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ORIGINAL ARTICLE

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The role of pioneer neurons in the development of mouse visual cortex and corpus callosum

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Abstract The primordial plexiform neuropil is very critical to neocortical development. The pioneer neurons, mainly Cajal-Retzius cells in the marginal zone, and subplate neurons in the subplate, differentiate from the primordial plexiform neuropil. In this study, the development of corpus callosum, visual cortex, and subcortical pathways has been observed in C57BL/6 mice with various methods, such as DiI labeling in vitro and in vivo, DiI and DiA in vitro double labeling, immunocytochemistry, and in vivo BrdU and Fast Blue labeling. As early as E14, the primordial plexiform neuropil can be found in the telencephalic wall, and it contains many pioneer neurons. On E15 the primordial plexiform neuropil differentiates into the marginal zone and the subplate. Cajal-Retzius cells exist in the marginal zone, and subplate neurons are in the subplate. Either Cajal-Retzius cells or subplate neurons have long projections toward the ganglionic eminence, suggesting that they migrate tangentially from the ganglionic eminence. Cajal-Retzius cells are involved in radial migration, and subplate neurons participate to guide pathfinding of subcortical pathways. This study reveals how the pioneer neurons, through radial and tangential migration, play an important role in neocortical formation and in the pathfinding of the corpus callosum and subcortical pathways. Furthermore, DiI labeling in vivo has demonstrated the presence of pioneer neurons all along the corpus callosum pathway, especially in the midline. This suggests that pioneer neurons may also play a role in guiding the pathfinding of the corpus callosum.

Keywords Primordial plexiform neuropil · Subplate · Cajal-Retzius · Pathfinding · Migration

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Introduction

Various functional properties have been suggested for corpus callosum (CC) connections, such as binocular convergence and stereoscopic vision (Cynader et al. 1986; Gazzaniga 2000). Other suggestions are that the CC provides interhemispheric transfer of learned visual discrimination, as well as midline fusion of the hemifields. Visual experience is required for normal stabilization of mouse CC connections (Schmidt and Lent 1987). In turn, the CC plays an important role in visual cortex development and normal physiology. For instance, the CC is a crucial participant in the numerous cortical functions requiring collaborative processing of information from both hemispheres. One such operation is to combine the two partial cortical maps of the visual field into a single, coherent representation (Houzel and Milleret 1999). Furthermore, Houzel and Milleret (1999) report that CC connections between the two hemispheres are not “point to point”. That is, one point has widespread arbors and terminates into a handful of distant, radially oriented tufts. Therefore, the firing of a single CC projection neuron (CCpn – cortical neurons, usually pyramidal cells in layers 2–6, that give rise to axons that cross the midline through the CC bundle) might influence several cortical columns within the opposite hemisphere. This physiological radial connection has special morphological and functional significance that strongly suggests that CCpn are a key to understanding the function and organization of the visual cortex, and that the CC is a very useful model in developmental neuroscience (Hilbig et al. 1997). The above studies, and our previous research that focused on elucidating CC development in visual cortex (Elberger 1979, 1993, 1994), were done in postnatal animals. The present study extends these analyses to the prenatal period to complete the morphological analysis of CCpn development.

In order to explain neocortical development, the ‘dual origin’ theory has been proposed. The main point of the theory is that the appearance of the cortical plate (CP) (future layers 2–6) depends on a structure named the primordial plexiform neuropil (PPN; Marin-Padilla 1978).

The CP is subsequently established between the superficial (marginal zone) and deep (subplate) components of PPN. This is similar to a sandwich model. Neurons in the early PPN are thought to play important roles in the formation of the cortex. For example, Cajal-Retzius (C-R) cells in the marginal zone (MZ) are instrumental in neuronal migration and laminar formation, whereas cells in the subplate (SP) are involved in the formation of cortical connections (Lavdas et al. 1999). Therefore, neuronal migration and laminar formation are critical to understanding the establishment of the CP. Neuronal migration occurs either radially or tangentially. In radial migration, radial glia assist post-mitotic neurons in migrating from the intermediate layer into the CP. Migrating neurons can be guided along radial glia to their destination in the CP (Lambert and Goffinet 1998), complying with the principal of " inside-out" in which the migrating neurons are first deposited into deeper regions and then into increasingly more superficial regions of the CP (Bayer and Altman 1991). However, tangential migration also is important for the development of the neocortex. Tangentially migrating neurons typically have a round or squamous shape, and their long axons point to the direction of migration (O'Rourke et al. 1997). Since tangential neuronal migration was first reported in the intermediate zone (IZ; Walsh and Cepko 1988, 1992), many studies have addressed this subject. Thus, the PPN is a useful concept for understanding neocortical development.

The success of cortical connections depends on the correct wiring of the individual parts. Thus, during development, axons have to find the way to make connections with their appropriate target cells. So far, the mechanism that determines the manner in which growth cones navigate through preexisting tissue to find their correct targets is not very well understood. Two hypotheses suggested for this pathfinding mechanism are mechanical guidance and chemical guidance (Ghysen and Chambly-Chaudiere 2000). The chemical guidance theory attributes axon navigation to chemical attractive or repulsive effects. Many molecular candidates for these effects are proposed, including cell adhesion molecule (CAM), neuron-glia CAM-related molecule (NGCAM), roundabout (Robo), and transiently expressed axonal surface glycoprotein 1 (TAG-1; Stoeckli and Landmesser 1998). In contrast, the mechanical guidance theory uses contacts, such as synaptic contact and cell body contact, to explain pathfinding (Rajnicek et al. 1997). One accepted example of mechanical guidance is the C-R cell, which was described 100 years ago, but its guidance character was not understood until recently (Frotscher 1998). The C-R cells can guide fibers in the proper direction through contact guidance. One pathway that uses this mechanical guidance for development is the entorhinal-hippocampal pathway (Ceranik et al. 1999, 2000). In addition, C-R cells can also secrete reelin to guide many radially migrating cells at the same time (Frotscher 1998). Therefore, cortical connectivity is aided by a number of factors.

Many studies have shown that radial and tangential migration of pioneer neurons is closely linked to the formation of cortex. However, most of these data are obtained using in vivo tracing techniques. While in vivo tracing provides direct evidence for radial and tangential migration, it is difficult to visualize the other structures of the developing cortex in the same material, which limits the completeness of the analysis. On the other hand, in vitro tracing fails to supply convincing evidence for actual cell migration. Recently, using in vitro immunocytochemistry and tracing techniques, tangentially migrating neurons were observed to usually have long projections extending toward the ganglionic eminence (GE; Del-Rio et al. 2000; Soria and Fairen 2000). The present study's use of in vitro DiI to label pioneer neurons and analyze their development is another innovation designed to enable a more complete analysis of development. This method not only simplifies the necessary technical procedures, but also provides the opportunity to observe cortical development in the same material. With DiI applied to the GE, the labeled pioneer neurons in the MZ, IZ and later in the SP shows migration from the GE. Furthermore, the ability in the present study to observe the differentiation of pioneer neurons, the development of the CC, its CCpn, and the neuron birth date in cortex provides an outline of the genesis of visual cortex. The additional benefit of determining the spatiotemporal relationship between migration of pioneer neurons and the development of the subcortical pathway and the CC further elucidates the roles of pioneer neurons in pathway finding. No doubt, the present results as well as our previous work (Elberger 1993, 1994, 1997) will be helpful to reach the above aims, leading to a more complete picture of the development of the CC and CC projection neurons in visual cortex.

Materials and methods

Animals

All experiments were carried out in accordance with institutional guidelines for animal welfare; the facility is AAALAC accredited. Male and female C57BL/6 mice (Jackson Labs) were placed in breeding cages in a standard laboratory animal housing environment with the light cycle of 12 h on, 12 h off. Embryonic (E = days postconception, E0 = the day of conception) or postnatal (P = days postnatal, P0 = the first 24 h after birth) offspring were produced from timed pregnancies. Pups were born on E18; prenatal animals were obtained by Caesarian section. A total of 151 pups at E13–18 and P0–7 were used for the study.

To obtain embryonic mice, at appropriate ages, the pregnant dams were anesthetized (sodium pentobarbital, 40 mg/kg, i.p.) and fetuses were excised. The fetal skulls were opened carefully. Whole brains were taken out of the skulls using a fine spatula and immersion-fixed in 4% paraformaldehyde in 0.1 M phosphate buffer (PB) (pH 7.2) for 1–2 days at 4°C. To obtain postnatal mice, at designated ages the postnatal pups were removed from the mother, anesthetized (sodium pentobarbital, 40 mg/kg, i.p.), and perfused transcardially with 4% paraformaldehyde in PB (pH 7.2). The brains were exposed and immersion-fixed in the skulls for 1–2 days at 4°C. After this, the brains were taken out of the skulls using a fine spatula.

DiI labeling in vitro

Two sites were chosen for application of DiI (1, 1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate, D-282, Molecular Probes, Oregon, USA). DiI crystal placement produces labeling by anterograde and retrograde diffusion of the dye within the lipid bilayer of cells that physically contact the crystal. In some cases, the two hemispheres were separated midsagittally along the longitudinal fissure by a sharp razor blade to expose the CC at the midline. Using a dissection microscope, one or two small DiI crystals (100-µm size) were implanted in the midsagittal CC to examine CC and CCpn development by direct observation of the occipital region. In other cases, a coronal cut removed the anterior one third of the brain in order to expose the ganglionic eminence (GE). Since the internal capsule penetrates through the GE, one crystal of DiI was placed into the GE of each hemisphere to retrogradely label migrating neurons in the IZ and also anterogradely label the subcortical path. After crystal placement, the brains were incubated in phosphate-buffered saline (PBS) with 0.2% sodium azide and 0.1% paraformaldehyde in the dark at 37°C for 1–2 weeks. The brains were coronally sectioned with a vibratome at 75 or 100 µm thickness. Serial sections were mounted on glass slides and coverslipped using 65% glycerin in PBS (Elberger and Honig 1990). Sections were observed and images captured using the 568 nm laser line on a confocal laser scanning microscope (CLSM, Bio-Rad MRC1024).

DiI and DiA double labeling in vitro

The same procedures for killing animals and fixing brains were used as described above. In some cases, crystals of DiI and DiA 4-(4-dihexadecylamino)styryl)-*N*-methylpyridinium iodide, Molecular Probes, D-3883) were implanted into opposite visual cortices from the dorsal surface in order to visualize the development of the CC bundle, including the midline crossing. In the cases with the anterior one third of the brain removed, DiI was inserted into the midsagittal CC above the exposed septum, and DiA was placed in the GE. This allowed observation of the development of CC projection neurons and SP neurons in the same visual cortex. The labeling process and incubation were carried out as described above. In the two conditions, some brains were horizontally sectioned to observe the CC bundle in or near the midline; other brains were coronally sectioned to study visual cortex development in detail.

Fast blue labeling in vivo

Mice at P0–P5 were anaesthetized (sodium pentobarbital, 40 mg/kg, i.p.). Pups were fixed in a perinatal head holder modified to be used with a stereotaxic apparatus (David Kopf Instruments, Tujunga, Calif., U.S.A.). The head skin was disinfected and incised. The skull was exposed and from bony landmarks, the approximate position of the left visual cortex was determined. Over this area, a small hole was made with fine scissors or a 30 gauge needle. Then 0.1 µl Fast Blue (3% Fast Blue, Sigma, St. Louis, MO., F-5756, dissolved in dH_2O) was injected into the CC deep to the visual cortex with a 0.5-µl-size Hamilton syringe. After suturing the skin, the pups survived in the mothers' care for 2 days. The transcardial perfusion and postfixation were carried out according to the DiI labeling protocol above. Using an epifluorescence microscope with an ultraviolet filter, 100-µm-thick coronal sections were examined to confirm the left side injection site and to examine the extent of contralateral visual cortex labeling.

Double labeling with DiI in vivo and immunocytochemistry

In P0–P2 mice, the supernatant of a centrifuged saturated solution of DiI (100 mg DiI dissolved in 2 ml 100% EtOH) was injected, and procedures were as described for Fast Blue, above. The animals were killed two days after injection. After transcardial perfusion as described above, the brains were immersion-fixed overnight. Coronal 100-µm-thick sections were made with a vibratome, and washed three times in 0.1 M PB. To block nonspecific binding, the sections were incubated in 0.5% normal goat serum (NGS, Sigma, St. Louis, Mo.) for 30 min. After rinsing (three times for 5 min) in PB, the primary antibody (dilution 1:10, mouse anti-vimentin monoclonal IgM antibody, produced by Alvarez-Buylla A, Hybridoma Bank) was added, and the sections were incubated overnight at 4°C. After rinsing in PB, the sections were incubated in goat anti-mouse fluorescein-conjugated IgM (dilution 1:100 in PB, Vector, Burlingame, Calif., FI-2020) for 1 h at room temperature. After rinsing in PB, sections were mounted onto glass slides and coverslipped in 65% glycerin in PBS, then examined and photographed using separate scans with the 488 and 568 nm laser lines of the CLSM.

5-Bromo-deoxyuridine (BrdU) staining

To determine neuronal birth date, BrdU, an S-phase specific tracer, was used. A solution of 7.5 mg BrdU (Sigma, St. Louis, Mo., B-5002) was dissolved in 500 µl sterile 0.007 N NaOH physiological saline. Using a dose of 5 µg/gm, the BrdU solution was injected into the peritoneal cavity of pregnant mice on E12–E18 and in pups P0–P7. To intensify the effect, a second dose was given 3 h later. Animals were killed 1–5 days after injection. The embryonic brains were immersion-fixed, but postnatal pups were transcardially perfused and brains were post-fixed in 4% paraformaldehyde in 0.1 M PB overnight at 4°C. After fixation, coronal slices 125-µm-thick were cut with a vibratome. To visualize the BrdU in nuclear DNA, sections were incubated in a 4 M HCl solution for 10 min to denature the DNA. Sections were then thoroughly rinsed five times in PB to wash away the HCl in tissue. Normal goat serum 0.5% was applied to block nonspecific binding. Sections were incubated in mouse anti-BrdU IgG (dilution 1:10, produced by Kaufman, SJ, Hybridoma Bank) overnight at 4°C. After washing, horse anti-mouse fluorescein-conjugated IgG was added, and sections were incubated at room temperature for 1 h. Sections were mounted onto glass slides and coverslipped in 65% glycerin in PBS. Sections were examined and images captured using the 488 nm laser line of a CLSM.

Immunocytochemistry

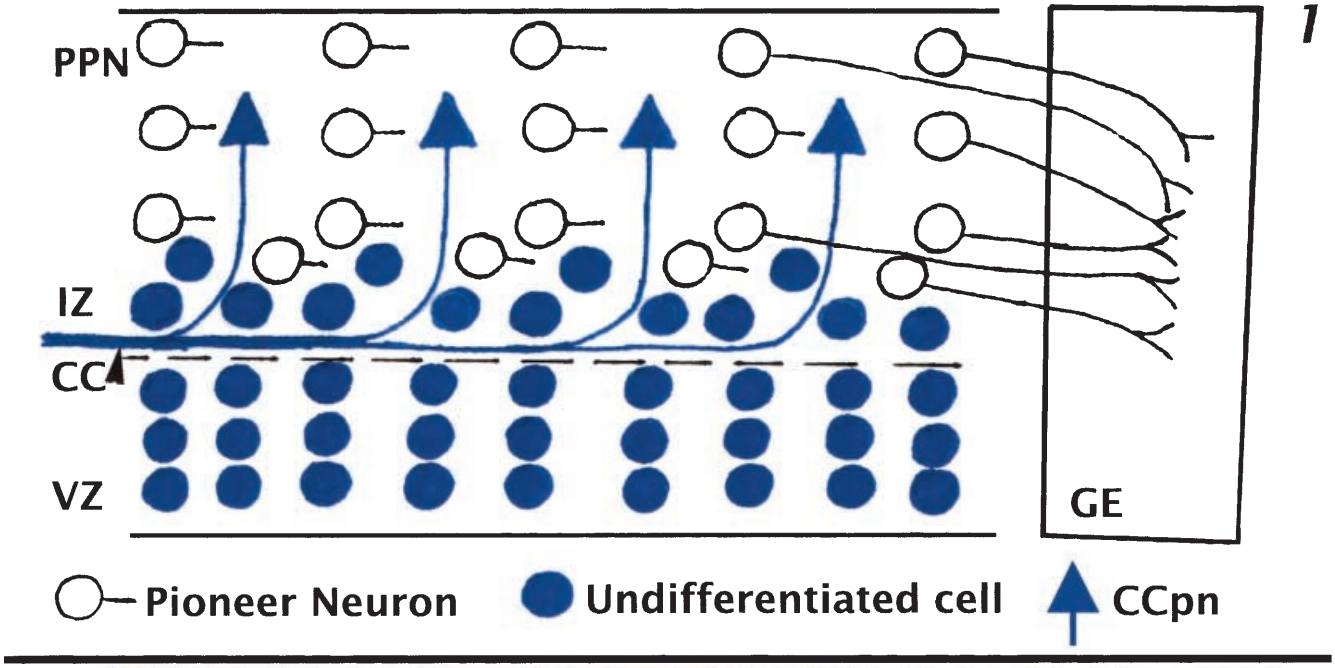
Neurofilament is an intracellular structure found in neurons, and is abundant in the cytoplasm of fibers. Specifically, it is one of the intermediate filaments in the neuron, and it is composed of polymer proteins (Graham 1999, Julien 1999, Herrmann and Aebi 2000). The dense fibers in the CC and subcortical pathways can be visualized with an antibody to neurofilament, so that axonal development in these pathways can be studied. Pregnant mice were used to produce E15–E18 embryos as described above. Brains were immersion-fixed in 4% paraformaldehyde for 1–2 days at 4°C, and 100-µm-thick sections were cut with a vibratome. Anti-neurofilament IgG (1:250 dilution, Chemicon, Temecula, Calif., MAB 1621) as primary antibody, and goat anti-mouse fluorescein-conjugated IgG (1:200 dilution, Chemicon, AP124F) were used in a routine immunocytochemistry protocol as described above. Sections were mounted and examined as above using the CLSM.

Results

Primordial plexiform neuropil (PPN) and the development of neocortex

DiI placement in the GE

The PPN concept and 'dual origin' theory are helpful to explain the development of cortex. The main point ex-



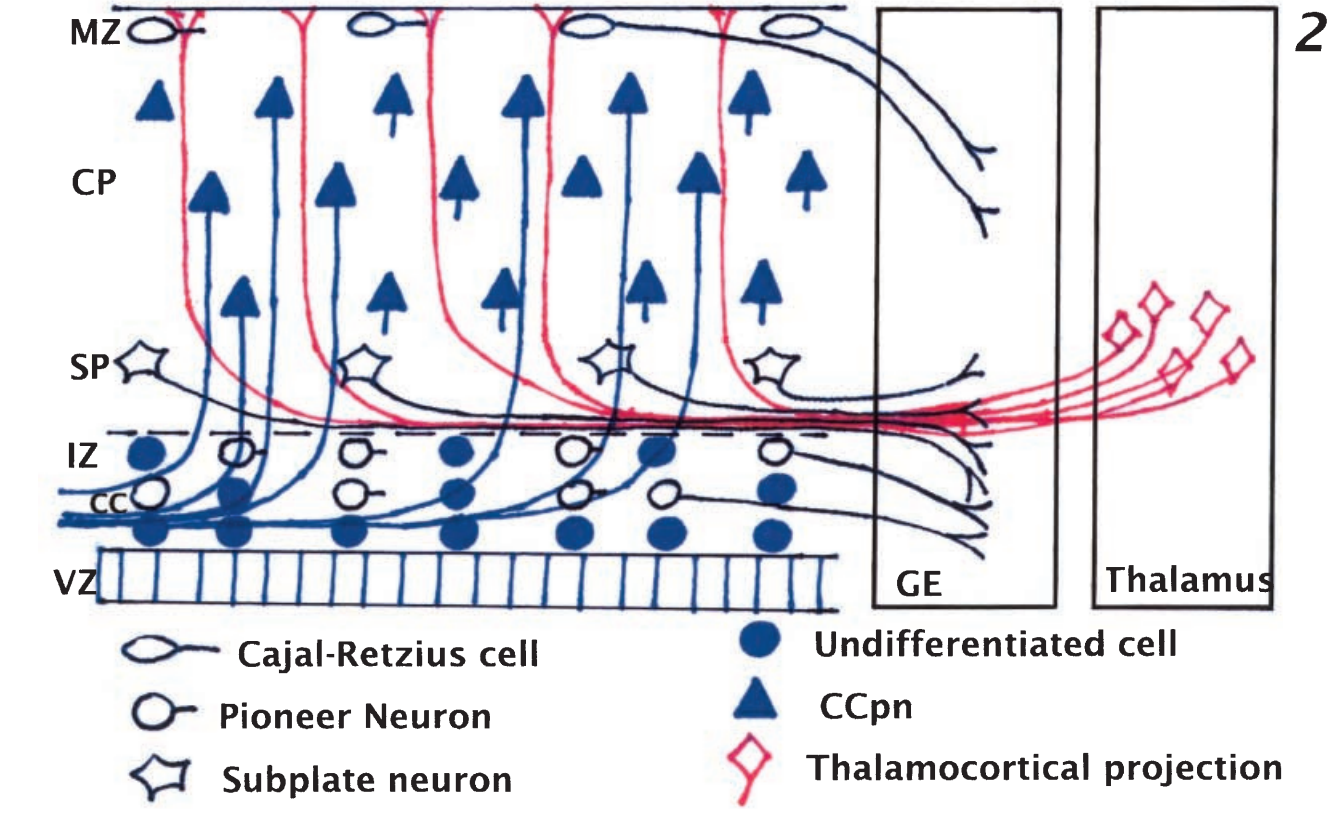


Fig. 1 This diagram represents the state of development at around E15. The PPN's function and the formation of the CP are illustrated. The PPN is superficial to (above) the VZ. There are many pioneer neurons inside the PPN. Their projections extend toward GE. At this early age, the ventricular zone is the anlage of neuron genesis. Inside the VZ there are many undifferentiated cells which can enter the IZ to participate in the formation of the CP. When some undifferentiated cells become postmitotic, they penetrate into the PPN. The initial 'sandwich' structure is the basement of the future neocortex. The superficial pioneer neurons differentiate into C-R cells. The deep pioneer neurons differentiate into SP cells. The postmitotic neurons in the middle develop into the pyramidal neurons in the CP. The pyramidal cells in this scheme refer to CCpn that have long projections toward the CC

Fig. 2 This diagram represents the state of development at around P0. This shows the functions of C-R cells and SP cells in cortical development and subcortical pathfinding. Pioneer neurons in the SP and MZ have differentiated SP neurons and C-R cells. The C-R cells participate in the radial neuron migration and subsequent lamination of the CP. This study shows the CCpn pyramidal cells. These DiI-labeled CCpn distribute in layers 3–5. SP neurons are inside the SP. SP neurons and their fibers guide the pathfinding of thalamocortical fibers. Because of different guidance mechanisms, the CC and subcortical pathways have their own distinct projection direction and location. The IZ contains many immature cells that are pioneer neurons from the GE, as well as undifferentiated cells from the VZ. At this time, the VZ has lost its proliferative function; therefore it reverts to a simple columnar epithelium

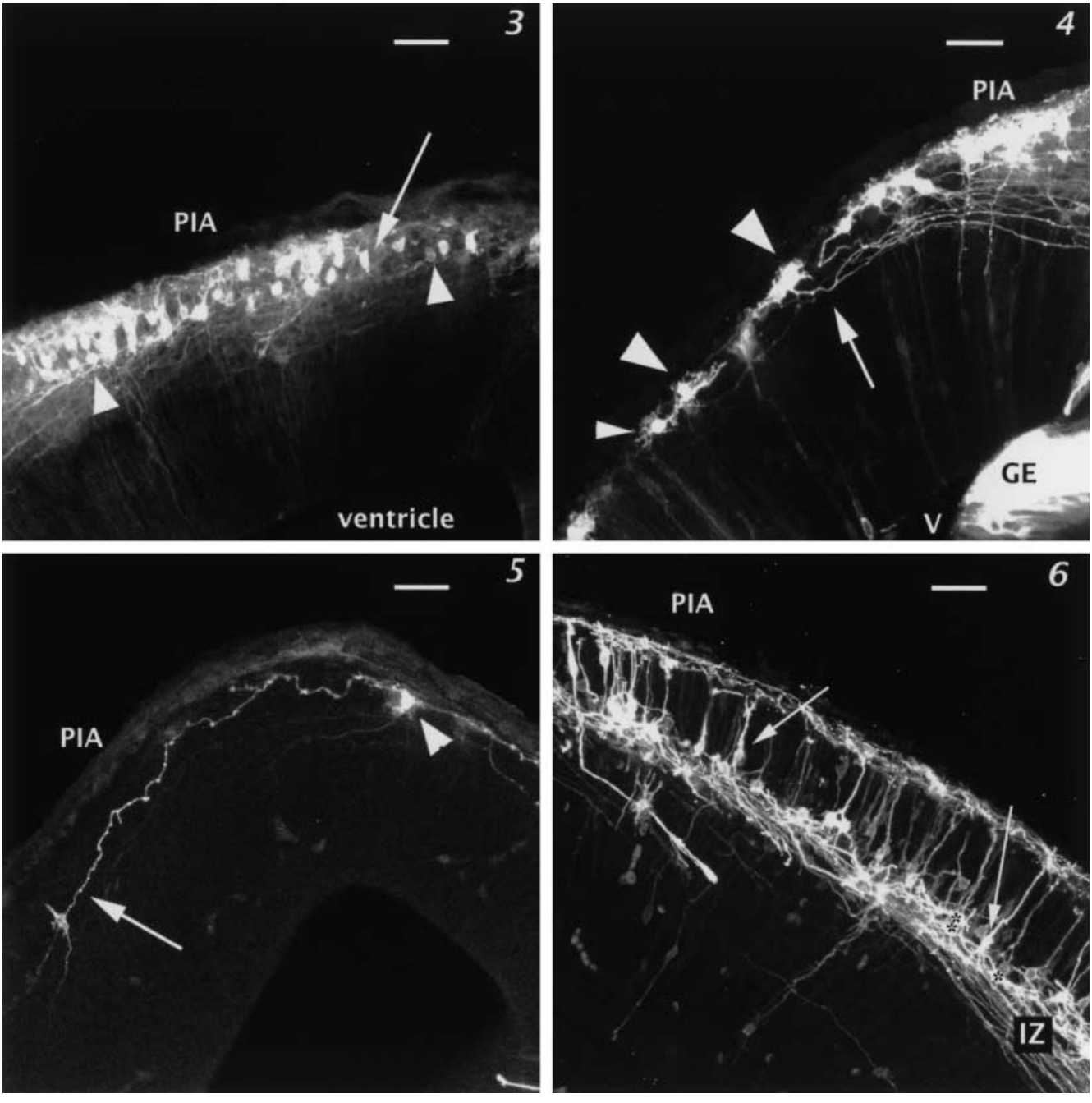


Fig. 3 E14 embryo. A crystal of DiI was placed into the GE. The pia and ventricle are indicated. The main labeled structure in the telencephalon is the PPN (*arrow*). At this age, the PPN is very thin. There are some pioneer neurons within the PPN. Pioneer neurons appear round, and have a long process projecting toward the GE (*arrowheads*). *Bar* 50 µm

Fig. 4 E15 embryo. A crystal of DiI was placed into the GE. The pia, GE and ventricle (*V*) are indicated. There is a layer of C-R cells along the MZ. The fusiform like C-R cells are oriented horizontally in the MZ, and are multipolar. The C-R cells have a long process (*arrow*) projecting toward the GE. Some short dendrites (*small arrowhead*) appear around the C-R cell bodies (*large arrowheads*). *Bar* 50 µm

Fig. 5 E17 embryo. A crystal of DiI was placed into the GE. One C-R cell (*arrowhead*) is in the MZ. The C-R cell has two long projections. One of them (*arrow*) extends to the GE, which is to the lower left but not visible. The pia is indicated. *Bar* 50 µm

Fig. 6 E15 embryo. A crystal of DiI was placed into the GE. In the IZ (*indicated*) there are many pioneer neurons (*asterisks*). Some pioneer neurons are beginning to differentiate into SP neurons (*arrows*). This process of differentiation is accompanied by significant changes in the shape of the cell body. For instance, the size has increased, the polarity has switched from horizontal to vertical, and the shape has become triangular or irregular. The mature SP neurons have apical dendrites extending toward the pia (*indicated*) and long axons extending toward the GE. *Bar* 50 µm

plored in this is that near the end of the second week of gestation, the PPN consists mainly of pioneer neurons, and at the same time, fibers exist superficial to the IZ. Later in development, the postmitotic neurons from the IZ migrate into the PPN in an 'inside-out' pattern to form the CP. In the present study, the processes involved in cortical development have been diagrammed (Figs. 1, 2).

With DiI placed in the GE, labeled cell bodies and their projections could be visualized as early as E14. The structure detected on E14 was the PPN located over the ventricular zone. The PPN contains many pioneer neurons that are small-sized, bipolar, and round or fusiform in shape (Fig. 3). Usually a long projection extends from the cell body of a pioneer neuron towards the GE. At this age, it is difficult to identify the IZ from the PPN. The ventricular zone (VZ) can be roughly identified from the tissue background. With increasing embryonic age, more and more pioneer neurons join the PPN from the IZ.

The main change on E15 is the differentiation of C-R cells and SP neurons. When the postmitotic neurons migrate into the PPN from the IZ, the CP is formed inside the PPN. At this age, the PPN is subdivided into two components (Fig. 1). The superficial component forms the MZ, and the deep component forms the SP. The pioneer neurons in the MZ differentiate into C-R cells, while the pioneer neurons in the SP differentiate into subplate neurons. The C-R cells, arranged horizontally within the MZ, are multipolar with squamous-shaped cell bodies (Figs. 4, 5). An axon extends from one side into the GE, and from the other side, short dendrites extend in all directions (Figs. 4, 5). The C-R cells can project over long distances, and are found in the MZ at prenatal and postnatal ages. In contrast, some pioneer neurons superficial to the IZ also begin to differentiate into SP neurons. These pioneer neurons initially gather in the superficial IZ. At first, these pioneer neurons form a thin sheet of SP cells. Then, with more and more pioneer neurons differentiating into SP neurons, the SP becomes thick and stratified. Generally the SP in ventrolateral cortex is thicker than in dorsal cortex and contains more neurons inside it than in cingulate and visual cortex. However, many pioneer neurons still remain in the IZ, and they keep their original morphology (Fig. 2). Compared with pioneer cells in the IZ, the neurons in the SP become relatively large, and they are of various shapes (Figs. 6, 7). Triangular and irregular shapes are common. With the maturity of SP neurons, the SP neurons generally change the polarity of their processes from horizontal to vertical (Figs. 6, 7). The apical dendrite extends to the pia, and the basal axon enters the GE. This morphology is similar to that of pyramidal cells in the CP, and therefore supports the theory that SP neurons eventually merge into the CP and change into pyramidal cells. With DiI placed in the GE, the pyramidal cells in the CP are not labeled, but the presence of a gap (cell-free zone) between the MZ and SP suggests that the CP begins to exist at this age (Figs. 6, 7). Interestingly, the telencephalic wall is thickest at the ventrolateral edge. The visual and cingulate cortices are thin and lack DiI-labeled cells. These observations strongly suggest that the telencephalic wall develops earlier near the ventrolateral edge than at the dorsomedial edge.

The obvious change on E16 is the appearance of subcortical fibers in the IZ. The telencephalic wall is thicker than on E15, and the subcortical fibers in the IZ become dense and crowded. There are still many migrating pioneer neurons among the fibers (Fig. 8). When the subcortical fibers are examined carefully, the afferent fibers, efferent fibers and migrating projection neurons can be identified. Efferent fibers originate from the SP neurons, and they enter the GE through the subcortical pathway. Afferent fibers come from the GE. Some afferent fibers reach the cortex through the subcortical pathway and extend to the cortical surface, but most afferent fibers are en route in the subcortical pathway. These latter fibers are easily identified by the growth cones on their tips (Fig. 8). Other observed fibers belong to the pioneer neurons in the IZ (Fig. 2).

On E17, more and more thalamocortical and corticospinal fibers join the subcortical pathway. As a result, the subcortical fibers become more numerous and they increase the thickness of the subcortical pathway. Before E17, the subcortical pathway usually is located in the outer IZ. After E17, the subcortical pathway is more dense, and it not only occupies the outer IZ, but also extends toward and through the SP. This leads to the neurons in the SP being surrounded by the dense fibers that now occupy the subcortical pathway, including the region where the SP cell bodies are located. By E17, the cell-free zone between the SP and the MZ becomes increasingly wide, which further increases the thickness of the CP (Fig. 9). Many fibers leave the subcortical pathway and turn vertically into the neocortex. These fibers are usually arranged radially in the neocortex (Figs. 2, 9).

DiI placement in midsagittal corpus callosum

After E15, the CC pathway can begin to be traced. At this age the CC fibers are few and thin, and they occupy the inner-intermediate and subventricular zones. Many growth cones can be found at the tips of the CC fibers. The CCpn in the CP are not easy to visualize until E17. Sometimes radial glia can be seen in the cortex. The cell bodies of radial glia, located in the ventricular zone, are oval in shape. They have two projections, one extending to the cortical surface and the other extending to the ventricular surface (Fig. 10). The basis for DiI labeling of the radial glia is uncertain. Some authors attribute this to DiI diffusion from the DiI placement site (Roberts et al. 1993). On E16 a few CCpn in the CP are faintly labeled, and they have an indeterminate shape (data not shown).

The major change at E17 is that the labeled CC fibers become denser, and CCpn begin to appear in the deep CP, the future layers 4 and 5 (Fig. 11). The CC fibers occupy the inner-intermediate and subventricular zones. The CCpn in visual cortex can be labeled from this age, onward. The CCpn are located in the deep CP and

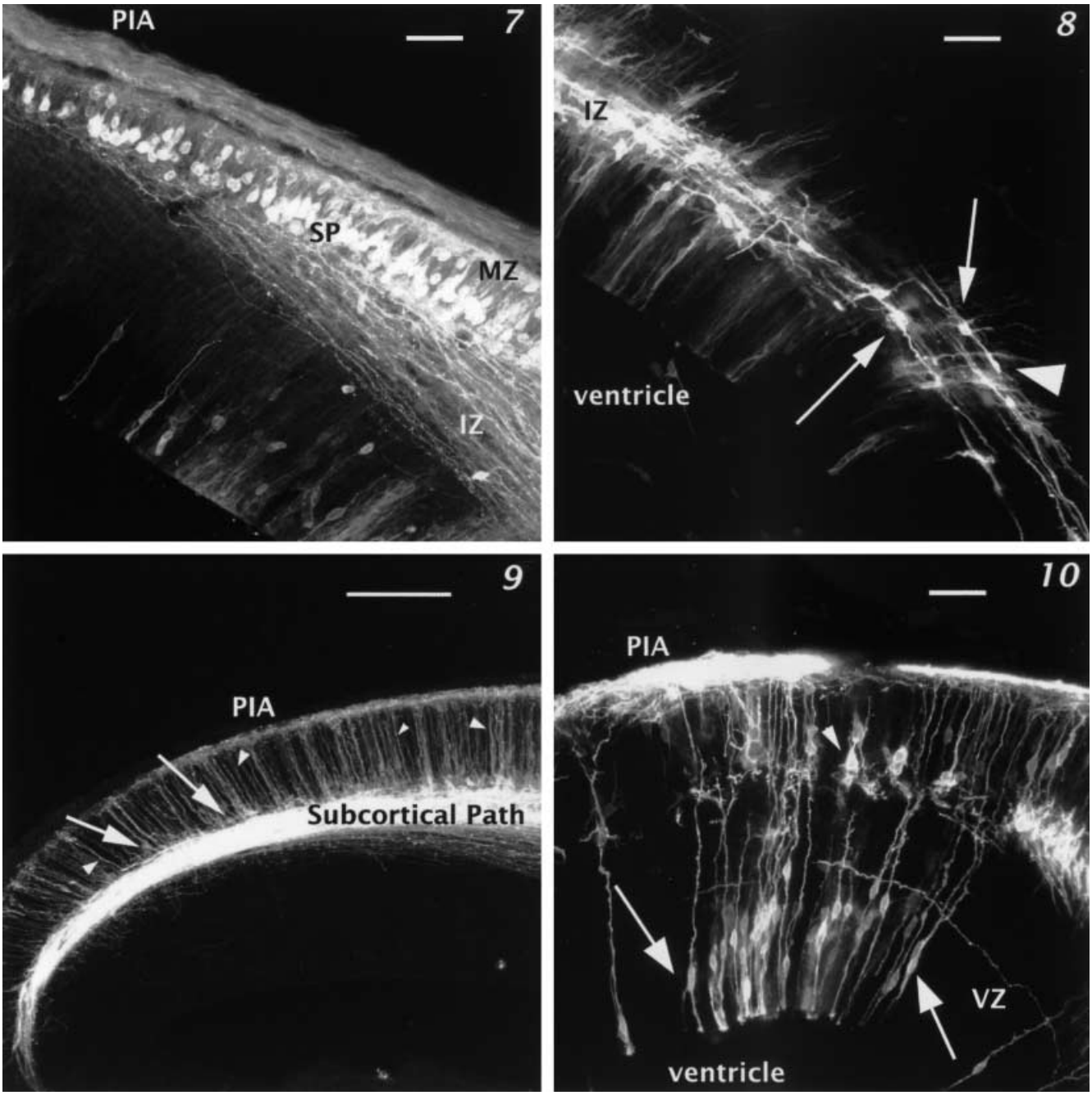


Fig. 7 E15 embryo. A crystal of DiI was placed into the GE. The telencephalic wall is visible. Subplate neurons are grouped superficial to the IZ (*indicated*). The pioneer neurons still within the IZ are small, round or fusiform in shape, and are horizontal. In comparison, the SP neurons are large, have a typical basket cell shape, and have more of a vertical orientation. The CP will eventually be formed between the MZ (*indicated*) and the SP (*indicated*). The pia is indicated. *Bar* 50 µm

Fig. 8 E16 embryo. A crystal of DiI was placed into the GE. The ventricle is indicated. At this age there are many pioneer neurons (*arrows*) and fibers in the IZ (*indicated*). Growth cones (*arrowhead*) can be seen on some fiber tips that extend toward the visual zone. *Bar* 50 µm

Fig. 9 E17 embryo. A crystal of DiI was placed into the GE. The pia is indicated. The subcortical path (*indicated*) in the IZ is intensely labeled. These abundant fibers (*arrowheads*) then leave the IZ and turn sharply to enter the neocortex and distribute themselves in the neocortex radially. Some SP neurons (*arrows*) are visible among the fibers. *Bar* 50 µm

Fig. 10 E17 embryo. A crystal of DiI was placed into the GE. The pia and ventricle are indicated. Radial glial cells (*arrows*) and SP cells (*arrowhead*) are visible. Radial glia can occasionally be observed in the telencephalic wall. Usually radial glia are located in the VZ (*indicated*). Their cell bodies are ovoid, and have one projection each toward pial and ventricular surfaces. It should be emphasized that radial glia are rarely labeled by DiI, and may have occurred in this tissue when DiI diffused a far distance from the crystal. *Bar* 50 µm

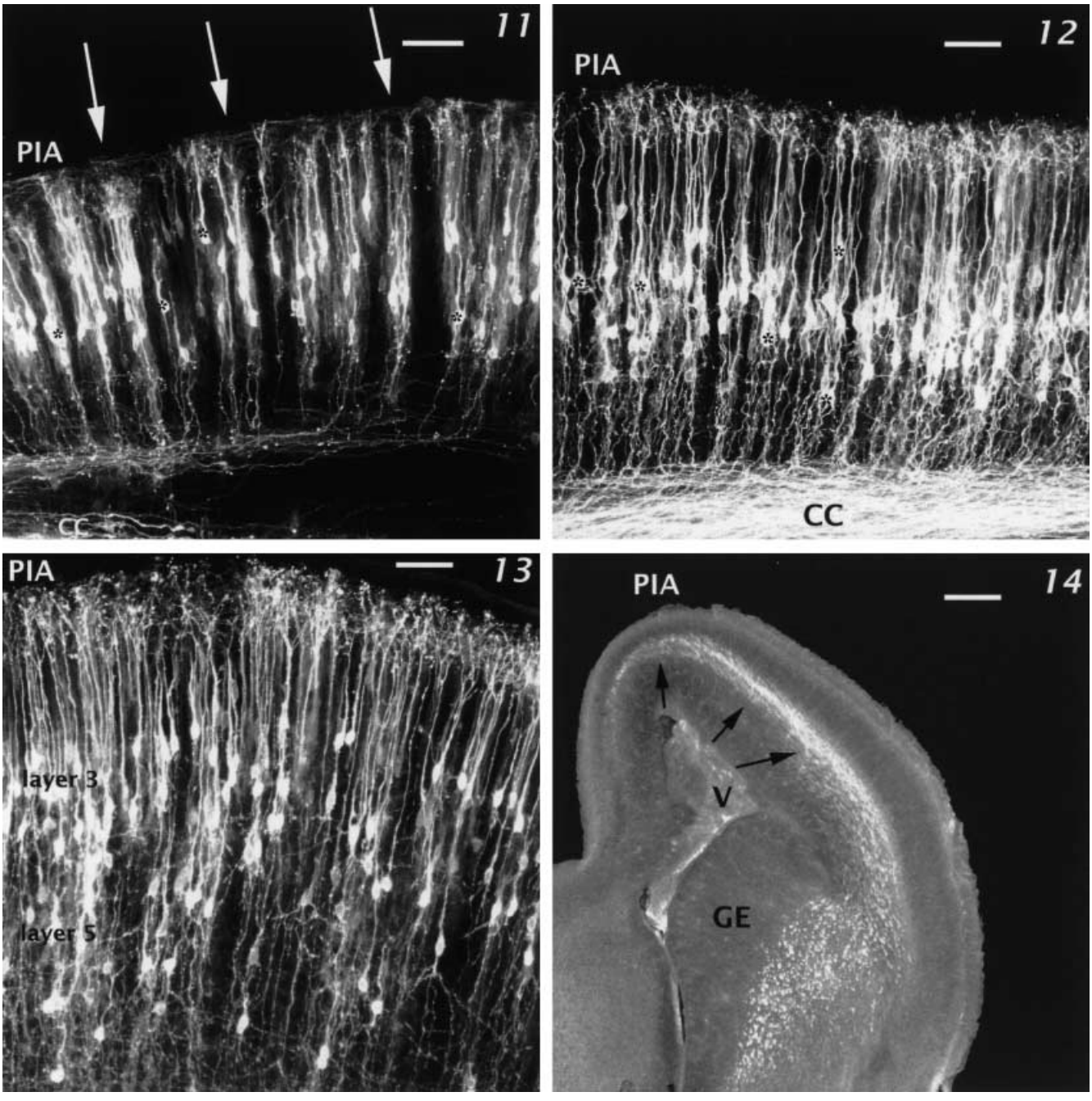


Fig. 11 E17 embryo. A crystal of DiI was placed into the midsagittal CC. The pia and labeled CC bundle are indicated. In the future visual zone; CC projection neurons show a columnar organization. The CC projection neurons (*asterisks*) located in the lower CP (future layers 4 and 5) show no distinct laminar organization. *Bar* 50 μm

Fig. 12 E18 embryo. A crystal of DiI was placed into the midsagittal CC. The pia and labeled CC bundle are indicated. In the future visual zone; CC projection neurons (*asterisks*) are distributed in the lower CP (future layers 4 and 5). No obvious CCpn neuronal lamination pattern can be seen. *Bar* 50 μm

Fig. 13 P0 embryo. A crystal of DiI was placed into the midsagittal CC. In the visual zone, CC projection neurons display a laminar arrangement. Many CC projection neurons migrate in 'inside-out' pattern to form layer 3 above layers 4 and 5. Other CCpn are scattered in layer 4 and layer 5. The pia, layer 3, and layer 5 are indicated. *Bar* 50 μm

Fig. 14 E18 embryo. A coronal section was immunocytochemically reacted for neurofilament. Neurofilament, which is present in axons, labels the CC/subcortical pathway (*arrows*). The pia, GE, and ventricle (*V*) are indicated. *Bar* 50 μm

packed together. Prior to birth, CCpn are small, and oval or round in shape. However, they have neuronal characteristics such as polarity and identifiable dendritic and axonal processes. The CCpn has an apical dendrite extending to the cortical surface. Near the end of the main trunk, this apical dendrite branches into many arbors extending to the cortical surface. A single axon extends in the direction opposite that of the apical dendrite, and finally converges into the CC bundle. In early cortical development, the radial arrangement of CCpn initially appears like a "cortical column" in which the cell bodies and projections cluster together in one column. Between columns there is an obvious gap (Fig. 11). With increasing age, the CCpn distribute tangentially in bands.

On E18, the CP has increased in thickness, and CCpn have matured. More and more DiI labeled CCpn are located in the inner CP, and the cell bodies are larger than on E17 (Fig. 12). The neuronal clustering or columnar distribution is hardly visible on E18 (Fig. 12). At E18 (Fig. 12) as well as E17 (Fig. 11), the CC projection neurons are concentrated in the inner CP, the future layers 4 and 5. Layer 3 will not be formed until P0. Morphologically and functionally, the visual cortex can be divided into areas 17 and 18. From cytoarchitectural landmarks, these two areas could be roughly identified in the present study, but there is no significant structural difference between them at these ages.

After birth, the CCpn have more typical neuronal characteristics. The CP is very thick, and CCpn in the CP are transitioning to laminar concentrations. On P0, postmitotic neurons migrate through the inner CP (future layers 4 and 5) into the outer CP to form a distinct layer 3 (Fig. 13). With the thickening CP, more and more neurons migrate through layers 4 and 5 to reach layer 3. As a result, layer 3 becomes dense and distinct from layers 4 and 5. At the same time, the inner CP (future layers 4 and 5) expands, and the CCpn are scattered loosely in layers 4 and 5 (Figs. 2, 13).

Neurofilament-positive fibers

Using a neuron-specific marker, the CC and subcortical pathways are both labeled in the same brain tissue. Because the CC and subcortical pathway fibers are closely adjacent, the CC can not be distinguished from the subcortical pathway by neurofilament immunostaining. Therefore, this is collectively called the CC/subcortical pathway. As early as E15, the CC/subcortical pathway is labeled in the IZ and subventricular space (data not shown). With increasing age, the CC/subcortical pathway becomes larger and denser. The fibers near the commissure are thinner than the fibers near the ventral region, as observed with DiI labeling. The conjoined CC/subcortical pathway fibers branch near the basal ganglia into two bundles. One branch continues into the intermediate and subcortical zones. This is the anlage of the external capsule. Another branch makes a turn into the basal ganglia. This is the anlage of the internal capsule (Fig. 14).

Double-labeled CC projection neurons and subplate neurons in the visual cortex

Using DiI and DiA double labeling, DiI-labeled CCpn and DiA-labeled subplate neurons can be compared in the same section. Both types of neurons are visible in the same tissue as early as E16, but at this age the subcortical fibers and subplate neurons are more prominent than CCpn and their fibers even though the DiA application site is farther from the visual zone than the DiI application site (Fig. 15). That means the subplate neurons and the subcortical pathway develop earlier than the CCpn and the CC pathway. On E18 the visual cortex is relatively mature. Both DiI- and DiA-labeled neurons are increased in number. The CCpn are located in the CP, and subplate neurons are located in the SP at E18 (Fig. 16). However, the subcortical pathway occupies the upper-intermediate zone, and the CC pathway occupies the low-IZ and subventricular space (Figs. 2, 16). Images with double labeling show that subplate neurons are irregular and triangular and usually larger than CCpn, which appear small and round.

Extension of CC fibers and midline crossing

DiI- and DiA-double labeling

Placement of DiI and DiA separately into opposite visual cortices provide a vivid analysis of the timing of CC fibers crossing between the hemispheres. The CC bundle forms as early as E15, when it extends to the ipsilateral cingulate cortex. The CC fiber tract appears like an arrowhead with many growth cones on the tips of fibers (data not shown). Some migrating cells can be found among the CC fibers, suggesting that the CC bundle is pathfinding to the contralateral telencephalon. Due to the longitudinal fissure, there are separate CC bundles at this age. However, on E16 the septum has formed, providing a bridge for the CC bundles to cross the midline. At this time, the CCs from the two hemispheres appear to contact each other (Fig. 17), but they never extend into the contralateral cortex until E17. The CC midline crossing begins on E17. Deep to the pia over the cingulate cortex, the CC penetrates to the contralateral telencephalon along the dorsal septum. On E18 the whole process is completed, and CC projection neurons are observed in contralateral cingulate cortex, even as far as the dorsal cortex (Fig. 18).

Fast blue tracing in vivo

To provide additional information on the development of CCpn distribution patterns, Fast Blue tracing in vivo was used. With DiI tracing in vitro, cell bodies, dendrites and axons are easily visible in groups or in single units. In contrast, Fast Blue mainly labels neurons retrogradely, however axons are only visible in large groups and den-

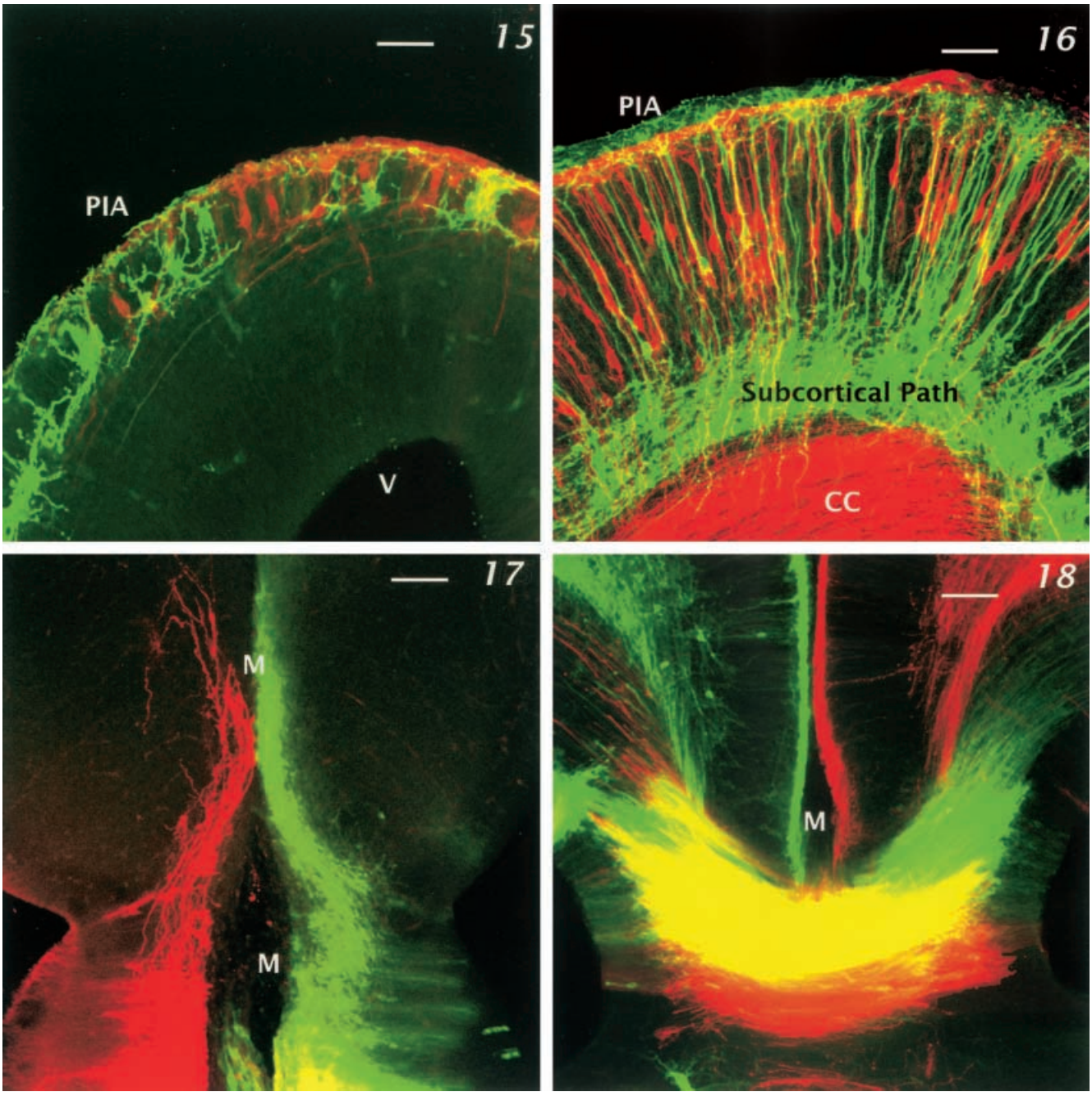


Fig. 15 E16 embryo. A crystal of DiI was placed into the midsagittal CC, while a crystal of DiA was placed into the GE. The pia and ventricle (*V*) are indicated. In the future visual zone, DiI (*red*) labels CC projection neurons, and DiA (*green*) labels SP neurons. The CC projection neurons are pyramidal-shaped, while the SP neurons are basket-shaped. Despite the closer distance from the crystal placement, only a few DiI-labeled cells appear in the future visual zone and cingulate cortex, and they are mainly located in the CP. However, at a farther distance from the crystal placement, DiA labeled many neurons in the subplate. *Bar* 50 μm

Fig. 16 E18 embryo. A crystal of DiI was placed into the midsagittal CC, while a crystal of DiA was placed into the GE. In the future visual zone, the CC and SP labeling are spatially separated. The CC projection neurons (*red*) are superficial to the SP neurons (*green*). Deep to these neuronal distributions, the subcortical pathway is superficial to the CC fibers. Thus, the superficial-deep arrangement of CC-SP neurons is reversed for the CC and subcortical pathways. Most SP neurons are interspersed within the subcortical pathway, and therefore are hidden behind subcortical fibers. The pia, subcortical pathway, and CC are indicated. *Bar* 50 μm

Fig. 17 E16 embryo. Crystals of DiI and DiA were placed in symmetrical locations on the cortical surface of the future visual zone in opposite hemispheres. Horizontal sections were viewed to determine the growth of the CC. The CC fibers are seen navigating toward the septum. The red (*DiI*) and (*DiA*) green fibers from opposite hemispheres physically contact each other, but do not cross the midline (*M*) toward the contralateral cortex. Growth cones can be seen on tips of the fibers. *Bar* 50 μm

Fig. 18 E18 embryo. Crystals of DiI and DiA were placed in symmetrical locations on the cortical surface of the future visual zone in opposite hemispheres. At this age, the red (*DiI*) and green (*DiA*) CC fibers have crossed the midline (*M*) into the opposite hemisphere. *Bar* 50 μm

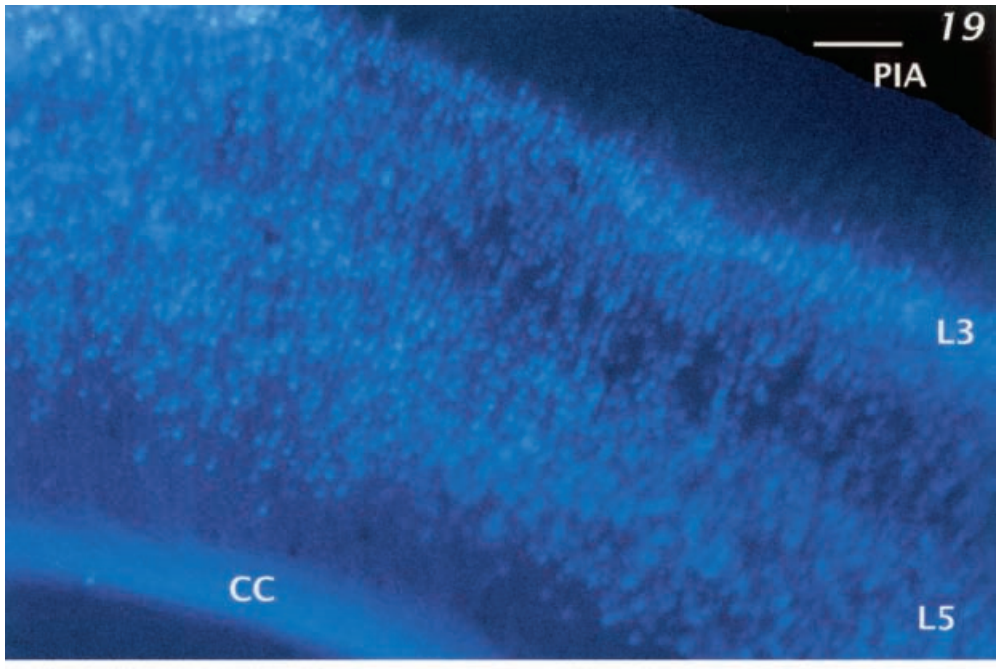


Fig. 19 P2 pup. Fast blue was injected in vivo into visual cortex. This coronal section of contralateral visual cortex shows CC projection neurons. They are densely distributed in the CP, and are located in layers 3–5, with concentrations in layer 3 (*L3*) and layer 5 (*L5*). The pia and CC are indicated. *Bar* 50 µm

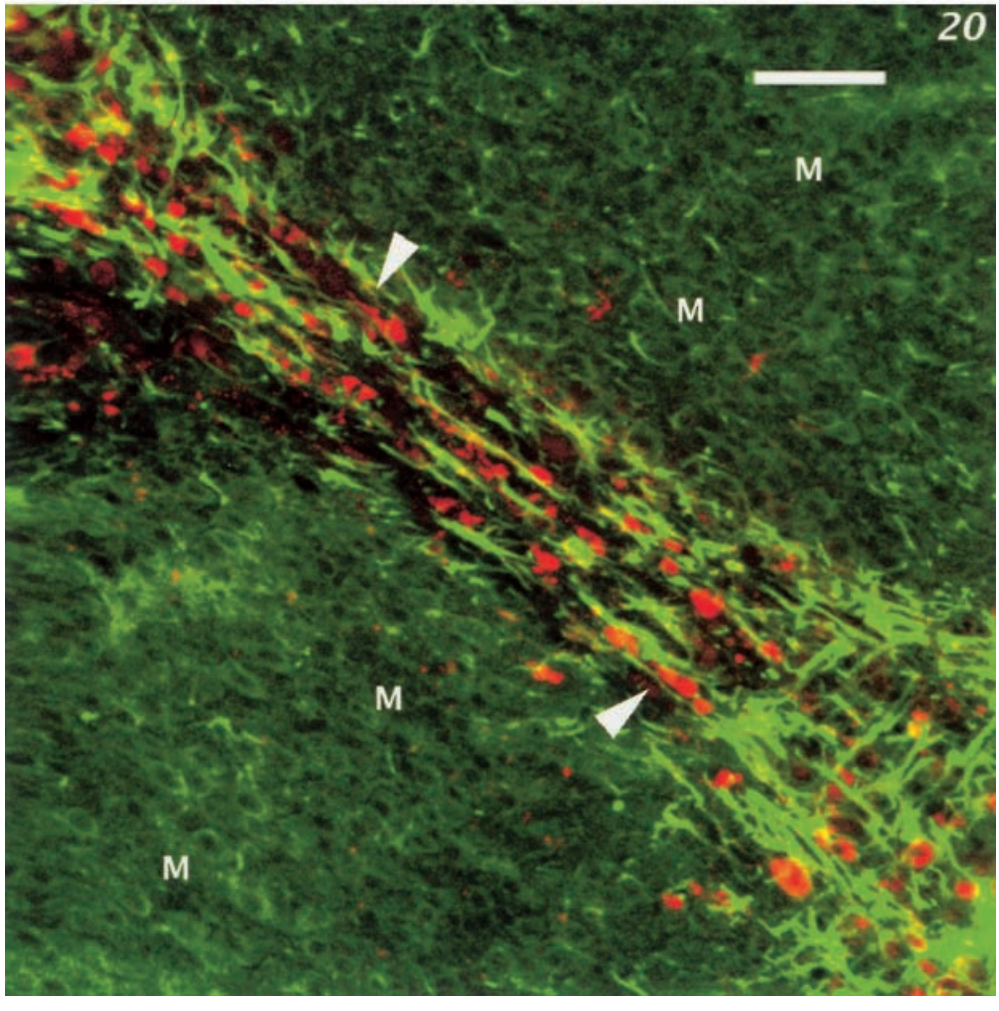
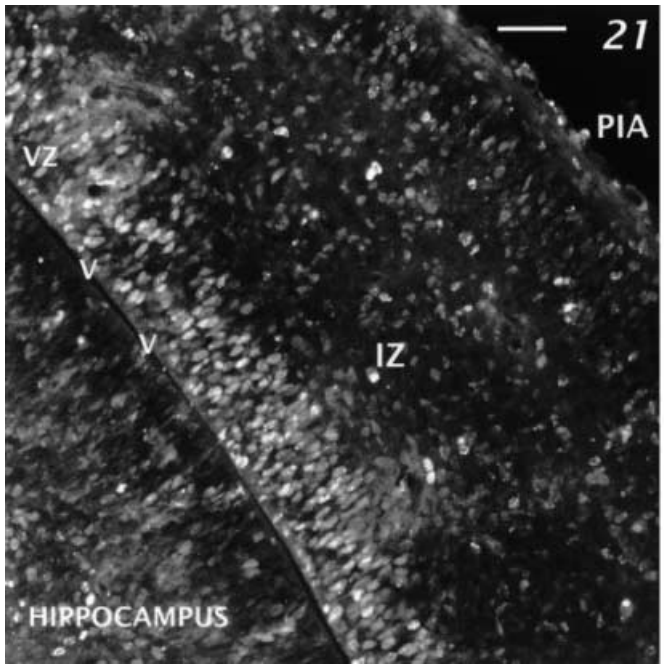


Fig. 20 P2 pup. A solution of DiI was injected into the visual cortex of one hemisphere. Coronal sections were immunocytochemically reacted for vimentin to label glial cells. At the midline of the CC, there are many DiI-labeled cell bodies (*in red*) that are not vimentin+, and therefore they are pioneer neurons. These neurons are ovoid, and some display a short process (*arrowheads*) in the direction of the CC fibers. However, vimentin+ glial fibers (*in green*) are also present at the CC midline (*M*) positioned obliquely here. *Bar* 50 µm



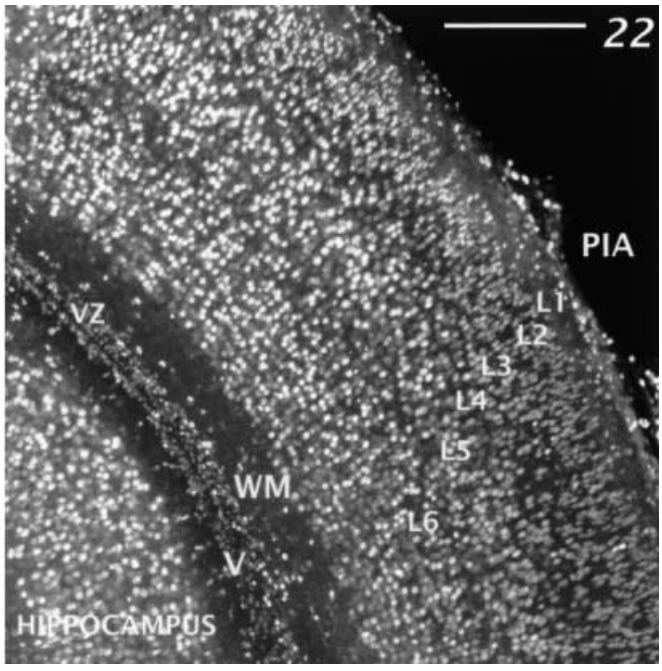


Fig. 21 An injection of BrdU was made in the pregnant mother when the embryo was E12, and then the mother was killed 3 days later; therefore, the tissue shown is from an E15 embryo. After Caesarian section the E15 embryo brain was coronally sectioned and immunocytochemically reacted for BrdU. The pia, ventricle (*V*), hippocampus, VZ and IZ are indicated. The BrdU+ nuclei are mainly located in the VZ. Few BrdU+ nuclei are located in the IZ. *Bar* 50 μm

Fig. 22 An injection of BrdU was made in the P0 pup, and then the pup was killed 2 days later; therefore, the tissue shown is from a P2 pup. The pia, white matter (*WM*), ventricle (*V*), hippocampus, and VZ are indicated. At P2 the BrdU+ nuclei are distributed radially through layers 2–6 in visual cortex. From the concentration of the BrdU+ nuclei, the 6-layer structure in visual cortex can be identified clearly. Locations of cortical layers 1 (*L1*) through 6 (*L6*) are marked. *Bar* 50 μm

drites can not be detected. Therefore, Fast Blue complements the DiI results by revealing a larger population of cell bodies without attached processes, so that neuronal distribution patterns are more easily distinguished. After a single injection into one hemisphere, many contralateral cell bodies are labeled. The fibers are too weakly labeled to identify single fibers; instead there is a labeled pathway in the SP and IZ. The CCpn are much more densely packed than revealed by DiI labeling in vitro. The CCpn can be seen in layers 3–5 of contralateral cortex as shown by DiI labeling (Fig. 19), moreover the subplate can be distinguished only in the cortex ipsilateral to the injection site (data not shown).

DiI and immunocytochemistry double labeling

In order to understand how the pioneer neurons guide the CC fibers to form the commissure, migrating cells were labeled in vivo with DiI. As anticipated, many migrating cells are found within the CC bundle. These cells are bipolar and fusiform, and are orientated horizontally as if to pass through the CC bundle. Sometime there are one or two short processes extending from the migrating cells. Double labeling with DiI and anti-vimentin immunocytochemistry was carried out to determine whether these cells are neuronal or glial. Many vimentin-positive glial cells and fibers are labeled in the CC bundle, but none of them are double-labeled with DiI (Fig. 20).

Neuron birth date

It is helpful to analyze the problems of radial migration and lamination in visual cortex while considering the birth date of the neurons. To determine the neuron birth date in the IZ and CP, mice from E12–P7 were injected with BrdU, and killed 1–5 days later. BrdU is a marker of the S phase in dividing cells, therefore it marks the nuclei of undifferentiated cells and postmitotic neurons at the age of injection. Conversely, BrdU does not mark the nuclei of mature neurons because they are no longer in this S phase state. In the present study, the ages at BrdU injection and analysis of labeling were different, therefore the analysis of results evaluated the developmental pattern before and after BrdU injection. This overlapping injection/sacrifice pattern provided various data sets to elucidate neuronal genesis.

To emphasize that age at both injection and sacrifice are important, the results for each age are described using an X/Y convention, where X is the age at injection and Y is the age at sacrifice. For BrdU at age E12/E13, only neuroepithelial nuclei are labeled. For BrdU at ages E12/E14 and E12/E15 (Fig. 21), the nuclei of neuroepithelium and IZ plus PPN neurons are labeled. For BrdU at ages E14/E15 and E14/E16, a thin subplate is labeled. For BrdU at ages E15/E17 and E16/E18, a more mature

subplate and CP are present. Interestingly, for BrdU at age E13/E18, there is a very different labeling pattern, with positive nuclei concentrated in the ventricular, subventricular and intermediate zones, but sparse in the subplate and CP because the neurons that occupy these latter structures were not born until after the BrdU was injected at E13. For BrdU at P0/P2, labeling reveals a visual cortex with a very typical structure of a 6-layer CP and an additional subplate (Fig. 22).

Taken together, it appears that neuroepithelium can proliferate as early as E12. The undifferentiated cells in neuroepithelium show significant mitosis and migration. On E13 progenitor cells start to migrate into the IZ. On E14 the cells in the ventricular zone not only migrate toward the IZ, but also begin to form the subplate. On E15 the CP begins to appear, and by P0 the visual cortex has the normal 6-layer structure. Therefore, these BrdU data showing neuron birth date and development of cortical lamination support the conclusions obtained from DiI labeling in vitro.

Discussion

The present study investigated the structural development of the mouse central nervous system using in vitro carbocyanine dyes for pathway tracing, a dye for in vivo pathway tracing, immunocytochemistry, and BrdU staining. This combination of methods produced new results related to how pioneer neurons in the PPN migrate from the GE, and how pioneer neurons systematically differentiate into C-R cells and SP neurons. New results have also been obtained related to the development of CC and visual cortex with extensive details regarding the CCpn that exist between the C-R cells and SP neurons. This study shows for the first time how CC axons and subcortical pathway axons navigate to their appropriate positions under the guidance of pioneer neurons. The results are discussed below according to the major findings observed.

Development of the primordial plexiform neuropil and formation of the visual cortex

The PPN is very crucial to neocortical formation. The PPN is formed in early fetal development, and is composed of afferent and efferent fibers and scattered neurons. The neurons in PPN are mainly pioneer neurons that migrate from the GE (Marin-Padilla 1998; Lavdas et al.1999). The PPN is initially superficial to the IZ, but then the postmitotic cells from the VZ incorporate within the PPN. At that time the PPN is split into two components. The superficial component forms the MZ which contains many C-R cells, while the deep component forms the subplate, which also contains pioneer cells (Marin-Padilla 1998). Pioneer neurons in both the MZ and subplate have migrated from the GE (Marin-Padilla 1998). The pioneer neurons are positive for GABA, calbindin, and calretinin (Soria and Fairen 2000). Pioneer neurons in the MZ and SP have different functions. The C-R cells guide radial migration, whereas pioneer cells in the subplate guide pathfinding (Del Rio et al. 2000). The PPN is critical for the lamination of cortical neurons and fibers, which is needed to establish the structure of the cortex.

The neurons that migrate radially to the CP are guided and moved along radial glial cells that span the entire thickness of the CP (Rakic 1990; Misson et al. 1991). The guidance cues for this radial migration remain unknown. A number of extracellular or cell-surface molecules such as reelin and astrotactin, or intracellular proteins such as filamin and disabled, have been implicated (D'Arcangelo et al.1995; Zheng et al.1996; Fox et al. 1998; Rice et al. 1998). Reelin is understood much better than other molecules for its molecular guidance mechanisms; however there are several theories regarding reelin's function. Reelin is secreted by C-R cells in the MZ (layer 1), and is crucial for radial migration (Frotscher 1998; Soriano et al.1997; Super et al. 2000). Interestingly, reelin is not secreted by radial glial cells or other cells in the CP. It is reported that reelin in the upper preplate and MZ is suitably positioned to provide a stop signal to cells at the end of their migration journey (Sheppard and Pearlman 1997; Pearlman et al.1998). Without reelin in the MZ, cortical neurons might reverse direction as they do in cell culture, or migrate away from the pia and accumulate beneath the preplate. Alternatively, reelin might serve as a scaffold for the attachment of peptide growth factors that would provide differentiation signals. Radial migration is believed to be the predominant pathway for migration in the CP. Studies have demonstrated that 80–90% of the billions of neuronal precursors in mammalian cortex migrate along glial fibers (Hatten 1999).

However, both in vivo and in vitro experiments have shown that migrating neurons can adopt tangential migratory paths toward their positions in the CP. Results supporting tangential migration have come from tracing studies of DiI-labeled postmitotic neurons (O'Rourke et al. 1995), from BrdU birthdating experiments, and from lineage analyses with retroviruses (Walsh and Cepko 1988, 1990; Austin and Cepko 1990; Mione et al. 1997). Sources of cortical neurons have been discovered in the lateral ganglionic eminence (LGE) and medial ganglionic eminence (MGE) of the ventral telencephalon, which are the primordia of the basal ganglia (Tamamaki et al. 1997; Lavdas et al. 1999; Zhu et al. 1999; Anderson et al. 2001). More recently, the presence of labeled cells in the cortex has been reported after placement of DiI in the LGE of embryonic rats and mice (Tamamaki et al. 1997). Labeled cells leave the LGE, cross over the corticostriatal boundary and follow tangential migratory routes to take up positions predominantly within the IZ. Many of these neurons are positive for GABA (Tamamaki et al. 1997). It has also been reported that tangential migrating neurons in the MZ come from the GE (Parnavelas et al. 2000). The mechanisms that guide tangentially migrating

neurons from the GE to the neocortex are currently the subject of intensive investigations. It has been hypothesized that chemoattractant cues present in the neocortex are responsible for the dorsal migration of cells that are born in the ventral telencephalon. Substances such as netrin (Metin et al. 1997) and Slit1 and Robo1 (Yuan et al. 1999) may be involved in guiding tangential migration. Tangential migration also can be guided by axons (Gray et al. 1990). It is known that in cortex there are two major classes of neurons, the excitatory pyramidal neuron, and the inhibitory non-pyramidal interneuron (Parnavelas 2000). These two neuronal classes have different origins. It is thought that pyramidal neurons arise from the VZ surrounding the telencephalic ventricles. From there, newly generated neurons migrate outward along the processes of radial glial cells to reach the CP where they accumulate in an 'inside-out' sequence to form the six-layered structure of the neocortex. In contrast, the non-pyramidal neurons arise in the GE of the ventral telencephalon, and these neurons follow tangential migratory routes to reach their destination in the developing cortex (Parnavelas 2000). Thus, tangentially migrating neurons are important in cortical development.

Long-projection pioneer neurons and their tangential migration

Using DiI labeling in vivo several authors have observed that pioneer neurons migrated from the GE tangentially (O'Rourke et al. 1995; Tamamaki et al. 1997; Parnavelas 2000). Recently Soria used immunocytochemistry in flattened cortex to demonstrate that tangentially migrating neurons in the IZ, SP and MZ have long projections that extend into the GE (Soria and Fairen 2000). Similar results were obtained by DiI tracing in E13 mice, which showed subplate neurons sending axons outside the neocortex toward the GE (Del-Rio et al. 2000).

The present study has utilized similar methods to visualize tangentially migrating neurons with DiI labeling in vitro. The combination of techniques has provided more accurate details to understand migration and development of these long-projection pioneer neurons in visual cortex. By examining the PPN from E14 onward, the present results observed that the pioneer neurons in the SP component of the PPN changed into nonpyramidal SP cells. Those pioneer neurons should be the same cells as Soria (2000) has observed with immunocytochemistry or other authors saw with DiI in vivo (Del-Rio et al. 2000). The results above strongly suggested that pioneer neurons in PPN or C-R cells in MZ, as well as subplate neurons in SP, migrated from the same site and shared the same origin (Figs. 1, 2).

The DiI labeling in the present study showed that the subplate has basket-like cells, large in size and with varying morphology. Basket cells are usually associated with interneurons and characteristically are GABA-positive (Costa 1998). This is in agreement with many studies that show that tangentially migrating neurons are GABA-positive nonpyramidal cells (Tamamaki et al. 1997; Lavdas et al. 1999; Del Rio et al. 2000). Because the SP will disappear in the adult, the fate of SP neurons is controversial; they may die in the future, or else they may be incorporated into neocortical layers 5 and 6 and perform interneuron functions (Super et al. 1998; Del-Rio et al. 2000; Parnavelas et al. 2000). Thus, the location and morphological characteristics of pioneer neurons, especially their long projection toward the GE, indicate that the pioneer neurons should tangentially migrate from the GE to form the PPN, where they are progenitors of C-R cells and subplate neurons (Figs. 1, 2).

Cortical lamination and the CC projection neuron's radial migration

The lamination of visual cortex can be established through radial migration. By examining visual cortex from E15 onward, the present study observed that the CP developed from the 'sandwich' formed by C-R cells and subplate pioneer neurons. By investigating visual cortical development, the process of radial migration and the C-R cell's function in radial migration is revealed. It is known that C-R cells depend on radial glia to carry out their function. In the present study, the C-R cells can be seen after E15, and are still observed in layer 1 on P7. Below C-R cells, radial glia span the whole telencephalic wall from the MZ to the VZ, and provide scaffolding for neuronal migration (Rakic 1990, 1995; Bentivoglio and Mazzarello 1999). The 'cortical column' that appears on E17 is good evidence for radial migration in visual cortex. Usually the column's members migrate along the same radial glial fiber (Walsh and Cepko 1990). In the present study it is possible that each column represents the group of migrating cortical neurons that are associated with a single radial glial cell that is also not labeled. The development of CCpn and cortical lamination also requires the process of radial migration. Before E18, the CCpn form a single cell layer in the deeper CP (prospective layers 4 and 5). After P0, postmitotic neurons start to migrate through the deep CP into the superficial CP (layer 3). This phenomenon, representing the 'inside-out' pattern of cortical development, places the CCpn in different cortical layers (Fig. 2). The BrdU experiment revealed neuronal differentiation and cortical lamination from another view. The BrdU-positive cells first appear in the VZ on E12, in the IZ on E13, in the SP on E14, and in the CP on E15. This labeling sequence is a reflection of the progression of developing neurons extending outward from the VZ. The BrdU and DiI labels show that in the embryonic period, lamination of the neurons in the CP does not yet exist. In the postnatal period the neurons in the CP start to laminate. For P2 mice with BrdU injected on P0, the CP shows a typical 6 layer structure; in comparison, the DiI labeling and Fast Blue tracing in vivo reveals only the CCpn subset of CP neurons, and shows pyramidal CCpn concentrated in layer 3, but also scattered in layers 4 and 5. The BrdU detects a

CP as early as E15, but the CCpn subset can not be visualized by DiI labeling until E17. Since BrdU-positive cells in visual cortex appear earlier than DiI-labeled CCpn, this suggests that the CC subset of cortical neurons develops later than the rest of the cortex. However, the first appearance of CCpn may be artificially delayed by the experimental limitation of labeling them with DiI only when their axons near the midline; thus the CCpn may develop at the same time as all other cortical neurons.

Corpus callosum, subcortical pathway, and pathfinding

It is well-known that chemical and mechanical guidance play an important role in pathfinding. Experiments focusing on long-range commissural pathfinding have explored how, when and why the commissural fibers cross the midline. The retinotectal system has traditionally received a great deal of attention, but although long-range, it is not commissural. Instead, the commissural axons in spinal cord and brain are extensively studied for pathfinding at the molecular level. Using a three-dimensional collagen-gel matrix system, Tessier-Lavigne and colleagues demonstrated a chemoattractive effect of floorplate explants on commissural axons (Tessier-Lavigne and Goodman 1996). With the discovery that the floorplate can secrete netrin-1 as a molecule for long-range guidance cues, this research has provided a new line of investigation into the mechanisms of commissural pathfinding. Studies of mice lacking the CC, hippocampal and/or anterior commissures show that netrins are severely defective in the midline (Serafini et al. 1996). Other substances such as CAM and immunoglobulin (Steockli and Landmesser 1998) provide short-range guidance cues that may play a role in commissural pathfinding. The role of mechanical guidance in commissural pathfinding has also been the subject of discussion (McConnell et al. 1989; Livy and Wahlsten 1997). The entorhino-hippocampal projection pathfinding appears to be guided by C-R cells over a long period of time; contact guidance appears to be involved (Frotscher 1998; Ceranik et al. 1999, 2000). Contact guidance also appears to be an important factor in brain development (Rajnicek et al. 1997). Another study showed that the cingulate cortex was a factor for CC commissure midline crossing (Koester and O'Leary 1993, 1994). Thus, there are many factors that appear to play a role in commissural, long-range pathfinding.

Subplate neurons guide the subcortical pathway

The present study has focused on pioneer cells' guidance effects on the subcortical pathway, and on CC formation and pathfinding. One possible explanation for the means by which subcortical pathway finding occurs is as follows. As early as E14, the fibers in the IZ are very sparse, and they mainly belong to the projections of tangential pioneer neurons extending toward the GE. On E15 the pioneer neurons in the outer IZ begin to differentiate into SP neurons. At this age, in the absence of afferent thalamic fibers, the subcortical pathway still is too sparse to be detected by DiI tracing from the GE. After E16, there is a dramatic change. At first the fibers in the subcortical pathway suddenly increase, and many growth cones appear on the fiber tips. The directions of the growth cones point to the cortex. After E17, the subcortical pathway becomes very dense and occupies the outer IZ and SP. Many afferent fibers leave the subcortical pathway and radially extend to the cortical surface. The subcortical pathway mainly contains the thalamocortical fibers, the afferent fibers from the internal capsule. The developmental spatiotemporal relationship between SP neurons and the subcortical pathway strongly suggests that projections of SP neurons pioneer the navigation of thalamocortical fibers through the subcortical pathway. It is possible that the SP neurons' projections guide the thalamocortical fibers' navigation as C-R cells do for the entorhinal-hippocampal pathway (Fig. 2). This hypothesis is consistent with the fact that netrin in the IZ is a direct factor that guides the subplate pathway out of the internal capsule (McConnell et al. 1989; Allendoerfer and Shatz 1994; Richards et al. 1997).

Pioneer neurons guide CC pathfinding

On the other hand, the CC is generated much later than the subcortical pathway. Many theories are available to explain CC pathfinding. Either chemical attraction or mechanical effects must eventually exert their functions through receptors. The functions of growth cones, usually present on fiber tips, are also linked to receptors. Chemoattractant and chemorepellant molecules can specifically act on receptors in the growth cone to influence growth cone movement (Isbister and O'Connor 2000). In the present study, the CC fibers on E16 can be labeled in the deep IZ and subventricular zone, but there are only a few CCpn in visual cortex and they are lightly labeled. The CC bundle, found along cingulate cortex, has many growth cones on the tips of CC fibers. The growth cones point to the commissural midline. On E16 the two sides of the CC bundle begin to contact each other in the septum, but the CC fibers do not extend into the contralateral telencephalon until E17. The Fast Blue injection in vivo shows that the postnatal CC fibers have reached contralateral cortex, and neurons located in layers 3–5 extend projections to form the CC. In addition, using DiI injection in vivo into the visual zone, many DiI-labeled cells migrate along the midline of the CC. Anti-vimentin immunocytochemistry demonstrates that these migrating DiI-labeled cells are not glia. What are these cells? According to their morphological and developmental features, they are probably pioneer cells. Where are they from? O'Rourke believed that progenitor cells in the VZ or IZ can either migrate into cortex radially or somewhere into IZ tangentially (O'Rourke et al. 1997). There-

fore, we may conclude that pioneer cells are first produced in the IZ or VZ in the visual zone. Later, along the CC bundle, the pioneer cells reach the commissural midline to provide the guidance necessary for CC pathfinding. What kind of substance the pioneer neuron secretes and how the substance affects growth cones' receptors are not known. Moreover, tangential migration even exists in the adult subventricular zone (Doetsch and Alvarez-Buylla 1996), indicating that tangential migration patterns are varied and complex. The subject clearly merits further research.

The present study has used a variety of techniques in the embryonic and early postnatal mouse to elucidate the time course and factors involved in the development of numerous brain pathways. The main points are: (1) long-projection pioneer neurons from GE are found in visual cortex, (2) these pioneer neurons are constitutive of PPNs that develop into neocortex, (3) both C-R cells and SP neurons differentiate from pioneer neurons in the PPN, (4) C-R cells participate in radial migration, but SP neurons participate to guide the subcortical pathway, (5) the pioneer neurons in the CC pathway probably play a role in CC pathfinding. Conclusions detail the complex and interrelated development of commissural and non-commissural pathways. Further studies are needed to explore the mechanisms of navigation and actions of chemical factors involved in commissural pathfinding.

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