed for GABA immunofluorescence as described above, and counterstained with DAPI.

Microscopy and image processing

Fluorescence labeling was viewed on an epifluorescence microscope and photographed digitally. Images were processed using Photoshop (Adobe, San Jose, CA, USA). For cell counting of BrdU/GABA or BrdU/Dlx double-labeled cells, three to eight images were collected from one or two brains for each BrdU birthday and postnatal age combination. The brightness and contrast of each image were optimized. The BrdU fluorescence channel

(green) was smoothed (Gaussian blur, two pixel radius), to enhance specificity in the identification of heavily labeled, first generation cells (Caviness et al., 1995). First generation cells were operationally identified by high-level (suprathreshold) BrdU labeling in more than half of the nucleus.

Cell counting and laminar position analysis

The positions of $BrdU^+$ (first generation) cells and Dlx^+ or $GABA^+$ cells were plotted using different color dots to mark $BrdU^+$ (green dots), Dlx^+ or $GABA^+$ (red dots), and double-labeled (yellow dots) cells. The cortex (layers 1–6b) was analyzed with a grid of 10 equal horizontal bins, with additional bins for the intermediate zone (iz) and vz/svz (for example, see Fig. 3). In normal cortex, bin 1 corresponded to the marginal zone (mz), and bin 10 approximately corresponded to layer 6b, which is the upper part of the subplate in rodents (Allendoerfer and Shatz, 1994; Valverde et al., 1995; McQuillen et al., 2002). The number of cells, and the area of each bin were measured using ImageJ (NIH).

Confocal microscopy

A laser scanning confocal microscope (Bio-Rad LS2000) was used to collect images from double-labeled specimens. TO-PRO-3 (Molecular Probes), an infrared fluorescent counterstain, was added (100 nM) in some experiments to show cell nuclei in confocal images.

RESULTS

Changing distributions of interneurons in normal and *reeler* postnatal cortex

To label interneurons, antibodies against two markers were used: (1) GABA, which is expressed in migrating and mature interneurons; and (2) Dlx transcription factors (pan-Dlx antibody), which are expressed in interneurons and their precursors (Anderson et al., 1997; Eisenstat et al., 1999). GABA and Dlx were both used, to ensure that all stages of interneuron maturation were detected. To minimize potential variations of laminar fate due to gradients of cortical development (Caviness et al., 2000), all experiments were done in parietal cortex.

$GABA^+$ interneurons. GABA was detected in the nucleus and cytoplasm of interneurons, as described previously (Hevner et al., 2003a). In normal P0.5 cortex, $GABA^+$ cells were most abundant in layer 5 and the mz (Fig. 1A). By P7.5, $GABA^+$ cells were greatly reduced in the mz, and were distributed relatively evenly throughout other cortical layers (Fig. 1B). This suggested that interneurons migrated out of the mz, and into the cortical plate during the first postnatal week. Other possibilities that could explain the changing distribution of interneurons, such as selective apoptosis in the mz, were considered but appeared unlikely, as discussed below.

In P0.5 *reeler* cortex (Fig. 1C), $GABA^+$ interneurons were concentrated mainly in the superficial half of the cortex, including the “superplate,” which is comprised of cells normally found in the mz and subplate (Caviness, 1982). In P7.5 *reeler* cortex (Fig. 1D), $GABA^+$ interneurons were much less abundant in the superplate, and were instead distributed somewhat evenly throughout the malformed cortical plate. This shift of interneuron positions suggested that interneurons migrated inward during the first postnatal week in *reeler*, as in normal mice.

Dlx^+ cells. Dlx, a homeobox transcription factor, was detected mainly in cell nuclei (Hevner et al., 2003a). In general, the positions and apparent migrations of Dlx^+ cells were highly similar to those of $GABA^+$ cells, suggesting that both markers generally labeled the same cells. In P0.5 normal cortex, Dlx^+ cells were concentrated mainly in the mz and layer 5 (Fig. 2A). In P7.5 cortex, Dlx^+ cells were relatively depleted from the mz, and were instead distributed throughout the cortical plate (Fig. 2B). In *reeler*, Dlx^+ cells were concentrated in the superficial cortical plate and superplate on P0.5 (Fig. 2C), but were mostly absent from the superplate, and were rather evenly scattered in the cortical plate, on P7.5 (Fig. 2D).

The only obvious discrepancy between Dlx and GABA labeling was in the iz and vz/svz. In these compartments, Dlx^+ cells were more abundant than $GABA^+$ cells, in normal as well as *reeler* cortex (compare Figs. 1 and 2). The apparent $Dlx^+/GABA^-$ cells were probably a mixture of immature interneurons (Xu et al., 2003) and glial progenitors (Marshall and Goldman, 2002). The abundance of Dlx^+ cells in the iz and vz/svz appeared to decrease between P0.5 and P7.5 (compare Fig. 2A and B), suggesting that many $Dlx^+/GABA^-$ precursors migrated “outward” into the cortex and matured into $Dlx^+/GABA^+$ interneurons.

Laminar fate analysis and interneuron birthdating

To determine if specific cohorts of interneurons (born during early, middle, or late corticogenesis) shifted positions during the first postnatal week, interneuron birthdating was done by double immunofluorescence for BrdU, a thymidine analog, and GABA or Dlx. Pregnant dams received a single dose of BrdU by i.p. injection on one day in corticogenesis between E10.5 and E16.5, and BrdU-labeled pups were killed on P0.5 or P7.5. Two-color immunofluorescence was then used to identify single- and double-labeled cells immunoreactive for BrdU, and for GABA or Dlx. Images of double labeled sections were collected by epifluorescence microscopy (e.g. Fig. 3A) or by laser scanning confocal microscopy (Fig. 3B and C). In addition, the birthdays of presumed projection neurons were inferred from BrdU labeling of Dlx^- or $GABA^-$ cells.

To analyze the laminar fates of labeled cells, the cortex was divided into horizontal “bins” (Fig. 3D). Binning is widely used to analyze laminar fate (e.g. Fairén et al., 1986; Polleux et al., 1998) because it is less subjective than the visual identification of cortical layers, which can be indistinct in neonatal mice. Also, binning facilitated laminar fate analysis in *reeler* cortex, which is malformed and does not contain recognizable layers other than the superplate. The cortical gray layers, consisting of layers 1–6b (or the superplate and cortical plate in *reeler*), were analyzed with a grid of 10 equally spaced cortical bins (Fig. 3D). In general, the cortical bins were highly correlated with layers observed by counterstaining with DAPI. The mz was represented by the superficial 10% of cortex, i.e. bin 1. The

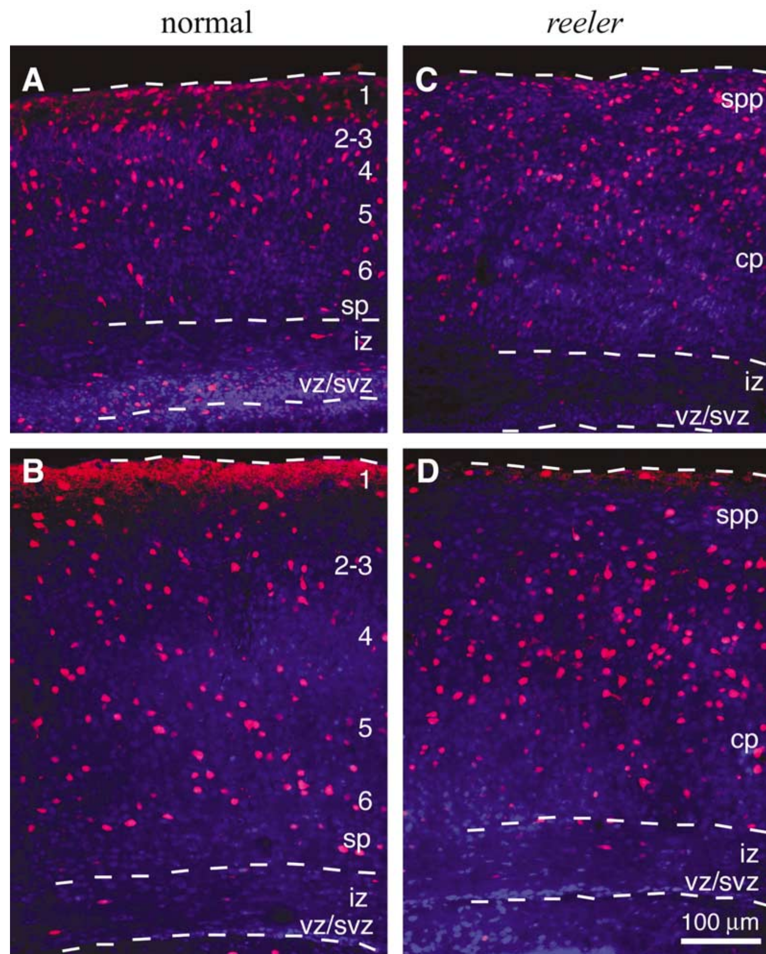


Fig. 1. GABA immunofluorescence (red) in control and *reeler* postnatal parietal cortex. (A) Normal parietal cortex, P0.5. $GABA^+$ interneurons were concentrated mainly in layers 1 (the mz) and 5, and scattered throughout other layers of the cortical plate. A few migrating $GABA^+$ interneurons were located in the iz, and in the vz/svz. (B) Normal parietal cortex, P7.5. $GABA^+$ interneurons were distributed relatively evenly among the cortical layers, except for low numbers in layer one and the subplate (sp). The diffuse immunoreactivity at the top of layer 1 was edge artifact. (C) *Reeler* parietal cortex, P0.5. $GABA^+$ cells were most abundant in the superplate (spp) and upper half of the cortical plate (cp). (D) *Reeler* parietal cortex, P7.5. $GABA^+$ cells were distributed throughout the cortical plate, with lower numbers in the superplate and the deepest part of the cortical plate. The blue fluorescent counterstain is DAPI. Scale bar=100 μm (all panels). Images were digitally optimized for brightness and contrast with Adobe Photoshop.

subplate (layer 6b) was represented by the deep 10% of cortex, i.e. bin 10. These and other correlations in the P7.5 cortex are shown in Fig. 3D. The vz/svz and the iz were assigned to separate bins below the cortical bins. The thickness of vz/svz and iz bins varied among sections, and generally did not equal the thickness of a cortical bin (Fig. 3D). For laminar fate analysis, single- and double-labeled cells were marked with different color dots and counted in each bin (Fig. 3D). Finally, cell counts were divided by bin areas, to calculate the areal density of cells in each bin (cells/mm^2).

Bin analysis of interneuron positions in control and *reeler* cortex

To rigorously analyze the distribution of interneurons in the cortex, $GABA^+$ cells and Dlx^+ cells were counted in binned sections, and cell densities were plotted in bar graphs (Fig. 4). The quantitative data confirmed that in normal P0.5 cortex, $GABA^+$ interneurons and Dlx^+ cells were most abundant in the mz and layer 5, with lower abundance in other layers (Fig. 4Aa and Ba). Notably, the densities of $GABA^+$ cells and Dlx^+ cells in each layer were quantitatively similar: for example, both $GABA^+$ cells and Dlx^+ cells numbered about 2800 cells per mm^2 in the mz (Fig. 4Aa top bin, and Fig. 4Ba top bin). Between P0.5 and P7.5 (Fig. 4Ab and Bb), the density of interneurons in the mz decreased in absolute and relative terms. On P0.5, the mz had the highest density of $GABA^+$ cells and Dlx^+ cells in the cortex proper (approximately 2800 cells per mm^2), but on P7.5, the mz (and subplate) had the lowest density of $GABA^+$ cells and Dlx^+ cells (approximately 200–250 cells per mm^2).

In P0.5 *reeler* mice, interneuron density was highest in the superplate, which held approximately 2600–2800 interneurons/mm^2 (Fig. 4Ac and Bc). In P7.5 *reeler* mice, the number of interneurons in the superplate decreased markedly to the lowest density in the cortex, approximately 200–250 cells per mm^2 (Fig. 4Ad and Bd). Thus, in *reeler*

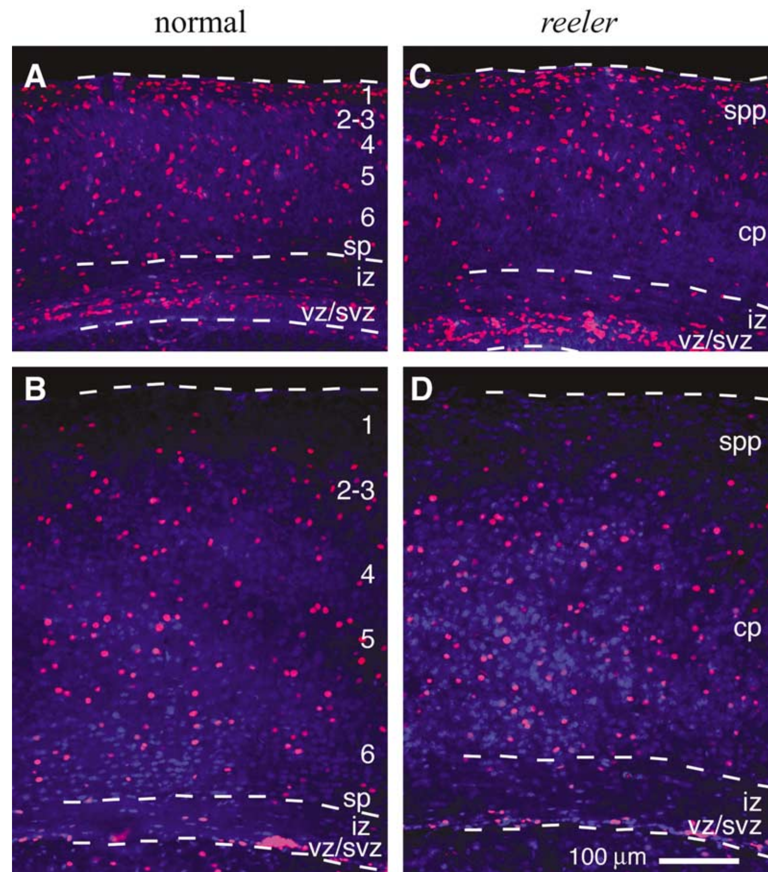


Fig. 2. Dlx immunofluorescence (red) in control and *reeler* postnatal parietal cortex. (A) Normal parietal cortex, P0.5. In the cortex proper (layers 1-subplate), Dlx$^+$ cells were most abundant in layers 1 and 5. Dlx$^+$ cells were also numerous in the iz and vz/svz. (B) Normal parietal cortex, P7.5. Dlx$^+$ cells were evenly distributed throughout most cortical layers, except the mz and subplate (sp), which contained few Dlx$^+$ cells. Also, many Dlx$^+$ cells persisted in the vz/svz; these may have been glial progenitors (Marshall and Goldman, 2002). (C) *Reeler* parietal cortex, P0.5. Dlx$^+$ cells were most abundant in the superplate (spp) and upper half of the cortical plate (cp), as well as the iz and vz/svz. (D) *Reeler* parietal cortex, P7.5. Dlx$^+$ cells were scattered throughout the cortical plate, but were relatively depleted from the superplate. The vz/svz also contained significant numbers of Dlx$^+$ cells. The blue fluorescent counterstain is DAPI. Scale bar=100 μm (all panels). Images were digitally optimized for brightness and contrast with Adobe Photoshop.

as in normal mice, the distribution of cortical interneurons shifted inward during the first postnatal week. Moreover, the magnitude of the shift was quantitatively similar in *reeler* as in normal cortex.

The density of Dlx$^+$ cells was similar to the density of GABA$^+$ cells in the cortex proper, but not in the iz and vz/svz. In these zones, Dlx$^+$ cells outnumbered GABA$^+$ cells by ratios of 2:1 or more, in normal as well as *reeler* cortex (e.g. compare Fig. 4Aa and Ba). As noted above, the Dlx$^+$/GABA$^-$ cells in the iz and vz/svz were probably a mixture of immature interneurons (Xu et al., 2003) and glial progenitors (Marshall and Goldman, 2002). The density of GABA$^+$ cells in the iz decreased about three-fold during the first postnatal week (from approximately 300 to approximately 100 cells per mm^2), and the density of GABA$^+$ cells in the vz/svz decreased even more (from approximately 700 to approximately 70 cells per mm^2), suggesting that many interneurons migrated upward from these zones into the cortex. The density of Dlx$^+$ cells in the iz also decreased substantially, from approximately 1200 to approximately 500 cells per mm^2. The density of Dlx$^+$ cells in the vz/svz actually *increased* between P0.5 and P7.5, presumably reflecting the persistence of numerous glial progenitors rather than interneuron precursors at the later age (Marshall and Goldman, 2002).

To estimate the extent of maturation from Dlx$^+$/GABA$^-$ to Dlx$^+$/GABA$^+$ phenotypes between P0.5 and P7.5, we calculated the overall ratio of Dlx$^+$ cells to GABA$^+$ cells throughout the cortex (including the cortical mantle, the iz, and the vz/svz) at both ages (Table 1). In P0.5 cortex, the ratio of Dlx$^+$ cells to GABA$^+$ cells was approximately 1.4, in both normal and *reeler* mice (Table 1). By P7.5, this ratio decreased to approximately 1.3. This decline in the ratio of Dlx$^+$ cells to GABA$^+$ cells suggested that maturation was a small but significant factor during the first postnatal week. As noted above, most of the Dlx$^+$/GABA$^-$ cells were located in the iz and vz/svz, and these cells evidently matured concurrently with migration into the cortical plate.

Overall, the density of interneurons throughout the entire cortex decreased almost two-fold during the first postnatal week, from approximately 1000 GABA$^+$ cells/mm^2 on P0.5, to approximately 550 GABA$^+$ cells/mm^2 on P7.5

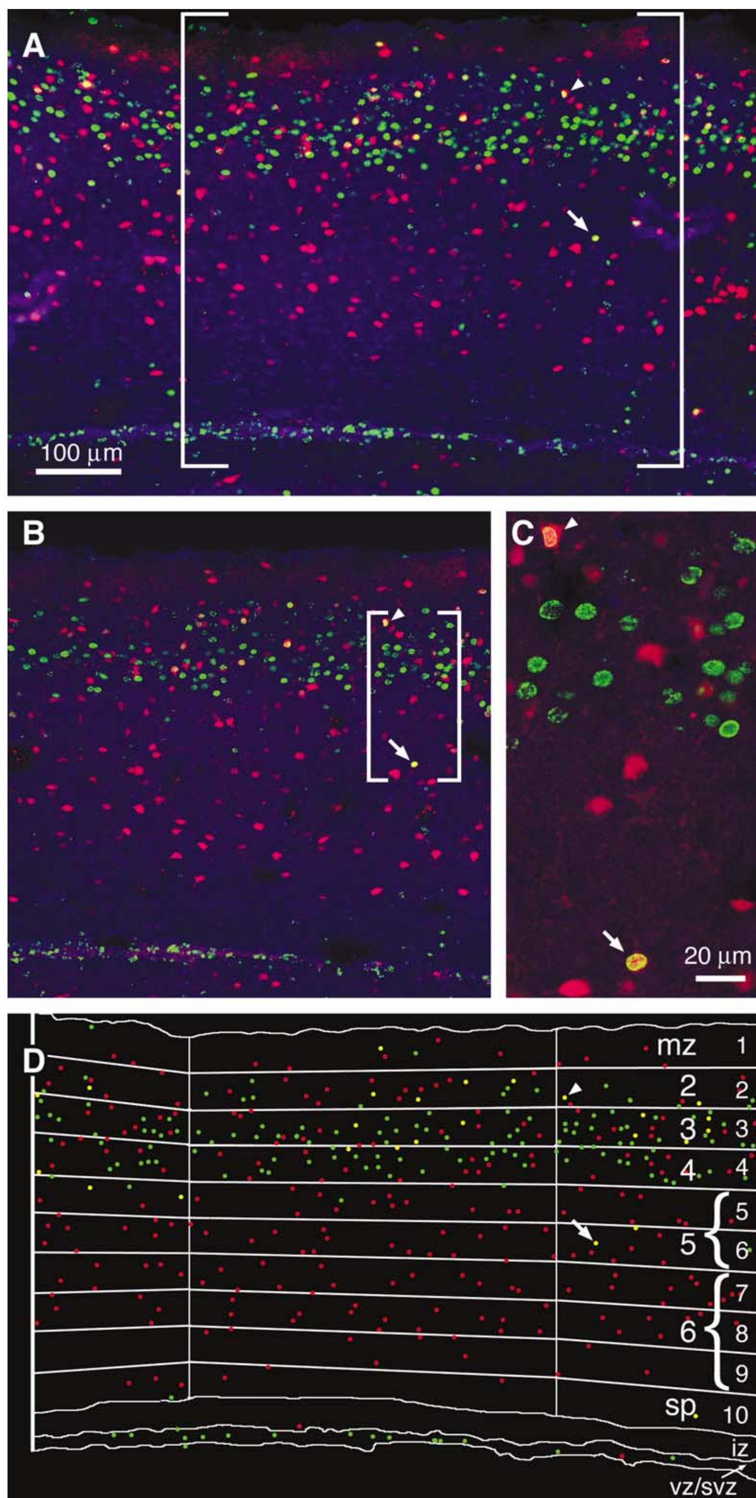


Fig. 3. Double immunofluorescence for GABA (red) and BrdU (green), and analysis of laminar fate, in normal P7.5 parietal cortex. BrdU was administered on E14.5. (A) Epifluorescence microscopy. Double-labeled cells (yellow) were located mainly in layers 2–4 (e.g. arrowhead), though a few were scattered in other layers (e.g. arrow). The blue fluorescent counterstain is DAPI. (B) Confocal microscopy of the bracketed area in (A). The thin optical section confirmed that cells were double labeled for GABA and BrdU (e.g. arrowhead, arrow). The blue (pseudocolor) fluorescent counterstain is TO-PRO-3. (C) Confocal image at higher magnification of the bracketed area in (B). BrdU labeling was strongest around the periphery of nuclei, and GABA was present in the nucleus as well as cytoplasm of interneurons. (D) Binning for laminar fate analysis. In the cortex proper (layers 1–6b), bin 1 was the top bin (mz), and bin 10 was the bottom bin (subplate). The radial positions of cells were plotted using colored dots to designate $GABA^{+}/BrdU^{-}$ (red), $GABA^{-}/BrdU^{+}$ (green), or $GABA^{+}/BrdU^{+}$ (yellow) labeling. Scale bars=100 μm for A, B, and D; 20 μm for C. Images were digitally adjusted for brightness and contrast with Adobe Photoshop.

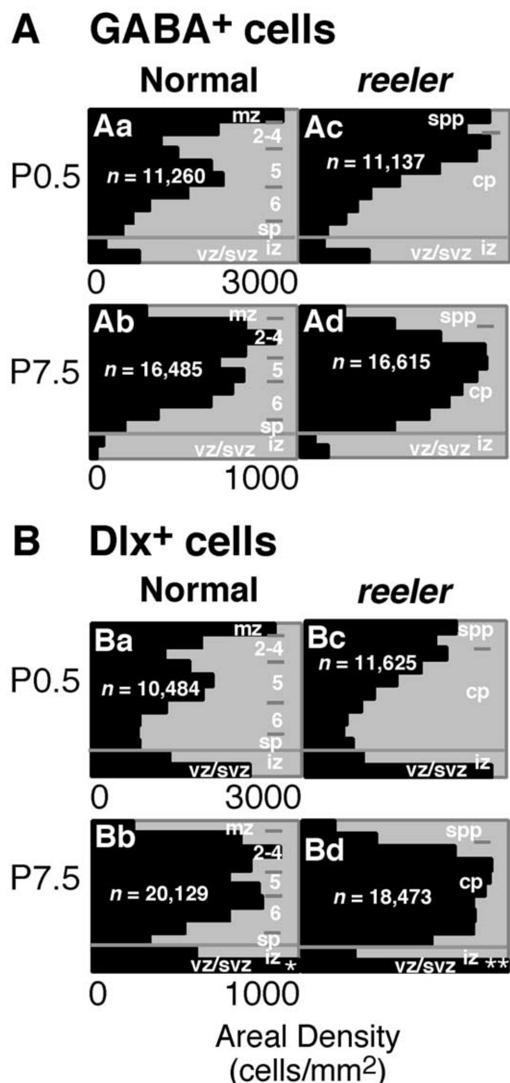


Fig. 4. Distribution of interneurons in control and *reeler* parietal cortex, P0.5 and P7.5. (A) GABA$^+$ interneurons. In normal cortex, the most prominent change between P0.5 (Aa) and P7.5 (Ab) was the shift of interneurons out of the P0.5 mz (bin 1), and into layers 2–4 (bins 2–4) of P7.5 cortex. In *reeler* cortex, GABA$^+$ cells shifted from positions in the superplate (bins 1–2) and upper part of the cortical plate (bin 3) on P0.5 (Ac), and into lower parts of the cortical plate on P7.5 (Ad). (B) Dlx$^+$ cells. The densities, radial distributions, and migrations of Dlx$^+$ cells were very similar to those of GABA$^+$ cells, in both normal (Ba and Bb) and *reeler* (Bc and Bd) cortex. The only exceptions were the iz and the vz/svz, where the density of Dlx$^+$ cells was higher than the density of GABA$^+$ cells. The Dlx$^+$ cell density in the vz/svz was quite high on P7.5, due partly to the thinness and small area of the vz/svz at this age: the Dlx$^+$ cell density was 5949 (*) in normal P7.5 vz/svz (Bb), and 4554 (**) in *reeler* P7.5 vz/svz (Bd). Numbers (*n*) are the total number of cells counted for each graph. Scales were the same for *reeler* as for normal cortex.

(Table 1). Previous work by Fairén et al. (1986) has shown that by adulthood, interneuron areal density in the mouse cortex decreases even further, to approximately 225 interneurons/mm^2. Decreasing interneuron density is attributed primarily to postnatal growth of the cortex (Price et al., 1997). Another possible explanation for decreasing interneuron densities, and for changing distributions of interneurons, could be apoptosis.

Table 1. Interneuron density in normal and *reeler* parietal cortex[a]

Marker	Control		*Reeler*	
	P0.5	P7.5	P0.5	P7.5
GABA	1024	537	901	551
Dlx	1399	697	1371	670

[a] Numbers indicate the average cell density (cells/mm^2) throughout the entire cortex from the ventricular to the pial surface (all bins).

Lack of interneuron apoptosis in postnatal mouse cortex

In principle, apoptosis could affect both the overall abundance and the apparent distribution of interneurons in the cortex. Using the TUNEL method to detect apoptotic cells in tissue sections, we found that the prevalence of apoptosis was quite low in the early postnatal cortex (Fig. 5). Most of the apoptotic cells were located in the upper cortical plate, at its boundary with the mz. Similar findings were reported in previous studies of the early postnatal cortex (Finlay and Slattery, 1983; Ferrer et al., 1992; Spreafico et al., 1995; Valverde et al., 1995; Thomaidou et al., 1997). To test whether interneurons undergo apoptosis, we used double fluorescence labeling for apoptosis and GABA immunoreactivity. Of more than 100 apoptotic cells identified by TUNEL in sections of P0.5 and P7.5 cortex, none contained GABA immunoreactivity (Fig. 5). Since apoptotic cells maintain intact cell membranes (Dikranian et al., 2001), the absence of GABA cannot be explained by cellular membrane permeabilization during apoptosis. These results suggest that very few or no mature interneurons are eliminated by apoptosis in the early postnatal cortex.

Radial migration of middle- and late-born interneurons in normal cortex

To investigate whether specific cohorts of interneurons (born on particular days in neurogenesis) migrated during the first postnatal week, we studied the positions of interneurons (GABA$^+$ cells and Dlx$^+$ cells) born on each day of corticogenesis from E10.5 to E16.5, in P0.5 and P7.5 cortex. Results were very similar using GABA or Dlx as markers; here we show the results from birthdating of Dlx$^+$ cells.

Interneuron production and laminar fates. Birthdating indicated that the overall production of Dlx$^+$ cells was relatively low during early corticogenesis (E10.5–E12.5), and increased several-fold during middle and late corticogenesis (E13.5–E16.5). This conclusion was evident whether examined in P0.5 or P7.5 cortex (Fig. 6). An acceleration of neurogenesis around E13.5 has previously been noted for the total complement of cortical neurons (Takahashi et al., 1999).

With regard to laminar fates, the distribution of each interneuron cohort varied according to cell birthday and postnatal age. Early-born interneurons (E10.5–E12.5)

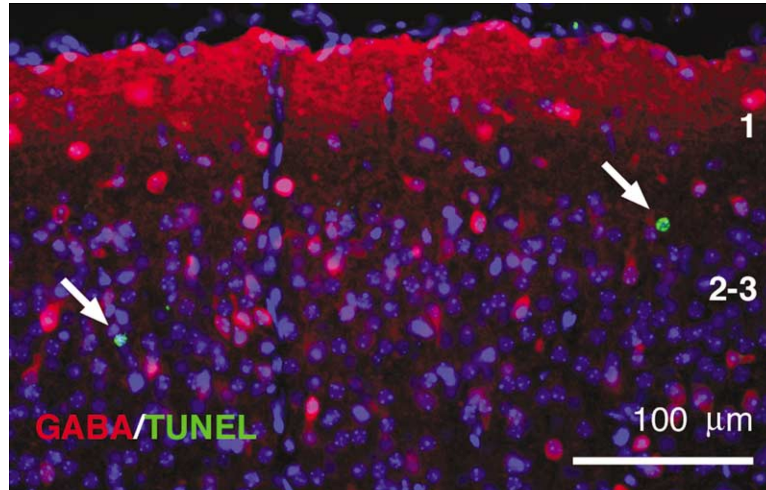
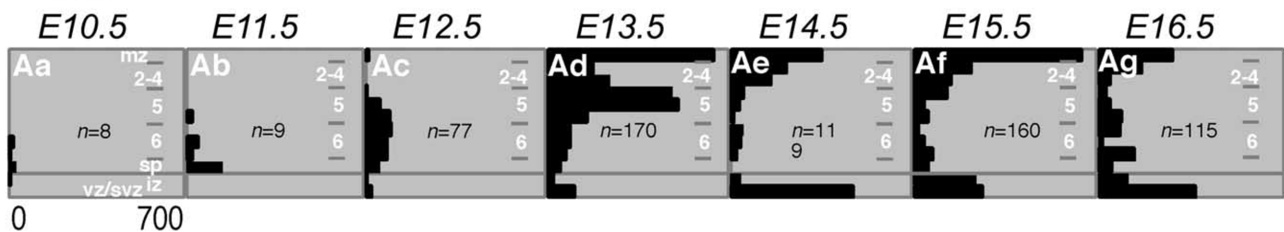


Fig. 5. Double labeling for apoptosis (TUNEL method, green) and GABA (red) in normal P7.5 parietal cortex. The most common location of apoptotic cells (arrows) was in layers 2–3, though they were overall infrequent (estimated <0.1%) in cortex on P7.5 or P0.5 (not shown). No apoptotic cells contained GABA. Results were similar in *reeler* cortex, except that TUNEL$^+$ cells were more scattered among layers in the cortical plate of *reeler*. The bright red fluorescence at the top of layer 1 was edge artifact. The blue counterstain is DAPI. Scale bar=100 μm. The image was adjusted for brightness and contrast with Adobe Photoshop.

were located in deep layers of the cortex on P0.5 (Fig. 6Aa–Ac) as well as P7.5 (Fig. 6Ba–Bc), indicating that they did not migrate during this period. On the other hand, middle- and late-born cohorts (E13.5–E16.5) showed clear shifts of laminar position between P0.5 and P7.5. Many middle- and late-born interneurons were located in the mz on P0.5 (Fig. 6Ad–Ag), but moved into the cortical plate by P7.5 (Fig. 6Bd–Bg). Within the P7.5 cortical plate, most middle- and late-born interneurons settled in positions that were consistent with the inside-out sequence of neurogenesis. Interneurons born on E13.5 occupied mainly layers 5–6 (Fig. 6Bd); interneurons born on E14.5 occupied mainly layers 2–5 (Fig. 6Be); and interneurons born on E15.5 and E16.5 occupied mainly layers 2–4 (Fig. 6Bf–Bg). These results suggested that upon leaving the mz, interneurons born on E13.5–E16.5 migrated to predeter-



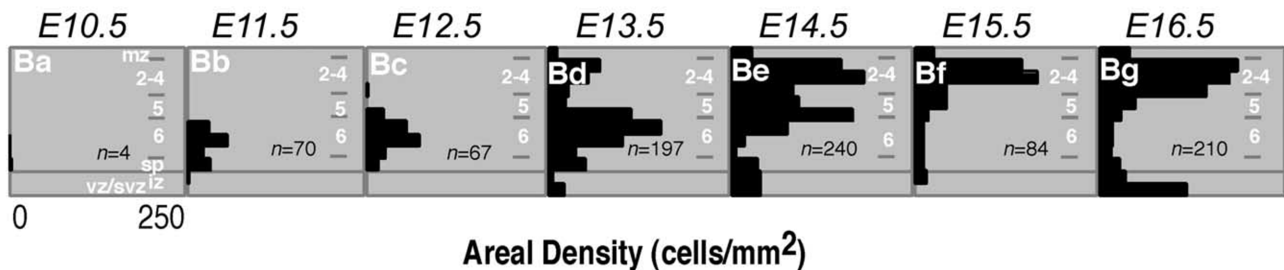
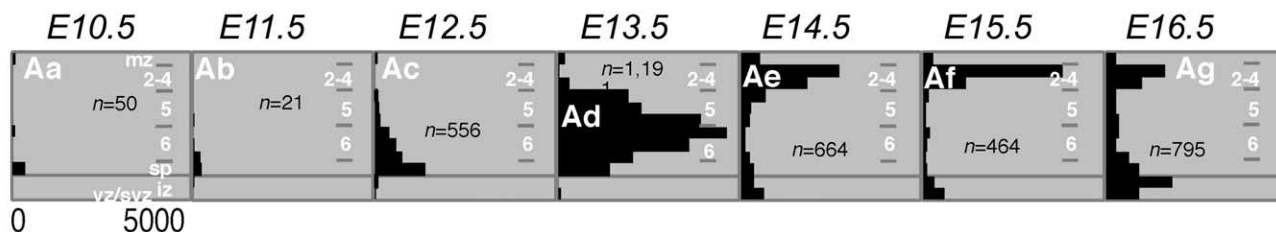


Fig. 6. Birthdays and laminar positions of Dlx$^+$ interneuron cohorts in normal parietal cortex. Graphs show the distribution of Dlx$^+$ cells born on each embryonic day from E10.5–16.5, in P0.5 cortex (A) or P7.5 cortex (B). Note the abundance of middle- and late-born Dlx$^+$ cells (born E13.5–E16.5) in the mz of P0.5 cortex (Ad–Ag), and the apparent redistribution of these cells out of the mz and into layers 2–5 by P7.5 (Bd–Bg). Note also the overall inside-out relation between cell birthday and laminar position in P7.5 cortex. Numbers (*n*) are the total number of double-labeled cells (BrdU$^+$/Dlx$^+$) counted for each graph. Data were produced by cell counting as described in the Experimental Procedures and illustrated in Fig. 3. All P0.5 graphs are plotted to the same scale, and all P7.5 graphs are plotted to the same scale. Approximate layers are indicated in graphs of E10.5-born cells (Aa and Ba).

A P0.5



B P7.5

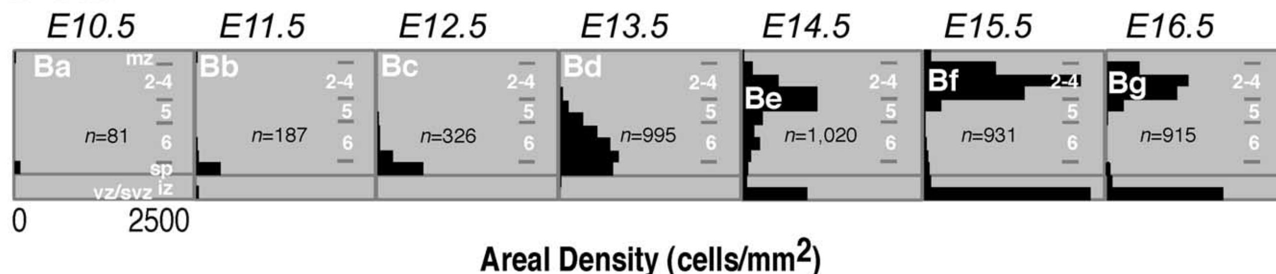


Fig. 7. Birthdays and laminar positions of Dlx$^-$ cell cohorts (presumed projection neurons) in normal parietal cortex. Graphs show the distribution of Dlx$^-$ cells born on each embryonic day from E10.5–16.5, in P0.5 cortex (A) or P7.5 cortex (B). The inside-out sequence of neurogenesis was evident at both postnatal ages. However, a proportion of Dlx$^-$ cells born on E15.5 and E16.5 shifted from positions in the iz, subplate, and layer 6 on P0.5 (Af and Ag), into upper cortical layers by P7.5 (Bf and Bg), suggesting that they migrated outward. Numbers (*n*) are the total number of BrdU$^+$/Dlx$^-$ cells counted for each graph. Data were produced and plotted as described in Fig. 6.

mined positions in the cortical plate. In addition, some middle- and late-born interneurons migrated outward, from the iz and vz/svz to the cortical plate (Fig. 6).

Presumed projection cell production and laminar fates. The production of Dlx$^-$ cells (presumed projection neurons), like Dlx$^+$ cells, was relatively low from E10.5 to E12.5, and increased several-fold thereafter. The laminar fates of presumed projection neurons (Dlx$^-$) generally conformed to the "inside-out" gradient of neurogenesis, whether assessed in P0.5 cortex (Fig. 7A) or P7.5 cortex (Fig. 7B). Broadly speaking, Dlx$^-$ cells born from E10.5 to E13.5 settled predominantly in deep cortical layers (5, 6, and subplate), and Dlx$^-$ cells born from E14.5 to E16.5 settled in upper layers (2–4). Few presumed projection neurons occupied the mz on either P0.5 or P7.5. Notable changes of laminar position were observed only among Dlx$^-$ cells born on E15.5–E16.5. A significant proportion of cells in these cohorts occupied the iz, subplate, and deep cortical plate on P0.5 (Fig. 7Af and Ag), and apparently migrated outward, into upper layers of the cortical plate by P7.5 (Fig. 7Bf and Bg).

Comparison of interneuron and presumed projection neuron laminar fates in P7.5 cortex. In general, interneuron cohorts had broader radial distributions than did the same cohorts of projection neurons. For example, E15.5-born Dlx$^+$ cells and Dlx$^-$ cells both had peaks of areal density in layers 2–4, but a larger proportion of interneurons settled outside the peak layers (compare Figs. 6 Bf and 7Bf). Likewise, projection neurons born on E11.5 were located almost exclusively in the subplate on P7.5 (Fig. 7Bb), but interneurons born on E11.5 occupied layer 6 as well as the subplate (Fig. 6Bb). The sharpest contrast was between interneurons and presumed projection neurons born in the middle of corticogenesis (E13.5–E14.5). Dlx$^+$ interneurons born on E13.5 and E14.5 had broad laminar fate profiles, with bimodal density peaks in layers 2–4 and 5–6 (Fig. 6Bd and Be), but Dlx$^-$ cells (presumed projection neurons) had unimodal density peaks. Dlx$^-$ cells born on E13.5 occupied mainly layer six (Fig. 7Bd), while Dlx$^-$ cells born on E14.5 occupied layers 3–5 (Fig. 7Be). Similar distinctions were noted in the adult mouse by Fairén et al. (1986), and in the adult ferret by Peduzzi (1988).

Radial migration of middle- and late-born cohorts in reeler cortex

Previous birthdating studies have shown that the cortical layers in adult *reeler* mice are relatively inverted, i.e. laminar fates follow an overall "outside-in" rather than "inside-out" progression (Caviness, 1982; Polleux et al., 1998). However, it is unknown if the outside-in sequence applies to interneurons in *reeler*, since overall birthdating is representative mainly of projection neurons, which outnumber interneurons by a ratio of four or 5:1 (Hendry et al., 1987; Meinecke and Peters, 1987; Peduzzi, 1988; Tamamaki et al., 2003).

Interneuron production and laminar fates in reeler. The neurogenesis of Dlx$^+$ cells in *reeler* proceeded similarly as in normal mice, with the same acceleration of cell production around E13.5 (Fig. 8). However, Dlx$^+$ cells did not migrate to normal laminar fates. In fact, it was clear that Dlx$^+$ cells followed an "outside-in" laminar fate sequence

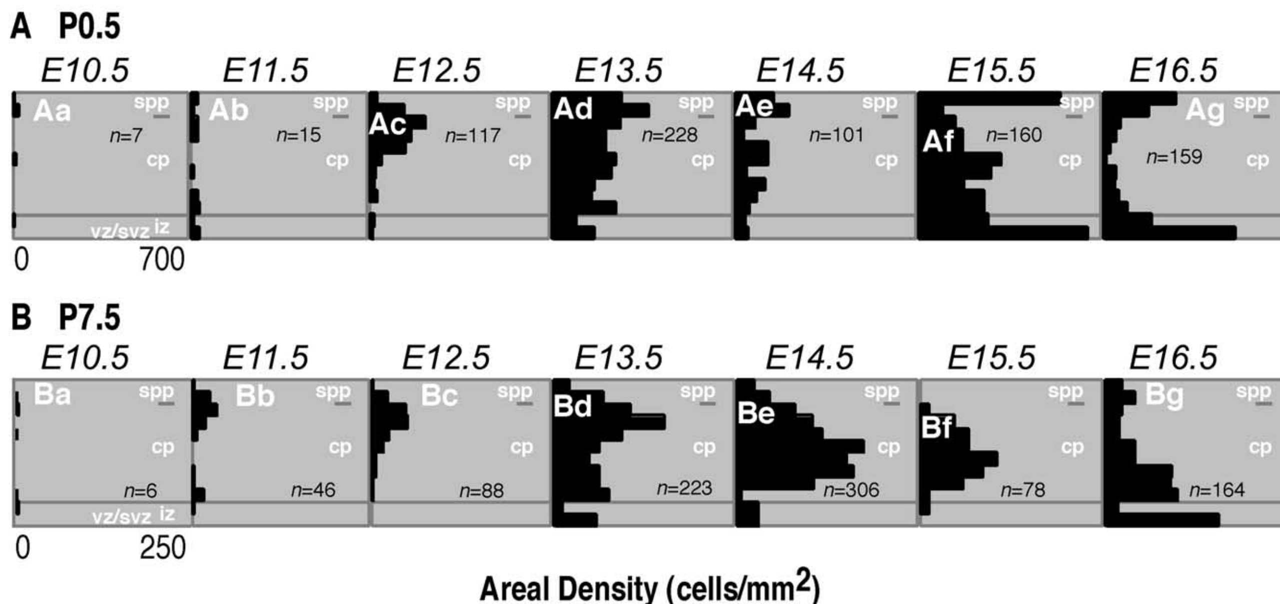


Fig. 8. Birthdays and laminar fates of Dlx$^+$ interneuron cohorts in *reeler* parietal cortex. Graphs show the distribution of Dlx$^+$ cells born on each embryonic day from E10.5–16.5, in *reeler* cortex on P0.5 (A) or P7.5 (B). Numerous middle- and late-born Dlx$^+$ cells (born E13.5–E16.5) were located in the superplate (bins 1–2) on P0.5 (Ad–Ag), but these cells shifted out of the superplate and into deep layers of the cortical plate by P7.5 (Bd–Bg). Note also the overall "outside-in" (inverted) relation between interneuron birthday and laminar position in P7.5 cortex. Numbers (*n*) are the total number of double-labeled cells (BrdU$^+$/Dlx$^+$) counted for each graph. Data were produced and plotted as described in Fig. 6.

in the P7.5 cortex (but not in the P0.5 cortex). This indicated that reelin deficiency did not uncouple interneuron laminar fates from projection cell laminar fates—a point to be taken up in the Discussion.

Interestingly, the laminar positions of middle- and late-born Dlx$^+$ cells shifted during the first postnatal week in *reeler*, as in normal mice. Early-born cohorts in *reeler* (E10.5–E12.5) did not shift positions, but remained in superficial layers (including the superplate) from P0.5 (Fig. 8Aa–Ac) to P7.5 (Fig. 8Ba–Bc). Many middle- and late-born Dlx$^+$ cells (E13.5–E16.5) shifted from positions in the superplate (top two bins) on P0.5 (Fig. 8Ad–Ag), to positions in the deep half of the cortical plate on P7.5 (Fig. 8Bd–Bg). This shift of middle- and late-born interneurons out of the superplate in *reeler* was reminiscent of the shift out of the mz by the same cohorts in normal mice. Also, some interneurons migrated outward from the iz and vz/svz to the cortical plate in *reeler*, as in normal mice (Fig. 8).

Presumed projection cell production and laminar fates in reeler. The overall production of presumed projection neurons was low from E10.5 to E12.5, and higher from E13.5 to E16.5, in *reeler* (Fig. 9) as in normal mice (Fig. 7). As expected, the laminar fates of Dlx$^-$ cells were roughly inverted in *reeler*, as previously described for the total complement of cortical cells (Caviness, 1982; Polleux et al., 1998). The outside-in sequence was evident for Dlx$^-$ cells in P0.5 cortex (Fig. 9A) as well as P7.5 cortex (Fig. 9B). The only obvious shift involved late-born Dlx$^-$ cells (E15.5 and E16.5), which were relatively abundant in the iz on P0.5 (Fig. 9Af and Ag), but sparse in the P7.5 iz (Fig. 9Bf and Bg). This suggested that late-born presumed projection neurons migrated outward from the iz into the cortical plate during the first postnatal week in *reeler*, similarly to normal mice (although destination positions within the cortical plate were obviously different).

Comparison of interneuron and presumed projection neuron laminar fates in reeler. Interneuron cohorts in *reeler* generally occupied broader distributions than the same cohorts of presumed projection neurons. For example, late-born interneurons and presumed projection neurons (born on E14.5 to E16.5) both had peak distributions in the deep half of the P7.5 cortical plate, but the interneuron distributions extended more broadly into the upper half of the cortical plate and the superplate (Figs. 8 and 9). This interesting parallel with normal mice suggested that despite the cortical inversion, underlying relations between the laminar fates of interneurons and projection neurons were generally preserved.

CONCLUSIONS

Our results suggest that interneurons and projection neurons undergo distinct radial migrations during the first postnatal week (Fig. 10). It appears that middle- and late-born interneurons migrate both "inward" from the mz, and "outward" from the iz and vz/svz, to predetermined positions in the cortical plate. In contrast, late-born projection neurons migrate mainly outward from the iz. Despite their different migrations, the same cohorts of interneurons and projection neurons converge on similar laminar fates by P7.5. Late-born cohorts converge on superficial layers (2–4) in normal mice, but on deep cortical plate positions in *reeler* mice.

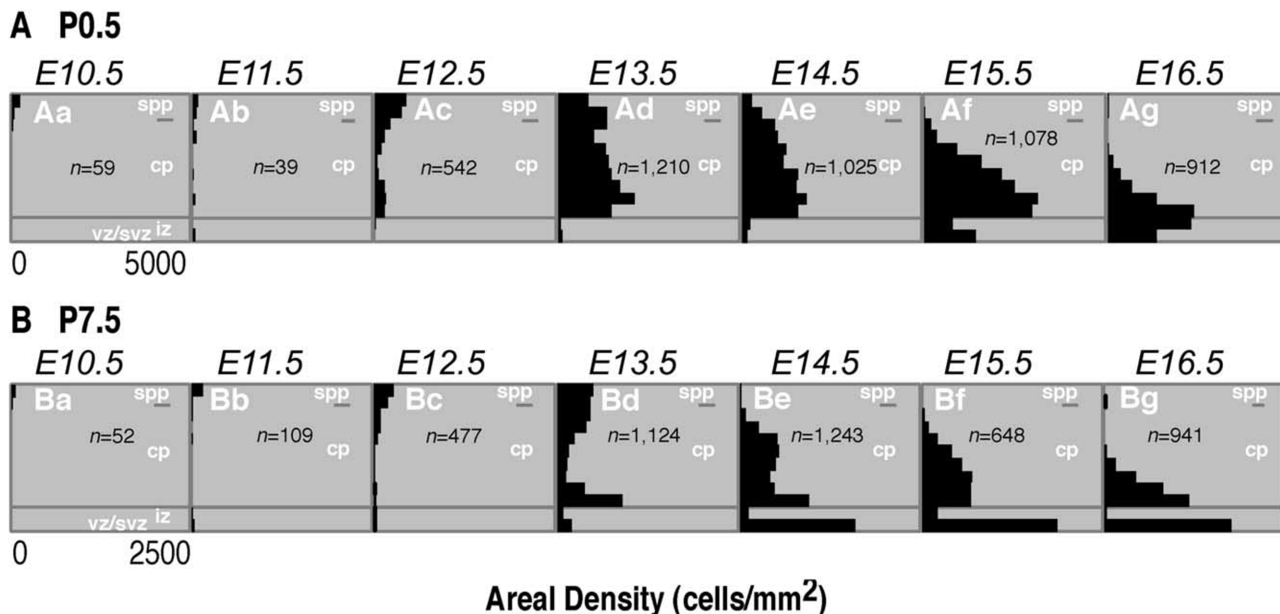


Fig. 9. Birthdays and laminar fates of Dlx^- cell cohorts (presumed projection neurons) in *reeler* parietal cortex. Graphs show the distribution of Dlx^- cells born on each embryonic day from E10.5–16.5, in *reeler* cortex on P0.5 (A) or P7.5 (B). The overall inverted, "outside-in" sequence of neurogenesis was evident at both postnatal time points. However, a proportion of Dlx^- cells born on E15.5 and E16.5 shifted positions out of the P0.5 iz (Af and Ag), and into deep layers of the cortical plate by P7.5 (Bf and Bg), suggesting that they migrated outward. Numbers (*n*) are the total number of $BrdU^+/Dlx^-$ cells counted for each graph. Data were produced and plotted as described in Fig. 6.

DISCUSSION

We used cell birthdating and double labeling for interneuron markers (GABA and Dlx) to test the hypothesis that cortical interneurons migrate radially during the first postnatal week. Previous studies have found that interneurons can migrate both toward and away from the ventricle during embryonic life, but the magnitude and duration of inward migration have been uncertain (Nadarajah et al., 2002, 2003; Polleux et al., 2002; Tanaka et al., 2003). Quite recently, studies by Ang et al. (2003) have suggested that the mz may be a primary route for tangential interneuron migration, and that inward migration may be an important mechanism for interneuron entry into the cortical plate (Ang et al., 2003).

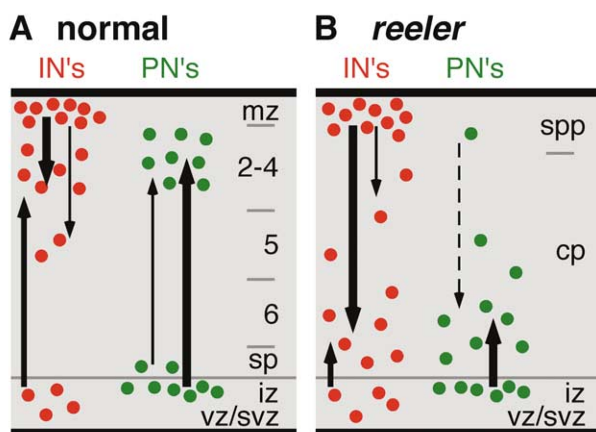


Fig. 10. Radial migrations in normal and *reeler* cortex during the first postnatal week. (A) In normal cortex, many middle- and late-born interneurons (IN′s) migrated inward, from the mz to upper cortical layers. In addition, some late-born interneurons and projection neurons (PN′s) migrated outward from the subplate (sp), iz and vz/svz to upper cortical layers. (B) In *reeler*, middle- and late-born interneurons migrated inward from the superplate to the deep cortical plate, and outward from the iz and vz/svz to the deep cortical plate. Late-born projection neurons migrated outward from the iz to the deep cortical plate. Dashed arrow indicates a small migration of projection neurons from the superplate to the deep cortical plate (Fig. 9). The width of each arrow represents the approximate magnitude of the migration.

The present study builds on previous work in several ways. Firstly, our results provide quantitative evidence, based on cell counting, that inward migration from the mz brings a major flux of interneurons into the cortical plate. Secondly, we demonstrate that interneuron radial migration (inward and outward) occurs not only prenatally, but also postnatally. Thirdly, we find that specific cohorts of interneurons (middle- and late-born cells) migrate radially during the first postnatal week. Fourthly, we show that in *reeler*, middle- and late-born interneurons migrate into the superplate (mz equivalent) on P0.5, but redistribute to form an outside-in gradient by P7.5. On the basis of these and other observations, we propose that interneurons and projection neurons use different mechanisms of radial migration, even though their laminar fates are ultimately coordinated with regard to cell birthday (Fairén et al., 1986; Peduzzi, 1988; Valcanis and Tan, 2003).

Shifts of interneuron distribution in the cortex

The results showed that interneuron distributions shifted during the first postnatal week, especially for middle- and late-born cohorts, in normal and *reeler* mice (Figs. 1, 2, 4, and 6–9). Several processes could explain these shifts, including

interneuron radial migration, maturation, proliferation, apoptosis, and tangential migration. We believe that the evidence favors radial migration, for the following reasons.

Maturation could not account for shifting interneuron distributions because the overall ratio of Dlx^+ cells to $GABA^+$ cells changed only slightly between P0.5 and P7.5 (Table 1). Moreover, the distributions of Dlx^+ cells and $GABA^+$ cells were virtually identical, except in the iz and vz/svz (Fig. 4). A significant number of $Dlx^+/GABA^-$ cells (presumed immature interneurons) were present in the iz and vz/svz on P0.5, and many of these cells appeared to mature concurrently with outward migration to the cortical plate by P7.5 (Fig. 4). However, there was no evidence that the mz contained many $Dlx^+/GABA^-$ cells, and thus maturation could not explain the marked decrement of $Dlx^+/GABA^+$ interneurons in the mz between P0.5 and P7.5 (Fig. 4). Overall, the results suggested that maturation had little impact on apparent interneuron distributions.

Interneuron cell birth (proliferation) and cell death (apoptosis) also could not account for the shifting interneuron distributions. Almost all cortical neurons are generated by E17.5, and neuron proliferation is not significant after P0.5 (Caviness et al., 1995; Levers et al., 2001). Moreover, our birthdating studies showed that specific cohorts of interneurons (middle- and late-born cells) shifted positions, and the apparent migration of these cohorts would not be affected by proliferation after P0.5. With regard to apoptosis, many studies have shown that apoptosis is infrequent in the early postnatal cortex, and only rarely involves cells in the mz (Finlay and Slattery, 1983; Ferrer et al., 1992; Spreafico et al., 1995; Valverde et al., 1995; Thomaidou et al., 1997; Kuan et al., 2000). The mz was the predominant layer where interneuron density decreased between P0.5 and P7.5 in the present study (Figs. 4, 6, and 8). [On the other hand, Blaschke et al. (1996) observed abundant apoptosis in the fetal cortex, but their study used a unique method and has not been replicated.] Finally, we detected no $GABA^+$ interneurons among >100 apoptotic cells (Fig. 5), suggesting that apoptosis of mature interneurons is exceedingly uncommon.

Tangential migration could potentially contribute to shifting profiles of interneuron radial distribution, if interneurons migrated tangentially out of (or into) specific cortical layers. However, whereas many studies have observed tangential migration through the *embryonic* cortex (De Carlos et al., 1996; Anderson et al., 1997; Tamamaki et al., 1997; Lavdas et al., 1999; Marín et al., 2001; Polleux et al., 2002; Ang et al., 2003; Tanaka et al., 2003), no studies have reported tangential migration in the *postnatal* mouse cortex. Indeed, tangentially migrating interneurons seem to be depleted from the neocortical iz by P0.5 (DeDiego et al., 1994). While it is conceivable that interneurons could migrate tangentially out of the neocortical mz and into the hippocampal molecular layer, this possibility has been discounted by a recent study showing that hippocampal interneurons also migrate inward during early postnatal development (Morozov and Freund, 2003). The parallel findings in hippocampus (Morozov and Freund, 2003) and neocortex (present study) suggest that inward radial migration of interneurons is a widespread phenomenon, and may occur throughout the early postnatal cortex.

Different mechanisms of radial migration by interneurons and projection neurons

What mechanism(s) could account for the robust inward migration of interneurons during the first postnatal week? Recent work by Stumm et al. (2003) suggests that a chemoattractant system involving stromal cell derived factor-1 (SDF-1), a chemokine secreted by the embryonic leptomeninges, and CXCR4, a receptor expressed by migrating interneurons, could play an important role. They found that SDF-1 was a potent chemoattractant for migrating interneurons, and that interneuron migration into upper cortical layers was dependent on the expression of SDF-1 and CXCR4. They also found that SDF-1 expression by meningeal cells was markedly down-regulated between P0 and P9, i.e. the same period when middle- and late-born interneurons shifted out of the mz and into upper cortical layers. We speculate that the down-regulation of meningeal SDF-1 expression reduces the outward attraction of interneurons and tips the balance toward inward migration, thus allowing interneurons to enter the cortical plate. Other, unknown factors would then be responsible for guiding interneurons to their appropriate laminar positions.

The role of reelin in regulating interneuron radial migration remains undefined. Whereas our present results demonstrated that interneurons, like projection neurons, followed an inverted, "outside-in" migration sequence in P7.5 *reeler* cortex (Figs. 8 and 9), reelin deficiency could have affected interneuron migrations directly or indirectly. Previously, reelin was found to directly regulate interneuron migration in the olfactory bulb (Hack et al., 2002), but direct effects on cortical interneuron migration have not been reported. Indeed, many interneurons migrate into the mz despite high levels of reelin, which seem to exclude cortical plate projection neurons from entering the mz (Hammond et al., 2001). In *reeler*, the lack of reelin did not impede interneuron migration into the superplate (mz equivalent) by P0.5, nor did reelin deficiency impede inward migration into the cortical plate by P7.5 (Fig. 8). Most significantly, the coordination of interneuron and projection neuron laminar fates was maintained in *reeler*, despite the cortical "inversion."

Are interneuron laminar fates regulated by signals from projection neurons?

The simplest explanation for our findings is that interneurons are guided to their ultimate laminar positions by signals derived from cortical plate projection neurons. We hypothesize that different cohorts of projection neurons express specific molecules that are recognized by interneurons with the same cell birthday. The cues from projection neurons could then signal interneurons to arrest radial migration in the appropriate layer. This hypothesis accounts for the coordination of interneuron and projection neuron laminar fates in normal as well as *reeler* mice (Fairén et al., 1986; Peduzzi, 1988; and the present study), and is consistent with the observation that interneuron and projection neuron laminar fates are specified at the time of

mitosis (McConnell, 1988; McConnell and Kaznowski, 1991; Valcanis and Tan, 2003). This hypothesis also explains the observation that interneuron positions depend on projection cell properties in chimeric *Dab1*$^{+/+}$/*Dab1*$^{-/-}$ mice (Hammond et al., 2001).

A similar hypothesis of cortical layer formation was recently proposed by Ang et al. (2003). They also suggested an alternative hypothesis, postulating that interneurons may receive cues for laminar fate from the cortical vz, which is contacted by some inwardly migrating interneurons (Nadarajah et al., 2002; Ang et al., 2003). However, the latter hypothesis would appear to conflict with the observation that interneuron laminar fates are determined at the time of mitosis (Valcanis and Tan, 2003), and with our present results from studying *reeler* mice. Since reelin is expressed only in the mz and cortical plate (Alcántara et al., 1998; Pesold et al., 1998; Hevner et al., 2003a), it is hard to imagine how the inversion of interneuron laminar fates could arise from changes in the postnatal vz of *reeler* mice.

Finally, one implication of our hypothesis is that projection neurons, guided by reelin and other signals, play the primary role in layer formation. Consistent with this idea, mutations that affect the development of projection neurons are often associated with cortical malformations (e.g. Schmahl et al., 1993; Mallamaci et al., 2000; Hevner et al., 2001; McEvilly et al., 2002; Sugitani et al., 2002), but mutations that disrupt interneuron development do not perturb cortical layers (Anderson et al., 1997; Sussel et al., 1999). Further experiments will be necessary to refine and test this hypothesis.

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