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Guidance of Developing Axons by Diffusible Chemoattractants

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One of the first steps in the formation of specific neuronal connections is the projection of axons to their targets through diverse and changing environments. The selection of pathways by neuronal growth cones occurs with a high degree of precision and appears to be under the control of locally acting guidance cues that direct growth cones to a succession of intermediate targets (Dodd and Jessell 1988; Harrelson and Goodman 1988). Many of the molecules that regulate growth cone extension and navigation are expressed in the extracellular matrix or on the surface of cells along the pathways taken by developing axons. Some of these molecules, such as laminin, are present in restricted regions of the developing nervous system and may influence the pathway choice of axons (Cohen et al. 1986; Riggott and Moody 1987; Tomasselli and Reichart 1989). Others, such as neural cell adhesion molecules N-CAM and N-cadherin, appear to act as adhesive substrates that promote growth cone extension (Edelman 1988; Jessell 1988; Takeichi 1988).

In addition to the recognition of cues provided by cells along the pathway of developing axons, it is possible that growth cones are oriented by gradients of chemoattractant molecules that are released selectively by intermediate or final cellular targets. Although chemotropism has been invoked on repeated occasions (Ramón y Cajal 1909; Trinkaus 1985), the evidence in favor of this mechanism of axon guidance has remained equivocal. A local source of the tropic molecule nerve growth factor (NGF) can reorient regenerating sensory axons in vitro (Letourneau 1978; Gundersen and Barret 1979) and appears to attract sympathetic axons in vivo (Menesini-Chen et al. 1978). However, it is unclear whether NGF normally exerts a tropic action in the guidance of axons within the developing embryo (see Davies 1987). More recent studies suggest that developing axons may be guided by chemotropic factors other than NGF. Trigeminal sensory neurons extend axons in vitro in response to a diffusible factor distinct from NGF that is secreted by one of their normal target tissues, the maxillary epithelium (Lumsden and Davies 1983, 1986). A similar in vitro analysis has suggested that the axons of corticospinal projection neurons extend collateral branches toward one of their final targets, the basilar pons, in response to a diffusible factor secreted by pontine tissue (Heffner et al. 1990).

We have been examining the cues responsible for the guidance of axons of commissural neurons in the embryonic rat spinal cord (see Fig. 1). Commissural neurons differentiate in the dorsal spinal cord and extend axons ventrally, close to the lateral edge of the spinal cord (Holley 1982; Altman and Bayer 1984; Wentworth 1984; Dodd et al. 1988). When commissural axons reach a point just dorsal to the motor column, they alter their trajectory and project through the motoneuron column toward the floor plate, a specialized group of epithelial cells located at the ventral midline of the spinal cord (Jessell et al. 1989). The directed growth of commissural axons to the ventral midline raises the possibility that they are guided by a chemotropic factor secreted by the floor plate.

In this paper, we discuss evidence that supports the idea that commissural axons are guided by a floor-plate-specific chemotropic factor (Tessier-Lavigne et al. 1988; Placzek et al. 1990a). Our studies show that floor plate cells secrete a diffusible factor(s) that influences the orientation of commissural axon growth in vitro without affecting other embryonic spinal cord axons. We also provide preliminary evidence that the floor plate can orient the growth of commissural axons in the developing chick embryo in vivo. The floor plate factor can diffuse considerable distances through the neural epithelium and can override intrinsic polarity cues within the epithelium to reorient essentially all commissural axons. The orienting effect of the floor plate occurs in the absence of any detectable effect on the survival or differentiation of commissural neurons or on the rate of axon growth. These findings provide support for the hypothesis that gradients of chemotropic factors guide developing axons to their targets in the vertebrate nervous system.

EXPERIMENTAL PROCEDURES

Culture. For explant cultures, one- to four-segment long pieces of dorsal spinal cord and floor plate from 24–27 somite stage embryos (E11–E11.5) were dissected and embedded in three-dimensional collagen gels (Fig. 1c) and cultured in supplemented Ham’s F12 medium (Tessier-Lavigne et al. 1988) at 37°C in a 5% CO2 environment. To generate conditioned medium, floor plates dissected from E11 and E13 rat embryos or the dorsal part of the E11 spinal cord were collected in L15 medium and plated in tissue culture dishes. After overnight incubation in supplemented Ham’s F12 containing 5% horse serum, tissues were cultured in...
Figure 1. (a, b) Trajectory of commissural axons in the embryonic rat spinal cord. (a) Schematic diagram of a transverse section of an E12 rat spinal cord showing the location of the first three classes of differentiated neurons and their prospective axonal trajectories. Motoneurons (M) differentiate in the ventral region of the spinal cord from E10.5 and extend axons to their target muscles. Commissural (C) and association (A) neurons differentiate from E11 in the dorsal region of the spinal cord, adjacent to the roof plate (R). Association axons project laterally to join the ipsilateral lateral funiculus (→). Commissural axons grow ventrally along the lateral margin of the spinal cord to the motor column, then alter their trajectory and course directly through the nascent motor column to the floor plate (F). After crossing the midline of the spinal cord at the floor plate, commissural axons turn by 90° to form longitudinal projections in the contralateral ventrolateral funiculus (→). (b) Transverse section of E12 rat spinal cord at the cervical level, labeled with a monoclonal antibody to the TAG-1 antigen. Motor and commissural axons but not association axons express TAG-1. Commissural axons project toward the floor plate. TAG-1+ neurons in the dorsal root ganglia (d) can also be seen. Note that the spinal cord shown here is at a more advanced stage of development than those used for explant cultures (see below). (c) Schematic diagram of the main experimental protocol. Each section of E11 spinal cord (see below) was dissected into three portions: a dorsal explant (D) comprising the dorsal third or half, a floor plate explant (F) comprising the ventral-most fifth, and the remaining ventral explant without floor plate (V), which contained most of the motoneurons. Explants were then embedded in three-dimensional collagen matrices in appropriate orientations. Calibration: 100 μm. (Reprinted, with permission, from Tessier-Lavigne et al. 1988.)

serum-free supplemented Ham’s F12 for a further 48–60 hours. Tissues dissected from 100 E11 or 40 E13 embryos were used to condition 1 ml of medium. Floor-plate-conditioned medium could be frozen at −20°C for periods up to a month without detectable loss of activity. Before use, conditioned medium was diluted with an equal volume of fresh supplemented Ham’s F12 medium containing 10% horse serum.

Immunocytochemistry and dye labeling. Explants cultured in collagen gels and sections of whole embryos were processed for immunocytochemistry as described previously (Dodd et al. 1988; Tessier-Lavigne et al. 1988). TAG-1, an axonal glycoprotein, was detected using monoclonal antibody 4D7 (Yamamoto et al. 1986) or with rabbit anti-TAG-1 antibodies (Dodd et al. 1988). Cellular retinoic acid binding protein (CRABP) was detected using affinity-purified turkey anti-CRABP antibodies (a gift from W. Blaner, Columbia University).

Retrograde or anterograde labeling of commissural neurons in explant culture was performed with DiI (1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate, molecular probes; 2.5 mg/ml in dimethylformamide) (see Honig and Hume 1986; Gode ment et al. 1987; Placzek et al. 1990). Explants were fixed in 4% paraformaldehyde in 0.12 M phosphate buffer (pH 7.4) for 24–48 hours and washed extensively in phosphate-buffered saline (PBS). The top layer of collagen above the region to be injected was removed and 5–30 nl of DiI was injected into the tissue using a glass microcapillary. DiI was allowed to diffuse at 4°C for 1–5 days, and explants were then examined under a Zeiss Axioplan with epifluorescence optics.

Chick embryo grafts. The vitelline membrane above the area to be operated was removed. An incision was made through ectoderm and mesoderm, leaving endoderm intact. An incision was made adjacent to the closing neural tube at nonsegmented levels. Grafts of chick or rat floor plate were positioned within the cut and embryos were allowed to develop for a further 40–48 hours at 38°C before analysis.

Assays of defined growth factors and cell-conditioned media. A panel of growth factors and media conditioned by primary cells or cell lines were tested for their ability to mimic the effect of the floor plate in evoking commissural axon outgrowth. Factors and conditioned media were added to E11 dorsal spinal cord explants grown alone in a collagen gel matrix. Axon outgrowth was assayed after 40 hours. The following factors and media did not mimic the effect of the floor plate. Factors: ciliary neurotrophic factor (rat) 10–100 ng/ml; brain-derived neurotrophic factor (porcine) 10–100 ng/ml; NGF (murine) 10–100 ng/ml; basic fibroblast growth factor (bFGF) (bovine rec. ± heparin) 1–100 ng/ml; aFGF (bovine rec. ± heparin) 10 ng/ml; transforming growth factor-α (TGF-α) (human rec.) 1–100 ng/ml; TGF-β1 (porcine rec.) 1–
100 ng/ml; TGF-β2 (porcine rec.) 1–100 ng/ml; platelet-derived growth factor (PDGF) (porcine rec.) 3 ng/ml; epidermal growth factor (EGF) (murine) 1–100 ng/ml; insulin-like growth factor (IGF-1) (human rec.) 10–100 ng/ml; IGF-II (human rec.) 10–100 ng/ml; interleukin-1 (IL-1) (human rec.) 1–10 ng/ml; IL-2(B) (human rec.) 0.1–1 units/ml; IL-3 (human rec.) 10–3 to 10–2 units; IL-4 (human rec.) 10–5 to 10–3 units; IL-6 (human rec.) 1–10 ng/ml; IL-3 (murine rec.) 1:1000 stock; IL-5 (murine rec.) 1:1000 stock; IL-6 (murine rec.) 1:1000 stock; G-colony-stimulating factor (G-CSF) (human rec.) 0.1–1 ng/ml; M-CSF (human rec.) 10–2 to 10–3 stock; tumor necrosis factor-α (TNF-α) (human rec.) 10–2 to 10–3 stock; TNF-β (human rec.) 10–2 to 10–3 stock; interferon-α (human rec.) 8–80 IU/ml; interferon-β (human rec.) 17–170 IU/ml; interferon-γ (human; leukocyte) 3–30 IU/ml; interferon-λ (human; fibroblast) 10–100 IU/ml; leukemia inhibitory factor (murine rec.) 1:1000 stock; retinoic acid 10–7 to 10–5 μ; scatter factor (murine rec.) 1:1000 stock; laminin (EHS sarcoma) (1–100 μg/ml); fibronectin (human) 1–100 μg/ml. Combinations of bFGF/TGF-β1; TGF-β1/TGF-β2/PDGF/EGF; TGF-β1/TGF-β2/PDGF/EGF/bFGF/IGF-1/IGF-II were also tested without effect. In all cases, factors were tested at concentrations necessary to reveal their primary activities. (rec. = recombinant-DNA-derived proteins). Cell-conditioned media: 10T-1/2, COS, 3T3, XTC, P19, PC12, P388D1, MRCS, C6, RD, primary astrocyte, spleen, thymus, bone marrow, and macrophage (all rat).

RESULTS

Evidence for a Floor-plate-derived Chemoattractant

The existence of a floor-plate-derived chemoattractant was established initially by examining the effect of the floor plate on axon outgrowth from explants of dorsal spinal cord cultured in three-dimensional collagen gels. Dorsal explants were taken from rat embryos at embryonic day (E) 11, the age at which commissural neurons begin to differentiate and extend processes. Dorsal explants cultured alone for 39–44 hours show little or no axon outgrowth (Fig. 2a). In contrast, dorsal explants cultured with an E11 floor plate explant placed 100–400 μm from their ventral-most edge show axon outgrowth within 20–24 hours. After 40–44 hours, a characteristic pattern of outgrowth is observed from all of these explants, with most axons projecting
in thick fascicles from the ventral-most edge to the floor plate (Fig. 2b). Axons that project into the collagen gel are not preceded or accompanied by migrating cells, as assessed by staining with nuclear dyes (Tessier-Lavigne et al. 1988). Marked outgrowth is also observed from dorsal explants cultured alone but exposed to medium conditioned by E11 or E13 floor plate; however, under these conditions, axons emerge from all edges of the explant (Fig. 3). These experiments suggest that the floor plate secretes a diffusible factor that promotes axon outgrowth from dorsal spinal cord explants. All of the axons that project from dorsal explants in the presence of floor plate appear to derive from commissural neurons as assessed by expression of the TAG-1 glycoprotein. Studies both in vivo and in vitro indicate that TAG-1 is selectively expressed by commissural neurons in the dorsal spinal cord of E11 rat embryos (Figs. 1b and 2c) (Dodd et al. 1988; Furley et al. 1990).

TAG-1-labeled axons are observed within control explants at a density equal to that in explants cocultured with the floor plate. These axons are oriented along the original dorsal-ventral axis of the explants (Fig. 2c,d). Instead of projecting into the collagen gel at the ventral most edge of the explant, however, these axons remain confined to the explants, extending along its inside perimeter (Fig. 2d). This suggests that the floor-plate-derived factor promotes commissural axon outgrowth from the neural epithelium into the collagen gel. Quantitative analysis of TAG-1-labeled neurons shows that the number and length of commissural axons are similar in dorsal explants cultured alone or with a floor plate (Placzek et al. 1990a). Thus, the floor-plate-derived factor does not appear to affect the survival or differentiation of commissural neurons or the rate of growth of commissural neurons.

The effect of the floor plate on axon outgrowth appears to be selective for commissural neurons (Table 1). Axons that do not express TAG-1 and that presumably derive from association neurons are also present within dorsal explants cultured for 40–44 hours with floor plate (Placzek et al. 1990a) but are not observed projecting from the explants toward the floor plate. In addition, motor axons extend into the collagen gel from ventral explants grown alone, but the extent and pattern of axon outgrowth is not altered when ventral explants are cultured with floor plate (Table 1) (Tessier-Lavigne et al. 1988).

The specificity of expression of this chemotropic factor in embryonic rat tissues was examined at different stages of development. Expression of the chemotropic factor is largely confined to the floor plate, over its entire rostrocaudal length, during the period in which commissural axons project to the ventral midline in vivo (E11–E14) (Table 2) (Tessier-Lavigne et al. 1988). Explants of E13-PO floor plate have the same effect as E11 floor plate in evoking outgrowth of TAG-1+ axons from dorsal explants, although lower activity is detected in floor plate explants from older embryos (Table 2). This effect is not mimicked by explants of E11–E14 dorsal spinal cord nor E11 ventral spinal cord (Table 2). However, E13 ventral spinal cord evokes the outgrowth of a small number of TAG-1-labeled axons. It is possible that ventral spinal cord cells at this age secrete low amounts of a factor that affects commissural axon outgrowth. Alternatively, the factor secreted by the floor plate may bind to the ventral spinal cord in vivo and be released slowly by the ventral explant in vitro. If the deviation in trajectory observed as commissural axons reach the dorsal aspect of the motor column indicates the point at which commissural axons first respond to the chemotropic factor, then the ventral spinal cord should contain activity detectable in the in vitro assay. It is clear though that the chemotropic activity of the floor plate always greatly exceeds that of adjacent ventral tissue. Thus, it is likely that at all

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**Figure 3.** Medium conditioned by the floor plate evokes commissural axon outgrowth from E11 dorsal spinal cord explants. (A) After 40 hr in culture in the presence of floor-plate-conditioned medium, fasciculated axons emerge from the lateral- and the ventral-most edges of the dorsal explant. (B) Occasionally, axons also emerge from the roof plate (r) of the dorsal explant. (C) No outgrowth is seen from dorsal explants cultured for 40 hr in the presence of medium conditioned by dorsal spinal cord. Calibrations: (A, B, C) 170 μm. (Reprinted, with permission, from Placzek et al. 1990a.)
relevant developmental stages, there is a concentration gradient of the factor in the spinal cord with its high point in or near the floor plate.

A large number of nonneural tissues, defined growth factors, and media conditioned by cultured cells fail to mimic the effect of the floor plate (Table 2, see also Experimental Procedures). Sparse outgrowth of commissural axons is, however, observed when dorsal spinal cord explants are cocultured with Rathke's pouch, embryonic limb bud mesenchyme, and dermomyotome (Table 2). It is not clear whether these tissues synthesize the floor plate chemoattractant or an unrelated activity that can also promote neurite outgrowth. Preliminary biochemical studies indicate that the floor plate chemoattractant is in a protein with a molecular weight of about 70,000 (M. Tessier-Lavigne et al. unpubl.).

### Orientation of Commissural Axons

The findings described in the previous section show that the floor plate can promote commissural axon outgrowth into a collagen matrix but do not reveal whether the floor plate has an orienting effect on axon growth. To provide information on the orientation of commissural axons in relation to a source of floor plate

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### Table 1. Specifity of Neuronal Response to Floor Plate Explants

<table>
<thead>
<tr>
<th>Neurons tested</th>
<th>Age</th>
<th>No. of explants</th>
<th>Response</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dorsal spinal cord</td>
<td>E11</td>
<td>&gt;200</td>
<td>directed outgrowth of commissural axons</td>
</tr>
<tr>
<td>Ventral spinal cord</td>
<td>E11</td>
<td>10</td>
<td>spontaneous axon outgrowth; not increased or oriented by the floor plate</td>
</tr>
<tr>
<td>Dorsal root ganglion</td>
<td>E13</td>
<td>11</td>
<td>no axon outgrowth</td>
</tr>
<tr>
<td>Trigeminal ganglion</td>
<td>E11, E13</td>
<td>4</td>
<td>no axon outgrowth</td>
</tr>
<tr>
<td>Superior cervical ganglion</td>
<td>PO</td>
<td>3</td>
<td>no axon outgrowth</td>
</tr>
<tr>
<td>Retina</td>
<td>E14, E17</td>
<td>5</td>
<td>sparse axon outgrowth; not increased or oriented by the floor plate</td>
</tr>
</tbody>
</table>

Neuronal explants were cocultured in the presence or absence of a piece of E11 floor plate in a collagen gel matrix, and the pattern of axon outgrowth was examined after 40 hr. Dorsal root, trigeminal and superior cervical ganglion explants were cultured in the absence of NGF. Spontaneous axon outgrowth, presumably from motoneurons, was observed from E11 ventral spinal cord explants, but the extent and orientation of axon growth were not affected by the presence of a nearby floor plate explant.

### Table 2. Ability of Embryonic Rat Tissue Explants to Evoke Commissural Axon Outgrowth

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Age</th>
<th>No. explants tested</th>
<th>Dorsal explants exhibiting axon outgrowth (%)</th>
<th>Average no. of axon bundles/positive explant</th>
</tr>
</thead>
<tbody>
<tr>
<td>Floor plate: spinal cord</td>
<td>E11</td>
<td>&gt;200</td>
<td>100</td>
<td>24</td>
</tr>
<tr>
<td></td>
<td>E13</td>
<td>13</td>
<td>100</td>
<td>31</td>
</tr>
<tr>
<td></td>
<td>E14–17</td>
<td>28</td>
<td>89</td>
<td>12.5</td>
</tr>
<tr>
<td></td>
<td>E18–PO</td>
<td>48</td>
<td>52</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>P6</td>
<td>8</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Floor plate: hindbrain</td>
<td>E11</td>
<td>2</td>
<td>100</td>
<td>21</td>
</tr>
<tr>
<td></td>
<td>E13</td>
<td>4</td>
<td>100</td>
<td>34</td>
</tr>
<tr>
<td>Floor plate: midbrain</td>
<td>E11</td>
<td>2</td>
<td>100</td>
<td>19.5</td>
</tr>
<tr>
<td></td>
<td>E13</td>
<td>6</td>
<td>100</td>
<td>28.5</td>
</tr>
<tr>
<td>Ventral spinal cord</td>
<td>E11</td>
<td>22</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>E13</td>
<td>21</td>
<td>38</td>
<td>3</td>
</tr>
<tr>
<td>Dorsal spinal cord</td>
<td>E11</td>
<td>22</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>E13</td>
<td>4</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Ventral midline: forebrain</td>
<td>E11</td>
<td>2</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>E13</td>
<td>4</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Rathke's pouch</td>
<td>E13, E14</td>
<td>26</td>
<td>30</td>
<td>8.5</td>
</tr>
<tr>
<td>Limb bud</td>
<td>E11</td>
<td>60</td>
<td>33</td>
<td>10</td>
</tr>
<tr>
<td>(some TAG-1+)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sclerotome</td>
<td>E11</td>
<td>27</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Dermomyotome</td>
<td>E11</td>
<td>30</td>
<td>95</td>
<td>5</td>
</tr>
<tr>
<td>Notochord</td>
<td>E10, E11</td>
<td>9</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>E13</td>
<td>6</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Tissues were tested for their ability to promote outgrowth of commissural axons from an E11 dorsal explant. Explants were cultured 200–400 μm from the ventral-most edge of a dorsal explant, and their effect was examined after 40 hr. In addition to the tissues shown in the table, explants of the following tissues were found not to promote commissural axon outgrowth: P9 skeletal muscle, E11 maxillary process, E11 mandibular process, E11 heart, E14 lung, E14 gut, E14 retina, E18 adrenal gland, E19 skin, P4 pituitary gland, adult choroid plexus, adult salivary gland, and adult testis.
factor, we examined the angle of emergence of axons from the ventral edge of dorsal explants under two conditions: (1) with a uniform concentration of the factor (in the form of conditioned medium) and (2) with a local source of the floor plate factor (achieved by placing a floor plate explant opposite or lateral to the ventral edge).

In the presence of conditioned medium, axons emerge from all edges of the explant (Fig. 3A,B). Axons emerge from the ventral edge of these explants with a mean angle of $5.4^\circ \pm 28.6^\circ$, where $0^\circ$ indicates an angle of emergence perpendicular to the cut edge of the explant (Fig. 4A). The range of angles is broad but exhibits an approximately Gaussian distribution centered around the perpendicular. The clustering of emerging axons around the perpendicular probably reflects the existence of intrinsic cues that direct axons along a dorsal-ventral trajectory within the explant (see below).

We also examined the extent to which the angle of axon emergence is altered when outgrowth is evoked by a localized floor plate explant (Fig. 4B). With a floor plate placed opposite the ventral edge of the dorsal explant, the mean angle of emergence is $0^\circ \pm 18.2^\circ$. The angles of emergence are more sharply clustered around the perpendicular, with about half (50.4%) of the bundles emerging within $\pm 10^\circ$ of the perpendicular, compared with 27.5% in the presence of conditioned medium. Thus, cues intrinsic to the dorsal explant direct commissural axons along a dorsal-ventral trajectory within the explant, but a localized source of floor plate factor with its high point opposite the ventral edge of the explant can sharpen the distribution of angles at which the axons emerge.

The ability of the floor plate to orient commissural axons is most obvious when the floor plate explant is positioned 200–400 $\mu$m from one of the lateral edges of the dorsal explant (Fig. 4C). Under these conditions, most (91.2%) of the bundles that emerge from the ventral edge are oriented toward the floor plate (Fig. 4C), with a mean angle of emergence of $27.0^\circ \pm 21.2^\circ$. Once in the collagen gel, axons do not markedly reorient their growth (Tessier-Lavigne et al. 1988). This contrasts with the behavior of individual trigeminal sensory and corticospinal axons, which reorient as they grow through a similar matrix in response to chemoattractants (Lumsden and Davies 1983; Heffner et al. 1990). The reason why commissural axons fail to reorient in the collagen gel is unclear but could result from their growth in thick fascicles.

Because axons extend through the collagen gel in fascicles, it is possible that only a subpopulation of commissural axons respond to the floor plate and that others follow these pioneers. To avoid problems of interpretation caused by the fasciculation of axons, we examined the effect of the floor plate on commissural axonal trajectories within the dorsal neural epithelium (Placzek et al. 1990a). A floor plate explant was opposed to one end of a long dorsal explant (Fig. 5A,B), and the complete trajectory of commissural axons was assessed by injecting DiI into the region of the neural epithelium and in adjacent to the roof plate. The initial growth of DiI-labelled axons originating from this region is along the dorsal-ventral axis. Axons located close to the floor plate deviate from their dorsal-ventral trajectory and reorient toward the floor plate (Fig. 5C).

![Figure 4. The floor plate orients the growth of commissural axons. Histograms of the angles of emergence of commissural axon bundles from the ventral-most edge of E11 dorsal spinal cord explants cultured either with E11 floor-plate-conditioned medium (A) with a two-segment piece of floor plate explant placed 200–400 $\mu$m from the ventral-most edge of the dorsal explant or with a long (700–1000 $\mu$m) floor plate explant placed at right angles to the roof plate of the dorsal explant at a distance of 200–400 $\mu$m (C). Schematic of the experimental protocol in each case, illustrating typical results after 40 hr in culture, is shown alongside each histogram. (A–C) Percentage of bundles emerging at each angle. (A) Angles of emergence from explants cultured in the presence of floor-plate-conditioned medium (271 bundles; 10 explants). The variance of this distribution is significantly smaller than in A ($p<0.001$, F test) (B) Angles of emergence from explants cultured with a floor plate explant placed opposite the ventral-most edge (274 bundles; 10 explants). (C) Angles of emergence from explants cultured with a floor plate explant to one side (113 bundles; 10 explants). The mean angle of emergence is significantly different from that in A and B; $p<0.0001$; t test. (C) Fewer fascicles extended from the ventral edge because (1) fascicles also emerged from the lateral edge (these bundles were not included in this analysis) and (2) these dorsal explants were only one segment in length, compared with the two segment stretches used in A and B. Part of the ventral spinal cord, which has no activity in isolation, was left attached to each floor plate explant to prevent the floor plate from curling. The angle at which each fascicle emerged from the ventral-most edge was measured from photographs (see diagram in A). Since the ventral-most edge of the explant tended to be convex, angles were measured from the tangent to the edge at each point. Axon bundles emerging to the left of the ordinate axis were ascribed values between $0^\circ$ and $-90^\circ$; those emerging to the right were given values between $0^\circ$ and $+90^\circ$. (Reprinted, with permission, from Placzek et al. 1990a.)](https://symposium.cshlp.org/article/284/1/13584/1/PLACZEK-ET-AL)
The distance from the floor plate over which axons turn ranges from 155 µm to 375 µm for different explants, with a mean of 243 ± 54 µm (s.d.; n = 15 explants). Within this range, all labeled axons reorient their growth so that they are directed toward the floor plate explant. At greater distances, axons fail to reorient toward the floor plate and continue to project along their normal dorsal-ventral route, until they reach the cut ventral edge (Fig. 5C). To determine whether the floor plate also affects commissural axons that originate from neurons located more ventrally, similar explants were sectioned and labeled with anti-TAG-1 antibodies. The pattern of TAG-1⁺ axons throughout the neural epithelium is consistent with that of Dil-labeled axons (Fig. 5D,E), indicating that the axons of neurons located ventrally within the explants respond to the floor plate in the same way as dorsal axons. TAG-1⁺ labeled axons reorient their growth when they are within 195 ± 47 µm (s.d.; n = 14 explants) of the floor plate. Thus, the floor plate can override the intrinsic dorsal-ventral polarity cues within the dorsal neural epithelium and appears to reorient all commissural axons.

It is possible that the floor plate factor diffuses within the neural epithelium for only a short distance with more distant axons reorienting their growth by fas-
circulating on axons closer to the floor plate, resulting in a contact-dependent cascade of fasciculation. This seems unlikely, since TAG-1 axons appear unfasciculated within the dorsal spinal cord explant (see also Holley et al. 1982; Holley and Silver 1987). However, this issue can be addressed directly by interposing a piece of E11 ventral spinal cord between the floor plate and the dorsal spinal cord. Commissural axons reorient toward the floor plate under conditions in which the nearest axons are separated from the floor plate by an interposed piece of ventral spinal cord 150–220 μm long (Placzek et al. 1990a). These results suggest that the floor plate factor can diffuse long distances through ventral neural epithelium to reorient the growth of commissural axons. The diffusion through ventral neural epithelium is pertinent, since this is the normal environment through which the factor would have to diffuse in the developing embryo. The distance at which reoriented axons are observed in these experiments could represent the maximum range of action of the factor or simply be the distance over which the factor can diffuse within the time of the experiment. Nevertheless, the point in vivo at which commissural axons break away from their initial trajectory in the dorsal spinal cord and grow to the floor plate is about 100–200 μm from the ventral midline, well within the range of action of the factor defined in vitro.

We do not know whether the orienting and outgrowth promoting actions of the floor plate are mediated by the same molecule(s). Because of this, the ability of different regions of the spinal cord to reorient commissural axon growth within neural epithelium was also tested (Placzek et al. 1990a). Commissural axons turn toward E11 and E13 floor plate. In contrast, axons do not deviate from their dorsal-ventral trajectory toward E11 dorsal or ventral spinal cord explants. Thus, at E11 only the floor plate appears to release the chemoattractant. Reorientation was not observed toward E13 dorsal spinal cord. However, TAG-1-labeled axons did reorient toward E13 ventral spinal cord from which the floor plate had been removed, but reorientation occurred only within about 10 μm of the ventral tissue and not within the remainder of the dorsal neuroepithelium (not shown). Thus, within the E11–E13 spinal cord, the regional expression of orienting activity parallels that of outgrowth promoting activity.

The results described above demonstrate that a localized source of factor, in the form of a tissue explant, can compete effectively with the intrinsic polarity cues within the dorsal explant. However, in the presence of floor-plate-conditioned medium, the pattern of TAG-1+ axons within dorsal explants is not detectably different from that in explants cultured alone (Placzek et al. 1990a). Thus, a uniform concentration of factor (in the form of conditioned medium) does not prevent axons from responding to the polarity cues within the neural epithelium. Yet, floor-plate-conditioned medium promotes the extension of axons located near the dorsal or lateral edges into the collagen gel. It is possible that a gradient of the factor is established at the border between the dorsal explant and the collagen gel. For example, the factor may be degraded or otherwise inactivated by neural tissue, creating a local concentration difference that may be sufficient to direct axon outgrowth from the explant into the collagen gel. Alternatively, the factor may have growth-promoting activity even when it is not presented in a concentration gradient with the consequence that axons respond to a uniform concentration of the factor by growing into collagen. At present, we cannot exclude that the outgrowth promoting and orienting effects of the floor plate are mediated by distinct molecules. However, the specificity of expression reported above indicates that if there are distinct factors they must be restricted to the floor plate in E11 embryos.

Chemotrophic Guidance of Commissural Axons In Vivo

To provide a further test of the role of the floor-plate-derived chemoattractant in axon guidance, we have examined whether commissural axons orient their growth toward an ectopically located floor plate in vivo. Small segments of E13 rat or stage 17 chick floor plate were grafted adjacent to the neural tube of stage 10 chick embryos. Embryos were incubated for a further 40–48 hours before examination of commissural axon trajectories. Since the chick homolog of TAG-1 has not yet been identified, we used antibodies against the CRABP as a marker of chick commissural axons (Maden et al. 1989). The location of the grafted tissue was determined using monoclonal antibody (MAb) 2E7 (Dodd and Jessell 1988) to identify rat floor plate and MAb SC1 (Tanaka and Obata 1984) to identify chick floor plate.

With a grafted floor plate located near to the lateral region of the spinal cord, commissural axons could be observed to deviate from their normal ventral trajectory at a point dorsal to the motoneuron column and to emerge from the neural tube toward the ectopic floor plate (Fig. 6). CRABP immunoreactive axons on the contralateral side of the spinal cord followed their usual path and projected medially through the motor column toward the floor plate (Fig. 6). Grafts of other regions of the neural tube did not result in the deflection of commissural axons (not shown), suggesting that this effect is specific to the floor plate. These results provide preliminary evidence that the floor plate is capable of orienting commissural axons in vivo as well as in vitro.

DISCUSSION

The movement of many cell types is directed by gradients of soluble molecules emanating from a point source. For example, cellular slime molds orient their growth by chemotaxis up a gradient of cAMP, and polymorphonuclear leukocytes are attracted to leukotrienes and complement peptides (Devreotes and Zigmond 1988). The directed migration of growth cones and the ability of axons to reach their targets via aber-
Figure 6. An ectopic floor plate alters the trajectory of chick commissural axons in vivo. Grafting a rat floor plate adjacent to the neural tube of a chick embryo causes the deviation of commissural axons from their normal trajectory. Commissural axons were identified by their expression of CRABP immunoreactivity. On the side of the spinal cord contralateral to the graft, commissural axons project ventromedially to the floor plate, but on the side adjacent to the floor plate graft no medially directed CRABP+ immunoreactive axons are present in the ventral spinal cord. Axons emerge from the spinal cord and project to the region of the ectopic floor plate graft (arrowheads). Similar results were obtained with chick floor plate grafts. (r) Roof plate; (f) floor plate; (drg) dorsal root ganglion. Bar: 200 μm.

The role of the factor might then be to provide a cue that ensures that commissural axons project directly through the motor column to the ventral midline, rather than fasciculate with motor axons extending out of the spinal cord. Identification of the floor-plate-derived chemotropic factor will be necessary to determine in more detail the contribution of chemotropism to the guidance of commissural axons in vivo. In recent studies (Placzek et al., 1990b), we have found that expression of floor-plate-specific chemotropic activity in cells of the neural plate and neural tube can be induced by the notochord. Moreover, removal of the notochord at early stages of neural tube development prevents the expression of this chemoattractant by cells at the ventral midline. These findings indicate that chemotropic activity and probably other specialized properties of the floor plate are induced by local signals that derive from axial mesodermal cells of the notochord.

Chemotropism is likely to be only one of several guidance mechanisms that operate successively or coordinately to define the trajectory of commissural axons in vivo. The initial ventral trajectory of these axons through the dorsal spinal cord may be determined by extracellular matrix cues, in particular laminin (D. Karagogeos et al., in prep.). Moreover, once commissural growth cones arrive at the midline, their subsequent guidance appears to be regulated by contact-dependent interactions with floor plate cells (Dodd et al. 1988; Bovolenta et al. 1988; Bovolenta and Dodd 1990; Kuwada et al. 1990; Yaginuma et al. 1990).

There is evidence for the existence of target-derived
diffusible factors capable of orienting developing axons in two other neural systems. The analysis of developing mouse trigeminal sensory axons has shown that one of their peripheral targets, the maxillary arch, secretes a factor that is capable of directing their growth. When trigeminal ganglia from E10–E12 embryos are confronted with age-matched maxillary processes and limb buds (an inappropriate target), there is directed axonal growth to the maxillary process (Lumsden and Davies 1983, 1986). NGF itself has no effect on axon outgrowth from E10 trigeminal ganglia cultured on twodimensional substrates and axon outgrowth is unaffected by antibodies to NGF. Moreover, NGF is not detected in the maxillary process until E11 (Davies 1987).

These observations indicate that the E10 maxillary process secretes a diffusible factor(s), distinct from NGF, that evokes outgrowth of trigeminal sensory axons. A diffusible factor has also been implicated in directing the growth of axons of developing corticopontine neurons (Heffner et al. 1990). In this system, evidence for chemotropism was again obtained by confronting explanted cortical tissue with pontine tissue in collagen matrices. Neurons extend axons along an apparently normal dorsal-ventral trajectory within explants of cortical tissue but project into the collagen matrix when they reach the ventral ventricular surface (Heffner et al. 1990, see also O’Leary et al., this volume). This pattern of growth is dramatically altered by explants of pontine tissue. Axons that emerge from the ventral edge of the explant reorient their growth within the collagen matrix to project toward the target. In addition, axons appear to give off collateral branches that project directly to the pontine target. These experiments provide evidence that diffusible chemotactic factors can both reorient growth cones and evoke directional branching of axons. As in the other two systems discussed above, the identity of the chemotropic factor released by the pontine target tissue is not known.

Collectively, the findings discussed in this paper suggest that chemotropism plays a more prominent role in the guidance of central and peripheral neurons than appreciated previously. At present, NGF is the only defined molecule for which a chemotactic action has been demonstrated, and it remains possible that NGF has a tropic function in vivo, in addition to its well-established neurotrophic role. In vertebrate neural tissues, chemotropic factors appear to guide axons over a distance of up to 300 μm, which is well within the theoretical limit of action of diffusible signals in embryonic tissues and consistent with the range over which diffusible factors have been proposed to influence cell patterning in other organisms (Wolpert 1969; Crick 1970). The sensitivity of growth cones in detecting diffusible gradients has not yet been determined. However, other vertebrate cells, for example leukocytes, can orient in response to soluble gradients of chemotactic factors that generate only 1% differences in concentration across the diameter of the cell (Zigmond 1977). It would not be surprising if growth cones exhibited a similar sensitivity in their ability to recognize cell-surface and diffusible guidance cues.

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