Timing and Origin of the First Cortical Axons to Project Through the Corpus Callosum and the Subsequent Emergence of Callosal Projection Cells in the Mouse

By: Hiroki S. Ozaki and Douglas Wahlsten

Abstract:

A precise knowledge of the timing and origin of the first cortical axons to project through the corpus callosum (CC) and of the subsequent emergence of callosal projection cells is essential for understanding the early ontogeny of this commissure. By using a series of mouse embryos and fetuses of the hybrid cross $B6D2F_2/J$ weighing from 0.36 g to 1.0 g (embryonic day E 15.75–E 17.25), we examined the spatial and temporal distribution of callosal projection cells by inserting crystals of the lipophilic dye (DiI: 1, 1'-diocta decyl-3,3,3',3'-tetram ethyl-indocarbocyanine perchlorate) into the contralateral white matter just lateral to the midsagit-tal plane. Around 0.4 g or E 15.8, retrogradely labeled cells were found restricted to a discrete cluster continuously distributed from the most ventral part of presumptive cingulate cortex to the hippocampus. During subsequent development, however, the tangential distribution of these labeled cells in ventromedial cortex did not extend further dorsally, and in fetuses where the CC became distinct from the hippocampal commissure (HC), labeled axons of cells in the ventral cingulate cortex were observed to intersect the callosal pathway and merge with labeled axons of the HC derived from cells in the hippocampus. The first cortical axons through the CC crossed the midline at about 0.64 g or E 16.4, and these axons originated from a scattered neuronal population in the dorsal to lateral part of the presumptive frontal cortex. The earliest callosal cells were consistently located in the cortical plate and showed an immature bipolar appearance, displaying an ovoid- or pearl-shaped perikaryon with an apical dendrite coursing in a zig-zagging manner toward the pial surface and a slender axon directed toward the underlying white matter. Callosal projection cells spread progressively with development across the tangential extent of the cerebral cortex in both lateral-to-medial and rostral-to-caudal directions. In any cortical region, the first labeled cells appeared in the cortical plate and their number in the subplate was insignificant compared to that in the cortical plate. Thus, these results clarify that the CC is pioneered by frontal cortical plate cells, and the subsequent ontogeny of callosal projection cells proceeds according to the gradient of cortical maturation.

Indexing terms: pioneer neurons; cortical plate cells; axonal guidance; hippocampal commissure; corticogenesis

Article:

Many neurons in the cerebral neocortex of placental mammals project to the contralateral hemisphere, and their axons form the midline structure, the corpus callosum (CC). A key event in the formation of this commissure is the first traverse of the interhemispheric fissure and subsequent cortical axon fasciculation in the fetal period. The introduction of postmortem tract tracing methods (Godement et al., 1987; Honig and Hume, 1989) has allowed much stronger statements about the ontogeny of this commissure by greatly improving the observation of fetal axon extension. In a previous study using lipophilic dyes as anterograde tracers, we have statistically ad-dressed the timing of the first crossing of the midplane by mouse callosal axons originating from four major neocortical regions (presumptive frontal, parietal, temporal, and occipital cortex) and found large rostral-caudal differences in the contribution of regions of the cerebral cortex to formation of the early CC at the midsagittal

plane (Ozaki and Wahlsten, 1992). The obtained estimate of the stage when the first CC axons arrive at the midplane is more convincing than several other estimates from mice based on silver stains (Glas, 1975; Silver et al., 1982), electron microscopy (Zaki, 1985) and a general purpose stain with eosin (Wahlsten, 1987), although our previous study did not exclude the possibility that a region other than presumptive frontal cortex is the source of earliest callosal projections to reach midplane.

More recently, Koester and O'Leary (1994) have re-ported in rat fetuses that a discrete cluster of cortical plate cells in the most ventral part of the cingulate cortex sends the first axons across the nascent CC. They noted that cingulate cells are the first to be found retrogradely labeled in the hemisphere contralateral to injections of the lipophilic tracer DiI (1, 1'-dioctadecyl-3,3,3',3'-tetram ethyl-indocarbocyanine perchlorate) in the ventromedial wall of the cerebral cortex. However, there is a problem with the interpretation of this observation. In rodents, the CC is closely apposed with the hippocampal commissure (HC) at the midplane early in development, and it is almost impossible to definitively discriminate between the two commissures until they are more mature (Valentino and Jones, 1982; Wahlsten, 1984). This topographical arrangement strongly implies that it is very difficult to localize injections of the tracer in the earliest CC without the involvement of the HC. Furthermore, the rodent CC forms at least 1 day after the HC and the first CC axons cross the midline by traversing the anterodorsal aspect of the preexisting HC (Valentino and Jones, 1982; Wahlsten, 1987; Livy and Wahlsten, 1997), which raises the possibility that cells of origin of the HC may be retrogradely labeled earlier than cells of origin of the CC in the contralateral cortex by such DiI injections.

Koester and O'Leary (1994) sought to determine whether neocortical subplate cells extend the first axons across the CC as they do through the internal capsule (McConnell et al., 1989; Blakemore and Molnar, 1990; De Carlos and O'Leary, 1992; Erzurumlu and Jhaveri, 1992). We now address the same question by using a series of embryos and fetuses of the hybrid cross B6D2F$_2$/J mice. The same F2 hybrid cross has been used to establish standards for normal callosal origins (Ozaki and Wahlsten, 1992) as well as several other aspects of the fetal brain (Wahlsten, 1981; Wahlsten and Smith, 1989). Our results show that cells extending the first axons across the nascent CC of mice reside in the cortical plate of the presumptive frontal cortex, not the ventral cingulate cortex, and that the subsequent development of callosal projection cells follows the tangential gradients of cortical maturation (Rickmann et al., 1977; Raedler et al., 1980; Smart and Smart, 1982; Floeter and Jones, 1985; Uylings et al., 1990; Bayer and Altman, 1991).

MATERIALS AND METHODS
Mice and mating
Subjects of the present study were the F$_2$ hybrid off-spring of B6D2F$_1$/J hybrid mice (cross of C57BL/6J female and DBA/2J male purchased from the Jackson Laboratory, Bar Harbor, ME). Methods of breeding were essentially the same as those employed by Ozaki and Wahlsten (1992, 1993). Briefly, females were checked for presence of a vaginal plug every 5 hours or after being mated overnight. Conception (0.0 day) was defined as the time midway between detection of a plug and the previous plug check. All of the experimental protocols used to complete the present study were approved by the animal experiment committee at the Toyama Medial and Pharmaceutical University and carried out in accordance with its guide on care and management of animals.

Neuronal labeling
To examine the early development of the CC, we used postmortem DiI (Molecular Probes, Inc., Eugene, OR) labeling in aldehyde-fixed brains (Godement et al., 1987; Honig and Hume, 1989). When used in aldehyde-fixed tissue, DiI applied to axons labels neurons in their entirety. Fetuses were removed from dams anesthetized with sodium pentobarbital (100 mg/kg maternal weight, intra-peritoneal injection) by caesarian section at embryonic (E) ages between E15.75 and E17.25. This time period includes the first outgrowth and crossing of the midplane by CC axons from the major neocortical regions (Ozaki and Wahlsten, 1992). After being weighed, each fetus was perfused transcardially with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.5), and its whole head without the scalp was immersed in fresh fixative for a few days. The cerebral cortex of one hemisphere was exposed by removing the skull, and four to eight crystals of DiI (each about 50-µm-long) were placed along the parasagittal plane (0.1–0.3 mm lateral from the midline; 1.0–2.0 mm caudal from the frontal

pole) with the aid of a stereoscope and a graticule in a 20× eyepiece, then pushed into the developing cortical white matter/intermediate zone (0.8- to 1.3-mm-deep from the pia mater) by using a glass microelectrode attached to a stereotaxic apparatus. These large dye injections were intended to intersect all axons that had already cross the midline through the CC, although the number of crystals and the coordinates for injections varied depending on size of the brain.

Viewing and data presentation

After the dye application, the heads were returned to fresh fixative, incubated in a 37°C oven for the first month, and then stored at room temperature for a subsequent 1 to 2 months (longer times for older fetuses) in the dark. The brains were dissected from the heads and cut at 60 μm in the coronalplane with a Microslicer (D.S.K., Kyoto, Japan, DTK-1000). Sections were collected in serial order in ice-cold 0.1 M phosphate buffer (pH 7.5), and examined, and photographed by using a uorescence microscope with rhodamine illumination. A total of 107 brains with successful DiI injections was selected for analysis. For each brain, a schematic drawing of the cerebral cortex from a superior view was reconstructed from serial sections by using a projector and a microscope equipped with a drawing tube, and the distribution of cells retrogradely labeled in the hemisphere contralateral to DiI injections was superimposed. In several sections of some brains, the DiI labeling was photoconverted to a brown, insoluble reaction product by exposing the section to green light in the presence of diaminobenzidine (DAB: 1.5 mg/ml in 50 mM Tris buffer, pH 8.2) according to the protocols described by Sandell and Masland (1988).
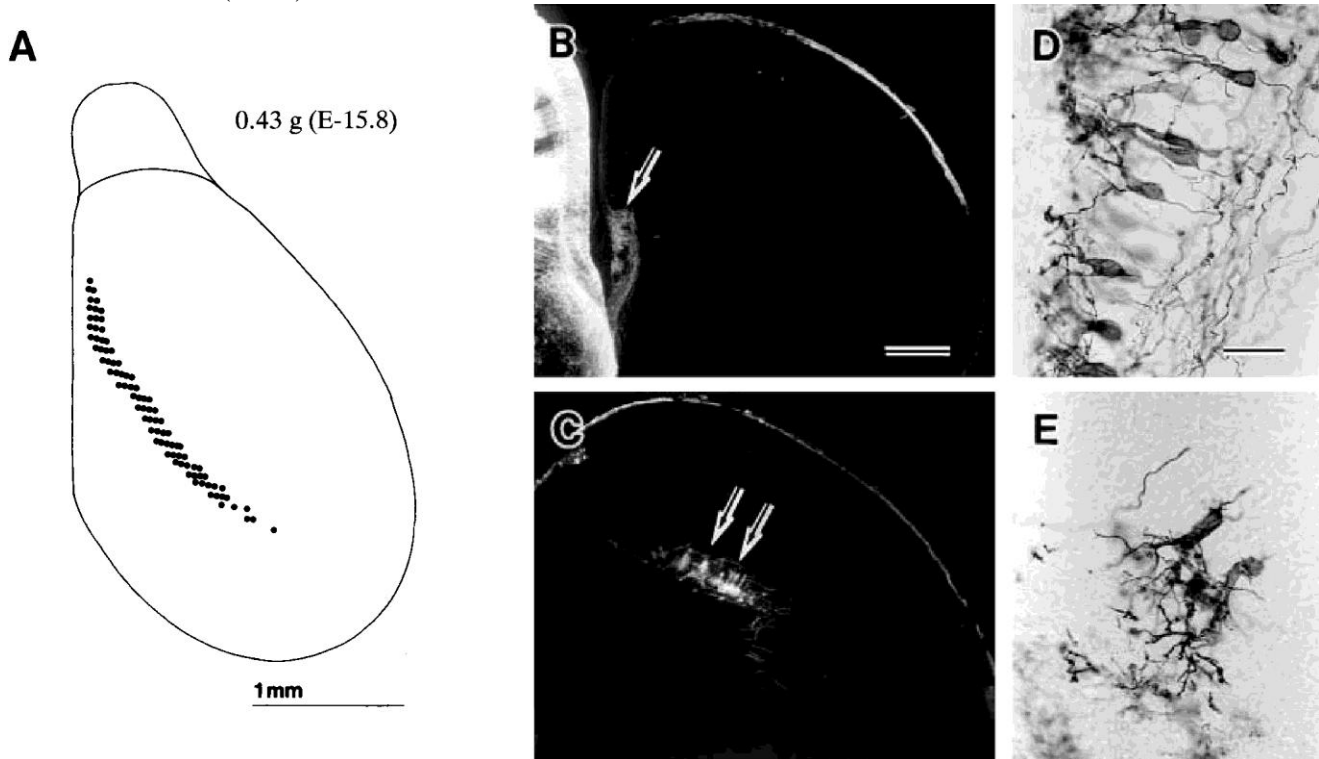

Fig. 1. Cells retrogradely labeled in a fetus weighing 0.43 g (embryonic day 15.8, E15.8) by 1,1′-dioctadecyl-3,3,3′,3′-tetramethyl-indocarbocyanine perchlorate (DiI) injections into the contralateral white matter. A: The distribution of retrogradely labeled cells superimposed on a schematic drawing of the cerebral cortex from a superior view. Filled circles represent labeled cells but one symbol does not necessarily correspond to one cell. B,C: Low-magni®cation ¯uores-cence photomicrographs showing the location of retrogradely labeled cells (arrows) in coronal sections. Note that retrogradely labeled cells are restricted to a discrete cluster in the most ventral aspect of cingulate cortex (B) and the hippocampus (C). D,E: Typical morphologies of photoconverted cells from the presumptive cingulate cells (D) and the hippocampus (E) at coronal plane (upper, dorsal; left, medial). Scale bars = 300 μm in B and C, 40 μm in D and E.

To describe the developmental stage of a mouse embryo or fetus, its chronological age (E16, for example) is conventionally used. However, because the prenatal growth rate of individuals varies substantially depending strongly on embryonic genotype and maternal environment (Wahlsten and Wainwright, 1977), the chronological age is not perfectly related to brain development (Wainwright et al., 1989). Previous studies have found that prior to birth, the body weight of a mouse is a better indicator of morphological maturity than age (Wahlsten and Smith, 1989; Bulman-Fleming and Wahlsten, 1991) and that more consistent results can be obtained by comparing brains of mice at the same body weight (Wahlsten, 1987; Ozaki and Wahlsten, 1992,

1993). Therefore, the prenatal development of the callosal pathway in this study was assessed in relation to fetal body weight. This simple metric allows convenient comparisons with other studies. For the present data from E15.75 to E17.25, the relation between body weight and chronological age was nearly linear (age = 14.77 + 2.49 × body weight, $R^2 = 0.69$).

RESULTS

Temporal distribution of cells retrogradely labeled by contralateral DiI injections

In B6D2F$_2$/J mouse fetuses, the first outgrowth of callosal axons from their cells of origin in four major neocortical regions (frontal, parietal, temporal, and occipital cortex) occurs at about 0.4 g body weight or 15.8 days after conception (Ozaki and Wahlsten, 1992); therefore, we chose to initiate the present study with fetuses around this body weight or age. Figure 1 illustrates a representative result obtained from those fetuses with DiI crystals applied into the developing cortical white matter/intermediate zone near the midplane. Cells retrogradely labeled in the contralateral hemisphere to the injection were restricted to a discrete cluster in ventromedial cortex, forming an arched band extending rostrocaudally (Fig. 1A) from the most ventral part of the presumptive cingulate cortex (Fig. 1B,D) to the hippocampus (Fig. 1C,E). Most of labeled cells in the ventral cingulate cortex were located in the cortical plate and had complex morphologies with extensively branched dendritic trees typically extending from large pyramidal-like soma superficially into the marginal zone beneath the pial surface (Fig. 1D). The morphological features of these cingulate cells resembled those described in rat fetuses for neurons in the corresponding cortical region that have been claimed to send the first axons coursing through the CC (Koester and O'Leary, 1994). During subsequent development, contralaterally labeled cells increased somewhat in the ventral cingulate cortex and hippocampus, but their overall distribution pattern did not change appreciably until fetuses reached about 0.64 g or E 16.4, when a spatially distinct population of cells was found retrogradely labeled in the rostral part of dorsal neocortex (Fig. 2A).

After their first appearance, retrogradely labeled cells in dorsal neocortex rapidly increased in number with body weight or age, accompanying the progressive expansion of their tangential distribution toward the medial and caudal directions (Figs. 2, 3). In contrast to these changes in dorsal neocortex, on the other hand, the number of labeled cells in ventromedial cortex neither increased significantly nor did their tangential distribution extend further dorsally with weight or age (compare Fig. 3D,E with Fig. 1B,C). Indeed, even in fetuses where retrogradely labeled cells were widely distributed across the mediolateral extent of neocortex, the dorsal part of the medial cerebral wall adjacent to the ventral cingulate cortex or the hippo-campus remained unlabeled. Thus, these findings imply that labeled cells in ventromedial cortex and dorsal neocortex are developmentally independent populations. In favor of this implication is an ontogenetic fact that the ventral part of cingulate cortex and the hippocampus are derived from the mesocortex and the allocortex, respectively, whereas the dorsal cingulate cortex and the adjacent dorsal neocortex originate from the isocortex (Hankin and Silver, 1988).

Source of earliest callosal projections

In all of the fetuses analyzed, cells in the hippocampus were consistently found retrogradely labeled from the contralateral hemisphere (Figs. 1C, 3E). Because the hippocampus is the major source of the HC (Voneida et al., 1981), it is evident that the HC is involved in our DiI injections. In photoconverted sections, we carefully examined axon trajectories of the clustered labeled cells in the ventral cingulate cortex because they were always seen to be continued caudally to labeled cells in the hippocampus (Figs. 1 A, 2A–C).

At embryonic ages approximately between E15.8 and E16.4, when contralaterally labeled cells were localized exclusively in ventromedial cortex, it was impossible to distinguish the CC from the HC (Fig. 1B). However, in fetuses where substantial numbers of cells were found retrogradely labeled in dorsal neocortex, we could distinguish the two commissures at the midplane with confidence (Fig. 4). In these fetuses, we found that axons originating ventrolaterally from a cluster of labeled cells in ventral cingulate cortex did not decussate in the CC; instead, they intersected the callosal pathway to merge with axons of the HC derived from labeled cells in the hippocampus (arrows in Fig. 4). These findings demonstrate the ventral cingulate cells to be of origin of the HC

as well as cells in the hippocampus, which in turn indicates that the source of earliest callosal projections to reach midplane is the rostral part of dorsal neocortex.

The indusium griseum is a midline structure overlying the CC. In mice this structure is suggested to be a displaced portion of the dentate gyrus and represents a phylogenetically old olfacto-recipient outpost of the hippo-campus (Adamek et al., 1984). Some indusium griseum cells were labeled in only a few cases where DiI crystals were injected very close to the midline (an arrowhead in Fig. 4A). In these materials, the indusium griseum cells lay just dorsal to the callosal fiber bundle near the midplane, apart from the ventral cingulate cells whose axons were observed to decussate the CC and join the HC. However, because cells of the indusium griseum were not retrogradely labeled in most cases by our DiI injections, it is unlikely that this structure contains early callosal cells.

Nature of earliest callosal cells
The stage when the earliest CC axons cross the midline is about 0.64 g body weight or E16.4, when a scattered population of retrogradely labeled cells is first found in the rostral part of dorsal neocortex (Fig. 2A). As illustrated in a coronal section in Figure 3A, this population of labeled cells resided in the dorsal to lateral part of the presumptive frontal cortex, although their distribution and position in the cerebral cortex varied slightly among fetuses. These earliest callosal cells were consistently located in the cortical plate, a cell-dense layer superficial to the cell-sparse intermediate zone, and had an ovoid- or pearl-shaped perikaryon with an apical dendrite coursing in a zig-zagging manner toward the pial surface and a slender axon directed toward the underlying white matter (Fig. 5A). Occasionally they displayed emerging branching of the apical dendrite below the pial surface (an arrowhead in inset of Fig. 5A), but no basal dendrites. Because these morphological features are slightly more mature than those of postmitotic migrating neurons which are generally fusiform spindle-shaped (Schwartz et al., 1991; Auladell et al., 1995), the earliest callosal cells in our materials might correspond to early postmigratory neurons. Indeed, in mouse the axons of migrating neurons are shown not to traverse the CC before E19 (Auladell et al., 1995). Callosal projection cells, which were retrogradely labeled later in development, generally exhibited a more mature appearance, i.e., a pyramidal-like perikaryon and a prominent apical dendrite (Fig. 5B,C).

Sequence in the development of callosal projection cells
In a given region of neocortex, callosal projection cells first appeared in the cortical plate, and two tangential gradients across the neocortex were recognized in the expansion of the distribution of callosal projection cells with development (Figs. 2, 3). One is a lateral-to-medial gradient: the appearance and subsequent accumulation of retrogradely labeled cells occur earlier in the dorsolateral part than in the dorsomedial part of neocortex at a given rostrocaudal level. This gradient is relatively prominent at frontal levels (Fig. 3A–C) but it is weak in parietal and occipital cortex where retrogradely labeled cells appear as a continuous band across its mediolateral extent from the beginning (Fig. 3D,E). The other is a more pronounced rostral-to-caudal gradient with the appearance of retrogradely labeled cells being earlier in the more rostral cortical region (Fig. 2). The approximate body weights (embryonic ages) of fetuses at which retrogradely labeled cells were first found in presumptive parietal and occipital cortex were 0.76 g (E 16.7) and 0.91 g (E 17.0), respectively.

Callosal projection from cells in the subplate layer
In neonatal ferrets and cats, neocortical subplate cells are shown to send axons to contralateral cortex (Chun et al., 1987; Antonini and Shatz, 1990). To know the contribution of subplate cells to the callosal projection, we examined the location and morphology of retrogradely labeled cells in neocortex.

With development, labeled cells in neocortex progressively spread vertically and tangentially (Fig. 3). However, most of these cells were located in the cortical plate and displayed morphological features characteristic of developing pyramidal cells with radially aligned apical dendrites toward the pial surface (Fig. 3D,E), rather than horizontal, multipolar, or inverted pyramidal morphology typical of subplate callosal neurons (Chun et al., 1987; Antonini and Shatz, 1990). Only a few cells were retrogradely labeled in the neocortical subplate by our

large DiI injections into the contralateral white matter near the midplane. This observation confirms a previous finding in rat fetuses (Koester and O'Leary, 1994) that a callosal projection from the subplate layer is minor.

A

0.64 g (E-16.4)

B

0.69 g (E-16.5)

C

0.76 g (E-16.7)
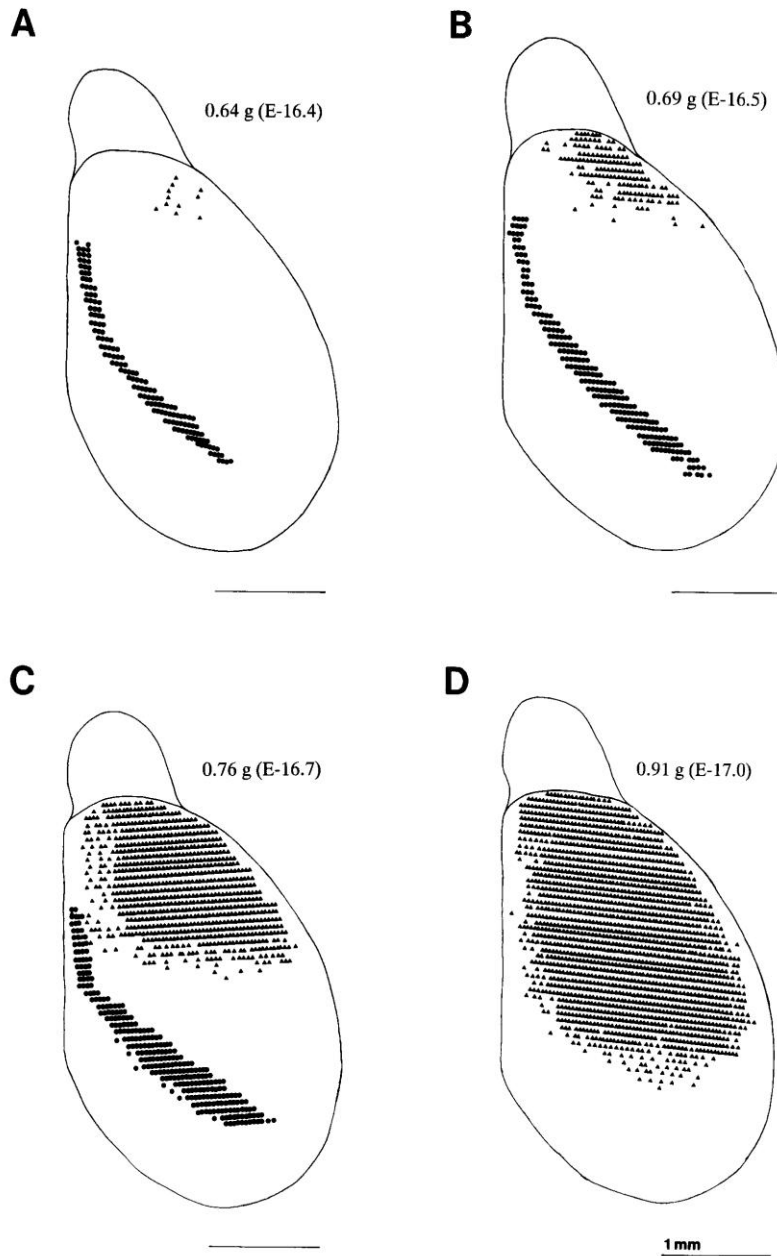
D

0.91 g (E-17.0)

1 mm

Fig. 2. The distribution of retrogradely labeled cells superimposed on a schematic drawing of the cerebral cortex from a superior view at different body weights or ages (A±D). Note the marked differences in the number and distribution of labeled cells between ventromedial cortex (indicated by filled circles) and dorsal neocortex (indicated by filled triangles). One symbol does not necessarily correspond to one cell. In D, labeled cells in ventromedial cortex are not shown because they overlap with those in dorsal neocortex, but their distribution is similar to C.

Collaterals from callosal projections

In fetuses with retrogradely labeled cells in neocortex, we sometimes observed several labeled axons leaving the cortical white matter to course into the internal capsule (white arrowheads in Fig. 3D). This raises the possibility that at early stages callosal projection cells also extend collaterals toward subcortical structures. Then, we carefully examined the trajectories of axons of labeled cells in neocortex.

Axons originating from neocortical labeled cells extended radially downward to reach the cortical white matter, where they changed their direction medially and coursed through the CC (Fig. 3D). At the superficial part of the white matter, a considerable number of these axons bifurcated to extend collaterals laterally (Fig. 5). However, these collaterals appeared to proceed toward ipsilateral cortical regions without descending into the internal capsule. Thus, labeled axons in the internal capsule could be collaterals from callosal projection cells, but more

likely represent the minor crossed corticostriatal projection through the CC documented in previous studies (Fisher et al., 1986; McGeorge and Faull, 1989; Morino et al., 1992). Our present data support the idea that in rodents, the population of callosal projection cells is segregated from subcortically projection populations early in development (Koester and O'Leary, 1993).
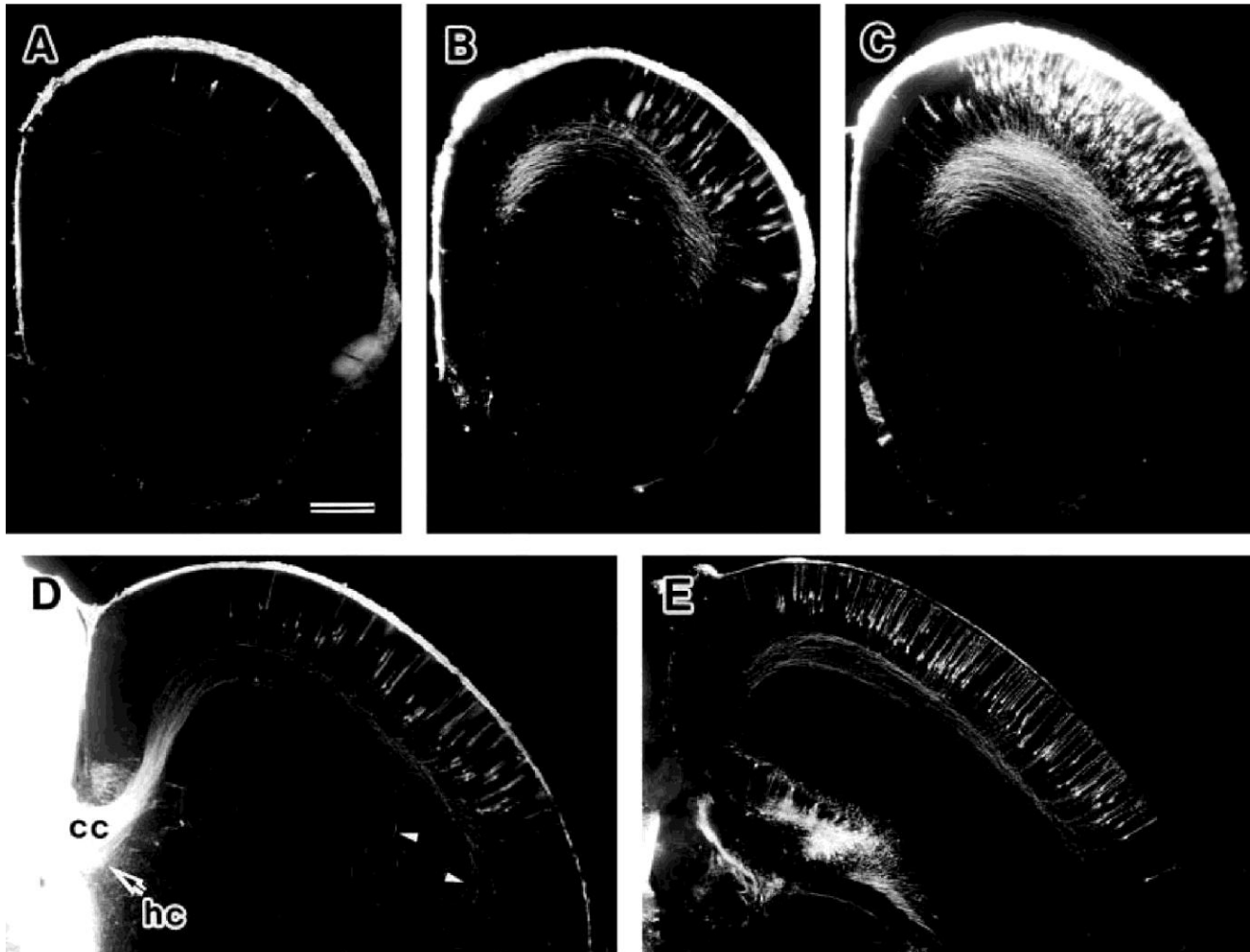

Fig. 3. Fluorescence photomicrographs of coronal sections at the level of the presumptive frontal (A±C), parietal (D), and occipital cortex (E). **A:** A fetus of 0.64 g (embryonic day [E]16.4). **B:** A fetus of 0.69 g (E16.5). **C,D:** A fetus of 0.76 g (E16.7). **E:** A fetus of 0.91 g (E17.0). Retrogradely labeled cells in dorsal neocortex ®rst appear as a scattered neuronal population in the dorsal to lateral aspect of the presumptive frontal cortex (A), and their distribution expands across the tangential extent of the cerebral cortex medially (B,C) and caudally (D,E) with brain development (see also Fig. 2). On the other hand, the tangential distribution of labeled cells in the presumptive cingulate cortex (D) and the hippocampus (E) does not extend further dorsally beyond that shown in Figure 1B and C, respectively. Note the presence of the unlabeled zone in the dorsal aspect of medial cortex (D,E). In D, the corpus callosum (cc) is de®nitively distinguishable from the hippocampal commissure (hc). White arrowheads indicate labeled axons leaving the cortical white matter to course downward in the internal capsule. Scale bar = 300 µm (applies to all).

DISCUSSION

Source of earliest callosal projections

Our results show that in B6D2F$_2$/J mice the first cortical cells to send axons across the nascent CC are found as a scattered neuronal population in the dorsal to lateral part of presumptive frontal cortex at about 0.64 g body weight or E16.4. This stage is consistent with our previous estimate of the first arrival at the midplane of frontal CC axons from the same hybrid mouse population based on anterograde tract tracing using lipophilic dyes (Ozaki and Wahlsten, 1992). The earliest callosal cells are cortical plate neurons, not subplate ones, and show an immature bipolar morphology resembling that of early postmigratory neurons. This morphology is somewhat different from a more mature pyramidal-like morphology of later callosal projection cells, whose order of axonal crossing approximately corresponds to the gradient of proliferation and maturation of cells within the cortex, which has been documented to be from rostral-lateral to caudal-medial (Rickmann et al., 1977; Raedler et al., 1980; Smart and Smart, 1982; Floeter and Jones, 1985; Uylings et al., 1990; Bayer and Altman, 199 1).
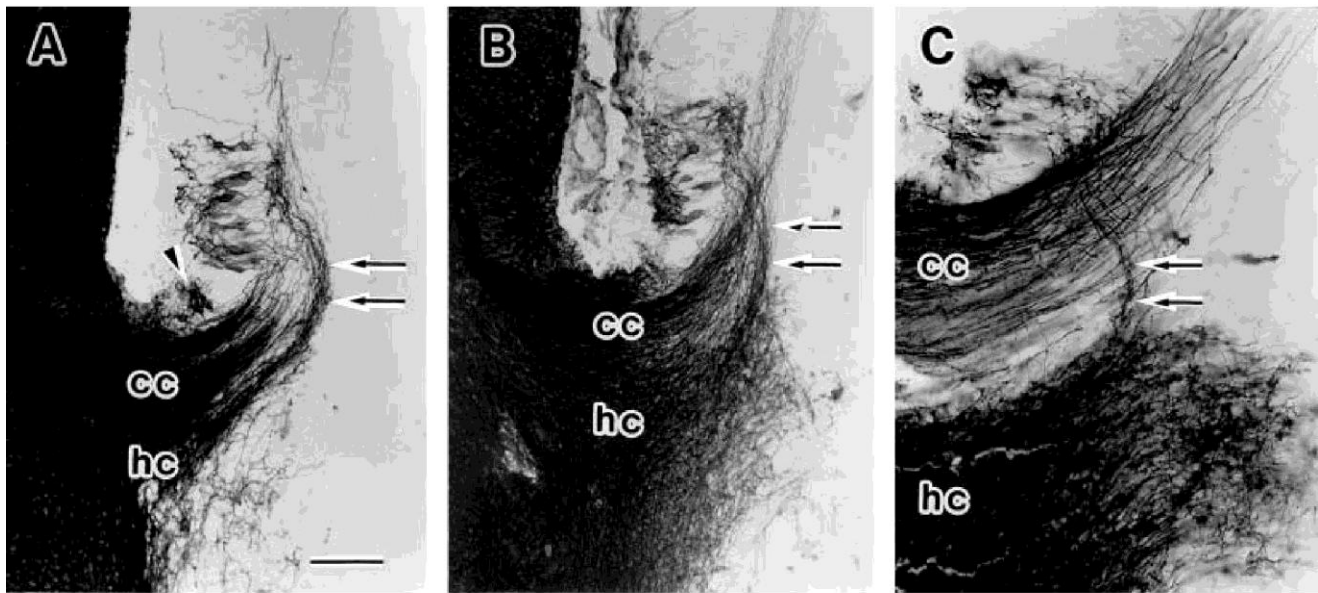
Fig. 4. Photoconverted coronal sections showing the trajectory of axons originating from a cluster of labeled cells in ventromedial cingulate cortex (A: 0.67 g or embryonic day [E]16.4; B: 0.76 g or E16.7; C: 0.89 g or E17.0). Note that axons (arrows) from cingulate cells do not join labeled axons of the corpus callosum (cc) but intersect the callosal pathway to merge with those of the hippocampal commissure (hc). An arrowhead in A indicates labeled indusium griseum cells. Scale bar = 100 μm (applies to all).
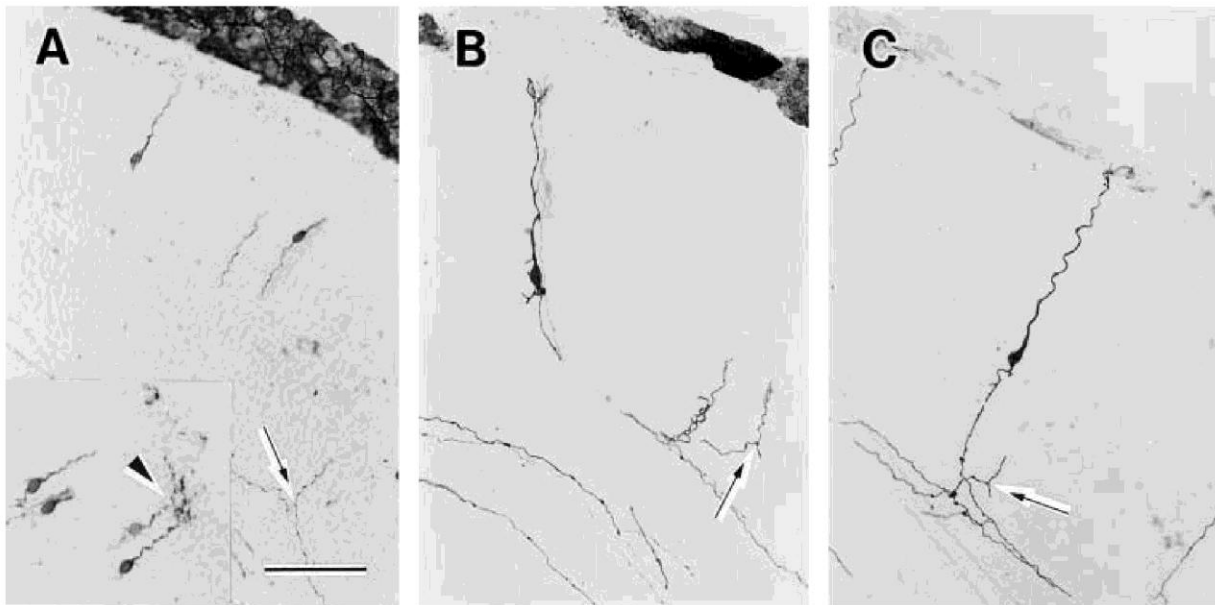

Fig. 5. Morphologies of photoconverted callosal cells in coronal sections. A: Earliest callosal cells found in the dorsal aspect of the presumptive frontal cortex at 0.64 g or embryonic day (E)16.4. The two cells are the same as the left two labeled cells shown in Figure 3A. An **inset** in the left lower side is another example of earliest callosal cells found in the lateral aspect of the presumptive frontal cortex at 0.65 g or E16.4, and an arrowhead indicates emerging branching of the apical dendrite below the pial surface. These earliest cells display an immature bipolar appearance. B,C: Callosal cells found in the dorsal aspect of the presumptive frontal cortex at 0.69 g or E16.5 (B; the level is somewhat caudal to that of A) and in the dorsolateral aspect of the presumptive parietal cortex at 0.74 g or E16.6 (C). The pyramidal-like morphologies are more mature than those seen in A. Arrows indicate the bifurcation of callosal axons (their cell bodies are located in adjacent sections) at the superficial part of the white matter. Upper, dorsal; left, medial. Scale bar = 100 μm (applies to all).

Regarding the cortical source of earliest callosal projections to reach midplane, our finding contrasts with that of a previous study in rat (Koester and O'Leary, 1994). We have confirmed in mouse the presence of a discrete cluster of cells in the most ventral part of presumptive cingulate cortex, which extend their axons to the contralateral hemisphere at least 1 day before cells in presumptive frontal neocortex and whose location and morphology are comparable to those for cells described in rat fetuses as callosal pioneers. At later embryonic stages, however, when the CC and HC become distinct from each other, axons of these cingulate cells intersect the CC and join the HC in the septal region. The anatomical projection pattern of these axons is characterized by the perpendicular orientation through the CC just lateral to the midsagittal plane, and their location in ventral cingulate cortex seems identical to that of the perforating fibers which originate from cells in the cingulate

cortex and project to not definitively known targets in the septum without decussating in the CC (Kappers et al., 1936; Yakovlev and Locke, 1961). It has been shown that in mouse the perforating fibers become identifiable in silver stained sections between embryonic days E15 and 16 (the plug date being E0), at least 1 day prior to the initial appearance of the callosal projection at the midplane (Hankin and Silver, 1988). The similarities in their anatomical features and time sequence of development suggest that the axons of ventromedial cingulate cells may represent a part of the perforating fibers (Kappers et al., 1936; Yakovlev and Locke, 1961). Although the perforating fiber pathway is currently believed to be ipsilateral, our results imply that this pathway includes interhemispheric projections through the HC.

Against our finding, it maybe argued that the ventromedial cingulate cells retrogradely labeled prior to cells in frontal neocortex are indeed the earliest callosal neurons, but their callosal axons are rapidly removed via either apoptotic cell death or axon elimination, and a different population of cells newly projecting through the HC are detected later in the same cortical region with retrograde labeling. However, because it has been shown that apoptotic cell death in the rodent cerebral cortex (Spreafico et al., 1995) and axon elimination in rodent callosal projection (O'Leary et al., 1981; Ivy and Killackey, 1981, 1982) occur postnatally, these phenomena seem to be inappropriate to account for our finding. Alternatively, it also may be possible that the labeled cells in ventromedial cingulate cortex are heterogeneous in projection and that, even if most of these cells project through the HC, a minor cell population projects through the CC. Although we cannot rule out this possibility, it should be stressed that the tangential distribution of labeled cells in this cortical region does not extend further dorsally with development, when labeled cells in neocortex increase greatly in number and tangential distribution with body weight or age. This observation, taken in conjunction with the ontogenetic fact that the ventral part of cingulate cortex is derived from the mesocortex, whereas the dorsal cingulate cortex and the adjacent neocortex originate from the isocortex (Hankin and Silver, 1988), strongly suggests that labeled cells in ventromedial cingulate cortex and dorsal neocortex are developmentally independent populations.

Potential role of pioneers in CC development
We have shown that cortical plate cells in the presumptive frontal cortex are the first neurons to send axons across the nascent CC. In other neural systems, invertebrate (Klose and Bentley, 1989) and vertebrate (Lamborghini, 1987; McConnell et al., 1994), early-developing populations of axons have been shown to pioneer axonal pathways in a way critical for the normal development of later-arising axonal projections. An intriguing issue is whether or not the callosal pioneers also serve a crucial role in the formation of this commissure.

The leading or "pioneering" growth cones are often described in many developing nervous systems to be larger and more complex than later-arising growth cones that fasciculate onto these lead axons (Lopresti et al., 1973; Kim et al., 1987; Nordlander, 1987; Yaginuma et al., 1991). To explain this phenomenon, it is assumed that pioneering growth cones are larger and more complex because they sample increased environmental cues to navigate and lay down a pathway, whereas later-arising cells use the previously established path represented by the pioneer axons, making more simple pathway decisions. In the developing CC, however, virtually all growth cones in the callosal white matter are large and complex with broad lamellipodial veils regardless of age or position (Norris and Kalil, 1990): there are no consistent differences in size or morphological complexity among growth cones growing out earlier in development and those following later; nor do growth cone size or morphology differ in different positions along the callosal pathway prior to crossing to the opposite hemisphere. These observations suggest that callosal axons navigate independently of one another. In fact, the possibility that regardless of cortical regions of their origin, any callosal axons may traverse the midline first to pioneer the CC is shown by our previous study (Ozaki and Wahlsten, 1993) in mice with the hereditary defects of the CC, where this structure is totally or partially absent due to a substrate defect responsible for axon guidance at the midsagittal plane early in development (Silver et al., 1982; Wahlsten, 1987).

Because the present study clarifies the ontogeny of callosal projection cells in a normal mouse fetus, we could address the role of pioneering frontal axons by examining the origin of earliest axons across the CC and the

subsequent sequence in which axons of callosal projection cells cross midplane in fetuses of acallosal strain mice.

Contribution of subplate neurons to CC formation

In some mammalian species, neurons of the neocortical subplate have been reported to pioneer the internal capsule by sending the first axons into this pathway (McConnell et al., 1989; Blakemore and Molnar, 1990; De Carlos and O'Leary, 1992; Erzurumlu and Jhaveri, 1992). Sub-plate neurons are the earliest generated neuronal population of the cerebral cortex and they have also been shown in the cat to project to the opposite hemisphere (Chun et al., 1987; Antonini and Shatz, 1990). Koester and O'Leary (1994) have reported in rat, however, that these neurons neither extend the first axons across the CC nor significantly contribute in the cortical projection through the CC to contralateral hemisphere. Consistent with this report, our results in mouse show that subplate neurons do not project through the CC prior to cells in the cortical plate and that at any given cortical region only a few cells in the subplate can be retrogradely labeled from large DiI injections into the contralateral white matter. Because cortical plate cells are generated from the neuroepithelium of the telencephalic vesicle later than subplate neurons, the late development of the CC relative to the internal capsule must be relevant to the differences in the class of pioneering neurons and their contributions to the formation of these major efferent pathways from the cerebral cortex.

Guidance cues for callosal axons

The CC is formed by cortical axons commissurating at the midplane over the septal region. Then, what are the cues for the early extension of callosal axons polarized medially toward the midline? One candidate is chemo-tropic substances emitted by cells along the callosal pathway. Axon guidance by chemotropism has been demonstrated in the development of several neural connections in the central nervous system (O'Leary, 1994). It seems, however, unlikely that cells of the dorsal septum release a diffusible substance attracting the growing callosal axons to the midplane. It has been demonstrated that in spite of the retarded growth of the dorsal septum (Wahlsten and Bulman-Fleming, 1994), callosal axons in acallosal mutant mice normally emerge from their cells of origin and grow medially toward their midline crossing point on schedule (Ozaki and Wahlsten, 1993). Whether diffusible or local guidance mechanisms operate in the medially polarized extension of callosal axons, further studies are needed to clarify the nature of guidance cues which appear to be expressed in a temporally regulated manner along the callosal pathway.

LITERATURE CITED

Adamek, G.D., M.T. Shipley, and M.S. Sanders (1984) The indusium griseum in the mouse: Architecture, Timm's histochemistry and some afferent connections. Brain Res. Bull. *12:*657–668.

Antonini, A. and C.J. Shatz (1990) Relation between putative transmitter phenotypes and connectivity of subplate neurons during cerebral cortical development. Exp. Neurol. *107:*36– 47.

Auladell, C., A. Martinez, S. Alcantara, H. Super, and E. Soriano (1995) Migrating neurons in the developing cerebral cortex of the mouse send callosal axons. Neuroscience *64:*1091–1103.

Bayer, S.A. and J. Altman (1991) Neocortical Development. New York: Raven Press.

Blakemore, C. and Z. Molnar (1990) Factors involved in the establish of specific interactions between thalamus and cerebral cortex. Cold Spring Harbor Symp. Quant. Biol. *55:*491–504.

Bulman-Fleming, B. and D. Wahlsten (1991) The effects of intrauterine position on the degree of corpus callosum deficiency in two substrains of BALB/c mice. Dev. Psychobiol. *24:*395–412.

Chun, J.J.M., M.J. Nakamura, and C.J. Shatz (1987) Transient cells of the developing mammalian telencephalon are peptide-immunoreactive neurons. Nature *325:*617–620.

De Carlos, J.A. and D.D.M. O'Leary (1992) Growth and targeting of subplate axons and establishment of major cortical pathways. J. Neurosci. *12:*1194–121 1.

Erzurumlu, R.S. and S. Jhaveri (1992) Emergence of connectivity in the embryonic rat parietal cortex. Cerebral Cortex *2:*336–352.

Fisher, R.S., M.K. Boylan, C.D. Hull, N.A. Buchwald, and M.S. Levine (1986) Branched projections of cat sensorimotor cortex: Multiple retro-grade labeling via commissural corticocortical, decussated corticostriatal and undecussated corticostriatal axons. Brain Res. *384:*395–400.

Floeter, M.K. and E.G. Jones (1985) The morphology and phased outgrowth of callosal axons in the fetal rat. Dev. Brain Res. *22:*7–18.

Glas, P. (1975) Onderzoek Naar de Vreoge Ontwikkeling van de Commissuren in het Mediane Gebied van het Telencephalon bij de Witte Muit. Groningen: Drukkerij Van Denderen B.V.

Godement, P., J. Vanselow, S. Thanos, and F. Bonhoeffer (1987) A study in developing visual systems with a new method of staining neurones and their processes in fixed tissue. Development *101:*697–713.

Hankin, M.H. and J. Silver (1988) Development of intersecting CNS fiber tracts: The corpus callosum and its perforating fiber pathway. J. Comp. Neurol. *272:*177–190.

Honig, M.G. and R.I. Hume (1989) DiI and DiO: Versatile fluorescent dyes for neuronal labelling and pathway tracing. Trends Neurosci. *12:*333– 341.

Ivy, G.O. and H.P. Killackey (1981) The ontogeny of the distribution of callosal projection neurons in the rat parietal cortex. J. Comp. Neurol. *195:*367–389.

Ivy, G.O. and H.P. Killackey (1982) Ontogenetic changes in the projections of neocortical neurons. J. Neurosci. *2:*735–743.

Kappers, C.U.A., G.C. Huber, and C.C. Crosby (1936) The Comparative Anatomy of the Nervous System of Vertebrates Including Man, Vol. II. New York: Dmacmillan Co.

Kim, G.J., C.J. Shatz, and S.K. McConnell (1991) Morphology of pioneer and follower growth cones in the developing cerebral cortex. J. Neurobiol. *22:*629–642.

Klose, M. and D. Bentley (1989) Transient pioneer neurons are essential for formation of an embryonic peripheral nerve. Science *1245:*982–984.

Koester, S.E. and D.D.M. O'Leary (1993) Connectional distinction between callosal and subcortically projecting cortical neurons is determined prior to axon extension. Dev. Biol. *160:*1–14.

Koester, S.E. and D.D.M. O'Leary (1994) Axons of early generated neurons in cingulate cortex pioneer the corpus callosum. J. Neurosci. *14:*6608– 6620.

Lamborghini, J.E. (1987) Disappearance of Rohon-Beard neurons from the spinal cord of larval *Xenopus laevis*. J. Comp. Neurol. *264:*47–55.

Livy, D.J. and D. Wahlsten (1997) Retarded formation of the hippocampal commissure in embryos from mouse strains lacking a corpus callosum. Hippocampus *7:*2–14.

Lopresti, V., E.R. Macagno, and C. Levinthal (1973) Structure and development of neuronal connections in isogenic organisms: Cellular interac-tions in the development of the optic lamina of *Daphnia magna.* Proc. Nat. Acad. Sci. USA *70:*433–437.

McConnell, S.K., A. Ghosh, and C.J. Shatz (1989) Subplate neurons pioneer the first axon pathway from the cerebral cortex. Science *245:*978–982.

McConnell, S.K., A. Ghosh, and C.J. Shatz (1994) Subplate pioneers and the formation of descending connections from cerebral cortex. J. Neurosci. *14:*1892–1907.

McGeorge, A.J. and R.L.M. Faull (1989) The organization of the projection from the cerebral cortex to the striatum in the rat. Neuroscience *29:*503–537.

Morino, P., M. Herrera-Marschitz, J.J. Meana, U. Ungerstedt, and T. Hökfelt (1992) Immunohistochemical evidence for a crossed cholecystokinin corticostriatal pathway in the rat. Neurosci. Lett. *148:*133–136.

Nordlander, R.H. (1987) Axonal growth cones in the developing amphibian spinal cord. J. Comp. Neurol. *263:*485–496.

Norris, C.R. and K. Kalil (1990) Morphology and cellular interactions of growth cones in the developing corpus callosum. J. Comp. Neurol. *293:*268–281.

O'Leary,D.D.M. (1994) Attractive guides for axons. Nature *371:*15–16.

O'Leary, D.D.M., B.B. Stanfield, and W.M. Cowan (1981) Evidence that the early postnatal restriction of the cells of origin of the callosal projection is due to the elimination of axonal collaterals rather than to the death of neurons. Dev. Brain Res. *1:*607–617.

Ozaki, H.S. and D. Wahlsten (1992) Prenatal formation of the normal mouse corpus callosum: A quantitative study with carbocyanine dyes. J. Comp. Neurol. *323:*81–90.

Ozaki, H.S. and D. Wahlsten (1993) Cortical axon trajectories and growth cone morphologies in fetuses of acallosal mouse strains. J. Comp. Neurol. *336:*595–604.

Raedler, E., A. Raedler, and S. Feldhaus (1980) Dynamical aspects of neocortical histogenesis in the rat. Anat. Embryol. *158:*253–269.

Rickmann, M., B.M. Chronwall, and J.R. Wolff (1977) On the development of non-pyramidal neurons and axons outside the cortical plate: The early marginal zone as a pallial anlage. Anat. Embryol. *151:*285–307.

Sandell, J.H. and R.H. Masland (1988) Photoconversion ofsome fluorescent markers to a diaminobenzidine reaction product. J. Histochem. Cytochem. *36:*555–559.

Schwartz, M.L., P. Rakic, and P.S. Goldman-Rakic (1991) Early phenotype expression of cortical neurons: Evidence that a subclass of migrating neurons have callosal axons. Proc. Nat. Acad. Sci. USA*88:*1354–1358.

Silver, J., S.E. Lorenz, D. Wahlsten, and J. Coughlin (1982) Axonal guidance during development of the great cerebral commissures: De-scriptive and experimental studies, in vivo, on the role of preformed glial pathways. J. Comp. Neurol. *210:*10–29.

Smart, I.H.M. and M. Smart (1982) Growth patterns in the lateral wall of the mouse telencephalon: I. Autoradiographic studies of the histogenesis of the isocortex and adjacent areas. J. Anat. *134:*273–298.

Spreafico, R., C. Frassoni, P. Arcelli, M. Selvaggio, and S. De Biasi (1995) In situ labeling of apoptotic cell death in the cerebral cortex and thalamus of rats during development. J. Comp. Neurol. *363:*281–295.

Uylings, H.B.M., C.G. Van Eden, J.G. Parnavelas, and A. Kalsbeek (1990) The prenatal and postnatal development of rat cerebral cortex. In B. Kolb and R.C. Tees (eds): The Cerebral Cortex of the Rat. Cambridge, MA: MIT Press, pp. 35–76.

Valentino, K.L. and E.G. Jones (1982) The early formation of the corpus callosum: A light and electron microscopic study in foetal and neonatal rats. J. Neurocytol. *11:*583–609.

Voneida, T.J., R.M. Vardaris, S.E. Fish, and C.T. Reiheld (198 1) The origin of the hippocampal commissure in the rat. Anat. Rec. *201:*91–103.

Wahlsten, D. (1981) Prenatal schedule of appearance of mouse brain commissures. Dev. Brain Res. *1:*461–473.

Wahlsten, D. (1984) Growth of the mouse corpus callosum. Dev. Brain Res. *15:*59–67.

Wahlsten, D. (1987). Defects of the fetal forebrain in mice with hereditary agenesis of the corpus callosum. J. Comp.Neurol. *262:*227–241. Wahlsten, D. and B. Bulman–Fleming (1994) Retarded growth of the medial septum: A major gene deficit in acallosal mice. Dev. Brain Res. *77:*203–214.

Wahlsten, D. and G. Smith (1989) Inheritance of retarded forebrain commissure development in fetal mice: Results from classical crosses and recombinant inbred strains. J. Hered. *80:*11–16.

Wahlsten, D. and P. Wainwright (1977) Application of a morphological time scale to hereditary differences in prenatal mouse development. J. Embryol. Exp. Morphol. *42:*79–92.

Wainwright, P., C. Pelkman, and D. Wahlsten (1989) The quantitative relationship between nutritional effects on preweaning growth and behavioural development in mice. Dev. Psychobiol. *22:*183–195.

Yaginuma, H., S. Homma, R. Kunzi, and R.W. Oppenheim (1991) Pathfinding by growth cones of commissural interneurons in the chick embryo spinal cord: A light and electron microscopic study. J. Comp. Neurol. *304:*78–102.

Yakovlev, P.I. and S. Locke (1961) Limbic nuclei of thalamus and connections of limbic cortex. III. Corticocortical connections of the anterior cingulate gyrus, the cingulum, and the subcallosal bundle in monkey. Arch. Neurol. *5:*364–400.

Zaki, W. (1985) Le processus dégénératif au cours du développement du corpus. Archiv. Anat. Micr. Morphol. Exper. *74:*133–149.