Guidance of commissural growth cones at the floor plate in embryonic rat spinal cord

PAOLA BOVOLENTA* and JANE DODD†
Department of Physiology & Cellular Biophysics and Center for Neurobiology & Behavior, Columbia University, 630 W 168th Street, New York, NY 10027, USA

*Present address: Instituto Ramon y Cajal, Consejo Superior de Investigacion Cientifica, Avenida Dr Arce 37, 28006, Madrid, Spain
†To whom correspondence should be addressed

Summary
The floor plate of the embryonic rat spinal cord has been proposed to act as an intermediate target that plays a role in the pattern of extension of commissural axons. To begin to examine the role of the floor plate in axon guidance at the midline, we have studied the precision of the commissural axon projection to and across the floor plate during development. To delineate the pathway, the fluorescent carbocyanine dye, Di-I, has been used as a probe. We show that commissural axons traverse the floor plate and turn rostrally at its contralateral border with remarkable precision. Axons were not observed to turn ipsilaterally and turned only upon reaching the contralateral edge of the floor plate. Virtually all commissural axons follow this route. The morphology of commissural growth cones was also examined. As they encountered the floor plate, commissural growth cones became larger and increased in complexity. The reorientation of axons in register with the floor plate boundary and the change in the morphological properties of commissural growth cones as they traverse the midline suggest that the floor plate may act as a guidepost with functions similar to cells that have been implicated in axon guidance in invertebrates.

Key words: axon guidance, intermediate target, growth cone, commissural neuron, floor plate, Di-I.

Introduction
The patterning of axonal projections in the developing nervous system is an early step in the formation of neuronal connections. Studies in vertebrates and invertebrates (Bate, 1976; Blair and Palka, 1985; Edwards, 1982; Eisen et al. 1986; Harris, 1984; Kuwada, 1986; Lance-Jones and Landmesser, 1981a,b; Raper et al. 1983; Scholes, 1979; Westerfield et al. 1986) have shown that the choice of axonal pathways is a selective process, suggesting the existence of guidance cues within the environment. Such cues may be associated with the neuroepithelium, with other axons or with intermediate or final cellular targets of developing axons.

Evidence for the existence of intermediate cellular targets that influence axonal trajectories comes from studies of developing invertebrate axonal projections. Landmark or guidepost cells serve as contact-mediated guidance cues for growth cones (Bastiani and Goodman, 1984, 1986; Bate, 1976; Bentley and Caudy, 1983a,b; Bentley and Keshishian, 1982; Taghert et al. 1982). The existence of cells that serve equivalent functions in vertebrates has not been established, although there are several sites that represent important decision regions in the projection of certain axons.

These regions include the lumbosacral plexus, an intermediate region in the pathway of motor axons (Lance-Jones and Landmesser, 1981a,b), and the optic chiasm, a decision region in the projection of retinal axons towards the lateral geniculate nucleus (Godement et al. 1990; Silver, 1984; Taylor, 1987; Wilson et al. 1988). However, the mechanism of axonal patterning at these intermediate targets does not appear to involve the interaction of growth cones with specific cell types that function equivalently to an invertebrate guidepost cell. In contrast, the floor plate of the embryonic rat spinal cord, that is an intermediate target in the projection of a class of commissural axons, may have properties similar to those of landmark or guidepost cells.

In the rodent, the earliest population of commissural neurons differentiate in the dorsal spinal cord and extend axons ventrally. The initial projection of these neurons is adjacent to the lateral edge of the spinal cord (Dodd et al. 1988; Holley, 1982; Windle and Baxter, 1936) but changes abruptly at a point just dorsal to the motor neuron column (see Fig. 1). At this point, the axons project medially and ventrally towards the midline floor plate. Commissural axons cross the midline by travelling through the floor plate, and afterwards turn from the transverse plane of the spinal cord into the
longitudinal plane to join the rostrally projecting contralateral ventral funiculus (Holley, 1982; Holley et al. 1982; Wentworth, 1984). The floor plate may act as an intermediate target in the trajectory of commissural axons in two ways. First, the floor plate has been shown to release a chemotropic factor that influences the direction of growth of commissural axons in vitro and may contribute to the navigation of commissural axons towards the ventral midline in vivo (Placzek et al. 1988; Tessier-Lavigne et al. 1988; Placzek et al. submitted). Second, the floor plate may provide local guidance cues for commissural growth cones at the midline. The floor plate expresses a number of surface antigens that are not expressed by the surrounding neuroepithelium and that may play a role in contact-mediated guidance of growth cones. In addition, the expression of the axonal glycoproteins TAG-1 and L1 by commissural axons is altered coincidently with passage of the axons across the floor plate (Dodd et al. 1988). Since both L1 and TAG-1 are thought to be involved in axon extension (Stallcup and Beasley, 1985; Fischer et al. 1986; Furley et al. 1990), the regulation of the spatial expression of these two axonal glycoproteins at the floor plate may be important for appropriate guidance. Finally, in the absence of the floor plate, the pathway of commissural axons at the midline is perturbed, suggesting that the floor plate plays an important role in commissural axon guidance (Bovolenta et al. 1988).

The functional interaction between commissural axons and the floor plate provides an opportunity to examine the role of a set of anatomically and antigenically defined cells in contact-mediated axon guidance in the vertebrate. The floor plate extends supraspinally through the mesencephalon (Kingsbury, 1930). Thus, it may be possible in this system to address more generally the issue of the nature of cues that mediate axonal passage across the midline of the central nervous system and the formation of commissural pathways.

To begin to determine whether the floor plate functions in vivo to provide local guidance cues, we have examined the morphology and growth pattern of commissural axons as they navigate towards and across the floor plate. We have used Di-I (1,1'-diotadecyl-3,3',3'-tetramethylindocarbocyanine perchlorate, Molecular Probes) to label commissural axons and analyze their trajectory and the behaviour of their growth cones as the axons encounter and navigate the floor plate. We find that the projection pattern of individual commissural axons is precise. Virtually all labelled axons project straight across the floor plate in the transverse plane of the spinal cord and make a right-angled turn to project longitudinally. The change in direction is made only upon reaching the contralateral edge and is exactly in register with this edge. During the initial part of the contralateral longitudinal projection, axons apparently maintain contact with the longitudinal face of the floor plate. These observations suggest that passage across the midline and into the ventral funiculus are dependent on the floor plate. The morphology of the growth cones of commissural axons changes as growth cones encounter distinct regions of the pathway and suggest that contact-mediated mechanisms of guidance operate at the midline.

Materials and methods

Di-I labelling of commissural axons

Spinal cords were dissected from rat embryos on embryonic days (E)11 to 14 (where E0=day of vaginal plug). The dissected tissue extended rostrally as far as the caudal tip of the developing fourth ventricle and caudally to the level of the hind limb bud. The spinal cord was split open longitudinally at the roof plate to obtain an 'open-book' configuration (see Fig. 1), and the preparation was then fixed by immersion in 4% paraformaldehyde (in 0.1M phosphate buffer, pH 7.4) at 4°C overnight, and washed in phosphate buffer containing 0.9% NaCl (PBS).

Glass micropipettes with tip diameters of 5–10 μm were filled with Di-I (1,1'-diotadecyl-3,3',3'-tetramethylindocarbocyanine perchlorate, Molecular Probes) (2.5 mg ml$^{-1}$ in DMSO) by capillarity. For each application 15–25 nl of Di-I was injected into the spinal cord using an automated micro-injection system (Eppendorff). Commisural neuron cell bodies were retrogradely labelled by pressure injection of the Di-I solution into the contralateral ventral funiculus or into the floor plate. Anterograde labelling of commissural axons was obtained by injecting the dye into the dorsal region shown to contain commissural neurons (see Fig. 1). After Di-I application, the tissue was again placed in 4% paraformaldehyde and kept in the dark, at room temperature, for 4–20 days, depending on the distance that the Di-I was required to travel (Godement et al. 1987). The preparations were then washed with PBS or Tris buffer, mounted with buffer on a glass slide, and viewed and photographed using phase contrast and epifluorescence optics (Zeiss). The position and boundaries of the floor plate were easily identified under phase-contrast optics (Fig. 1). In addition, in some experiments the extent of the floor plate was established using monoclonal antibodies that selectively label the floor plate (Dodd et al. unpublished) (Fig. 5). The projections of labelled commissural axons were observed in whole mounts of the entire embryonic spinal cord in the open-book configuration described above (see Fig. 1). An advantage of this configuration is that it permits the visualization in the same optical plane of the entire pathway, with the exception of the earliest extension from the cell body that is anyway obscured by diffusion of Di-I around the injection site.

Characterization of injection site and extent of axonal filling

In order to determine the position of commissural neuron cell bodies within the whole-mount preparation at different developmental stages, Di-I was applied to the floor plate or to the contralateral ventral funiculus as described above (data not shown). Commisural neuron cell bodies were mainly restricted to the dorsal region (lateral part of the preparation) of the spinal cord. In younger embryos, the commissural neurons were clustered more dorsally than in older embryos. The information obtained from retrograde labelling was used to standardize the position of injections of Di-I into the ventral-most region of dorsal spinal cord such that maximum numbers of cell bodies and proximal axons of commissural neurons developing at any given stage or rostrocaudal level should be exposed to Di-I. The time necessary to fill the entire trajectory of a commissural axon at each age was assessed.
Fig. 1. Trajectory of commissural axons in the embryonic rat spinal cord: orientation of the 'open-book' preparation.
(A) Schematic diagram of a section of an E12 rat spinal cord cut in the transverse plane, showing the location of commissural neurons (c) in the dorsal spinal cord, close to the roof plate (r). Commisural axons extend initially towards the ventral spinal cord parallel to the external limiting membrane and then extend ventromedially towards the midline floor plate (f). They cross the floor plate and turn rostrally into the ventral funiculus. (B) Double-exposed phase-contrast and fluorescence micrograph of a transverse vibratome section of an E13 rat spinal cord. (Ventral region of one side shown only). Commisural axons (c) have been labelled with Di-I by injection into the dorsal part of the intact spinal cord. Axons have almost reached the floor plate (f). (C) Schematic diagram and (D) phase-contrast and fluorescence micrograph of a spinal cord such as that in A, in the open-book configuration. In this preparation, the floor plate (f) occupies the midline (dotted line in D indicates the ipsilateral edge) and the dorsal spinal cord is lateral. The projection of commissural axons towards and across the floor plate and within the ventral funiculus can be observed in the same optical plane. Di-I was injected at widely spaced points along the dorsal spinal cord as indicated by arrows. X= B 400; D 100. Diagrams are not drawn to scale.
4-5 days of incubation were required to visualize completely the axons of commissural neurons that had not yet extended as far as the floor plate, while longer times (6-20 days) were required for detection of growth cones in the contralateral portion of the spinal cord. The commissural neuron population differentiates over a relatively protracted period (Altman and Bayer, 1984). Thus, in preparations in which the leading axons had reached the contralateral side of the spinal cord, younger axons had extended less far. It was therefore possible to observe the growth cones and axons of commissural neurons at several developmental stages in a single preparation.

**Photoconversion of the fluorescent labelling**

DiI-labelling permitted visualization of detailed growth cone morphology. Examination of fluorescence images indicated that the morphology of commissural growth cones changed markedly as they encountered distinct regions in the pathway. However, since these images cannot be used to obtain quantitative information about growth cone complexity, we used the fluorescence emission from Di-I to oxidize diaminobenzidine (DAB) and generate an insoluble reaction product following the procedures described for Lucifer Yellow by Maranto (1982) and for other fluorescent markers by Sandell and Masland (1986).

Briefly, the tissue was rinsed with Tris Buffer (0.1 M, pH 8.2) and placed on a microconavity slide. The tissue was covered with a drop of DAB solution (1.5 mg ml⁻¹ Tris buffer) and the slide was placed on the microscope stage and exposed to 528 nm illumination focussed through a 20x long working distance objective (Nikon). The DAB solution was replaced every 10 min. This procedure resulted in the fading of the fluorescent signal and the generation of a brown DAB reaction product. The photoconversion took approximately 20-30 min, after which the tissue was rinsed in buffer, dehydrated through alcohols, cleared in xylene and embedded in Permount. This procedure resulted in a stable brown reaction product at sites of Di-I label and permitted details of the growth cone to be assessed. The dimensions of each growth cone were measured on camera lucida drawings of DAB-labelled growth cones as described below.

**Quantitation of growth cone size and morphology**

We measured the shape of each growth cone by making camera lucida drawings of preparations in which photoconversion of DiI had been performed. The number of filopodia was counted and the maximum width in the horizontal plane and the maximum length were measured. The area was calculated as the product of these two measurements. Although the depth or thickness of each growth cone was not measured, a subjective appraisal of the three-dimensional complexity was made. The growth cones fell into two classes. We define simple growth cones as those of small size (between 9-12 μm in length and 2-4 μm in width), thin and blunt and with no more than two filopodia. Growth cones that were designated complex had two or more filopodia and were expanded in area (between 12-18 μm in length and 4-9 μm in width) and volume.

**Results**

The development of the commissural pathway was analysed by observing individual commissural axons at various stages of growth in fixed embryos of different ages. In rats, the commissural neuron population differentiates over a protracted period of approximately 36 h (Altman and Bayer, 1984). At stages in which the leading axons had reached the contralateral spinal cord younger neurons had extended axons over a shorter distance towards the floor plate. Therefore, two stages in the development of commissural neurons could be distinguished. During the first phase, the earliest commissural axons extend through an environment consisting of undifferentiated neuroepithelial cells and motor neurons, while at later stages of development, the older commissural axons are already established in the environment through which new axons extend.

The behaviour of axons and the morphology of growth cones observed at distinct points in the pathway were essentially independent of position of neurons along the rostrocaudal axis. However, since there is a rostral-to-caudal gradient of development in the spinal cord, the embryonic age at which events were observed depended on position in the rostrocaudal axis. Information with respect to the age at which observations were made is given below for axons at an approximately midthoracic level, except where stated otherwise.

**The projection of commissural axons towards the floor plate in the transverse plane of the spinal cord**

We first examined the projection of commissural axons during their extension towards the floor plate as they travelled in the transverse plane of the spinal cord (see Fig. 1). The axons extend initially ventrally, close to the lateral edge of the spinal cord, as far as the motor neuron column. At this point they alter direction and grow ventromedially towards the floor plate (Fig. 1A,B). It was difficult in this study to identify single axons or initial axonal segments in the most dorsal region of the pathway because Di-I diffuses from the site of injection. We therefore confined our analysis of the initial part of the pathway to the ventro-medial projection from the motor column to the floor plate.

**Timing and pattern of growth towards the floor plate**

To determine whether the earliest commissural axons that project towards the floor plate extend independently of other commissural axons, the pattern of this early growth was examined.

The first axons reached the floor plate by E11.5. In preparations taken from E11.5 to E12 a range of axons, extended over different distances towards the floor plate, was observed (Fig. 2A). Some of the leading commissural axons had grown through the entire ipsilateral half of the spinal cord and had reached the floor plate (Fig. 2B). Other axons had not yet reached the floor plate but had grown some distance towards the ventral midline. Although these axons extended through an environment containing the axons of the oldest commissural neurons, the overall density of axons was low (with axons spaced up to 30 μm apart), so that most axons probably extended independently of each other at this stage. Injections of larger volumes of Di-I resulted in a similar density of labelled axons. The axons grew in the transverse plane towards the floor
Axon patterning at the floor plate

439

Fig. 2. The extension of commissural axons towards the floor plate. Whole mounts of an E11.5 (A) and a caudal E12 (B,C) spinal cord open-book preparation showing the earliest axons growing towards the floor plate (dotted lines indicate the ipsilateral edge of the floor plate in each case) in the transverse plane (see Fig. 1C). The axons extend towards the floor plate with little deviation. The growth cones are simple with single filopodia. The filopodium on the more advanced axon in B has just reached the ipsilateral floor plate edge, as shown by the phase contrast view. x350. Photomontage has been used in A and B to show details that would otherwise be out of focus.

plate so that axons at different rostrocaudal levels were parallel to one another.

In older embryos (E12.5–13), axons travelling towards the floor plate took a similar course to those in younger embryos and were intermingled loosely with the axons that had already reached the floor plate (Figs 3,4). While the axons did not appear to be tightly apposed to one another it was not possible with the resolution of light microscopy on whole mount preparations to determine whether the growth cones of late commissural axons fasciculated with each other or upon older axons that had already reached the floor plate.

The pathway taken by commissural axons at the floor plate is highly directed

Commissural axon growth on arrival at the floor plate

We examined the precision of the commissural axon pathway into and across the floor plate. The following questions were addressed: (i) do axons grow straight into the floor plate when they reach the ipsilateral edge?; (ii) do axons pause or extend in other directions before projecting forwards into the floor plate? and (iii) do all axons follow the same course?

Examination of preparations fixed at E11.5–12 (n=115 embryos) revealed growth cones at the ipsilateral edge of the floor plate and growth cones that had entered the floor plate (Figs 2,3). Axons projected straight across the floor plate edge (Fig. 3). Only 5 axons, out of hundreds observed, turned to project longitudinally at the ipsilateral floor plate boundary. Thus virtually all axons projected into the floor plate perpendicular to its longitudinal axis without deviation from the transverse plane. Although it was not possible to determine directly whether commissural axons paused at the ipsilateral edge, we could not detect a build-up of axons at this boundary at any developmental stage observed.

Passage of commissural axons across the floor plate of the spinal cord

Within the floor plate, commissural axons continued to grow perpendicular to the longitudinal axis of the floor plate without major deviation in the rostral or caudal directions (Figs 3,4). All labelled axons were found in the ventral-most third of the floor plate with the most recently crossed fibres closest to the basal edge. All axons that crossed the floor plate took the same route from the ipsilateral to the contralateral boundaries.

Commissural axons alter the direction of their projection at the contralateral edge of the floor plate

We examined the nature and position of the change in
Fig. 3. Commissural axons as they cross the floor plate. E12.5–13 preparations showing commissural axons that have entered the floor plate (both edges indicated by dotted lines in A; contralateral edge shown in B). The axons do not change direction at the ipsilateral edge of the floor plate but extend straight into the floor plate. The growth cones are expanded in size and bear several filopodia. B shows a photo-oxidized preparation. The meandering appearance of the axons is an artifact of tissue shrinkage upon post-fixation and dehydration. Details of the growth cone morphology are discernible using this technique. X: (A) 350; (B) 800.

Fig. 4. Commissural axons turn at the contralateral floor plate edge. Fluorescence micrographs of E12.5–13 preparations. In each case, a single axon has made a sharp turn at the contralateral edge of the floor plate (indicated by a dotted line). Rostral is to the right. In A and B, the growth cone of a second axon has just arrived at the contralateral edge. After the turn in A the axon initially follows the floor plate-neuroepithelium boundary before moving laterally. In B and C, the preparations were flattened slightly to facilitate photography and this resulted in the exposure of the longitudinal face of the floor plate, along which the axons have travelled. Arrow in C shows a small, caudally directed protrusion from the axon at the contralateral edge X: A, B, 350; C, 250. Photomontage has been used in D.

course taken by both early and late populations of commissural axons after they had crossed the floor plate.

(i) The position of the turn coincides with the edge of the floor plate. Preparations fixed at E12–13 contained axons that had extended as far as the contralateral edge of the floor plate with some entering the ventral funiculus (Fig. 4). Arrival of commissural axons at the contralateral edge coincided with an orthogonal turn in the axonal projection (Fig. 4). The transition in plane of axon growth from transverse to longitudinal occurred precisely at the boundary between the floor plate and the contralateral neuroepithelium. Axons were never observed to turn before reaching the contralateral edge of the floor plate.

(ii) The earliest axons appear to turn independently of one another. In the preparations that showed the earliest axons crossing the contralateral edge of the floor plate, labelled axons were spaced up to 30 μm
Fig. 5. Axons crossing the floor plate at E13.5–14 follow the same route as earlier axons. All commissural axons appear to turn at the contralateral floor plate edge. (A, C) Fluorescence micrographs illustrate the position and precision of the turn. The position of the floor plate can be verified by examination of the preparation under phase contrast optics or by labelling with monoclonal antibodies that bind to antigens expressed selectively by the floor plate. B shows such labelling in a separate preparation, using the antibody 2E7. D shows a fluorescence and phase contrast double exposed micrograph of the preparation shown in C. As in Fig. 4, this preparation was flattened slightly for photographic purposes, such that the longitudinal face of the floor plate can be seen. ×: A, B 150; C, D 115.
apart. Thus the turn made by a single axon could often be observed. Under conditions in which the majority of the axons in the field were thought to be labelled (as judged from immunohistochemical labelling with antibodies that label commissural axons selectively (Dodd et al. 1988)), many individual axons appeared to turn independently of each other and in contact with the floor plate edge (Fig. 4C). Later developing commissural axons also crossed the floor plate and changed their direction of growth at the contralateral edge (Fig. 5). The earliest of these sometimes appeared to be in contact with axons that had already turned. The density of the later axons was such that it was not possible to tell whether they turned in contact with floor plate cells or by fasciculating with other commissural axons. Examination at the EM level will be necessary to determine the cellular interactions of the growth cones at this site.

Axon extension in the longitudinal plane is rostrally directed

With very few exceptions, all labelled commissural axons turned rostrally when they made the transition into the longitudinal plane. For the first 30–100 μm of the rostral projection, the axons appeared to maintain close contact with the longitudinal face of the floor plate, growing along the boundary between the floor plate and the contralateral neuroepithelium (Figs 4, 5). Axons were never observed to reenter the floor plate although after projecting rostrally for 30–100 μm, axons occupied more lateral positions in the funiculus (Figs 4, 5).

The organization of commissural axons within the funiculus appeared to depend on both the axial level and the timing of origin of the axons. The older axons at any given rostrocaudal level of spinal cord were located more laterally than younger axons. Axons originating caudally were found in a ventral position relative to more rostral axons. We have not studied the organization of axons within the funiculus in further detail.

The precision of the projection

Virtually all labelled commissural axons showed the projection pattern described above. We observed only very infrequent deviations (less than ten examples in each case out of a total of thousands of axons observed in the study), all at later stages of development. These deviations in the pathway fell into three classes: axons that turned rostrally on the ipsilateral side of the floor plate; axons that turned caudally instead of rostrally at the contralateral edge of the floor plate; axons that did not alter direction at the contralateral edge of the floor plate but projected straight (and dorsally) into the contralateral spinal cord. Axons were never observed to alter direction within the floor plate.

The morphology of commissural growth cones changes with position on the pathway

Growth cone morphology has been shown to change at decision regions of an axonal pathway in situ (Bovolenta and Mason, 1987; Caudy and Bentley, 1986a; Harris et al. 1987; Nordlander, 1987; Ramon y Cajal, 1909; Taghert et al. 1982; Tosney and Landmesser, 1985). We therefore examined whether commissural growth cone morphology and dimensions alter as they traverse distinct regions of the pathway.

Growth cone morphology during projection in the transverse plane towards the floor plate

Throughout their course towards the floor plate, the majority of commissural growth cones (78.5%, n=42) had simple morphologies (Figs 2, 6). They were small (average area 24.5±9.5 μm² s.e.m., n=8) (Fig. 7), elongated (average length 11.1±1.3 μm s.e.m. and 2.15±0.8 μm s.e.m.) and rarely exhibited filopodia. The few filopodia observed in this part of the pathway were one or two per growth cone and were short when compared to those displayed at later stages (Figs 3, 6). These filopodia extended either forwards from the tip of the growth cone or from the side and were not observed to project backwards. The axon behind the growth cone was smooth and filopodia or similar extensions were not observed in this part of the pathway.

Growth cone morphology at the floor plate

As commissural growth cones reached the ipsilateral edge of the floor plate, their morphology changed dramatically. Several filopodia were extended and the bodies of the growth cones became expanded and complex (Figs 2, 3, 6). The filopodia extended from both the tips and the sides of the growth cones. Filopodia extending from the sides emerged at right angles to the growth cones or were angled forwards. In a few cases, a filopodium that extended backwards was seen (example in Fig. 6G). Filopodial length was variable, ranging from 3–20 μm. 89% (n=19) of the growth cones observed at the ipsilateral edge of the floor plate displayed this morphology. Complex features were also displayed as growth cones traversed the floor plate (Figs 3, 6). However, only 68% (n=38) of the growth cones found in the body of the floor plate fell into the complex category. At the contralateral edge of the floor plate, the great majority of the growth cones observed (96%, n=22) had complex morphology (Fig. 7). Thus a larger percentage of growth cones were complex at each edge of the floor plate than within the floor plate.

On arrival at the contralateral edge, growth cones extended filopodia that were directed forwards (towards the neuroepithelium) and to each side (rostrally and caudally) in apparent contact with the floor plate cells. The change in direction of axonal growth at the contralateral border of the floor plate was accompanied by a predominantly rostral extension of filopodia. Although it was not possible to determine whether the change in direction of filopodial extension preceded the axonal turn, in some cases a short protrusion was observed extending caudally at the site of the turn (arrow in Fig. 4).

Within the floor plate region (i.e. at either edge of the floor plate or in between) the average length and...
average width of growth cones were 15±2.3 μm and 6.5±1.9 μm (S.E.M.) respectively, and the average area of the growth cones was 97.6±34 μm² (S.E.M.), n=10 (See Fig. 7).

Fig. 6. morphology of commissural growth cones in different regions of the pathway. (A) The position of each growth cone illustrated in B, is shown in the schematic representation of the spinal cord. (B) Commisural growth cones are simple as they extend in the ipsilateral neuroepithelium (a,b). Growth cones are larger and have several filopodia as they encounter and cross the ipsilateral edge of the floor plate (c–f), within the floor plate (h,k) and at the contralateral edge (g) of the floor plate. ×: 1000.

Growth cone morphology of axons travelling in the longitudinal plane
The growth cones of axons that had turned into the ventral funiculus were difficult to visualize individually
Axon patterning at the floor plate

Fig. 7. Histogram comparing the areas of growth cones examined in the ipsilateral neuroepithelium (open, first column in each case) and in contact with the floor plate (hatched, second column in each case. This category includes growth cones at both edges of and within the floor plate.). Growth cones with areas <40 \( \mu m^2 \) were not observed in contact with the floor plate. Growth cones with areas >40 \( \mu m^2 \) were not detected in the ipsilateral neuroepithelium. (growth cones on axons extending towards the floor plate: \( n=8 \), mean length=11.1±1.3 \( \mu m \) (S.E.M.), mean width=2.15±0.8 \( \mu m \) (S.E.M.), mean area=24.5±9.5 \( \mu m^2 \) (S.E.M.). Growth cones in contact with the floor plate: \( n=10 \), mean length=15.1±2.3 \( \mu m \) (S.E.M.), mean width=6.5±1.9 \( \mu m \) (S.E.M.), mean area=97.6±2\( \mu m^2 \) (S.E.M.). These measurements were made from photooxidized preparations.

because the axons were close together in this part of the pathway. Since it was not possible to outline a single growth cone with certainty, measurements of their dimensions were not made.

Lamellipodia, recognisable in situ as thin veil-like expansions of the central core of a growth cone (Roberts and Taylor, 1983; Bovolenta and Mason, 1987; Nordlander, 1987), did not appear to be a major feature of commissural growth cones at any part of the pathway. Since lamellipodia have been observed in other studies in which the same conditions of fixation and labelling were used, it is unlikely that the absence of lamellipodia in this study was artefactual.

Discussion

The floor plate of the embryonic rat spinal cord appears to be an intermediate target in the pathway of one set of commissural axons towards supraspinal sites (Dodd et al. 1988; Tessier-Lavigne et al. 1988). Histological (Ramon y Cajal, 1909; Windle and Baxter, 1936; Wentworth, 1984), immunocytochemical (Dodd et al. 1988) and electron microscopic (Holley, 1982; Holley et al. 1982) studies in rodents have shown that as commissural axons reach the ventral half of the spinal cord they alter their direction of growth within the transverse plane of the spinal cord to project medially towards the floor plate. A chemotropic factor released by the floor plate may guide commissural axons towards the midline (Tessier-Lavigne et al. 1988). At the midline, commissural axons cross the floor plate and turn orthogonally to join the contralateral ventral funiculus. Although the floor plate represents the anatomical landmark at which commissural axons alter the direction of their growth to project rostrally, it is not clear from existing studies whether the floor plate influences the choice of pathway of commissural axons at the midline.

This paper describes the pattern and precision of the pathway taken by commissural axons as they traverse the floor plate of the embryonic rat spinal cord and change the orientation of their growth in register with the contralateral boundary of the floor plate. We show the following. (i) Virtually all axons of this population project contralaterally. Axons arriving at the floor plate and contacting the ipsilateral edge appeared not to pause and did not alter their course, thus projecting directly across the floor plate. (ii) Arrival at the contralateral boundary between the edge of the floor plate and the spinal cord neuroepithelium coincided precisely with the orthogonal transition from the transverse to the longitudinal plane of commissural axon extension. Axons were never observed to change direction within the floor plate. (iii) Axons projected rostrally in the longitudinal plane. (iv) Growth cones underwent morphological changes as they navigated regions of the pathway that differ in their biochemical and cell surface properties.

Almost every axon in the labelled population displayed the stereotyped course described above. This finding, coupled with the observed precision of the pathway, suggests that commissural axons are guided across the midline and into the ventral funiculus. The fact that the projection pattern coincides precisely with the contralateral boundary of the floor plate suggests that the floor plate may provide the signals responsible for this guidance. Examination of growth cone morphology and size as commissural axons extend along the pathway supports this idea.

Growth cone morphology

Commissural growth cones underwent marked changes in morphology as they extended through different regions of the pathway. In other systems, in vivo, growth cones have been shown to undergo marked and stereotypic morphological changes as axons traverse distinct cellular environments (Bovolenta and Mason, 1987; Caudy and Bentley, 1986a,b; Godement et al. 1990; Harris et al. 1987; Nordlander, 1987; Ramon y Cajal, 1909; Taghert et al. 1982; Tosney and Landmesser, 1985). Although the details of growth cone shape may differ according to neuronal type (Bovolenta and Mason, 1987; Caudy and Bentley, 1986a; Hayden et al. 1985; Nordlander, 1987; Tosney and Landmesser, 1985), there is general agreement that growth cones become more elaborate at decision regions or choice points, displaying filopodia and lamellipodia, whereas in regions where they have a common pathway they have been found to be simple (Bovolenta and Mason,
A number of surface molecules are expressed by the floor plate and may mediate interactions with commissural axons. Both the floor plate and commissural axons express high levels of NCAM which may have a role in growth cone-floor plate interactions. However, NCAM is also expressed at high levels by motor neurons at a time when NCAM-positive commissural axons grow through the motor neuron column in close proximity to motor neuron cell bodies. The axons are not deflected from their ventromedial course towards the floor plate as they pass the motor neurons, suggesting that the expression of NCAM may be involved but is not sufficient for a group of cells to act as an intermediate target.

Other surface molecules expressed selectively by floor plate cells at the embryonic stages when commissural axons cross the midline include P84, a glycoprotein shown to subserve adhesion of cerebellar neurons in vitro (Chuang and Lagenaour, 1990) and GP90, a glycoprotein of the cadherin family (Moss and White, 1989; Ranscht and Dours, 1989 and personal communication). In addition, several surface molecules expressed exclusively in the floor plate from early stages and identified with monoclonal antibodies also represent potential adhesion molecules (Fig. 5; Dodd et al. 1988 and unpublished obs).

An adhesive interaction between commissural growth cones and floor plate cells could account for the distinct patterns of growth at the two boundaries of the floor plate

The behaviour of a growth cone at a boundary between distinct substrates may depend on the order in which cues of different adhesive strength or interactive capacity are presented. Growth cones of peripheral neurons, given a choice in vitro between substrates that are permissive for growth but that differ in their adhesive properties, have been shown to extend onto the more adhesive substrate (Burmeister and Goldberg, 1988; Hammarback and Letourneau, 1986; Letourneau, 1975). Recent studies in vitro have shown that axons encountering a border between differentially adhesive substrates turn if travelling from the more to the less adhesive while they grow straight across the border when travelling from the less to the more adhesive (Burmeister and Goldberg, 1988). A difference in adhesive properties of the floor plate and the surrounding neuroepithelium may therefore mediate the extension of axons straight across the ipsilateral boundary. At the contralateral edge, however, the order in which substrates are presented is reversed. To cross this border, growth cones would be required to travel from a more adhesive to a less adhesive substrate. The physical constraints of the axonal cytoskeleton may decrease the likelihood of axons turning back on themselves at the boundary (Letourneau et al. 1986; Katz, 1985). One way for axons to maintain contact with the floor plate would be to turn orthogonally and travel along the lateral face.

The mechanism by which a growth cone changes
direction of extension is unclear at present (Bray, 1987; Bray and Chapman, 1985; Bray and Hollenbeck, 1988: Burmeister and Goldberg, 1988; Lamoureux et al. 1989; Marsh and Letourneau, 1984) and could not be addressed with the resolution of the present study.

Behaviour of commissural axons within the ventral funiculus

Commissural axons within the ventral funiculus never reenter the floor plate despite the fact that individual axons appear to travel rostrally in the funiculus as far as 100 μm in contact with the floor plate lateral edge. This may result from axon–axon interactions within the funiculus. As axons enter the contralateral ventral funiculus, they begin to express L1 (Dodd et al. 1988). This protein has been shown to mediate axon–axon fasciculation in other systems (Stallcup and Beasley, 1985; Fischer et al. 1986) and may subserve commissural axon fasciculation within the ventral funiculus, decreasing the probability that axons will reenter the floor plate. The timing of expression of L1 may be critical to appropriate guidance of the axons in the funiculus.

The rostrally oriented projection of commissural axons

The information required for commissural axons to turn at the contralateral edge of the floor plate may be independent from the cues that direct them to turn rostrally. With almost no exceptions, commissural axons labelled in this study project rostrally. This is in contrast to results in non-mammalian vertebrates, in which commissural axons have been observed to project both rostrally and caudally (Kuwada et al. 1990; Nornes et al. 1980; Oppenheim et al. 1988; Roberts et al. 1988; Windle and Baxter, 1936; Oppenheim, personal communication). The axons of commissural neurons differentiating over the developmental stages of this study can be followed for several segments within the funiculus and in many instances reach the hindbrain, suggesting that they represent supraspinally projecting relay neurons (see also Altman and Bayer, 1984). However, in the chick, the frog and the fish there appears to be a greater variety of intraspinal and supraspinally projecting commissural neurons than in mammals (Altman and Bayer, 1984; Kuwada et al. 1990; Oppenheim et al. 1988; Roberts et al. 1988; Wentworth, 1984; Windle and Baxter, 1936; Oppenheim personal communication), and it is probable that equivalent populations of commissural axons have not been compared.

Neither the nature nor the source of a putative rostral cue are known. The rostral-to-caudal gradient of development that is observed in the embryonic spinal cord (Wentworth, 1984) may contribute to the establishment of a gradient of a rostral signal. The possibility that a graded developmental increase in a factor released from the floor plate itself directs axonal growth rostrally can be tested experimentally. The symmetry of the commissural projection suggests that a rostral cue is likely to be expressed bilaterally (Dodd et al. 1988). Thus the finding that axons turn rostrally only at the contralateral edge of the floor plate may indicate that the cues directing axons across the ipsilateral border of the floor plate are stronger than the rostral signal. At the contralateral edge, however, where the effect of local cues may be to cause axons to turn, the influence of a rostral cue would ensure that all axons turn rostrally. Hierarchies of guidance cues have previously been suggested to order axonal pathways (Berlot and Goodman, 1984; Caudy and Bentley, 1986b; Fraser, 1980; Jessell, 1988; Letourneau, 1975).

These observations raise the possibility that the floor plate functions as a vertebrate counterpart of the guidepost or landmark cells that influence the trajectory of invertebrate axons (Bate, 1976; Bentley and Keshishian, 1982; Taghert et al. 1982). The selective elimination of guidepost cells, either by cell ablation or by genetic manipulation, leads to selective perturbation of the pathways of axons that normally contact and extend towards the cells (Bastiani and Goodman, 1986; Bentley and Caudy, 1983; 1988; Thomas et al. 1988). While selective ablation of neural cells is difficult to achieve in mammalian embryos, examination of the mouse mutant, Danforth’s short tail (Sd), revealed the potential role of the floor plate as a group of guidepost cells (Bovolenta et al. 1988). In this mutant, the floor plate is absent from the caudal spinal cord and in this region of the spinal cord the commissural pathway is perturbed. The observations described in this paper are consistent with a role for the floor plate as an intermediate target and suggest that the floor plate provides contact-mediated guidance to commissural axons. The similarity of the behaviour of commissural axons at the floor plate and of invertebrate axons encountering a guidepost or landmark cell is striking and emphasizes the idea (Dodd and Jessell, 1988) that many of the mechanisms employed to guide axons by the developing invertebrate and vertebrate nervous systems may be common.

We thank Eric Hubel for photographic assistance. We are also grateful to Cliff Hume, Tom Jessell and Carol Mason for critically reading the manuscript and Andrew Lumsden for discussions at an early stage of the project. We are also particularly indebted to Drs P. Godement, J. Kuwada, C. Lagenaur and R. Oppenheim for providing us with preprints of their related work. Funding was provided by NSF (BNS 8910027), The Esther A and Joseph P Klingenstein Fund, The Irma T Hirschl Foundation and MDA.

References

Axon patterning at the floor plate


(Accepted 20 March 1990)