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Development of the Perforating Pathway: An Ipsilaterally Projecting Pathway Between the Medial Septum/ Diagonal Band of Broca and the Cingulate Cortex That Intersects the Corpus Callosum

TIANZHI SHU, WEI-BIN SHEN, AND LINDA J. RICHARDS*
Department of Anatomy and Neurobiology and the Program in Neuroscience, School of Medicine, The University of Maryland, Baltimore, Baltimore, Maryland 21201

ABSTRACT

The perforating pathway (PFP) intersects the corpus callosum perpendicularly at the midline in the dorsoventral axis. Therefore axons in either the PFP or the corpus callosum make different axonal guidance decisions in the same anatomical region of the developing cortical midline. The mechanisms underlying these axonal choices are not known. To begin to identify these guidance mechanisms, we characterized the development of these two pathways in detail. The development of the corpus callosum and its pioneering projections has been described elsewhere (Shu and Richards [2001] J. Neurosci. 21:2749–2758; Rash and Richards [2001] J. Comp. Neurol. 434:147–157). Here we examine the development, origins, and projections of axons that make up the PFP. The majority of axons within the PFP originate from neurons in the medial septum and diagonal band of Broca complex. These neurons project in a topographic manner to the cingulate cortex. In contrast to previous reports, we find that a much smaller projection originating from the cingulate cortex also contributes to this pathway. The pioneering projections of the PFP and the corpus callosum arrive at the corticoseptal boundary at around the same developmental stage. These findings show that ipsilaterally projecting PFP axons and contralaterally projecting callosal axons make distinct guidance decisions at the same developmental stage when they reach the corticoseptal boundary. J. Comp. Neurol. 436:411–422, 2001.

Indexing terms: neocortex; septum; cortico-septal boundary; midline; glial wedge; glial sling; DiI

The nervous system is wired up by axonal projections that respond to environmental guidance cues and/or fasciculate with other axon bundles to locate their targets. These guidance cues may be chemo-attractive, -repulsive, -permissive, or -suppressive and presented as either diffusible or membrane-bound molecules (Tessier-Lavigne and Goodman, 1996). A very interesting phenomenon is that at some axonal choice points in the nervous system, axon bundles intersect one another without being misled and changing their original trajectory. Such axonal decisions often occur at the midline of embryos where axons choose either to remain ipsilateral or to cross the midline and project contralaterally.

In addition to guidance by molecules diffusing or bound within the axonal environment, axon-axon interactions are also important for axonal guidance during the targeting phase of growth, as in the case of axonal guidance by pioneering axons. Pioneering axons have been shown to play a role in the development of efferent cortical projections both through the internal capsule (McConnell et al., 1989, 1994; De Carlos and O'Leary, 1992; Erzurumlu and

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*Correspondence to: Linda J. Richards, Ph.D., Department of Anatomy and Neurobiology, The University of Maryland, Baltimore, HSF 222, 685 West Baltimore St, Baltimore MD, 21201.
E-mail: lrich001@umaryland.edu

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Jhaveri, 1992) and across the corpus callosum (Koester and O'Leary, 1994; Ozaki and Wahlsten, 1998; Rash and Richards, 2001).

The embryonic midline is an axonal choice point in a number of different developmental systems (Van Hartesveldt et al., 1986; Tessier-Lavigne et al., 1988; Jacobs and Goodman, 1989; Mori et al., 1990; Marcus et al., 1995; Wang et al., 1995; Hummel et al., 1999). Here, axons of different pathways intersect and then project either ipsi- or contralaterally. In the mouse visual system all ganglion cell axons converge at the optic chiasm. Retinal ganglion cells from ventrotemporal retina leave the chiasm and project ipsilaterally to the lateral geniculate nucleus; retinal ganglion cells distributed throughout other parts of the retina leave the chiasm and project contralaterally (for review, see Guillery et al., 1995). Cells within the region of the optic chiasm therefore may play a role in guiding the ipsi- or contralateral projections (Wizenmann et al., 1993; Godement et al., 1994; Sretavan et al., 1994, 1995; Marcus et al., 1995, 1999; Wang et al., 1995; Erskine et al., 2000), but the molecular mechanisms underlying this guidance decision are not fully understood (for review, see Mason and Sretavan, 1997).

In the developing cortex, axons interact at the midline and project either ipsilaterally within the perforating pathway (PFP; Hankin and Silver, 1988) or contralaterally via the corpus callosum. The PFP is so named because it intersects (perforates) the corpus callosum perpendicularly during development (Hankin and Silver, 1988). The intersection of the two pathways has been previously described (Kölliker, 1894; Smith, 1897; Kappers et al., 1936; Yakovlev and Locke, 1961; Hankin and Silver, 1988), but reports on the origin and the target of PFP are not consistent. Earlier studies suggested that neurons in the hippocampus gave rise to the PFP (Kolliker, 1894; Smith, 1897), whereas other studies suggested that the PFP arises in the cingulate cortex (Kappers et al., 1936; Yakovlev and Locke, 1961; Hankin and Silver, 1988).

In adult rodents a septocingulum pathway exists that projects from the medial septum and the vertical and horizontal limbs of the diagonal band of Broca (MSDB) complex. The axons from neurons within the MSDB cross the genu of the corpus callosum and innervate the cingulate cortex (Swanson and Cowan, 1979; Bigl et al., 1982; Milner et al., 1983; Rye et al., 1984; Woolf et al., 1986; Freund and Gulyas, 1991). How this septocingulum pathway in adults relates to the PFP during development and at what stage neurons in the MSDB might form this pathway are unknown.

In this study, we first describe the projection pattern of the PFP and propose a morphological definition of it. Second, we clarify the origin and possible target of the PFP using carbocyanine dye tracing. Third, we describe the developmental time course of the axons that pioneer the PFP and relate this to the development of axons that pioneer the corpus callosum. Finally, we quantify and map the origin and projection of neurons whose axons make up the PFP to determine the relative contributions of axons from MSDB and cingulate cortical neurons to this pathway. These results provide a basis for further analysis of the mechanisms of axonal guidance at the cortical midline.

MATERIALS AND METHODS

Animals

C57BL/6J mice (Jackson Labs, Bar Harbor, ME) were mated, and the embryos were staged based on a plug date of embryonic day (E) 0. Embryos were used between E15 and E17. Mice were housed at the University of Maryland School of Medicine animal facility. Pregnant dams were anesthetized with sodium phenobarbital (Nembutal; Abbott Laboratories, IL) at 0.07 mg/g body weight and placed on a warming pad to maintain body temperature while their pups were perfused one at a time. Procedures related to animal use were approved by the Animal Care and Use Committee at the University of Maryland, Baltimore.

Immunohistochemistry

All embryos were transcardially perfused with saline, followed by 4% paraformaldehyde, and then postfixed in 4% paraformaldehyde for 2–3 days. Brains were blocked in 3% agar and cut at 45 μm on a Vibratome (Leica, Deerfield, IL). Immunohistochemistry was performed as described before with minor modifications (Shu et al., 2000). Briefly, sections were washed three times in phosphate-buffered saline (PBS; pH 7.4), blocked in a solution of 2% normal goat serum (for polyclonal antibodies, v/v; Vector, Burlingame, CA) or donkey serum (for monoclonal neurofilament [NF] antibody; v/v; Jackson ImmunoResearch, West Grove, PA) and 0.2% Triton X-100 (v/v; Sigma, St. Louis, MO) in PBS for 2 hours, and then incubated in primary antibody (rabbit anti-NF [145 kD] polyclonal antibody [Chemicon, Temecula, CA], used at 1:1000 for fluorescent Cy2 detection; rabbit anti-glial fibrillary acidic protein [GFAP] polyclonal antibody [DAKO, Copenhagen, Denmark], used at 1:1000 for fluorescent Cy3 detection; mouse anti-NF [145 kD; Chemicon], used at 1:10,000 for nickel-diaminobenzidine [DAB] reaction or 1:1000 for fluorescent labeling) overnight.

On the following day, sections for nickel-DAB detection were washed three times in PBS and then incubated in biotinylated-donkey anti-mouse secondary antibody (1:600, Jackson ImmunoResearch) for 1 hour. After three washes in PBS, sections were incubated in avidin/biotin solution (1:500, Vector) for 1 hour, followed by washing three times. Sections were then immersed in the nickel-DAB chromogen solution (2.5% nickel sulphate and 0.02% DAB in 0.175 M sodium acetate) activated with 0.01% (v/v) H_2O_2 until a dark purple/black precipitate formed. Sections were washed in PBS, dehydrated through increasing concentrations of ethanol, immersed in two changes of 100% xylene and coverslipped in DePeX (Sigma) mounting medium. Sections for fluorescent detection were incubated in Cy2-conjugated donkey anti-rabbit IgG(H+L) (1:400, Jackson ImmunoResearch) for 2 hours. For double-labeling of NF and GFAP, the sections were labeled with rabbit anti-GFAP polyclonal antibody (DAKO; used at 1:1000) and Cy3-conjugated donkey anti-rabbit IgG(H+L) (1:400; Jackson ImmunoResearch) first as described above, washed extensively with PBS and then labeled with the mouse anti-NF (145 kD; Chemicon) and Cy2-conjugated donkey anti-mouse IgG (H+L) (1:400; Jackson ImmunoResearch). After three washes in PBS, fluorescently labeled sections were mounted with PVA/DABCO mounting medium for confocal microscopy.

Tract tracing

Carbocyanine dye injections were performed on embryonic brains of C57BL/6J mice perfused with 0.9% saline followed by 4% paraformaldehyde and then postfixed in 4% paraformaldehyde until injection. A 10% solution of 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (DiI; Molecular Probes, Eugene, OR) in dimethylformamide (Sigma) was injected into brains using a fine-tipped glass micropipette attached to a PicoSpritzer (General Valve, Fairfield, NJ). Serial injections were made in either the cingulate cortex along the midline or the medial septum and diagonal band of Broca complex (MSDB) from rostral to caudal. Single injections were also made in each of these regions at different ages. Brains were stored in the dark at room temperature for at least 4 weeks to allow for DiI diffusion. Brains were then blocked and cut at 45 μm on a vibratome. Injection sites were verified after sectioning by the presence of a fluorescent bolus and a needle track. The sections were washed, mounted, and coverslipped with poly vinyl alcohol (PVA)/DABCO mounting medium and analyzed by confocal microscopy.

Quantification and statistics

To quantify the number of neurons projecting within the PFP from either the MSDB or the cingulate cortex, injections of DiI were made in individual brains. Retrogradely labeled neurons were viewed by fluorescence microscopy and counted in every section that contained either the MSDB or the cingulate cortex. Neurons within either rostral or caudal regions of the MSDB and the cingulate cortex were counted and grouped by their location. The mean number of neurons in each region was calculated, but we also included the raw counts, as there was some variability in the size of each injection. In addition, because there may be some differences in dye diffusion, we employed a repeated measurement t-test to analyze our data. This test treated the data sets as dependent data sets, thus enabling us to compare the number of neurons in different regions of the same brain (from the same single injection).

Preparation of figures

Mounted sections were analyzed with an upright light microscope (Leica). Images were scanned with a PowerPhase digital camera (Phase-one, Coppenhagen, Denmark) into Adobe PhotoShop software (version 4.0) on a Macintosh G3 computer. Sections labeled with fluorescent Cy2, Cy3, or DiI were analyzed by confocal microscopy (Olympus, Fluoview). Argon and krypton lasers with appropriate filters were used to detect the different signals. Sections were scanned at 2–5-μm optical section thickness and in some cases were collapsed into "z-series" photomicrographs. Images were imported into Adobe PhotoShop where they were labeled and the figures assembled.

RESULTS

Projection pattern of the perforating pathway

We found that the PFP is preferentially labeled by either a monoclonal or polyclonal anti-NF (145 kD) antibody (Chemicon) early in development (Fig. 1). Between E15 and E17, NF labeled both the PFP and the laterally projecting cortical axons, but not the medially projecting callosal axons (Fig. 1; Shu et al., 2000). In both sagittal (Fig. 1A and depicted schematically in 1B) and coronal (Fig. 1C–E) sections at E17, the PFP can be seen projecting in the ventrodorsal axis between the MSDB and the cingulate cortex. The perforating axons project ipsilaterally along the midline (Fig. 1C), cross the corpus callosum perpendicularly (Figs. 1D, 3; Hankin and Silver, 1988), and avoid the glial wedge (Fig. 1F–I), a midline glial population that expresses slit-2 and guides callosal axons at the midline (Shu and Richards, 2001). Two regions of the PFP were evident (Fig. 1A,B): a region that projected rostral to the corpus callosum for up to 310 μm (average of six animals), which we have called the precallosal PFP (labeled arrow in Fig. 1A; Fig. 1F,G); and a region that crosses the genu and body of the corpus callosum perpendicularly for approximately 300 μm (average from six animals), which we have called the callosal PFP (labeled arrowheads in Fig. 1A; Fig. 1C,D). Both regions together make up the PFP. After the perforating axons cross the corpus callosum, they extend caudally and probably join the cingulum bundle, which projects within the rostrocaudal axis of the cingulate cortex (Hankin and Silver, 1988; see NF labeling in Fig. 1A between the labels CC and CgCtx).

Figure 1A also shows a projection from the MSDB to the fornix (asterisk in Fig. 1A), which is the septohippocampal pathway (Linke and Frotscher, 1993; Super and Soriano, 1994). The septohippocampal projection is most likely the caudal extension of the PFP as we have defined it here. We did not include the septohippocampal projection in our analysis of the PFP because it does not perforate the corpus callosum and has been previously described in developing rat and mouse (Linke and Frotscher, 1993; Super and Soriano, 1994).

A large number of neurons within the MSDB complex labeled with NF (Figs. 1E, 4) displayed characteristics typical of MSDB neurons. A gradation in the size of neurons from dorsal to ventral regions exists within the adult MSDB, with larger neurons found within the HDB (horizontal limb of the diagonal band of Broca; Dinopoulos et al., 1988; Brauer et al., 1988). A similar gradation in the size of neurons was observed in our sections of developing brain.

Origin and projection of the perforating pathway

Earlier studies have suggested that neurons in the cingulate cortex give rise to the PFP (Kappers et al., 1936; Yakovlev and Locke, 1961). However, we did not observe any neurons in the cingulate cortex labeled by NF (using either the polyclonal or monoclonal anti-NF [145 kD] antibodies) at E17, whereas we observed many neurons labeled in the MSDB (as shown above). In adult rats many brain regions including the hippocampus and the entorhinal, insular, pyriform, infralimbic, and cingulate cortices receive inputs from the MSDB (Luiten et al., 1985, 1987; Woolf et al., 1986; Tomimoto et al., 1987; Gaykema, 1990). Furthermore, axons projecting from the MSDB to the cingulate cortex have been reported in adult rats to cross the genu of the corpus callosum and join the cingulum bundle to form a septocingulum projection (Swanson and Cowan, 1979; Milner et al., 1983). Given the observation that MSDB neurons are labeled in association with the PFP, the septocingulum projection found in adults might be

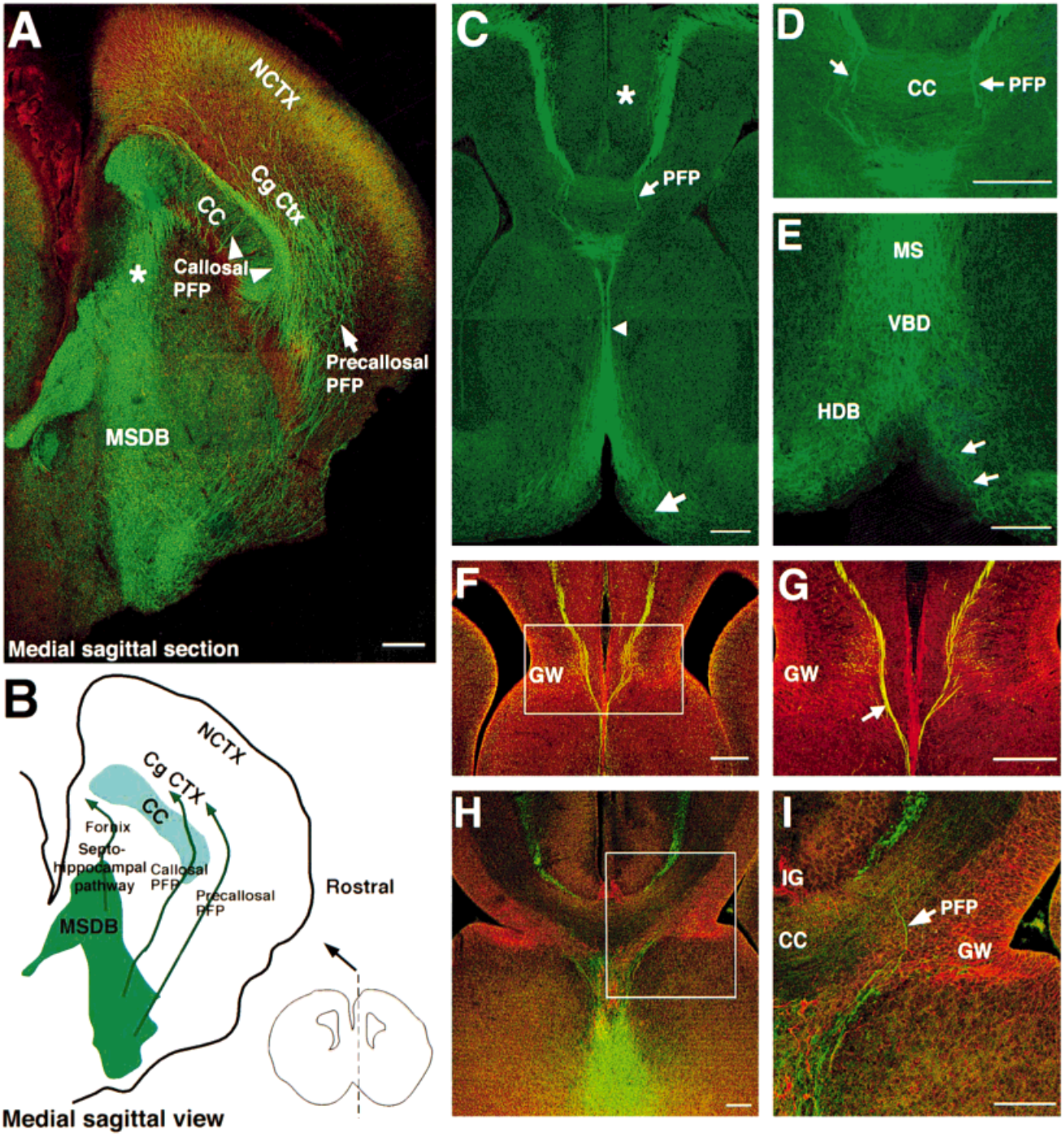

Fig. 1. Axonal projections of the perforating pathway on embryonic day (E) 17 labeled with anti-neurofilament (NF) antibody. The PFP was examined in both sagittal (A) and coronal (C–I) sections with anti-NF antibody at E17 in mouse. The polyclonal anti-NF antibody was used in panels A, C, D, and E, and the monoclonal anti-NF antibody was used in panels F–I; both antibodies gave the same labeling pattern. **A:** A medial, sagittal section. **B:** Schematic of the same section. A schematic of a frontal section (inset in B) shows the approximate region of the sagittal section taken for A and B. NF labeling shows the perforating pathway (PFP) projecting between the medial septum and the diagonal band of Broca complex (MSDB) and the cingulate cortex (Cg Ctx). Two regions of the pathway are evident: axons projecting rostral to the corpus callosum, which we have called the precallosal PFP (arrow labeled precallosal PFP in A); and axons that cross (perforate) the corpus callosum (CC), which we have called the callosal PFP (arrowheads labeled callosal PFP in A). A projection from the MSDB to the fornix (the septo-hippocampal pathway) is labeled with an asterisk in A and was not included as part of the PFP in our study. **C:** In coronal sections, the callosal PFP connects the MSDB (large arrow) and the cingulate cortex (asterisk) ipsilaterally. The axons project along the midline (arrowhead) and cross the corpus callosum (small arrow labeled with PFP) before entering the cingulate cortex. **D:** The perforating axon bundles intersect the corpus callosum perpendicularly (arrows, and labeled PFP). **E:** MSDB neurons are labeled in association with the PFP (some neurons in HBD are indicated by arrows; D and E are high-power views of C). **F:** The precallosal PFP projects through the taenia tecta region connecting the rostral MSDB with the cingulate cortex. **G:** A higher power view of the boxed region in F shows that axons in the precallosal PFP (green staining indicated by the arrow labeled with the monoclonal anti-NF antibody) avoid the glial wedge (red structure labeled GW, stained with an anti-GFAP antibody). **H:** The callosal PFP penetrates the genu of the corpus callosum connecting the caudal MSDB with the cingulate cortex. **I:** The axons of the PFP make a sharp turn as they encounter the glial wedge (arrow labeled PFP; I is a higher power view of the boxed region in H). HDB, horizontal limb of the diagonal band of Broca; IG, indusium griseum; MS, medial septum; NCTX, neocortex; VBD, vertical limb of the diagonal band of Broca. Scale bars = 200 μm.

derived from the PFP, and the MSDB may be the origin of the PFP. Alternatively, the absence of neurons labeled by NF within the cingulate cortex could also be due to the antibody itself and therefore we could not exclude the possibility that cingulate neurons also contribute to the PFP. To address this issue and to verify the origin and projection of PFP, we anterogradely and retrogradely labeled the PFP using the lipophilic dye tracer DiI.

To test directly whether neurons in the MSDB project to the cingulate cortex through the PFP, multiple injections of DiI were made along the rostral-caudal cingulate cortex of E17 brains to label neurons retrogradely in the MSDB (Fig. 2A,C, with one injection site indicated by the asterisk in each). The projection pattern labeled by DiI was largely identical to that observed with NF (compare Fig. 2A and C with Fig. 1F and C, respectively). Approximately 280 neurons (total) were retrogradely labeled in the MSDB in approximately 12 (50-μm) sections/brain (Fig. 2B,D; average from three animals). Again, neurons in the HDB were larger in size (Fig. 2B) than those in the MS. Therefore neurons in the MSDB do project to the cingulate cortex via the PFP.

To determine whether cingulate cortical neurons also contribute to the PFP, we performed multiple DiI injections into the MSDB (specifically into the vertical and horizontal limbs of the diagonal band of Broca) of E17 mouse brains to label neurons retrogradely in the cingulate cortex. Axons labeled in the PFP consisted of both anterogradely labeled MSDB axons and retrogradely labeled cingulate axons (Fig. 3A,C). Labeling of precallosal PFP axons (arrows in Fig. 3A,B) retrogradely labeled 11 neurons in the cingulate cortex in approximately 7 (50-μm) sections/brain (arrowheads and inset in Fig. 3B; average count from three animals), and labeling of callosal PFP axons (arrows in Fig. 3C,D) retrogradely labeled eight neurons in the cingulate cortex in 6 (50-μm) sections/brain (arrowheads and inset in Fig. 3D, average count from three animals). All of these neurons were found in the ventral region of the cingulate cortex.

It was possible that a larger number of axons from the cingulate cortex contributed to the PFP but terminated in more dorsal regions of the MS and therefore would not be labeled by ventral injections in the MSDB. To investigate this possibility, deeper and larger injections into the MS and ventral MSDB were made (Fig. 3E). Injections labeling the precallosal PFP labeled 23 neurons in approximately 7 (50-μm) sections/brain (average count from three animals), and injections labeling the callosal PFP labeled 21 neurons in approximately 6 (50-μm) sections/brain (average count from three animals) in the cingulate cortex (Fig. 3F). Again, all retrogradely labeled neurons were found in the ventral cingulate cortex. Therefore ventral cingulate neurons also give rise to the PFP and project to the MSDB, but they contribute fewer axons to the PFP than neurons in the MSDB ($P < 0.05$, t-test for independent samples).

These results demonstrate that the PFP is indeed a bidirectional fiber tract that contains axons projecting between the MSDB and the cingulate cortex.

Temporal development of the perforating pathway

When does the PFP first form? On E15, the first pioneering axons of the PFP project between the MSDB and the cingulate cortex (Fig. 4A,B), as labeled by NF and by DiI injected into the MSDB (Fig. 4G–I). Neuronal cell bodies in the MSDB were also labeled by NF (Fig. 4C,D). In addition, in both the rostral (arrow in Fig. 4G) and caudal (arrows in Fig. 4H,I) regions of the ventral cingulate cortex, neurons were retrogradely labeled with DiI, indicating that some cingulate neurons project to the MSDB as early as E15 even though they were not labeled with NF. On E16, more fibers join the pioneering axons forming a bundle that extends into the cingulate cortex (Fig. 4E,F). These results are consistent with previous findings showing that the PFP can first be identified between E15 and E16 (Hankin and Silver, 1988). Callosal axons begin to cross the midline at late E15/early E16 (Ozaki and Whalsten, 1998; Rash and Richards, 2001). Therefore both pathways meet at the corticoseptal boundary at late E15/early E16. We have confirmed this result by performing different colored carbocyanine dye injections in either the MSDB or the neocortex of the same brain (data not shown).

Axonal topography of the perforating pathway

As shown by NF and DiI labeling, the PFP projects ipsilaterally and extends approximately 600 μm from rostral to caudal (as we have defined it). Our previous experiments demonstrated the origin of the PFP and the relative contributions of axons from the cingulate cortex and the MSDB. However, they did not address whether there was any topographic specificity between different rostrocaudal regions of the PFP. To investigate this we made single injections into either the MSDB (Figs. 5A–F) or the cingulate cortex (Fig. 5G–L) at E17 and counted the number of neurons retrogradely labeled in either rostral or caudal target regions.

Single injections along the rostral-caudal axis of the MSDB retrogradely labeled neurons in both the rostral (Fig. 5B,E) and caudal (Fig. 5C,F) cingulate cortex; however, significantly more neurons in the rostral cingulate cortex projected to the MSDB than the caudal cingulate cortex (37 vs. 8, $P < 0.001$, t-test for correlated samples, $n = 9$; Table 1 and Fig. 6A). No topographic specificity was found between projections from either the rostral or caudal cingulate cortex to either the rostral or caudal MSDB (20 vs. 17; 5 vs. 3, respectively; Table 1 and Fig. 6A).

Axonal projections from the MSDB were studied in a similar manner by making single injections along the rostral-caudal axis of the cingulate cortex (Fig. 5G–L). Both the rostral and caudal MSDB projected to the entire cingulate cortex and contributed similar numbers of axons to the PFP (113 vs. 147, average from nine individual animals; Table 1 and Fig. 6B). No projection preference from the rostral MSDB to either the rostral or the caudal cingulate cortex was observed (58 vs. 55; Fig. 6B). However, neurons in the caudal MSDB projected preferentially to the caudal cingulate cortex (117 vs. 29, $P < 0.05$, t-test for independent samples; Table 1 and Fig. 6B). Furthermore, the caudal cingulate cortex received a significantly larger projection from the caudal MSDB than did the rostral cingulate cortex (117 vs. 55, $P < 0.05$, t-test for correlated samples; Table 1 and Fig. 6B). In addition, the rostral cingulate cortex received a larger (but not significant) projection from the rostral MSDB than did the caudal cingulate cortex (58 vs. 29; Fig. 6B). Therefore at least some topographic specificity is evident in the PFP rostrocaudally from the MSDB to the cingulate cortex. Figure 6 summarizes these projections.

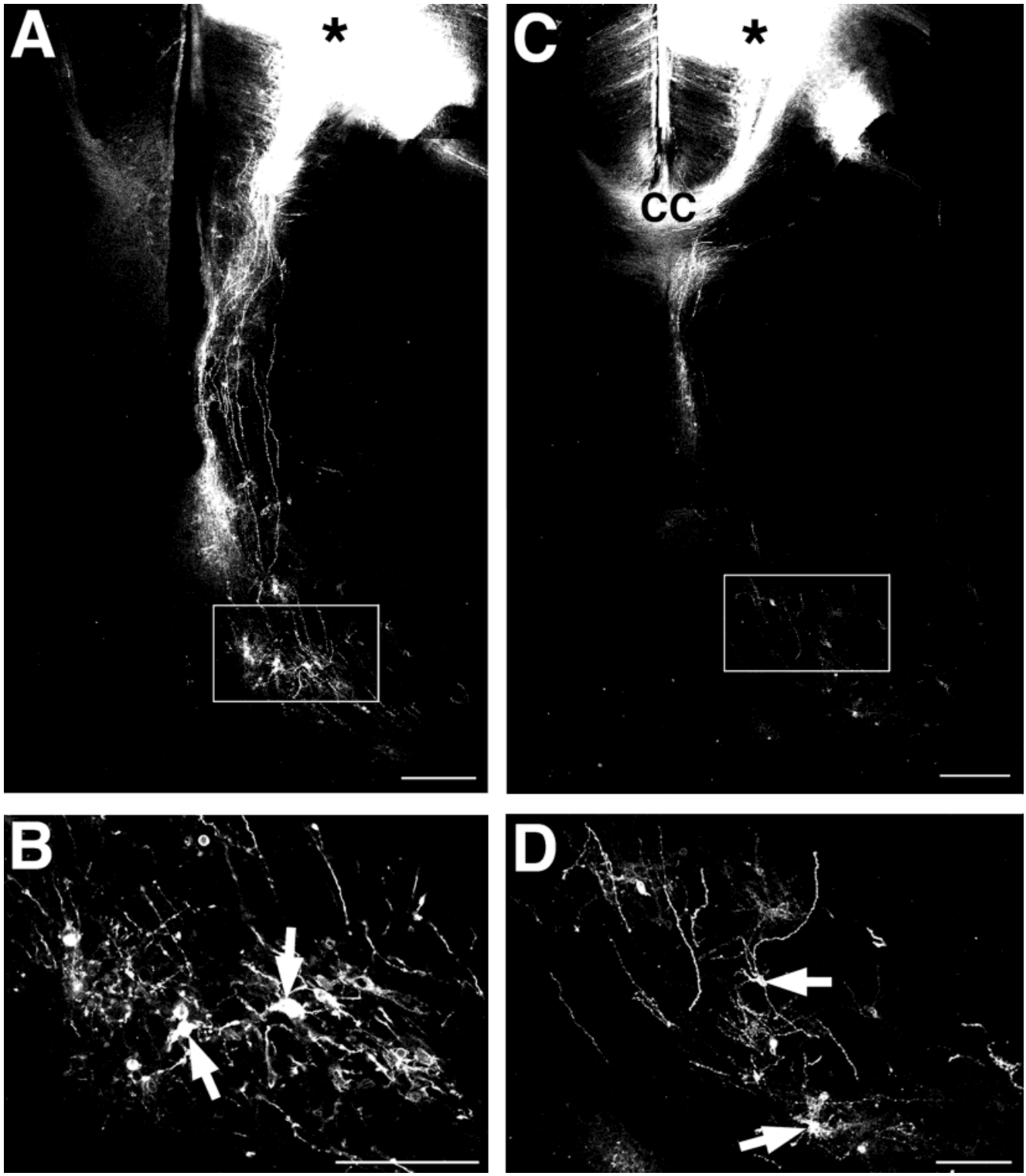

Fig. 2. MSDB neurons project to the cingulate cortex via the perforating pathway. Multiple DiI injections were made into the cingulate cortex along the rostral-caudal axis of E17 mouse brains. **A,B:** Neurons in the rostral MSDB are retrogradely labeled by an ipsilateral injection in the cingulate cortex (injection site shown by the asterisk in A). B is a higher power view of the boxed region in A detailing the cell bodies (arrows in B) of neurons in the HDB projecting within the PFP to the cingulate cortex. **C,D:** Neurons in the caudal MSDB are retrogradely labeled by an ipsilateral injection in the cingulate cortex. D is a higher power view of the boxed region in C showing retrogradely labeled neurons in the caudal MSDB (arrows in D). Asterisk in C indicates the injection site. Scale bars = 200 μm in A,B; 100 μm in C,D.

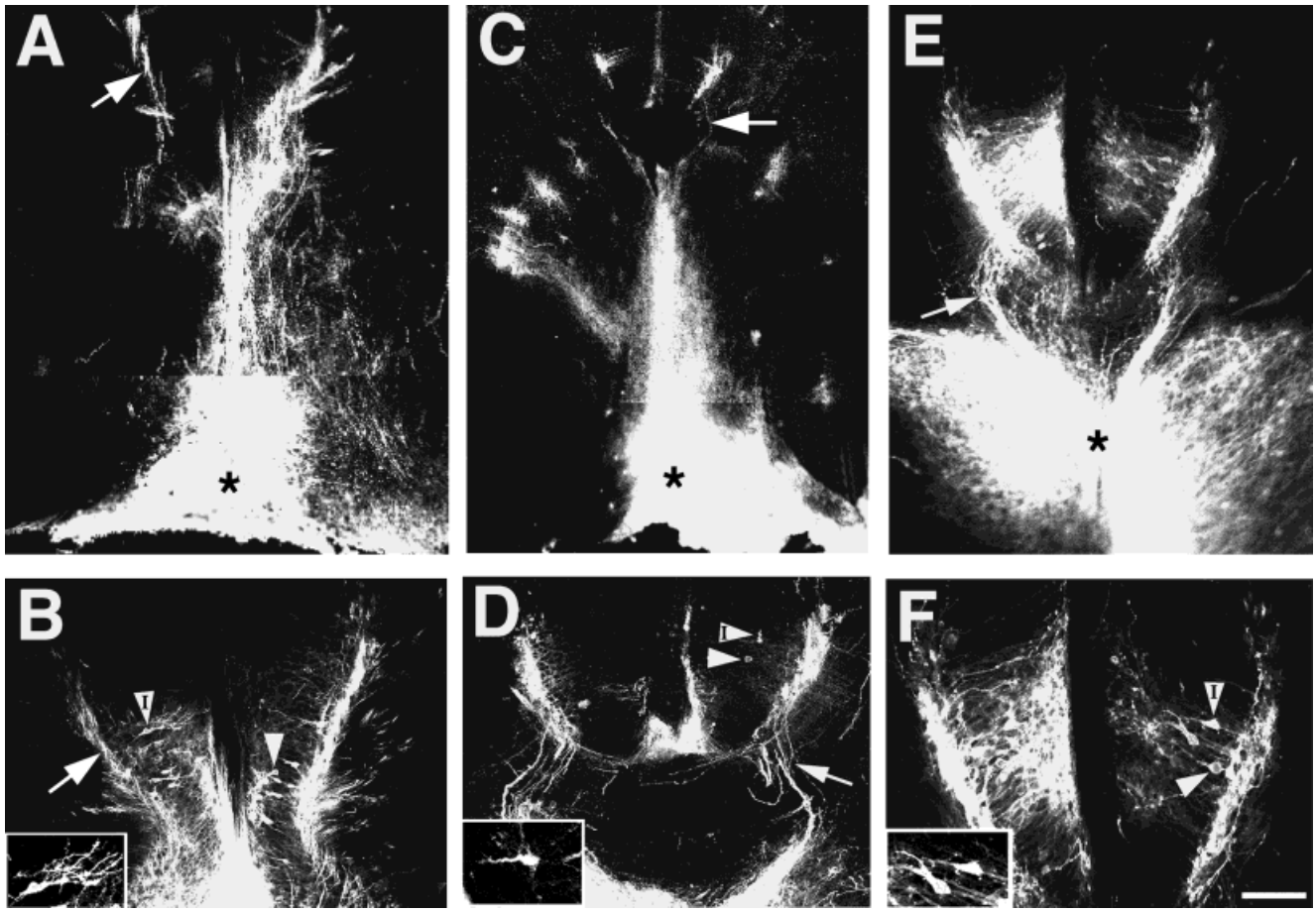

Fig. 3. Ventral cingulate neurons project to the MSDB via the perforating pathway. Multiple DiI injections were made into the MSDB of E17 mouse brains. (B, D, and F are higher power views of A, C, and E, respectively; insets in B, D, and F are higher power views of cells marked by an I in the arrowhead of each panel, respectively). **A,B:** Neurons in the rostral cingulate cortex are retrogradely labeled; arrowheads in B indicate two retrogradely labeled neurons in the ventral part of the rostral cingulate cortex. **C,D:** Neurons in the caudal cingulate cortex are retrogradely labeled (arrowheads in D). **E,F:** A greater number of neurons are labeled in the ventral cingulate cortex when a larger and more deeply placed injection is performed in the medial septum. Arrows in A and B indicate the PFP as it penetrates the taenia tecta rostral to the corpus callosum; arrows in C, D, and E indicate the PFP as it penetrates the corpus callosum. Asterisks in A, C, and E indicate the injection sites. DiI diffused to both sides of the MSDB, thus resulting in labeling of the PFP on both sides of the brain. Scale bar in F = 120 μm in A, 200 μm in C, 90 μm in B,D, 100 μm in E, 70 μm in F.

DISCUSSION

The present study investigated the trajectory, origin, and probable targets of the PFP, a midline pathway that intersects the corpus callosum. We found that this pathway projects ipsilaterally and connects the cingulate cortex and MSDB in a bidirectional fashion. A larger proportion of axons project from the MSDB to the cingulate cortex than vice versa. Our results suggest that the PFP may correspond to the septocingulum projection described in the adult (Swanson and Cowan, 1979; Milner et al., 1983), which forms a crucial part of the limbic circuitry. In adult rodents, two major subtypes of septal neurons have been identified, cholinergic and γ-aminobutyric acid (GABA)ergic neurons (Brauer et al., 1991; Milner, 1991). Both subtypes project to telencephalic regions including the hippocampus and the neocortex (Bigl et al., 1982; Rye et al., 1984; Brashear et al., 1986; Freund and Antal, 1988; Freund and Gulyas, 1991). Our results show that the PFP axons project to the cingulate cortex at embryonic stages. These axons may continue to grow past the cingulate cortex and into the neocortex at postnatal stages. If so, these axons may form the septocortical projection seen in the adult (Bigl et al., 1982; Rye et al., 1984; Freund and Gulyas, 1991).

Our study of the PFP parallels similar studies of the development of the septohippocampal pathway in rat and mouse (Linke and Frotscher, 1993; Super and Soriano, 1994). Given that the cingulate cortex and the hippocampus both arise from the medial wall of the telencephalon, it is likely that the septohippocampal pathway and the PFP described here are extensions of the same fiber tract. Both pathways also labeled with NF in our study (see Fig. 1A). We found that the first PFP axons to reach the cingulate cortex do so at late E15/early E16. The first axons to reach the hippocampus from the septum arrive at E17/E18 in the mouse, and injections in the hippocampus do not retrogradely label neurons in the septum until E17/E18 (Super and Soriano, 1994). These observations, to-

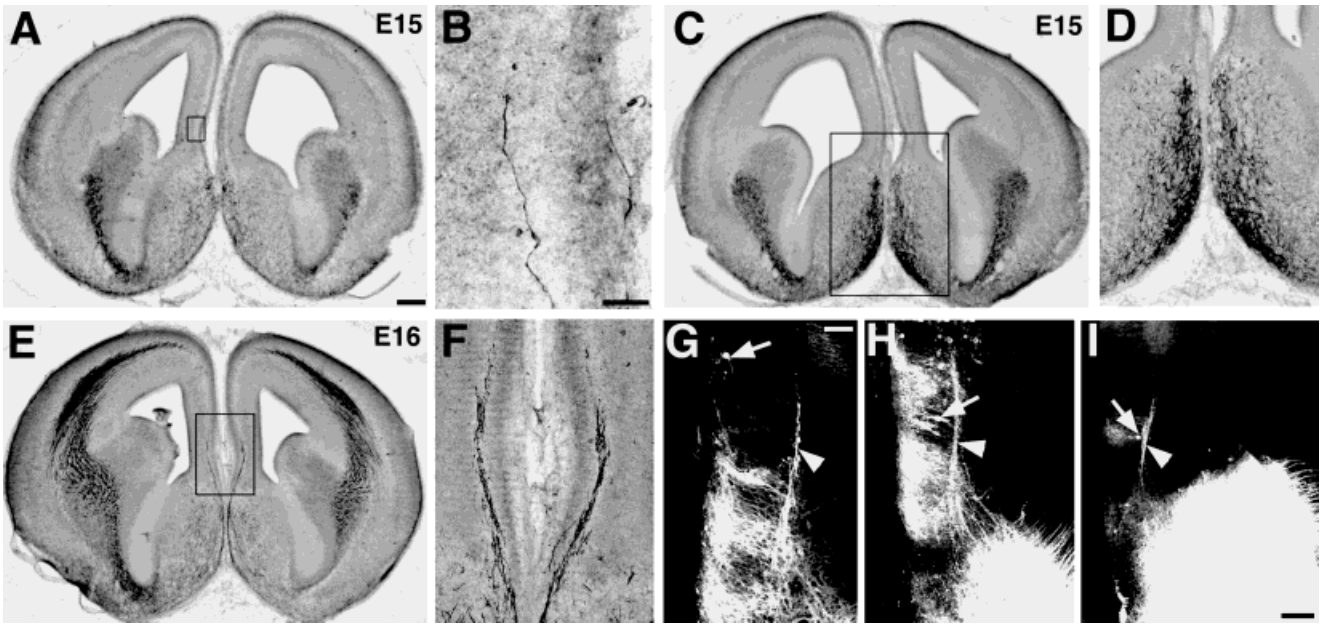

Fig. 4. Formation of the perforating pathway during development. Perforating axons were labeled either with the monoclonal anti-neurofilament antibody (A–F) or DiI (G–I) at E15 (A–D, G, H, and I) or E16 (F and G). **A,B:** On E15, the first PFP axons project between the MSDB and the cingulate cortex. B is a higher power view of the boxed region in A. **C,D:** Neurons in the MSDB are labeled with neurofilament on E15. D is a high-power view of the boxed region in C. **E,F:** On E16, more perforating fibers join the pioneering axons and form a larger axon bundle crossing the cingulum-septal boundary toward the cingulate cortex (compare F with B). F is a higher power view of the boxed region in E. **G–I:** On E15, pioneering perforating axons are labeled by DiI injections in the MSDB (arrowheads in G–I). Injections into either the rMSDB (G) or the cMSDB (H and I are two individual animals) retrogradely labeled neurons in the ventral cingulate cortex (indicated by arrow). Thus, on E15, neurons in both the MSDB and the cingulate cortex project axons bi-directionally to form the PFP. Scale bar in A = 200 μm in A,C,E, 100 μm in D. Scale bar in B = 30 μm in B, 100 μm in F. Scale bar in G = 50 μm in G. Scale bar in I = 60 μm in H, 90 μm in I.

gether with our data, indicate that the PFP forms before the septohippocampal pathway. Axons from the hippocampus arrive at the septum at E15/E16 and have been proposed to pioneer a pathway for the septohippocampal projection (Linke and Frotscher, 1993; Super and Soriano, 1994). The delay in the formation of the septohippocampal projection, relative to the PFP, may be because they require the presence of the pioneering axons from the hippocampus to arrive at the septum.

Here we used two methods of labeling the PFP: NF immunohistochemistry and DiI. Both methods gave the same labeling pattern of the MSDB and the PFP from the septum to the cingulate cortex; however, neurons in the cingulate cortex were not labeled by NF but were retrogradely labeled by DiI injections in the MSDB. We have previously found that the NF antibodies (both the polyclonal and monoclonal anti-NF (145-kD) antibodies have identical labeling patterns) also do not label the corpus callosum embryonically but do label many other axonal tracts, including the lateral neocortical projection (Shu et al., 2000). However, the NF antibodies do label the corpus callosum and the PFP at P0 (Preston et al., 2000). Therefore the lack of NF staining in the cingulate-septal projection and the corpus callosum embryonically may indicate that these medial cortical projections utilize different neurofilament subunits, such as NF-L (68 kD) or NF-H (200 kD), or phosphorylation states of the subunits, that are not recognized by these antibodies during embryonic stages of development. Maturation differences in the expression of different neurofilament subunits have been reported previously in the corpus callosum (Figlewicz et al., 1988), and differences in neurofilament phosphorylation have been shown to occur during axonal maturation (Dahl and Bignami, 1986). Further studies are required to determine whether this result is relevant for the development of the PFP.

The pioneering axons for both the PFP and the corpus callosum reach the midline at the same time during development, but end up growing along very different pathways. How are these two pathways differentially guided at the corticoseptal boundary? Three midline glial populations have been identified at the corticoseptal boundary: the glial sling (Silver et al., 1982), the glial wedge, and glia within the indusium griseum (Shu and Richards, 2001). Glial sling cells migrate from the lateral ventricular zone, forming a bridge-like structure that underlies the developing corpus callosum (Silver et al., 1982). Ablation and rescue experiments have shown that the glial sling is important for the formation of the corpus callosum (Silver et al., 1982; Silver and Ogawa, 1983). The sling is hypothesized to provide a substrate over which the callosal axons cross the midline and a barrier that prevents them from entering the septum (Silver et al., 1982). Callosal axons follow the sling and cross the midline of the brain without penetrating the sling and invading the septum (Silver et al., 1982; Katz et al., 1983). In contrast, axons of the PFP project ipsilaterally through the glial sling, in the dorsoventral axis. Although the first axons of the PFP project at

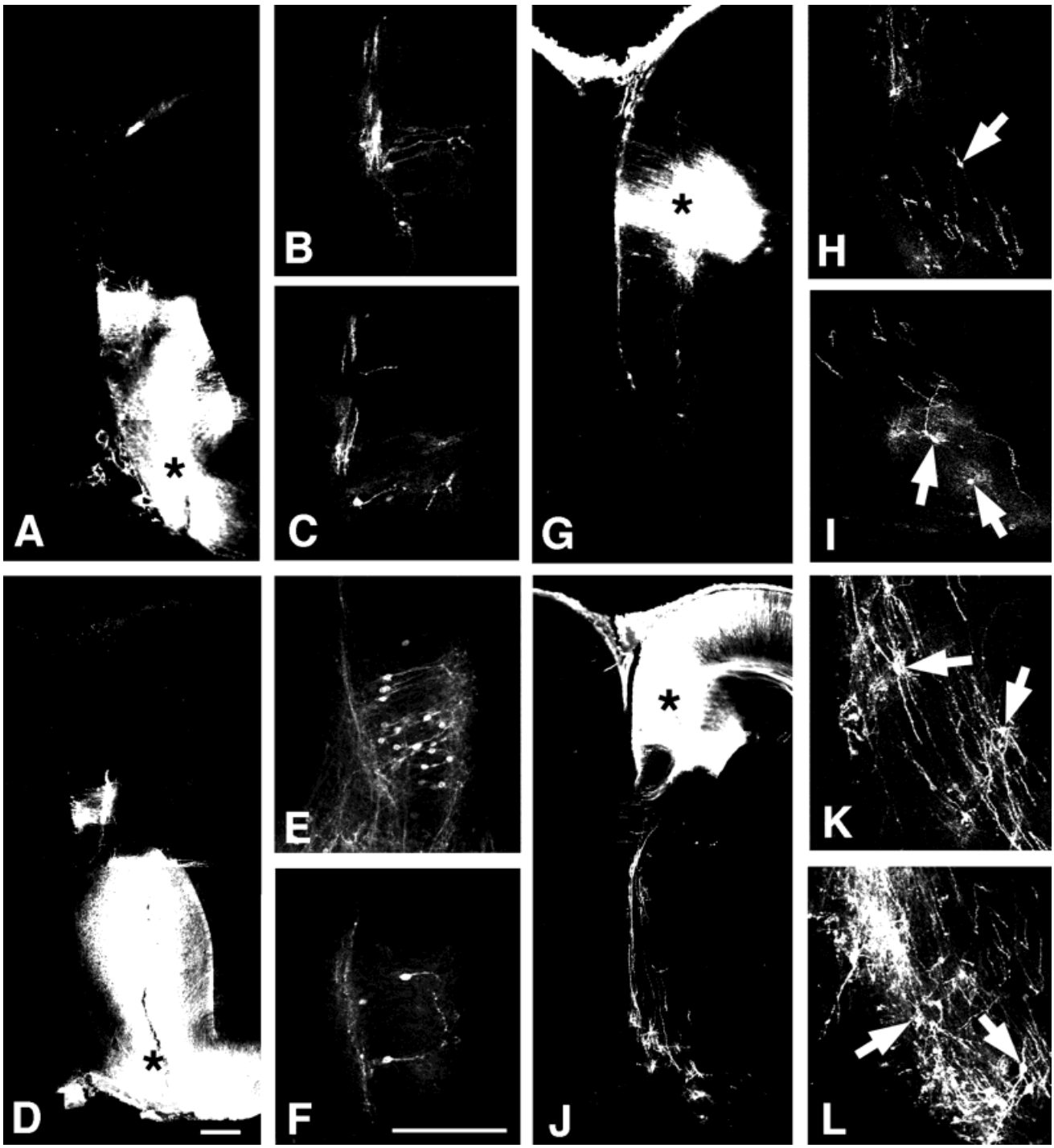

Fig. 5. **A–L:** MSDB, but not cingulate neurons project topographically within the PFP. To examine the development of projections from different rostrocaudal regions of the MSDB and the cingulate cortex, we made single DiI injections into different E17 brains. The injection sites are shown by an asterisk in A (rMSDB), D (cMSDB), G (rostral cingulate cortex), and J (caudal cingulate cortex). We have defined rostral MSDB and cingulate cortex as being rostral to the genu of the corpus callosum, whereas caudal MSDB and cingulate cortex are defined as those regions ventral to the genu and body of the corpus callosum. All other panels show examples of retrogradely labeled cells in either rostral (B,E,H,K) or caudal (C,F,I,L) regions of the target that were counted and analyzed for their projection preference. Note the small number of retrogradely labeled cells in the caudal cingulate cortex (C and F) from injections in either the rostral or caudal MSDB, respectively. In contrast, many more neurons were retrogradely labeled in the MSDB (arrows in H,I,K,L) from injections in the cingulate cortex. Scale bar in D = 200 μm in A,D,G,J. Scale bar in F = 200 μm in all other panels.

TABLE 1. Counts of Labeled Neurons From Injections in Either MSDB or Cingulate Cortex at E17[1]

	No. of retrogradely labeled neurons in the:			
Injection site	Rostral cingulate cortex	Caudal cingulate cortex	Rostral MSDB	Caudal MSDB
Rostral MSDB (n = 5)	20 (mean of 14, 32, 23, 11, 22)	5 (mean of 5, 3, 7, 3, 9)		
Caudal MSDB (n = 4)	17 (mean of 4, 15, 35, 13)	3 (mean of 0, 4, 6, 0)		
Rostral cingulate cortex (n = 4)			58 (mean of 88, 54, 20, 68)	29 (mean of 33, 50, 15, 19)
Caudal cingulate cortex (n = 5)			55 (mean of 24, 37, 74, 31, 108)	117 (mean of 101, 70, 199, 65, 151)

Retrogradely labeled neurons were counted in serial sections of brains injected with DiI.
[1]The mean and the individual counts are included since the number of cells labeled between brains varies depending on the size of the injection and the diffusion of the dye. However, the relative numbers of neurons labeled in either rostral or caudal regions of the same brain (with a single injection) may be directly compared. MSDB, medial septum and diagonal band of Broca complex.

E15 before the glial sling has fully formed at E17 (Silver et al., 1982), later arriving PFP axons must grow through the glial sling. Therefore, unlike the corpus callosum, the glial sling is not a barrier for axons of the PFP, indicating that differential guidance mechanisms may be utilized by the PFP and the corpus callosum to navigate the corticoseptal boundary.

Two other populations of glia that arise at the corticoseptal boundary are the glial wedge and glia within the indusium griseum (Shu and Richards, 2001). These glia express the chemorepulsive molecule slit-2 and guide callosal axons toward the midline through a suppressive/repulsive mechanism (Shu and Richards, 2001). The trajectory of the PFP in vivo suggests that it may be influenced by guidance molecules derived from the septum and from the glial wedge. In the septum the PFP forms a fasciculated bundle of axons that remain confined just lateral to the midline until they reach the glial wedge at the corticoseptal boundary. When the PFP reaches the glial wedge, it follows the outline of the wedge laterally before turning into the cingulate cortex. The axons neither spread out into the lateral septum nor invade the glial wedge (Fig. 1I). This finding suggests that the lateral septum and/or the glial wedge might produce either nonpermissive or inhibitory molecules that guide the perforating axons and maintain them in a fasciculated bundle. Alternatively, the boundary of the glial wedge may provide a restricted growth-permissive area for the PFP axons. A similar mechanism has been shown to occur in the guidance of retinal ganglion cells axons at the optic chiasm (Marcus et al., 1999, 2000).

Because the corpus callosum and the PFP intersect close to the glial wedge, the glial wedge may play a role in sorting these axons and directing their respective trajectories either ipsilaterally or contralaterally. Although the PFP axons avoid the glial wedge in vivo, they are not strongly repelled from it. Furthermore, the MSDB neurons do not express either of the slit receptors, robo1 or robo2 (data not shown). Thus it is unlikely that the perforating axons would respond to slit-2 expressed by the glial wedge, but instead may be influenced by additional molecules expressed by the glial wedge. Recent experiments in *Drosophila* show that robo and robo2 are responsible for mediating an "antilingering" response of contralaterally projecting growth cones to slit (Simpson et al., 2000; Rajagopalan et al., 2000). If the same mechanism is operating here, then slit and robo1/2 may be responsible for repelling callosal axons across the midline but not PFP axons, thus defining contralaterally from ipsilaterally projecting axons.

In addition to soluble guidance cues, the PFP may be guided by bound guidance cues associated with cellular membranes or within the extracellular matrix (see Kaprielian et al., 2000, for a review of axon guidance molecules at the midline). One possibility is that the callosal axons express membrane surface molecules that cause them to fasciculate with other callosal axons, but not with the perforating axons and vice versa. DCC (deleted in colorectal cancer), a receptor of Netrin-1 (Keino-Masu et al., 1996), is present on the callosal axons (Shu et al., 2000), but is not expressed on the PFP axons (data not shown). DCC has been shown to mediate axon guidance in a number of systems (Keino-Masu et al., 1996; Fazeli et al., 1997; Anderson et al., 2000). In addition, other Ig-like membrane proteins such as L1 and TAG-1 are also expressed by callosal axons, but not PFP axons (Wolfer et al., 1994; Demyanenko et al., 1999; Fujimori et al., 2000). The differential expression of these cell surface proteins and others such as those belonging to the neural cell adhesion molecule (N-CAM) family may be responsible for the different trajectories of these two independent pathways.

Our examination of the projection preference of axons in either the cingulate cortex or the MSDB revealed that MSDB neurons contribute significantly more axons to the PFP than neurons in the cingulate cortex. In addition, neurons in the MSDB preferentially send their axons to regions of the same rostrocaudal level of the cingulate cortex. This may indicate that guidance cues may be present in the cingulate cortex that guide axons from the MSDB to specific rostrocaudal regions of the cingulate cortex. Topographic guidance cues such as those important in retinotectal targeting (reviewed in Goodhill and Richards, 1999) may also be involved in axonal targeting between the MSDB and the cingulate cortex, but the actual cues remain to be identified. Our results also show that some neurons from the MSDB do not project topographically, indicating that if topographic guidance cues do exist, they are specific to some neurons in the MSDB and not others.

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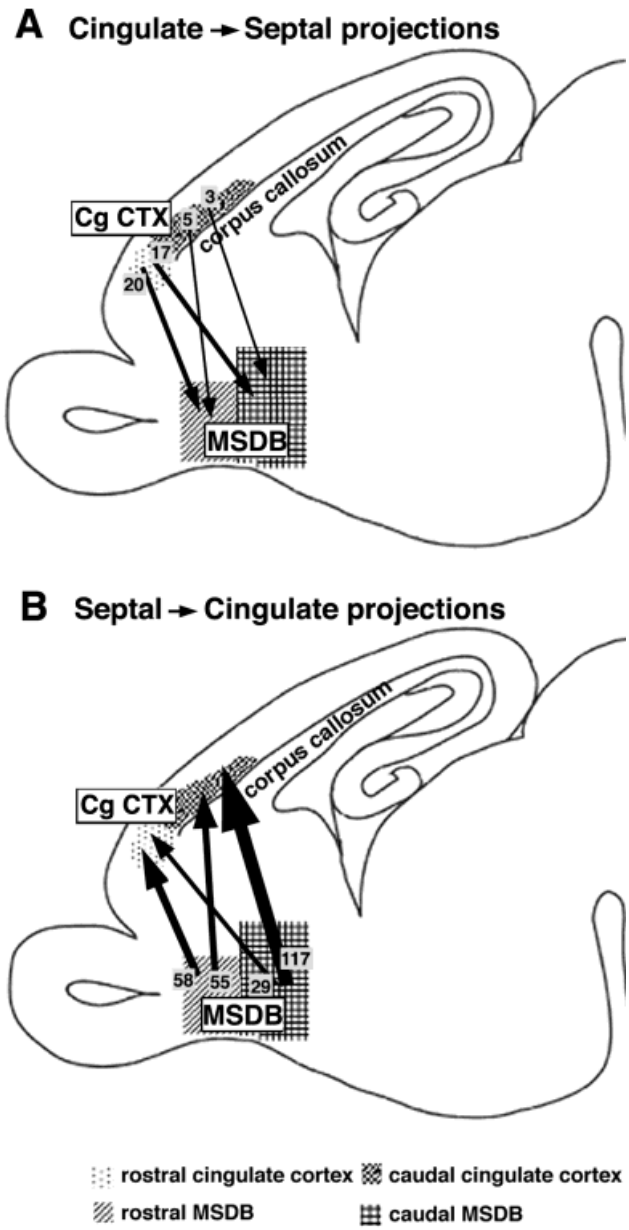

Fig. 6. Analysis of axonal projection preference in the PFP. Schematics of sagittal E17 sections are shown to give a rostrocaudal perspective of the PFP. Neurons labeled by the injection paradigm shown in figure 5 were counted and grouped according to their axonal projection preference. **A,B:** Schematic representations of the direction of axonal projections (arrows; based on the retrograde transport of DiI) and the number of neurons counted in each group. The thickness of the arrows is indicative of the relative size of the projection from each region. A shows the cingulate-septal projections of the PFP, and B shows the septal-cingulate component. Note that the medial septum and diagonal band of Broca complex (MSDB) contributes relatively more axons to the PFP than the cingulate cortex (Cg CTX) and also that the cMSDB projects preferentially to the caudal cingulate cortex than to the rostral cingulate cortex.

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