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Guidance of Cerebellofugal Axons in the Rat Embryo: Directed Growth toward the Floor Plate and Subsequent Elongation along the Longitudinal Axis

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Summary

To elucidate guidance mechanisms of brain commissural axons, we examined the navigation of cerebellofugal axons. Axons were labeled by implantation of the fluorescent tracer DiI into the cerebellar plate (CP) of fixed, flat whole-mount embryonic rat brain. Axons initially grew straight toward the ventral midline floor plate (FP) in the rostral hindbrain and then, after crossing it, made a right-angled turn to grow either caudally or rostrally along the longitudinal axis. In collagen gel culture, CP axons showed directed growth toward both FP explants and heterologous cells expressing netrin-1, a FP-derived chemoattractant for spinal commissural axons. These results suggest that CP axons are guided to the midline by FP-derived chemoattractant(s) and then reoriented, possibly by another guidance cue, for longitudinal extension. Considering that the basic structures of the neural tube, including the FP, extend up to the caudal diencephalon, these results suggest that common guidance mechanisms operate for ventrally decussating commissural axons in both the brain and spinal cord.

Introduction

During development of the vertebrate CNS, a number of decussating axons develop through the ventral and dorsal midline structures that connect the two lateral halves of the bilaterally symmetrical neural tube. Recent studies have detailed pathfinding behavior and underlying guidance mechanisms of spinal cord commissural axons that cross the ventral midline during development (Holley, 1987; Dodd et al., 1988; Tessier-Lavigne et al., 1988; Bovolenta and Dodd, 1990; Placzek et al., 1990a, 1990b; Yaginuma et al., 1991; Yaginuma and Oppenheim, 1991; Serafini et al., 1994; Kennedy et al., 1994; Bernhardt, 1994). These spinal cord axons initially course circumferentially toward the ventral midline floor plate (FP) and then grow longitudinally after crossing it (Holley and Silver, 1987; Bovolenta and Dodd, 1990; Kuwada et al., 1990; Yaginuma et al., 1991). The finding that the basic structures of the neural tube, such as the floor, alar, and basal plates, are relatively well conserved from the spinal cord to the level of the caudal diencephalon (Kingsbury, 1930; Puelles et al., 1987) raises the possibility that commissural neurons in the brain and spinal cord share common axonal guidance mechanisms. This idea is supported by the fact that the projection patterns of commissural axons are maintained between spinal cord and hindbrain segments in both chick and zebrafish embryos (Trevarrow et al., 1990; Clarke and Lumsden, 1993; Glover, 1993; for review, see Kimmel, 1993).

Guidance of cerebellofugal projections, however, would seem to vary from other commissural projections in the brain based on their adult projection patterns. Axons from two of the cerebellar nuclei, the interposed and lateral nuclei, initially course rostrally and then turn medially toward the midline (Chan-Palay, 1977; Ito, 1984; Brodal, 1992), as opposed to taking the circumferential pathway followed by spinal cord commissural axons. Although such projection patterns in the adult may indicate that these axons follow guidance mechanisms distinct from those for the spinal commissural axons, it is also possible that they are simply a consequence of brain development following the completion of the projections.

In the present study, we examined the early development of cerebellofugal axons in order to clarify whether common axonal guidance mechanisms operate for commissural neurons in the brain and spinal cord. For this purpose, we first analyzed cerebellofugal axon pathfinding using fixed, flat whole-mount embryonic rat brain preparations, which enable ready recognition of the entire trajectories of labeled axons, together with their relation to the circumferential and longitudinal axes of the developing brain (Lumsden and Keynes, 1989; Bovolenta and Dodd, 1990; Yaginuma et al., 1991). Cerebellofugal axons were labeled by implanting small crystals of the fluorescent carbocyanine dye, 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (DiI), into the cerebellar primordium (cerebellar plate [CP]). We found that these axons initially coursed circumferentially toward the ventral midline FP in the rostral hindbrain; then, after crossing it, they made a right-angled turn to grow either caudally or rostrally along the longitudinal axis at a distance from the FP. Next, to examine whether cerebellofugal axons are guided to the FP by its chemotropic activity in the same way as are commissural axons in the spinal cord (Tessier-Lavigne et al., 1988; Placzek et al., 1990a, 1990b), we cocultured, using collagen gel matrix, CP explants with FP explants in the rostral hindbrain or with aggregates of COS cells expressing netrin-1, a recently identified FP-derived chemoattractant for spinal commissural axons (Serafini et al., 1994; Kennedy et al., 1994). We found that CP axons showed directed growth toward the FP as well as toward netrin-secreting COS cell aggregates. These results suggest that guidance mechanisms for ventrally decussating commissural axons are common to both the brain and spinal cord.

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Results

Appearance of Flat Whole-Mount Brain Preparations

In the flat whole-mount brain preparation, the FP can be recognized as a transparent longitudinal stripe running down the center (Figure 1). The CP can be observed as a gently arced protuberance located on both sides of the rostral hindbrain, thus allowing accurate implantation of DiI. The tectum (T) in the midbrain is located rostral to the CP; the isthmus (Is) region (midbrain–hindbrain boundary) can be recognized as a transparent transverse line. This preparation permitted examination of entire axonal trajectories from soma to growth cone without tissue sectioning. Moreover, it allowed us to monitor the initial outgrowth of cerebellofugal axons in early embryos, which are difficult to access by tissue sectioning. Similar preparations have been successfully employed in the study of axonal projection development in rat and chick embryos (Lumsden and Keynes, 1989; Bovolenta and Dodd, 1990; Yaginuma et al., 1991).

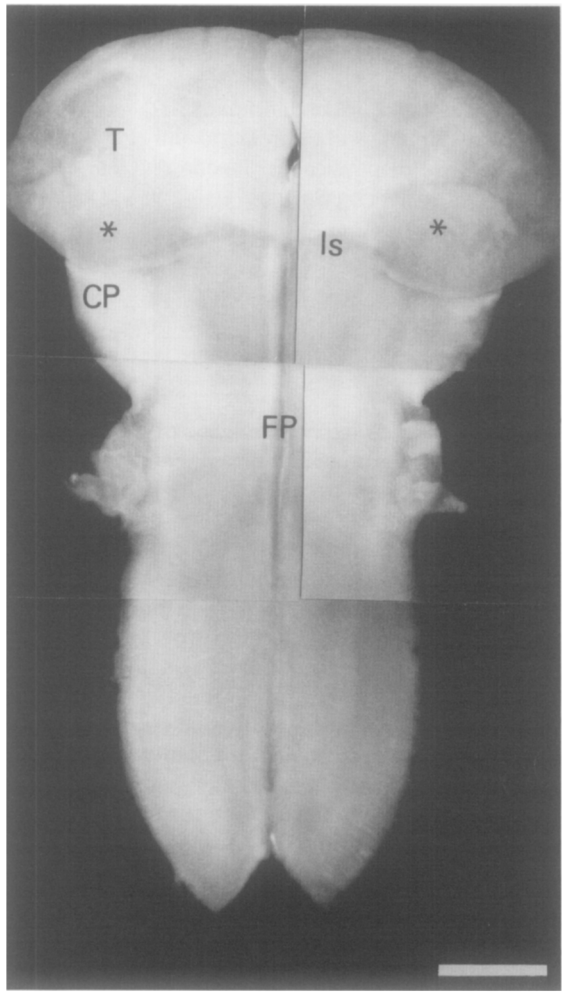

Figure 1. Ventral View of a Flat Whole-Mount Brain Preparation That Includes the Region Extending from the Midbrain through to the Caudal End of the Hindbrain

The ventral midline floor plate (FP) is located at the center, and the isthmus (Is) can be seen as a transparent transverse line at the midbrain–hindbrain boundary. The cerebellar plate (CP) can be observed as a gently arced protuberance situated on both sides of the rostral hindbrain. Asterisks indicate regions of the tectum (T) that overlie the CP. E14 rat embryo. A dark-field light micrograph. Anterior is up. Bar, 1 mm.

Initial Axon Outgrowth from the CP

CP axon outgrowth began at embryonic days 12–13 (E12–E13). At E12, the earliest stage at which we were successfully able to implant DiI into the CP, labeled axons were mostly confined to the CP (data not shown). At E13, CP axons left the CP and headed straight toward the ventral midline in the rostral hindbrain (Figures 2A and 2B). This contrasts with the trajectories of interposed and lateral nuclei axons in adult rats, which initially run rostrally after leaving the cerebellum (e.g., Faull and Carman, 1978). The leading tips of cerebellofugal axons exhibited typical growth cone morphology, with many filopodia-like protrusions (Figure 2C).

Multiple implants of tiny DiI crystals into the lateral (i.e., dorsal) margin of the CP revealed that initial axon growth followed a direction perpendicular to the lateral edge (Figure 2D).

Axon Arrival at the Ventral Midline

CP axons projected straight toward the ventral midline FP at around E14 (Figures 3A and 3B), growing perpendicular to the longitudinal axis of the brain. Upon entering the FP, they did not change growth direction. Growing axons that arrived later tended to run closer to the pial surface than earlier ones (Figure 3C). Growth cones extended several filopodia around the medial longitudinal fasciculus on both sides (Figure 3E), while most growth cones were simple in morphology, without notable filopodia and lamellipodia, as they grew through the FP (Figure 3D).

Developmental Translocation of Deep Cerebellar Nuclei Neurons

At E15 or later, implantation of DiI near the lateral margin of the CP occasionally failed to label cerebellofugal axons. This failure might be due to a displacement of cerebellar nuclei cells within the CP. In support of this view, during the early developmental stages examined here, cerebellar deep nuclear neurons, from which cerebellofugal axons originate, were reported to migrate from superficial to deep regions of the CP (Altman and Bayer, 1985a, 1985b). To confirm this possibility, we implanted small DiI crystals into the site of commissure formation in rostral hindbrain and examined the location of retrogradely labeled cells in the CP. Labeled cells were dispersed between the lateral margin and the medial regions of the CP at E14 (Figures 4A and 4B), but were confined to regions a greater distance from the lateral margin at E15 and later (Figures 4C and 4D). These results suggest that cerebellar nuclear neurons are translocated to deep regions in the CP at around E15. In preparations at E15 and later, we therefore implanted DiI into corresponding regions of the CP to follow axonal trajectories (see following sections).

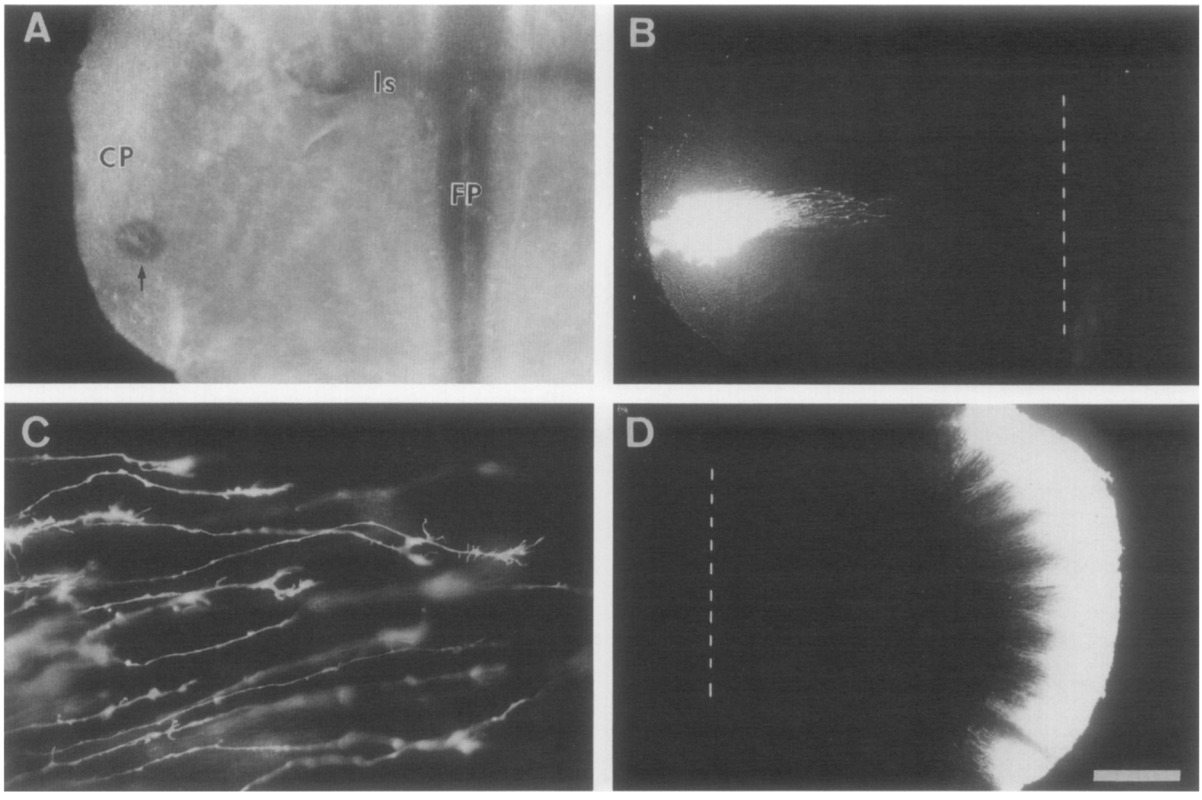

Figure 2. Outgrowth of Axons from the CP at E13

(A) Dark-field micrograph of E13 rat embryo implanted with DiI (arrow) into the left CP. Is, isthmus.

(B) The same view as (A), but under epifluorescent illumination. Cerebellofugal axons can be seen growing straight toward the ventral midline (dashed line in [B] and [D]).

(C) Higher magnification of leading tips of cerebellofugal axons shown in (B).

(D) Multiple implants of DiI crystals were made along the lateral margin of the right CP. Note that initial axonal outgrowth occurs perpendicular to the lateral edge.

Bar, 400 μm (A, B, and D), 40 μm (C).

Pathway Selection after Crossing the FP

At E15, CP axons grew beyond the FP (Figure 5A). The earliest growing axons extended for some distance from the FP before abruptly turning at a right angle to extend caudally (Figure 5B). The growth cones of these caudally growing axons occasionally extended short processes, which were directed caudally as well as laterally at the turning point, but virtually lacked lamellipodial veils (Figure 5D).

Axons arriving at the FP somewhat later also made a right-angled turn, but thereafter projected rostrally (Figure 5C). These later growing, ascending axons followed a path that ran closer to the fourth ventricle and turned more medially than that of earlier descending ones (Figures 5B and 5C). The morphology of their growth cones appeared more complex at the turning point than that of descending axons (Figures 5D and 5E).

The rostrally growing axons reached the midbrain at around E16, in agreement with a previous report (Cholley et al., 1989) (Figure 6A). At this stage, another mass of descending axons occurred, running along the same longitudinal axis as the ascending ones (Figure 6B, arrow). These descending axons appeared to follow a more restricted course than those that descended initially. At the turning point, axons occasionally bifurcated into branches extending both caudally and rostrally (Figure 6C, arrows). The caudally elongating component occurred somewhat later, after the rostrally growing axons had proceeded for some distance (data not shown). In a few cases, uncrossed axons that grew longitudinally were observed (data not shown).

Chemotropic Guidance of CP Axons toward the Ventral Midline In Vitro

Cerebellofugal axons elongated straight toward and then through the FP. Such axon behavior is reminiscent of that of spinal cord commissural axons (Bovolenta and Dodd, 1990; Kuwada et al., 1990; Yaginuma et al., 1991), whose guidance to the ventral midline is suggested to occur via the action of a diffusible chemoattractant released from the FP (Tessier-Lavigne et al., 1988; Placzek et al., 1990a, 1990b; Yaginuma and Oppenheim, 1991; Kennedy et al., 1994; Serafini et al., 1994), thus raising the possibility that a similar guidance mechanism operates for CP axons.

To examine this possibility, we cocultured CP explants with FP explants from E13 or E14 embryos in collagen

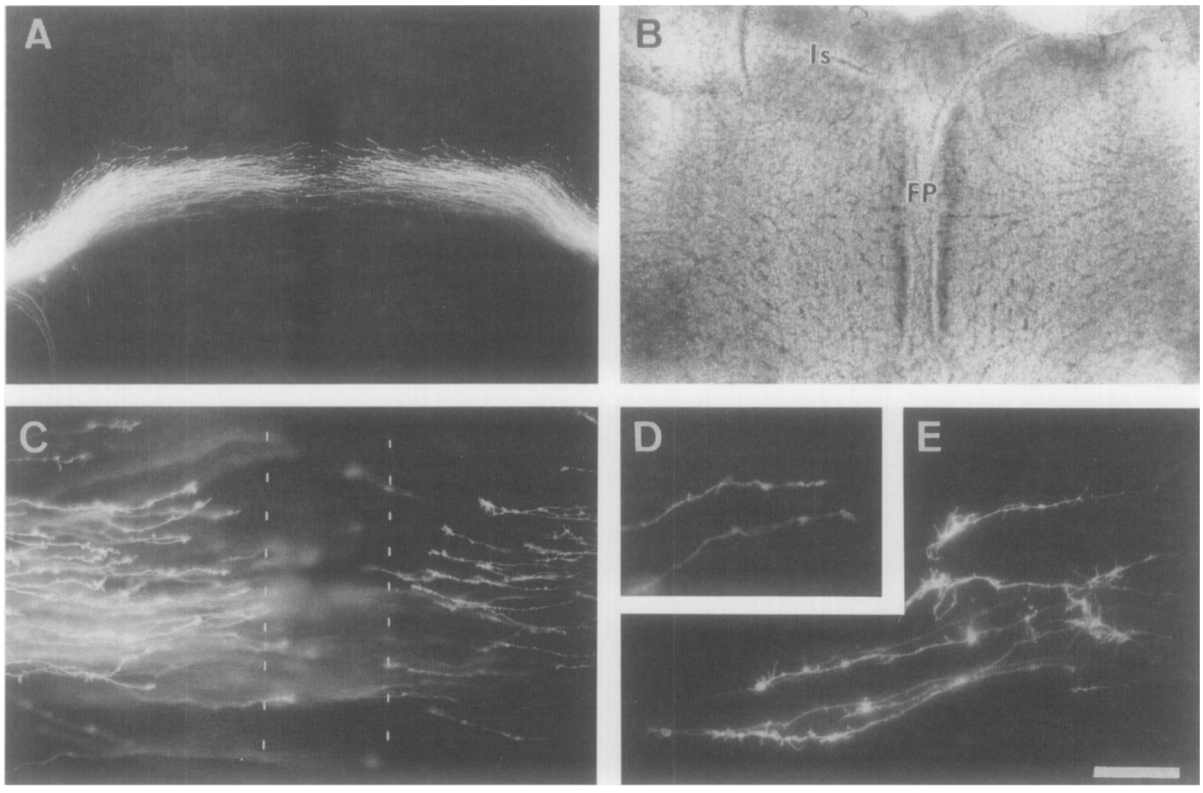

Figure 3. Cerebellofugal Axons Crossing the Ventral Midline at E14

Epifluorescent (A) and bright-field (B) light micrographs of E14 rat hindbrain showing that CP axons growing from both sides encounter each other at the ventral midline. DiI was placed into the CP on both sides. (C), (D) and (E) are higher power epifluorescent micrographs of the growing tips of the axons shown in (A). Within the FP, later crossing axons run closer to the pial side than early ones, which are out of focus in (C). Dashed lines in (C) denote the edges of the FP. Growth cones within the FP tend to be simple in morphology (D), whereas those near the edges of the FP (dotted lines in [C]) exhibit a complex morphology, with many filopodia (E). Is, isthmus. Bar, 400 μm (A and B), 80 μm (C), 40 μm (D and E).

gels, which enable detection of diffusible chemotropic activity (Ebendal and Jacobson, 1977; Lumsden and Davies, 1983, 1986). FP explants were taken from the region of the rostral hindbrain where CP axons are expected to decussate (Figure 7A). CP explants, when cultured alone, showed some neurite outgrowth from the cut (i.e., medial or ventral in normal rat brain) edge but not from the lateral (i.e., dorsal in normal rat brain) surface (13/13 explants tested; Figure 7B). In contrast, when FP explants were closely apposed to the cut edge of CP explants (data not shown) or their lateral surface, thick bundles of neurites grew extensively from the CP explant toward the FP explant (22/22 explants; Figure 7C). However, when CP explants were cocultured with other CP explants or with the dorsal thalamus, a major target of ascending cerebellofugal axons, no neurite outgrowth occurred from the lateral surface (8/8 explants in both cases; data not shown).

Next, to ascertain whether the FP in the rostral hindbrain is capable of reorienting CP axons, FP explants were juxtaposed aside CP explants, with the cut edge oriented perpendicular to the longitudinal axis of the FP. As shown in Figure 7D, DiI-stained CP axons located near the FP explant turned, within the CP, toward the FP, deflecting from their in vivo growth direction (12/12 explants; see Figures 2B and 2D). These results support the notion that the ventral midline FP in the rostral hindbrain releases a diffusible chemoattractant that guides CP axons toward the ventral midline.

Recently, diffusible chemotropic factors that may be responsible for guidance of spinal cord commissural axons toward the FP have been purified from chick embryonic brain (Serafini et al., 1994; Kennedy et al., 1994). mRNA of one of these molecules, netrin-1, was found to be expressed in the FP at all axial levels from the spinal cord into the caudal diencephalon (Kennedy et al., 1994). Thus, these findings, together with the present results, further raise the possibility that the same molecule, netrin-1, mediates the guidance of CP axons toward the ventral midline. COS cells expressing recombinant netrin-1 were previously reported to secrete diffusible forms of netrin-1 proteins (Kennedy et al., 1994). We therefore examined this possibility by coculturing CP explants with aggregates of transfected COS cells expressing *netrin-1* cDNAs in collagen gels. When aggregates of transfected COS cells expressing netrin-1 were closely apposed to the lateral surface of CP explants, CP axons grew extensively toward COS cell aggregates in the same fashion as FP explants (12/12 explants tested; Figure 7E). However, when CP

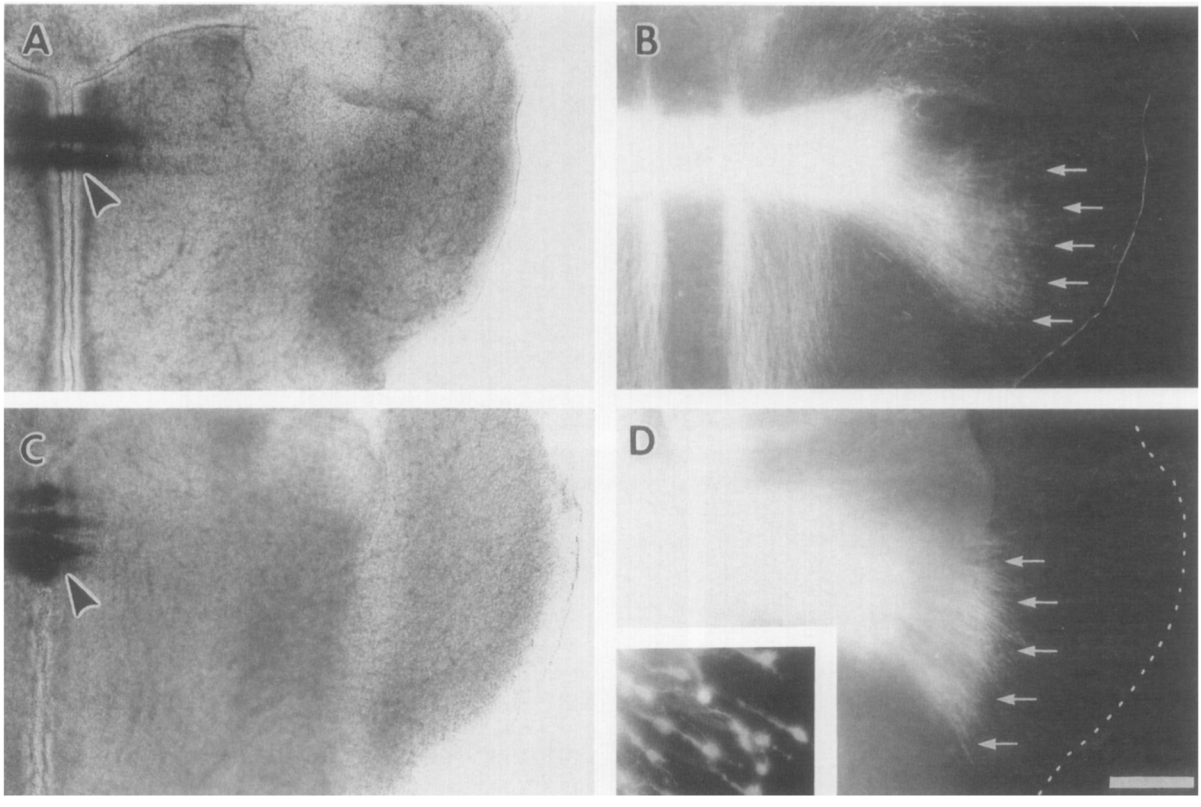

Figure 4. Retrograde Labeling of CP Axons

DiI crystals were placed in the loci of cerebellofugal axon decussation. Retrogradely labeled cells (arrows) in the CP of an E14 embryo (A and B) are located in a more lateral region of the CP than those of an E15 embryo (C and D). Inset in (D) shows a high power micrograph of such cells. Dotted lines indicate the lateral margin of the CP. Anterior is up and lateral is to the right. Arrowheads indicate DiI placement sites. (A and C) Bright-field micrographs; (B and D) the same views as (A) and (C), but under epifluorescent illumination. Bar, 400 μm (A–D), 80 μm (inset in [D]).

explants were cocultured with aggregates of control COS cells, no axon bundles emerged from the lateral surface (6/6 explants tested; Figure 7F). These results suggest that the same molecule guides developing commissural axons toward the ventral midline in both the brain and spinal cord.

Discussion

Utilizing flat whole-mount brain preparations permitted reliable and reproducible labeling of developing CP axons. DiI placement into the CP demonstrated that CP axon outgrowth is initiated at E12–E13 (Figure 8A, part i) and that, after crossing the ventral midline FP at around E14 (Figure 8A, part ii), axons make a right-angled turn to descend caudally or ascend rostrally along longitudinal paths at a distance from the FP at E15–E16 (Figure 8A, part iii). In collagen gel culture, extensive neurite outgrowth occurred from CP explants toward FP explants, suggesting that CP axons are guided toward the ventral midline by diffusible chemoattractant(s) released from the FP (Figure 8B). These results suggest the existence of guidance cues for brain commissural axons at the ventral midline FP, and possibly at a distance lateral to it (Figure 8C).

Correspondence of Labeled Projections to Cerebellofugal Projections

Restricted implantation of small DiI crystals was made into the lateral margin of the CP at early stages (E12–E14). Considering that this corresponds to the region of emergence of cerebellar deep nuclear neurons (Altman and Bayer, 1978, 1985a), and that deep nuclear neurons are generated at E12–E14 (Altman and Bayer, 1978), it is highly likely that the axons labeled in the present study were cerebellar nuclear neuron axons.

DiI placement into the midline area of the rostral hindbrain at E14 and later resulted in retrograde labeling of CP cells that were translocated from a superficial location to a deep region during development. This is consistent with results from tritium thymidine studies of deep nuclear neurons by Altman and Bayer (1985a, 1985b) and further suggests that the labeled axons derive from cerebellar deep nuclear neurons. Consistent with this view, all projection patterns of labeled axons corresponded to those of interpositofugal and dentatofugal projections in adult mammals: ascending axons to the thalamus and brainstem and descending axons to the inferior olive, the pons, and the medulla oblongata (Chan-Palay, 1977; Ito, 1984; Brodal, 1992). Considering that ascending axons give off de-

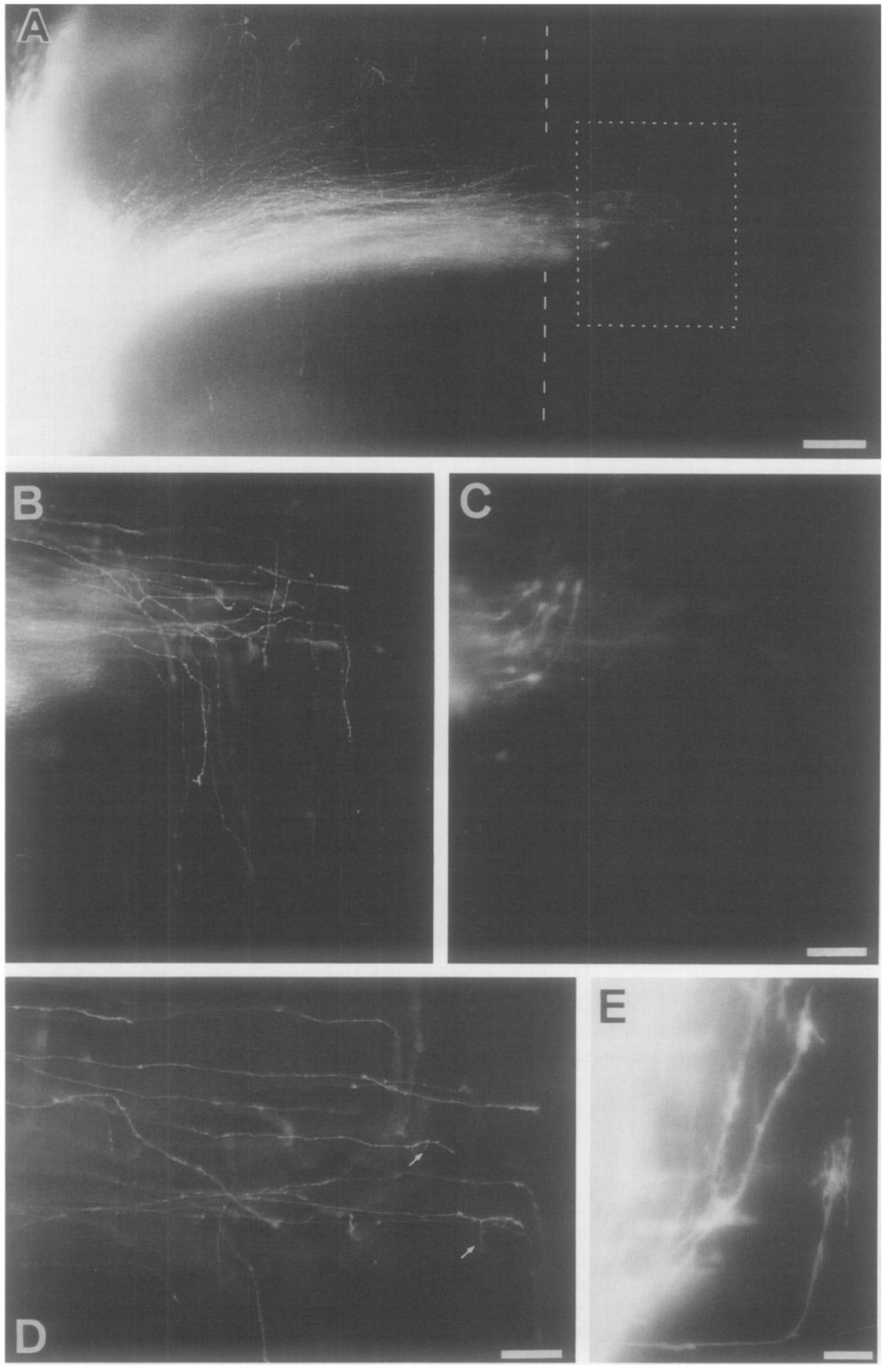

Figure 5. Cerebellofugal Axons Growing along Longitudinal Pathways after Crossing the Ventral Midline in E15 Rat Embryo

(A) Cerebellofugal axons crossing the ventral midline at E15. Dashed line indicates the ventral midline.

(B and C) Higher magnification of the area within the dotted rectangle in (A). These are from the same microscopic field but of different focal planes. Later arriving, rostrally ascending axons (C) run closer to the ventricle than earlier growing ones (B). After crossing the ventral midline, both groups of axons make a right-angled turn, although later growing axons follow a more medial longitudinal path than do earlier growing, caudally descending ones. The diffuse appearance of axons in (C) is due to their greater depth within the tissue.

(D) High power micrograph of the caudally projecting axons shown in (B). Growth cones at the turning point occasionally extend short protrusions or branches directed caudally (arrows) as well as laterally.

(E) Growth cones of rostrally projecting axons around the turning point are more complex in morphology.

Rostral is up and lateral is to the right. Bars, 200 μm (A), 100 μm (B and C), 50 μm (D), 20 μm (E).

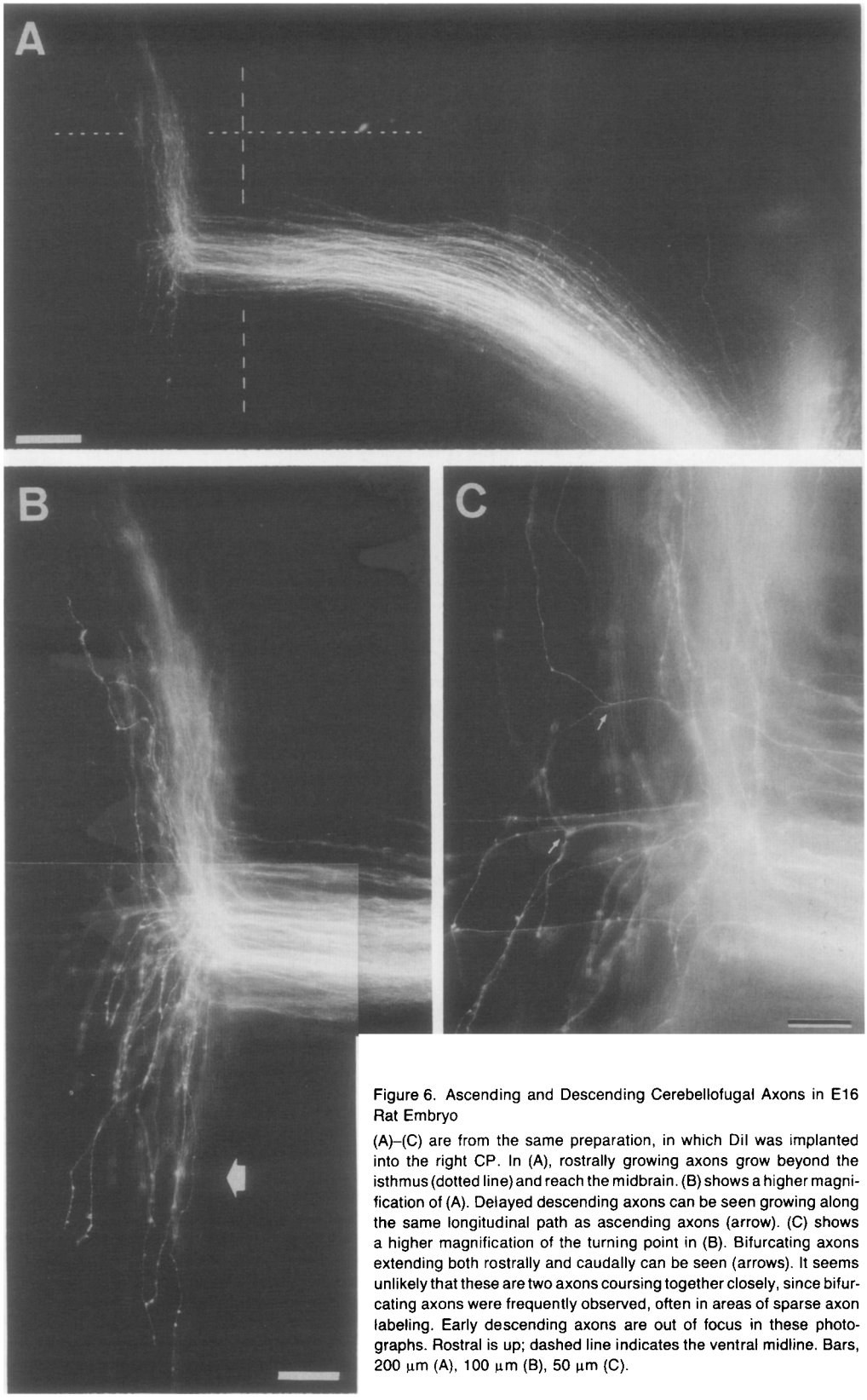

Figure 6. Ascending and Descending Cerebellofugal Axons in E16 Rat Embryo

(A)–(C) are from the same preparation, in which DiI was implanted into the right CP. In (A), rostrally growing axons grow beyond the isthmus (dotted line) and reach the midbrain. (B) shows a higher magnification of (A). Delayed descending axons can be seen growing along the same longitudinal path as ascending axons (arrow). (C) shows a higher magnification of the turning point in (B). Bifurcating axons extending both rostrally and caudally can be seen (arrows). It seems unlikely that these are two axons coursing together closely, since bifurcating axons were frequently observed, often in areas of sparse axon labeling. Early descending axons are out of focus in these photographs. Rostral is up; dashed line indicates the ventral midline. Bars, 200 μm (A), 100 μm (B), 50 μm (C).

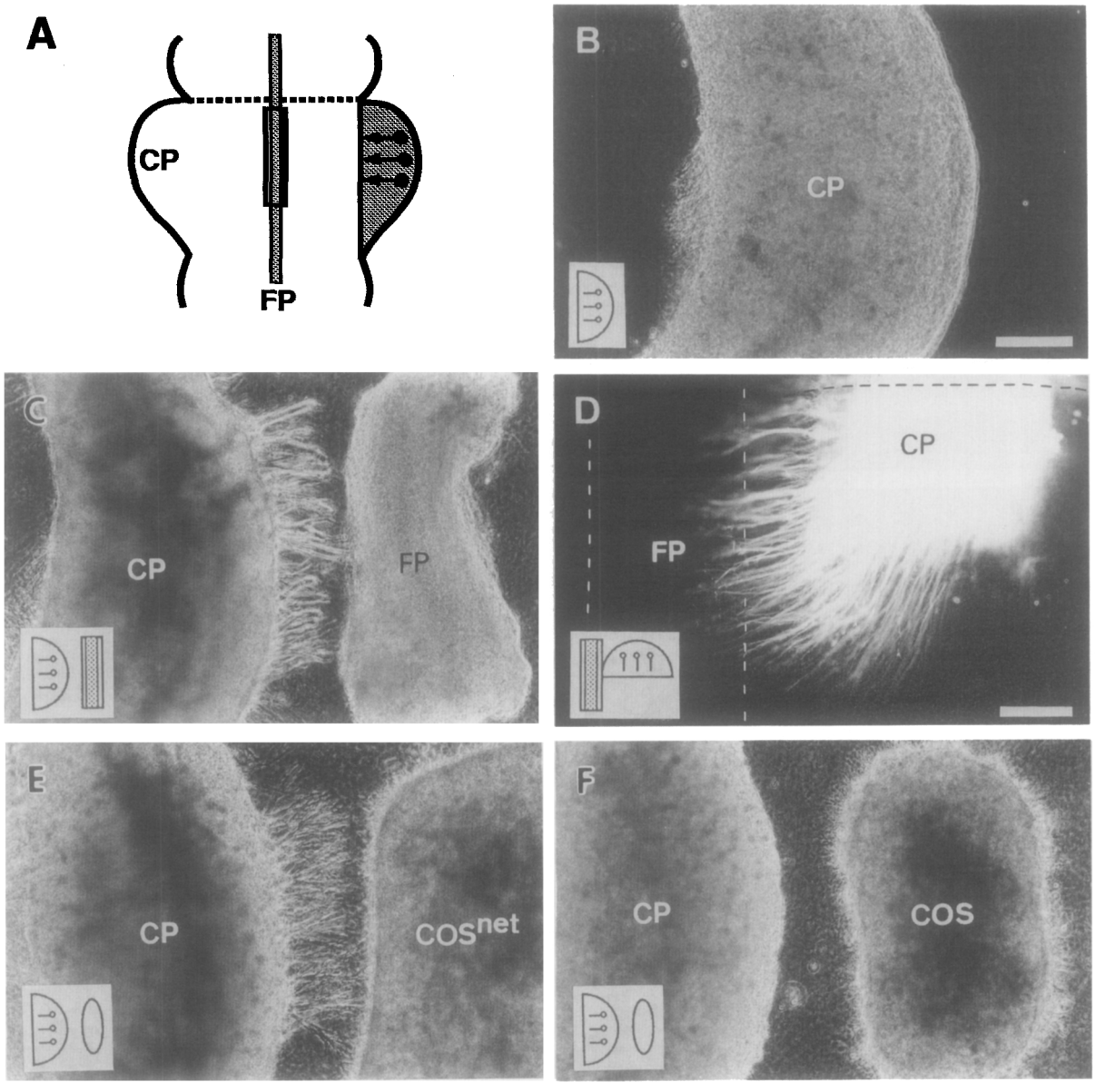

Figure 7. Directed Growth of CP Axons toward FP Explants and Aggregated COS Cells Secreting Netrin-1 in Collagen Gel Culture

(A) Diagram showing a flat whole-mount preparation of embryonic rat brain and the areas used for explant coculture experiments (rectangle at center and shaded area to the right) described below. Arrows show axon orientation in the CP.

(B) CP explant cultured alone. Note that no axons emanate from the lateral surface of the CP. Lateral is to the right.

(C) Coculture of a CP explant with a FP explant from the rostral hindbrain. Lateral surface of the CP explant was faced with the FP. Profuse neurites can be seen growing from the CP toward the FP explant.

(D) Fluorescent micrograph of DiI-labeled CP axons cocultured in close contact with a FP explant. Note that in the CP DiI-labeled axons deviate from in situ axonal orientation (see Figures 2B and 2D) and reorient toward the FP explant. White dashed line indicates edges of FP tissue. Black dashed line denotes the lateral surface of the CP. DiI crystals were inserted into the lateral margin of the CP.

(E) Coculture of a CP explant with an aggregate of transfected COS cells expressing netrin-1. The lateral surface of the CP explant was faced with the aggregate of COS cells. Neurite outgrowth is elicited from a CP explant toward an aggregate of netrin-expressing COS cells.

(F) Coculture with an aggregate of control nontransfected COS cells. No such outgrowth is observed from a CP explant.

Outgrowth was scored as positive if any axon bundles extended from the lateral surface of the CP explant toward the COS cell aggregate. (B, C, E, and F) Phase-contrast photomicrographs. Insets at lower left diagram arrangement and orientation of explants. COS^{net}, aggregated COS cells expressing netrin-1; COS, aggregate of nontransfected COS cells. Bars, 200 μm (B, C, E, and F), 150 μm (D).

scending collaterals in adult rat (Bentivoglio and Kuypers, 1982) and cat (McCrea et al., 1978; Tolbert et al., 1978), the late growing component that bifurcated after midline decussation may correspond to this subset of axons. On the other hand, earlier growing descending axons may presumably correspond to inhibitory nucleo-olivary projections (Fredette and Mugnaini, 1991), which are distinct from the above-mentioned excitatory projections.

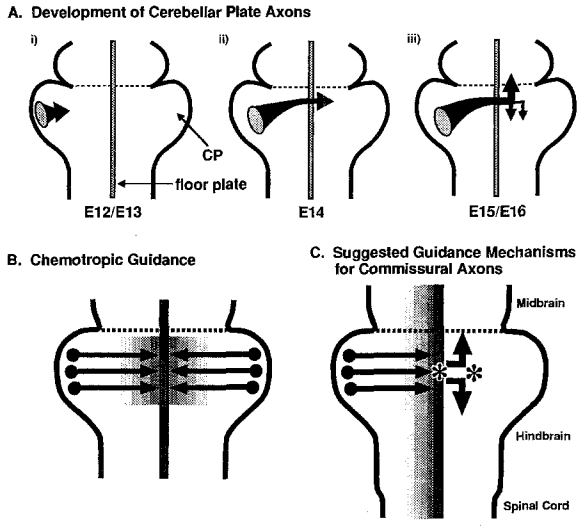

Figure 8. Diagrams Summarizing the Present Findings

(A) Developmental sequence of cerebellofugal axons. Cerebellofugal axons begin to grow toward the ventral midline in rostral hindbrain at E12/E13 (i), cross the ventral midline FP at around E14 (ii), and then make a right-angled turn to elongate along longitudinal paths at a distance from the FP at E15/E16 (iii).

(B) Suggested guidance mechanism of CP axons toward the ventral midline. Cerebellofugal axons may be guided by diffusible chemoattractant(s) that may be secreted from the FP. Netrin-1 may be responsible for this guidance.

(C) Suggested guidance mechanisms for ventrally decussating commissural axons in the brain and spinal cord. Locations of suggested guidance cues for circumferential and longitudinal growth of commissural axons are indicated by asterisks. Black arrows represent cerebellofugal axons.

Moreover, the present results are consistent with the previous finding that axons from deep nuclear neurons arrive at the midbrain at E16 (Cholley et al., 1989).

Comparison of Labeled Axonal Trajectories to Those in Adult

The DiI-labeled CP axons in flat whole-mount preparations grew straight toward the midline after leaving the CP. This means that CP axons follow a circumferential course during initial development in the rat embryo. In contrast, cerebellofugal axons in the adult rat initially course rostrally and then turn medially toward the midline. This apparent difference in axonal trajectories may be explained by the fact that, by the time of observation, the anteroposterior axis of the embryonic brain had been extensively undulated owing to the formation of the mesencephalic, pontine, and cervical flexures, thereby obscuring the circumferential axis. This axis nevertheless became recognizable as the embryonic brain was unfolded and flattened. Longitudinal morphogenic movements that lead to the rostral displacement of ventral aspects of the neural tube and caudal displacement of dorsal aspects (Hallonet et al., 1990), together with the disproportional development of the brain that occurs following the period of the present investigation, may also contribute to the bending of projection trajectories during development. We suspect that in adult rat the rostral orientation of initial axonal trajectory and subsequent turning toward the midline are partially the result of disproportional development of the brain that follows the establishment of the projections. Axonal projection patterns in adult that appear superficially quite distinct may arise from similar embryonic trajectories.

Growth of CP Axons toward the FP

Growth Patterns In Vivo

This study has revealed that the pattern of CP axon development is similar to that of commissural axons in the spinal cord in the following respects: initial circumferential course, midline decussation via the FP, straight path toward and within the FP, right-angled turn after midline decussation followed by longitudinal growth, and complex growth cone morphology around both edges of the FP (cf. Bovolenta and Dodd, 1990; Kuwada et al., 1990; Yaginuma et al., 1991).

Oriented Growth In Vitro

The finding that neurites from dorsal spinal cord explant grow toward the FP in collagen gel matrix led to the suggestion that commissural axons are guided to the ventral midline by a FP-derived chemoattractant (Tessier-Lavigne et al., 1988; Placzek et al., 1990a, 1990b). In the present coculture experiments, neurites from CP explants similarly grew toward FP explants in collagen gel matrix (Figure 7). Moreover, the axons from both the dorsal spinal cord (Placzek et al., 1990a, 1990b) and the CP showed reoriented growth toward FP explants placed in close apposition. It is therefore likely that axons of deep cerebellar nuclear neurons are guided to the ventral midline by a chemoattractant presumably released from FP cells, in the same way as are commissural axons in the spinal cord (Tessier-Lavigne et al., 1988; Placzek et al., 1990a, 1990b). In terms of the chemoattractant, netrin-1 may be a strong candidate, since recombinant netrin-1 expressed by COS cells that were shown to secrete diffusible forms of netrin-1 proteins (Kennedy et al., 1994) elicited neurite outgrowth from CP explants (Figures 7E and 7F) at a distance, in the same manner as FP explants.

Thus, cerebellofugal axons appear to be strikingly similar to spinal cord commissural axons with regard to both developmental features and in vitro responsiveness to the FP.

Growth beyond the FP

Having arrived at the FP, axons then require the action of a different mechanism to leave it. Growth cone encounters with the FP might cause changes in the expression of cell surface molecules, leading to alteration in growth cone responsiveness to environmental cues. In fact, it has been shown that spinal cord commissural axons express the TAG-1 molecule until they reach the FP, but thereafter express L1 (Dodd et al., 1988). Another intriguing possibility is that there may be an interaction at the midline between axons growing from both sides of the cerebellum that permits them to grow beyond it. In support of this notion is the observation that ablation of a commissural neuron on one side resulted in the failure of a contralateral counterpart to cross the midline in grasshopper embryos (Myers and Bastiani, 1993). We are currently examining this possibility by unilateral removal of the CP. Preliminary

results seem to indicate that CP axons can progress beyond the midline in the absence of their contralateral counterparts (Shirasaki et al., 1994).

Right-Angled Turn of Axons after Crossing the Midline

Commissural axons of the rat spinal cord turn rostrally, maintaining close contact with the longitudinal face of the FP and growing along the boundary between the FP and the contralateral epithelium (Bovolenta and Dodd, 1990). Based on these observations, the FP has been suggested to play an additional role in reorienting commissural axons and causing them to grow along a longitudinal plane, probably because the FP is a more adhesive substrate than adjacent regions of the cord (Bovolenta and Dodd, 1990; Chuang and Lagenaur, 1990; Hunter et al., 1992; Klar et al., 1992). CP axons showed behavior strikingly similar to that of spinal commissural axons, with some exceptions: though the majority of cerebellar axons turned rostrally, a subset turned caudally instead; neither descending nor ascending axons appeared to contact the longitudinal face of the FP, but rather grew along two different longitudinal paths at a distance from the FP; at the location of rostral turning, CP axons occasionally gave rise to bifurcations, a phenomenon not reported in rat spinal cord. These observations suggest the possibility that somewhat different mechanisms are responsible for CP axon and spinal cord commissural axon reorientation after midline decussation.

The mechanism by which CP axons alter growth direction remains unclear. The right-angled turn of CP axons cannot be explained solely by the action of chemoattractive (Lumsden and Davies, 1986; Tessier-Lavigne et al., 1988; Heffner et al., 1990; Placzek et al., 1990a, 1990b; Sato et al., 1994) or chemorepulsive (Fitzgerald et al., 1993; Pini, 1993; Colamarino and Tessier-Lavigne, 1994, Soc. Neurosci., abstract; Tamada et al., 1994) factors, because such diffusible substances should result in the gradual turning of CP axons.

The finding that the growth cones of ascending axons show a complex morphology (Figure 5E) near the region of turning implies that this is a decision point for ascending axons (for review, see Mason and Godement, 1992). On the other hand, this hypothetical decision point was ignored by early descending axons, which instead turned more laterally, extending processes both caudally and laterally before advancing caudally (Figure 5D). This suggests that, at the turning points, the growth cones of descending axons respond to distinct environmental cues.

The nature of presumptive environmental cues responsible for turning remains unknown, but recent studies suggest that growing axons recognize and follow specific scaffolds of high affinity preexisting tracts or neuroepithelial cells (Bastiani et al., 1984; Raper et al., 1984; Kuwada, 1986; Yaginuma et al., 1990; Chitnis et al., 1992). This might be the case for CP axons, since there appear to be preexisting tracts running along the longitudinal axis that correspond to CP axon tracts (Shirasaki et al., unpublished data).

Common Axonal Guidance Mechanisms for Commissural Neurons in the Brain and the Spinal Cord

The present results suggest that the FP plays a crucial role in the formation of commissural projections in the brain. Consistent with this view is the finding that the FP extends from the spinal cord through to the caudal diencephalon in vertebrates (Kingsbury, 1930; Puelles et al., 1987). Moreover, the FP generally appears to have chemotropic activity that is effective on ventrally decussating commissural axons in different regions along the anteroposterior axis of the CNS, because the FP in both the midbrain and hindbrain as well as that in the spinal cord can induce extensive axon outgrowth from dorsal spinal cord tissue in vitro (Placzek et al., 1990b), and because our preliminary coculture experiments using collagen gels indicate that CP axons are attracted not only by FP explants from the rostral hindbrain but also by those from caudal hindbrain, midbrain, and spinal cord (Tamada et al., 1993). In support of this view, the ventral axonal pathway is disrupted throughout the CNS in a FP-deficient zebrafish mutant (Hatta, 1992). The experiment with netrin-1 provides further evidence for the role of FP-derived chemoattraction in the guidance of commissural axons in the brain. Netrin-1 is a recently identified, diffusible molecule that may be responsible for the guidance of spinal cord commissural axons (Serafini et al., 1994; Kennedy et al., 1994). This molecule, when expressed in transfected COS cells, not only promotes but also redirects growth of spinal commissural axons in vitro (Kennedy et al., 1994). In the present study, we found that netrin-1, when similarly expressed, caused extensive growth of CP axons toward the COS cell aggregate (Figures 7E and 7F), indicating that netrin-1 plays a role in the guidance of CP axons toward the ventral midline. In support of the above view, *netrin-1* RNA is expressed in the FP at all axial levels of the neural tube up to the level of the caudal diencephalon (Kennedy et al., 1994), suggesting the possibility that this molecule acts on a variety of ventrally decussating axons at different axial levels of the neural tube.

In conclusion, while the appearance of commissural projections in the adult varies in different regions of the vertebrate CNS, it is likely that many of the ventrally decussating commissural projections are established following common axonal guidance mechanisms (Figure 8C).

Experimental Procedures

Flat Whole-Mount Embryonic Rat Brain Preparation

E12–E16 rat embryos were used (n = 120). Timed pregnant Wistar rats were obtained from Charles River Japan, Inc., where females that had been caged with males in the evening were checked for sperm positivity (vaginal plug detection) the next morning. This day was termed E0.

Pregnant rats were deeply anesthetized by intraperitoneal injection of sodium pentobarbital (50 mg/kg). The abdomen was opened and the uterus exposed. Embryos were then removed, and the midbrain and hindbrain were dissected out. After the midbrain and hindbrain were cut along the dorsal midline, meninges were removed, and the remainder of the brain was opened and flat whole-mounted with the

ventricular side down (Figure 1). All dissection procedures were performed in cold Hanks' solution. The brain was then fixed by immersion in 4% paraformaldehyde in 0.12 M phosphate buffer (pH 7.4) and stored at 4°C for several days.

Following delivery of embryos, adult rats were euthanized by an overdose of sodium pentobarbital.

DiI Labeling

The fluorescent lipophilic dye DiI (Molecular Probes, cat. no. D-282) was employed for tract tracing (Godement et al., 1987; Honig and Hume, 1989). Small DiI crystals were placed using an acute tungsten needle into E12–E16 rat CP (n = 95), which corresponds to the rhombic lip located at the lateral (the term "lateral" is used in terms of the opened brain preparation and would thus be retermed "dorsal" when referring to normal preparations) margin of the alar plate of the rostral hindbrain, and is surrounded rostrocaudally by the midbrain–hindbrain boundary and the pontine flexure. For retrograde labeling of cerebellar deep nuclear neurons, DiI crystals were implanted into the ventral midline in the rostral hindbrain of E14–E16 rat embryos (n = 20). After implantation of DiI, the whole-mount preparations were rinsed to remove unattached DiI particles and again placed in 4% paraformaldehyde. They were stored in the dark at room temperature for one-half to 7 days, depending on the required DiI diffusion distance. Thereafter, they were mounted on glass slides with a small volume of the fixative and observed with an epifluorescent microscope through a set of rhodamine filters (Olympus BH2-DMG). The labeled axons were photographed on Fuji Neopan 400 Presto film.

Explant Culture Preparations

E13–E14 rat embryos were dissected in DME/F12 medium (Sigma; cat. no D-8900) with additional (3.85 mg/ml) glucose. CP and FP explants were removed from longitudinally opened brain using acute tungsten needles. The FP was recognized as a thin, longitudinally running stripe. FP at the same rostrocaudal level as the CP was dissected out, since cerebellofugal axons grow through this region. After trimming, the explants were embedded together (separation < 500 μm) in collagen gel matrix, which may establish a gradient of diffusible substances (Ebendal and Jacobson, 1977; Lumsden and Davies, 1983, 1986). Collagen was obtained from rat tail tendon (Ebendal and Jacobson, 1977). CP explants were cocultured at 37°C in 5% CO_2 for 24–30 hr with FP explants or for 30–35 hr with COS cell aggregates. The culture medium was DME/F12 (Sigma) supplemented with 3.85 mg/ml glucose, 10 μg/ml streptomycin, 100 μg/ml transferrin, 5 μg/ml insulin, 5.29 ng/ml sodium selenite, 16.4 μg/ml putrescine dihydrochloride, 6.29 ng/ml progesterone, 7.40 ng/ml hydrocortisone, and 10% fetal bovine serum. Following fixation, explant cultures were observed and photographed in phase contrast.

For anterograde labeling of CP axons, DiI crystals were inserted into the lateral margin of CP explants after coculture with FP explants for 40–48 hr. After DiI diffusion, the explants were processed as described above (see DiI labeling).

Expression of Netrin-1 in COS Cells

Transfection of pGNET1^{myc} (*netrin-1* cDNA; gift from Marc Tessier-Lavigne) into COS7 cells was performed using LipofectAMINE (GIBCO BRL) as directed. Aggregates of transfected COS cells were prepared as previously described (Kennedy et al., 1994), except that hanging drop cultures for COS cell aggregation were incubated for 24–30 hr; aggregates of COS cells were harvested into warm DME/F12 medium containing 10% heat-inactivated fetal bovine serum.

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