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Floor Plate Chemoattracts Crossed Axons and Chemorepels Uncrossed Axons in the Vertebrate Brain

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Summary

In the bilaterally symmetrical vertebrate CNS, all developing axons must choose between remaining on the same side of the midline or growing across it. The mechanism underlying this axonal pathfinding is, however, poorly understood. Here we demonstrate that the ventral midline floor plate (FP) chemorepels two types of ipsilaterally projecting axons, one from the alar plate and another from the basal plate in the mesencephalon. We further demonstrate that the FP chemoattracts contralaterally projecting myelencephalic as well as metencephalic axons. The FP at all axial levels displayed both chemoattractive and chemorepellent activities, suggesting that FP chemoattraction and chemorepulsion may be at work throughout the neuraxis. Chemotropic guidance by the FP may therefore play a key role in the establishment of neuronal projection laterality.

Introduction

A fundamental characteristic of the vertebrate CNS is bilateral symmetry along the midline. This symmetrical structure divides neuronal projections into two types, uncrossed projections to ipsilateral targets and crossed projections to contralateral targets. Why some axons cross the midline while others do not is a key issue in the patterning of axonal projections. Over the past decade, the mechanism involved in the formation of crossed projections has been studied extensively in spinal cord commissural projections. Spinal commissural axons, which derive from the dorsal part of the neural tube (alar plate [AP]), initially project ventrally along the circumferential axis and then cross the ventral midline (Holley and Silver, 1987; Bovolenta and Dodd, 1990; Kuwada et al., 1990; Yaginuma et al., 1991). Accumulating evidence indicates that these axons are guided by a diffusible molecule released from the floor plate (FP), a structure situated along the ventral midline of the neural tube. When cocultured with FP explants in collagen gels, which establish a gradient of diffusible substances (Ebendal and Jacobson, 1977; Lumsden and Davies, 1983, 1986), spinal commissural axons show reoriented growth toward FP explants (Tessier-Lavigne et al., 1988; Placzek et al., 1990a, 1990b). The chemoattractive activity of the FP was recently shown to be mimicked by netrin-1, a molecule that appears to be released from the FP (Kennedy et al., 1994; Serafini et al., 1994). Consistent with these in vitro findings are the in vivo observations that spinal commissural axons in chick embryo grow toward ectopically transplanted FP (Placzek et al., 1990b; Yaginuma and Oppenheim, 1991) and that these axons show abnormal trajectories in the FP-deficient zebrafish mutant *cyclops* (Bernhardt et al., 1992a, 1992b), the FP-depleted zebrafish (Bernhardt et al., 1992a), and the mouse mutant *Danforth's short tail* (Bovolenta and Dodd, 1991).

In the *cyclops* mutant, midline-decussating axons also exhibit anomalous trajectories in the brain (Hatta, 1992). Taking into account the fact that the FP extends continuously up to the level of the caudal diencephalon (Kingsbury, 1930; Puelles et al., 1987) and that the brain contains a variety of crossed axons coursing through the FP (e.g., Bourrat and Sotelo, 1988, 1990; Cholley et al., 1989; Wassef et al., 1992; Kandler and Friauf, 1993; Shirasaki et al., 1995 [this issue of *Neuron*]), it would be tempting to assume that the FP in the brain provides a chemoattractive guidance cue for crossed axons, just as spinal FP does for spinal commissural axons. In favor of this view, cerebellofugal axons in early development have recently been shown to exhibit trajectories strikingly similar to those of spinal commissural axons (Shirasaki et al., 1995). In vitro, these axons were shown to be chemoattracted by FP explants from the metencephalon, where the axons were to have crossed the midline (Shirasaki et al., 1995), suggesting that chemoattraction by metencephalic FP plays a role in the guidance of cerebellofugal axons. There are a variety of crossed axons throughout the brain, however, and it remains to be clarified whether these axons are also chemoattracted by the FP at corresponding axial levels. Moreover, if this is the case, the question that naturally arises is whether or not these crossed axons share common chemoattractive guidance mechanisms.

On the other hand, normally uncrossed axons in the spinal cord (Bernhardt et al., 1992a) and the hindbrain (Hatta, 1992) often cross the ventral midline in the *cyclops* mutant. Although these studies suggest that the FP is also involved in the guidance of uncrossed axons, the nature of the FP cue for uncrossed axons remains to be elucidated. An intriguing possibility is that the FP releases a diffusible chemorepellent(s) that prevents axons from crossing the midline. This idea emerged from the discovery of diffusible inhibitory or repellent activities in other systems (Fitzgerald et al., 1993; Pini, 1993). Considering that the FP chemoattracts crossed axons (Tessier-Lavigne et al., 1988; Placzek et al., 1990a, 1990b; Shirasaki et al., 1995), the possibility is likely that the same structure exerts the opposite activity on an opposite axon type, uncrossed axons.

In the present study, we first tested to determine whether or not the FP chemorepels uncrossed axons. We then

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examined the role of FP chemoattraction in the guidance of crossed axons in the brain. Finally, we examined whether the FP at all axial levels possesses chemoattractive and chemorepellent activities. Our results demonstrate that the FP repels and reorients, from a distance in collagen gel, uncrossed AP and basal plate (BP) axons in the mesencephalon. With regard to chemoattraction in the brain, we found that myelencephalic and metencephalic AP axons projecting contralaterally in vivo were attracted by the FP. Such chemoattractive and chemorepellent FP activities occurred at all anteroposterior levels of the neuraxis, suggesting that common chemotropic axonal guidance mechanisms operate throughout different levels of the neuraxis. We propose that FP chemoattraction and chemorepulsion may play a role in determining the direction of axonal growth along the circumferential axis, thereby establishing neuronal projection laterality in the CNS.

Results

Mesencephalic Dorsal-Most AP Axons Project Ipsilaterally without Crossing the Ventral Midline

Investigating the possibility that the formation of uncrossed projections is related to FP chemorepulsion requires the examination of a region containing neurons projecting exclusively ipsilaterally. Implantation of a lipophilic tracer, 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (DiI), into various loci of the brain revealed that axons from the dorsal-most AP in the mesencephalon (DAPms) satisfy this requirement (Figure 1). We implanted DiI into the DAP in the mesencephalon and the caudal diencephalon of a whole-mounted E15 rat brain (Figure 1A) that was flattened after cutting along both the dorsal and ventral midlines (Figures 1B–1D). Dorsal to the implantation site, cells were retrogradely labeled near the dorsal midline, ipsilateral to the implantation (Figures 1B and 1C). In the caudal diencephalon, labeled axons crossed the dorsal midline through the presumptive posterior commissure (Figure 1B). Most of the anterogradely labeled axons initially projected straight toward the ventral midline FP along the circumferential axis, but then gradually turned caudally at the ventral portion of the AP and eventually coursed toward the ipsilateral metencephalon along the longitudinal axis (Figures 1B and 1D). A small axonal population crossed the FP at the level of the caudal diencephalon (Figure 1D). DiI implantation into the mesencephalic ventral AP (VAPms) labeled FP-crossing axons, which presumably correspond to crossed tectofugal axons (data not shown). These results indicate that the DAPms region is composed mainly of ipsilaterally projecting cells. In E13 rat, axons were found to initiate ventral growth from the DAPms (data not shown). We therefore used DAPms from E12–E13 rat embryos in the following in vitro assay.

Mesencephalic Dorsal-Most AP Axons Turn Away from FP Explants In Vitro

DAPms axons failed to cross the midline in spite of their initial ventral growth, turning gradually at a distance from the FP as shown schematically in Figure 1A and Figure 2A. This raises the possibility that the FP possesses chemorepellent activity effective against DAPms axons. To examine this possibility, DAPms explants were cocultured in collagen gel with FP explants from the mesencephalon (FPms; Figure 2). Longitudinal DAPms explants containing the roof plate (RP) were taken from the dorsal-most AP region (Figures 2A and 2B). When DAPms explants

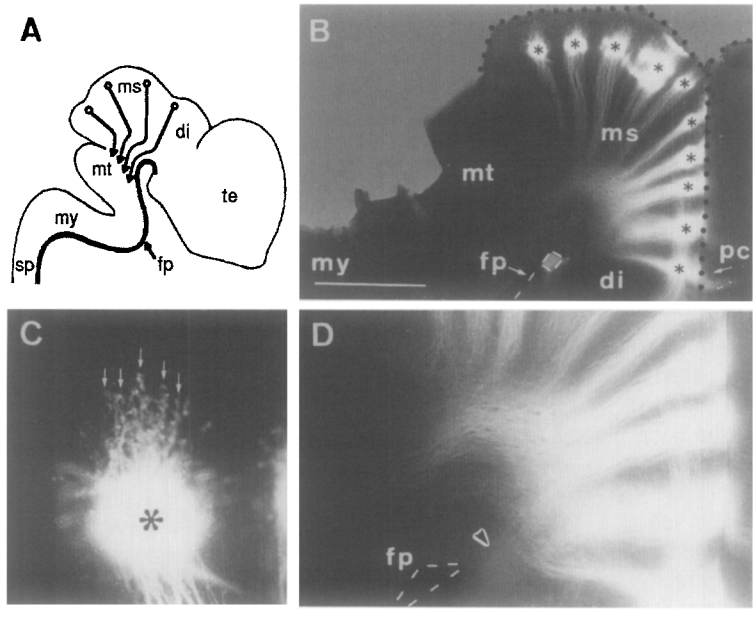

Figure 1. Growth of DAPms Axons In Vivo

(A) Schematic lateral view of rat embryonic CNS, including trajectory of DAPms axons. Open circles, DAPms cells; curved arrows, DAPms axons.

(B–D) Fluorescence photomicrographs of flat-mounted E15 rat brain cut along the dorsal (black dotted line) and ventral midlines and implanted with DiI into the DAP of the mesencephalon and caudal diencephalon. In this preparation, the two hemispheres are partially connected by the dorsal midline at the rostral mesencephalon and caudal diencephalon. Note that the cephalic flexure at the bottom of the mesencephalon makes the two orthogonal axes of the neural tube superficially complex at this stage: the circumferential axis converges toward the concave ventral midline, while the longitudinal axis curves along it. DiI injections (asterisks) retrogradely labeled cells (shown at high magnification and indicated by arrows in [C]) near the dorsal midline and anterogradely labeled axons that initially run ventrally along the circumferential axis toward the invaginated ventral midline FP (white dashed line) and then gradually turn caudally, constantly maintaining a distance from the FP (B). The axons turned at varying distances from the FP. The labeled axons are shown at high magnification in (D). The arrowhead in (D) indicates FP-crossing axons. di, diencephalon; ms, mesencephalon; mt, metencephalon; my, myelencephalon; pc, posterior commissure; sp, spinal cord; te, telencephalon. Bar, 1 mm (B), 200 μm (C), 500 μm (D).

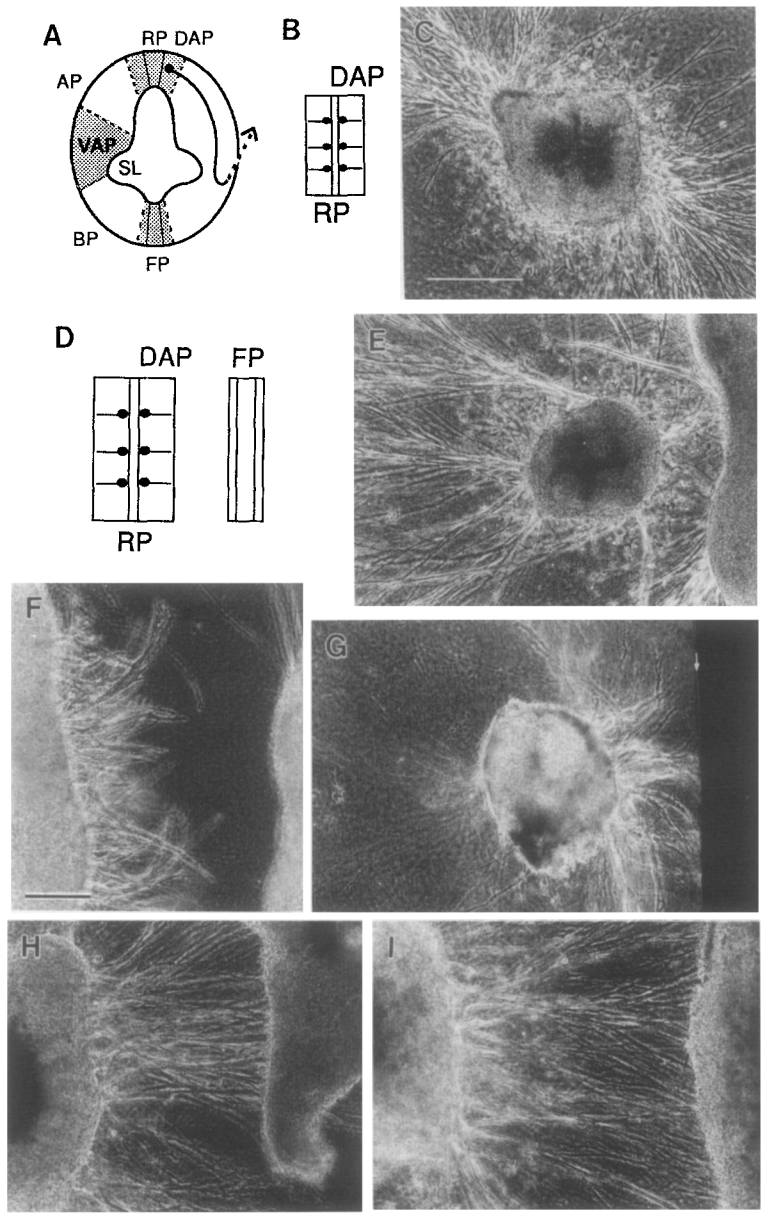

Figure 2. Turning of DAPms Axons Away from FPms Explants in Collagen Gel Culture

The diagram representing a coronal view of the mesencephalon (A) shows explant origins (shaded areas). (B) and (D) contain diagrams showing orientation and arrangement of explants ([B] corresponds to [C]; [D] to [E]–[H]). DAPms neurons and the direction of axonal growth in vivo are shown by closed circles with short bars (B and D). Phase-contrast photomicrographs show DAPms explants cultured alone (C) or cocultured with live FPms (E–G), heat-treated FPms (H), or VAPms (I) explants. In (G), DAPms and FPms explants were separated by a permeable membrane filter (arrow shows the edge of the filter). Filters alone did not affect the growth of the axons (n = 3). Note that DAPms axons turn away from FPms explants (E–G) without contacting them or their processes (F and G). AP, alar plate; BP, basal plate; RP, roof plate; SL, sulcus limitans, the AP–BP boundary; VAP, ventral alar plate. Bars, 500 μm (C and E), 200 μm (F–I).

were cultured alone for 2–4 days (n = 9; Figure 2B), they emitted axons straight from the edges of the explants in a radial pattern (Figure 2C). Most of the elongating axons grew straight and remained on the same focal plane as the explants in three-dimensional gel matrix (Figure 2C).

When cocultured with FPms explants (n = 18; Figure 2D), however, DAPms explants extended axons that gradually turned, in gel, away from FPms explants (Figures 2E–2G). These axons appeared to be more extensively fasciculated than those from DAPms explants cultured alone (compare Figures 2E–2G with Figure 2C). Many axons were out of focus because they not only turned on the horizontal plane but also deflected upwards in three-dimensional gel (Figures 2E–2G). The DAPms axons turned even in cases when explants were fixed after a short period of time (before contacting FPms explants or their processes; Figure 2F), and when permeable membrane filters were placed between the two explants (n = 5; Figure 2G). Thus, the turning of DAPms axons can be ascribed to a diffusible substance. FPms explants retained their turning activity even when adjacent BP tissue was completely removed (n = 3; data not shown). This suggests that the FPms is capable of inducing turning all on its own. In control cocultures of DAPms explants with heat-treated FPms (n = 10; Figure 2H) or ventral AP explants (VAPms; n = 11, Figure 2I) taken from the area of the DAPms axon pathway (Figure 2A), DAPms axons grew straight in a radial pattern on the same focal plane.

Quantitatively, the angle of axon fascicle turning in cocultures with live FPms (39° ± 27°, mean ± SD; n = 297; Figure 3A) was significantly greater ($p < .0001$, Mann-Whitney test) than that with heat-treated FPms (17° ±

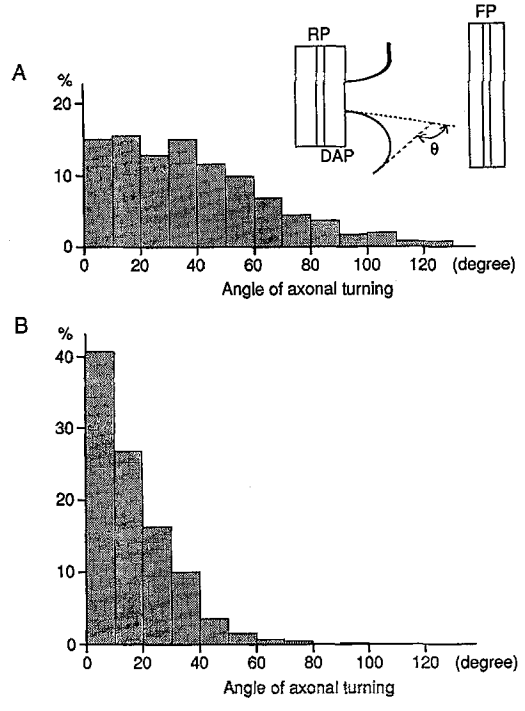

Figure 3. Histograms Showing Turning Angle of DAPms Axons Cocultured with FPms Explants

Turning angles of axon bundles in the gels were measured from the DAPms explant surface facing FPms explants. The difference between the direction of an axon at its point of emergence and that at its termination (schematically shown in the inset of [A]) was plotted on a histogram. DAPms explants were cocultured with live (A) or heat-treated (B) FPms explants. The angle in cocultures with live FPms (39° ± 27°, mean ± SD; n = 297) was significantly greater ($p < .0001$, Mann-Whitney test) than that with heat-treated FPms (17° ± 14°; n = 460).

14°; n = 460; Figure 3B). These results suggest that the FPms chemorepels DAPms axons.

Laterally Growing Mesencephalic BP Axons Also Turn Away from FP Explants

We next examined whether FP chemorepulsion is involved in the guidance of axons other than those from the DAPms. When E12–E13 mesencephalic BP (BPms) explants, taken from the region rostral to the mesencephalic–metencephalic boundary (isthmus) and caudal to the level of the posterior commissure (Figure 4A), were cultured alone (n = 12), axons grew straight from the lateral edge, with fewer axons emerging from the medial aspect (Figure 4B). In contrast, when FPms explants were apposed to the lateral edge of the BPms explants (n = 24), most of the axons, while emerging from the lateral edge of the BPms, turned away from the FPms explants (Figures 4C and 4D). As observed with DAPms axons, BPms axons in coculture appeared to be more extensively fasciculated than those from BPms explants cultured alone. Turning of BPms axons was not observed in cocultures with APms explants (n = 14) or heat-treated FPms (n = 4; data not shown). As was the case in DAPms–FPms cocultures, BPms axons turned even when no contact was made with FPms explants or their processes. These results suggest that another type of axon, the BP axon in the mesencephalon, is chemorepelled by the FPms.

Immunostaining of cocultured BPms explants using anti-tyrosine hydroxylase (TH) antibodies revealed that a major population of the turning axons expressed TH (data not shown), suggesting that BPms axons repelled by the FPms include axons of TH-positive mesencephalic dopaminergic neurons.

FP chemorepulsion of BP axons in explants caudal to

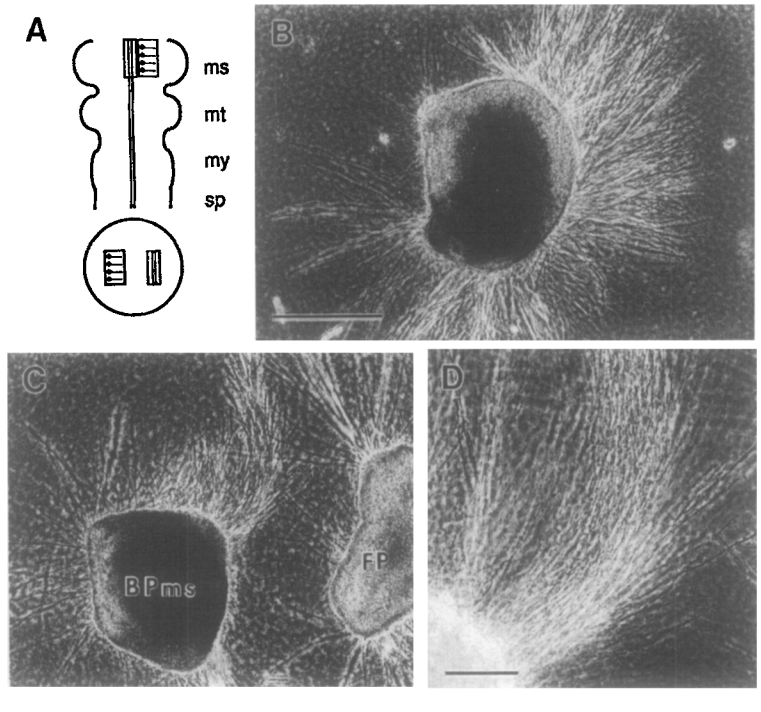

Figure 4. Turning of BPms Axons Away from FPms Explants

The diagram of a flat-mounted embryonic brain (A) shows the location of BPms and FPms explants taken for coculture (upper panel) and their arrangement in culture (lower panel; see Figure 1 for abbreviations). BPms explants were cultured alone (B) or with FPms explants (C) apposed to the lateral edge of BPms explants as diagrammed in (A). (D) shows a higher magnification of turning axon bundles shown in (C). Medial (ventral) is to the left in all photographs of BPms explants. Note that BPms axons emerging from the lateral edge turn away from FPms explants (C and D), while they grow radially when cultured alone (B). FPms explants not contaminated by adjacent BP tissue also induced turning (n = 5). BPms axons turned even when BPms and FPms explants were separated by a filter (n = 5), which alone had no effect on the axonal growth (n = 3). Bars, 500 μm (B and C), 100 μm (D).

the mesencephalon was also tested by coculturing E12–E13 metencephalic (n = 14), myelencephalic (n = 6), and spinal (n = 5) BP explants with FP explants from corresponding axial levels. Unlike BPms axons, axons emerging from the lateral aspect of the BP explants did not display evident turning (data not shown).

Rhombencephalic AP Axons Grow toward FP Explants

To test whether axons in the brain are chemoattracted by the FP, we cocultured AP explants from the rhombencephalon (metencephalon and myelencephalon) with FP explants from corresponding levels of the neuraxis (Figure 5). When metencephalic AP (APmt; n = 9; Figure 5A) and myelencephalic AP (APmy; n = 7; Figure 5D) were cultured alone, axons emanated from the cut ventral (medial) edge of the explants (data not shown) but not from the intact dorsal (lateral) surface (Figures 5B and 5E). In contrast, when metencephalic FP (FPmt; n = 14) and myelencephalic FP (FPmy; n = 7) explants were apposed to the dorsal surface of the APmt and APmy explants, respectively, each AP explant extended axons from the dorsal surface (Figures 5C and 5F) in the direction opposite to in vivo axon growth (Shirasaki et al., 1995). Similar axonal outgrowth occurred from the dorsal surface of the AP explants at all anteroposterior levels of the rhombencephalon (data not shown). The AP in the rhombencephalon would thus appear to contain axons that are chemoattracted by the FP at the corresponding axial level.

FP at All Axial Levels Attracts Metencephalic AP Axons but Repels Mesencephalic DAP and BP Axons

Finally, to test whether FP chemoattractive and chemorepellent activities occur at all anteroposterior levels of the neuraxis, AP or BP explants were cocultured with FP explants from noncorresponding anteroposterior levels (Figure 6). We found that DAPms axons turned away from FPmy (n = 9; Figure 6A) and FP from the spinal cord (FPsp; n = 7; Figure 6B); that BPms axons turned away from FPmy (n = 18; Figure 6C) and FPsp (n = 7; Figure 6D); and that APmt axons grew toward FPms (n = 7; Figure 6E) and FPsp (n = 7; Figure 6F). All turning and directed growth of axons occurred at a distance from FP explants.

To summarize, the FP at all anteroposterior levels along the neuraxis possesses both chemoattractive and chemorepellent activities. These results suggest that the reaction of an axon can be attributed to a difference in axon response rather than a difference in FP activity. These results further imply that FP chemoattraction and chemorepulsion may be effective on a variety of axons at all axial levels.

Discussion

Chemoattraction by the FP in the Brain

The present results suggest that the FP directs the growth of rhombencephalic AP axons toward itself. Moreover,

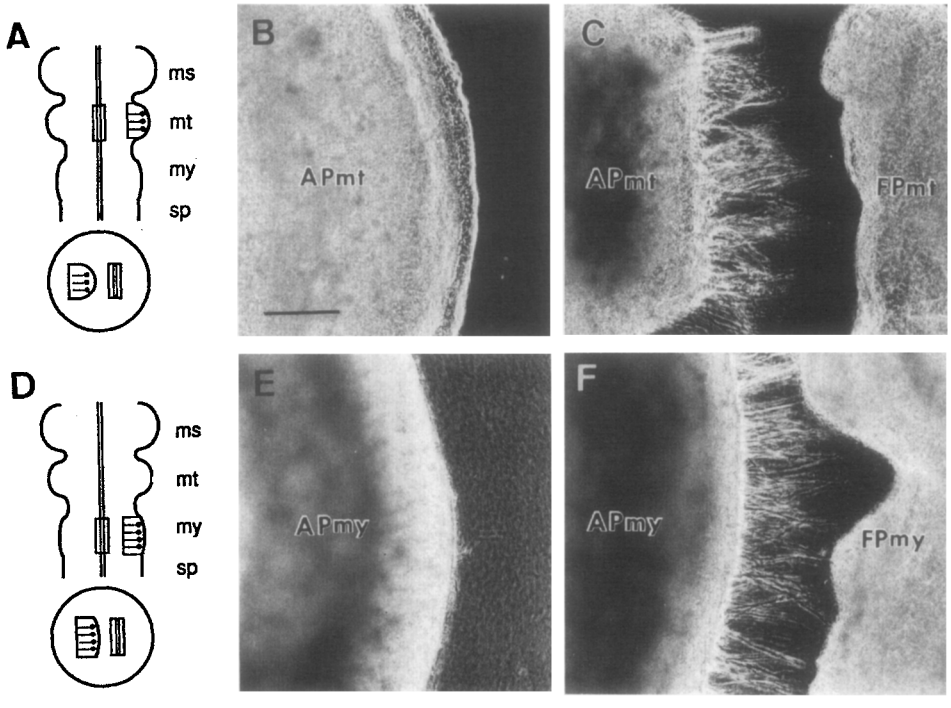

Figure 5. Growth of Rhombencephalic AP Axons toward FP Explants

As diagrammed in (A) and (D), AP explants from the metencephalon (APmt; B and C) and myelencephalon (APmy; E and F) were cultured alone (B and E) or with FP explants from the corresponding anteroposterior level (FPmt [C] and FPmy [F]) apposed to the dorsal (lateral) surface of AP explant. Dorsal (lateral) is to the right. Note that with both APmt and APmy explants, axons grow toward FP explants in coculture (C and F), whereas no axonal outgrowth occurs when the explants are cultured alone (B and E). Bar, 200 μm.

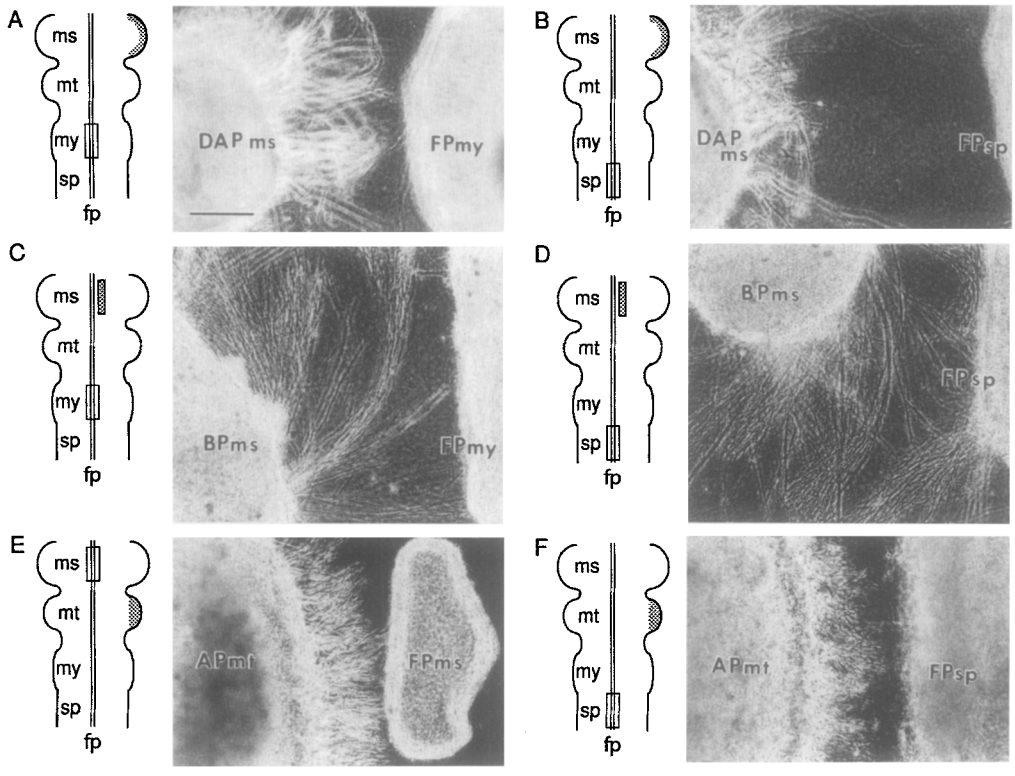

Figure 6. Attractive and Repellent Activity of the FP at Various Axial Levels

DAPms (A and B), BPms (C and D), and APmt (E and F) explants were cocultured with FP explants taken from noncorresponding anteroposterior levels. Photomicrographs show DAPms with FPmy (A) and spinal FP (FPsp; B); BPms with FPmy (C) and FPsp (D); and APmt with FPms (E) and FPsp (F). Locations of explants used in culture are schematically shown in the diagram at the left of each panel. Bar, 200 μm.

APmt axons, though failing to turn in collagen gel, turn within APmt explants toward FP explants (Shirasaki et al., 1995), suggesting that these axons are reoriented toward the FP. Together, these results indicate that the FP in the rhombencephalon may chemoattract rhombencephalic AP axons just as spinal cord FP does spinal commissural axons (Tessier-Lavigne et al., 1988; Placzek et al., 1990a, 1990b; Kennedy et al., 1994; Serafini et al., 1994) (Figure 7A).

The APmt used in the present study corresponds to the cerebellar primordium (cerebellar plate). Deep cerebellar nuclei neurons that are generated here at the stage of the present experiments (Altman and Bayer, 1978a, 1985a, 1985b) extend axons that grow ventrally straight toward the FP, then cross it to project contralaterally (Shirasaki et al., 1995). At around the same stage, a variety of neurons projecting through the FP are generated in the APmy; these include secondary sensory relay neurons (Kandler and Friauf, 1993) and neurons of the precerebellar nuclei (Altman and Bayer, 1978b, 1987; Bourrat and Sotelo, 1988, 1990; Wassef et al., 1992). In accordance with the literature, DiI injection into the APmy labeled axons that grow toward the ventral midline (Tamada et al., unpublished data). Thus, both APmt and APmy appear to contain neurons that project toward the FP. It is therefore likely that the FP chemoattracts various kinds of crossed axons in both the metencephalon and myelencephalon, probably contributing to the formation of crossed projections in these regions (Figure 7B).

Chemorepulsion by the FP

Mesencephalic AP Axons

The present in vitro finding that DAPms axons turn away from FPms explants without contacting them suggests that the FPms releases a diffusible repellent factor (chemorepellent) that reorients DAPms axons away from it. The present in vivo DiI labeling showed that a major population of DAPms neurons project ipsilaterally. The growth pattern of these axons suggests that these correspond to uncrossed tectobulbar axons (Kröger and Schwartz, 1990; Clarke and Lumsden, 1993; Shepherd and Taylor, 1995) or axons from the mesencephalic nucleus of the trigeminal nerve (Stainier and Gilbert, 1991; Easter et al., 1993; Chédotal et al., 1995). Together, the present findings suggest that FP chemorepulsion plays a role in in vivo axonal guidance; the FP may prevent these axons from crossing the ventral midline and reorient them to grow ipsilaterally along the longitudinal axis (Figure 7A). FP chemorepulsion may presumably contribute to the guidance of AP axons that initially grow ventrally along the circumferential axis but do not cross the ventral midline (Figure 7B). It is to be noted, however, that other cues are likely at work in initially directing ventral growth and in the subsequent de-

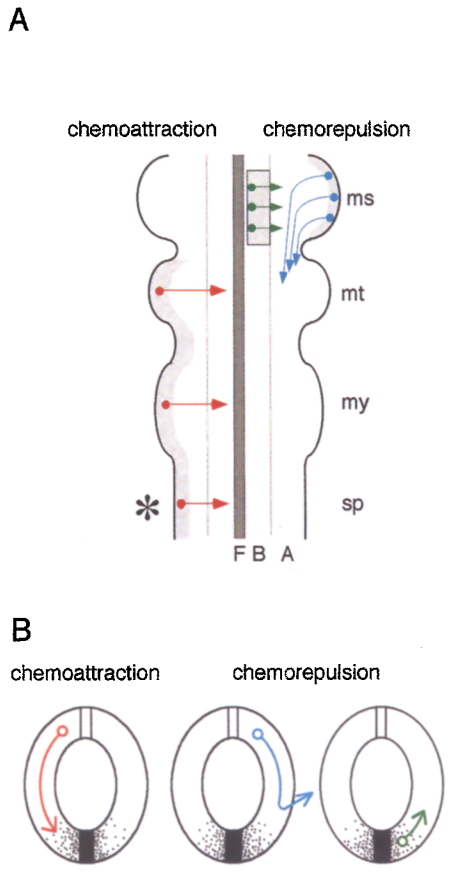

Figure 7. Summary Diagram and Proposed Mechanisms for Axonal Guidance by FP Chemoattraction and Chemorepulsion

(A) Diagram summarizing the present findings combined with the findings of previous reports on spinal commissural projections (asterisk; Tessier-Lavigne et al., 1988; Placzek et al., 1990a, 1990b) and on cerebellofugal projections (Shirasaki et al., 1995). The FP, shown at center, possesses both chemoattractive and chemorepellent activities along its entire length. Regions (stippled) containing chemoattracted axons (red), including that of the spinal cord AP, are shown on the left, while those containing chemorepelled axons (DAPms, blue; BPms, green) are shown on the right. Arrows show growth patterns of axons in these regions.

(B) Coronal view of the neural tube showing proposed axonal guidance of crossed AP axons by FP chemoattraction (left, red), and of uncrossed AP (center, blue) and BP (right, green) axons by chemorepulsion. Presumable release of diffusible factors from the FP is represented by dots. FP chemorepulsion may act in two ways: to prevent ventrally directed axons from crossing the ventral midline, allowing them to grow along the anteroposterior axis (center), and to direct BP axons away from the ventral midline (right).

cision regarding growth direction along the anteroposterior axis.

Mesencephalic BP Axons

Axonal outgrowth from the lateral edge of BPms explants cultured alone implies that these axons correspond to axons projecting laterally away from the ventral midline FP in vivo. It appears that BP axonal growth may have already been directed laterally at the time of dissection. Turning of laterally growing BPms axons away from FPms explants suggests that these axons are also chemorepelled by the FPms (Figure 7A). Possible candidates for the chemorepelled BPms axons are the mesencephalic dopaminergic neurons (e.g., Specht et al., 1981; Hynes et al., 1995) and the oculomotor neurons (Puelles and Privat, 1977; Puelles, 1978), as both of these neuronal types appear to be generated here at the stage of the present experiments (Altman and Bayer, 1981). Expression of TH in these axons in vitro suggests that a significant portion of the BPms axons chemorepelled by the FP originate from mesencephalic dopaminergic neurons. In vivo, TH-positive axons grow laterally away from the FP (Specht et al., 1981; Shirasaki et al., unpublished data). Together, the present findings suggest an additional role for FP chemorepulsion in the guidance of uncrossed axons in vivo; it may be that the FP determines the initial direction of BP axon outgrowth and then directs the axons away from the ventral midline (Figure 7B).

Turning of Axons in Collagen Gel

Inhibition of axonal growth from a distance was previously demonstrated in dorsal root ganglia cocultured with ventral spinal cord explants using a collagen gel culture assay (Fitzgerald et al., 1993). In this case, however, it is unclear whether the ventral spinal cord possesses chemorepellent activity, since the turning of axons away from the spinal cord was not demonstrated. Chemorepellent activity was clearly demonstrated in the olfactory system (Pini, 1993); olfactory bulb explants, when cultured with septal explants at a distance, extended axons in the direction opposite to the septal explants, with a lack of axonal outgrowth from their septal-facing aspect. These olfactory axons did not exhibit turning in collagen gel, though they turned within the olfactory explants. In contrast to the olfactory system study, the present study revealed that both DAPms and BPms axons emanate from explants and then gradually turn in gel away from FPms explants. Although the reason for the difference between the results of the present and previous studies remains unclear, the following explanations can be speculated: DAPms and BPms neurons may possess the capacity for straighter and more vigorous extension than olfactory neurons, and extensive fasciculation of DAPms and BPms axons compared with olfactory bulb axons may enable them to grow out of the explants even in the presence of a chemorepellent in the gel.

Relevance to Studies on Other Types of Axons

In zebrafish, axons of VeLD neurons in the spinal cord and subsets of reticulospinal neurons in the hindbrain, for example, grow from the BP toward the ventral midline, but do not cross it. These BP axons, however, abnormally cross the midline in the FP-deficient mutant *cyclops* (Bernhardt et al., 1992a; Hatta, 1992), suggesting the involvement of the FP in uncrossed axon guidance. It may not be possible to ascribe the midline decussation failure of these axons to diffusible FP cues, since they continue to grow medially until they approach the FP, whereabouts they abruptly change growth direction to course caudally. This contrasts with DAPms axon trajectories, which gradually change course as they approach the FP. It would therefore seem likely that these ventrally directed, uncrossed BP axons respond to FP cues other than diffusible chemorepellents. BP axons, such as the reticular axons, show growth patterns somewhat distinct from those of AP ax-

ons. For example, whereas crossed AP axons project straight toward the FP and cross it perpendicularly (Holley and Silver, 1987; Bovolenta and Dodd, 1990; Kuwada et al., 1990; Trevarrow et al., 1990; Yaginuma et al., 1991; Shirasaki et al., 1995), contralaterally projecting BP reticular axons tend to cross the FP obliquely (Metcalfe et al., 1986; Glover and Petursdottir, 1991; Chang and Raible, 1994). This contrast in growth pattern also suggests that AP axons and BP reticular axons may respond to distinct types of FP guidance cues.

Patterning of CNS Axonal Projections by the FP

The vertebrate CNS develops from the neural tube, which consists of common structures aligned along the circumferential axis: the RP, AP, BP, and FP (Kingsbury, 1930; Puelles et al., 1987). Although the neural tube differentiates along the anteroposterior axis via subdivision by segmentation (Puelles et al., 1987; Hanneman et al., 1988; Lumsden and Keynes, 1989), each segment appears to share a common conserved developmental plan. Segments in the hindbrain (rhombomeres), for example, contain sets of neurons with similar axonal projection patterns (Metcalfe et al., 1986; Hanneman et al., 1988; Lumsden and Keynes, 1989; Trevarrow et al., 1990; Clarke and Lumsden, 1993; reviewed in Kimmel, 1993). This repetition of axonal projection patterns along the anteroposterior axis suggests the intriguing hypothesis that FP chemoattraction and chemorepulsion play a common role in the formation of crossed and uncrossed projections in different axial levels of the CNS.

The present and previous findings concerning chemoattraction of crossed axons support this idea: both APmt (Shirasaki et al., 1995; and the present study) and APmy (present study) axons as well as spinal cord commissural axons (Tessier-Lavigne et al., 1988; Placzek et al., 1990a, 1990b) are chemoattracted by the FP at the corresponding axial level; the FP at all anteroposterior levels of the CNS shows similar chemoattractive activity toward both APmt axons (present study) and spinal commissural axons (Placzek et al., 1990b); mRNA for netrin-1, a molecule responsible for chemoattraction of spinal commissural axons (Kennedy et al., 1994; Serafini et al., 1994), is expressed in the FP at all levels (Kennedy et al., 1994), and recombinant netrin-1 mimics the chemoattractive activity of the FP toward cerebellofugal axons (Shirasaki et al., 1995).

With regard to chemorepulsion of uncrossed axons, although we failed to observe FP chemorepulsion in BP regions other than the mesencephalon, this does not preclude the possibility that axons in these regions are in fact chemorepelled. We assayed for chemorepulsion by examining axon turning in gel, which might fail to detect turning within BP explants. Moreover, since we cultured whole BP explants that may contain heterogeneous cells, a small axonal population that did turn in gel, if present, might be masked by the large axonal population that is unaffected by the FP. Indeed, such BP regions contain a variety of uncrossed axons that might be chemorepelled by the FP, such as branchiomotor axons in the rhombencephalic BP, which project laterally away from the FP (Lumsden and Keynes, 1989; Chang et al., 1992; Guthrie and Lumsden, 1992). The aforementioned hypothesis that FP chemotropism plays a common role in axon guidance is supported by several lines of evidence: the FP at all levels of the neuraxis exhibits similar chemorepellent activity against both DAPms and BPms axons (present study); the FP, at a distance in vitro, repels the axons of trochlear motoneurons (S. A. Colamarino and M. Tessier-Lavigne, personal communication), which originate from the BP in the rostral-most rhombencephalon and project laterally away from the FP; and when FP was transplanted ectopically into embryonic chick hindbrain in vivo, motor axons avoided the transplanted tissue, and experiments in which rat or chick FP and BP were cocultured within collagen gels showed that the FP released a diffusible chemorepellent of axonal growth (S. Guthrie and A. Pini, personal communication). The FP therefore appears to chemorepel a variety of uncrossed axons in the CNS, such as DAPms axons, BPms axons (including dopaminergic axons), and motor axons in the rhombencephalon and spinal cord.

Together with these data, our results suggest that FP chemoattraction and chemorepulsion likely play an important role in the guidance of crossed and uncrossed axons in the CNS, thereby establishing neuronal projection laterality.

Although the FP is not discernible in regions rostral to the caudal diencephalon (Kingsbury, 1930; Puelles et al., 1987), the FP-deficient zebrafish mutant *cyclops* shows a more severe phenotype in the rostral portions of the brain (Hatta et al., 1991, 1994; Hatta, 1992), such as the ventral forebrain (Hatta et al., 1994) and middiencephalic region (Macdonald et al., 1994). *Sonic hedgehog*, which appears to be involved in patterning of the ventral CNS (Echelard et al., 1993; Krauss et al., 1993; Riddle et al., 1993; Roelink et al., 1994), is expressed in these regions (Echelard et al., 1993; Krauss et al., 1993; Macdonald et al., 1994; Roelink et al., 1994) in addition to the FP, suggesting that these regions may share organizing activity similar to that of the FP. Like *sonic hedgehog*, *netrin-1* mRNA was detected diffusely in the ventral forebrain (Kennedy et al., 1994). These findings, together with the proposal for the presence of neuromeres in the forebrain (Puelles et al., 1987; Figdor and Stern, 1993; Puelles and Rubenstein, 1993), raise the possibility that these forebrain regions affect axonal growth patterns via chemoattractive and chemorepellent activities as the FP does in lower regions of the CNS.

Since the mature vertebrate brain is a highly complex structure that contains neurons exhibiting diverse projection patterns, it has not generally been speculated that the mechanisms responsible for the construction of neuronal circuits would operate universally throughout the brain. Evidence exists, however, to indicate that projection patterns that appear superficially distinct in adults arise from similar embryonic trajectories (Shirasaki et al., 1995). This raises the possibility that the guidance of a variety of CNS axonal projections can be achieved with relatively few sets

of commonly acting cues. Chemotropic FP cues, as demonstrated in the present study, may serve such a function in establishing neural patterns in the vertebrate CNS.

Experimental Procedures

DiI Labeling

All procedures followed those of Shirasaki et al. (1995), with some modifications. Briefly, the brains of E13–E15 Wistar rat embryos were cut along the ventral and dorsal midlines, whole-mounted with the ventricular side down, and fixed with 4% paraformaldehyde. Small crystals of DiI (Godement et al., 1987; Honig and Hume, 1989) were placed into the DAP of the mesencephalon and caudal diencephalon. At 1–7 days after DiI implantation, the preparations were observed with an epifluorescent microscope and photographed.

Explant Culture

E12–E14 rat embryos were used. Procedures for explant culture also followed those of Shirasaki et al. (1995). In the DAPms chemorepulsion assay, the following explants were dissected from E12–E13 mesencephalic regions between the mesencephalic–metencephalic boundary and posterior commissure: DAPms explants from the dorsal midline area (including the RP and DAP), FPms explants from the ventral midline area, and VAPms from the area dorsal to the sulcus limitans. We attempted to dissect DAPms regions adjacent to the RP to exclude possible contamination with contralaterally projecting tectofugal neurons. DAPms explants contained meninges, which could not be removed because the DAPms region is extremely thin at this stage. In the BPms repulsion assay, BPms explants were dissected from E12–E13 mesencephalon by cutting both the sulcus limitans and the lateral edge of the FPms. In both assays, explants were embedded in collagen gel matrix, which may establish a gradient of diffusible substances (Ebendal and Jacobson, 1977; Lumsden and Davies, 1983, 1986). To verify that FP repulsion was due to diffusible activity, DAPms or BPms explants were cocultured with FP explants, separated by a permeable barrier (polycarbonate filter, 0.2 μm pore size; Millipore, Ireland). DAPms or BPms explants were cocultured for 2–4 days with FP explants from corresponding or different anteroposterior levels (separation < 1 mm).

In the chemoattraction assay, APmt and APmy explants were dissected from the dorsal AP of E13–E14 rat embryos by cutting ventrally, leaving the dorsolateral surface intact. They were cultured alone or with FP explants placed at a distance (separation < 500 μm) in collagen gels for 24–30 hr. In coculture, FP explants were faced with the intact dorsolateral surface of APmt and APmy explants.

Following fixation, explant cultures were observed and photographed in phase contrast.

Quantification of Chemorepellent Activity

For quantitation of chemorepellent activity, we measured the turning angle of axon bundles from the DAPms explant surface facing the FPms explants. The difference between the direction of axons at their point of emergence and at their terminus was plotted on a histogram. Axons were excluded from the analysis when either of these points or the direction of growth was indiscernible. A total of 297 and 460 bundles were measured from five and three randomly sampled cocultures with live and heat-treated FPms explants, respectively.

Immunocytochemistry

Following fixation, whole BPms explants were immunostained using rabbit (1:250; Chemicon) or mouse (1:100; Boehringer Mannheim) anti-TH antibodies as primary antibodies, biotinylated secondary antibodies (1:200; Vector), and then Cy3-conjugated Streptavidin (1:500; Jackson ImmunoResearch).

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