Crossing the Floor Plate Triggers Sharp Turning of Commissural Axons

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During development of the vertebrate CNS, commissural axons initially grow circumferentially toward the ventral midline floor plate. After crossing the floor plate, they abruptly change their trajectory from the circumferential to the longitudinal axis. Although recent studies have unraveled the mechanisms that control navigation of these axons along the circumferential axis, those that result in the transition from circumferential to longitudinal trajectory remain unknown. Here, we examined whether an interaction with the floor plate is a prerequisite for the initiation of trajectory transition of commissural axons, using in vitro preparations of the rat metencephalon. We found that commissural axons in the metencephalon, once having crossed the floor plate, turned sharply to grow longitudinally. In contrast, axons extending in floor plate-deleted preparations, continued to grow circumferentially, ignoring the hypothetical turning point. These results suggest that a prior interaction of commissural axons with floor plate cells is a key step for these axons to activate a navigation program required for their change in axonal trajectory from the circumferential to the longitudinal axis.

Key Words: axon guidance; floor plate; commissural axons; sharp turn; longitudinal growth; intermediate target; navigation program; rat; hindbrain.

INTRODUCTION

During development of the nervous system, axons navigate considerable distances toward their final targets in a highly stereotyped and directed manner. In many cases, however, these axons do not grow straight toward their final targets from the beginning, but extend via a variety of intermediate targets or guidepost cells that divide their overall journey into several steps (Tessier-Lavigne and Goodman, 1996; Mueller, 1999). Commissural axons, originating from the alar plate of the vertebrate neural tube, for example, have at least three discrete steps of pathfinding to reach their final targets (Colamarino and Tessier-Lavigne, 1995). They initially navigate ventrally toward the ventral midline along the circumferential axis, then cross the ventral midline floor plate, to project contralaterally. On the contralateral side, the commissural axons abruptly change their growth direction to extend either rostrally or caudally along the longitudinal axis. These pathfinding behaviors are common among commissural axons at all axial levels of the CNS where floor plate cells are found (i.e., from the spinal cord rostrally to the midbrain) (Colamarino and Tessier-Lavigne, 1995; Murakami and Shirasaki, 1997).

Recent studies have begun to unravel the mechanisms that control the navigation of commissural axons toward and at the floor plate: ventral navigation toward the floor plate is, at least in part, mediated by floor plate chemotraction (Cook et al., 1998), and when they reach this intermediate target, the encounter of commissural axons with the floor plate abolishes their responsiveness to the floor plate-derived chemoattractant, allowing the axons to continue their pathfinding (Shirasaki et al., 1998). Changes in growth cone responsiveness to a midline repellent also seem to help prevent crossing axons from recrossing the
midline (Zou et al., 2000). In addition, a balance between positive and negative signals expressed by floor plate cells is also thought to contribute to preventing these axons from inappropriately recrossing the midline (Stoeckli and Landmesser, 1998; Van Vactor and Flanagan, 1999).

However, our understanding of the mechanism underlying the next pathfinding step (i.e., the change in axonal trajectory from the circumferential to the longitudinal axis after having crossed the floor plate) is quite limited. In a mouse mutant, Danforth’s short-tail, in which the floor plate is absent from a large region of the spinal cord (Bovolenta and Dodd, 1991) and in notochordless Xenopus embryos that lack the floor plate altogether (Clarke et al., 1991), spinal commissural axons, having crossed the midline, continue to extend circumferentially without making a longitudinal turn (Bovolenta and Dodd, 1991; Clarke et al., 1991). These observations raise the possibility that commissural axons’ encounter with the floor plate plays a critical role in turning of these axons on the contralateral side. Interpretation of these results, however, may be complicated because the mutation also disrupts normal differentiation of ventral neurons including motor neurons (Bovolenta and Dodd, 1991), leading to changes in the extracellular environment where commissural axons make a sharp turn.

In the present study, we directly tested whether an interaction of commissural axons with the floor plate is required for inducing subsequent turning of these axons, using in vitro preparations of the rat metencephalon, which can reproduce the sharp turning of commissural axons in the metencephalon. The results show that an encounter of commissural axons with the floor plate triggers a navigation program required for the change in their trajectory from the circumferential to the longitudinal axis.

**MATERIALS AND METHODS**

**Whole-Mount In Vitro Preparations of the Rat Metencephalon**

Procedures for tissue dissection and explant culture followed those of Shirasaki et al. (1998), with some modifications. The brains of embryonic day 13 (E13) Wistar rats were used because commissural axons in the metencephalon leave the cerebellar plate (CP) at around E13 (Shirasaki et al., 1995). These commissural axons are referred to hereafter as CP axons. A region from the level of the trigeminal ganglion to midbrain–hindbrain boundary (isthmus), which therefore includes the entire metencephalon, was used for the cultures. A 0.5- to 1-mm-wide circumferential metencephalic strip that contained the circumferential trajectory of CP axons was dissected from the metencephalon. A longitudinal strip with a width of about 0.5 mm was taken from the basal plate of the entire metencephalon. These explants were dissected under a binocular microscope (Olympus SZ-ST 4045). Under the microscope, the floor plate can be recognized as a transparent stripe, which permitted discrimination between the floor plate and the adjacent basal plate.

When circumferential strips including the floor plate were prepared, care was taken to exclude the contralateral basal plate. In the metencephalon, CP axons, after having crossed the floor plate, make a sharp turn at a distance from the floor plate (Figs. 1A and 1B) (see also Shirasaki et al., 1995). This is in marked contrast with the rat spinal commissural axons, which make a rostral turn, maintaining a close contact with the contralateral border of the floor plate (e.g., Bovolenta and Dodd, 1990). However, the distance between longitudinal trajectory of CP axons and the floor plate permitted us to perform microsurgical manipulations, such as dissection of circumferential strips with or without the floor plate and of longitudinal strips, without disrupting the longitudinal pathway of CP axons. The circumferential and longitudinal strips were placed on a polycarbonate isopore membrane filter (Millipore, Tokyo; pore size 5 μm), with the ventricular side of the explants down. These explants were put together to fuse the cut aspects of the preparations, followed by embedding in collagen gels. The explants on the filter were cultured for 2–3 days. CP axons tended to grow along the explant border when the cut edges of the strips did not fuse well. Such preparations were excluded from the analysis.

**Fluorescent Dye Labeling and Immunostaining**

After fixation with 4% paraformaldehyde, CP axons were labeled by implanting microfluidic devices of the fluorescent tracer 1,1'-dioctadecyl-3,3',3'-tetramethylindocarbocyanine perchlorate (DiI; Molecular Probes, Eugene, OR) and 3,3'-dioctadecyloxacarbocyanine perchlorate (DiO; Molecular Probes) (Godement et al., 1987; Honig and Hume, 1989) into the CP. The preparations were then stored in the dark at room temperature for 3–5 days. Whole-mount immunohistochemistry was performed on cultured explants in collagen gels, with a monoclonal antibody to TAG-1 (4D7) (Yamamoto et al., 1986), as previously described (Shirasaki et al., 1996, 1998).
Assessment of CP Axon Turning within Cultured Explants

In the experiments in which CP axon turning was examined using longitudinal strips, the preparations were cultured for 3 days to allow the axons to grow for a sufficiently long distance for the analysis. After the culture, preparations that meet the following criteria were considered to exhibit “turning”: (1) At least one CP axon showed an abrupt shift of trajectory from the circumferential to the longitudinal axis within the longitudinal strips, but not along the cut edges of the strips. In preparations that satisfied this criterion, a major population of CP axons showed a sharp turning to grow longitudinally, with some axons apparently stalling around the turning point. (2) CP axons that did not show a turn appeared to stall around the region of the turning point without reaching the far edge of the longitudinal strips. These behaviors of CP axons were in sharp contrast to those that had not encountered the floor plate; such axons continued to grow circumferentially within the longitudinal strips, thus reaching the far edge of the strips.

RESULTS

CP Axon Turning after Crossing the Floor Plate In Vitro

In our previous assay that utilized a narrow (0.5–1 mm width) circumferential strip of the metencephalon, CP axons did not consistently turn after crossing the floor plate (Shirasaki et al., 1998), even though CP axons in vivo make a sharp turn on the contralateral side of the basal plate in the metencephalon (Figs. 1A and 1B) (see also Shirasaki et al., 1995). In the present study, we were able to recapitulate sharp turning of CP axons by dissecting a larger region encompassing the entire metencephalon for in vitro assay. After 2 days in culture, CP axons were traced using expression of TAG-1, an axonal surface glycoprotein, as a molecular marker for these axons (Shirasaki et al., 1996, 1998). We found that TAG-1-positive (TAG-1+) CP axons grew straight toward the floor plate, showing no sign of longitu-
dinal growth before reaching the floor plate (n = 5; Fig. 2A). Because TAG-1 expression is reduced around the floor plate (Shirasaki et al., 1996), CP axons were also labeled with the fluorescent tracer DiI to follow their trajectories after they had crossed the floor plate. We found that most DiI-labeled axons that had crossed the floor plate made a sharp turn to extend longitudinally (n = 9; Figs. 2B–2D). Thus, turning of CP axons does occur in our in vitro preparation after crossing the floor plate.

Basal Plate on Both Sides Can Induce CP Axon Turning

Two possible mechanisms may be speculated for CP axon turning after midline crossing: (1) preferred substrate for CP axons in the basal plate region; (2) inhibitory influence from region dorsal to the turning point. To test these ideas, we dissected longitudinal strips composed only of the floor plate and the adjacent basal plate, and juxtaposed them to CP explants to fuse the cut aspects of the preparations.
FIG. 4. CP axons, once having crossed the floor plate, turn sharply to grow longitudinally. (A) Diagram showing an experimental manipulation. A circumferential strip extending up to the contralateral border of the floor plate (hatched region in the upper left panel) and a longitudinal strip taken from the contralateral basal plate (hatched region in the lower left panel) were put together to fuse the cut aspects of the preparations (middle right panel). (B) Bright-field micrograph of the coculture preparation after a 3-day culture. Asterisks in B and C indicate Dil injection site. (C) Fluorescent micrograph of the same preparation as shown in B. (D) High-power micrograph of the area shown by an arrowhead in C. Many CP axons abruptly change their axonal trajectory from the circumferential to longitudinal axis within the longitudinal strip. (E) Diagram showing an experimental manipulation similarly prepared as in A, except the longitudinal strip was taken from the ipsilateral side (hatched region in the lower left panel). (F) Bright-field micrograph of the manipulated preparation after a 3-day culture. Asterisks in F and G indicate Dil injection site. (G) Fluorescent micrograph of the same preparation as shown in F. (H) High-power micrograph of the area shown by arrowhead in G. CP axons, after having crossed the floor plate, show trajectory transition from the circumferential to longitudinal axis. Scale bar, 250 μm (B, C, F, and G) and 100 μm (D and H).
In this study we have examined the behavior of CP axons after they have crossed the floor plate in vitro. We traced CP axon trajectory using TAG-1 immunostaining and DiI labeling. Although Dil implantation also labeled some non-crossing axons, Dil implantation into the alar plate of the metencephalon-labeled axons that cross the midline and thereafter show a sharp turn to grow either rostrally or caudally along the longitudinal axis, a characteristic feature of CP axons (Shirasaki et al., 1995). These axons in vitro were previously shown to be attracted by the floor plate (Shirasaki et al., 1998), which is a major characteristic of
alar plate-derived commissural axons that express TAG-1 (Shirasaki et al., 1996, 1998). It is therefore likely that the Dil-labeled midline-crossing axons and those labeled by TAG-1 before crossing the midline belong to the same axonal population and represent commissural axons that turn on the contralateral side. Moreover, the turning of the Dil-labeled axons occurred at a distance from the floor plate as they do in vivo (Shirasaki et al., 1995). We therefore conclude that CP axon turning after crossing the floor plate was well reproduced in the present culture preparation.

In our previous culture conditions in which a narrow circumferential strip of the metencephalon was used, turning of CP axons after crossing the floor plate was not consistently reproduced (Shirasaki et al., 1998). In the

![Diagram](image1.png)

**FIG. 5.** Without encountering the floor plate, CP axons ignore the hypothetical turning point. (A) Diagram showing an experimental manipulation. The region that includes the CP (hatched region in the upper left panel) and a longitudinal strip taken from the contralateral side (hatched region in the lower left panel) were put together (middle right panel). (B) Bright-field micrograph of the manipulated preparation after a 3-day culture. (C) Fluorescent micrograph of the same preparation as shown in B. Asterisks in B and C indicate Dil injection site. (D) High-power micrograph of the area shown by an arrowhead in C, where most Dil-labeled CP axons continue to grow circumferentially. (E and F) Fluorescent micrograph of a similarly manipulated preparation stained for expression of TAG-1. In this preparation, a circumferential strip was similarly prepared as in A, but the circumferential strip was made by cutting near the outer border of the floor plate on the ipsilateral side. This resulted in longer growth distance between the CP and the contralateral longitudinal strip, compared to the case in A. TAG-1⁺ axons around white asterisk in E are commissural axons at around the level of the trigeminal ganglion. (F) Higher-magnification view of the area shown by arrows in E. Note that expression of TAG-1 on CP axons is maintained in the contralateral longitudinal strip. Without an encounter with the floor plate, TAG-1⁺ axons continue to grow circumferentially, ignoring the hypothetical turning point, although the possibility that an encounter of CP axons with the ipsilateral basal plate proximal to the floor plate contributes to triggering CP axon turning cannot be excluded. Scale bar, 250 μm (B, C, and E) and 100 μm (D and F).
In an in vitro assay, we dissected a region encompassing the entire metencephalon or a longitudinal strip of the metencephalic basal plate. Although the reason for the difference between the present and the previous results remains unknown, our finding that the present metencephalic in vitro preparations included many longitudinal axons extending around the basal plate (R. Shirasaki and F. Murakami, unpublished observation) raises the possibility that these longitudinal axons provide cues for axon turning.

Downregulation of TAG-1 expression was also observed for CP axons that had crossed the floor plate in the narrow circumferential strip (Shirasaki et al., 1998). It is therefore unlikely that the downregulation of TAG-1 expression at the floor plate is causally related to CP axon turning.
although we cannot rule out the possibility that turning off TAG-1 expression is prerequisite for the initiation of subsequent sharp turning of CP axons.

**Switch in Commissural Axon Responsiveness to Guidance Cues by the Floor Plate**

CP axons, having crossed the floor plate, turned even in an explant taken from the ipsilateral side (Figs. 4E–4H). Considering that the vertebrate CNS is bilaterally symmetrical along the midline, guidance cues for commissural axon turning should be present on both sides of the midline. In this context, commissural axons should first encounter the cues that induce longitudinal turning on the ipsilateral side. However, commissural axons do not turn before encountering the floor plate, which can be explained by assuming that they do not have responsiveness to the cues for sharp turning. Our finding that commissural axons continued to grow straight, ignoring the hypothetical turning point, in floor plate-deleted in vitro preparations (Fig. 5) is consistent with the view that an encounter with the floor plate results in sensitization of commissural axons to subsequently encountered cues required for their longitudinal navigation.

Studies in invertebrates lend support to the idea that interaction between commissural axons and midline cells is likely to play a key role in initiating specific events after crossing the midline. In the grasshopper CNS, for example, serotonergic neurons develop serotonin uptake activity immediately after the growth cones have crossed the midline. Interestingly, the expression of fibroblast growth factor (FGF) receptor on commissural axons appears to be induced when they cross the midline, which in turn induces the uptake activity (Condron, 1999). Related to this, commissural axons fail to turn at the contralateral longitudinal pathway, if expression of new molecules on the growth cones at the midline is perturbed by transcriptional blockade (Von Bernhardi and Bastiani, 1995). Another example can be seen in the Drosophila CNS: commissural axons initiate expression of Roundabout (Robo), a receptor for the midline repellent Slit, after they have crossed the midline (Kidd et al., 1998, 1999). This upregulation of Slit/Robo signaling on the contralateral side is thought to contribute to preventing commissural axons from inappropriately recrossing the midline.

**Molecular Mechanism of Commissural Axon Turning**

F-spondin, a secreted molecule expressed by floor plate cells (Klar et al., 1992), was recently shown to be involved in the guidance of commissural axons at the floor plate of the embryonic chick spinal cord. Blocking the function of F-spondin causes lateral drifting of commissural axons from the contralateral floor plate boundary, but it did not affect rostral turning of the axons after crossing the floor plate (Burstyn-Cohen et al., 1999). This suggests that F-spondin may control the location of commissural axon turning along the circumferential axis, although it may not be responsible for the initiation of commissural axon turning.

In the developing rodent spinal cord, Slit, a secreted repulsive ligand for Robo receptor, is expressed not only in floor plate cells but also in motor neurons (Holmes et al., 1998; Brose et al., 1999; Li et al., 1999; Yuan et al., 1999). This has led to a speculation that Slit repellent activity functions not only in preventing Robo-expressing commissural axons from recrossing the midline but in inducing these axons to turn longitudinally (Li et al., 1999). In this context, if a longitudinally aligned inhibitory region exists at a distance from the floor plate dorsal to the site of axon turning, it could contribute to commissural axon turning. Recently, Zou et al. (2000) found that spinal commissural axons in vitro become responsive to a repellent activity secreted by both the floor plate and the ventral spinal cord after midline crossing and proposed an interesting hypothesis that these repellent activities squeeze commissural axons into surrounding fiber tracts. However, it does not seem to be the case in the rostral hindbrain of rodents because Slit does not seem to be expressed in the region adjacent to the floor plate, unlike in the spinal cord (Holmes et al., 1998). In addition, we have shown that CP axons, after having crossed the floor plate, turn in longitudinal strips, irrespective of whether they enter from the dorsal or ventral side of the strips (Fig. 4). Thus, it is unlikely that repulsive molecules such as Slits are responsible for longitudinal navigation of CP axons. In this light, as a result of contact between commissural axons and the floor plate, upregulation of ligand/receptor signaling other than Slit/Robo might be involved in the trajectory transition of CP axons.

In conclusion, our results suggest that an encounter of commissural axons with the floor plate, an intermediate target of these axons, triggers a navigation program required for their change in axonal trajectory from the circumferential to the longitudinal axis. This may explain the difference of commissural axon behaviors before and after crossing the midline of the bilaterally symmetrical vertebrate CNS.

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