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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 chemoattractants 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

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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; Stoeckli 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 chemoattractive 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 Ca^{2+}/Mg^{2+} 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/II (monoclonal JDR.3B8, 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 phalloidin (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 Volocity software (Improvision). The angle of approach to the

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