Development 126, 3649-3659 (1999)
Printed in Great Britain © The Company of Biologists Limited 1999
DEV8619

Ventral midline cells are required for the local control of commissural axon guidance in the mouse spinal cord

Michael P. Matise[1,*], Marc Lustig[3], Takeshi Sakurai[3], Martin Grumet[3] and Alexandra L. Joyner[1,2]

[1]Howard Hughes Medical Institute and Skirball Institute of Biomolecular Medicine, [2]Departments of Cell Biology and Physiology & Neuroscience, [3]Department of Pharmacology, New York University School of Medicine, 540 First Avenue, New York, NY 10016, USA

*Author for correspondence (e-mail: matise@saturn.med.nyu.edu)

Accepted 9 June; published on WWW 19 July 1999

SUMMARY

Specialized cells at the midline of the central nervous system have been implicated in controlling axon projections in both invertebrates and vertebrates. To address the requirement for ventral midline cells in providing cues to commissural axons in mice, we have analyzed *Gli2* mouse mutants, which lack specifically the floor plate and immediately adjacent interneurons. We show that a *Dbx1* enhancer drives *tau-lacZ* expression in a subpopulation of commissural axons and, using a reporter line generated from this construct, as well as DiI tracing, we find that commissural axons projected to the ventral midline in *Gli2*$^{-/-}$ embryos. *Netrin1* mRNA expression was detected in *Gli2*$^{-/-}$ embryos and, although much weaker than in wild-type embryos, was found in a dorsally decreasing gradient. This result demonstrates that while the floor plate can serve as a source of long-range cues for C-axons in vitro, it is not required in vivo for the guidance of commissural axons to the ventral midline in the mouse spinal cord. After reaching the ventral midline, most commissural axons remained clustered in *Gli2*$^{-/-}$ embryos, although some were able to extend longitudinally. Interestingly, some of the longitudinally projecting axons in *Gli2*$^{-/-}$ embryos extended caudally and others rostrally at the ventral midline, in contrast to normal embryos in which virtually all commissural axons turn rostrally after crossing the midline. This finding indicates a critical role for ventral midline cells in regulating the rostral polarity choice made by commissural axons after they cross the midline. In addition, we provide evidence that interactions between commissural axons and floor plate cells are required to modulate the localization of Nr-CAM and TAG-1 proteins on axons at the midline. Finally, we show that the floor plate is not required for the early trajectory of motoneurons or axons of the posterior commissure, whose projections are directed away from the ventral midline in both WT and *Gli2*$^{-/-}$ embryos, although they are less well organized in *Gli2*$^{-/-}$ mutants.

Key words: Gli2, Floor plate, Cell adhesion molecule, Nr-CAM, TAG-1, Netrin-1, Mouse, Dbx1

INTRODUCTION

In both invertebrates and vertebrates, there is substantial evidence that cells at the midline of the central nervous system (CNS) play an important role in controlling axon projections (reviewed in Tessier-Lavigne and Goodman, 1996). In the ventral midline of the midbrain, hindbrain and spinal cord of vertebrates lies the floor plate, a specialized structure found transiently during development. In vivo and in vitro experiments have demonstrated that the floor plate can exert a strong influence over axon projections, possessing both attractive and repulsive influences on growing axons (reviewed in Colamarino and Tessier-Lavigne, 1995).

An important population of axons that are attracted by the floor plate in vitro are commissural (C-) axons, whose early projections in vivo are directed ventrally toward the floor plate. An attractive activity of the floor plate on C-axons has been shown to be encoded by the Netrin family of secreted proteins (Kennedy et al., 1994; Serafini et al., 1994). Netrins comprise a small family of laminin-related proteins that are highly conserved from worms to mammals (reviewed in Tessier-Lavigne and Goodman, 1996). In the midline of the developing mouse CNS, *Netrin1* is expressed at high levels in the floor plate and more weakly in the adjacent ventral ventricular zone (VZ) in a dorsally decreasing gradient (Kennedy et al., 1994; Serafini et al., 1996). In vitro, Netrin1 can mimic the activity of the floor plate in attracting C-axons (Kennedy et al., 1994) and most of these axons fail to project ventrally in *Netrin1*-deficient mouse mutants (Serafini et al., 1996). Thus, *Netrin1* may largely, if not entirely, account for the chemoattractive activity of the floor plate on C-axons in the mouse.

In the rodent spinal cord, C-axons turn rostrally 90° to project longitudinally in the ventral funiculus (VF) after crossing through the floor plate in the ventral commissure (Bovolenta and Dodd, 1990a). It has been demonstrated in vitro that C-axons are no longer attracted by *Netrin1* after crossing

the ventral midline (Shirasaki et al., 1998). This result provides a possible explanation for the ability of these axons to exit the floor plate and project longitudinally in the VF. In addition, in rodents, the turning of C-axons into the longitudinal axis is correlated with a switch in adhesion molecule localization from TAG-1 to L1 on these axons (Dodd et al., 1988), providing evidence that guidance mechanisms differ along the transverse and longitudinal axes. Furthermore, these data suggest at least two distinct roles for ventral midline cells as an intermediate target of C-axons: attracting these axons to the midline, and modifying their responsiveness to cues that initially control this process allowing them to extend longitudinally.

The floor plate has also been shown to exert a repulsive influence on some axons whose trajectories are directed away from the ventral midline (Guthrie and Pini, 1995). Netrins have been implicated in this process, although not all repulsive activities seem to be mediated by Netrins (Shirasaki et al., 1996; Varela-Echavarria et al., 1997; Stoeckli et al., 1997), suggesting that other molecules expressed in the floor plate or ventral midline cells may influence axon guidance. However, the requirement for ventral midline cells in providing repulsive cues to axons in vivo has not been addressed. In addition, C-axons, which are initially attracted to the midline, do not re-cross after projecting through the floor plate, but instead project longitudinally along its lateral margin, a behavior consistent with the suggestion that C-axons acquire a contact-mediated repulsive response to the floor plate after they cross.

Recent experiments have provided clues as to the molecular basis for the functionally opposite influence of ventral midline cells on different aspects of C-axon guidance. In chick, perturbation of Nr-CAM, which is expressed in the floor plate in chick embryos, using blocking antibodies results in the failure of many C-axons to enter the floor plate (Stoeckli et al., 1997). These results suggest that adhesion molecules can control whether C-axons are attracted or inhibited by floor plate cells. In flies, a number of mutations that disrupt or prevent the development of midline cell types also disrupt C-axon guidance at the midline (Thomas et al., 1988; Seeger et al., 1993). For example, in *Drosophila sim* mutants, axon commissures do not form and the lateral longitudinal tracts are collapsed in the midline (Thomas et al., 1988). In *Drosophila* embryos mutant for *comm*, which encodes a putative transmembrane protein that is expressed on midline cells, C-axons do not cross the midline (Seeger et al., 1993; Tear et al., 1996). Genetic evidence suggests that *comm* interacts with another transmembrane protein, robo, that is found on C-axons (Kidd et al., 1998). In *robo* mutants, C-axons cross the midline but then re-cross, leading to the suggestion that *robo* mediates a repulsive response of C-axons to midline cells. Furthermore, epistasis analysis of *robo:comm* double mutants suggests that *comm* acts downstream of *robo*, perhaps locally, to overcome a *robo*-mediated repulsive response to midline cells (Kidd et al., 1998; Tessier-Lavigne and Goodman, 1996). Recently, another molecule that may provide inhibitory cues to C-axons has been identified, Slit. Slit proteins are expressed on midline cells in embryonic flies and rats (Kidd et al., 1999; Brose et al., 1999), and one of the three *Drosophila* Slit homologues found in rat, rSlit2, can repel motor axons in vitro (Brose et al., 1999). Slit proteins are ligands for robo receptors (Kidd et al., 1999; Brose et al., 1999; Li et al., 1999), and may thus play a role in the robo-mediated repulsion that postcommissural C-axons and motor axons display towards the floor plate in vitro.

Although the role of ventral midline cells in providing axon guidance cues has been studied in the *Drosophila* nervous system (Tessier-Lavigne and Goodman, 1996) and in the spinal cord of zebrafish *cyclops* (*cyc*) mutants (Bernhardt et al., 1992; Greenspoon et al., 1995), it has not previously been possible to address their specific requirement in higher vertebrates. The one mouse mutant that has been studied, Danforth's short-tail (*Sd*), lacks a floor plate, but the mutation also generally disrupts ventral neuron, notochord and motoneuron differentiation (Bovolenta and Dodd, 1990b). We have analyzed axon projections in *Gli2* mutant mouse embryos that lack precisely the floor plate and immediately adjacent interneurons (ventral intermediate region-VIR-cells) in the midbrain, hindbrain and spinal cord (Matise et al., 1998; Ding et al., 1998). Despite the absence of a floor plate and *Sonic hedgehog* (*Shh*) expression, notochord signalling is apparently normal, and the overall pattern of neuronal differentiation outside of the ventral midline region in *Gli2*$^{-/-}$ mutants is similar to wild-type (WT) embryos (Matise et al., 1998). Thus, the selective nature of the midline CNS phenotype of *Gli2*$^{-/-}$ embryos provides a unique opportunity to examine the requirement in higher vertebrates of ventral midline cells, including the floor plate, in providing cues that influence axon guidance.

Our results demonstrate that C-axons are able to make their initial projections toward the midline despite the absence of ventral midline cells. Since *Netrin1* mRNA expression persisted in *Gli2*$^{-/-}$ embryos, but only at very low levels, this suggests that a gradient of Netrin1 protein could be sufficient to attract C-axons to the ventral midline in *Gli2*$^{-/-}$ embryos. While most C-axons appear to remain in the ventral midline region, a few, but not most, can project longitudinally after reaching the ventral midline in *Gli2*$^{-/-}$ mutants. However, those C-axons that can extend longitudinally in *Gli2*$^{-/-}$ mutants make rostrocaudal polarity errors and project abnormally in the midline. We also studied the axonal localization of the cell adhesion molecule (CAM) proteins Nr-CAM and TAG-1 on C-axons. We show here that Nr-CAM is detected at moderate levels on C-axons as they project toward the floor plate at E10-11, and becomes highly enriched on C-axons specifically in the VC, but then drops to lower levels in the contralateral VF. In *Gli2*$^{-/-}$ embryos, we found that Nr-CAM was also detected at a moderate level on C-axons as they projected ventrally towards the floor plate, but was not enriched at the ventral midline. In addition, TAG-1, which is not detected on C-axons after leaving the VC to project longitudinally in the VF in WT embryos (Dodd et al., 1988), was detected on the rare longitudinal axons in *Gli2*$^{-/-}$ embryos. However, L1 staining, which is normally confined to axons in the VC and VF in WT embryos, was also only detected in the ventral midline and VF in *Gli2*$^{-/-}$ embryos. In contrast to the defects in C-axons, two other classes of axons whose projections are directed away from the ventral midline, spinal motoneurons and the dorsally directed projections of the posterior commissure were similar in WT and *Gli2*$^{-/-}$ mutant embryos. Thus, the floor plate, while being sufficient in vitro to repel motoneuron and posterior commisure axons, is not required in vivo to control the polarity of their axonal projections. Together, these results demonstrate an important requirement for the floor plate in providing local,

but not long-range, control of specific axon populations in the mouse spinal cord.

MATERIALS AND METHODS

Mice

The generation and genotyping of *Gli2* mutant mice was performed as described (Mo et al., 1997). Transgenic *Dbx1/tauLacZ* reporter mice were generated using standard methods (Hogan et al., 1994). The transgene construct consisted of a 5.7 kb DNA fragment upstream of the *Dbx1* enhancer and promoter (Lu et al., 1996) followed by an expression cassette containing a *tau-lacZ*-polyA with an ATG translation start site (Mombaerts et al., 1996). This DNA construct was microinjected into fertilized eggs obtained from a Swiss Webster (SW) × SW/*Gli2*$^{+/–}$ cross. Two transgenic founders were obtained that transmitted the transgene, both of which expressed β-galactosidase in a pattern similar to that previously reported (Lu et al., 1996). One transgenic *Dbx1/tauLacZ; Gli2*$^{+/–}$ male founder was bred with heterozygous *Gli2*$^{+/–}$ females to obtain *Gli2*$^{–/–}$ homozygous offspring transgenic for *Dbx/tauLacZ*. The morning of vaginal plug detection was designated as E0.5.

Axon tracing

DiI (1.1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate) in dimethyl formamide (3 mg/ml) was pressure injected into the dissected spinal cords of fixed (4% paraformaldehyde at 4°C, overnight) tissue and incubated at room temperature for 2-5 days.

Antibodies

Anti-Nr-CAM polyclonal antibody (837) was generated by injecting rabbits with an Fc fusion protein consisting of the six Ig-like domains and first two Fn III repeats of mouse Nr-CAM; immunoblotting extracts of brain membranes confirmed reactivity with the major 140 kDa form of mouse Nr-CAM (M. L., T. S. and M. G., unpublished data). Rabbit anti-L1 antibody (371) was generated against rat L1 purified by monoclonal affinity column chromatography (Friedlander et al., 1994). Immunoblotting confirmed reactivity with the major L1 species of ~200 and 140 kDa.

RNA in situ hybridization, histochemistry and antibody staining

Whole-mount RNA in situ hybridization was performed with a *Netrin1* probe (Serafini et al., 1996) as described (Matise and Joyner, 1997). Antibody staining, β-gal histochemistry and sectioning was as described (Matise and Joyner, 1997). Antibodies used and dilutions were: Nr-CAM (837) and L-1 (371), 1:300-500; TAG-1 (4D7), 1:2; β-gal (5′-3′, Inc.), 1:500; DCC (Calbiochem), 1:10,000. Cy3- and fluorescein-conjugated secondary antibodies (Jackson Immunochemicals) were used at 1:100-250. β-gal staining was performed as described (Matise and Joyner, 1997). Cell nuclei were visualized with Hoechst 33258 dye (Sigma) by adding it to Gelmount (Biomeda) coverslipping medium at 1:1000.

For ‘open-book’ preparations, spinal cords were dissected from embryos and cut open along the dorsal midline. The tissue was then flat-mounted under a coverslip on a glass microscope slide in Gelmount, so that the ventral midline was in a medial location and the dorsal midline was located at either lateral margin (see Fig. 2I,J).

The turning of C-axons at the ventral midline in *Gli2* mutants was quantified by observing the trajectory of anterogradely labeled C-axons after dorsal injections of DiI. Only axons that turned and traveled for at least 1/4 the length of a spinal segment in the same longitudinal direction were counted. To arrive at totals, counts were pooled from four *Gli2* mutant and two WT embryos in which 2-4 separate injections were made.

Microscopy

Whole-mount-stained embryos were photographed in flat-mounted preparations on a Leica stereomicroscope. Bright-field and some fluorescent images were obtained on a Leica DMR XE compound microscope equipped with epifluorescence. Some fluorescent images were collected on a Leitz compound microscope equipped with a Princeton Instruments cooled CCD camera and processed in Metamorph imaging software (Universal Imaging, Inc.). Confocal images were collected on a Leica TCS NT laser scanning microscope. Images from 3-5 optical sections were superimposed in Adobe Photoshop. Some images were processed for color balance and/or cropped in Adobe Photoshop. Schematics were drawn in Canvas and composite figures were assembled in QuarkXpress.

RESULTS

Spinal cord commissural axon projections

The ventral commissure does not form in *Gli2*$^{–/–}$ mutant embryos

When we examined *Gli2*$^{–/–}$ mutants at embryonic day 11 (E11), we found that the ventral commissure (VC), which is the crossing tract for C-axons and is distinguishable by its characteristic morphology at this stage in WT embryos, was not seen in the ventral midline of the spinal cord (Fig. 1A, B, arrowheads). To determine whether C-axon projections were made in these mutants, we labeled C-axons in the spinal cord and hindbrain anterogradely with the lipophilic fluorescent dye, DiI. In WT embryos, DiI-labeled C-axons can be visualized projecting ventrally in the transverse plane toward the midline, where they enter the floor plate ipsilaterally and cross, forming the ventral commissure (Fig. 1C,E). Upon reaching the contralateral side of the ventral midline, C-axons normally turn rostrally to leave the transverse plane and project longitudinally in the ventral funiculus (VF; Bovolenta and Dodd, 1990a). In *Gli2*$^{–/–}$ mutants, numerous ventrally projecting DiI-labeled axons were also detected, and the trajectory of these labeled axons toward the ventral midline in the transverse plane was similar to WT embryos (*n*=8; Fig. 1C-F). However, at the ventral midline of *Gli2*$^{–/–}$ mutants, DiI-labeled axons did not form a ventral commissure, but instead occupied a large area where normally the floor plate and VIR cells differentiate (Fig. 1F). Thus, while C-axons were able to reach the ventral midline despite the absence of floor plate and VIR cells in *Gli2*$^{–/–}$ mutants, the organization of these axons once they reached this point was abnormal.

A *Dbx1* enhancer labels a subpopulation of commissural neurons in the spinal cord and hindbrain that project abnormally in *Gli2*$^{–/–}$ mutants

Our DiI axon tracing results suggested that the organization of most, if not all, dorsal C-axons at the ventral midline of *Gli2*$^{–/–}$ mutants was disrupted. To study the behavior of C-axons in more detail in *Gli2*$^{–/–}$ mutants, we sought to develop an independent means of marking these axons. To do this, we generated a new transgenic mouse line in which *tau-lacZ* (TLZ) sequences (Callahan and Thomas, 1994; Mombaerts et al., 1996) were placed downstream of a 5.7 kb *Dbx1* (Dbx1) enhancer/promoter fragment (Lu et al., 1996; Fig. 2). In transgenic (Tg) Dbx1/TLZ embryos at E10-11, β-galactosidase (β-gal) expression was detected in cells and axons whose soma were located in the region where the endogenous *Dbx1* gene is expressed (Shoji et al., 1996), in the sulcus limitans region throughout the spinal cord and hindbrain (Fig. 2A,G). β-gal-

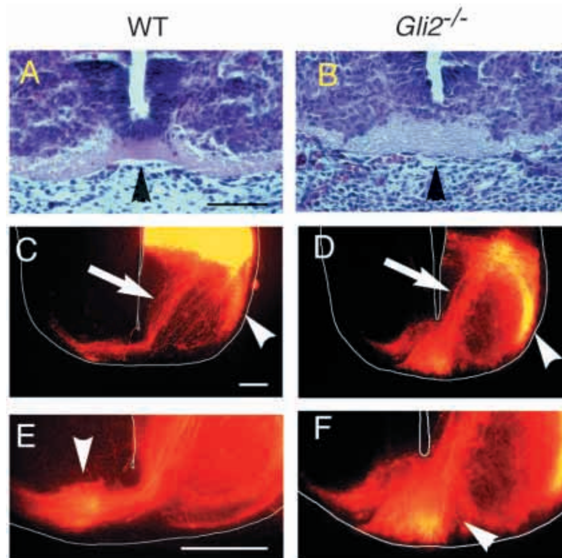

Fig. 1. The ventral commissure does not form, but C-axons reach the ventral midline in *Gli2*$^{-/-}$ embryos. (A,B) Transverse sections through thoracic spinal cord at E11 showing that the ventral commissure (medium purple stained structure at spinal cord margin above arrowhead in A) does not form in *Gli2*$^{-/-}$ embryos (arrowhead in B). (C-F) Transverse sections through mid-thoracic spinal cord after ipsilateral DiI injections into the dorsal cord at E11. C-axons project towards the ventral midline in *Gli2*$^{-/-}$ embryos (D, arrow) following a similar trajectory as in WT embryos (C, arrow). In *Gli2*$^{-/-}$ embryos, DiI-labeled axons do not cross uniformly into the contralateral ventral funiculus as in WT embryos (arrowhead in E); instead, C-axons collect in the midline (arrowhead in F). Note ipsilaterally projecting dorsal association neurons (white arrowhead in C and D) appear to project normally in *Gli2*$^{-/-}$ embryos. Scale bar in A, 60 μm for A,B; in C, 60 μm for C,D; in E, 60 μm for E,F.

expressing cells were also detected more dorsally in the rostral spinal cord and hindbrain (Fig. 2A,G, asterisks), in a region where *Dbx1* gene expression has not been detected (Shoji et al., 1996), suggesting that the 5.7 kb enhancer fragment is lacking sequences that repress endogenous *Dbx1* transcription in this region. β-gal expression was no longer detected in the spinal cord of Dbx1/TLZ embryos after E12.

In TgDbx1/TLZ embryos, β-gal-expressing axons projected towards the ventral midline and crossed the floor plate to enter the contralateral VF, where they turned rostrally to extend longitudinally (Figs 2B,I, 3A). At E11, many β-gal-expressing axons in the ventral commissure co-expressed TAG-1, a marker of C-axons at this stage (Fig. 2C; Dodd et al., 1988). Thus, the 5.7 kb *Dbx1* enhancer labels ventral C-neurons in the hindbrain and spinal cord, as well as a dorsal population in the caudal hindbrain/rostral spinal cord of TgDbx1/TLZ embryos. In addition, a few β-gal-expressing axons were seen in the lateral funiculus, suggesting that some ipsilaterally projecting cells are also labeled in the spinal cord and hindbrain in TgDbx1/TLZ mice (Fig. 2A, white arrowhead).

TgDbx1/TLZ transgenic mice provide an opportunity to study the projections of a ventral class of C-neuron that would otherwise be difficult to selectively label in vivo using anatomical tracing techniques, as well as providing an independent marker for C-axons in *Gli2* mutant spinal cords. Therefore, we crossed TgDbx1/TLZ reporter mice with *Gli2* mutants. When we examined TgDbx1/TLZ; *Gli2*$^{-/-}$ embryos at E10-11, we found β-gal-expressing C-axons directed towards the ventral midline in the transverse plane (Fig. 2D), as in WT TgDbx1/TLZ embryos, extending our results obtained with DiI labeling of dorsal C-neurons. As with DiI-labeled axons, the projections of β-gal-labeled ventral C-axons accumulated in the ventral midline region in TgDbx1/TLZ; *Gli2*$^{-/-}$ embryos (Fig. 2D,E). Thus, specific groups of both dorsally and ventrally derived C-axons can project to the ventral midline in *Gli2*$^{-/-}$ embryos, but do not project normally after reaching this point.

β-gal staining in TgDbx1/TLZ; *Gli2*$^{-/-}$ embryos revealed another aspect of C-axon projection defects in mutant embryos not obvious using DiI tracing. In contrast to WT embryos, β-gal-labeled C-axons collected in clusters along the rostrocaudal axis in the ventral midline region of the spinal cord in *Gli2*$^{-/-}$ embryos (Fig. 2H,J). In addition, in some regions of the spinal cord, the ventral trajectory of dorsal C-axons toward the ventral midline could be seen to divert rostrally or caudally toward an axon cluster (Fig. 2F). Notably, β-gal-expressing axons were not detected in the ventral midline between some of these clusters (Fig. 2H,J), raising the possibility that many C-axons failed to make organized longitudinal projections in these mice.

Reduced *Netrin1* expression in the ventral spinal cord of *Gli2*$^{-/-}$ mutant embryos

It has previously been shown that *Netrin1* is both necessary and sufficient to attract most C-axons ventrally (Kennedy et al., 1994; Serafini et al., 1996). The finding that C-axons were able to project to the ventral midline despite the absence of a floor plate in *Gli2*$^{-/-}$ mutants thus raised the question of whether *Netrin1* was expressed in the ventral spinal cord of these embryos.

In WT embryos at E10-12, *Netrin1* mRNA expression was detected at high levels in the floor plate and in a dorsally decreasing gradient in the ventral ventricular zone (Fig. 3A; Kennedy et al., 1994). In the spinal cord and hindbrain of *Gli2*$^{-/-}$ embryos, *Netrin1* expression was also detected, but only in the VZ, and at lower overall levels than in WT embryos (Fig. 3B). Notably, in *Gli2*$^{-/-}$ embryos, the remaining *Netrin1* expression in the VZ was seen in a dorsally decreasing gradient, similar to the expression dorsal to the floor plate in the VZ of WT embryos (n=3; Fig. 3B). Thus, graded but overall much lower levels of *Netrin1* mRNA expression in the VZ of *Gli2*$^{-/-}$ embryos likely results in sufficient Netrin1 protein production to attract and/or guide C-axons to the ventral midline in these embryos (Fig. 3C,D). Interestingly, *Netrin1* expression in the VZ of *Gli2*$^{-/-}$ embryos was uniform along the rostrocaudal axis of the spinal cord and hindbrain (data not shown), so C-axon clustering is not correlated with any observable differences in *Netrin1* expression along this axis.

Only a subset of C-axons project longitudinally in *Gli2*$^{-/-}$ embryos

The presence of C-axon clusters and the absence of clearly visible axon projections between some clusters suggested the possibility that most or all C-axons remained in the ventral midline and failed to project longitudinally in *Gli2*$^{-/-}$ embryos. To address this possibility, we examined axon trajectories in the ventral midline of *Gli2*$^{-/-}$ embryos more closely using fluorescent-tagged antibodies to β-gal in TgDbx1/TLZ; *Gli2*$^{-/-}$ embryos and confocal microscopy, and ventral midline injections of DiI. In TgDbx1/TLZ; *Gli2*$^{-/-}$ embryos, we found

that longitudinally directed axons were detected in some restricted regions of the spinal cord at E11 (for example, Fig. 4A,B; *n*=5). These longitudinally projecting axons were found primarily between some of the axon clusters in the ventral midline; however, the large number of misdirected axons within clusters prevented a detailed analysis of C-axon trajectories in these regions. Thus, despite the absence of the floor plate and a well-organized VC in *Gli2*$^{-/-}$ mutants, rare C-axons that reached the ventral midline were capable of changing their direction to project longitudinally.

Longitudinally projecting C-axons turn both rostrally and caudally and are disorganized at the ventral midline of *Gli2*$^{-/-}$ mutants

Examination of the longitudinally directed C-axons in *Gli2*$^{-/-}$ embryos revealed that their trajectories in this axis were abnormal. In WT embryos, C-axons project in a well-fasciculated manner in the VF immediately adjacent to the floor plate (Fig. 4C, short arrow). At regular intervals, axons leave this fascicle and project laterally toward the ventral horn (Fig. 4C, long arrows). Longitudinally projecting axons are never seen in the midline in WT embryos. In contrast, in *Gli2*$^{-/-}$ embryos most longitudinally directed axons were found in the ventral midline region and did not travel in a well-fasciculated tract as in WT embryos (Fig. 4B,D; *n*=2).

The finding that some C-axons can project longitudinally in *Gli2*$^{-/-}$ mutants raised the question of whether C- axons made correct rostrocaudal turning decisions at the ventral midline. To investigate this, we examined C-axon projections at the ventral midline after making ipsilateral DiI injections into the dorsal spinal cord in *Gli2*$^{-/-}$ mutants. In striking contrast to WT embryos in which nearly 100% of C-axons turn rostrally after crossing the floor plate (Table 1, Fig. 4A,E; also see Bovolenta and Dodd, 1990a), caudally turning axons were also seen in addition to rostrally turning axons in the ventral midline in *Gli2*$^{-/-}$ embryos in roughly equivalent numbers (Table 1; Fig. 2F). These results show that, while most, or all, C-axons reached the ventral midline in *Gli2*$^{-/-}$ mutants, only a small subset extended longitudinally, and those that did seemed to make random rostrocaudal polarity choices and projected in a disorganized, undirected manner in this axis.

The localization of Nr-CAM and TAG-1 cell adhesion molecules on C-axons is abnormal in *Gli2*$^{-/-}$ mutants

To address whether interactions with the floor plate are necessary to modulate cell-adhesion molecule localization on

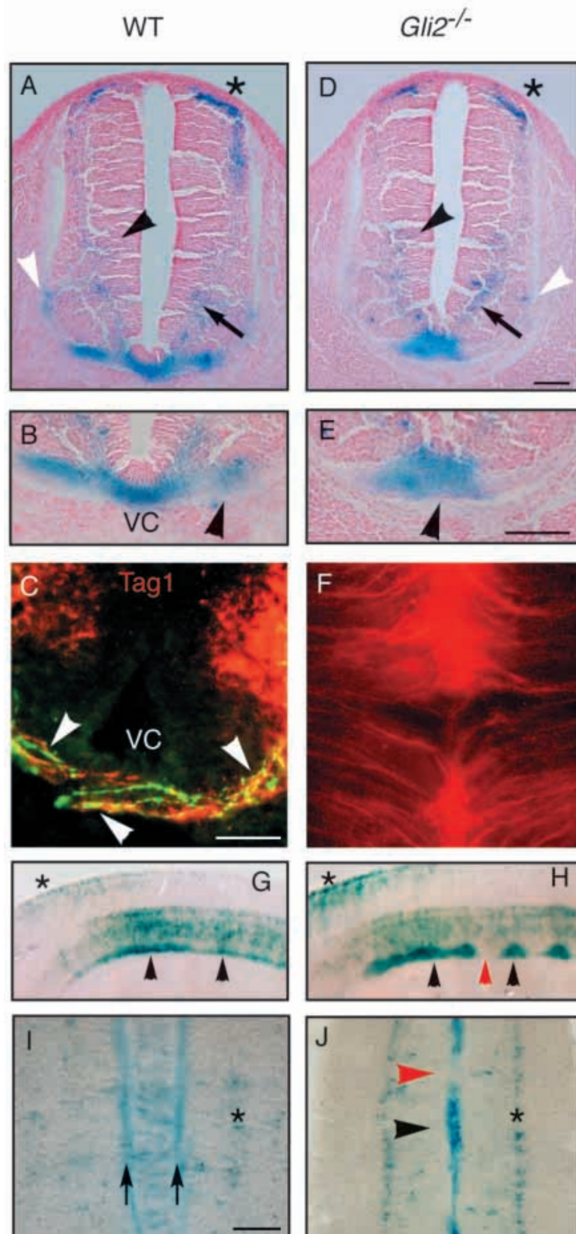

Fig. 2. A *Dbx1* enhancer labels a subset of commissural neurons in the spinal cord and hindbrain and reveals abnormalities in C-axon projections in *Gli2*$^{-/-}$ embryos. (A-E) Transverse sections through the cervical spinal cord of WT (A-C) and *Gli2*$^{-/-}$ (D,E) TgDbx1/TLZ embryos at E11. Some dorsal C-neurons are labeled in the cervical spinal cord/caudal hindbrain in these mice (asterisks in A,D,G,H), as well as ventrally located cells along the entire hindbrain and spinal cord (black arrowheads in A,D; asterisks in I,J). In WT embryos, labeled axons cross at the ventral commissure (VC, B) and turn rostrally to project longitudinally in the ventral funiculus (B, arrowhead). (C) Most or all β-gal-labeled axons in the VC (green) co-express TAG-1 (red) at E11 (yellow), a marker of C-axons at this stage. (D,E) In TgDbx1/TLZ; *Gli2*$^{-/-}$ embryos, β-gal is detected in C-cells (D, black arrowhead) and axons projecting toward the midline (D, arrow), similar to WT embryos. β-gal-expressing axons occupy a large area in the ventral midline of *Gli2*$^{-/-}$ embryos (E, arrowhead). (F) Flat-mount 'open-book' preparation of the ventral midline region of a *Gli2*$^{-/-}$ embryo at E11, stained with TAG-1. Rostral is to the top. Some C-axons divert rostrally while others turn caudally as they approach the midline. (G,H) Lateral view of rostral spinal cords and caudal hindbrain of TgDbx1/TLZ embryos. Rostral is to the left and dorsal to the top. In *Gli2*$^{-/-}$ embryos, many β-gal-expressing C-axons collect in large clusters at the ventral midline (H, black arrowheads) instead of projecting more uniformly to the midline as they do in WT embryos (G, arrowheads). β-gal expression is absent between some clusters (H, red arrowhead). (I,J) Flat-mounted open-book preparations of thoracic spinal cord from TgDbx1/TLZ WT (I) and *Gli2*$^{-/-}$ (J) embryos at E11. (I) Longitudinally projecting axons labeled with β-gal are located immediately lateral to the floor plate, whose margins are indicated by arrows. (J) In *Gli2*$^{-/-}$ embryos, β-gal-expressing axons are not detected between some clusters (red arrowhead) and axons collect in the midline (black arrowheads). Asterisks in I, J indicate location of ventral C-cells labeled by the 5.7 kb *Dbx1* enhancer. Rostral is to the top in F,I,J. Scale bar in D, 50 μm for A,D; in E, 50 μm for B,D; in C, 20 μm, in I, 60 μm for I,J.

Table 1. Turning of C-axons at the ventral midline

	Rostral	Caudal	*n*
Wild type	45	2	2
Gli2$^{-/-}$	41	34	4

C-axons at the ventral midline, we examined the expression of three proteins that are detected at different levels on pre- and postcommissural C-axons: Nr-CAM, TAG-1 and L1.

Nr-CAM (Ng-CAM related CAM; Grumet, 1997) protein is detected in a dynamic pattern on C-axons in the mouse spinal cord (Fig. 5A). Nr-CAM is detected at moderate levels on C-axons projecting to the ventral midline and on longitudinal axons in the VF (Fig. 5A, arrowhead). At the ventral midline, Nr-CAM becomes highly enriched on C-axons in the VC (Fig. 5A, arrow). Nr-CAM staining is also detected on floor plate cells (Fig. 5A, red arrowhead), whose cell bodies are situated dorsal to C-axons in the VC at this stage (Fig. 5A). In contrast, in *Gli2*$^{-/-}$ mutants, we found that Nr-CAM was detected at a constant, moderate level on C-axons (Fig. 5B, arrow) and was not enriched at the ventral midline, as in WT embryos. These observations suggest that contact with floor plate cells is necessary for the local enrichment of Nr-CAM protein on C-axons in the VC.

TAG-1 is also normally detected on C-axons projecting ventrally towards the floor plate, but decreases as axons cross the midline in the VC and is not detected on longitudinal axons in the VF (Fig. 5C, G; also see Dodd et al., 1988). In *Gli2*$^{-/-}$ mutants, TAG-1 protein was detected on C-axons projecting towards the midline, but was also seen on the (rare) longitudinally directed C-axons in *Gli2*$^{-/-}$ mutants (Fig. 5D,H,I). Since TAG-1 is never detected on longitudinal axons in WT embryos at this stage, these results suggest that ventral midline cells are required for the proper modulation of TAG-1 localization on C-axons and furthermore provide evidence that TAG-1 expression per se is not incompatible with longitudinal C-axon growth in the mouse spinal cord.

L1 is detected only on C-axons in the VC and VF of WT embryos. In the VF, L1 also labels many axons that are not commissural (Fig. 5E; Dodd et al., 1988). In *Gli2*$^{-/-}$ mutants, L1 was also only detected on axons in the ventral midline and more laterally in the VF (Fig. 5F). Thus, localization of L1 protein at the midline and on the longitudinal projections of C-axons does not appear to require interactions with floor plate or VIR cells in the mouse.

Normal C-neuron differentiation in *Gli2*$^{-/-}$ mutants embryos

Previously, we have shown that the differentiation of cells dorsal to the ventral midline appears normal in *Gli2*$^{-/-}$ embryos (Matise et al., 1998). However, to address whether *Gli2* is specifically required for aspects of dorsal or ventral commissural neuron differentiation relevant to axon guidance, we examined expression of TAG-1 (Dodd et al., 1988) and DCC, a receptor for Netrin1 (Keino-Masu et al., 1996), on dorsal commissural cells in the spinal cord and hindbrain of *Gli2*$^{-/-}$ embryos at E10-11. We found that the expression patterns of these markers was similar in WT and *Gli2*$^{-/-}$ embryos (data not shown). These results suggest that, even though *Gli2* is normally expressed in the ventricular zone during the time commissural neurons are generated (Hui et al., 1994; Sasaki et al., 1997), the abnormalities in C-axon projections at the ventral midline in *Gli2*$^{-/-}$ mutants are not likely due to a requirement for *Gli2* in commissural neuronal precursors, but rather indicate that these defects result primarily from the absence of ventral midline cells in these embryos.

Axons whose trajectories are directed away from the floor plate

Ventral midline cells are not required for motoneurons to project into the periphery

One cell population whose projections may be repulsed by the floor plate are motoneurons, whose axons are directed away from the ventral midline in vivo and are repulsed by the floor plate in vitro (Guthrie and Pini, 1995). To examine early motoneuron projections, we back-filled motoneurons in the spinal cord at E11-12 by peripheral nerve injections of DiI. In WT embryos, these injections retrogradely labeled motoneurons in the lateral motor column of the spinal cord and their axons projecting out the ventral roots (Fig. 6A). In *Gli2*$^{-/-}$ embryos, DiI-labeled motor axons were also detected exiting the spinal cord, but instead of forming a discrete ventral root as in WT embryos, motor axons projected into the periphery broadly from the ventrolateral and ventromedial spinal cord (Fig. 6B). Despite exiting the CNS in abnormal positions, motoneuron axons joined dorsal root nerves to form normal-appearing spinal nerves in the periphery (Fig. 6B, and data not shown). These findings suggest that proper ventral root formation requires the normal development of ventral midline cells in the mouse CNS, although many or most motoneurons apparently do not require the presence of the floor plate to exit the CNS or form spinal nerves.

Whereas in the spinal cord all classes of motor axons exit via the ventral roots, in the hindbrain only somatic motoneurons project out the ventral roots, while visceral and branchiomotor neuron axons exit dorsally. Since it has been proposed that the floor plate may provide chemorepellant cues that govern the divergent dorsoventral (d/v) axon exit points of different motoneuron classes in the hindbrain (Guthrie and Pini, 1995), we examined cranial nerve formation in the hindbrain of *Gli2*$^{-/-}$ embryos at E11. Using neurofilament staining to identify hindbrain cranial nerves and their CNS exit points, we found that all cranial nerves appeared normal in the hindbrain and midbrain of *Gli2*$^{-/-}$ embryos (*n*=4; data not shown), with the exception of the IIIrd and IVth, which were absent (Matise et al., 1998). These results suggest that cues that may be provided by ventral midline cells are not required to establish the d/v exit points of most hindbrain motoneurons.

We next addressed whether the absence of the occulomotor nerve (CNIII) and the trochlear nerve (CNIV) was the result of aberrant axon projections or disrupted motoneuron development using DiI tracing. CNIII can be labeled anterogradely by DiI injection into the ventral cephalic flexure region where its cell bodies originate, while CNIV is labeled by ventral injections immediately rostral to the isthmus. In *Gli2*$^{-/-}$ embryos, DiI injections into these regions failed to label any aberrant axon tracts that might be identified as mis-projecting CNIII or CNIV (data not shown), suggesting that these nerves do not form in *Gli2*$^{-/-}$ embryos. We cannot, however, rule out the possibility that some occulomotor or trochlear motoneurons extend axon projections that join pre-

existing axon tracts within the CNS, owing to the lack of independent markers for these neurons and their projections in WT and *Gli2*$^{-/-}$ mouse embryos.

Axons of the posterior commissure

It has previously been shown that the dorsally directed axons of the posterior commissure (PC) are repulsed by floor-plate-derived signals in vitro (Shirasaki et al., 1996). However, Netrin1 alone does not appear to be responsible for this repulsion (Shirasaki et al., 1996), suggesting that other molecules expressed in the floor plate might repel PC axons in vivo. To address this possibility, we examined the formation of the PC in TgDbx1/TLZ WT and *Gli2*$^{-/-}$ mutant embryos, as PC axons are labeled in these mice (Fig. 6C). We found that PC axons, which project across the dorsal midline near the prosomere (p)1/p2 border, were seen in a similar location in *Gli2*$^{-/-}$ embryos (Fig. 6D). There were qualitatively similar numbers of axons crossing the dorsal midline in WT and *Gli2*$^{-/-}$ embryos (*n*=6), however, these axons were not as discretely organized as in WT embryos and so were less densely fasciculated. Thus, while the floor plate may express molecules that are sufficient to repel PC axons in vitro, the floor plate alone is not required for a dorsally directed trajectory in vivo.

DISCUSSION

In this study, we have examined axon guidance in the midbrain, hindbrain and spinal cord of *Gli2*$^{-/-}$ mutant embryos in which ventral midline cells fail to develop. We have focused primarily on the requirement for ventral midline cells (floor plate and adjacent VIR interneurons) in providing guidance cues to C-axons in the spinal cord. We have shown that a *Dbx1* enhancer/promoter (Lu et al., 1996) labels primarily ventrally derived C-neurons in the spinal cord and hindbrain, and that these axons, along with dorsal C-axons labeled with DiI, can project to the ventral midline despite the absence of a floor plate and VIR cells. Since there is a persistence of weak *Netrin1* mRNA expression in the VZ in *Gli2*$^{-/-}$ mutant embryos, this suggests that lower but graded expression of *Netrin1* is sufficient to attract C-axons to the midline in these embryos. However, ventral midline cells are required for the correct organization of C-axons at the midline, since the VC did not form in *Gli2*$^{-/-}$ mutant embryos, and most C-axons remained in clusters at the ventral midline, despite homogeneous *Netrin1* expression along the rostrocaudal axis. While only a small number of C-axons projected longitudinally in *Gli2*$^{-/-}$ embryos, those that did made random rostrocaudal polarity choices and grew in a disorganized manner in this axis. We have also shown that the floor plate and/or VIR cells are required to control the localization of Nr-CAM and TAG-1, but not likely L1, proteins on C-axons at the ventral midline. Notably, two classes of axons that are normally directed away from the ventral midline, motoneuron axons and posterior commissure (PC) axons, were directed away from the midline in *Gli2*$^{-/-}$ mutants, although they projected in a less organized manner. This result shows that the floor plate, while being sufficient to repel motor and PC axons in vitro (Guthrie and Pini, 1995; Shirasaki et al., 1996), is not required for the polarity of their trajectories in vivo.

The floor plate and/or VIR cells are not required to provide long-range chemoattractant cues to C-axons or chemorepulsive cues to motor or posterior commissure axons

We have shown that most, if not all, C-axons project to the ventral midline in the spinal cord of *Gli2*$^{-/-}$ embryos. We also found that *Netrin1* was expressed, but at much lower levels in *Gli2*$^{-/-}$ embryos than in WT embryos, because the floor plate is absent in these embryos, the normal site of highest *Netrin1* expression. Since *Netrin1* has been shown to be both necessary and sufficient to guide C-axons to the ventral midline in vivo and in vitro (Serafini et al., 1994; Kennedy et al., 1994; Serafini et al., 1996), and since only one *Netrin* is expressed in the spinal cord of mice at these stages (Serafini et al., 1996), our findings suggest that the preservation of even weak expression of *Netrin1* is sufficient to account for the ability of C-axons to reach the midline in *Gli2*$^{-/-}$ embryos. Since we have not examined Netrin1 protein levels in WT and *Gli2*$^{-/-}$ embryos, it is unknown whether lower *Netrin1* mRNA levels lead to decreased in Netrin1 protein in *Gli2*$^{-/-}$ embryos. However, studies in chick embryos with Netrin1 antibodies demonstrated a close correspondence between protein and mRNA localization (MacLennan et al., 1997). Thus, unless the situation is drastically different in mouse, a similar correlation should hold.

While we cannot rule out that the floor plate provides an important source of *Netrin1* in WT embryos which is sufficient on its own to attract C-axons to the midline, our results argue against the idea that the floor plate alone accounts for the guidance of C-axons projections to the ventral midline. An alternative possibility, revealed by our observations of C-axon trajectories to the ventral midline, is that C-axons are also attracted by Netrin1 produced by VZ cells, suggesting a more important role for VZ cells in guiding C-axons ventrally than has been previously thought. If the VZ normally serves a similar role in guiding C-axons in WT embryos as in *Gli2*$^{-/-}$ mutants, it is possible that the shape of the ventricular zone in part dictates the ventral trajectory of C-axons at early stages of neural differentiation. That the VZ may also serve this role in WT embryos is supported by our observation that the ventral trajectory of C-axons towards the ventral midline in *Gli2*$^{-/-}$ mutants was similar to WT embryos, namely along the lateral margins of the ventral VZ (see Figs 1, 2). Thus, despite the absence of very high *Netrin1* expression in the floor plate, C-axons in *Gli2*$^{-/-}$ mutants still projected directly to the ventral midline rather than meandering toward it, as some axons do in *Netrin1* mutants (Serafini et al., 1996).

Furthermore, this model predicts that C-axons must find cells within the VZ inhibitory and that this inhibition prevents them from entering into a region where *Netrin1* expression levels are high (see Fig. 3C,D). Several observations support this hypothesis. First, during the early stages of C-axon projections, most axons are detected at the margins of, and never within, the VZ. Second, occasionally C-axons can be seen to extend an axon before their cell bodies leave the VZ. In these cases, the axon turns sharply only after leaving the VZ, rather than projecting directly towards the ventral midline, which would take it through the VZ (data not shown). Third, in *Gli2*$^{-/-}$ mutants, C-axons accumulate in the ventral midline ventral to the VZ, near the site of highest *Netrin1* expression in these mutants, whereas in WT embryos C-axons cross

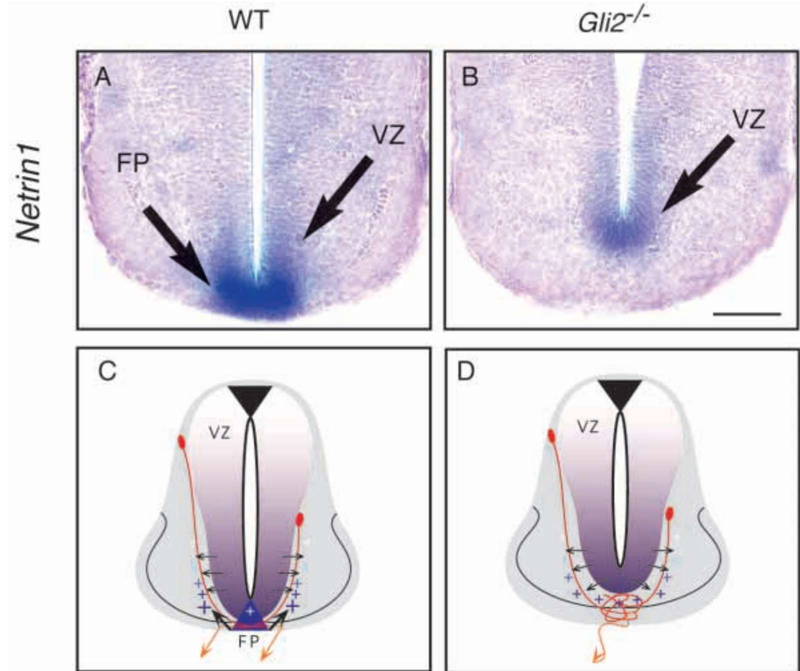

Fig. 3. Persistent low-level *Netrin1* expression in *Gli2*$^{-/-}$ mutant embryos. (A,B) Transverse sections through the lumbar spinal cord at E11. (A) In WT embryos, *Netrin1* expression is normally seen at high levels in the floor plate (FP) and in a dorsally decreasing gradient in the ventral ventricular zone (VZ). (B) In *Gli2*$^{-/-}$ embryos that lack a FP, only low-level VZ *Netrin1* expression is detected (arrow). (C,D) Summary of *Netrin1* results and possible explanation for the ability of C-axons to reach the ventral midline in *Gli2*$^{-/-}$ embryos. *Netrin1* expression is shown in purple. (C) WT embryos. Dorsal and ventral C-axons (red) are attracted to the midline by the coordinate expression of *Netrin1* in both the FP and VZ, likely giving rise to a corresponding Netrin1 protein gradient (plus marks). (D) In *Gli2*$^{-/-}$ embryos, the lower level of *Netrin1* expression is sufficient to attract/guide C-axons to the ventral midline, perhaps owing to the persistence of a gradient of expression. In both cases, additional mechanisms must prevent C-axons from entering into the VZ, where *Netrin1* expression is relatively high, but not from entering the FP. Scale bar in B, 50 μm for A,B.

through the floor plate (which might modify their responsiveness to *Netrin1*; Shirasaki et al., 1998).

Can the VZ simultaneously provide both attractive and repulsive cues to C-axons? Recent experiments have shown that axon-substratum interactions can convert a number of chemotactic signals from attractive to inhibitory (Song et al., 1998), and that similar mechanisms might function to modulate *Netrin1* responses as well (Ming et al., 1997). Thus, Netrin1 expression in the VZ may account for both the attractive and repulsive response of ventrally projecting C-axons to the VZ. Since C-axons enter the floor plate ipsilaterally but do not re-cross, it is possible that these same mechanisms function to

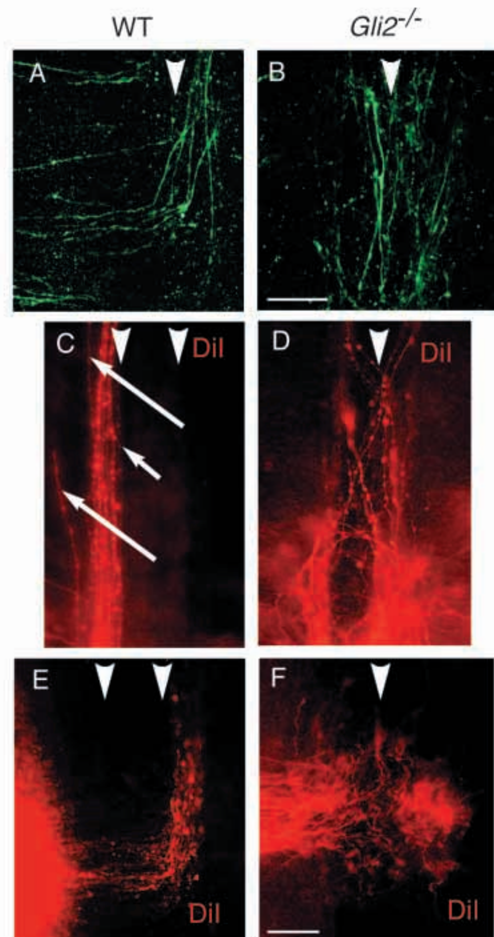

Fig. 4. Some C-axons project longitudinally in *Gli2*$^{-/-}$ embryos. Arrowheads delineate the margins of the floor plate in WT embryos (A,C,E) and the approximate location of the ventral midline in *Gli2*$^{-/-}$ mutant embryos (B,D,F). (A,B) Superimposed confocal images of ventral midline region in flat-mounted open-book spinal cords. (A) In WT embryos, longitudinally projecting β-gal-expressing C-axons are normally found lateral to the ventral midline. (B) In *Gli2*$^{-/-}$ embryos, longitudinally projecting C-axons can be detected in the midline (arrowhead) in some regions. (C,D) View of the ventral midline of WT (C) and *Gli2*$^{-/-}$ embryo (D) after DiI injections immediately lateral to (in C) or directly into the ventral midline (in D). (C) In WT embryos, longitudinally projecting axons that travel immediately adjacent to the floor plate (marked by arrowheads) can be seen. At least two different types of longitudinal projections can be distinguished (arrows; see text for details). (D) In *Gli2*$^{-/-}$ embryos, longitudinally directed axons can also be detected, but these axons are abnormally located in the ventral midline (arrowhead) and are not well-organized as in WT embryos. More lateral injections gave similar results. (E) In WT embryos, ipsilateral DiI injections into the dorsal spinal cord label C-axons as they project towards the ventral midline and then turn rostrally in the contralateral VF after crossing the floor plate (between arrowheads). (F) In *Gli2*$^{-/-}$ embryos, C-axons project to the ventral midline (indicated by an arrowhead), but most display ‘confused’ trajectories, and fail to extend longitudinally. Some of the axons that turn longitudinally in *Gli2*$^{-/-}$ embryos turn caudally, while others turn rostrally. Rostral is to the top in all figures. Scale bar in B, 10 μm for A, B; in F, 50 μm for E,F and 32 μm for C,D.

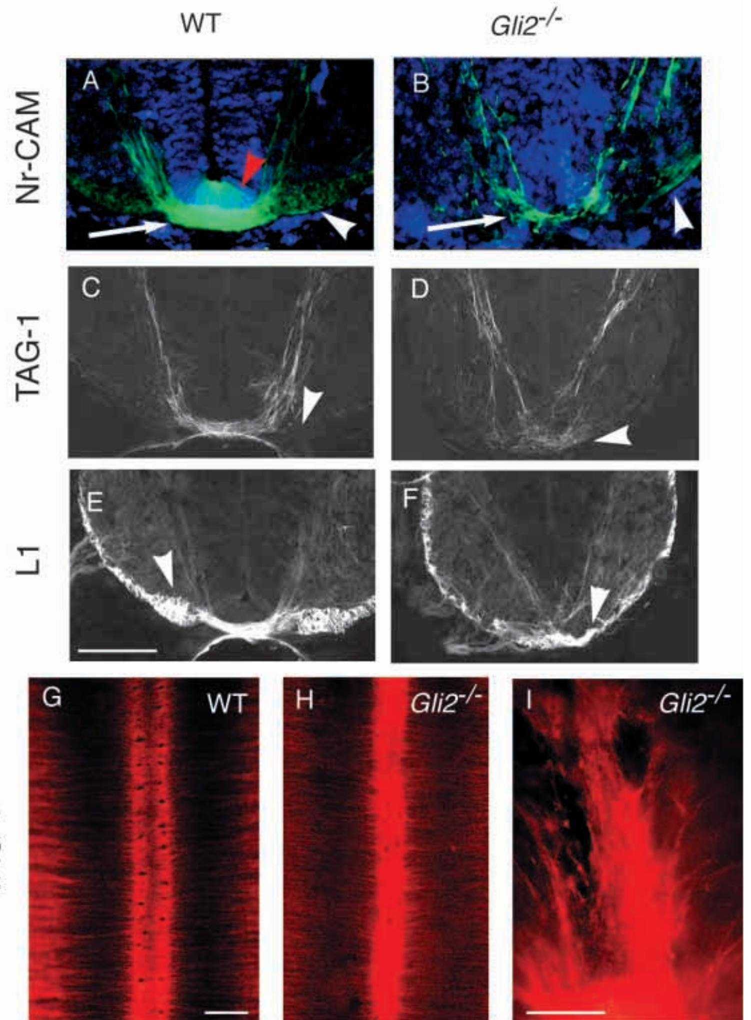

Fig. 5. Abnormal localization of NrCAM and TAG-1 cell adhesion molecule proteins on C-axons at the ventral midline of *Gli2*$^{-/-}$ embryos. Transverse sections through the mid-thoracic region of WT (A,C,E) and *Gli2*$^{-/-}$ (B,D,F) embryo spinal cords at E11. Nuclei in A and B have been stained with Hoechst 33258 dye. (A) In WT embryos, Nr-CAM staining (green) is detected on C-axons as they project toward the ventral midline. As C-axons enter the VC (arrow), Nr-CAM expression becomes highly enriched. Nr-CAM expression is also detected on floor plate cells (red arrowhead). Nr-CAM expression is weak in the VF on postcommissural C-axons (white arrowhead). (B) In *Gli2*$^{-/-}$ mutant embryos, the precommissural level of Nr-CAM protein on C-axons was similar to WT embryos. However, expression levels did not increase at the ventral midline in *Gli2*$^{-/-}$ mutants (arrow). Nr-CAM staining was also detected at weak levels in some regions of the VF in *Gli2*$^{-/-}$ embryos (arrowhead). (C) In WT embryos, TAG-1 expression is detected on C-axons before and within the VC, but is not detected postcommissurally in the VF (arrowhead). (D) In *Gli2*$^{-/-}$ embryos, TAG-1 is detected on C-axons projecting towards, and at the ventral midline (arrowhead; see also I). (E) In WT embryos, L1 expression marks longitudinal axons in the VC and VF (arrowhead). (F) In *Gli2*$^{-/-}$ embryos, L1 is also only detected on axons in the ventral midline and VF (arrowhead), although they are not well organized. (G-I) Ventral midline region in flat-mounted open-book preparations of E11 spinal cords stained in whole-mount for TAG-1. (G) In WT embryos, TAG-1 staining shows precommissural C-axon tracts at the ventral midline. TAG-1 staining is extinguished as axons cross through floor plate, resulting in stronger staining at the lateral margins of the floor plate where contralateral and ipsilateral C-axons meet. (H) In *Gli2*$^{-/-}$ embryos, TAG-1 staining is strong in the ventral midline region where C-axons from both sides of the spinal cord meet one another and become disorganized. (I) High magnification of ventral midline region showing that TAG-1 staining can be detected on longitudinally projecting C-axons in *Gli2*$^{-/-}$ embryos. Top of figure is dorsal in A-F and rostral in G-I. Scale bar in E, 60 μm for A-F; in G, 75 μm for G,H; in I, 30 μm.

repel C-axons after crossing the ventral midline. Alternatively, distinct mechanisms may operate to control whether C-axons find the floor plate attractive or repulsive.

Our finding that motoneuron axons and posterior commissure projections formed in *Gli2*$^{-/-}$ mutants suggests that the floor plate is not required for the trajectory of these axons away from the ventral midline in vivo, although since they are not as well organized as in WT embryos ventral midline cells may play a modulatory role. It has been shown in vitro that the floor plate is sufficient, however, to repel these classes of axons (Guthrie and Pini, 1995; Shirasaki et al., 1996) and, in *cyc* mutant zebrafish embryos, the PC does not form (Hatta et al., 1994). Recently, it has been shown that rSLit-2,

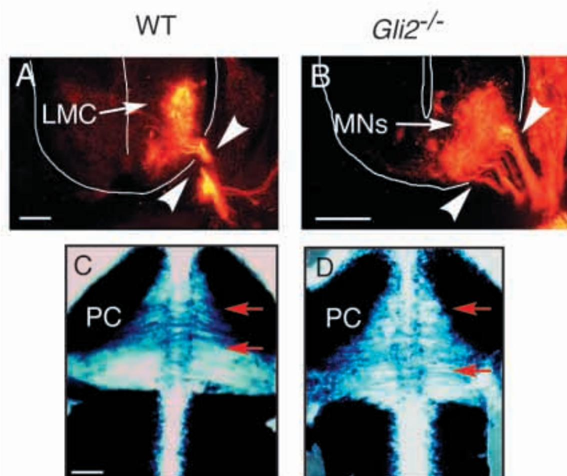

Fig. 6. Some axons whose trajectories are directed away from the ventral midline project similarly in WT and *Gli2*$^{-/-}$ embryos. (A,B) Transverse sections though the lower thoracic spinal cords after DiI injections into spinal nerves in the periphery. (A) Retrograde transport of DiI labels motoneurons in the lateral motor column (LMC) and their discrete ventral root exit (arrowheads) in WT embryos. (B) In *Gli2*$^{-/-}$ embryos, motoneurons are also labeled (MNs), but these cells exit the CNS from an abnormally broad ventral root (arrowheads). (C,D) Flat-mounts of dorsal diencephalon/mesencephalon region in Tg/Dbx1TLZ embryos at E12 stained with X-gal showing the projections of the posterior commissure (PC; between red arrows) cross the dorsal midline at this point in both WT (C) and *Gli2*$^{-/-}$ embryos (D). Note that the PC is not as discretely organized in *Gli2*$^{-/-}$ embryos as it is in WT embryos, resulting in less densely organized axons. Top of figure is dorsal in A,B and rostral in C,D. Scale bars, 60 μm in A,B; 100 μm in C for C,D.

which is expressed in the rat floor plate, can repel motoneurons axons in vitro (Brose et al., 1999). However, our results suggest that there may be redundancy with other repulsive molecules whose expression is not confined to the floor plate in the mouse CNS and that these putative chemorepulsive cues must be expressed in *Gli2*$^{-/-}$ mutants, similar to *Netrin1* expression in the VZ. Consistent with this, *rSlit-1* is expressed in both the floor plate and ventral VZ (Brose et al., 1999).

The floor plate provides local modulation of adhesion molecule localization on C-axons

Our results provide convincing in vivo evidence that ventral midline cells are required to alter the localization of CAMs on C-axons. We found that, in WT embryos, Nr-CAM levels are normally enriched on C-axons precisely in the VC, whereas in *Gli2*$^{-/-}$ mutants, Nr-CAM was maintained at its lower precommissural level at the ventral midline. These results suggest that the floor plate may directly induce the local accumulation of Nr-CAM on axons in the VC (M. L., T. S. and M. G., unpublished data), and that this accumulation is prevented in *Gli2*$^{-/-}$ embryos that lack floor plate cells. We also found TAG-1 protein on the rare longitudinal axons in *Gli2*$^{-/-}$ embryo spinal cords at E11, unlike in WT embryos at this stage in which TAG-1 is not detected on axons in the VF. This finding suggests that ventral midline cells may, directly or indirectly, control the disappearance of TAG1 on C-axons at the ventral midline. In contrast, our finding that L1 expression was detected in the ventral midline of *Gli2*$^{-/-}$ embryos suggests that its localization may be primarily controlled by axon-axon interactions or other cues present in the midline. Thus, the floor plate and/or VIR cells appear to exert specific modulatory influences on different CAMs.

It has recently been shown in vitro that C-axons are no longer responsive to Netrin1 chemoattraction after crossing through the floor plate and ventral midline (Shirasaki et al., 1998). Our finding that most C-axons remained at the ventral midline in *Gli2*$^{-/-}$ embryos is consistent with the idea that C-axons remain responsive to Netrin1 in these mutants. Indeed, C-axon clusters form at the midline in a region near the VZ with the highest remaining *Netrin1* expression in *Gli2*$^{-/-}$ embryos.

The role of ventral midline cells in C-axon guidance

In *Gli2*$^{-/-}$ mutant embryos, two distinct types of ventral midline cells are absent: floor plate and VIR cells. It is not clear from our studies whether these cell types play similar or distinct roles in providing cues to C-axons.

The closest parallel in vertebrates to the phenotype in *Gli2*$^{-/-}$ mutants comes from zebrafish. In *cyclops* (*cyc*) mutants, the single midline floor plate cell fails to develop but cells that flank the floor plate ('lateral floor plate cells') are present (Hatta et al., 1991). The role of lateral floor plate cells in providing guidance cues to C-axons has been studied by ablating these cells in WT or *cyc* mutant embryos (Bernhardt et al., 1992). In zebrafish, lateral floor plate cells express *Nk2.2* (R. Karlstrom and M. P. M., unpublished data) and could be analogous to mouse VIR cells, which are also defined by their expression of the mouse homolog *Nkx2.2* (Ericson et al., 1997; Matise et al., 1998). Thus, ablation of these lateral cells in *cyc* mutants creates a situation similar to *Gli2*$^{-/-}$ mouse mutants. One caveat in these experiments is that it is not clear whether lateral floor plate cells were re-induced after ablation, due to the lack of specific markers for these cells at the time that the experiments were done. Nevertheless, in both *cyc* mutants and *cyc*+lateral-floor-plate-cell ablation mutants, C-axons were found to be capable of projecting to the ventral midline (Bernhardt et al., 1992). Thus, despite species differences in the trajectory that C-axons take to reach the ventral midline, and in the greater cellular and spatial complexity of the mouse compared to the zebrafish CNS (Bernhardt et al., 1992; see also Colamarino and Tessier-Lavigne, 1995), the floor plate or immediately adjacent cells, are not required for the long-range attraction of C-axons to the midline in either species.

In contrast to our findings in *Gli2*$^{-/-}$ mutant embryos, in *cyc* mutants and in *cyc*+lateral-floor-plate-cell ablation mutants, the vast majority of C-axons turned rostrally to project longitudinally, as in WT embryos (Bernhardt et al., 1992). These results suggest that the floor plate or adjacent cells are not involved in the polarity decisions made by C-axons at the ventral midline in zebrafish. However, one difference between the experiments in the mouse and zebrafish is that, in zebrafish, cell ablations were only performed in restricted regions of the spinal cord whereas, in *Gli2*$^{-/-}$ mutants, floor plate and VIR cells are absent throughout the neuraxis. Taken together, these studies in fish and mice indicate that ventral midline cells may play a more important role in controlling the longitudinal projections and polarity of C-axons in the mouse spinal cord.

One possibility is that *Gli2*$^{-/-}$ mutant embryos have additional defects that might affect longitudinal C-axon guidance, for example, defects in the paraxial mesoderm. However, since rostral turning of C-axons after crossing the midline can occur in isolated mouse spinal cord explants grown in culture (R. Imondi and Z. Kaprelian, *Soc. Neurosci. Abstracts* **24**, 287, 1998), it is likely that polarity cues sufficient for the longitudinal growth of C-axons are contained within the CNS of the mouse. Our results do not distinguish, however, whether ventral midline cells provide rostrocaudal polarity cues, or rather control the response of C-axons to cues provided by other cells or tissues in the spinal cord in the mouse.

Summary

In the mouse spinal cord, the early projections of C-axons can be divided into a number of discrete steps. First, axons project ventrally to the midline in the transverse plane. Second, they cross the midline, forming the VC. Third, they turn 90° rostrally in the contralateral VF. Fourth, they extend longitudinally in the VF toward their ultimate target. Our studies of C-axon guidance in *Gli2*$^{-/-}$ mouse embryos demonstrate that ventral midline cells in the mouse CNS are primarily required for the last two steps in this process, as well as for the formation of an organized VC. We have shown that neither ventral midline cells nor high levels of *Netrin1* expression in the floor plate are required for the guidance of C-axons to the ventral midline in *Gli2*$^{-/-}$ mutant mice. Taken together with studies of *Netrin1* mouse mutants, our results furthermore suggest that a weak Netrin1 gradient is sufficient to provide ventral guidance cues to C-axons. In addition, longitudinal guidance of C-axons is not absolutely dependent on the presence of ventral midline cells, although since many fewer axons extend longitudinally in *Gli2*$^{-/-}$ mutants, these cells likely play an important role in facilitating their extension along this axis. Ventral midline cells do, however, appear to be required for the modulation of CAM localization on C-axons and for the rostral turning of these axons in the contralateral VF. These studies thus demonstrate

an important local, but not long-range, requirement for the floor plate in controlling C-axon projections in the mouse spinal cord.

We wish to thank J. Dodd, T. Jessell, P. Mombaerts, F. Ruddle, M. Tessier-Lavigne for reagants, K. Losos for generating the Dbx1/TLZ transgenics, Heidi Park for help with breeding *Gli2* mice, and Jane Dodd, Steve Burden and Gord Fishell for helpful comments on the manuscript. M. P. M. was partially supported by an NRSA postdoctoral fellowship, A. L. J. is an investigator of the Howard Hughes Medical Institute. This work was supported by grants from the HFSF and NICHD.

REFERENCES

Bernhardt, R. R., Nguyen, N. and Kuwada, J. (1992). Growth cone guidance by floor plate cells in the spinal cord of zebrafish embryos. *Neuron* **8**, 869-882.

Bovolenta, P. and Dodd, J. (1990a). Guidance of commissural growth cones at the floor plate in embryonic rat spinal cord. *Development* **109**, 435-447.

Bovolenta, P. and Dodd, J. (1990b). 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. *Development* **113**, 625-639.

Brose, K., Bland, K. S., Wang, K. H., Arnott, D., Henzel, W., Goodman, C. S., Tessier-Lavigne, M. and Kidd, T. (1999). Slit proteins bind Robo receptors and have an evolutionarily conserved role in repulsive axon guidance. *Cell* **96**, 795-806.

Callahan, C. A. and Thomas, J. B. (1994). Tau-β-galactosidase, an axon-targeted fusion protein. *Proc. Natl Acad. Sci.* **91**, 5972-5976.

Colamarino, S. A. and Tessier-Lavigne, M. (1995). The role of the floor plate in axon guidance. *Annual Review Neurosci.* **18**, 497-529.

Ding, Q., Motoyama, J., Gasca, S., Mo, R., Sasaki, H., Rossant, J. and Hui, C.-c. (1998). Diminished Sonic hedgehog signaling and lack of floor plate differentiation in *Gli2* mutant mice. *Development* **125**, 2533-2543.

Dodd, J., Morton, S. B., Karagogeos, D., Yamamoto, M. and Jessell, T. M. (1988). Spatial regulation of axonal glycoprotein expression on subsets of embryonic spinal neurons. *Neuron* **1**, 105-116.

Ericson, J., Rashbass, P., Schedl, A., Brenner-Morton, S., Kawakami, A., van Heynigen, V., Jessell, T. M. and Briscoe, J. (1997). Pax6 controls progenitor cell identity and neuronal fate in response to graded Shh signaling. *Cell* **90**, 169-180.

Friedlander, D. R., Milev, P., Karthikeyan, L., Margolis, R. K., Margolis, R. U. and Grumet, M. (1994). The neuronal chondroitin sulfate proteoglycan neurocan binds to the neural cell adhesion molecules Ng-CAM/L1/NILE and N-CAM, and inhibits neuronal adhesion and neurite outgrowth. *J. Cell Biol.* **125**, 669-680.

Greenspoon, S., Patel, C. K., Hashmi, S., Bernhardt, R. R. and Kuwada, J. Y. (1995). The notochord and floor plate guide growth cones in the zebrafish spinal cord. *J. Neurosci.* **15**, 5956-5965.

Grumet, M. (1997). Nr-CAM: a cell adhesion molecule with ligand and receptor functions. *Cell Tissue Research* **290**, 423-428.

Guthrie, S. and Pini, A. (1995). Chemorepulsion of developing motor axons by the floor plate. *Neuron* **14**, 1117-1130.

Hatta, K., Kimmel, C. B., Ho, R. K. and Walker, C. (1991). The cyclops mutation blocks specification of the floor plate of the zebrafish central nervous system. *Nature* **350**, 339-341.

Hatta, K., Puschel, A. W. and Kimmel, C. B. (1994). Midline signalling in the primordium of the zebrafish anterior central nervous system. *Proc. Natn. Acad. Sci., USA* **91**, 2061-2065.

Hogan, B., Beddington, R., Constantini, F. and Lacy, E. (1994). *Manipulating the Mouse Embryo: A Laboratory Manual.* Cold Spring Harbor, New York: CSHL Press.

Hui, C.-c., Slusarski, D., Platt, K. A., Holmgren, R. and Joyner, A. L. (1994). Expression of three mouse homologs of the *Drosophila* sement polarity gene *cutibus interruptus*, *Gli*, *Gli-2*, and *Gli-3*, in ectoderm-and mesoderm-derived tissues suggests multiple roles during postimplantation development. *Dev. Biol.* **162**, 402-413.

Keino-Masu, K., Masu, M., Hinck, L., Leonardo, E. D., Chan, S. S.-Y., Culotti, J. G. and Tessier-Lavigne, M. (1996). *Deleted in Colorectal Cancer* (DCC) encodes a netrin receptor. *Cell* **87**, 175-185.

Kennedy, T. E., Serafini, T., de la Torre, J. R. and Tessier-Lavigne, M. (1994). Netrins are diffusible chemotropic factors for commissural axons in the embryonic spinal cord. *Cell* **78**, 425-435.

Kidd, T., Bland, K. S. and Goodman, C. S. (1999). Slit is the midline repellant for the Robo receptor in Drosophila. *Cell* **96**, 785-794.

Kidd, T., Brose, K., Mitchell, K. J., Fetter, R. D., Tessier-Lavigne, M., Goodman, C. S. and Tear, G. (1998). Roundabout controls axon crossing of the CNS midline and defines a novel subfamily of evolutionarily conserved guidance receptors. *Cell* **92**, 205-215.

Li, H.-s., Chen, J.-h., Wu, W., Fagaly, T., Zhou, L., Yuan, W., Dupuis, S., Jiang, Z.-h., Nash, W., Gick, C. et al. (1999). Vertebrate Slit, a secreted ligand for the transmembrane protein roundabout, is a repellant for olfactory bulb axons. *Cell* **96**, 807-818.

Lu, S., Shashikant, C. S. and Ruddle, F. H. (1996). Separate *cis*-acting elements determine the expression of mouse *Dbx* gene in multiple spatial domains of the central nervous system. *Mech. Dev.* **58**, 193-202.

MacLennan, A. J., McLaurin, D. L., Marks, L., Vinson, E. N., Pfeifer, M., Szulc, S. V., Heaton, M. B. and Lee, N. (1997). Immunihistochemical localization of Netrin-1 in the embryonic chick nervous system. *J. Neurosci.* **17**, 5466-5479.

Matise, M. P., Epstein, D. J., Park, H. L., Platt, K. P. and Joyner, A. L. (1998). *Gli2* is required for induction of floor plate but not most ventral neurons in the mouse central nervous system. *Development* **125**, 2759.

Matise, M. P. and Joyner, A. L. (1997). Expression patterns of developmental control genes in normal and *Engrailed-1* mutant mouse spinal cord reveal early diversity in developing interneurons. *J. Neurosci.* **17**, 7805-7816.

Ming, G. L., Song, H. J., Behnringer, B., Holt, C. E., Tessier-Lavigne, M. and Poo, M. M. (1997). cAMP-dependent growth cone guidance by netrin-1. *Neuron* **19**, 1225-1235.

Mo, R., Freer, A. M., Zinyk, D. L., Crackower, M. A., Michaud, J., Heng, H. H.-Q., Chik, K. W., Shi, X.-M., Tsui, L.-C., Cheng, S. H. et al. (1997). Specific and redundant functions of *Gli2* and *Gli3* zinc finger genes in skeletal patterning and development. *Development* **124**, 113-123.

Mombaerts, P., Wang, F., Dulac, C., Chao, S. K., Nemes, A., Mendelsohn, M., Edmonson, J. and Axel, R. (1996). Visualizing an olfactory sensory map. *Cell* **87**, 675-686.

Sasaki, H., Hui, C.-c., Nakafuku, M. and Kondoh, H. (1997). A binding site for Gli proteins is essential for HNF-3β floor plate enhancer activity in transgenics and can respond to Shh in vitro. *Development* **124**, 1313-1322.

Seeger, M., Tear, G., Ferres-Marco, D. and Goodman, C. S. (1993). Mutations affecting growth cone guidance in *Drosophila*: Genes necessary for guidance toward or away from the midline. *Neuron* **10**, 409-426.

Serafini, T., Colamarino, S. A., Leonardo, E. D., Wang, H., Beddington, R., Skarnes, W. C. and Tessier-Lavigne, M. (1996). Netrin-1 is required for commissural axon guidance in the developing vertebrate neurvous system. *Cell* **87**, 1001-1014.

Serafini, T., Kennedy, T. E., Galko, M. J., Mirzayan, C., Jessell, T. M. and Tessier-Lavigne, M. (1994). The Netrins define a family of axon outgrowth-promoting proteins homologous to C. elegans UNC-6. *Cell* **78**, 409-424.

Shirasaki, R., Katsumata, R. and Murakami, F. (1998). Change in chemoattractant responsiveness of developing axons at an intermediate target. *Science* **279**, 105-107.

Shirasaki, R., Mirzayan, C., Tessier-Lavigne, M. and Murakami, F. (1996). Guidance of circumferentially growing axons by Netrin-dependent and –independent floor plate chemotropism in the vertebrate brain. *Neuron* **17**, 1079-1088.

Shoji, H., Ito, T., Wakamatsu, Y., Hayasaka, N., Ohsaki, K., Oyanagi, M., Kominami, R., Kondoh, H. and Takahashi, N. (1996). Regionalized expression of the *Dbx* family of homeobox genes in the embryonic CNS of the mouse. *Mech. Dev.* **56**, 25-39.

Song, H., Ming, G., He, Z., Lehmann, M., Tessier-Lavigne, M. and Poo, M. (1998). Conversion of neuronal growth cone responses from repulsion to attraction by cyclic nucleotides. *Science* **281**, 1515-1518.

Stoeckli, E. T., Sonderegger, P., Pollerberg, G. E. and Landmesser, L. T. (1997). Interference with axonin-1 and NrCAM interactions unmasks a floor-plate activity inhibitory for commissural axons. *Neuron* **18**, 209-221.

Tear, G., Harris, R., Sutaria, S., Kilomanski, K., Goodman, C. S. and Seeger, M. A. (1996). *commissureless* controls growth cone guidance across the CNS midline in *Drosophila* and encodes a novel membrane protein. *Neuron* **16**, 501-514.

Tessier-Lavigne, M. and Goodman, C. S. (1996). The molecular biology of axon guidance. *Science* **274**, 1123-1133.

Thomas, J. B., Crews, S. T. and Goodman, C. S. (1988). Molecular genetics of the *single-minded* locus: a gene involved in the development of the *drosophila* nervous system. *Cell* **52**, 133-141.

Varela-Echavarria, A., Tucher, A., Puschel, A. W. and Guthrie, S. (1997). Motor axon subpopulations respond differentially to the chemorepellants Netrin-1 and Semaphorin D. *Neuron* **18**, 193-207.