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Perturbation of neuronal differentiation and axon guidance in the spinal cord of mouse embryos lacking a floor plate: analysis of Danforth's short-tail mutation

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Summary

The floor plate of the vertebrate nervous system has been implicated in the guidance of commissural axons at the ventral midline. Experiments in chick have also suggested that at earlier stages of development the floor plate induces the differentiation of motor neurons and other neurons of the ventral spinal cord. Here we have examined the development of the spinal cord in a mouse mutant, Danforth's short-tail, in which the floor plate is absent from caudal regions of the neuraxis. In affected regions of the spinal cord, commissural axons exhibited aberrant projection patterns as they reached and crossed the ventral midline. In addition, motor neurons were absent or markedly reduced in number in regions of the spinal cord lacking a floor plate. Our results suggest that the floor plate is indeed an intermediate target in the projection of commissural axons and support the idea that several different mechanisms operate in concert in the guidance of axons to their cellular targets in the developing nervous system. In addition, these experiments suggest that the mechanisms that govern the differentiation of the floor plate and other ventral cell types in the neural tube are common to mammals and lower vertebrates.

Key words: Danforth's short-tail, commissural axons, floor plate, motor neurons, axon guidance, spinal cord.

Introduction

The development of the nervous system depends on cellular interactions that control the identity of neurons and the initial pattern of axonal projections. The guidance of developing axons appears to involve interactions between molecules on the surface of axonal growth cones and in the neuronal environment (Dodd and Jessell, 1988). Several classes of environmental cues have been implicated in axonal guidance; these include cell surface and extracellular matrix adhesion molecules (Jessell, 1988; Takeichi, 1988; Tomaselli and Reichardt, 1989), molecules that have inhibitory actions on growth cones (Kapfhammer *et al.* 1986; Patterson, 1988; Walter at al. 1990), diffusible chemoattractants (Menesini-Chen *et al.* 1978; Lumsden and Davies, 1986; Tessier-Lavigne *et al.* 1988) and cells that serve as intermediate targets for growth cones (Bate, 1976; Bentley and Caudy, 1983; Bastiani and Goodman, 1986).

In the vertebrate nervous system, cells of the floor plate, a structure that occupies the ventral midline of the spinal cord, hindbrain and midbrain, have been implicated in axon guidance (Ramon y Cajal, 1909; Kingsbury, 1930; Jessell *et al.* 1989). The floor plate secretes a diffusible chemoattractant that orients the growth of spinal commissural axons *in vitro* (Tessier-Lavigne *et al.* 1988; Placzek *et al.* 1990*a*), and may have a role *in vivo* in the growth of these axons to the ventral midline (Placzek *et al.* 1990*b*). Contact-mediated interactions between commissural growth cones and floor plate cells may guide axons at the midline (Bovolenta and Dodd, 1990; Kuwada *et al.* 1990; Yaginuma *et al.* 1991). In addition, the expression of cell surface glycoproteins of the immunoglobulin superfamily is altered on commissural axons as they pass through the floor plate (Dodd *et al.* 1988; Furley *et al.* 1990). These observations suggest that the floor plate is an intermediate target of developing commissural axons.

Intermediate target cells have been implicated in axon guidance in invertebrates. Misrouting of axons is observed after ablation of target cells (Bentley and Caudy, 1983; Klose and Bentley, 1989) and in genetic mutants in which target cells fail to differentiate (Thomas *et al.* 1988). Examination of axonal projection patterns after laser ablation of the floor plate in zebra fish (Bernhardt and Kuwada, 1990) or in a mutant in

which floor plate cells are missing (Hatta *et al.* 1990; Kuwada and Hatta, 1990) have also provided preliminary evidence that the floor plate may contribute to axon guidance. To examine further the role of the floor plate in axon guidance in the mammalian central nervous system, we have analysed commissural axon trajectories in the mouse mutation, Danforth's short-tail (*Sd*) (Dunn *et al.* 1940) in which the floor plate is absent from a large region of the spinal cord (Theiler, 1959). The absence of the notochord through most of the axis in *Sd* mice may be the primary defect in this mutant (Gruneberg, 1958; Theiler, 1959) and may account for the absence of the floor plate (Placzek *et al.* 1990*c*).

In embryos affected by the *Sd* mutation, the floor plate was absent from the lumbosacral region of the spinal cord. In the affected region, commissural axons exhibited aberrant trajectories as they reached and crossed the ventral midline of the spinal cord. These results suggest that the floor plate contributes to the guidance of commissural axons at the midline. In addition, we found that there was a marked reduction in the number of motor neurons in the spinal cord at segmental levels in which the notochord and floor plate were absent. These findings extend studies in chick embryos which implicate the floor plate not only in axon guidance but also in the control of neuronal cell differentiation in the embryonic CNS (Yamada *et al.* 1991). Preliminary results of this work have been published in abstract form (Bovolenta *et al.* 1988).

Materials and methods

Animals

CD-1, C57BL/6 and C3H/HEH mice carrying the Danforth's short-tail (*Sd*) mutation were analysed. Mice were obtained as heterozygotes from Jackson Laboratories, USA and from B. Cattanach, Harwell, England. The mice also carried either the *Re* and A^J (Jackson Laboratory strain) or one of the following mutations that affect the colour and appearance of the coat: *Re, Ra,* W^c, A^w (Cattanach strains). We cannot exclude that the neural phenotypes obtained reflect in part the influence of mutations other than *Sd*. One argument against this, however, is that the histological appearance of the spinal cord in affected embryos was the same, regardless of which other mutations were present. In addition, as described below, in regions of the spinal cord where the floor plate was present, the neural phenotype was normal and no other defects were observed.

Heterozygotes were crossed and pregnant females were killed and embryos recovered on embryonic days (E) 10 to 20, where E0 is the day of detection of the vaginal plug. The appearance of the tail was noted and the embryos were fixed by immersion in paraformaldehyde (4% in 0.1 M phosphate buffer (PB), pH 7.4, at 4°C, for 1.5–3 h). Embryos were then immersed in 30% sucrose in PB overnight for subsequent cryostat sectioning or in phosphate-buffered saline (PBS) for plastic embedding and sectioning.

External appearance of embryo

Before E11, it is not possible to distinguish between wild-type and mutant embryos based solely on external features (Gluecksohn-Schoenheimer, 1945; Gruneberg, 1958). However, from E11 onwards, the tails of mutant animals were shrunken in diameter and length when compared with the tails of wild-type littermates. Gluecksohn-Schoenheimer (1945) and Gruneberg (1958) have previously shown that animals with more affected tails are homozygous for the *Sd* mutation while those less affected are heterozygotes.

We examined 48 *Sd*/+, 22 *Sd*/*Sd* and 43 wild-type embryos in 22 litters. We observed no differences in the expression of antigens or behaviour of axons in affected regions of homozygotes and heterozygotes. In a few cases, gross distortion of the hind quarters of young embryos (E11–13) were observed. These embryos were not included in the study.

Immunohistochemistry

After immersion in sucrose for 24–36 h, embryos were embedded in Tissue Tek and frozen. Serial transverse sections (10–15 μm) were cut on a cryostat and collected onto gelatin/chrome alum-subbed slides. Sections were washed with PBS and incubated with monoclonal or polyclonal antibodies diluted in PBS containing 1% heat-inactivated goat serum (HIGS) and 0.1% Triton X-100, overnight at 4°C. Sections were washed with PBS containing 1% HIGS and incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies overnight at 4°C, or FITC-conjugated secondary antibodies, for 30 min at 22°C. Secondary antibodies (Boehringer-Mannheim or TAGO) were diluted 1:100 in PBS/HIGS. Sections incubated with FITC-conjugated secondary antibodies were then washed with PBS and coverslipped with glycerol 50% in sodium carbonate buffer, pH 9.0, containing 0.04% paraphenylenediamine, and viewed on a Zeiss axioplan fluorescence microscope. Sections incubated with HRP-conjugated secondary antibodies were washed with PBS, incubated for 5 min with non-activated diaminobenzidine (DAB) 0.5 mg ml^{-1}, then incubated with DAB activated with 0.0003% H_2O_2. Sections were washed, dehydrated through graded alcohols and xylene and coverslipped in xylene/permount (Fisher).

Antibodies

Available antibodies that label the floor plate selectively in chick and rat do not cross-react with mouse floor plate. The following antibodies were used in this study. 2H3, a mouse IgG1 that labels the 155×10^3 *Mr* neurofilament subunit in rodents (Dodd *et al.* 1988), 4D7, a mouse IgM that recognizes TAG-1 (Yamamoto *et al.* 1986; Dodd *et al.* 1988), 5A5, a mouse IgM that identifies polysialylated NCAM (PSA-NCAM) (Dodd *et al.* 1988), 4C11 and 1C6, mouse IgMs that identify components of the basement membrane underlying the floor plate in the embryonic rodent spinal cord (Wang and Dodd, unpublished).

Histology

Plastic sections of spinal cord were used for counting motor neurons. Embryos were initially embedded in agar (1% in PBS) and 100 μm transverse sections were cut on a vibratome. These sections were washed in PBS, lightly osmicated, dehydrated and embedded in Epox (Fullam) between plastic slides and allowed to polymerize for 48 h at 60°C. 4 μm serial sections were cut on a Leitz 20/50 Histocut, and stained with toluidine blue, using standard procedures. For acetylcholinesterase (AChE), staining was performed on frozen and Epon sections according to Karnovsky and Roots (1964).

Motor neuron identification

mAb 4D7 recognizes the TAG-1 glycoprotein which first appears in the spinal cord on differentiating motor neurons

(Yamamoto *et al.* 1986; Dodd *et al.* 1988). At later stages of spinal cord development, TAG-1 is expressed by developing commissural neurons. However, at early stages of ventral spinal cord development, the expression of TAG-1 can be used to identify motor neurons (Dodd *et al.* 1988). Motor neurons also express PSA-NCAM, 2H3-labelled neurofilaments and AChE. These markers are of less diagnostic value for motor neurons because floor plate cells express PSA-NCAM and AChE, and differentiated neurons other than motor neurons express PSA-NCAM and 2H3. However, the loss of the markers in ventral spinal cord in affected embryos suggested that motor neurons were absent.

Motor neuron quantification

Motor neurons were identified by the size of their nuclei and somata as described by Lance-Jones (1982). A profile was counted only if the nucleolus was present and the Abercrombie correction factor (Abercrombie, 1946) was applied to the results to give final figures.

Results

Perturbation of notochord and floor plate development in affected embryos

Serial transverse sections through the length of the spinal cord from sacral to cervical levels in E11 to E20 embryos were used to assess the development of the notochord and floor plate in affected and wild-type embryos. In embryos exhibiting severe tail defects, the notochord was absent from the entire rostrocaudal extent of the neuraxis, whereas in embryos with less severe tail defects the notochord was absent caudally but present in patches of a few cells in cervical and thoracic regions. The differentiation of the floor plate in chick is dependent solely on inductive signals from the notochord (Placzek *et al.* 1990*c*). We therefore examined whether the floor plate was absent in *Sd* mice using several criteria: (i) cells of the floor plate have a characteristic wedge-shaped appearance (van Straaten *et al.* 1988; Smith and Schoenwolf, 1989) (Fig. 1A,B); (ii) at E11 and E12, the medial cells of the mouse floor plate express the polysialylated form of NCAM (PSA-NCAM) recognized by the mAb, 5A5 (Fig. 2A); (iii) mouse floor plate cells express acetylcholinesterase (AChE) (Fig. 3A,B); (iv) the basement membrane immediately underlying the floor plate expresses antigens recognized by mAbs 4C11 (Fig. 4A) and 1C6 (not shown). Low levels of the 4C11- and 1C6-reactive antigens are also expressed by floor plate cells in culture (J. Wang and J. Dodd, unpublished data) providing evidence that the floor plate is responsible for the secretion and deposition of these antigens in the subjacent basal lamina.

Cells at the ventral midline of the caudal-most neural tube in Sd mice did not express the characteristic morphological features of the floor plate (Fig. 1C,D). PSA-NCAM (Fig. 2B) and AChE activity (Fig. 3C,D) were not present in cells at the ventral midline of the spinal cord. Similarly, the floor plate-associated basal lamina antigens 4C11 (Fig. 4C) and 1C6 (not shown) were absent in caudal regions of affected embryos. These results provide evidence that the floor plate is absent at caudal levels in *Sd* mice. In heterozygotes, the floor plate was absent over a 200–400 μm stretch while, in homozygotes, the floor plate was absent for 600–800 μm.

In rostral regions of the spinal cord of affected embryos, the morphological and histological properties of the floor plate were detected even though the notochord was absent (Fig. 4D). Despite the absence of the notochord, coronal sections of the rostral *Sd* spinal cord appeared identical to those taken from sibling wild-type embryos at the same rostrocaudal level (Figs 4D and 8C,F). It is likely that the notochord was present in rostral regions at earlier stages of development (see discussion).

The ventral spinal cord exhibits a more generalized disruption in Sd *embryos*

At levels of the spinal cord in which the floor plate was absent the dorsal spinal cord appeared normal by morphological criteria. To determine whether the disruption of ventral spinal cord was confined to the floor plate, we examined the distribution of motor neurons in wild-type and affected embryos.

The number of presumptive motor neurons, identified by immunohistochemistry, and their position within the spinal cord were markedly different in E10 and E11 wild-type and *Sd* embryos. In normal embryos, two discrete, bilaterally located columns of motor neurons were detected at all levels of the neuraxis (Figs 5A–D, 6A–C). In contrast, at the most caudal levels of affected homozygous embryos no 4D7- or 5A5-reactive cells were observed (Figs 5E, 6D). AChE staining (shown at E12.5 in Fig. 3C) and 2H3 labelling (not shown) were also absent in the ventral spinal cord in these regions. At slightly more rostral levels, but still within the affected region, presumptive motor neurons were observed even though the floor plate was absent (Figs 5F, 6E). However, the number of labelled neurons appeared to be decreased in comparison to those in wild-type spinal cord at the same axial level and were not arranged in bilateral columns but were grouped together at the ventral midline (Figs 5F, 6E and 10). Few $4D7^+$ or $2H3^+$ cells were observed in dorsal regions of the caudal spinal cord of wild-type and *Sd* embryos at E11, suggesting that the differentiation of commissural neurons in caudal spinal cord was just beginning. $4D7^+$ neurons at the ventral midline are therefore likely to be motor neurons, despite their ectopic position. At progressively more rostral levels, there was a gradual resumption of the bilateral organization of labelled cells and bilaterally paired ventral roots that coincided with the reappearance of the floor plate (see Fig. 5G,H). Thus the absence of the floor plate in homozygous *Sd* embryos is associated with a marked loss of presumptive motor neurons most caudally and a decrease in number and a change in the position of presumptive motor neurons in more rostral regions of the affected spinal cord (Fig. 10).

It is possible that motor neurons continued to differentiate in *Sd* embryos without expressing the antigenic markers characteristic of motor neurons. We

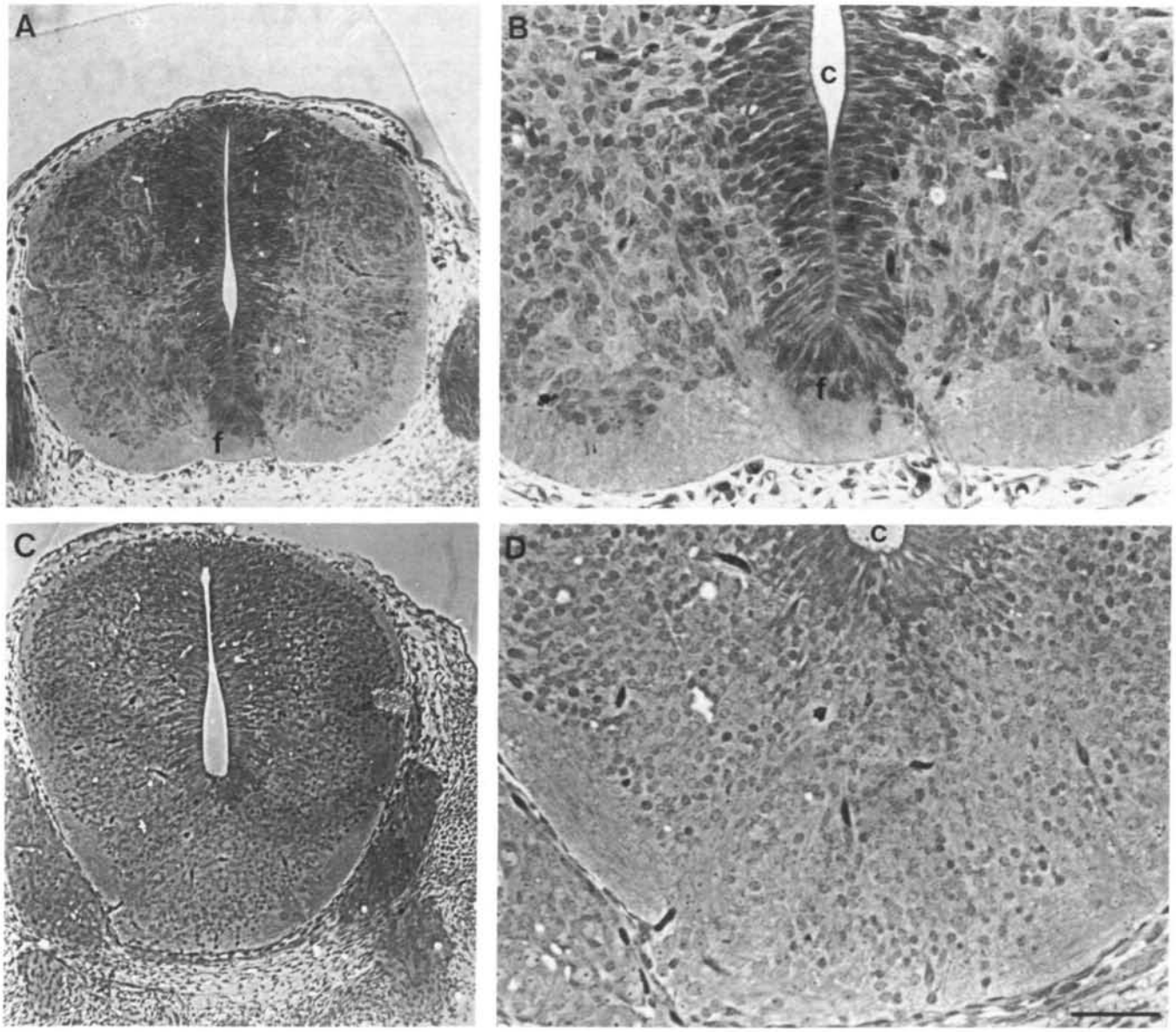


Fig. 1. The morphology of the ventral spinal cord in affected regions of the *Sd* mouse differs from the wild type. (A) 4 μm plastic section of the E13 spinal cord of a wild-type mouse showing a ventral midline floor plate (f). (B) A similar section through the spinal cord of a sibling *Sd* mouse shows that a morphologically identifiable floor plate is absent. (C,D) High-power micrographs of the ventral midline of the sections shown in A and B. m=motor neuron pool. Abbreviations: f, floor plate; c, central canal. Calibration bar: A and C=180 μm; B and D=60 μm.

therefore used morphological criteria to identify and count motor neurons in serial 4 μm plastic sections through the caudal spinal cord of normal and *Sd* embryos. Counts from two such E13 embryos are available. There was a marked reduction in the number of motor neuron profiles in a heterozygote *Sd* embryo in comparison to a wild-type littermate. In a 300 μm stretch of spinal cord in which the floor plate was absent, presumptive motor neurons were displaced to the ventral midline (Fig. 7) and reduced in number to 36% of the number present in sections taken from the same rostrocaudal level of a wild-type embryo (Fig. 7 and Table 1). In the 100 μm that includes the emerging caudal end of the floor plate, the number of motor neuron profiles was reduced to 53%. Thus, in the affected region of the spinal cord near to the caudal end of the floor plate, motor neurons were reduced in number and displaced medially. Motor neuron numbers were not reduced to zero in this *Sd* embryo presumably because the embryo was a heterozygote and the region lacking a floor plate was relatively short (probably corresponding to regions F and G in Fig. 5). These results, together with the immunohistochemical data at E11 and E12, suggest that motor neurons do not differentiate in the ventral spinal cord in the absence of the floor plate and at distances over 500 μm away from

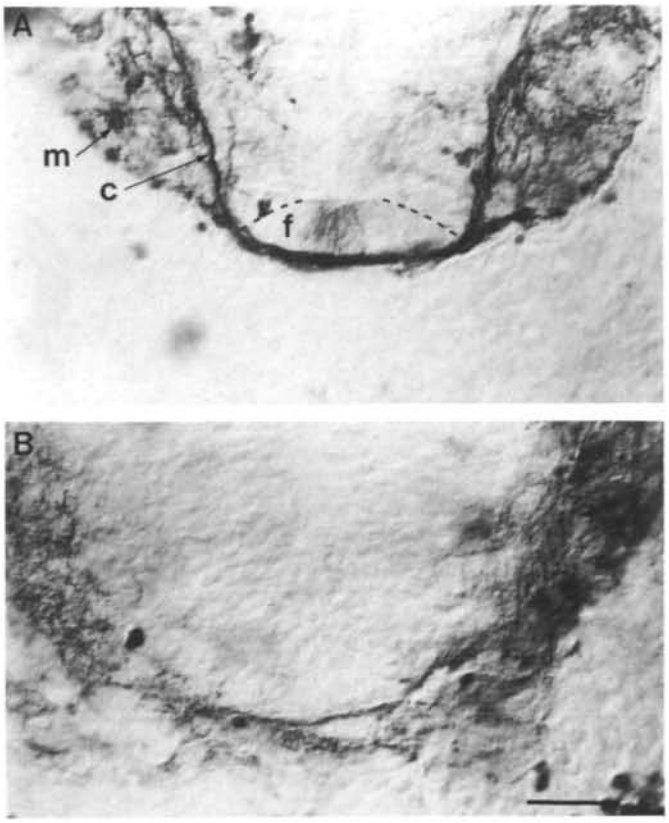


Fig. 2. Expression of PSA-NCAM in the ventral midline is absent in the affected regions of the *Sd* mouse. (A) Ventral region of a normal E12 mouse spinal cord. The midline region of the floor plate (f) is labelled with mAb 5A5. The lateral extent of the floor plate is identified morphologically and is indicated by dashed lines. 5A5 also labels the motor neurons (m) and the commissural axons (c) that cross the floor plate. (B) Ventral region of a littermate *Sd/Sd* mouse. Although 5A5 labels motor neurons and commissural axons, there is no midline labelling of the neuroepithelium. Note that the sections in A and B are taken from the same level in the rostrocaudal axis. Calibration: 45 μm. We examined the expression of 5A5 in 9 *Sd/Sd*, 16 *Sd/+* and 20 +/+ embryos at E11–13.

Table 1. *Number of motor neurons in equivalent spinal cord sections in the presence and absence of the floor plate*

100 μm regions examined: caudal to rostral	Mean no. of motor neurons per 100 μm		% decrease in motor neuron no. in *Sd/+*
	Sd/+	+/+	
1	487.5 (8*)	1257.5 (11)	61.2
2	387.5 (16)	1057.5 (11)	63.4
3	330 (11)	1032.5 (8)	68
4	587.5 (9)	1100 (6)	46.6

Motor neuron profiles were counted in 4 μm sections in equivalent serial 100 μm regions of an E13 *Sd/+* mouse and a +/+ littermate. Region 1 is the most caudal analysed whereas region 4 is the most rostral and includes the region in which the floor plate is first observed in the affected mouse.

* number of 4 μm sections counted.

the caudal end of the floor plate. In regions that lack a ventral floor plate but that are within 400 μm of the caudal end of the floor plate, motor neurons are reduced in number and displaced towards the midline. Motor neurons appear to differentiate in an arc at a distance from, but around, the caudal end of the floor plate. These data suggest that cells further than 500 μm from the floor plate do not receive a motor neuron-inducing signal. Studies in chick embryos from which the notochord has been removed have also provided evidence that motor neuron differentiation and position are dependent on the floor plate and/or the notochord (Yamada *et al.* 1991 see Discussion).

Alterations in commissural axon trajectory in the spinal cord of Sd *embryos*

We next examined the axonal projection pattern of commissural neurons in the embryonic spinal cord of normal and *Sd* embryos from E12 to E14 using mAbs 4D7 against the TAG-1 glycoprotein and 2H3 against neurofilaments. At E12, TAG-1 expression on motor neurons is low or absent but is present at high levels on commissural axons. In embryonic rat and mouse spinal cord, TAG-1 is expressed on the cell bodies of commissural neurons in the extreme dorsal region of the spinal cord before axon extension (Yamamoto *et al.* 1986; Dodd *et al.* 1988). In normal embryos, commissural axons initially project ventrally, close to the lateral edge of the spinal cord until they reach the motor neuron column. Axons then project ventrally and medially through the motor neuron column towards the midline floor plate (Fig. 8A,D). On arrival at the midline, axons cross the floor plate and turn rostrally in register with the contralateral face of the floor plate, to join the longitudinally projecting ventral funiculus (Holley, 1982; Dodd *et al.* 1988; Bovolenta and Dodd, 1990).

There was no obvious change in the location and number of TAG-1$^+$ neurons in the dorsal spinal cord of wild-type and affected embryos. Moreover, the initial ventral trajectory of commissural axons was similar in normal and affected embryos. However, as axons reached more ventral regions, their projection patterns diverged. In normal embryos, axons projected medially and ventrally (Fig. 8A,D), whereas in affected regions of *Sd* spinal cords commissural axons extended to the ventral midline near to the lateral edge of the spinal cord without projecting medially through the neuroepithelium (Fig. 8B,E). Commissural axons in *Sd* embryos that reached the ventral midline *via* this circumferential route then took one of two abnormal paths. Some axons crossed the midline and continued to project in the transverse plane such that they extended towards the dorsal region of the contralateral side of the spinal cord (Figs 8E, 9A,B). Other axons projected out of the spinal cord near the ventral midline forming an ectopic bundle of axons that projected a considerable distance into the mesenchyme surrounding the ventral spinal cord (Fig. 8B). Thus there is a marked perturbation of

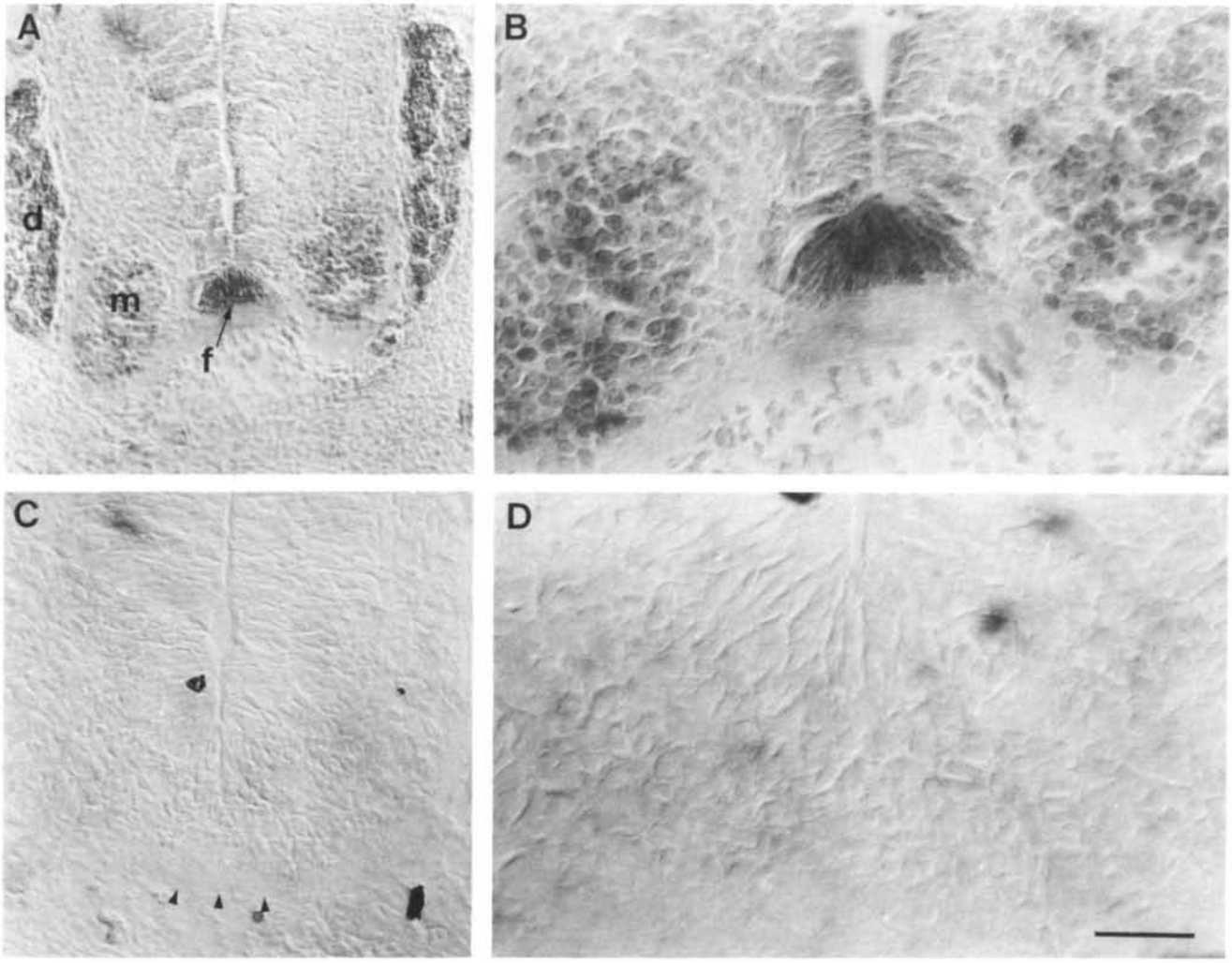


Fig. 3. Acetylcholinesterase labelling is absent from the ventral midline of spinal cord in affected embryos. (A,B) AChE staining is present in the floor plate (f) and motor neurons (m) in the E12.5 wild-type mouse spinal cord. (C,D) AChE staining is absent from the ventral spinal cord in the E12.5 *Sd/Sd* mouse. The sections in A and C are taken from similar axial levels in wild-type and *Sd* mice. B and D show high power views of the ventral midline from A and B respectively. Abbreviations: f, floor plate; m, motor neuron pool; d, dorsal root ganglion. Arrowheads in C indicate the ventral edge of the spinal cord. Calibration: A and C=200 μm; B and D=45 μm. The distribution of AChE was examined in the spinal cords of 3 *Sd/Sd*, 6 *Sd/+* and 9 *+/+* embryos at E12–20.

the commissural axon trajectory at the ventral midline in the absence of a floor plate.

In rostral regions of Sd *embryos the commissural axon trajectory is normal*

As described above, in *Sd* embryos, the floor plate is absent only in the lumbosacral region of the spinal cord and in thoracic and cervical regions a floor plate is present even in the absence of an underlying notochord (Fig. 8C,F). In these rostral regions, the axons of commissural neurons exhibit the trajectory observed in wild-type embryos (Fig. 8C,F). This result suggests that the *Sd* and other mutations do not affect the commissural axon trajectory directly. The correlation between the absence of a floor plate and the perturbation of the axon pathway suggests that the guidance of commissural axons at the midline is dependent on an intact ventral spinal cord.

Reorientation of commissural axons towards the floor plate

The floor plate orients the growth of commissural axons *in vitro* by the release of a diffusible chemoattractant (Tessier-Lavigne *et al.* 1988; Placzek *et al.* 1990*a*). One test of whether axons orient towards the floor plate *in vivo* is to monitor the trajectory of commissural axons in the most rostral part of the region of spinal cord lacking a floor plate. At distances of more than approximately 120 μm caudal to the floor plate the projection pattern of commissural axons at the midline was perturbed, as described above (Figs 8B,E; 9A,B). However, within 120 μm of the area containing a floor plate TAG-1-

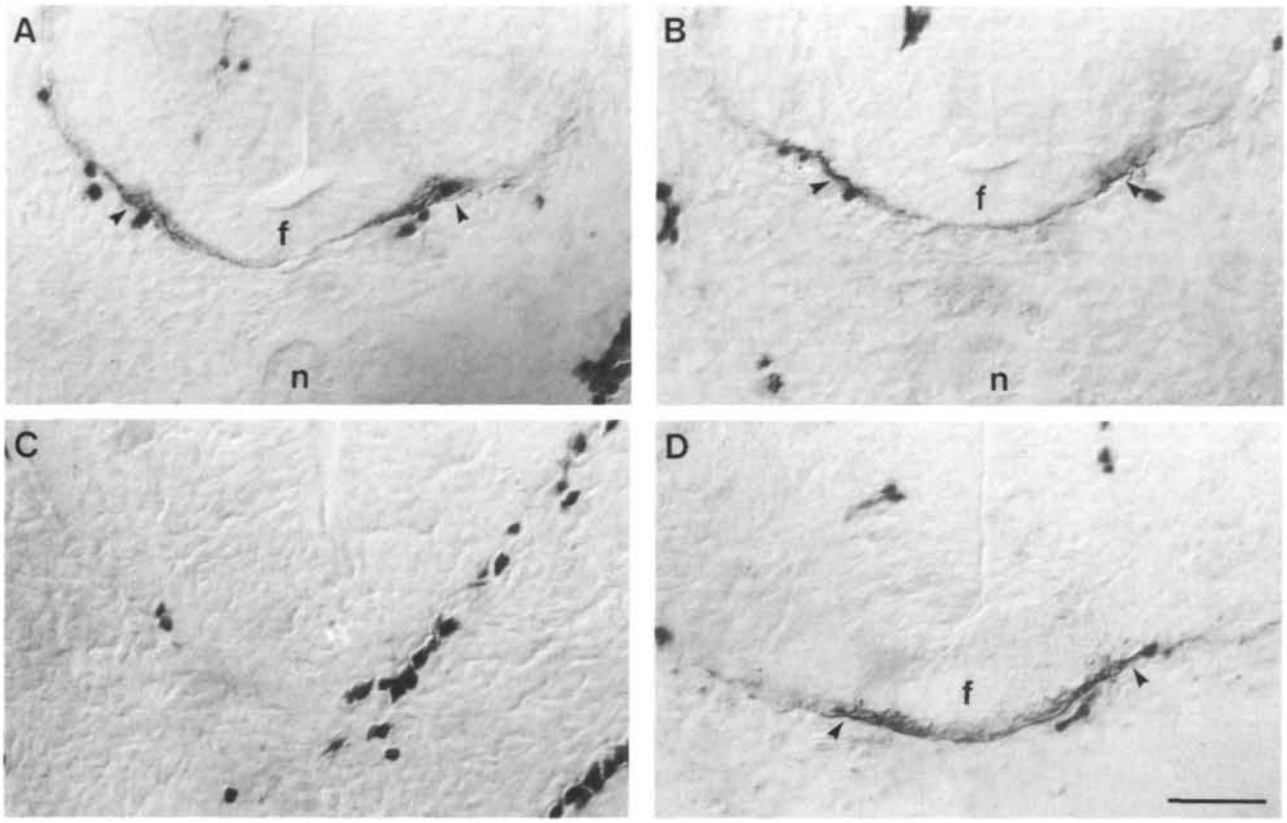


Fig. 4. Components of the basal lamina underlying the floor plate are absent in caudal regions of the *Sd* mouse. Labelling with mAb 4C11 is shown in sections of spinal cord taken from caudal (A) and rostral (B) regions of an E12 wild-type mouse and caudal (C) and rostral (D) sections of an *Sd* sibling embryo. 4C11 labelling is present at all levels of the neuraxis in normal embryos containing a notochord (n) but is not present in the absence of a floor plate (C). (D) 4C11 labelling is observed in rostral *Sd* spinal cord where the floor plate is present (f), despite the absence of a notochord. Abbreviations: f, floor plate; n, notochord; arrowheads indicate lateral extent of the floor plate. Calibration: 60 μm. 4C11 labelling was examined in 6 *Sd/Sd*, 22 *Sd/+* and 21 +/+ embryos at E11 and E12.

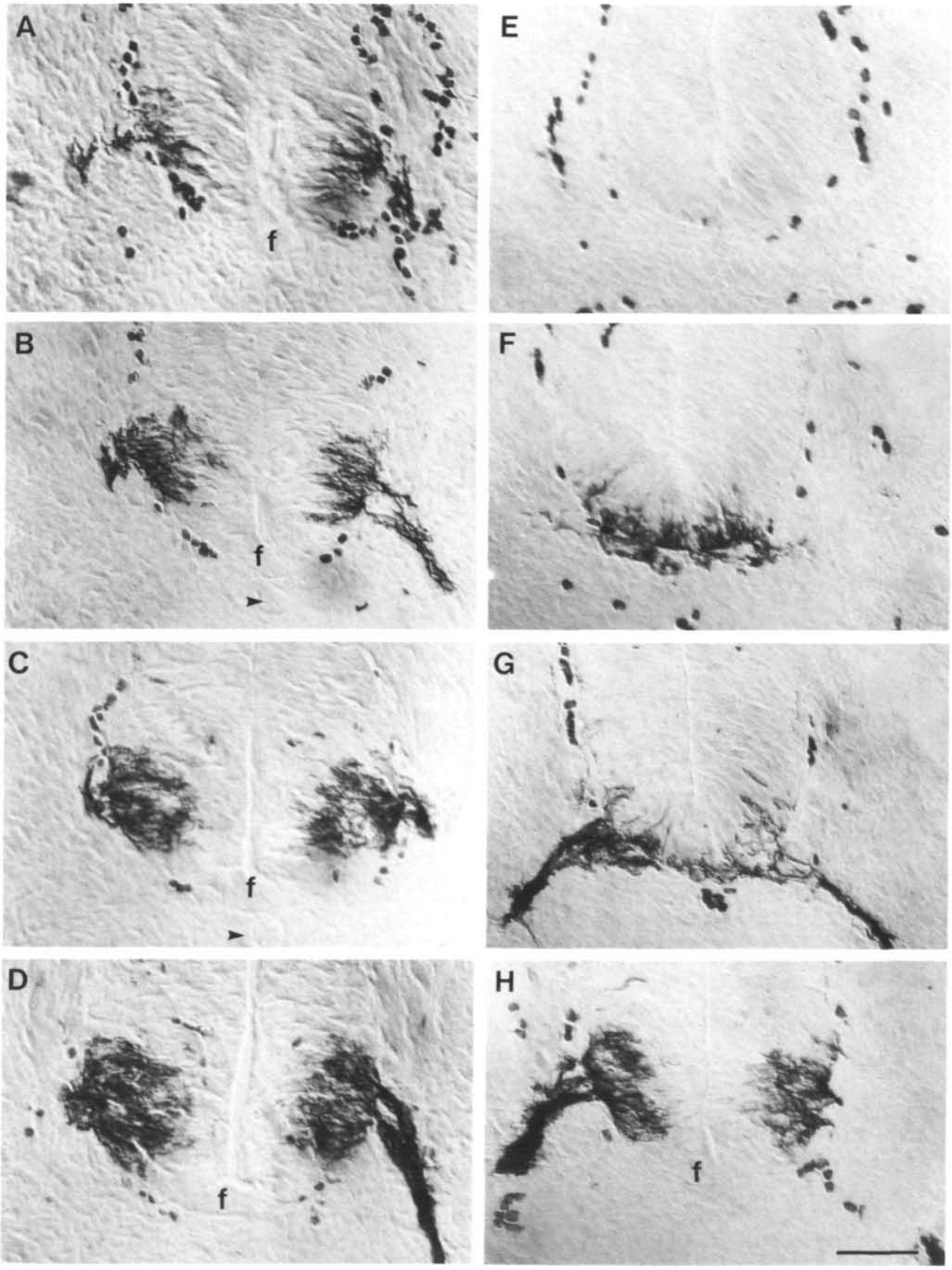
labelled commissural axons turned at the ventral midline to project longitudinally towards the caudal end of the floor plate (Figs 9C,D; 10). This finding suggests that growth cones are oriented towards the floor plate and is consistent with the response of growth cones to a chemoattractant emanating from the floor plate *in vivo*. A more detailed analysis of the paths taken by individual commissural axons is required to confirm this possibility and to define distances over which such a chemoattractant can act *in vivo*.

TAG-1 expression on commissural axons in Sd *embryos*

In normal embryos, TAG-1 is expressed on the ipsilateral axonal segment of commissural neurons (Dodd *et al.* 1988). At early developmental stages, such as in caudal levels of E12 embryos, the loss of TAG-1 expression occurs as commissural axons cross the floor plate. Thus no TAG-1 immunoreactivity is observed in the ventral funiculi (arrowheads in Fig. 8A,D). As the spinal cord develops, TAG-1 is lost only as axons enter the ventral funiculi, resulting in some labelling in the medial part of the ventral funiculi at rostral levels (Fig. 8C,D) and at caudal levels in older embryos. We examined whether TAG-1 expression by commissural axons was altered in affected regions of *Sd* embryos. In the absence of the floor plate, TAG-1 appeared to be expressed on commissural axons for a greater extent of their trajectory. Axons that had extended out of the spinal cord (Fig. 8B), into the contralateral spinal cord (Figs 8E, 9A,B) or turned at the ventral midline (Fig. 9C,D) expressed TAG-1. In rostral regions of affected embryos, where the floor plate was present but the notochord was absent, the expression pattern of TAG-1 on motor and commissural neurons was normal (Figs 8C,F and 9E).

Discussion

The present studies provide further evidence that the notochord and floor plate exert important influences on the development of the mammalian central nervous system. First, the absence of the notochord throughout the neuraxis is accompanied by the failure of floor plate differentiation in lumbosacral regions, as defined by several morphological and antigenic criteria. This result suggests that induction of the floor plate by the notochord, which has been demonstrated or inferred in


A
f
E
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f
F
C
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G
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Fig. 5. Changes in the number and position of presumptive motor neurons in *Sd* embryos. E11 normal (A–D) and *Sd* (E–H) embryos were labelled with mAb 4D7 to demonstrate the presence and position of motor neurons at different locations along the neuraxis. A–D show the position of motor neurons in a wild-type embryo in caudal spinal cord. E–H show sections taken from approximately the same levels as those in A–D. A and E are the most caudal sections and are 600–700 μm caudal to the sections shown in D and H respectively. (E) Motor neurons cannot be detected at the most caudal levels in the absence of the floor plate. F and G show sections more rostral than E but in which the floor plate is still absent. Motor neurons have developed but are found in a midline location. (H) With the emergence of the floor plate, motor neurons acquire a bilateral position. Abbreviations: f, floor plate. Arrowhead in B and C indicates the notochord. Calibration: 120 μm. Motor neuron distribution was examined in 6 *Sd/Sd*, 13 *Sd/+* and 9 +/+ E11 embryos using 4D7, 5A5 and 2H3.

chick (Watterson *et al.* 1955; van Straaten *et al.* 1985, 1988; Smith and Schoenwolf, 1989; Placzek *et al.* 1990*c*), occurs also during mammalian neural tube development. Second, the number of motor neurons appears to be markedly reduced at axial levels in which the notochord and floor plate are absent (Yamada *et al.* 1991). Signals from the notochord and/or from the floor plate may therefore be responsible for ventral spinal cord organization in mammals as well as in birds (Yamada *et al.* 1991). Third, commissural axons grow along abnormal pathways as they reach the ventral midline of the spinal cord at levels lacking a floor plate. However, the initial ventral trajectory of these axons is not obviously disrupted, supporting the idea that guidance cues other than the floor plate control the initial growth of commissural axons (Placzek *et al.* 1990*a*).

The *Sd* mutation was first identified by Danforth (1930) and described in detail by Dunn *et al.* (1940). The earliest observable deficit in *Sd* embryos is the degeneration of the notochord, such that homozygous embryos, E11 or older, lack a recognizable notochord (Theiler, 1954, 1959; Gruneberg, 1958). We have observed that in lumbosacral regions of E11 embryos the neural tube failed to exhibit any morphological specialization or antigenic markers characteristic of the floor plate in wild-type embryos. However, at more rostral levels a floor plate was present, even though the notochord was absent. One likely explanation for the presence of the floor plate rostrally is that notochord degeneration occurs after induction of the floor plate in rostral regions is completed. Several studies in other vertebrate species have shown that, following induction, floor plate differentiation proceeds independent of the notochord from a time soon after neural tube closure (Horstadius, 1944; Kitchin, 1949; Watterson *et al.* 1955; Yamada *et al.* 1991). In chick embryos, floor plate differentiation in caudal regions of the neuraxis is delayed with respect to rostral regions (Placzek *et al.*

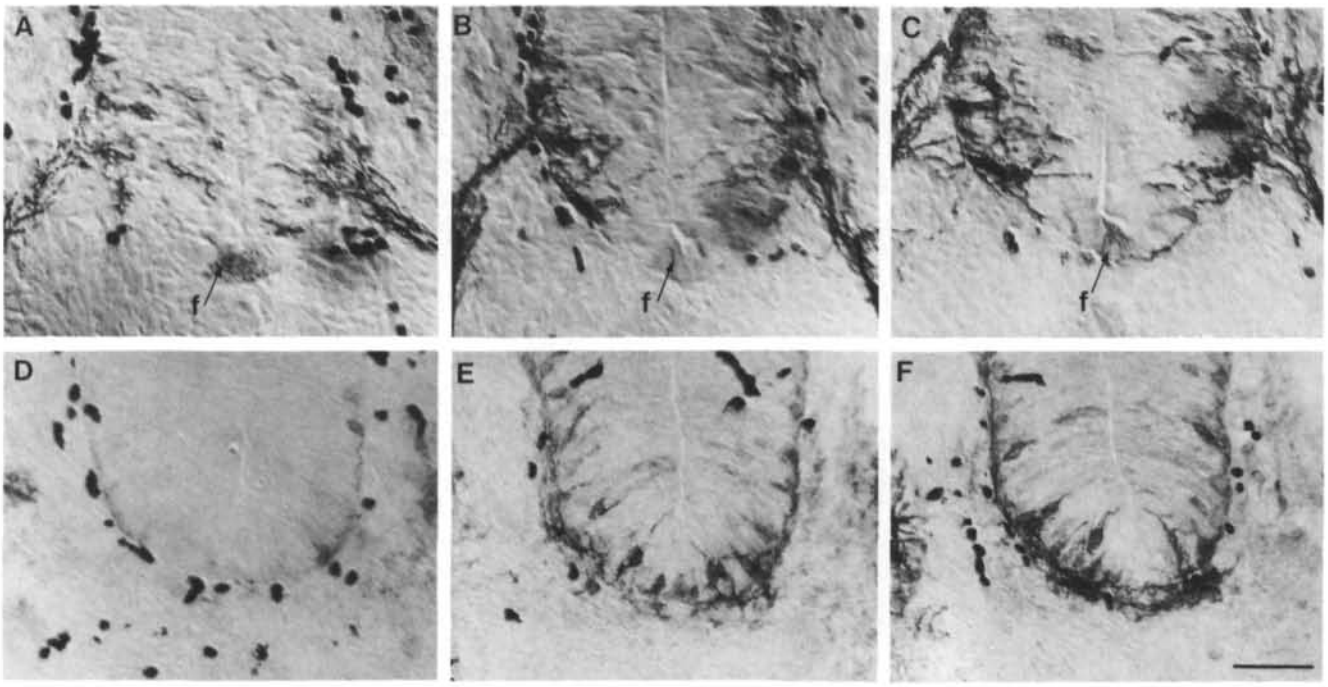
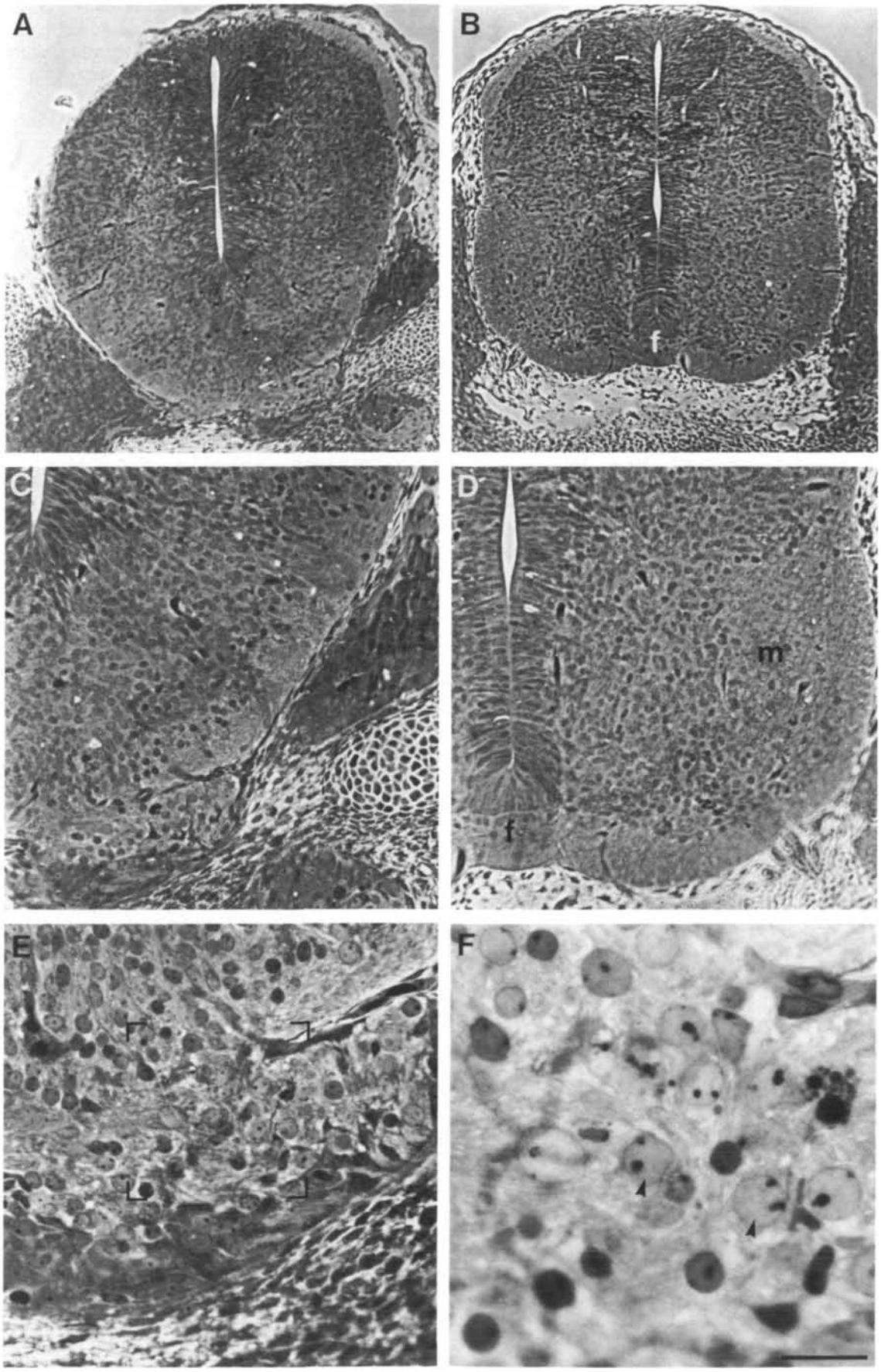


Fig. 6. PSA-NCAM expression demonstrates the loss of motor neurons in caudal regions of *Sd* embryos. E11 normal (A–C) and *Sd* (D–F) sibling embryos were labelled with mAb 5A5 to demonstrate the presence and position of PSA-NCAM. A and D are the most caudal sections in each series and the distance between A and C and between D and F was approximately 200 μm. A–C show the bilateral position of motor neurons in normal spinal cord taken from caudal regions of a wild-type embryo. Note the labelling of the floor plate by 5A5 in A–C. D–F show three sections at similar caudal levels in a sibling *Sd/Sd* embryo. Motor neurons are absent from the most caudal region (D) and decreased in number and positioned close to the midline at more rostral levels though still in the absence of the floor plate (E,F). f=floor plate. Calibration: 80 μm.


A
B
f
C
D
m
f
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F

Fig. 7. The position and number of morphologically identified motor neurons are perturbed in *Sd* mice. 4 μm plastic sections were used to count motor neurons at E13. A and B show the *Sd* (A) and normal (B) spinal cords demonstrating the presence in wild-type of large bilateral pools of motor neurons. C and D show comparable high-power micrographs of the regions containing motor neurons in *Sd* (C) and wild-type (D) spinal cords. E and F show increasingly higher power views of the ventral regions of the *Sd* spinal cord to illustrate the profiles counted as motor neurons. Examples of motor neuron profiles are indicated by arrows in F. The region shown in F is the same region that is enclosed by corner brackets in E. Calibrations: A,B=200 μm; C,D=100 μm; E=30 μm; F=15 μm. Abbreviations: m, wild-type motor neuron pool; f, floor plate.

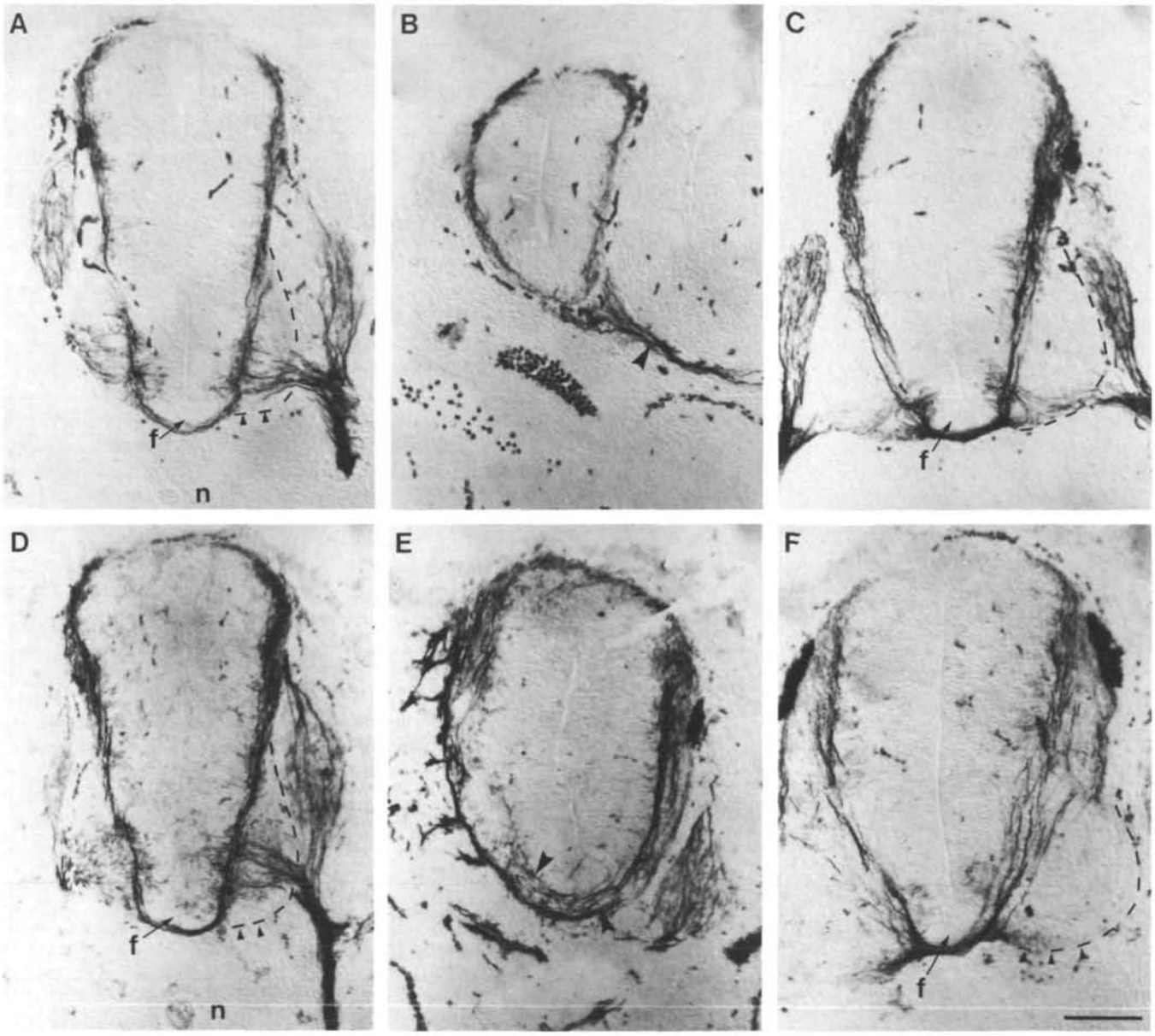

Fig. 8. The projection of commissural axons is perturbed in the absence of a floor plate. (A) Section of caudal spinal cord from a wild-type E12 mouse embryo. (B,C) Sections from a sibling *Sd/Sd* embryo showing caudal (B) and rostral (C) spinal cord. (D) Section through caudal spinal cord of a wild-type late E12 embryo. (E,F) Sections through caudal (E) and rostral (F) spinal cord of a sibling *Sd/Sd* embryo. All sections have been labelled with mAb 4D7 to illustrate the projection of commissural axons. Axons in rostral regions of the affected embryos (where a floor plate (f) is present) project normally. In caudal regions of affected embryos, the projection is perturbed (compare A with B and D with E) such that axons leave the spinal cord (arrowhead in B) or extend into the contralateral side of the spinal cord without making a rostral turn (arrowheads in E). Small arrowheads in A,D and F indicate the ventral edge of the ventral funiculus. Dashed lines in A,C,D and F indicate the lateral edge of the spinal cord. Abbreviations: f, floor plate; n, notochord. Calibration: 120 μm. We examined the trajectory of commissural axons in sections from 15 *Sd/Sd*, 32 *Sd/+* and 31 +/+ embryos at E12 and E13.

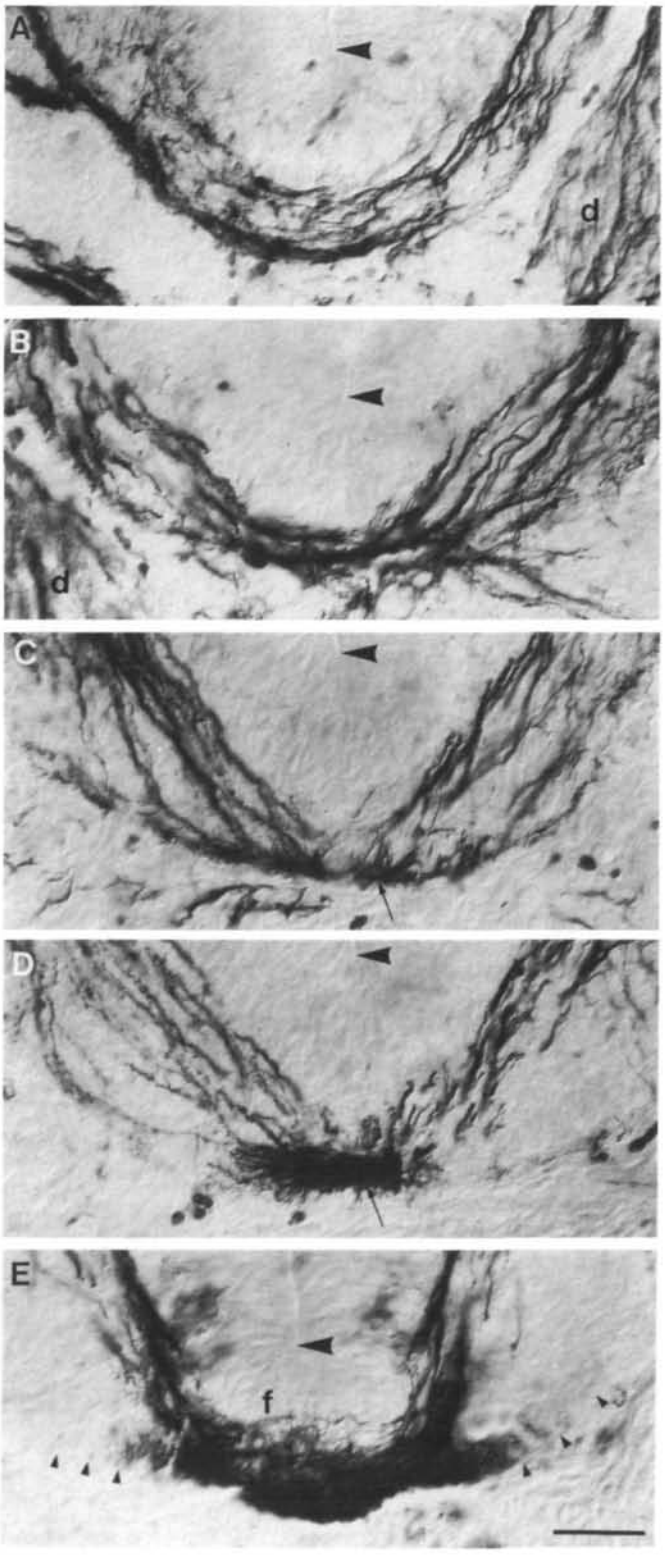

Fig. 9. Commissural axons in floor plate-deficient regions turn rostrally towards the caudal tip of the floor plate. A–E show successively more rostral sections of caudal spinal cord from a *Sd/Sd* E12 embryo. (A,B) At 300 μm (A) and 180 μm (B) caudal to the end of the floor plate axons extend along the lateral edge of the spinal cord towards the midline and continue into the contralateral side of the spinal cord. (C,D) At positions closer to the caudal end of the floor plate (120 μm and 60 μm, respectively) axons take a more directed course towards the ventral midline and turn at the midline towards the caudal tip of the floor plate (small arrows in C and D indicate bundles of axons that have turned rostrally). (E) The floor plate (f) is present in the ventral midline 60 μm rostral to the section shown in D. Axons take the normal trajectory in the presence of the floor plate. Large arrowheads in each photograph indicate the ventral tip of the central canal, providing a marker of the midline. Small arrows in E indicate the ventral funiculi. d=dorsal root ganglion. Calibration: 45 μm.

1990*c*). Thus it is possible that in caudal regions the floor plate is absent because the notochord is not present for long enough to induce and stabilize a floor plate or that the notochord is never present caudally.

Motor neuron differentiation and position are perturbed in the Sd *mouse*

The analysis of presumptive motor neuron differentiation presented here provides preliminary evidence that the absence of the notochord and/or the floor plate results in a marked reduction in motor neuron number. It is possible that the number of motor neurons reflects a decrease in the total cell number within the mutant spinal cord. Although we have not performed total cell counts, this seems unlikely to account fully for the observed decrease since the position of motor neuron profiles was also altered. Furthermore, the distribution of motor neurons at the midline in regions just caudal to the floor plate suggests that signals from the floor plate can travel caudally as well as laterally within the neuroepithelium. Since the nature and expression pattern of the *Sd* gene has not been determined, it is difficult to exclude the possibility that the reduction in motor neuron number results from a direct effect of the mutant gene on caudal motor neurons rather than from the absence of the floor plate and notochord. However, studies of embryonic chick spinal cord have shown that surgical removal of the notochord leads to the absence of both floor plate cells and motor neurons (Yamada *et al.* 1991). Moreover, the presence of an ectopic floor plate in chick spinal cord results in the induction of motor neurons in adjacent lateral and caudal neuroepithelium (Yamada *et al.* 1991). The studies presented here suggest that common mechanisms control the differentiation of ventral spinal cord cells in birds and mammals.

Recent studies in *Xenopus* and zebrafish embryos have also examined the relationship between the notochord, the floor plate and motor neurons. UV treatment of fertilized *Xenopus* embryos at the one-cell stage results in a graded series of axial defects, which include embryos in which the neural tube forms but in which the notochord and the floor plate are absent (Youn and Malacinski, 1981; Clarke *et al.* 1991). In these embryos, there is an 85% reduction in the number of identified motor neurons. Segregation of

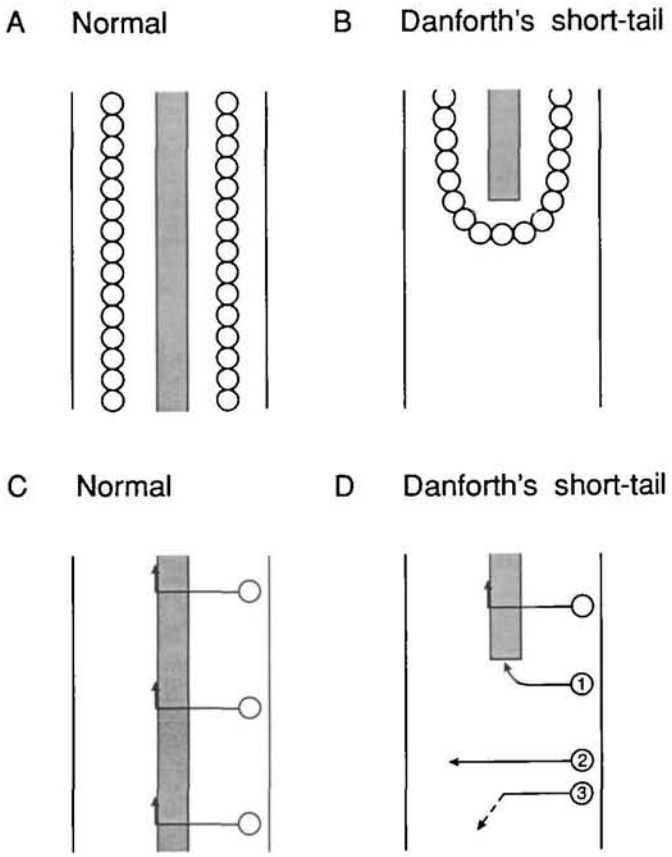

Fig. 10. Diagram to illustrate the behaviour of axons and the expression of motor neurons in normal (A,C) and affected (B,D) mice. (A) In normal embryos, the floor plate is present throughout the length of the spinal cord and motor neurons are located in bilaterally symmetrical pools adjacent to the floor plate. (B) In rostral regions of the affected embryos, the position of motor neurons is normal. In caudal regions of the spinal cord lacking a floor plate, motor neurons are absent. Close to the caudal end of the floor plate, in regions of the spinal cord without a floor plate, motor neurons are present, in decreased numbers, but are located close to or at the midline. (C) In wild-type embryos, the floor plate is present throughout the length of the spinal cord and axons cross the floor plate then turn rostrally and project longitudinally within the ventral funiculus adjacent to the floor plate. (D) In caudal regions of the spinal cord in *Sd* mice, at distances greater than 120 μm from the floor plate, commissural axons fail to make the longitudinal turn into the ventral funiculus, instead projecting into the contralateral spinal cord (2) or extending out of the spinal cord (dashed arrow on neuron 3). Within 120 μm of the caudal end of the floor plate, commissural axons take aberrant pathways but appear to project towards the floor plate (1). In rostral regions, where the floor plate is present, the projection path is normal.

primary and secondary motor neurons on the basis of somatic size indicates that the primary motor neurons are maintained while there appears to be a complete elimination of secondary motor neurons (Clarke *et al.* 1991). Similarly, in the zebrafish mutant, *cyc-1,* primary motor neurons are not altered by the absence of a floor plate (see Eisen, 1991). The persistence of primary motor neurons in *Xenopus* and zebrafish suggests that the differentiation of these neurons is controlled by signals that arise before the differentiation of the notochord and floor plate. In avian and mammalian species, motor neurons may correspond more closely to secondary than to primary motor neurons in lower vertebrates.

Commissural axon guidance in Sd *mice*

Several previous studies have implicated the floor plate as an intermediate target in the long-range and contact-mediated guidance of commissural axons in the developing spinal cord (Tessier-Lavigne *et al.* 1988; Bovolenta and Dodd, 1990; Kuwada *et al.* 1990; Placzek *et al.* 1990*a*; Clarke *et al.* 1991; Yaginuma *et al.* 1991). The *Sd* embryo provides an opportunity to examine commissural axon trajectories in the absence of this putative intermediate target. However, the more generalized perturbation of cell differentiation in the ventral spinal cord, including the reduction in motor neuron number, means that it is not possible to correlate changes in axon trajectory solely with the absence of the floor plate. Despite this, observation of commissural axon guidance in *Sd* embryos has provided evidence to support a role for the floor plate in commissural axon guidance *in vivo*.

(i) The initial projection of commissural axons is unaffected by the absence of the floor plate

In *Sd* and unaffected embryos, commissural axons initially projected ventrally, suggesting the presence of guidance cues that are independent of the floor plate. Commissural axons in the spinal cord of floor plate-deficient chick embryos have a similar initial ventral projection (Placzek *et al.* 1991). Moreover, commissural axons in rat dorsal spinal cord explants project ventrally in the absence of a floor plate (Placzek *et al.* 1990*a*). It is possible that signals from the roof plate, a dorsal midline structure which persists in *Sd* embryos, contribute to the establishment of dorsoventral differences in the neural epithelium that are recognized by commissural growth cones. Alternatively, commissural axons may perceive the ventral neuroepithelium as dorsal. In the chick, the absence of the notochord and floor plate results in the expression of dorsal antigens throughout the dorsoventral axis of the spinal cord (Yamada *et al.* 1991).

(ii) The floor plate-derived chemoattractant may be necessary for ventromedial growth of commissural axons

The first detectable differences in commissural axon trajectory in *Sd* and wild-type spinal cords occur as growth cones enter the ventral region of the spinal cord. In wild-type embryos, commissural axons projected ventromedially, through the motor column to the floor plate. Studies *in vitro* have provided evidence that commissural axons may be guided to the ventral midline by the release of a diffusible chemoattractant from the floor plate (Tessier-Lavigne *et al.* 1988;

Placzek *et al.* 1990*a*). Commissural axons may respond to this factor, projecting directly across the motor column to the ventral midline, rather than fasciculating with motor axons that project out of the spinal cord (Placzek *et al.* 1990*a*,*b*). The failure of commissural axons to project medially through neuroepithelium in *Sd* embryos may result from the absence of the floor plate or from the absence of motor neurons. The absence or ventromedial displacement of motor neurons may permit the circumferential projection of commissural axons. The projection of commissural axons out of the spinal cord at the midline in *Sd* embryos may therefore be due, in part, to fasciculation of commissural axons with the axons of residual motor neurons that form a single ventral nerve root.

(iii) The chemotropic factor may operate in vivo

Preliminary evidence suggests that the floor plate-derived chemoattractant operates *in vivo*. Grafts of the floor plate adjacent to the chick neural tube lead to the growth of commissural axons out of the spinal cord to the vicinity of the graft (Placzek *et al.* 1990*b*). In addition, in regions of chick embryos in which the spinal cord has been experimentally rotated, spinal axons have been observed to project in the direction of the ectopically placed floor plate (H. Yaginuma and R. W. Oppenheim, personal communication). The behaviour of commissural axons in *Sd* embryos in the region of the spinal cord immediately caudal to the level at which the floor plate is present provides further evidence that the floor plate can orient axons *in vivo*. Commissural axons at spinal levels lacking a floor plate turned rostrally at the midline, to form a single ventral fasciculus, possibly in response to the chemoattractant released from the caudal end of the floor plate.

(iv) Cues on floor plate cells or in the basal lamina may guide commissural axons at the midline

The major deviation in commissural axon trajectory was observed as commissural axons reached the ventral midline. The transverse projection of commissural axons into the contralateral side of the spinal cord suggests that the floor plate has a role in the transition from circumferential to longitudinal growth of commissural axons. In rat embryos, this transition occurs near to the contralateral edge of the floor plate (Dodd *et al.* 1988; Bovolenta and Dodd, 1990) suggesting that contact between commissural growth cones and the floor plate initiates the switch to growth in the longitudinal plane. The patterns of growth cone extension by commissural axons in lower vertebrates are also perturbed in the absence of a floor plate (Bernhardt and Kuwada, 1990; Kuwada and Hatta, 1990; Clarke *et al.* 1991).

The floor plate expresses several surface antigens that may act as adhesion molecules, providing contact-mediated cues at the midline (Dodd and Jessell, 1988; Bovolenta and Dodd, 1990; Chang and Lagenaur, 1990). Commissural axons cross the midline through the ventral-most part of the floor plate (Bovolenta and Dodd, 1990) and, in the chick and zebrafish, the growth cones of commissural axons have been shown to contact the basement membrane under the floor plate (Kuwada *et al.* 1990; Yaginuma *et al.* 1991). Antigens present in the basement membrane underlying the basal surface of floor plate cells may contribute to the onset of longitudinal growth. Two basement membrane antigens identified by mAbs 4C11 and 1C6 that are found in the basement membrane ventral to the floor plate in normal embryos are absent in *Sd* embryos in regions lacking the floor plate. These or similar floor-plate-associated antigens may play a role in commissural axon guidance at the floor plate.

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