The Notochord and Floor Plate Guide Growth Cones in the Zebrafish Spinal Cord

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Elimination of the floor plate, a row of distinctive cells at the ventral midline of the spinal cord, dramatically increased the frequency of errors made by specific growth cones in the zebrafish embryo. This demonstrated that the floor plate participated in guiding specific growth cones at the ventral midline of the spinal cord. However, since a significant proportion of these growth cones followed their normal pathway despite the absence of the floor plate, we hypothesized that a second source of cues must exist at or near the ventral midline. We tested whether the notochord, which is located just ventral to the spinal cord, was an additional source of pathfinding cues by eliminating it prior to axonogenesis. Laser ablation of the notochord in wildtype embryos increased errors by spinal growth cones. Likewise, spinal growth cones made errors in *no tail* mutants that are missing the notochord but not the floor plate. These results demonstrate that the notochord also participates in guiding growth cones at the ventral midline. Furthermore, removal of both the floor plate and notochord resulted in a higher error rate than removal of either one alone. These results suggest that the notochord and floor plate normally act in concert to guide spinal growth cones at the ventral midline.

[Key words: growth cone, spinal cord, zebrafish, notochord]

Neurons make precise connections with each other by projecting growth cones that extend along cell specific pathways to reach their destinations in developing embryos (Dodd and Jessell, 1988; Goodman and Shatz, 1993). Growth cones navigate through embryos by sampling cues found in their local environment. A variety of cues that guide growth cones have been characterized in a number of different embryos. Cues can be bound to the surfaces of cells, embedded in the extracellular matrix, or diffusible; cues can induce growth cones to extend or inhibit them from extending; and cues can affect specific growth cones or affect all growth cones. Recent investigations of guidance cues suggest that several cues may act redundantly at specific

sites in embryos to insure that growth cones follow their correct pathways. We have now directly tested this hypothesis at the ventral midline of the zebrafish spinal cord by examining the pathways growth cones follow in the absence of several putative sources of guidance cues.

The ventral midline of the spinal cord is occupied by a set of distinctive nonneuronal cells known as the floor plate cells (Baker, 1927; Jessell et al., 1989). The floor plate plays a prominent role in guiding spinal growth cones in a variety of embryos. It attracts the growth cones of commissural neurons to the ventral midline of the spinal cord in mammals and chicks (Tessier-Lavigne et al., 1988; Bovolenta and Dodd, 1991; Yaginuma and Oppenheim, 1991). Furthermore, the floor plate participates in guiding specific growth cones at the ventral midline of the zebrafish spinal cord and hindbrain (Bernhardt et al., 1992a,b; Hatta, 1992). In the zebrafish embryo, the removal of the floor plate either by laser ablation or by the *cyclops* mutation induces the growth cones of a specific subset of spinal neurons including a specific commissural neuron, the CoPA (commissural primary ascending) neuron (Fig. 1), to follow aberrant pathways at the ventral midline. The *cyclops* mutation is a pleiotropic, recessive mutation that is 92% penetrant and deletes the floor plate cells (Hatta et al., 1991). CoPA growth cones follow aberrant pathways 57% of the time in the absence of the floor plate compared with 1% in normal embryos.

Although the dramatic increase in errors at the ventral midline of the zebrafish spinal cord demonstrates that the floor plate is a source of guidance cues, these same results suggested that additional sources of guidance cues must exist at the ventral midline since a significant proportion of CoPA neurons followed their correct pathway despite the absence of the floor plate (Bernhardt et al., 1992a,b). Redundancy in guidance cues has been inferred in many other systems where it was observed that growth cones could follow either normal or aberrant pathways following elimination of a source of guidance cues (Raper et al., 1984; Schubiger and Palka, 1985; Harrelson and Goodman, 1988; Hedgecock et al., 1990; Chitnis and Kuwada, 1991).

A good candidate as an additional source of guidance cues at the ventral midline of the zebrafish spinal cord is the notochord. In the zebrafish, the notochord lies just ventral to the floor plate and remains in contact with the spinal cord throughout much of axonogenesis (e.g., see Fig. 2 in this article and Fig. 1 in Halpern et al., 1993). When crossing the ventral midline, the CoPA growth cone extends between the floor plate cell and the basal lamina that separates the spinal cord from the notochord (Bernhardt et al., 1992a). Thus the notochord is located in a position where it could readily affect growth cones at the ventral midline

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Figure 1. Schematic diagram of the spinal cord in an early zebrafish embryo shown in a "open-book configuration." In this configuration the spinal cord is shown as if it was cut longitudinally along the dorsal midline, opened up, and flattened. In this as in the following figures, unless noted otherwise, anterior is to the left. Seven classes of neurons, the commissural primary ascending (CoPA), commissural secondary ascending (CoSA), Kolmer-Agduhr (KA), ventral longitudinal descending (VeLD), Rohon-Beard (RB), dorsal longitudinal ascending (DoLA), and caudal primary motor (CaP) neuron; the two early longitudinal axon tracts, the dorsal (DLF) and ventral (VLF) longitudinal fasciculus; and the midline floor plate (FP) cells are illustrated. The dashed line represents the ventral midline.

of the spinal cord by secreting a guidance cue. Moreover, the notochord does affect development of the ventral spinal cord by inducing the floor plate and motor neurons (Bovolenta and Dodd, 1991; Clarke et al., 1991; Yamada et al., 1991; Roelink et al., 1994).

If the notochord is another source of pathfinding cues, then elimination of the notochord should induce errors in pathfinding and elimination of both the notochord and floor plate may induce a higher rate of errors than would either the elimination of the floor plate or notochord alone. In fact, disruptions in the normal pattern of axons do occur in *Xenopus* and mouse embryos when the notochord and floor plate are missing (Bovolenta and Dodd, 1991; Clarke et al., 1991). Ultraviolet irradiation of the vegetal poles of *Xenopus* embryos prior to the first cleavage produces embryos that lack both the notochord and floor plate. Likewise in the caudal regions of the neuraxis of embryos afflicted with the Danforth's short tail (*Sd*) mutation the notochord is missing and the spinal cord is missing the floor plate. In both cases, some spinal axons are disarrayed. However, it is not presently possible to ascribe these defects to either the missing floor plate or the notochord or both since both the notochord and the floor plate were absent.

In the anterior region of the neuraxis of the *Sd* embryos the spinal cords sometimes are missing the notochord but not the floor plate (Bovolenta and Dodd, 1991). In these regions the pattern of commissural axons in cross sections of the spinal cord appeared identical to those taken from sibling wild-type embryos. This likely demonstrates that the initial circumferential portions of commissural axons to the floor plate were not obviously perturbed. However, the trajectories of these axons once at the floor plate is difficult to discern from cross-sections of spinal cords. For example, mistakes such as turning ipsilaterally rather than contralaterally or posteriorly rather than anteriorly in the floor plate region by some but not all axons would be difficult to detect. Since it is unclear whether commissural axons have abnormal trajectories at the ventral midline in the absence of the notochord alone, the notochord's role for guidance of growth cones in the floor plate region remains an open question.

We tested whether the notochord and the floor plate are both

sources of guidance cues for CoPA growth cones in the zebrafish spinal cord by examining CoPA growth cones in the absence of the notochord and in the absence of the notochord and floor plate. Notochord cells were removed by laser ablation in wild-type embryos and by the *no tail* mutation in which the floor plate is present but the notochord fails to differentiate (Halpern et al., 1993). The notochord and floor plate were eliminated by laser ablating notochord cells in *cyclops* embryos. The results demonstrate that the notochord and floor plate operate in concert to guide growth cones at the ventral midline of the spinal cord.

Materials and Methods

Animals. Zebrafish embryos were collected from a laboratory breeding colony and maintained according to procedures described previously (Myers et al., 1986).

Labeling neurons. In most embryos, the CoPA neurons were labeled with MAb CON1 at 24–27 hr PF as previously described (Bernhardt et al., 1990). MAb CON1 selectively labels the CoPA and RB neurons (cell bodies and axons) in the early spinal cord. These two classes of neurons are easily distinguishable based upon their soma shapes and location. In some wild-type embryos, axons in the embryos were labeled with a MAb against acetylated α -tubulin (Piperno and Fuller, 1985; Bernhardt et al., 1990) following laser ablation of the notochord. Some *cyclops* embryos in which the notochord was laser ablated were doubly labeled with MAb CON1 and the MAb against acetylated α -tubulin. This was accomplished by incubating embryos with anti-acetylated α -tubulin followed by incubating with MAb CON1. In the spinal cord anti-acetylated α -tubulin primarily labels the axons with little labeling of the cell bodies with the exception of the Rohon-Beard (RB) neurons in which both the axons and cell bodies were well labeled (Bernhardt et al., 1990). Thus, in double-labeled embryos, all axons were labeled, but the CoPA axons were distinguishable from others because the CoPA cell bodies and axons were more darkly labeled than other neurons due to the double labeling by MAb CON1 and anti-acetylated α -tubulin.

GABAergic neurons were labeled in notochord ablated *cyclops* embryos as previously described (Bernhardt et al., 1992b). Cells were classified based upon cell soma shape, size and position as well as their axonal trajectories in the spinal cord.

In 12 *cyclops* embryos CoPA neurons were filled intracellularly with Lucifer yellow (LY) dye in the regions of the spinal cord dorsal to the where the notochord had been ablated. Camera lucida drawings were made and photographs taken to record the trajectories of all neurons labeled by LY or antibodies.

Laser ablations. Notochord cells were ablated by focusing a laser microbeam as previously described (Bernhardt et al., 1992a). Notochord cells were laser ablated at 12–14 hr PF embryos (2–4 hr prior to the beginning of axonogenesis by CoPA neurons), in segments 10–14. In embryos in which somites 10–14 were all not yet evident, the extent of the unformed segments was estimated and the notochord cells in this region was ablated. Since in early embryos the notochord consists of a single row of cylindrical cells, the laser beam was focused on the ventral portion of the notochord to avoid damage to the floor plate and ventral spinal cord. Notochord cells showed obvious signs of damage immediately after exposure to the laser microbeam and after several minutes had begun to break up. All experimental animals were examined with DIC optics 10–30 min postablation to ensure that the floor plate was intact in wild-type embryos, the spinal cord was undamaged in both wild-type and *cyclops* embryos, and the notochord cells in experimental regions were successfully destroyed.

In *cyclops* embryos in which the GABAergic neurons were assayed following laser ablation of the notochord, the notochord was ablated in segments 8 through 14.

Histological examination of notochord ablated embryos. In six wild type embryos the specificity and efficacy of the laser ablation procedure was measured by labeling all nuclei with Hoechst dye 2 hr following laser ablation of the notochord cells. Hoechst dye labels the nuclei of cells by intercalating into the DNA and fluorescing when excited by UV light (Critser and First, 1986). Additionally, semithin and ultrathin sections of embryos were prepared and analyzed as described previously (Kuwada et al., 1990).

Analysis of CoPA axon trajectories. The aberrant trajectories followed by CoPA axons in the absence of the notochord were categorized

Figure 2. The notochord can be efficiently and selectively ablated. Comparison of control wild-type embryos (A–C) with wildtype embryos following laser ablation of the notochord (D–F). *A*, DIC micrograph showing the row of midline floor plate cells (between arrows) and the notochord (*N*) in a side view of a whole-mounted control embryo at 17 hr PF. *B*, Light micrograph of a transverse section of a 16 hr PF control embryo showing the midline floor plate cell (arrow) in the spinal cord (*S*). This section was obliquely cut relative to the longitudinal axis so that numerous notochord nuclei are visible in the same plane. The oblique section makes the profile of the control spinal cord appear larger than that of the experimental spinal cord shown in *E*, which was cut orthogonal to the longitudinal axis. Examination of control versus experimental spinal cords in whole-mounted embryos confirmed that the size of the spinal cord was unchanged by notochord ablations. *C*, EM micrograph showing the midline floor plate cell (asterisk) and the basal lamina (*BL* and between arrowheads) between the spinal cord and notochord from the same embryo shown in *B*. *D*, DIC micrograph of a side view of a whole-mounted 14 hr PF embryo 30 min following laser ablation of the notochord showing that the notochord cells (*N*) have been destroyed but the overlying midline floor plate cells (between arrows) are intact. *E*, Light micrograph of a cross-section of a 16 hr PF embryo 2 hr following ablation of the notochord (*N*) showing that the midline floor plate cell (arrow) is intact and that the spinal cord appears normal. *F*, EM micrograph showing the damaged notochord (*N*) and the intact midline floor plate cell (asterisk) and basal lamina (*BL* and between arrowheads) from the same embryo shown in *E*. Scale bars: *A* and *D*, 50 μ m; *B* and *E*, 25 μ m; *C* and *F*, 5 μ m.

into the same classes previously defined for CoPA axons in the absence of the floor plate (Bernhardt et al., 1992a). (1) Axons were classified as normal when they extended to the ventral midline, crossed the midline, turned anterior, reached the halfway point between the ventral midline and the dorsal longitudinal fasciculus (DLF) by two segments, and ascended in the DLF. (2) Axons were classified as ipsilateral/ascending errors when they extended to the midline, turned and extended anteriorly, but did so on the ipsilateral side, that is, failed to cross the midline. (3) Axons were classified as directional errors when they failed to turn anteriorly at the ventral midline. This category includes axons that crossed to the contralateral side and descended; axons that failed to cross the midline and descended; axons that bifurcated with one axon descending and the other ascending; and axons that crossed the midline, turned posterior, but then changed directions to ascend by making a loop or kink. (4) Axons were classified as ventral/ascending errors when they extended to the ventral midline, crossed to the contralateral side, turned anterior, but remained in the ventral half of the spinal cord for an abnormally long distance (more than two segments). (5) Axons were categorized as peripheral errors when they extended out of the spinal

cord near the ventral midline. (6) Axons were categorized as following a circuitous route to the ventral midline when they followed a semicircular or wavy route to the ventral midline. These axons then crossed the midline and ran normally. (7) Axons were categorized as stalled when their growth cones remained near the ventral midline rather than extend further. (8) Axons were categorized as longitudinal when they initially projected and extended longitudinally from either the anterior or posterior pole of the cell body before extending ventrally. These growth cones were unusually large and complex. These categories of CoPA trajectories correspond to trajectories 1–8 in Figure 4.

Results

The notochord can be selectively ablated in embryos without damaging the overlying floor plate

Since the floor plate is known to guide CoPA growth cones and these growth cones extend across the ventral midline of the spinal cord by inserting between the floor plate cell and the basal

lamina that delimits the cord (Bernhardt et al., 1992a), it was important to demonstrate that the notochord could be selectively ablated with a laser microbeam without damaging the overlying floor plate or the basal lamina. Notochord cells in wild-type embryos (12–14 hr PF) were destroyed with a laser microbeam 2–4 hr prior to axonogenesis by CoPA neurons. Examination of these embryos ($n = 8$) 2 hr later with DIC optics demonstrated that the notochord cells were successfully ablated and the floor plate cells were intact (Fig. 2A,D). This was confirmed by examination of light and electron micrographs of cross-sectioned embryos ($n = 3$) (Fig. 2). These cross-sections showed that following exposure of notochord cells to the laser microbeam these cells were destroyed but the basal lamina that surrounds the notochord remained intact. Importantly, electron microscopic comparison of the spinal cord basal lamina in control versus laser ablated embryos revealed no obvious morphological evidence of damage to the basal lamina following laser ablation of the notochord cells. These results demonstrate that the notochord could be selectively removed with a laser microbeam without damaging the overlying floor plate and basal lamina.

In order to quantify the efficacy and selectivity of notochord cell ablations, the notochord was laser ablated in 14 hr PF wild-type embryos, the embryos allowed to develop 2 hr, then fixed and all cell nuclei were labeled with Hoechts dye (data not shown). The experimental segments had the same number of floor plate nuclei as control segments: experimental segments contained 6.75 ± 1.2 nuclei/segment ($n = 24$ segments in 6 embryos) while control segments contained 6.65 ± 1.3 nuclei/segment ($n = 58$ segments in 16 embryos). In the notochord, there were 20 ± 2.5 nuclei/segment in control segments but only 5.86 ± 5.5 nuclei/segment in experimental segments. Therefore, on average our ablation technique destroyed 71% of the notochord cells but did not destroy any floor plate cells.

CoPA axons follow either normal or aberrant trajectories in the absence of the notochord

Loss of the floor plate, either by the *cyclops* mutation or laser ablation, caused CoPA growth cones as well as the growth cones of other specific neurons to dramatically increase pathfinding error rates at the ventral midline (Bernhardt et al., 1992a,b). However, a significant percentage (43%) of CoPA growth cones still followed their normal pathway despite the absence of the floor plate. Based upon this result we hypothesized that a second source of pathfinding cues for CoPA growth cones must exist at the ventral midline. We tested whether the notochord was another source of pathfinding cues for CoPA growth cones by eliminating the notochord either via laser ablation in wildtype embryos or genetically by examining *no tail* mutants in which the notochord fails to differentiate (Halpern et al., 1993).

The notochord cells in five segments of wildtype embryos ($n = 25$) were selectively ablated with a laser microbeam at 12–14 hr PF, 2–4 hr prior to the start of axonogenesis by CoPA neurons. Thirteen to 15 hr later effects on CoPA growth cones were assayed by labeling them with MAb CON1. MAb CON1 selectively labels the cell body, axon, and growth cone of the CoPA (strongly) and Rohon-Beard (RB) (weakly) neurons in the early spinal cord (Bernhardt et al., 1990). Ablation of the notochord resulted in a 26-fold increase in pathfinding errors. In experimental segments nearly all of the 149 CoPA axons assayed extended properly to the ventral midline of the spinal cord but then 26% followed aberrant trajectories once at the midline (see Fig. 4B) compared with 1% of CoPA axons in control em-

bryos (Bernhardt et al., 1992a). For statistical analysis, the proportions of all aberrant CoPAs were arc sine-transformed and the means compared with the Student's *t* test. The proportion of errors following notochord ablations in wild-type embryos was significantly different from that in control, wildtype embryos ($t = 2.72$, $df = 39$, and $P < 0.01$). The classes of errors were comparable to those found in *cyclops* embryos, but the proportion of axons which fell into each class varied from *cyclops* embryos. Most notably a higher proportion of the axons that followed aberrant pathways made apparent directional errors at the ventral midline.

Pathfinding errors were also made by CoPA growth cones when the notochord was eliminated genetically. The notochord fails to differentiate in *no tail* mutant embryos (Halpern et al., 1993). The spinal cord with the exception of the posterior-most segments of these mutants contains a floor plate presumably because notochord precursor cells that express *sonic hedgehog* (Krauss et al., 1993) although unable to differentiate into notochord are able to induce the floor plate (Halpern et al., 1993). In those segments missing the notochord but not the floor plate 38% of CoPA axons assayed with MAb CON1 (73 axons from 10 embryos) had aberrant trajectories (see Fig. 4C). Again nearly all the axons initially extended correctly to the ventral midline, but then some extended aberrantly. The proportion of errors in *no tail* embryos as expected was significantly different from that in control, wild-type embryos ($t = 6.39$, $df = 11$, and $P < 0.005$). The types of aberrant trajectories followed varied from those seen in the absence of the floor plate and in wild-type embryos following laser ablation of the notochord. In *no tail* embryos CoPA growth cones often exited the spinal cord or they stalled near the ventral midline of the spinal cord. However, like wild-type embryos following laser ablation of the notochord, the largest category of errors was the directional category. At present, it is unclear why the types of errors made varied somewhat in *no tail* mutants compared to those seen following laser ablation of the notochord in wild-type embryos. However, the fact that removal of the notochord either genetically or by laser ablation leads to an increase in errors by CoPA axons near the ventral midline demonstrates that the notochord is required for normal error free pathfinding by CoPA growth cones. This conclusion suggests that the notochord is a second source of guidance cues at the ventral midline for CoPA growth cones.

Removal of notochord and floor plate cells induces a higher proportion of CoPA growth cones to follow aberrant pathways than removal of either one alone

To see if the effects on CoPA growth cones by the floor plate and the notochord were additive, CoPA trajectories were examined in the absence of both notochord and floor plate cells. This was accomplished by laser ablating notochord cells in *cyclops* embryos ($n = 26$) and subsequently assaying CoPA axons by labeling them with MAb CON1, anti-acetylated α -tubulin, or intracellular LY injections. In the absence of both the notochord and floor plate 73% of 171 CoPA axons had aberrant trajectories (Figs. 3, 4D). The range of aberrant pathways followed by CoPA growth cones was for the most part similar to those seen in the absence of the floor plate alone. However, 5% of CoPA axons extended longitudinally before extending ventrally towards the ventral midline (Fig. 3B). Nearly all the other CoPA axons extended directly to the ventral midline. Although this class of errors had previously not been observed of CoPA axons, they were observed in another class of commissural neu-

Figure 3. CoPA growth cones follow aberrant pathways in the absence of both the notochord and floor plate. The notochord and floor plate were eliminated by laser ablating the notochord approximately 13 hr prior to assaying CoPA axons in *cyclops* embryos. All panels are DIC micrographs of side views from a 27 hr PF whole-mounted embryos except *C* is from a 24 hr PF embryo. *A*, CoPA neurons (arrows) labeled with MAb CON1 in control wild-type embryos. Shown are the ventrally directed portions of the axons of CoPA neurons that extend to the ventral midline of the spinal cord which is just dorsal to the notochord (*N*). The longitudinal axons that run across the CoPA cell bodies are the ascending axons of CoPA neurons from the contralateral side of the spinal cord. *B*, Aberrant CoPA axons labeled with MAb CON1 following notochord ablation in a *cyclops* embryo. The white star denotes a CoPA neuron whose axon followed an abnormal ventral/ascending trajectory (out of plane of focus). Arrows denote the cell body and the longitudinally projecting axon of a CoPA neuron. Although the axon extends out of the focal plane, it ran into and followed the axon of the nearby CoPA neuron (white star) to the ventral midline, turned anterior, and ascended ipsilaterally. Arrowheads denote two descending growth cones that originated from CoPA neurons from more anterior segments on the contralateral side of the spinal cord. *N* indicates the position of the notochord. The arrow adjacent to the *N* marks the posterior end of the notochord ablated region. All the aberrant CoPA neurons resided in the experimental segments. *C*, An aberrant CoPA neuron from an experimental segment filled with LY in a *cyclops* embryo following notochord ablation. The axon extended to and crossed the ventral midline, but then bifurcated (open arrows) with one branch ascending and the other branch descending. The cell body and ventrally directed portion of the axon is out of the focal plane. *D*, An aberrant MAb CON1 labeled CoPA neuron (asterisk) among other axons labeled with anti-acetylated α -tubulin in an experimental segment of a *cyclops* embryo following notochord ablation. The axon (arrowheads) of this CoPA neuron followed an abnormal ipsilateral/ascending trajectory in segments in which the motor axons (arrows) ipsilateral to the CoPA neuron were normal. *E*, Micrograph of the contralateral side of the same region seen in *D*. The motor axons (arrows) contralateral to the aberrant CoPA shown in *D* are also normal. The axons in the contralateral spinal cord labeled by anti-acetylated α -tubulin are out of the focal plane. Scale bar (for all panels), 50 μ m.

rons, the CoSA neurons, in *cyclops* embryos (Bernhardt, et al., 1992b). Why some CoSA axons in *cyclops* embryos and some CoPA axons in the absence of the floor plate and notochord extend longitudinally is at present unclear. However, it is clear that the action of the notochord and floor plate on pathfinding by CoPA growth cones are roughly additive. CoPA growth cones made errors 73% of the time in the absence of both the notochord and floor plate, but only 57% of the time in absence of the floor plate and 26–38% of the time in the absence of the notochord. The proportion of errors following notochord ablations in *cyclops* embryos was significantly different from that in control *cyclops* embryos ($t = 2.40$, $df = 46$, and $P < 0.01$).

The effects on pathfinding due to the removal of the notochord are cell specific

To verify that the effects on pathfinding following elimination of the notochord are cell specific, neurons whose growth cones are not affected by the elimination of the floor plate were assayed following ablation of the notochord in wild-type ($n = 7$) and *cyclops* ($n = 9$) embryos. First, labeling axon tracts with anti-acetylated α -tubulin (Piperno and Fuller, 1985; Bernhardt et al., 1990) showed that the major spinal tracts, the medial longitudinal fasciculus and the dorsal longitudinal fasciculus, appeared normal (data not shown). Second, the Rohon-Beard (RB) neurons that are located in the dorsal cord and whose axons do

Figure 4. A schematic diagram depicting in an open book configuration the incidence of CoPA trajectories in *cyclops* embryos (A), wild-type embryos following ablation of the notochord (B), *no tail* embryos (C), and *cyclops* embryos following ablation of the notochord (D). The percentage of CoPA axons that had aberrant trajectories is denoted at the top of each panel. In these diagrams, anterior is to the left and the ventral midline is shown besides each cell. Shown are normal CoPA axons (1); ipsilateral/ascending axons (2); axons that have made a directional error (3); ventral/ascending axons (4); axons that exited out of the spinal cord (5); axons that followed a circuitous route to the ventral midline, but then were normal (6); axons that stopped at the midline (7); and axons that initially projected longitudinally in either direction (8). See Materials and Methods. The number of CoPA axons analyzed for each diagram was 201 for A, 149 for B, 73 for C, and 171 for D. The total (99%) in C does not add up 100% due to rounding.

not extend into the ventral cord had axons that followed their normal trajectories (data not shown). Third, for the most part the motor nerves appeared normal suggesting that the motor neurons which are located in the ventral cord and whose growth cones extend near the ventral midline (Eisen et al., 1986; Myers et al., 1986) extended normally in the absence of the notochord (Fig. 3D,E). These results suggest that the effects of notochord removal are cell specific just as they are following the elimination of the floor plate (Bernhardt et al., 1992a).

Occasionally motor nerves were absent or not as thick in experimental segments following notochord ablations (data not shown). This could be due to inadvertent damage of the ventral cord by the laser microbeam or to the normal variability seen in motor nerves at the stages (27 hr PF) assayed. Therefore, to ensure that pathfinding errors made by CoPA growth cones were not due to inadvertent damage of the ventral spinal cord, CoPA axons were assayed only in experimental segments in which both motor nerves appeared normal following ablation of the notochord in both wild-type ($n = 7$) and *cyclops* ($n = 9$) embryos. In these segments the percentage of CoPA growth cones that followed aberrant pathways was 33% in wild-type embryos and 73% in *cyclops* embryos. Therefore, the error rates seen were comparable to those obtained when CoPA growth cones were assayed in embryos in which state of the motor roots was unknown. These results confirm that it is the absence of the notochord rather than inadvertent damage to the ventral cord that is responsible for the pathfinding errors.

The organization of the spinal cord is not affected by notochord ablations

The notochord plays an important role in the dorsal/ventral patterning of the spinal cord by inducing ventral cell types in

a variety of embryos (Placzek et al., 1990). Because of this it is possible that removal of the notochord disrupts the normal cellular organization of the spinal cord in our experiments. The resultant abnormal environment might then induce CoPA growth cones to follow aberrant pathways. This is unlikely for several reasons. First, the ventral spinal cords in the trunk of *no tail* mutants are apparently normal; the cords contain correctly located floor plate cells and differentiation by motor neurons is apparently normal (Halpern et al., 1993). Second, in wild-type and *cyclops* embryos the notochord cells were destroyed relatively late in development (14–16 hr PF) making it unlikely that their ablation disrupted any inductive activity. The examination of notochord precursor cells in *no tail* embryos suggests that floor plate induction may normally occur at the tail bud stage (10 hr PF) (Halpern et al., 1993; Krauss et al., 1993). We confirmed that the cellular organization of the spinal cord following notochord ablations was relatively normal in both wildtype ($n = 7$) and *cyclops* ($n = 9$) embryos. Anti-acetylated α -tubulin labeling of these embryos showed that the RB neurons were located properly in the dorsal cord (data not shown), and for the most part the motor axons appeared normal suggesting that the motor neurons were normal in the absence of the notochord (Fig. 3D,E). Furthermore, labeling these embryos with anti-GABA demonstrated that the four classes of GABA-immunoreactive neurons (the VeLD, DoLA, KA, and a subset of CoSA neurons) found in the early spinal cord (Bernhardt et al., 1992b) were unchanged in location and number when compared with GABA-immunoreactive neurons in control *cyclops* embryos (Fig. 5A,B; Table 1). The fact that the VeLD and KA cells are located in the ventral cord corroborate the contention that patterning in the ventral spinal cord is not disrupted by the ab-

Figure 5. Ablation of the notochord does not change the location and number of specific classes of spinal neurons in *cyclops* embryos. Shown in *A* and *B* are sideviews of spinal cords centered on S9 from whole-mounted 24 hr PF embryos labeled with anti-GABA. The distribution of GABA-immunoreactive neurons are similar in control, *cyclops* (*A*) and notochord ablated, *cyclops* (*B*) embryos. The notochord cells were ablated at 14 hr PF in S8–S14 in *B*. Arrows denote GABA-immunoreactive cells at the ventral midline that are based upon their position likely to be KA cells, and arrowheads denote GABA-immunoreactive cells that are more dorsal than the ventral medial cells and are likely to be VeLD neurons. The dorsally located DA and CoSA neurons are out of the plane of focus in both *A* and *B*. *N* depicts the notochord in *A* and the remnant of the notochord in *B*. The distribution of anti-CON1 labeled CoPA neurons are similar in control, *cyclops* (*C*) and notochord ablated, *cyclops* (*D*) embryos. Shown in *C* and *D* are sideviews of spinal cords centered on S11 from whole-mounted 27 hr PF embryos labeled with anti-CON1. The notochord cells were ablated at 14 hr PF in S10–S14 in *D*. Arrows denote the ventrally running portions of the CoPA axons. In *D* the arrowhead marks the longitudinal axons of the CoPA neurons from the contralateral side. The diagonally extending portions of axons from contralateral CoPA neurons are labeled with anti-CON1 in both *C* and *D*. Asterisk in *D* denotes the remnant of the previously ablated notochord. Scale bar (for all panels), 40 μ m.

lation of the notochord. Furthermore, labeling notochord ablated, wild-type and notochord ablated, *cyclops* embryos with anti-CON 1 showed that the location and number of CoPA neurons was not affected by removal of the notochord (Fig. 5*C,D*; Table 2). These data suggest that in our experiments elimination of the notochord did not alter patterning within the spinal cord much like elimination of the floor plate, which is also known to play an important role in dorsal/ventral pattern-

ing within the spinal cord (Yamada et al., 1991), did not alter patterning (Bernhardt et al., 1992a). Therefore, the increase in pathfinding errors by CoPA growth cones in the absence of the notochord was likely due to the loss of notochord derived guidance cues. This suggestion in conjunction with the guidance activity of the floor plate, further suggests that proper pathfinding at the ventral midline of the spinal cord requires cues from both the notochord and floor plate.

Table 1. Distribution of GABA-immunoreactive spinal neurons in notochord ablated and notochord intact *cyclops* embryos

	CoSA	VeLD	DoLA	KA
Notochord ablated	3.2 ± 1.4	0.5 ± 0.2	1.1 ± 0.9	4.7 ± 2.0
Notochord intact	3.8 ± 0.7	0.7 ± 0.3	1.9 ± 0.9	4.8 ± 1.4

Ablation of the notochord does not change the number of GABA-immunoreactive neurons in the spinal cords of *cyclops* embryos. For each class of GABA-immunoreactive neurons the average number and the standard deviation of GABA-immunoreactive neurons per segment are given. Data were compiled from five notochord ablated *cyclops* embryos and five control *cyclops* embryos. Cells were classified based upon cell soma shape, size, and position as well as in some cases their axonal trajectories in the spinal cord (Bernhardt et al., 1992b).

Discussion

The notochord is a source of local guidance cues for growth cones at the ventral midline of the spinal cord

CoPA growth cones increased their error rate once they arrived in the region of the ventral midline of the spinal cord when the notochord was missing. This was observed in both wild-type embryos following the laser ablation of the notochord and in *no tail* mutants. Previously, no obvious effects on axonal trajectories was noted in the spinal cords of *no tail* mutants (Halpern et al., 1993). Using MAb CON1 that selectively labels CoPA neurons, we found that CoPA growth cones followed aberrant pathways 38% of the time in *no tail* mutants despite the fact that the overall pattern of axonal tracts was relatively normal. Similarly, CoPA growth cones followed aberrant pathways 26% of the time in wildtype embryos following the ablation of the notochord. Furthermore, the types of errors made in *no tail* mutants varied somewhat from those made following laser ablation of the notochord in wild-type embryos. The lower error rate and differences in types of errors in wildtype embryos is likely due to several factors. First, the laser ablation technique destroyed on average 71% of the notochord cells while no notochord cells differentiate in *no tail* mutants. The residual notochord cells may have contributed enough guidance cues so that fewer CoPA growth cones made errors compared with *no tail* mutants. Second, although notochord cells were killed several hours prior to axonogenesis by CoPA neurons this was after they had already begun to differentiate into notochord cells. Therefore, the notochord may have already produced some guidance cues that may have been retained in the ventral midline region of the spinal cord following ablation of the notochord. These residual cues may then account for the differences in error rates and types. Alternatively, the *no tail* mutation may directly affect CoPA neurons and, therefore, lead to a higher incidence of path-finding errors or to certain types of errors compared to wild-type embryos following ablation of the notochord. This is unlikely since CoPA neurons still express the CON1 antigen in *no tail* mutants, and the *no tail* gene which is the zebrafish homologue to the mammalian *T* gene is not expressed in the spinal cord (Schulte-Merker et al., 1992). Regardless of the reasons for the discrepancy in error rates and types between *no tail* and wildtype embryos following ablation of the notochord, both sets of results suggest that the notochord participates in guiding CoPA growth cones at the ventral midline of the cord. Since the notochord is separated from the growth cones within the spinal cord by two sets of basal laminae, the notochord derived cue is likely to be a locally secreted one that directs growth cones at or near the ventral midline of the spinal cord.

Table 2. Distribution of CoPA neurons in notochord ablated wild-type and *cyclops* embryos

	Proportion of cells in their normal position	Average cells per segment
Wild-type (<i>n</i> = 23)	142/142	1.2 ± 0.2
<i>cyclops</i> (<i>n</i> = 11)	76/76	1.4 ± 0.3

Ablation of the notochord does not change the number or location of CoPA neurons assayed with anti-CON1 in S10–S14 at 27–29 hr PF. CoPA neurons were determined to be normally located along the dorsal/ventral axis if their cell bodies were located in the dorsal third of the spinal cord. Control, wild-type embryos have 1.7 CoPA neurons per segment in S6–S11 at 28 hr PF (Bernhardt et al., 1990). *n* denotes the number of embryos that were assayed.

An alternative explanation to notochord derived guidance cues in our experiments is that removal of the notochord may prevent the floor plate from fully differentiating. This could result in a morphologically normal floor plate that fails to express guidance cues, and therefore result in CoPA errors. This seems unlikely for two reasons. First, the alternative hypothesis predicts that the error rate of CoPA axons in the absence of the notochord would be equal to that seen in the absence of the floor plate. However, the error rate for CoPA axons in the absence of the notochord (26–38%) is much less than that seen in the absence of the floor plate (57%). Second, the alternative hypothesis predicts that the error rate for CoPA axons in the absence of the notochord and floor plate should be equal to that seen in the absence of the floor plate alone. However, we found that laser ablation of the notochord in *cyclops* embryos induced a much higher error rate (73%) than that seen in control *cyclops* embryos (57%). These results suggest that although it is possible that the notochord is still required for complete differentiation of the floor plate, expression of guidance cues by floor plate cells does not require continued presence of the notochord. Therefore, it seems that our results are more consistent with a notochord derived guidance cue for CoPA growth cones.

Our conclusion that the notochord produces guidance cues for spinal growth cones may also apply to the commissural growth cones in mouse embryos. In some *Sd* mice, anterior regions of the embryos lack a notochord, yet retain a floor plate (Bovolenta and Dodd, 1991). In these regions of the spinal cord commissural axons extended properly to the floor plate, but their trajectories at the ventral midline was unclear. If some of the commissural axons followed aberrant pathways at the ventral midline, then the participation of notochord derived guidance cues would not be limited to the zebrafish.

Does the notochord secrete a chemotropic factor that attracts growth cones to the ventral midline?

The mammalian floor plate but not the notochord secretes a chemotropic factor that attracts the growth cones of commissural neurons (Tessier-Lavigne et al., 1988; Placzek et al., 1990; Bovolenta and Dodd, 1991; Kennedy et al., 1994; Serafini et al., 1994). For the most part errors by CoPA growth cones in the absence of the notochord were limited to the region of the ventral midline in the zebrafish embryo; extension by CoPA growth cones to the ventral midline appeared not be affected. This observation is similar to that following elimination of the floor plate (Bernhardt et al., 1992a), and suggests that neither the floor plate nor the notochord is required for extension to the ventral midline. This may mean that neither emits an chemoattractant

in the zebrafish. However, in these experiments it is possible that the absence of these structures may have actually decreased the rate of extension to the ventral midline by CoPA growth cones. Therefore, a role for a putative floor plate and/or the notochord derived chemoattractant cannot be ruled out in the zebrafish. Consistent with this contention is the fact that a small proportion of CoPA growth cones extended longitudinally before extending ventrally when both the floor plate and the notochord were absent. Furthermore, some CoSA growth cones also extended longitudinally before extending ventrally in the absence of the floor plate (Bernhardt et al., 1992b).

The notochord and floor plate act in concert to guide growth cones at the ventral midline of the spinal cord

Increased error rates by growth cones due to the elimination of the notochord and/or floor plate demonstrate that both these structures participate in guiding growth cones in the zebrafish spinal cord. What sorts of molecules derived from these cells may serve as local guidance cues at the ventral midline? First, the fact that the notochord is separated from the spinal cord by a basal lamina suggests that the notochord secretes a guidance molecule. On the other hand, the floor plate derived cues may be cell surface or secreted molecules (Bernhardt et al., 1992a). Second, the notochord and floor plate may utilize different molecules or utilize the same guidance molecule. At present our findings do not allow one to distinguish between these possibilities. In principle the delineation of how and what growth cones are affected by these two cell types may yield some clues regarding the nature of the guidance molecules at the ventral midline. For example, if the notochord and the floor plate utilized the same molecule, then one might expect that removal of the notochord would induce the same types of error as removal of the floor plate. Alternatively, if different molecules were employed, then different types of errors may be induced by the removal of the notochord compared to removal of the floor plate. In fact, the types of errors observed in CoPA growth cones in the absence of the notochord were not identical to those previously observed in the absence of the floor plate (Bernhardt et al., 1992a,b). This may indicate that the guidance molecules derived from the notochord are not exactly the same as those derived from the floor plate. Similarly, if the same molecule is utilized, then one might expect that the two structures would guide the same subset of growth cones. Alternatively, if different molecules were employed, then different growth cones would be guided by the two structures. The floor plate guides a subset of the early growth cones in addition to the CoPA growth cones. These are the growth cones of the CoSA, KA, and VeLD neurons (Bernhardt et al., 1992a,b). All these growth cones normally contact the ventral midline region of the spinal cord and, therefore, were thought to interact with the floor plate. In this study, only the CoPA neurons were assayed following the removal of the notochord. How the growth cones of the CoSA, KA, and VeLD neurons are affected by the absence of the notochord is at present unknown.

If the same guidance molecule is used by the floor plate and notochord, then both cell types may be sources of the molecule or the notochord may secrete the molecule which then attaches to the surface of the floor plate and perhaps the basal lamina. However, it is unlikely that the notochord is the sole source of guidance cues. If it were, then one would expect the growth cone error rate in *no tail* embryos that never differentiate notochord cells (Halpern et al., 1993) to be greater than in *cyclops*

embryos that never differentiate floor plate cells (Hatta et al., 1991). In fact the error rate is higher in *cyclops* embryos compared to *no tail* embryos. Thus the notochord and floor plate must both be sources of the guidance molecule if the same molecule is utilized.

A variety of putative growth cone guidance molecules are expressed by the notochord and floor plate in a variety of embryos and could potentially serve as local guidance cues at the ventral midline. For example, tenascin which has been demonstrated both to support and inhibit axonal extension *in vitro* (Faissner and Kruse, 1990; Wehrle and Chiquet, 1990; Lochter et al., 1991) is a secreted molecule found in the basal lamina surrounding the notochord (Mackie et al., 1988; Riou et al., 1988). The floor plate expresses a number of secreted and cell surface molecules known to promote axonal extension *in vitro*. These include f-spondin (Klar et al., 1992), P84 (Chuang and Lagenaur, 1990), and s-laminin (Sanes et al., 1990). Interestingly, both the floor plate and the notochord express netrin-1 (Kennedy et al., 1994; Serafini et al., 1994) and DM-GRASP (also known as SC1, BEN, and JC7) in the chick (Burns et al., 1991). However, an apparent zebrafish homologue to chick DM-GRASP is not expressed by the floor plate in the early cord (Kanki et al., 1994), but antibodies against goldfish neurolin (also a homolog to chick DM-GRASP) apparently recognize the floor plate in both goldfish and zebrafish (Paschke et al., 1992; Laessing et al., 1994). Despite this wealth of information regarding the expression and *in vitro* action of these molecules, so far their actual *in vivo* function within the spinal cord has not been elucidated. The challenge for the future will be to utilize the ability to manipulate genes in zebrafish (Weinberg, 1992; Mullins et al., 1994) to actually identify the molecules that control growth cones and determine how they function in the well characterized spinal cord.

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