phoresis (SDS-PAGE). The proteins were transferred to a polyvinylidene difluoride membrane (Millipore) and incubated with mAb 12CA5 (BABCO, Richmond, CA) followed by an alkaline phosphatase–conjugated antibody to murine immunoglobulin G (Promega). Membranes were developed using a stabilized substrate for alkaline phosphatase (Promega).
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17. Transfection procedures: HeLa cells were co-transfected with 1 μg of cytomegalovirus (CMV)–β-Gal (Stratagene) and 5 μg of pCI vector (Promega), pCI-P35 (*18*), or pCI–MC066L-A mixed with lipofectin. Thirty hours after transfection, the cells were treated with UV (312 nm) light for 15 s at room temperature or continuously with 100 μM hydrogen peroxide or cycloheximide (1 μg/ml; Sigma) and TNF-α (10 ng/ml; Boehringer Mannheim) or cycloheximide and anti-Fas (1.25 μg/ml; CH-11 antibody, Kamiya Biomedical, Seattle, WA). Twelve hours later, cells were fixed, stained with X-Gal (Promega), and examined microscopically. The control wells contained 100 to 200 flat, blue cells per microscopic field. The number of flat, blue cells in six fields of the untreated well of each transfectant was compared to the number of flat, blue cells in six fields of the treated well, and the percent viability was determined. The HaCaT cells were co-transfected with 0.33 μg of CMV–β-Gal and 1.67 μg of pCI, pCI-P35, or pCI–MC066L-A mixed with lipofectamine reagent (Life Technologies). Thirty hours after transfection, the cells were treated for 30 s with UV (312 nm) light or 10 μM hydrogen peroxide. Cells were fixed, stained, and scored for viability as described for HeLa cells.
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# Change in Chemoattractant Responsiveness of Developing Axons at an Intermediate Target

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Developing axons reach their final targets as a result of a series of axonal projections to successive intermediate targets. Long-range chemoattraction by intermediate targets plays a key role in this process. Growing axons, however, do not stall at the intermediate targets, where the chemoattractant concentration is expected to be maximal. Commissural axons in the metencephalon, initially attracted by a chemoattractant released from the floor plate, were shown to lose responsiveness to the chemoattractant when they crossed the floor plate in vitro. Such changes in axon responsiveness to chemoattractants may enable developing axons to continue to navigate toward their final destinations.

In the developing nervous system, axons navigate considerable distances toward their final targets in a highly stereotyped and directed manner. This process is achieved by a series of axonal projections to successive intermediate targets under the influence of local guidance cues (*1*). Accumulating evidence has indicated the importance of long-range chemoattraction in guiding developing axons not only to final (*2*) but also to intermediate targets (*3–10*). Commissural axons originating from the alar plate of the vertebrate central nervous system, for example, initially grow ventrally, attracted by a diffusible chemotropic molecule secreted from the ventral midline floor plate (*3–9*), an intermediate target of these axons (*6*, *11*). These axons, however, grow past the floor plate to extend contralaterally. Since the first demonstration of the existence of the floor plate–derived chemoattractant (*3*), an intriguing question has been why growing axons do not stall at their intermediate targets, where the chemoattractant concentration is expected to be maximal. Here, we provide evidence for a change in the chemoattractant responsiveness of growing axons during their growth across an intermediate target.

Metencephalon commissural axons, which originate from the cerebellar plate (CP) of the rat embryo, initially grow circumferentially toward floor plate cells at the ventral midline of the metencephalon (*6*). In vitro studies have suggested that these commissural axons (referred to hereafter as CP axons) are guided toward the midline by a diffusible chemoattractant released from floor plate cells (*6*, *7*, *9*). Here, we used an in vitro preparation that reproduces the crossing of the midline floor plate by CP axons to examine possible changes in the chemoattractant responsiveness of CP axons when they cross the floor plate (*12*). When a strip of the rostral metencephalon that included the entire circumferential trajectory of CP axons (Fig. 1A) was cultured alone in collagen gel, axons originating from the CP grew across the midline floor plate to extend contralaterally ($n$ = 16) (Fig. 1B) (*13*). We next tested whether CP axons, after they have crossed the floor plate, are attracted by an ectopic floor plate explant. We juxtaposed a floor plate explant to the metencephalic strip on one side (Fig. 1A) and examined the behavior of CP axons by implanting the fluorescent tracer 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (DiI) into the CP contralateral to the explant. Under such conditions, CP axons that had crossed the midline floor plate did not show directed growth toward the ectopic floor plate explant (Fig. 1C). To compare directly the behavior of CP axons extending from both sides, we implanted DiI crystals into the contralateral CP and 3,3′-dioctadecyloxacarbocyanine perchlorate (DiO) crystals into the ipsilateral CP of the same strip preparations (Fig. 1D). Although DiO-labeled CP axons that had not crossed the midline floor plate showed directed growth toward the ectopic explant, DiI-labeled axons that had crossed showed no sign of directed growth (Fig. 1, D and E).

Because implantation of DiI or DiO into the CP might also label axonal populations other than those from commissural neurons, such as longitudinally growing axons (Fig. 1C) (*6*), we next assessed chemoattraction of CP axons by labeling them with a molecular marker for commissural axons (*14*). Commissural axons at all axial levels from the spinal cord to the mesencephalon express TAG-1, an axonal surface glycoprotein, during their circumferential growth until they reach the floor plate in vivo (*9*, *15*). Moreover, TAG-1–positive (TAG-1$^+$) commissural axons including CP axons are attracted by the floor plate in vitro (*3*, *9*). We found that most TAG-1$^+$ CP axons in control strip preparations grew straight along the circumferential axis ($n$ = 15) (Fig. 1F), although some TAG-1$^+$ CP axons near the cut edges of the prep-

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arations spontaneously exited the preparations, deflected from their circumferential trajectory. However, TAG-1$^+$ CP axons at a distance of >0.16 mm away from the cut edges were never observed to exit from control strip preparations. Thus, we judged CP axons to be attracted by an ectopic floor plate explant if CP axons at a distance of >0.16 mm away from the cut edge facing the explant showed turning toward and penetration into the explant. We found that, although TAG-1$^+$ CP axons even at a distance of 0.56 ± 0.02 mm (mean ± SEM, $n$ = 17) away from the explant showed turning toward the ipsilaterally placed explant (Fig. 1G and Table 1), DiI-labeled CP axons, once they had crossed the floor plate, were not attracted by the explant (Table 1) (*16*). Thus, CP axons, which are attracted by the chemoattractant until they reach the floor plate, lack responsiveness to it after they cross the floor plate.

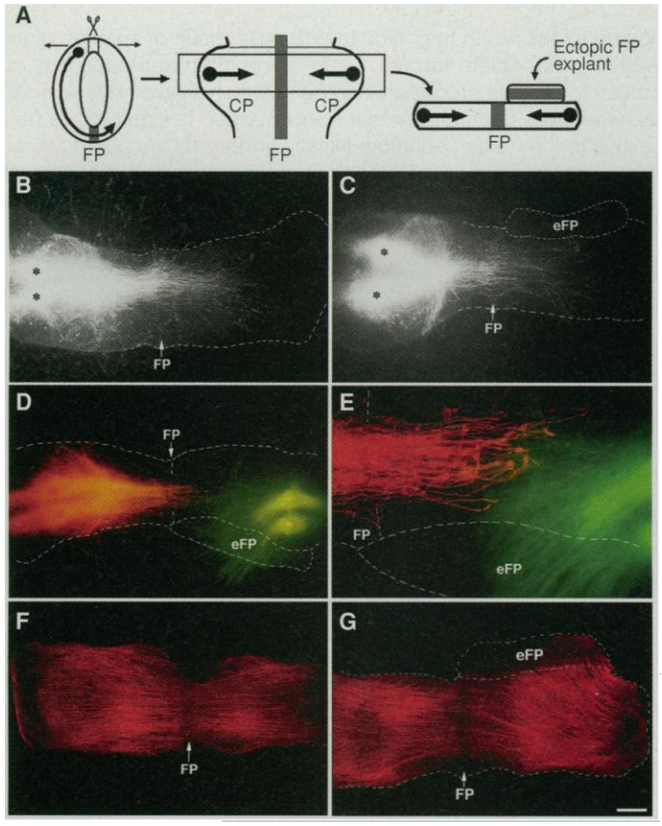


**Fig. 1.** Loss of CP axon responsiveness to a floor plate (FP)–derived chemoattractant when the CP axons cross the floor plate. (**A**) (Left) A diagrammatical view of the coronal plane of the neural tube with the trajectory of CP axons. (Center) Flat, whole-mount preparation that was obtained by cutting the dorsal midline of the neural tube. (Right) A metencephalic strip preparation of E13 rat brain including the circumferential trajectory of CP axons was further dissected from the whole-mount preparation as represented in the center diagram. An ectopic floor plate (eFP) explant was placed on one side of the preparation. (**B**) Fluorescent view of DiI-labeled CP axons in a metencephalic strip preparation that had been cultured for 2 days. (**C**) Fluorescent micrograph of DiI-labeled CP axons in a strip preparation with an ectopic floor plate explant on one side. (**D** and **E**) Different behaviors of CP axons in the same preparation with an ectopic floor plate explant on one side (lower right). DiO crystals were implanted into one side (right) and DiI crystals into the other side (left). (E) is a higher magnification view of (D). (**F**) Immunofluorescence micrograph of a strip preparation after staining with antibody to TAG-1. (**G**) In the presence of an ectopic floor plate explant, ipsilateral TAG-1$^+$ CP axons are deflected toward the explant. Asterisks in (B) and (C) indicate DiI injection sites, and arrows indicate the ventral midline floor plate (B to D, F, and G). Scale bar, 250 μm (B to D, F, and G) and 100 μm (E).

At least two possible mechanisms can be considered regarding this change in growth cone responsiveness: one is a cell-autonomous loss of responsiveness at the floor plate, and the other is a change caused by an interaction of commissural axons with floor plate cells. If the latter is the case, one would predict that the growth cones extending into the contralateral side without encountering the midline floor plate should retain responsiveness to the chemoattractant. We found that when a ventral region including the floor plate was surgically removed from the strip preparation (Fig. 2A), CP axons growing into the contralateral part showed directed growth toward the ectopic floor plate explant (Fig. 2B and Table 1). In contrast, when a ventral region that did not contain the floor plate was removed (Fig. 2C), CP axons, once they had crossed the midline floor plate, grew circumferentially without being attracted by the ectopic floor plate explant (Fig. 2D and Table 1). These results support the view that the growth cone encounter with floor plate cells abolishes its responsiveness to the floor plate–derived chemoattractant.

We next tested whether CP axons, after crossing the floor plate, continue to have responsiveness to netrin-1, a laminin-related

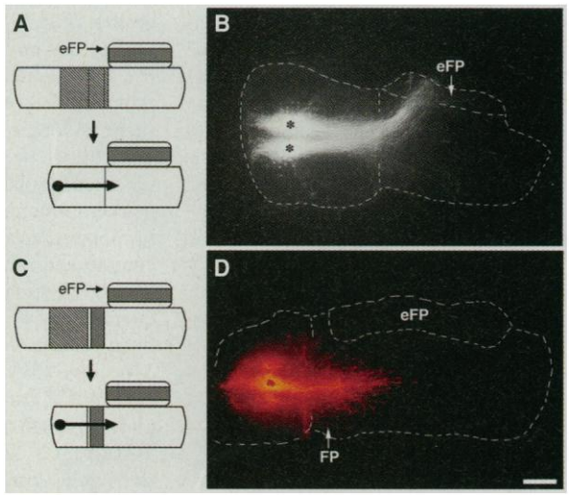


**Fig. 2.** Abolishment of chemoattractant responsiveness of CP axons by the midline floor plate. (**A** and **C**) Diagrams showing experimental manipulations. The hatched regions were removed from the strip preparation, and the remaining explants were put together to fuse the cut aspects of the preparation. (**B**) Fluorescent micrograph of a culture preparation after the experimental manipulation shown in (A), demonstrating that DiI-labeled CP axons growing into the contralateral part are attracted by an ectopic floor plate explant. Asterisks indicate DiI injection sites. (**D**) When a ventral region excluding the midline floor plate is removed, DiI-labeled CP axons, once they cross the midline floor plate, grow circumferentially on the contralateral side, showing no sign of being attracted by an ectopic floor plate explant. Scale bar, 250 μm.

**Table 1.** Chemoattraction of CP axons in metencephalic strip preparations. Culture preparations were scored as positive if they contained any CP axons that were qualified as "attraction" by the criteria described in the text. FP, floor plate.

| Explant apposed to one side of strip preparation | Fraction of preparations in which attraction of CP axons was observed |
|---|---|
| FP explant* | 17/17 |
| FP explant† | 0/20‡ |
| FP explant† (preparation without FP) | 12/12 |
| FP explant† (shortcut preparation) | 0/20 |
| Netrin-1–secreting aggregate* | 12/12 |
| Netrin-1–secreting aggregate† | 0/12 |

*Effect on ipsilateral CP axons. †Effect on contralateral CP axons. ‡In 7 out of 20 preparations, CP axons extending from both sides were labeled in the same preparations.

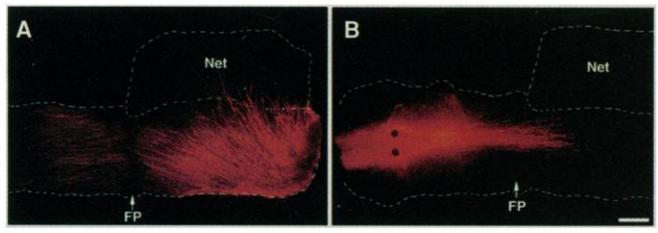


**Fig. 3.** Loss of CP axon responsiveness to netrin-1 when the CP axons cross the floor plate. A netrin-1–secreting cell aggregate was placed on one side of the strip preparation. (**A**) Immunofluorescence micrograph showing that TAG-1$^+$ CP axons are attracted by ipsilaterally placed aggregates of netrin-1–secreting cells (Net). (**B**) DiI-labeled CP axons are not attracted by netrin-1–secreting cell aggregates after they cross the floor plate. Asterisks indicate DiI injection sites. Scale bar, 250 μm.

diffusible protein secreted by floor plate cells (*5*, *17*). Netrin-1 can attract commissural axons at all axial levels from the spinal cord to the mesencephalon in vitro (*5*, *6*, *9*). We found that CP axons, which are attracted by aggregates of heterologous cells secreting netrin-1 (Fig. 3A and Table 1) (*18*), were not attracted by the aggregates after they had crossed the floor plate (Fig. 3B and Table 1) (*19*), indicating that CP axons lose responsiveness to netrin-1 during their growth across the floor plate (*20*).

Our results show that floor plate chemoattraction of commissural axons is effective only until they arrive at the floor plate. Although it remains unknown how modification of growth cone responsiveness to the chemoattractant is achieved, one possible mechanism is that the interaction of commissural axons with floor plate cells directly changes the chemoattractant responsiveness of the axons by modifying chemoattractant-receptor mechanisms locally in the growth cones. Alternatively, the modification might be mediated by changes in transcription of the receptor or one or more receptor-related molecules. In any case, receptor mechanisms mediated by netrin-1 may in some way be involved. Deleted in Colorectal Cancer (DCC) is a receptor or a component of a receptor that mediates the effect of netrin-1 on spinal commissural axons (*21*, *22*) and is also expressed on CP axons during their growth toward the floor plate in vivo (*23*). Thus, down-regulation of DCC expression at the floor plate may underlie the loss of the chemoattractant responsiveness. However, DCC appears to be expressed on commissural axons both as they project toward the floor plate and after they cross the floor plate in the developing rat spinal cord (*21*), suggesting that another as yet unidentified component of the netrin-1 receptor may be involved (*9*, *21*, *24*). It is also possible that the transfer of proteins from midline cells to crossing axons at the midline (*25*) is in some way involved in the regulation of the chemoattractant responsiveness of the axons.

After they cross the floor plate, commissural axons at all axial levels from the spinal cord to the mesencephalon abruptly change their growth direction to extend longitudinally (*6*, *11*, *26*). Although the molecular mechanisms subserving such a change in growth direction remain unknown, changes in growth cone responsiveness to guidance cues as demonstrated here might explain why commissural axons can grow along the longitudinal axis only on the contralateral side, despite the existence of identical ipsilateral cues.

In conclusion, loss of chemoattractant responsiveness of developing axons at intermediate targets as described here may contribute to their leaving the intermediate targets. In addition, encounters with their intermediate targets might also cause sensitization of growing axons to subsequently encountered cues that guide them to their final destinations.

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12. Experimental procedures for tissue dissection and explant culture followed those of Shirasaki *et al.* (*6*), with some modifications. In all the chemoattraction assays, the brains of embryonic day 13 (E13) Wistar rats were used, because CP axons leave the CP and head straight toward the floor plate at around E13 (*6*). A 0.5 to 1 mm wide metencephalic strip containing the entire circumferential trajectory of CP axons was dissected from the rostral hindbrain (Fig. 1A). The metencephalic strip and a floor plate explant, taken from the hindbrain, were placed on a polycarbonate isopore membrane filter (Millipore, Tokyo, Japan; pore size, 5.0 μm), with the ventricular side of the explants down, followed by embedding in collagen gels (*6*). The explants on the filter were then cultured for 2 days as previously described (*9*).
13. After fixation with 4% paraformaldehyde, we labeled CP axons by implanting fluorescent tracer DiI or DiO crystals (Molecular Probes, Eugene, OR) into the lateral margin of the preparation, from which these axons are known to emanate (*6*).
14. Whole-mount immunohistochemistry was performed on cultured explants in collagen gels, with an antibody to TAG-1 (monoclonal 4D7 supernatant, diluted 1:3), as described (*9*), except that each antibody incubation was performed for 1 day at 4°C.
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16. Because TAG-1 expression on CP axons is reduced around the floor plate (*9*), TAG-1$^+$ axons could not be followed to the contralateral side. To assess the chemoattraction of CP axons after they cross the floor plate, we labeled CP axons by implanting DiI into the CP contralateral to an ectopic explant.
17. To examine responsiveness to netrin-1, we used aggregates of 293-EBNA cells secreting recombinant netrin-1 in place of the ectopic floor plate explants. These aggregates were prepared by the hanging drop culture method as described (*9*). Generation of the 293-EBNA cell line stably secreting netrin-1 has been described (*9*).
18. CP axons growing within a distance of 0.58 ± 0.01 mm (mean ± SEM, $n$ = 12) from the aggregates showed directed growth toward them.
19. To ascertain whether the floor plate also mediates the change in the netrin-1 responsiveness of CP axons, we repeated the experiments of Fig. 2 but with a netrin-1–secreting cell aggregate in place of an ectopic floor plate explant. Although CP axons showed directed growth toward and penetration into the aggregate in Fig. 2A–type preparations ($n$ = 5), they did not do so in Fig. 2C–type preparations ($n$ = 6). These results support the view that an encounter with the floor plate by CP axons abolishes their responsiveness to netrin-1.
20. It should be noted, however, that there is evidence that floor plate cells also secrete a chemoattractant other than netrin-1 (*8*).
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26. I. T. Shepherd and J. S. H. Taylor, *J. Comp. Neurol.* **354**, 501 (1995). In our culture preparations, abrupt turning of CP axons after they crossed the floor plate and their growth along the longitudinal axis were not consistently reproduced.


27. We thank E. S. Ruthazer, N. Yamamoto, A. Tamada, Y. Oda, W.-J. Song, and H. Katsumaru for critical reading of the manuscript; M. Yamamoto for the 4D7 supernatant; M. Tessier-Lavigne for the 293 cell line; and E. S. Ruthazer for grammatical and stylistic improvement of the manuscript. Supported by a grant for priority areas (07279101) from the Ministry of Education, Science, Sports and Culture of Japan (to F.M.) and by a Postdoctoral Research Fellowship from the Japan Society for the Promotion of Science (to R.S.). F.M. is a Core Research for Evolutional Science and Technology Project researcher.

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