Adjacent pioneer commissural interneuron growth cones switch from contact avoidance to axon fasciculation after midline crossing

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Abstract

Commissural interneurons (CI) of the vertebrate spinal cord are guided ventrally toward the floor plate, but subsequently cross the midline and select a longitudinal fascicle at specific dorsal–ventral (D–V) positions. We examined at high resolution the detailed behaviors of individual pathfinding CI growth cones on the ipsilateral and contralateral sides of the spinal cord of living Xenopus embryos. We find that pre-crossing CI growth cones exhibit distinct pathfinding behaviors compared to post-crossing axons and that the behavioral switch occurs immediately upon crossing to the contralateral side. Groups of pioneer commissural axons typically extend simultaneously toward the ventral midline following discrete paths with separation between adjacent commissurals apparently maintained through contact inhibition. In contrast, shortly after crossing the midline, commissural axons turn longitudinally and begin to fasciculate with other crossed CIs. However, growth cones of crossed commissurals often select their final D–V longitudinal track through a series of rapid step-like dorsal adjustments that may be due to differential fasciculation with longitudinal axons. Together, our results suggest that guidance of commissural axons is controlled in part through interactions among CIs that switch rapidly from avoidance to fasciculation after midline crossing.

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Introduction

Commissural interneurons (CIs) transfer sensory information and help coordinate movements through connections onto neurons in the contralateral spinal cord. During their development, commissural axons locate their final synaptic partners through a multi-step process that involves guidance toward a series of intermediate target sites (Goodman and Tessier-Lavigne, 1997). Initially, CI cell bodies in the dorsal spinal cord extend their axons circumferentially toward the floor plate at the ventral midline. Guidance toward the midline is believed to result from the opposing influences of chemorepellents secreted from the roof plate and chemotactracts released from cells in the floor plate (Kaprielian et al., 2001). Subsequently, CI growth cones exit the floor plate and begin to extend into the intermediate regions of the contralateral spinal cord. However, shortly after crossing the midline, commissural axons turn to extend along a longitudinal fascicle, suggesting that pathway selectivity is rapidly modulated.

Several molecules that guide CI growth cones at intermediate choice points are known. For example, cells in the floor plate express chemoattractants such as Netrin and Sonic Hedgehog (SHH) that promote the ventral navigation of commissural axons toward the floor plate (Huber et al., 2003; Schnorrer and Dickson, 2004). Although molecules in the floor plate attract commissural axons, growth cones of CIs do not remain at the midline but project into the contralateral spinal cord due to both a loss of sensitivity to midline attractants and gain of sensitivity to midline repellents (Lyuksyutova et al., 2003; Kaprielian et al., 2001; Shirasaki et al., 1998). Increased responsiveness to midline repellents such as Slit, semaphorins and B class ephrins may not only promote commissural axon extension beyond the floor plate, but may help determine the D–V position of longitudinal growth and prevent recrossing of the midline (Simpson et al., 2000; Zou et al., 2000). Once across the midline, Wnt proteins and SHH appear to control the anterior–posterior (A–P) direction of outgrowth (Bourikas et al., 2005; Lyuksyutova et al., 2003), while Ephrins may form a...
dorsal boundary to growth (Imondi and Kaprielian, 2001). Interestingly, interactions with cues in the floor plate are required to alter responsiveness of CI growth cones to guidance cues subsequently encountered on the contralateral side of the spinal cord (Shirasaki and Murakami, 2001).

Commissural axon trajectories have been analyzed within fixed and labeled spinal cord preparations in several species (Dodd et al., 1988; Imondi and Kaprielian, 2001; Stoeccki et al., 1997). Although the details of ipsilateral growth is complicated by the large number of axons that extend toward the midline, following midline crossing most CIs appear to turn abruptly to ascend (although some descend or branch in *Xenopus*, Zebrafish and mouse) longitudinally along the ventral fascicle at the contralateral margin of the floor plate of the spinal cord (Kadison and Kaprielian, 2004; Kuwada et al., 1990; Roberts and Clarke, 1982). More recent studies showed that a majority of longitudinal axons eventually deviate away from the midline to extend along a more dorsal fascicle (Imondi and Kaprielian, 2001; Kadison and Kaprielian, 2004). These static views of fixed axons provide a read-out of pathways followed by axons and reveal the morphologies of growth cones at points along those pathways. However, static images provide no indication about the dynamic behavior of pathfinding growth cones. Live cell imaging of individual growing commissural axons before and after crossing the ventral midline would show how changes in growth cone motility lead to pathfinding behaviors and also allow for comparison with artificial guidance assays performed in vitro. For example, guidance of axons up gradients of chemotactic factors such as Netrin in vitro occurs by an adaptive process involving cyclical periods of desensitization followed by resensitization (Ming et al., 2002). Cyclical changes in growth cone sensitivity toward Netrin results in a zig-zag pattern of growth up gradients of guidance cues in vitro. It has been suggested that such an adaptive mechanism may be a common feature of chemotropic guidance where growth cones must continually readjust their sensitivity within a gradient and may therefore be evident during ventral commissural axon outgrowth in vivo. Although the behavior of crossing axons has been described at several midline choice points in live embryos (Bak and Fraser, 2003; Halloran and Kalil, 1994; Hutson and Chien, 2002; Mason and Wang, 1997; Sretavan and Reichardt, 1993), the growth patterns of living spinal CIs has not been assessed.

In this study, we examine pathfinding behaviors of individual CI growth cones before and after crossing the ventral midline. First, we quantified commissural axon projection patterns in three-dimensional reconstructions of fixed embryos. Next, we assayed the behavior of live, fluorescent CI growth cones during guidance toward and away from the midline on the ipsilateral and contralateral sides of the spinal cord. We find that pioneering pre-crossing commissurals rarely fasciculate and exhibit highly serpentine projections to the floor plate. Live imaging shows that CI growth cones often make extensive filopodial contacts with adjacent commissural axons. Filopodial contact between neighboring CIs does not lead to fasciculation, but rather appears to prevent veil protrusion between contacting filopodia. However, filopodial adhesion is often maintained between commissural axons during guidance to the floor plate. Once across the midline, most CIs turn sharply to fasciculate along a longitudinal fascicle. In contrast to commissurals on the ipsilateral side of the spinal cord, filopodial contact between neighboring CIs on the contralateral side of the spinal cord often leads to veil protrusion and fasciculation. Together, our findings suggest that distinct CI pathfinding behaviors on each side of the spinal cord are the result of interactions among commissural axons that switch from contact avoidance to close association after midline crossing.

Materials and methods

Whole-mount immunocytochemistry

Staged embryos were fixed in a 4% paraformaldehyde/4% sucrose solution in Ca²⁺/Mg²⁺ free phosphate-buffered saline (CMF-PBS) overnight at 4°C. After rinsing extensively in CMF-PBS, the spinal cord was exposed by dissecting somites and notochord. Embryos were blocked and permeabilized with several exchanges of GDB buffer (0.5% Fish Gelatin (Sigma), 0.2% Triton X-100) in CMF-PBS over a 1 h period. Primary antibodies (diluted in GDB) to β-tubulin isotype I/I (monoclonal JD3.3D8, 1:500; Sigma) and neurofilament (monoclonal 3A10; 1:25, Developmental Studies Hybridoma Bank) were applied overnight at 4°C. After several washes in GDB over 1 h at room temperature, embryos were incubated in Alexa-Fluor (Alexa-488, -546 or -647; Molecular Probes) conjugated secondary antibodies (1:250 in GDB) for at least 2 h at room temperature. After extensive rinsing in GBD, filamentous actin was labeled by incubating embryos in Alexa-Fluor conjugated phallolidin (Molecular Probes) for 15 min. Embryos were given a final wash in CMF-PBS then mounted by pinning laterally onto Sylgard (Dow Corning) dishes for confocal imaging.

Blastomere injection and embryo preparation for live spinal cord imaging

An EGFP expression construct in the pCS2+ vector for RNA synthesis (Dave Turner, University of Michigan) was provided by Maureen Ruchhoeft. One ventral blastomere (targets dorsal spinal cord) at the 8-cell stage was injected with 0.1–1 ng of capped mRNA transcribed in vitro (mMessage machine, Ambion). GFP fluorescent embryos at 24 h post fertilization (hpf) were pinned laterally onto a Sylgard dish containing 1 × MR. The spinal cord was exposed on one side by dissecting the skin and underlying axial myotome as described (Gomez and Robles, 2003). After extensive rinsing in MR solution, embryos were positioned for confocal imaging.

Confocal microscopy

Fluorescence imaging was performed on an Olympus FluoView 500 confocal imaging system mounted on an AX70 upright microscope using 40 × (NA 0.8) or 60 × (NA 0.9) water immersion objectives. Fluorophores were excited using 488, 543 and 647 nm laser lines. Pinholes were set at or below one Airy disc size for optimal optical sectioning of immunofluorescently labeled spinal cords. For imaging fixed preparations, the depth of the spinal cord on one side was collected at 1–2 um Z-steps for each wavelength using sequential imaging to minimize fluorescence bleed-through. Images were collected over the entire length of the spinal cord containing commissural axons viewed from the left, right and ventral aspect. To image the ventral surface, the spinal cord was separated from the embryo and pinned floor plate up using insect pins. For live imaging of GFP signals, deeper optical sections (>1 Airy disc) were collected using fewer individual z-steps per time point to minimize light exposure. For live cell imaging, image stacks were collected at 5-min intervals.

Image analysis

Maximum projection reconstructions and 3D rendering of image stacks was performed using Velocity software (Improvision). The angle of approach to the...
ipsilateral ventral fascicle, the ventral midline and to the contralateral ventral fascicle was determined by measuring the angle the distal 20 μm of commissural axons made with respect to the long axis at these positions. Length measurements and 3D coordinates of axon paths were determined by hand selecting axon positions from Z-planes using MetaMorph software (Universal Imaging). Individual 3A10 positive CIs could typically be followed even within fascicles by careful inspection of images planes of lateral views. The tortuosity of axon paths was calculated using a 3D analysis package, which measures the sum of curvature along each curve normalized for path length (Bullitt et al., 2003). Measurements of fluorescence intensities and growth cone morphologies were made from maximum projections using ImageJ software (W. Rasband, NIH). Spreadsheet (Excel), statistical analysis (InStat), graphics (Cricket Graph) and desktop publishing (Photoshop) software were used to create final figures.

Results

Three-dimensional reconstruction of CI projections in the Xenopus spinal cord

To begin to assess normal axon pathfinding by commissural axons we examined immunofluorescently labeled whole mount neural tube stage embryos. The 3A10 neurofilament antibody specifically labels CIs but not other classes of spinal neurons, however only after initial axon extension (Serafini et al., 1996; Shim et al., 2005). On the other hand, the β-tubulin antibody labels all spinal neurons from the earliest stage of process formation (Moody et al., 1996). Fluorescent phallloidin labels filamentous actin, which highlights all terminal growth cones. Triple labeled spinal cords were viewed laterally and ventrally by confocal microscopy along the length of the spinal cord (Fig. 1). This approach allowed us to accurately quantify CI growth patterns in comparison to other classes of neurons in three dimensions. Although we cannot distinguish between different types of CI, we can be confident we are viewing all CIs in spinal cords based on their immunolabeling pattern, cell body position and axonal projection pattern. Moreover, since the spinal cord develops rostrocaudally, CIs at different developmental stages are identified by viewing the entire extent of the early embryonic spinal cord. For example, at the anterior end of the mid neural tube stage spinal cord, crossed CIs are identified as neurofilament and β-tubulin positive axons that turn to extend longitudinally between the dorsal and ventral longitudinal fascicles.

Fig. 1. Whole mount Xenopus spinal cord fluorescently labeled and viewed laterally and ventrally by confocal z-series reconstructions. (A) Schematic representation of the spinal cord viewed laterally with approximate distances between dorsal and ventral fascicles and the midline indicated in microns. (B–D) Maximum projections of confocal z-series image stacks of a 28 hpf (NF stage 25) whole mount spinal cord viewed laterally (B—left side, D—right side) and ventrally (C). Rostral is to the left in all images and dorsal is up in (B) and (D). All neurons are detected with antibodies to β-tubulin (blue), while more mature CIs only are detected with antibodies against neurofilament (red). F-actin is detected using fluorescent phallloidin (green), where it is concentrated in terminal growth cones of all neurons (boxed region in C). The dorsal longitudinal fascicle (DLF), composed primarily of Rohon–Beard sensory neuron axons, and the ventral longitudinal fascicle (VLF), composed primarily of axons of motoneurons (MNs) and interneurons are indicated (white arrowheads in B and D). CIs extend ventrally and at this relatively late stage of development, some CIs appear as pairs, with one CI following closely behind its pioneer (red arrowheads in B, C and boxed region in B). The ventral view of the spinal cord allows visualization of the midline with CI axons crossing from both the right and left halves of the spinal cord. Dashed lines perpendicular to the longitudinal axis in (C) indicate cross-sectional views shown in (E). (E) Three cross-sections taken at successive rostral to caudal positions along the ventrally oriented spinal cord (at positions marked by dashed lines in C) show the cord narrows posteriorly. These images also show the peripheral nerve roots (arrowheads). (F) High magnification image of boxed region in (C) shows large flattened growth cones near the midline, which are reminiscent of paused growth cones in vitro. (G–I) High magnification image of boxed region in (B) shows the close association of a pioneer CI together with a follower commissural axon. Note the pioneer axon expresses both neurofilament (G, arrow) and β-tubulin (H, arrow), whereas the follower only expresses β-tubulin (H, arrowhead). Actin in merged image (I, green) shows the growth cone of the follower CI (I, arrowhead). Scale bar, 50 μm in (B–E), 15 μm in (F) and 18 μm in (G–I).
ventral fascicles (Fig. 1, fuchsia axons). At the same time, developmentally less mature neurofilament negative and β-tubulin positive CIs with growth cones still on the ipsilateral side of the spinal cord are observed more posteriorly (Fig. 1, blue axons). Some commissural axons with growth cones near the midline are also observed among the crossed axons in the anterior spinal cord, suggesting that CIs continue to extend axons at all A–P positions throughout development. Imaging the ventral aspect of the spinal cord allows us to visualize CI axons extending within the floor plate near the ventral midline. From this perspective, many large CI growth cones are detected within the floor plate (Fig. 1F), consistent with previous studies examining growth cones near the midline (Bovolenta and Dodd, 1990; Bovolenta and Mason, 1987). 3D rendering of Z-stacks allows complete rotation of the spinal cord and therefore visualization of neurons at any perspective (Movie 1).

CI axons follow distinct paths toward and away from the ventral midline

To begin to assess commissural axon pathfinding in the Xenopus spinal cord, we immunolabeled a time series of spinal cords fixed at 1 h intervals beginning at the earliest stages of outgrowth and aligned the anterior limit of each cord as described in the Method section. Although spinal cord development proceeds in a rostral to caudal fashion, at the earliest stages of commissural axogenesis, many pioneering CIs typically extend axons simultaneously toward the ventral midline (Figs. 2A, B). Since β-tubulin antibodies label all neurons beginning at axon initiation, we can be certain pioneers are detected by this assay (Moody et al., 1996; see below). These pioneering CIs can be viewed as parallel processes aligned perpendicular to the axis in a highly regular array. Adjacent pioneering commissural axons often extend toward the midline in close proximity (mean separation 20.0 ± 15 μm, n = 101 CIs from 3 spinal cords), suggesting that CI growth cones may interact during ventral pathfinding. Overlapping phalloidin labeling of adjacent growth cones suggests filopodial contacts between growth cones are common (Figs. 2A, B; insets), however, fasciculation of pioneering commissurals before crossing the midline was never observed. This is in contrast to Rohon–Beard and motoneuron (MN) axons which fasciculate closely within longitudinal fascicles even at the earliest stages of outgrowth (Figs. 1 and 2A). Unlike the pioneer commissural axon segments, individual axons often cannot be resolved within the tight fascicles of the longitudinal tracts. The separation between neighboring CIs is maintained as growth cones pass across the midline, but occasionally commissural axons do intersect and cross over one another (Fig. 2C). The rare cases in which ipsilateral commissural axons intersect are typically those that exhibit an A–P directional bias before crossing the midline. However, at later stages of development, follower axons appear to fasciculate along CI pioneers (Fig. 1G) so that regular spacing between commissural neighbors is maintained.

It was previously reported that commissural axons turn abruptly anteriorly or posteriorly to join a longitudinal track shortly after crossing the midline (Yoshida et al., 1998). These findings suggest the CIs become responsive to longitudinal fascicles and A–P guidance cues soon after crossing into the contralateral spinal cord. Importantly, CI growth cones must contact the floor plate to become responsive to contralateral guidance cues (Shirasaki and Murakami, 2001). This suggests
that commissural axon turning begins only after growth cones reach the floor plate, but the precise location of CI turning has not been determined. We examined a developmental time series of the ventral aspect of young spinal cords to track the complete path of the pioneer crossing CIs. Since all neurons are labeled with β-tubulin antibodies, we can be confident that only pioneering axons are examined. However, given that young CIs are not 3A10 positive, we must rely on cell body position and axon project pattern to identify pre-crossing neurons as CIs. To assess where turning begins, the angle of commissural axon crossing the ipsilateral ventral fascicle, the midline and the contralateral ventral fascicle was measured in embryos over several stages of development. To assess whether there was any directional bias at each of these choice points, individual CIs were grouped into ascending (180°–90°) and descending (90°–0°) axons. We find that commissural axons extend toward and across the ipsilateral ventral fascicle and midline nearly perpendicular to the longitudinal axis with no apparent directional bias (Fig. 2, Table 1). However, once across the midline, axons begin turning anteriorly or posteriorly, with the majority turning to ascend (Table 1). Axon turning begins at varying positions after crossing the midline, with some CIs turning before the contralateral ventral fascicle and other axons extending to a more dorsal position before turning (Fig. 3; see also Figs. 5 and 8). Since some crossed commissural axons approach the contralateral ventral fascicle at an acute trajectory, it appears that turning began before extensive contact with the fascicle (Table 1).

Although the overall direction of commissural axon extension is nearly perpendicular to the axis on the ipsilateral side of the spinal cord, close examination suggests that CI neurites follow highly tortuous paths during ventral guidance. To analyze this effect we quantified the mean curvature of CI and non-commissural axons. We compared the trajectories of commissural axons that extend perpendicular to the axis with neurons that extend longitudinal axons, including commissural axons that have turned onto a longitudinal fascicle. To quantify mean axon curvature, 3D coordinates of axon paths were determined from image stacks using MetaMorph software. These coordinates were then used to calculate curvature using 3D tortuosity analysis software (see Materials and methods) (Bullitt et al., 2003). Comparison of axon curvatures shows that CIs that extend perpendicular to the longitudinal axis are significantly more tortuous than longitudinally projecting axons (Table 2). A cell-specific difference in the degree of axon tortuosity also argues that differences are not due to fixation artifact. This behavior is specific for axons crossing perpendicular to the axis, as commissural axons extend along relatively straight paths within the contralateral longitudinal fascicles. The serpentine appearance of many commissural axons could be the result of either periodic side-to-side turning of growth cones during process extension or from the local bending of previously extended axons. The later possibility may be caused by adhesion to other cells such as intersecting axons, which directly displace perpendicular axons. However, local displacement is not likely responsible for all serpentine axons, as some neurites are highly curved even in regions

![Image](image-url)

**Fig. 3.** Crossed CI axons turn longitudinally at various points before or after contact with the contralateral ventral fascicle. (A–B) Lateral view of a triple-labeled embryonic spinal cord showing the first crossing CIs (arrowheads mark the ventral fascicle). More mature commissural axons are labeled with antibodies to neurofilament (A), while F-actin, which marks growth cones, is labeled with fluorescent phalloidin (B). (C) Merge of β-tubulin labeling (blue), F-actin labeling in (green) and neurofilament labeling of CIs (red). Some CIs approach the contralateral ventral fascicle at acute angles (arrows in C) indicating that turning began within the floor plate. In addition, a growth cone near the ventral fascicle has F-actin localized to one side (arrow in B), suggesting turning has begun. However, other crossed commissural axons extend beyond the ventral fascicle before turning (yellow arrow in C). Scale bar, 40 μm.

Table 1

<table>
<thead>
<tr>
<th>Ipsilateral ventral fascicle</th>
<th>Contralateral ventral fascicle</th>
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<tbody>
<tr>
<td>Average angle (ascend)</td>
<td>103.1 ± 11.8</td>
</tr>
<tr>
<td>Average angle (descend)</td>
<td>80.4 ± 9.0</td>
</tr>
<tr>
<td>Average angle (total)</td>
<td>94.1 ± 15.5</td>
</tr>
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The direction of CI extension was determined at three successive positions along CI pathways (see Materials and methods). CIs were binned into processes with ascending (<90°) and descending (>90°) bias at each position. The total average angle of approach was also determined at each position. There is no apparent directional bias until CI axons reach the contralateral ventral fascicle, where the direction of outgrowth begins to turn anteriorly and posteriorly. N = 145 CIs from 5 embryos.

* P < 0.005.
devoid of longitudinal axons, such as within the floor plate (Fig. 2D). Interestingly, periodic turning of axons is predicted for chemotropic guidance of CIs by a gradient of Netrin in vitro (Ming et al., 2002).

Spinal commissural axon outgrowth slows during midline crossing

Previously it was shown that pioneering forebrain commissural axons slow near the midline (Bak and Fraser, 2003; Mason and Wang, 1997). To determine if similar behavior was likely of *Xenopus* spinal cord CIs, we estimated the rate of commissural axon extension from the aligned developmental time series of immunolabeled spinal cords (Fig. 2). The distance of CI growth cones from the midline was determined for both uncrossed and crossed axons from the first appearance of tubulin-positive processes through turning at a contralateral longitudinal fascicle. The position of all growth cones relative to the ventral midline was measured along the length of the spinal cord beginning with the anterior most CIs within aligned spinal cords (Fig. 4). As described previously, many CIs initiate axons over several segments of the spinal cord simultaneously, although a developmental gradient is clear as growth cones are more advanced anteriorly. Between 22 and 23 hpf, axons extend rapidly toward the midline on the ipsilateral side (Fig. 4). Once at the midline, axon outgrowth appears to slow, as suggested by clustering of growth cones near the midline at all developmental time points. After crossing the floor plate, commissural axons accelerate to extend rapidly away from the midline, suggested by the scattering of growth cones on the contralateral side of the anterior spinal cord. The extension of secondary commissural axons (anterior ipsilateral points) after the pioneers likely contributes the large variability of growth cone positions in later embryos. Since developmental progression can vary among individual embryos, it was important to confirm this analysis with live imaging.

Growth cones become morphologically more elaborate after midline crossing

Previous studies suggest that growth cones become larger and more complex at decision regions (Halloran and Kalil, 1994; Mason and Wang, 1997; Skaliora et al., 2000; Tosney and Landmesser, 1985). To assess the morphology of CI growth cones at points along their pathway, we reconstructed at high resolution the spinal cords of fixed embryos expressing EGFP in CIs. GFP labels the entire volume of individual neurons including fine filopodia and veils, which are often not detected with phalloidin labeling in vivo. Furthermore, through targeted blastomere injections, only one side of the dorsal spinal cord is labeled, allowing us to visualize individual CIs in the absence of overlapping fluorescence from neighboring or crossed CIs. To help localize GFP-expressing CI growth cones to positions along their pathway, spinal cords were colabeled for neurofilaments and filamentous actin (Figs. 5A–C). We find that growth cones exhibit a dramatic increase in complexity after crossing into the contralateral spinal cord as determined by the formula

![Fig. 4. CI axon outgrowth slows at the midline, but accelerates after crossing.](image-url)
This is in contrast to growth cones within the floor plate and on the ipsilateral side of the spinal cord, which can be large, but typically appear rounded and have few filopodia (Figs. 1F and 5B). The greater complexity of crossed CI growth cones is evident by their increased number and length of filopodia as well as increased branch points. Although most crossed commissural axons do not maintain permanent branches after turning longitudinally (Yoshida et al., 1998), during turning small branchlets often form at intersection points with the contralateral fascicles (Fig. 5C). These types of branch points were not observed in commissural axons that intersect with fascicles on the ipsilateral side of the spinal cord, although small filopodia are evident along the shaft of some crossing axons (Fig. 5B). Once within a longitudinal fascicle, CI growth cones simplify, but remain highly filopodial (not shown). We also quantified the relative F-actin content of growth cones near the ipsilateral ventral fascicle, midline and contralateral ventral fascicle by measuring the intensity of fluorescent phalloidin staining. We find that the amount of F-actin parallels growth cone complexity, as growth cones turning at the contralateral ventral fascicle have significantly greater F-actin as compared to pre-crossing CI growth cones (Figs. 5D, E and F).

Alignment of adjacent commissural axons by contact avoidance on the ipsilateral side of the spinal cord

Next, we examined the extension of live CIs in early neural tube stage embryos during pioneering extension toward the floor plate. For this, EGFP mRNA was again injected into individual blastomeres, targeting dorsal neurons based on established lineage maps (Jacobson and Hirose, 1981). Although only subsets of CIs are labeled by blastomere injection, the axons observed in these experiments are likely pioneers at the early stages of development studied (e.g. see...
commissural axons grew slowly toward the midline and stalled midline varies widely among individual CIs (0.58 ± 0.22 μm/min, n = 13) on the ipsilateral side of the spinal cord. Some commissural axons grew slowly toward the midline and stalled or retracted briefly both at the ipsilateral ventral fascicle and within the floor plate (Fig. 6). Slowly growing CIs frequently exhibited marked retraction upon contact with longitudinal axons within the ventral fascicle (Fig. 6). Other commissural axons extended rapidly and at a constant rate of outgrowth toward the floor plate. The reason for these differences is uncertain, but may be related to the prevalence of neighboring axons, which appear to inhibit growth cone motility on contact (see below).

Chemosotropic guidance of growth cones up a gradient of Netrin in vitro occurs through an adaptive process that involves repeated periods of desensitization and resensitization. The result of adaptive chemotaxis toward Netrin is the serpentine turning of axons with a periodicity of around 20 min (Ming et al., 2002). Since CIs are thought to be guided toward the midline by a gradient of floor plate-derived Netrin in vivo, growing axons would be expected to display a similar adaptive behavior in the spinal cord. To illustrate the turning behavior of growth cones during guidance to the ventral midline, we plotted the x–y coordinates of the leading edge of commissural axons over time (Fig. 7A). Coordinate plots show that pioneering CI growth cones take highly tortuous paths toward the floor plate. Typically, growth cones migrating toward the midline exhibit repeated sharp changes in their direction of outgrowth. When measured as absolute angles with respect to the longitudinal axis, we find that the direction of growth changes dramatically during extension (mean ± standard deviation: 9.1 ± 10.6°/5 min, n = 13 CIs at 324 time points). However, individual CI growth cones do not follow stereotypical paths, rather axons undergo seemingly random changes in direction (Fig. 7A). Moreover, changes in the direction of outgrowth do not occur with a regular temporal periodicity as predicted for an adaptive process in a chemotropic gradient. Instead, turning often occurs immediately following growth cone filopodial contact with adjacent commissural axons (Fig. 7B). Interactions between neighboring commissural axons occurred repeatedly during guidance to the floor plate (mean ± standard deviation: 7.6 ± 3.8 contacts/CI trajectory, n = 5), but in no instances did pioneering axons fasciculate. Although we cannot be certain that all CIs were labeled in these experiments, the density of labeled processes (mean separation between adjacent labeled axons = 13.9 ± 2.1 μm, n = 8) was not significantly different than that observed in fixed immunostained spinal cords at a comparable stage. Therefore, as predicted from immunostaining of embryonic spinal cords, it appears that CIs use heterotypic avoidance with adjacent extending processes to maintain ventral projections. Interestingly, although filopodial contact with adjacent commissural axons seems to prevent fasciculation, contacts between filopodia and CI axons appear highly adhesive. Adhesion between filopodia and neighboring commissural axons is apparent as CI axons are pulled toward retracting filopodia resulting in the local bending of contacted axons (Fig. 7F). Despite this apparent contradiction, long lasting adhesive contacts can be maintained after growth cone collapse (Zimmer et al., 2003).
Pioneer commissural axons turn and fasciculate together after midline crossing

Studies of axon pathfinding by spinal CIs in fixed tissues have demonstrated that the projection patterns of crossed axons on the contralateral side of the spinal cord are distinct from pre-crossing axons (Bernhardt, 1994; Bovolenta and Dodd, 1990; Imondi and Kaprielian, 2001). Although guidance decisions of crossed CIs are known to be due in part to the gain and loss of responsiveness to certain floor plate-derived cues as well as A–P gradient cues (Bourikas et al., 2005; Lyuksyutova et al., 2003), it is not clear how cellular interactions change after midline crossing. To assess dynamic changes in commissural axon extension on the contralateral side of the spinal cord, we expressed GFP in CIs on the ipsilateral side of the spinal cord and viewed axons crossing into the unlabeled side. Visualizing contralateral commissural axon outgrowth is ideal as fluorescent processes

Fig. 7. CI axons exhibit contact-mediated repulsive turning during ventral extension on the ipsilateral side of the spinal cord. (A) x–y Cartesian coordinate plots of the position of ipsilateral CI growth cones extending toward the ventral midline. The locations of five example growth cones with normalized longitudinal (x coordinate) starting positions are shown. The spinal cord–notochord boundary is the bottom this plot. Growth cones undergo seemingly random and often sharp direction changes during ventral growth sampled at 5-min intervals. The yellow plot line illustrates the path taken by the example growth cone shown in (B, yellow arrowhead). (B–G) GFP-expressing CI imaged with 4D confocal microscopy during extension of its axon toward the ventral midline. Maximum projections of confocal z-series image stacks are displayed at 10–20 min intervals. Note multiple filopodial contacts with neighboring CI axon (C, E, F). These contacts do not result in fasciculation, rather are often followed by turning away from the contact site suggesting avoidance (D, G). However, filopodial contacts appear adhesive since they can locally displace the contacted axon (arrow in F). Scale, 20 µm.

Fig. 8. CIs exhibit distinct pathfinding behaviors after crossing to the contralateral side of the spinal cord. (A–I) Time series of GFP expressing CI axons extending into the contralateral spinal cord. Dashed line in (A) indicates the spinal cord–notochord border. Dorsal is up and rostral is to the left in all images. CI growth cones (arrowheads in A) are observed as they first emerge from the opposite side of the spinal cord at 0 min. Many growth cones turn anteriorly or posteriorly shortly after crossing and extend longitudinally in the ventral spinal cord (arrows in D). However, some axons extend further dorsally before turning longitudinally (arrowheads in D). Dorsally projecting axons turn longitudinally at varying positions and often exhibit transient branches (arrow in B and H) often appear at turning points. However, longitudinally extending CI axons often make D–V positional adjustments by rapid ventral shifts in position (matched lettered arrowheads in G–I). As axons continue to cross to opposite side of the spinal cord they begin fasciculating upon one another (arrows in E, G and I). A dorsal barrier to growth also becomes apparent over time marked by turning axons with stabilized filopodial contacts (arrow in F). Scale, 50 µm.
are easily distinguished extending into the unlabeled side of the spinal cord.

We first quantified the rate of contralateral commissural axon outgrowth and found that it also varied among individual neurons. The average rate of crossed CI outgrowth (0.66 ± 0.25 μm/min, n = 10) was not significantly different than ipsilateral CIs (P > 0.05). However, unlike on the ipsilateral side, individual growth rates did not slow consistently at discrete locations during pathfinding, except at the dorsal boundary to growth (Fig. 8) where axon outgrowth consistently slowed (not shown).

The behavior of crossed CI growth cones is dramatically different from the same population just prior to midline crossing. Consistent with our observations in fixed preparations, axons of crossing CIs begin turning longitudinally at variable D–V positions within the contralateral spinal cord. We find that 55% (n = 60 from 8 embryos) of ascending and descending axons begin turning immediately after crossing to the contralateral side of the spinal cord (Fig. 8). The first turning commissural axons begin fasciculating immediately as they emerge from the floor plate, suggesting that CIs rapidly switch from being non-permissive to being permissive toward one another. The close and persistent contacts between growth cones and axons (fasciculation) were distinct from the avoidance behaviors observed on the ipsilateral side of the spinal cord. In addition, since pioneering CIs do not fasciculate within the floor plate as determined by ventral images of immunolabeled spinal cords (Fig. 2), changes in CI self-affinity must occur over a very short distance and brief time period. However, not all commissural axons turn at the ventral fascicle. Rather some extend further dorsally before turning onto a longitudinal fascicle. These axons typically extend diagonally with respect to the longitudinal axis, suggesting their motility is influenced by both D–V and A–P guidance cues. As axons extend dorsally we observe both transient and permanent branching near the ventral fascicle (Fig. 8), which was not observed during ventral extension of CIs on the ipsilateral side of the spinal cord. Importantly, dorsally projecting axons always turn sharply and occasionally retract briefly near the dorsal fascicle. We find that axons that make their final turn at this apparent dorsal inhibitory boundary occasionally form stable and presumably adherent filopodial contacts with this dorsal region (Fig. 8).

Although some crossed CIs project directly to their final D–V position, many others reach their final longitudinal tract only after a series of short D–V position shifts. As described previously, many axons turn longitudinally onto the ventral fascicle shortly after midline crossing. However, these axons do not always remain associated with the ventral fascicle. At least 30% (n = 33) of these ventral longitudinal axons defasciculate transiently, only to reorient longitudinally along a more dorsal fascicle (Fig. 8). This is likely an underestimate, as the entire trajectory followed by crossed CIs was not always observed. Defasciculation occurs by a sharp dorsal turn so commissural axons are again extending nearly perpendicular to the longitudinal axis. This process may repeat several times as axons adjust to their proper longitudinal track, leaving a “staircase” impression of contralateral commissural axons. The outcome of this pattern of outgrowth is diagonally directed contralateral CI projections to the dorsal spinal cord, which is prominent in the developing mammalian (Kadison and Kaprielian, 2004), avian (Imondi and Kaprielian, 2001) and zebrafish (Kuwada et al., 1990) spinal cords.

Discussion

In this study, we used high-resolution 3D confocal microscopy to examine the cellular behaviors of individual CI pioneers during midline crossing in both fixed and live spinal cords of early Xenopus embryos. Before crossing the midline, CI growth cones exhibit contact mediated heterotypic avoidance for neighboring commissural axons during ventral extension. The apparent repulsion between neighboring axons may promote the highly regular alignment of non-overlapping CI processes that extend perpendicular to the A–P axis of the spinal cord. Moreover, live imaging shows that individual CI growth cones follow irregular paths during ventral guidance due to contact-dependent turning. These observations suggest that pioneering CIs exhibit contact-mediated heterotypic avoidance (homotypic being avoidance with self) toward adjacent commissural axons during ventral pathfinding. Such collateral avoidance may support the collective guidance of CIs toward floor plate derived chemottractants, in that individual axons that become misguided can be corrected by properly guided neighbors. By viewing the ventral surface of the spinal cord we find that the behavior of CI growth cones changes shortly after crossing the ventral midline. Some crossing commissural axons begin turning before reaching the contralateral ventral fascicle, which could be due to filopodial contact with this fascicle. Other crossed commissural axons select their proper D–V longitudinal tract through a series of step-like dorsal adjustments in position. Importantly, avoidance among pre-crossing CIs is negated rapidly as crossed CI axons fasciculate closely upon one another on the contralateral side of the spinal cord. Our results suggest that dynamic regulation of fasciculation between commissural axons controls CI pathfinding behaviors on the ipsilateral and contralateral sides of the spinal cord.

Time-lapse imaging has been used to study the behavior of living growth cones during midline crossing in several systems (Bak and Fraser, 2003; Hutson and Chien, 2002; Mason and Wang, 1997; Sretavan and Reichardt, 1993). Most of these studies find that axon outgrowth slows or stalls near the midline and that growth cones become larger and more complex at this site. Although we cannot image live CI growth cones nearest the midline due to presence of the closely apposed notochord, analysis of fixed spinal cords at several developmental stages suggests that pioneer commissural growth cones also slow and become larger at this decision region. However, unlike midline crossing at the mammalian optic chiasm, we find that CI growth cones do not become morphologically more complex until they exit the floor plate and reach the contralateral longitudinal fascicles.
This difference is likely due in part to how we measure complexity, which rates highly filopodial and branched growth cones as most complex and round growth cones as least complex. Given that spinal CIs do not select between alternative pathways within the floor plate, it is not surprising that complexity does not increase until after growth cones reach the contralateral side of the spinal cord. Once at the contralateral longitudinal fascicles, growth cones turn and branch, which undoubtedly contributes to their increased complexity. This result is consistent with observations made of cortical neuron growth cones after crossing the corpus callosum (Halloran and Kalil, 1994). Importantly, in our study, we have focused on pioneering commissural axon outgrowth. As reported by Bak and Fraser (2003), it is likely that follower CIs do not slow or become larger at the midline, but may increase in complexity on the contralateral side of the spinal cord as they select their proper longitudinal track and direction of outgrowth.

The ventral floor plate of the spinal cord is an important intermediate target site that initiates changes in CI growth cones that prevent axon recrossing and promote distinct pathfinding behaviors on the contralateral side of the spinal cord (Colamarino and Tessier-Lavigne, 1995; Kapielian et al., 2001). For example, CI growth cones are believed to lose responsiveness to the chemoattractant Netrin and gain responsiveness to the chemorepellents Slit, semaphorins and ephrins at the midline. Loss of responsiveness to Netrin may be due to Slit-mediated silencing of Netrin (Stein and Tessier-Lavigne, 2001), while gain of responsiveness to Slit (Zou et al., 2000) may be due to temporally and spatially precise insertion of Robo receptors into the plasma membrane of growth cones (Long et al., 2004; Sabatier et al., 2004). These changes function to repel commissural axons out of the floor plate and prevent recrossing of crossed axons. However, CIs must undergo additional changes that facilitate distinct axon pathfinding behaviors on the contralateral side of the spinal cord. These changes should allow growth cones to respond to A—P and D—V guidance cues such as Wnts and SHH (Bourikas et al., 2005; Luksyutova et al., 2003). Since crossed CIs in the Xenopus spinal cord may either ascend or descend and do so at varying D—V longitudinal tracts, contact with floor plate-derived cues may activate unique programs in distinct populations of CIs.

We find that pioneering CIs change their responsiveness toward each other from contact avoidance to fasciculation shortly after midline crossing. This change appears to occur rapidly since CIs close to the midline typically maintain a discrete distance from adjacent axons, but fasciculate closely with neighbors during longitudinal turning (Fig. 2). If we conservatively estimate that fasciculation begins at the contralateral ventral fascicle and a rate of outgrowth of 50 μm/h, then CI growth cones must switch their responsiveness for one another within 1 h. The molecular basis of this switch is unknown, but it may due to changes in the surface expression of guidance cue receptors or modulation of intracellular second messengers that regulate the effects of existing receptors. Spatial and temporal control of receptor expression may involve new protein synthesis (Brittis et al., 2002; Piper et al., 2005) or insertion of pre-existing receptors (Bouchard et al., 2004). Changes in the expression of cell adhesion molecules that control axon fasciculation may best explain our results. For example, CIs were shown to upregulate L1 and NrCAM upon contact with floor plate cells (Dodd et al., 1988; Matise et al., 1999; Stoeckli and Landmesser, 1995). However, more recent evidence suggests L1 may be expressed even in pre-crossing CIs (Tran and Phelps, 2000). As changes in L1 expression may be most evident on the initial crossing axons that do not fasciculate until after midline crossing, L1 expression should be reexamined in these individual pioneering commissural axons.

What may be the function of heterotypic avoidance of ipsilateral CIs in early developing CI pathways? One possibility is that contact inhibition between adjacent CIs allows for error corrections not achieved by chemotropism toward Netrin alone. Since CI growth cones often reorient ventrally after contact with neighboring CIs, this type of error correction appears common. Thus, chemotropic guidance of ipsilateral pioneering commissural axons may be supported by the population of CIs, where large stochastic deviations in ventral extension by individual CIs are corrected by properly aligned neighbors. However, if later extending commissural axons fasciculate upon particular pioneering CIs, then establishing discrete commissural tracks early in development may be maintained later in development. In fact, the first follower CIs often fasciculate closely with pioneers (Figs. 1B, G), so organization appears to be maintained at these early stages. The formation of distinct CI tracts may provide ordered extension into the contralateral spinal cord for subsequent guidance decisions. For example, in order to respond properly to contralateral A—P guidance cues, CI growth cones may need to extend onto the contralateral side of the spinal cord at specific longitudinal positions.

Important outstanding questions remain unanswered regarding commissural axon guidance. For example, what short-range molecules at the midline reprogram growth cones to respond differentially to guidance cues on the contralateral side of the spinal cord and how is growth cone responsiveness to these cues altered? Candidate proteins would have to be highly localized so they would affect only post-crossing CIs. One possibility is that commissural growth cone contact with extracellular matrix molecules within the basal lamina underlying the floor plate alters responsiveness to contralateral cues. In fact, CI growth cones have been shown by electron microscopy to first contact the basal lamina near the floor plate (Bernhardt, 1994; Yaginuma et al., 1991). How interactions with ECM components might alter growth cone behavior on the contralateral side of the spinal cord is not clear, but as discussed previously, new protein synthesis or receptor insertion by growth cones may be important factors. As an alternative, the rapid changes we observe in growth cone behaviors after crossing the midline may be accounted for by changes in intracellular signaling. Support for this comes from findings that the cell-substrata can regulate responsiveness to...
soluble axon guidance cues (Hopker et al., 1999). Future studies should examine whether intracellular signaling can also acutely modulate axon fasciculation.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ydbio.2005.09.049.

References


