THE JOURNAL OF COMPARATIVE NEUROLOGY 434:147–157 (2001)

A Role for Cingulate Pioneering Axons in the Development of the Corpus Callosum

BRIAN G. RASH AND LINDA J. RICHARDS*
The University of Maryland, Baltimore, School of Medicine, Department of Anatomy and Neurobiology, and the Program in Neuroscience, Baltimore, Maryland 21201

ABSTRACT
In many vertebrate and invertebrate systems, pioneering axons play a crucial role in establishing large axon tracts. Previous studies have addressed whether the first axons to cross the midline to from the corpus callosum arise from neurons in either the cingulate cortex (Koester and O'Leary [1994] J. Neurosci. 11:6608–6620) or the rostrolateral neocortex (Ozaki and Wahlsten [1998] J. Comp. Neurol. 400:197–206). However, these studies have not provided a consensus on which populations pioneer the corpus callosum. We have found that neurons within the cingulate cortex project axons that cross the midline and enter the contralateral hemisphere at E15.5. By using different carbocyanine dyes injected into either the cingulate cortex or the neocortex of the same brain, we found that cingulate axons crossed the midline before neocortical axons and projected into the contralateral cortex. Furthermore, the first neocortical axons to reach the midline crossed within the tract formed by these cingulate callosal axons, and appeared to fasciculate with them as they crossed the midline. These data indicate that axons from the cingulate cortex might pioneer a pathway for later arriving neocortical axons that form the corpus callosum. We also found that a small number of cingulate axons project to the septum as well as to the ipsilateral hippocampus via the fornix. In addition, we found that neurons in the cingulate cortex projected laterally to the rostrolateral neocortex at least 1 day before the neocortical axons reach the midline. Because the rostrolateral neocortex is the first neocortical region to develop, it sends the first neocortical axons to the midline to form the corpus callosum. We postulate that, together, both laterally and medially projecting cingulate axons may pioneer a path for the medially directed neocortical axons, thus helping to guide these axons toward and across the midline during the formation of the corpus callosum. J. Comp. Neurol. 434:147–157, 2001. © 2001 Wiley-Liss, Inc.

Indexing terms: axon guidance; fasciculation; midline; neocortex; mouse

During development the two telencephalic vesicles are derived from the prosencephalon. The rostral region of the prosencephalon is called the lamina terminalis and contains the commissural plate or lamina reuniens (Rakic and Yakolev, 1968, and references therein). Rostral to the lamina terminalis, midline glia "zipper" the midline together (Silver et al., 1993) possibly forming the equivalent of the massa commissuralis in humans (Rakic and Yakovlev, 1968), a region of cellular continuity within the dorsal interhemispheric fissure. The corpus callosum forms in the dorsomedial region of the cerebral cortex, at the corticoseptal boundary, in a region that was cellularly discontinuous just 1 day earlier in development in mice (Silver et al., 1993). Therefore, the first axons to cross the midline at this point (rostral to the hippocampal commissure) must traverse between the two cerebral hemispheres, pioneering an axon tract within this newly formed cellular region. The majority of axons that make up the corpus callosum arise from neurons within the neocortex (Yorke and Caviness, 1975; Wise and Jones 1976; Ivy and Killackey, 1981), but the corpus callosum may be pioneered by neurons in the cingulate cortex

Grant sponsor: The March of Dimes Foundation for Birth Defects; Grant number: 5-FY99-842; Grant sponsor: NIH/NINDS; Grant number: NS37792

*Correspondence to: Linda J Richards, University of Maryland, Baltimore, School of Medicine, Department of Anatomy and Neurobiology, 685 West Baltimore Street, Baltimore, MD 21201.
E-mail: lrich001@umaryland.edu

Received 1 November 2000; Revised 13 February 2001; Accepted 16 February 2001

© 2001 WILEY-LISS, INC.

(Koester and O'Leary, 1994). The first neocortical axons to cross the midline in the rodent arise from neurons in the rostrolateral neocortex (Ozaki and Wahlsten, 1992, 1998). These neocortical axons have been postulated to project the first axons across the nascent corpus callosum in mice (Ozaki and Wahlsten, 1998). Therefore, a discrepancy exists within the literature as to whether the corpus callosum is pioneered by cingulate or neocortical axons.

Pioneering axons have been shown to guide later developing axons to their targets in invertebrates (Bate, 1976; Bastiani et al., 1984; Klose and Bentley, 1989), *Xenopus* (Bork et al., 1987), zebrafish (Kuwada, 1986; Wilson and Easter, 1991), chick (Holley, 1982), mouse (Super et al., 1998), rat (De Carlos and O'Leary, 1992), and cat (McConnell et al., 1989). In many of these species researchers have asked the question: Are the pioneering axons really required for the development of later projecting axons? In simpler organisms such as grasshopper (Klose and Bentley, 1989) and zebrafish (Kuwada, 1986), this question has been addressed by specifically ablating the pioneering population by either heat-shock treatment or laser ablation, respectively. In these experiments, it was found that later developing axons were unable to follow their correct path of growth and innervate their normal targets. Thus, pioneering axons are necessary for the proper formation of the nervous system in these organisms.

In mammals, the corticothalamic projection is pioneered by neurons in the subplate layer of the neocortex that project the first neocortical axons into the internal capsule (McConnell et al., 1989; Catalano et al., 1991; De Carlos and O'Leary, 1992). Subplate ablation experiments have shown that subplate axons are required for the development of both the corticothalamic (McConnell et al., 1994) and thalamocortical pathways (Ghosh et al., 1990; Ghosh and Shatz, 1993). Therefore, subplate pioneering axons help to establish connections between the thalamus and the cortex during development.

Although the subplate plays a critical role in guiding laterally projecting neocortical axons through the internal capsule, it does not seem to play a role in guiding medially projecting neocortical axons across the corpus callosum. The evidence for this is twofold: (1) there is not a significant subplate projection across the corpus callosum in rodents (Koester and O'Leary, 1994; Ozaki and Wahlsten, 1998), and (2) callosal development is unaffected by subplate ablations in cats (McConnell et al., 1994). The first axons to cross the rostral cortical midline (rostral to the hippocampal commissure) are derived from neurons in the cingulate cortex (Koester and O'Leary, 1994). These axons begin to cross the midline at embryonic day (E) 17 in the rat and E15.5 in the mouse. Whether these cingulate axons project to the contralateral cortical hemisphere and whether they project before the neocortical axons has not been resolved. One study has suggested that the cingulate axons do not pioneer the corpus callosum because they do not project to the contralateral cortex but instead project through the hippocampal commissure to the hippocampus (Ozaki and Wahlsten, 1998). Such ambiguity may be possible because more caudal regions of the corpus callosum develop in close proximity to the hippocampal commissure (Valentino and Jones, 1982; Livy and Wahlsten, 1997). In previous studies, this pathway was investigated by using retrograde labeling techniques that label a single projection in isolation (Koester and O'Leary, 1994; Ozaki and Wahlsten, 1998). Here, we use anterograde labeling to investigate multiple axonal projections of the cingulate cortex during development. We also use double dye labeling to address whether neurons of the cingulate cortex cross the midline before neocortical axons, thus pioneering the pathway of the corpus callosum.

MATERIALS AND METHODS

Animals

Female C57BL/6J mice were mated after 5 PM and inspected the following morning for the presence of a vaginal plug. The day of detection was designated E0. Mice were weighed on this day and again 10 days later, when a weight increase of 20% indicated possible pregnancy. Embryos were staged based on conventional plug-dating correlated with embryo weight as described by Wahlsten, 1987; E16, E17, and E18 embryos had a mean weight of 0.476 g (n = 10), 0.621 g (n = 27), and 1.24 g (n = 6), respectively. Embryos of age E15 through E19, including ages E15.5 and E16.5, were used. Mice were housed at the University of Maryland, Baltimore, School of Medicine animal facility. Procedures involving animals were approved by the University of Maryland, Baltimore's animal care and use committee.

Fixation

On the required day of gestation, pregnant dams were anesthetized with sodium pentobarbital (Nembutal; Abbott Laboratories, IL) at 0.07 mg/g body weight and placed on a warming pad to maintain body temperature. Once deeply anesthetized, the mother's abdomen was opened to expose the uterus. Pups were removed sequentially and placed on ice, if older than E16, until deeply anesthetized. Intracardial perfusion for 1 minute with phosphate buffered saline (PBS, pH 7.4) preceded perfusion for 4–5 minutes with a solution of 4% paraformaldehyde in PBS. Heads were then removed and their skulls opened along the midsagittal and lambdoid sutures and subsequently stored in fixative at 4°C until carbocyanine dye injections.

Carbocyanine dye injections

The skulls were removed to expose the underlying cortex. Exposed brain tissue was kept moist with fixative, except for where the injection was made. Injections were made with pulled glass pipettes attached to a pressure injector (Picospritzer II, Parker Instrumentation) and pressurized nitrogen tank. Interior tip diameter averaged approximately 8 μm. Finer pipettes were used for injections in cingulate cortex. Pipettes were filled with solutions of either 10% DiI (Molecular Probes, catalog no. D-282) or saturating amounts of DiA (approximately 5% solutions; Molecular Probes, catalog no. D-3883) in dimethylformamide. Injections were made into the cingulate cortex (DiI), the dorsolateral neocortex (DiA), or both. To ensure that our observations were not due to fibers of passage from the neocortex labeled as they grew through the injection site in the cingulate cortex, we carefully examined brains for the presence of retrogradely labeled cell bodies in the neocortex. Because DiI (and DiA) diffuses both retrogradely and anterogradely, this is an effective method for detecting whether fibers of passage have been labeled. For hippocampal injections, brains were bisected coronally, exposing the hippocampal field, so that injections could be made directly into the hip-

10969861, 2001, 2, Downloaded from https://onlinelibrary.wiley.com/doi/10.1002/cne.1170 by Washington University School, Wiley Online Library on [21/07/2023]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

pocampus without penetrating the neocortex. Injected brains were stored at 37°C in darkness for at least 3 weeks (average of 5 weeks) before sectioning to allow for dye transport.

Sectioning and imaging

Brains were sectioned on a vibrating-blade microtome (Leica) at a thickness of 50 μm. Sections were mounted in order in a mounting medium containing 2.5% PVA/2.5% DABCO in glycerol and analyzed by using a laser scanning microscope (LSM; Olympus Fluoview 2.0). DiI and DiA were excited with a krypton laser (568-nm emission) and an argon laser (488-nm emission), respectively. Due to the broad emission spectrum of DiA, which emits significantly in the detection range used for DiI (602 nm ± 20 nm), single-laser illumination of the sample was achieved by the addition of barrier filters (Chroma). Because DiI is not excited by the argon laser, it was possible to produce unambiguous detection of DiA signals by blocking krypton excitation. Each image frame was scanned twice, once for each dye. These images were then superimposed. This procedure allowed us to take advantage of the relatively strong emission of DiA at the detection wavelength of 520 nm ± 20 nm (green). Image analysis and compilation was done with Adobe Photoshop 4.0 on a Macintosh G3 computer.

RESULTS

Neurons in the cingulate cortex project medially to the contralateral cortex as well as to the fornix and septum

To investigate the developmental timing of axonal projections from the cingulate cortex, we made small injections of DiI from E15 onward into the rostral cingulate cortex to anterogradely label cingulate axons (injection site shown as + in inset of Fig. 1A). In the same brains, we also injected DiA into the dorsal neocortex (injection site shown as * in inset in Fig. 1A) to label the first medially projecting neocortical axons passing through the DiA injection site. E15.5 (embryonic body weight 0.47 g) was the first time point at which we observed any axons crossing the midline from our injections (arrowhead in Fig. 1A). These axons were labeled with DiI, indicating that they originated from the cingulate cortex. The cingulate axons crossed the midline and turned dorsally toward the contralateral cortical hemisphere. We also observed two additional bundles of DiI-labeled axons projecting medially (arrows in Fig. 1A). One projected ventromedially toward the fornix, whereas the other projected lateral to the midline toward the septum within the perforating pathway (Hankin and Silver, 1988; Shu and Richards, unpublished observation). This result indicated that neurons in the cingulate cortex do project across the midline, entering both the contralateral cortex, the fornix, and the septum. The cingulate projection into the contralateral cortex became even more evident at E16.5 as these pathways became more distinct (arrowhead in Fig. 1B). As shown in Figure 1A, no DiA-labeled axons from the neocortex were present at the midline at E15.5 (in n = 4 brains with the same injection protocol). DiA-labeled axons from the neocortex first reached the midline between E16 and E16.5 (Figs. 1C, 3), concurrent with the finding of Ozaki and Whalsten, (1998).

Callosally projecting cingulate axons form a separate pathway to cross the midline

As development proceeded, anterogradely labeled cingulate axons continued to enter the contralateral cortical hemisphere, projecting dorsally (arrowheads in Fig. 1B,D). At E16.5, we observed the first distinction between the callosal cingulate pathway and the ventrally projecting cingulate axons within the white matter underlying the cingulate cortex (Fig. 1C). The contralaterally projecting axons remained dorsal, projecting toward and across the midline, whereas the ventrally projecting populations crossed over these axons (large arrow in Fig. 1C). DiA-labeled axons from the neocortex that approached the midline remained within the dorsal region of the tract in the same region as the contralaterally projecting cingulate axons (small arrow in Fig. 1C). By E18, the axon pathways from the cingulate cortex became even more physically separated, making it easy to distinguish both a contralateral (callosal) projection (arrowhead in Fig. 1D) and the ipsilateral projections (arrow in Fig. 1D).

Occasionally, we also observed retrogradely labeled cell bodies within the contralateral cingulate cortex as previously described (Koester and O'Leary, 1994). In addition, we observed retrogradely labeled cell bodies within the ipsilateral ventral septum, corresponding to neurons in the horizontal limb of the diagonal band of Broca (MSDB; small arrows in Fig. 1B), which give rise to the perforating pathway (Hankin and Silver, 1988; Shu and Richards, unpublished observation).

Injections in the hippocampus retrogradely label cells in the ipsilateral cingulate cortex

A previous study had shown that carbocyanine injections in medial cortex retrogradely labeled cells in the hippocampus (Ozaki and Whalsten, 1998). To determine whether axons from the cingulate cortex project to the hippocampus, we performed large injections of DiA in the hippocampus, filling the entire hippocampal field. Because the fornix and the hippocampal commissure form at least 1 day before the corpus callosum (Wahlsten, 1981; Valentino and Jones, 1982), this injection labeled the ipsilateral fornix at E16 (Fig. 2A). In the same brains injected with DiA in the hippocampus, we injected DiI in the cingulate cortex. From E16, the corpus callosum and the fornix/hippocampal commissure were quite distinct when independently labeled in this manner. In caudal regions of the developing corpus callosum, cingulate pioneering axons labeled with DiI projected dorsal to the fornix (arrowhead in Fig. 2A). In contrast, hippocampally projecting axons labeled by the cingulate injection clearly entered the fornix (arrow in Fig. 2A) and appeared yellow as they overlapped with the DiA-labeled axons within the fornix.

We then counted cell bodies retrogradely labeled by the hippocampal injections in serial sections throughout the cingulate cortex (Fig. 2B,C). At E16 (n = 3 brains), E17 (n = 3 brains), and E19 (n = 2 brains), we found a mean of only 164 (E16), 71 (E17), and 38 (E19) cells labeled in the cingulate cortex ipsilateral to the injection site. We found only five cells total in the contralateral cingulate cortex at E19 and none at E16 or E17. Therefore, the number of cingulate cortical neurons projecting to the hippocampus probably represents a small population of

10969861, 2001, 2, Downloaded from https://onlinelibrary.wiley.com/doi/10.1002/cne.1170 by Washington University School, Wiley Online Library on [21/07/2023]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

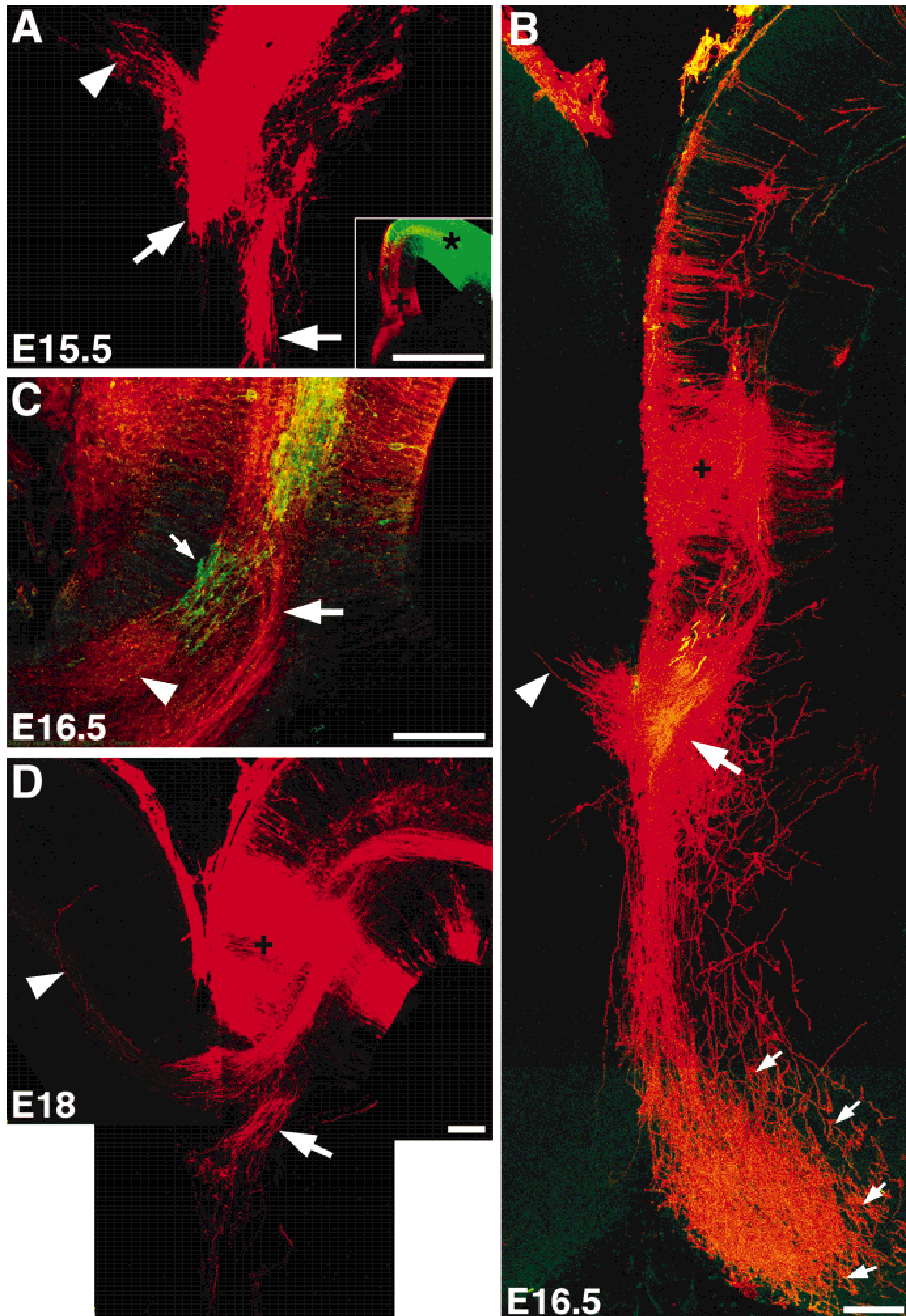

Fig. 1. Neurons in the cingulate cortex project axons across the midline and into the contralateral cortex. **A:** DiI injections (+ in inset) in the cingulate cortex labeled multiple axon pathways as early as embryonic day (E) 15.5. Three medial pathways could be distinguished: axons crossing the midline, growing up into the contralateral cortex (arrowhead in A); axons within the fornix; and axons within the perforating pathway (arrows in A). **B:** An injection of DiI (+) into the cingulate cortex at E16.5 labeled many axons crossing the midline, pioneering the corpus callosum (arrowhead) and projecting within the septum and fornix (large arrow). This injection also retrogradely labeled many cell bodies within the medial septum and the diagonal band of Broca complex (small arrows) forming the perforating pathway. **C:** Just lateral to the midline, the callosal pathway (arrowhead) was beginning to distinguish itself from the perforating pathway (large arrow), which crossed over the callosal pathway at this point. DiA-labeled axons from the neocortex followed the callosal pathway (green axons indicated by the small arrow). **D:** By E18, these different pathways became more spatially separated. At the midline, the callosal pathway (arrowhead) was present more dorsal to the perforating/fornix pathways (arrow). Scale bars = 200 μm in A–D.

10969861, 2001, 2, Downloaded from https://onlinelibrary.wiley.com/doi/10.1002/cne.1170 by Washington University School, Wiley Online Library on [21/07/2023]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

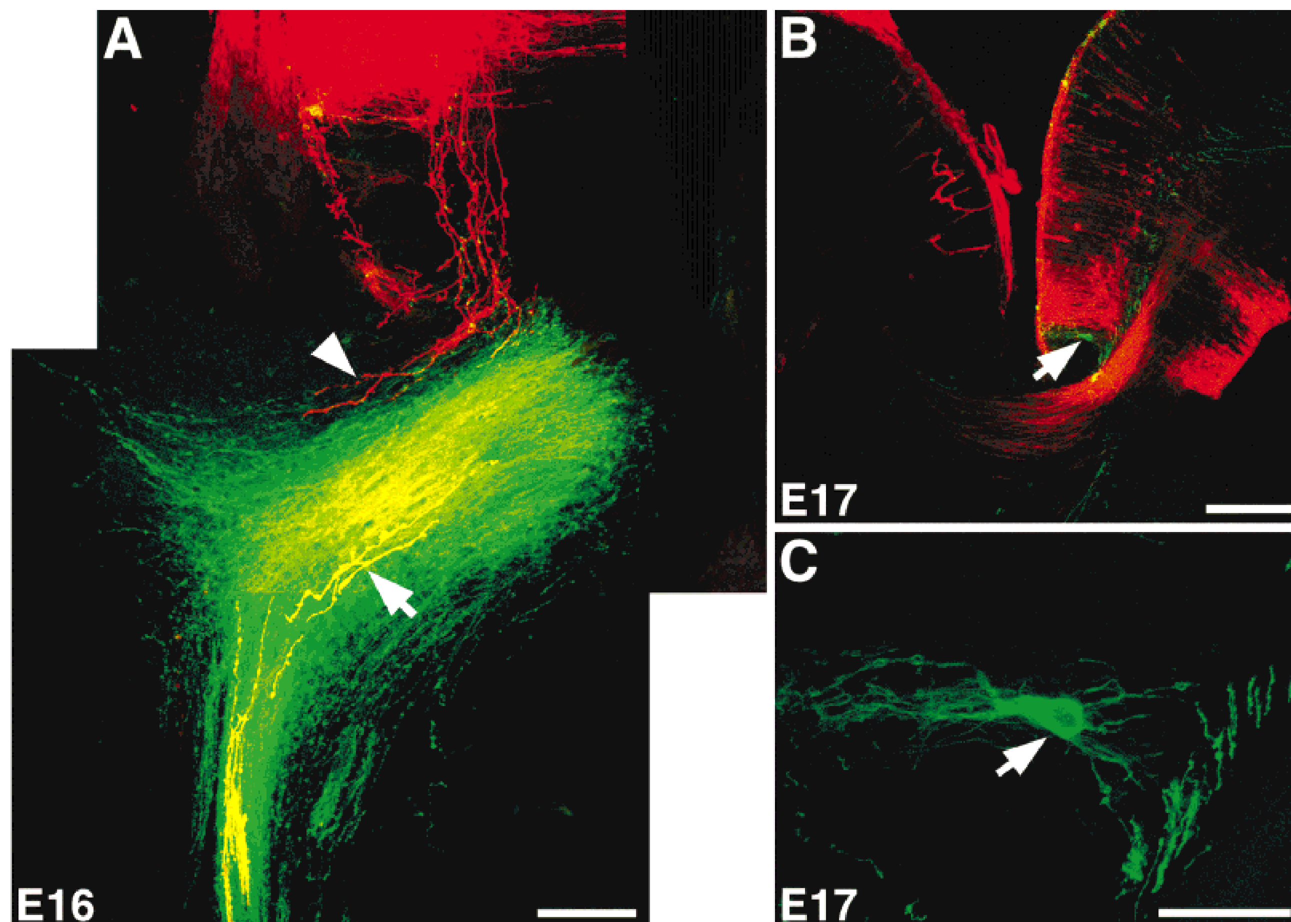

Fig. 2. Injections in the hippocampus and the cingulate cortex reveal separate pathways. Brains were injected with DiI in the cingulate cortex and DiA in the hippocampus. **A:** Callosally projecting axons from the cingulate cortex project dorsal to the fornix/hippocampal commissure (red axons indicated by the arrowhead). The fornix is entirely labeled with DiA and contains axons either retrogradely or anterogradely labeled by the cingulate injection (yellow axons within the fornix indicated by the arrow in A). Some pyramidal shaped neurons were retrogradely labeled by the DiA injection in the hippocampus, indicating that some neurons within the cingulate cortex do project to the hippocampus (arrow in **B**,**C**; C is a higher power view of B). Scale bars = 100 μm in A; 200 μm in B; 50 μm in C.

the total number of medially projecting neurons in the cingulate cortex at this stage. In addition, the majority of these hippocampally projecting cingulate axons project ipsilaterally rather than contralaterally, indicating that they do not cross the midline.

Neocortical axons cross the midline after cingulate cortical axons and may fasciculate with the cingulate cortical "pioneers"

The first neocortical neurons to send their axons across the corpus callosum lie in the rostrolateral neocortex (Ozaki and Wahlsten, 1998). To determine whether the callosal projection from the cingulate cortex crossed before the first neocortical axons, we double-labeled brains with DiI in the cingulate cortex (injection site shown in Fig. 3A) and DiA in the rostrodorsal neocortex (injection site shown in Fig. 3B; 3A and 3B are injections in the same brain, but at different rostrocaudal levels). In some cases, we placed a row of 4–5 injections along the dorsal neocortex (Fig. 3F) to label all the neocortical axons projecting to the midline, including those in the rostrolateral regions. Neurons in the rostrolateral region of the neocortex were both retrogradely labeled (cell bodies indicated by an arrow in Fig. 3B) and anterogradely labeled (axons indicated by arrow in Fig. 3C) as they passed through the injection site. In every case (n = 15 total brains from E15.5 [n = 4], E16 [n = 6], and E16.5 [n = 5]), DiA-labeled neocortical axons crossed the midline after a substantial number of DiI-labeled cingulate cortical axons had already crossed (Fig. 3C–G). The first DiA-labeled neocortical axons began to cross the midline at E16 (Fig. 3D,E,G). At this stage, very few DiA-labeled axons had reached the midline and those leading axons that had crossed within the tract already formed by the cingulate axons. In addition, neocortical axons appeared to fasciculate with the cingulate axons as they crossed the midline (Fig. 3E). The exact timing of when neocortical axons reached the midline varied somewhat by half a day between animals, with some neocortical axons not crossing the midline until E16.5 (Fig. 3F,G). In no brains did the neocortical axons cross before E16 and in every case (n = 15) followed a large group of cingulate axons across the midline.

10969861, 2001, 2, Downloaded from https://onlinelibrary.wiley.com/doi/10.1002/cne.1170 by Washington University School, Wiley Online Library on [21/07/2023]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

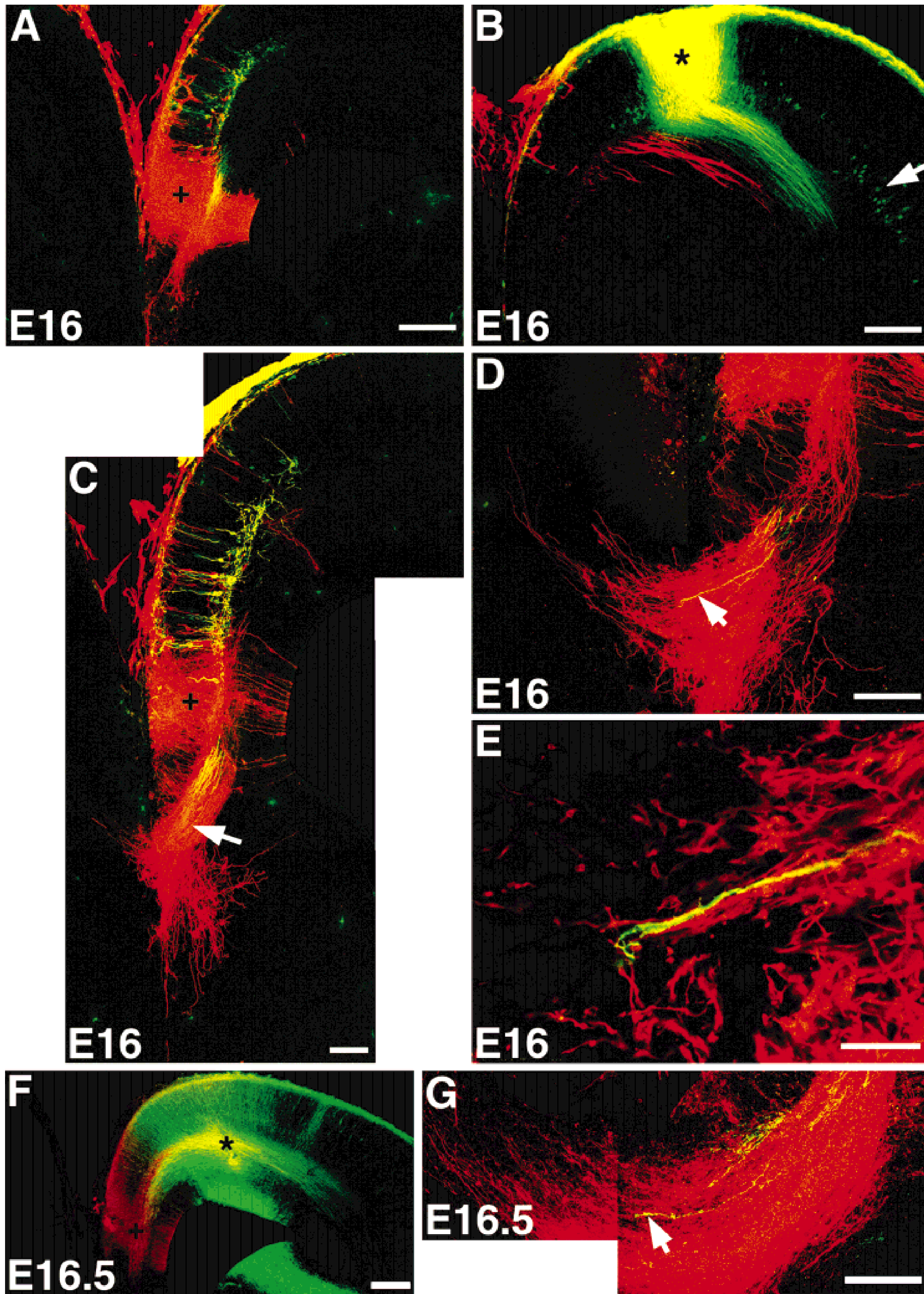

Fig. 3. Neocortical axons cross the midline within the pathway formed by the cingulate pioneering axons. Injections in both the cingulate cortex (+ in **A**) and the rostral neocortex (* in **B**) of the same brain (A–E are sections of the same brain) indicated that neurons in the cingulate cortex always projected axons across the midline before the arrival of neurons in the neocortex. **C:** Large bundles of DiI-labeled axons crossed the midline in advance of DiA-labeled neocortical axons (arrow). **D:** Leading DiA-labeled axons cross the midline within the tract formed by the DiI-labeled cingulate axons. **E:** A thin (10 μm) optical section shows a DiA-labeled neocortical axon extending along DiI-labeled cingulate axons (E is a higher power view of D). **F,G:** At embryonic day 16.5, even more cingulate axons have crossed the midline and the first neocortical axons are beginning to cross the midline (G is a higher power view of F; F shows the injection sites). Scale bar = 200 μm in A,B,F; 100 μm in C,D; 25 μm in E; 50 μm in G.

10969861, 2001, 2, Downloaded from https://onlinelibrary.wiley.com/doi/10.1002/cne.1170 by Washington University School, Wiley Online Library on [21/07/2023]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

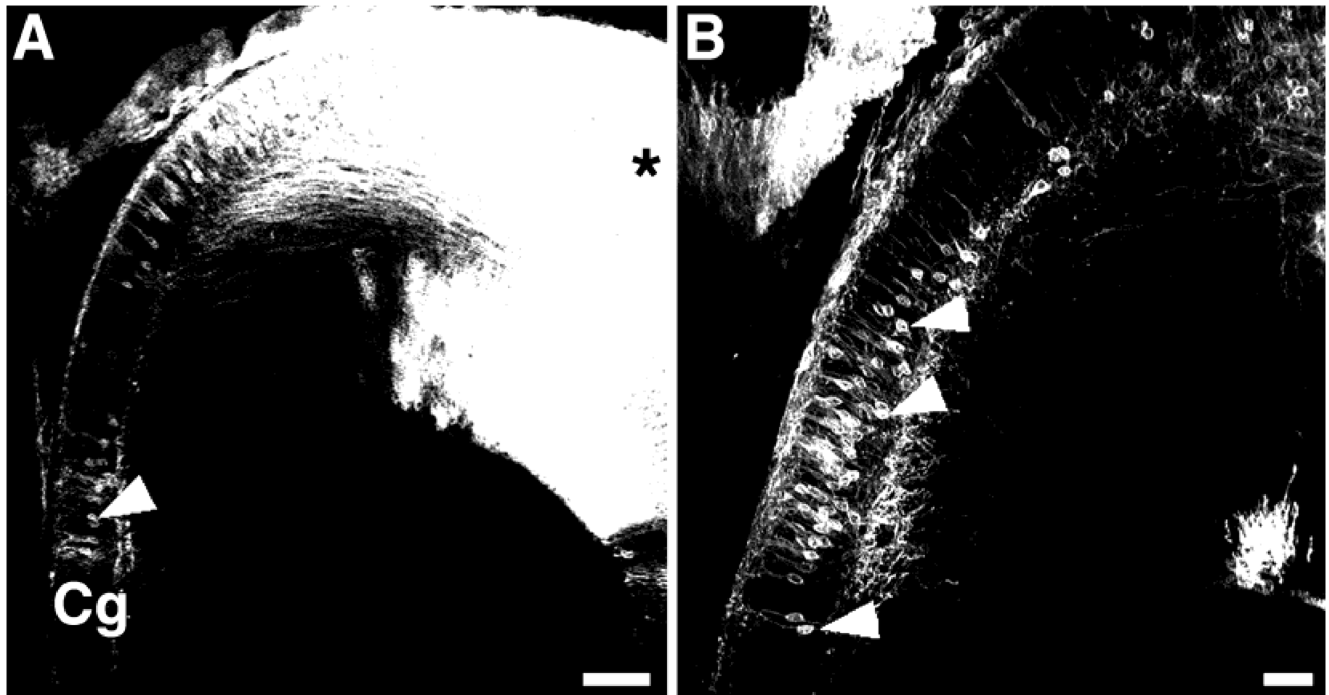

Fig. 4. **A,B:** Cingulate axons project laterally at E15. E15 brains were injected with DiA in the dorsolateral neocortex (* in A). Cell bodies within the cingulate cortex (Cg) were retrogradely labeled with this injection (arrowheads in A,B; B is a higher power view of a more rostral section of the same brain shown in A). Scale bar = 200 μm in A; 100 μm in B.

Neurons in the cingulate cortex also project laterally to the rostrolateral neocortex

During experiments to label medially projecting neocortical axons, we also noticed a large number of retrogradely labeled DiA-positive neurons in the cingulate cortex that were projecting rostrolaterally through the injection site. Figure 4 shows that, at E15, neurons within the cingulate cortex are retrogradely labeled by an injection in rostrolateral neocortex. Thus, neurons within the cingulate cortex also projected rostrolaterally in the opposite direction to the first medially projecting neocortical axons. These axons arrive at the intermediate zone of the rostrolateral neocortex at least 0.5 days before the first neocortical axons have reached the midline. Injections at E14 did not label this laterally projecting cingulate population (not shown); therefore, cingulate axons arrive at the lateral neocortex between E14 and E15. What we cannot determine from these experiments is whether the lateral cingulate projection arrives before the time when the first neocortical axons begin to extend from this region (see Discussion section).

In brains with a single DiA injection dorsally, cingulate neurons were labeled in caudal sections throughout the cingulate cortex as far as 1,350 μm away at E16 (Fig. 5, arrowheads). Analysis of serial sections demonstrates some neurons within the cingulate cortex project rostrally and laterally within the neocortex toward the site where the first neocortical neurons to project axons toward the midline reside (Ozaki and Wahlsten, 1998). By E18, both the laterally projecting cingulate neuronal cell bodies and the medially projecting neocortical axons can be seen in the same section with a single dorsolateral injection (Fig. 6A,B). Figure 6B also shows that neurons within the indusium griseum are not retrogradely labeled by the injection; thus, the lateral projection is specific to the cingulate cortex.

DISCUSSION

The role of cingulate pioneers in the formation of the corpus callosum

We have shown that axons from the cingulate cortex do enter the contralateral hemisphere, pioneering a path for callosal axons. These axons cross before axons from the neocortex reach the midline. In addition, neocortical axons grow within the tract formed by the cingulate axon bundle, possibly fasciculating with the cingulate axons. These results confirm the findings of Koester and O'Leary (1994) showing that axons from the cingulate cortex are the first to cross the midline and enter the contralateral cortex.

One definition of a pioneering axon may be that it is the first axon to grow along a specific trajectory. According to this definition, the cingulate axons that cross the midline to the contralateral cortex described here are pioneering axons for the corpus callosum. A further definition may include an even greater role of the pioneering axon in actually directing the growth of later developing axons, in this case from the neocortex, by direct fasciculation with the pioneering axon. Here, we have shown evidence that neocortical axons grow within the tract formed by the cingulate pioneers and at the midline do appear to be fasciculating with the pioneer axons. This hypothesis remains to be formally tested by investigating the develop-

10969861, 2001, 2, Downloaded from https://onlinelibrary.wiley.com/doi/10.1002/cne.1170 by Washington University School, Wiley Online Library on [21/07/2023]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

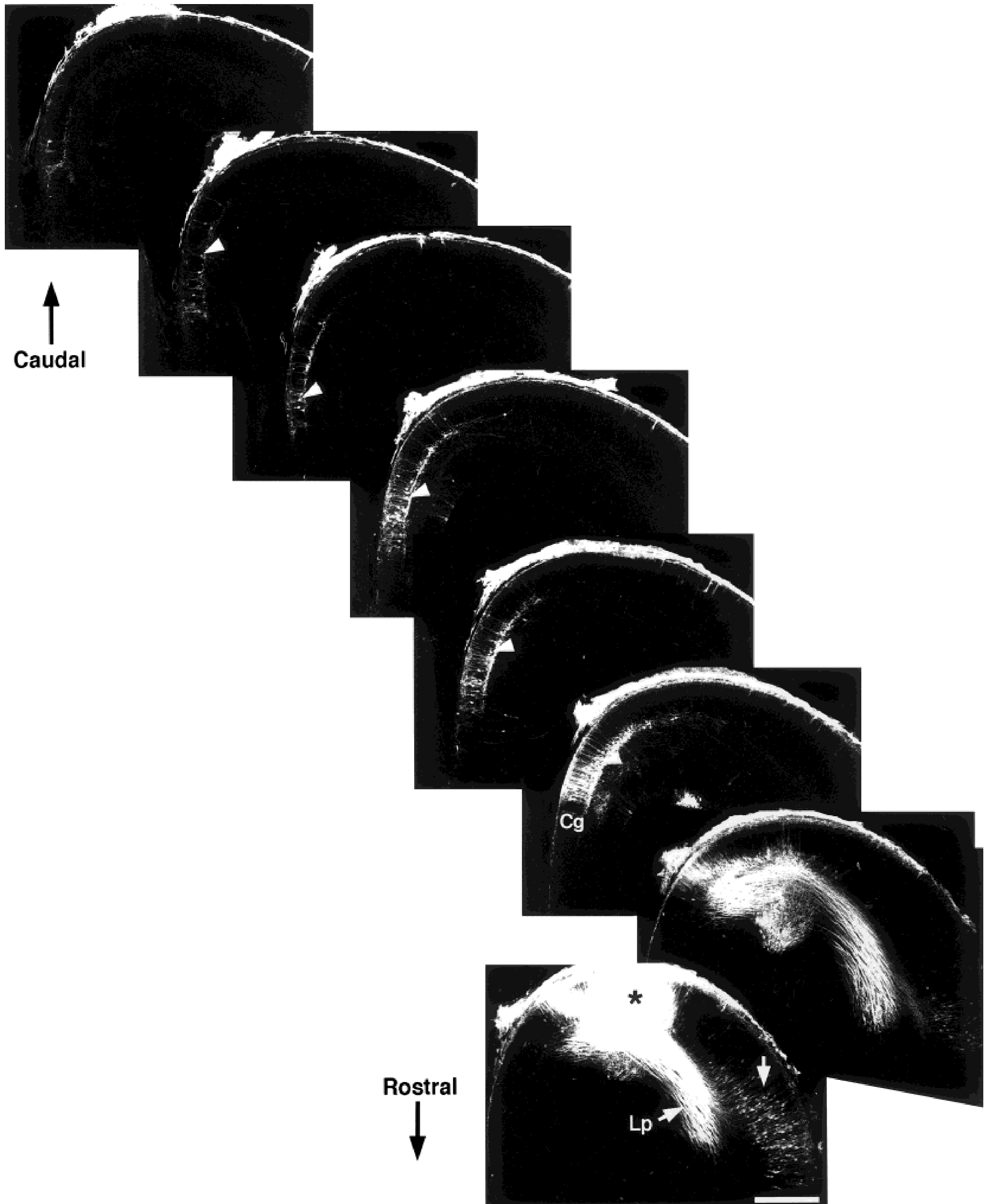

Fig. 5. Cingulate axons project rostrally and laterally in the neocortex. Serial sections are shown from the same brain (50 μm apart, every third section is shown). An injection at E16 in rostral neocortex (injection site shown by *) labels medially projecting neocortical axons (arrow in most rostral panel) and retrogradely labels neurons within the cingulate cortex (Cg) in caudal sections as far as 1,350 μm away (arrowheads). Lp, lateral projection. Scale bar = 400 μm.

10969861, 2001, 2, Downloaded from https://onlinelibrary.wiley.com/doi/10.1002/cne.1170 by Washington University School, Wiley Online Library on [21/07/2023]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

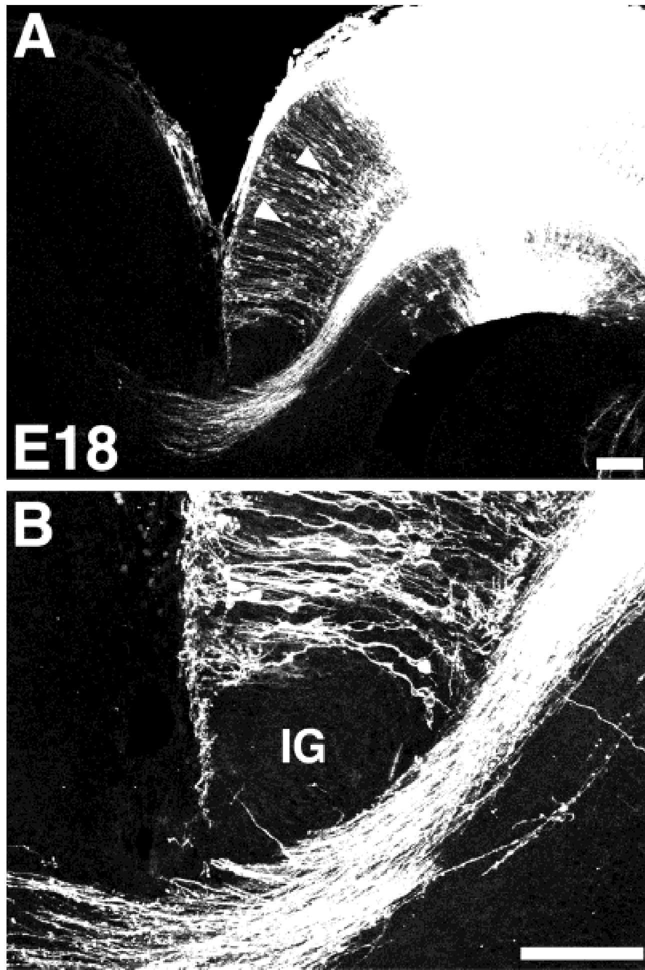

Fig. 6. **A,B:** Injections at E18 label both laterally projecting cingulate neurons and callosally projecting neocortical axons. Injections into the dorsorostral neocortex are sufficient to label both projections once they have formed. Retrogradely labeled cingulate neurons are shown (arrowheads in A; B is a higher power view of A). IG, indusium griseum. Scale bars = 200 μm.

ment of the corpus callosum in the absence of the cingulate pioneers.

Development of medial projections from the cingulate cortex

Because the hippocampal commissure and the fornix form approximately 1 day before the corpus callosum, Ozaki and Wahlsten (1998) put forward the idea that Koester and O'Leary (1994) may have labeled axons projecting to the hippocampus rather than to the contralateral cortex. By using anterograde labeling, we find that neurons in the cingulate cortex project to both the contralateral cortex and to the hippocampus. However, in most cases, these axons projected to the ipsilateral hippocampus and not to the contralateral hippocampus. In addition, the number of cingulate axons that projected to the hippocampus was low compared with the number of DiI-labeled axons we observed within the fornix and medial septal areas. Many of these axons were probably part of the perforating pathway containing afferent axons to the cingulate cortex from the MSDB that were retrogradely labeled by this injection into the cingulate cortex (Shu and Richards, unpublished observation). Further evidence of this is shown in Figure 1B by the large number of cell bodies retrogradely labeled in the MSDB (small arrows in this figure). Thus, although some cingulate axons project to the hippocampus, most do not project across the hippocampal commissure. This finding also makes it unlikely that Koester and O'Leary (1994) mistakenly labeled axons of the hippocampal commissure with their injections.

Previous investigators (Valentino and Jones, 1982; Livy and Wahlsten, 1997) have believed that the pioneering fibers of the corpus callosum grow along the preformed hippocampal commissure. However, many cingulate axons cross the midline rostral to the hippocampal commissure. In more caudal regions, we observed a clear demarcation of those axons from the cingulate cortex that will enter the fornix/perforating pathway from those that will form the corpus callosum. In addition, we have shown that callosal pioneering axons cross the midline dorsal to the fornix and hippocampal commissure and not within the tract formed by these axons, although these two pathways do form close together. It is possible that, in these more caudal regions, cingulate axons use the preformed hippocampal commissure to cross the midline. However, some mutant mice, such as the *Emx-1* knockout (Qui et al., 1996; Yoshida et al., 1997), fail to form a corpus callosum even in the presence of a hippocampal commissure, indicating that the presence of a hippocampal commissure is not sufficient for callosal axons to cross the midline.

Axons projecting to the contralateral hemisphere form a separate axon bundle from the hippocampally projecting population. As previously described (Hankin and Silver, 1988), the perforating pathway intersects the corpus callosum. Ozaki and Wahlsten (1998) found that axons projecting from the medial cortex into the fornix travel within the perforating pathway. Our results show that medially projecting axons from the cingulate cortex enter either one of two pathways as they grow into the intermediate zone; growing across the midline to the contralateral hemisphere or growing over the callosal axons in a separate bundle (arrows in Fig. 1C). These latter axons are met by afferent axons to the cingulate cortex from both the MSDB and the hippocampus. Therefore, the decision of which axon pathway to take seems to occur as soon as the cingulate axons enter the intermediate zone. The decision to cross over the callosal axons may be influenced by afferent axons from the hippocampus and the perforating pathway (Shu and Richards, unpublished observations) coming into the cingulate cortex as both the afferent and the efferent pathways form simultaneously. However, the decision to cross to the contralateral hemisphere is clearly not influenced by the presence of other axons as these cross before the arrival of neocortical callosal axons. Therefore, the cingulate callosal axons may be more influenced by cues present within their environment, because they are the first axons to form the nascent corpus callosum.

Guidance of the pioneering axons

The diverse trajectories of the cingulate populations suggests that they are differentially influenced by guidance signals at the corticoseptal boundary. Several structures with apparent guidance activities have been described within the corticoseptal region (Silver et al., 1993),

10969861, 2001, 2, Downloaded from https://onlinelibrary.wiley.com/doi/10.1002/cne.1170 by Washington University School, Wiley Online Library on [21/07/2023]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

including the glial sling (Silver et al., 1982), the glial wedge, and glia within the indusium griseum (Shu and Richards, 2001). The fact that the cingulate pioneers cross the midline at E15.5 before the complete formation of the glial sling at E17 suggests that the glial sling may not provide guidance activity for the pioneering axons. However, glial sling cells do begin to migrate toward the midline at E15; therefore, it is possible that sling cells may provide guidance for the callosal pioneers during their migration toward the midline, although there is currently no evidence for this hypothesis.

Glial wedge cells begin to express GFAP at E14, forming long processes toward the midline (also shown in cat at E34; Silver et al., 1993). By E15, these glial processes have coalesced into a wedge-shaped structure on either side of the midline lying directly ventral to where the corpus callosum will form (Shu and Richards, 2001). Therefore, it is likely that the glial wedge may play a role in guiding these pioneering axons from the cingulate cortex. Glia within the indusium griseum begin to express GFAP at E17, after the cingulate pioneers have crossed. Therefore, the indusium griseum glia seem not to be involved in guiding the cingulate callosal pioneers. An alternative hypothesis is that the indusium griseum glia are present before their expression of GFAP and are providing guidance activity for the pioneers at an earlier stage.

Development of the lateral projection from the cingulate cortex

In addition to the development of medial cingulate projections, neurons in the cingulate cortex project laterally at a very early stage of development (E15). Previous experiments have described this pathway in adult cats (Cavada and Reinoso-Suarez, 1985; Musil and Olson, 1988). Therefore, at least in cats, this projection is not transient. What has not been established from our experiments is whether the same cells are projecting both medially and laterally. Callosal axons from the neocortex largely project to homotopic regions in the contralateral hemisphere of the mouse (Yorke and Caviness, 1975). We also noticed that contralaterally projecting cingulate axons appeared to project into the homotopic cingulate cortex (Fig. 1D). Although we did not study this projection in detail, previous reports show that cingulate axons project to the contralateral cingulate cortex (Locke and Kruper, 1964; Locke and Yakolev, 1965). Our finding that cingulate axons also project laterally early in development suggests there may be several different cingulate populations that project to distinct targets. The only other alternative is that the same neuron projects to more than one target. If this were the case, we would have expected to see double-labeled neurons in regions dorsolateral to the cingulate injections and medial to the neocortical injections. We saw few, if any, double-labeled cells present, indicating that the laterally and medially projecting cingulate neurons may be separate populations. Further experiments are required to definitively address this issue and similarly to address whether the hippocampally and septally projecting cingulate axons also represent different populations of neurons.

The indusium griseum does not contribute to the lateral cingulate projection to the rostrofrontal neocortex. Given our injection paradigm, it was not possible to determine whether neurons of the indusium griseum act as callosal pioneers. Koester and O'Leary (1994) observed axons from the indusium griseum crossing the midline when they injected very medial regions. This finding may indicate that axons of the indusium griseum also project homotopically, but whether they act as callosal pioneers has not been definitively examined. Given the small size of the indusium griseum, and its proximity to the callosal fibers, this determination remains a technical challenge.

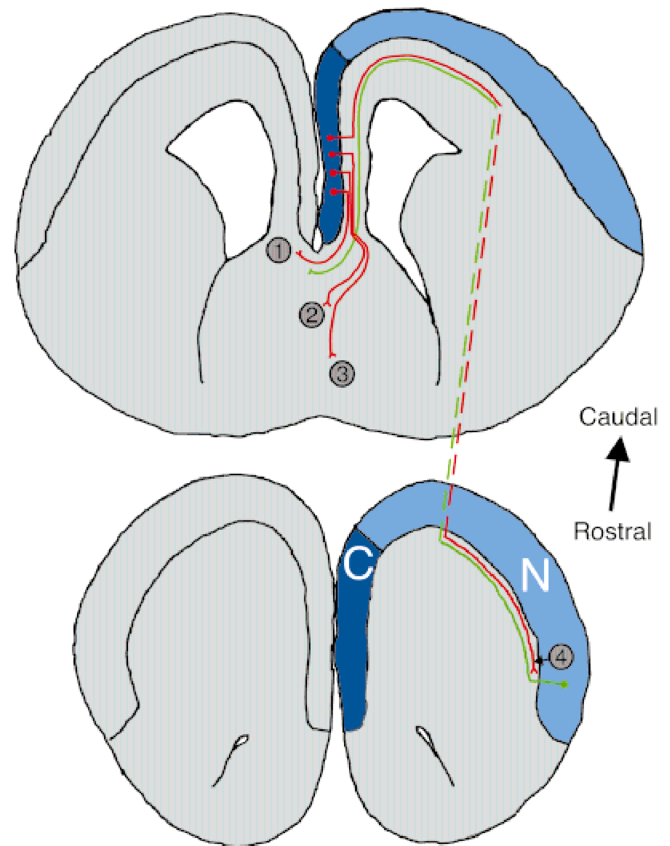

Fig. 7. Model of the role of cingulate pioneers in the development of the corpus callosum. Schematic of two coronal sections of the same brain. Cells within the cingulate cortex (dark blue region and labeled as C) send axons (labeled in red) medially to three targets (1) across the midline to the contralateral cortex, (2) by means of the fornix to the hippocampus, (3) to the septum, and (4) laterally to the rostrolateral neocortex. Neurons within the neocortex (light blue region and labeled as N) project axons across the corpus callosum (axons labeled in green). Callosal neocortical axons may fasciculate with the cingulate pioneers and use them as a guide to extend both toward the midline and across the midline. The dotted red and green lines indicate axons projecting between different rostrocaudal regions and do not depict the actual trajectory taken by the axons.

A model of interaction between cingulate and neocortical axons

Our results support the hypothesis that medial projections from the cingulate cortex pioneer a pathway for neocortical axons. Furthermore, neocortical axons grow in close apposition to the cingulate axons, indicating that they may use them as a guide to cross the midline. Finally, neurons within the cingulate cortex project laterally very early in development. These axons project to the rostrolateral neocortex, the site where neurons that will send the first neocortical axons across the midline reside. The

10969861, 2001, 2, Downloaded from https://onlinelibrary.wiley.com/doi/10.1002/cne.1170 by Washington University School, Wiley Online Library on [21/07/2023]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

timing and location of these projections may be coincidental. Alternatively neocortical axons may use the laterally projecting cingulate axons to reach the midline and the medially projecting cingulate axons to cross the midline (Fig. 7). Further evidence is required to determine whether neocortical axons fasciculate with the laterally projecting cingulate axons as they enter the intermediate zone and project medially. We have found that the lateral cingulate projection arrives at the rostrolateral neocortex at E15 but is not present at E14 (data not shown). Therefore, the lateral cingulate projection probably arrives at around the same time that the neocortical axons begin to project (given that they arrive at the midline at E16). By using the injection technique described here, it is impossible for us to analyze the neocortical axons as they first project, because the injection itself causes an intense fluorescence at the injection site. Therefore, by using the current protocol, axonal projections have to be analyzed at a distance. At E15 this method is a problem because we cannot be sure that axons from each population would be independently labeled. In addition, we have no evidence that the pioneers are required for neocortical axons to reach or cross the midline and, therefore, present our model as a working hypothesis.

Our data have resolved several controversies: (1) whether cingulate axons project to the contralateral cortex, (2) whether they arrive before axons from the neocortex, (3) whether neocortical axons cross in the same pathway as the cingulate pioneers, (4) whether there is a significant developmental projection to the hippocampus from the cingulate cortex, and (5) whether the cingulate projection to the hippocampus is ipsilateral or contralateral. It now remains to be tested whether these cingulate pioneers have functional significance in the development of the corpus callosum.

ACKNOWLEDGMENTS

We thank Kimberly Valentino, MSc, and Ning Zhang for excellent technical assistance. L.J.R. received a Basil O'Connor Starter Research Award from The March of Dimes Foundation for Birth Defects.

LITERATURE CITED

Bastiani MJ, Raper JA, Goodman CS. 1984. Pathfinding by neuronal growth cones in grasshopper embryos: III. Selective affinity of the G growth cone for the P cells within the A/P fascicle. J Neurosci 4:2311–2328.

Bate CM. 1976. Pioneer neurones in an insect embryo. Nature 260:54–56.

Bork T, Schabtach E, Grant P. 1987. Factors guiding optic fibers in developing *Xenopus* retina. J Comp Neurol 264:147–158.

Catalano SM, Robertson RT, Killackey HP. 1991. Early ingrowth of thalamocortical afferents to the neocortex of the prenatal rat. Proc Natl Acad Sci USA 88:2999–3003.

Cavada C, Reinoso-Suarez F. 1985. Topographical organization of the cortical afferent connections of the prefrontal cortex in the cat. J Comp Neurol 242:293–324.

De Carlos JA, O'Leary DD. 1992. Growth and targeting of subplate axons and establishment of major cortical pathways. J Neurosci 4:1194–1211.

Ghosh A, Shatz CJ. 1993. A role for subplate neurons in the patterning of connections from thalamus to neocortex. Development 117:1031–1047.

Ghosh A, Antonini A, McConnell SK, Shatz CJ. 1990. Requirement for subplate neurons in the formation of thalamocortical connections. Nature 347:179–181.

Hankin MH, Silver J. 1988. Development of intersecting CNS fiber tracts: the corpus callosum and its perforating fiber pathway. J Comp Neurol 272:177–190.

Holley JA. 1982. Early development of the circumferential axonal pathway in mouse and chick spinal cord. J Comp Neurol 205:371–382.

Ivy GO, Killackey HP. 1981. The ontogeny of the distribution of callosal projection neurons in the rat parietal cortex. J Comp Neurol 195:367–389.

Klose M, Bentley D. 1989. Transient pioneer neurons are essential for formation of an embryonic peripheral nerve. Science 245:982–984.

Koester SE, O'Leary DD. 1994. Axons of early generated neurons in cingulate cortex pioneer the corpus callosum. J Neurosci 11:6608–6620.

Kuwada JY. 1986. Cell recognition by neuronal growth cones in a simple vertebrate embryo. Science 233:740–746.

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

Locke S, Kruper DC. 1964. Transcallosal connections of the cingulate gyrus in monkey. Anat Rec 153:377–362.

Locke S, Yakolev PI. 1965. Transcallosal connections of the cingulum of man. Arch Neurol 13:471–476.

McConnell SK, Ghosh A, Shatz CJ. 1989. Subplate neurons pioneer the first axon pathway from the cerebral cortex. Science 245:978–982.

McConnell SK, Ghosh A, Shatz CJ. 1994. Subplate pioneers and the formation of descending connections from cerebral cortex. J Neurosci 14:1892–1907.

Musil SY, Olson CR. 1988. Organization of cortical and subcortical projections to anterior cingulate cortex in the cat. J Comp Neurol 272:203–218.

Ozaki HS, Wahlsten D. 1992. Prenatal formation of the normal mouse corpus callosum: a quantitative study with carbocyanine dyes. J Comp Neurol 323:81–90.

Ozaki HS, Wahlsten D. 1998. Timing and origin of the first cortical axons to project through the corpus callosum and the subsequent emergence of callosal projection cells in mouse. J Comp Neurol 400:197–206.

Qui M, Anderson S, Chen S, Meneses JJ, Hevner R, Kuwana E, Pedersen RA, Rubenstein JLR. 1996. Mutation of the *Emx-1* homeobox gene disrupts the corpus callosum. Dev Biol 178:17.

Rakic P, Yakovlev PI. 1968. Development of the corpus callosum and cavum septi in man. J Comp Neurol 132:45–72.

Shu T, Richards LJ. 2001. Cortical axon guidance by the glial wedge during the development of the corpus callosum. J Neurosci (in press).

Silver J, Lorenz SE, Wahlsten D, Coughlin J. 1982. Axonal guidance during development of the great cerebral commissures: descriptive and experimental studies in vivo, on the role of preformed glial pathways. J Comp Neurol 210:10–29.

Silver J, Edwards MA, Levitt P. 1993. Immunocytochemical demonstration of early appearing astroglial structures that form boundaries and pathways along axon tracts in the fetal brain. J Comp Neurol 328:415–436.

Super H, Martinez A, Del Rio JA, Soriano E. 1998. Involvement of distinct pioneer neurons in the formation of layer-specific connections in the hippocampus. J Neurosci 18:4616–4626.

Valentino KL, Jones EG. 1982. The early formation of the corpus callosum: a light and electron microscope study in fetal and neonatal rats. J Neurocytol 11:583–609.

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

Wilson SW, Easter SS Jr. 1991. A pioneering growth cone in the embryonic zebrafish brain. Proc Natl Acad Sci USA 88:2293–2296.

Wise SP, Jones EG. 1976. The organization and postnatal development of the commissural projection of the rat somatic sensory cortex. J Comp Neurol 168:313–344.

Yorke CH Jr, Caviness VS Jr. 1975. Interhemispheric neocortical connections of the corpus callosum in the normal mouse: a study based on anterograde and retrograde methods. J Comp Neurol 164:233–245.

Yoshida M, Suda Y, Matsuo I, Miyamoto N, Takeda N, Kuratani S, Aizawa S. 1997. *Emx-1* and *Emx-2* functions in development of dorsal telencephalon. Development 124:101–111.

10969861, 2001, 2, Downloaded from https://onlinelibrary.wiley.com/doi/10.1002/cne.1170 by Washington University School, Wiley Online Library on [21/07/2023]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License