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

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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 chemoaactive 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 postcommisural 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 Gl2 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 Gl2-/- mutants is similar to wild-type (WT) embryos (Matise et al., 1998). Thus, the selective nature of the midline CNS phenotype of Gl2-/- 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 Gl2-/- 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 Gl2-/- 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 Gl2-/- mutants. However, these C-axons that can extend longitudinally in Gl2-/- 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 Gl2-/- 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 Gl2-/- 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 Gl2-/- 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 Gl2-/- mutant embryos. Thus, the floor plate, while being sufficient in vitro to repel motoneuron and posterior commissure 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<sup>+/−</sup> 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<sup>+/−</sup> male founder was bred with heterozygous Gli2<sup>+/−</sup> females to obtain Gli2<sup>+/−</sup> homozygous offspring transgenic for Dbx1/tauLacZ. The morning of vaginal plug detection was designated as E0.5.

Axon tracing
DiI (1.1'-dioctadecyl-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 (Biomed) 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. 2J/L). The turning of C-axons at the ventral midline in Gli2 mutants was quantified by observing the trajectory of anterogradely labeled C-axons and 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<sup>+/−</sup> 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<sup>+/−</sup> 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<sup>+/−</sup> 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<sup>+/−</sup> mutants
Our DiI axon tracing results suggested that the organization of most, if not all, dorsal C-axons at the ventral midline of Gli2<sup>+/−</sup> mutants was disrupted. To study the behavior of C-axons in more detail in Gli2<sup>+/−</sup> 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-
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 across 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 C-axons, we examined the localization of Nr-CAM and TAG-1 adhesion molecules. In WT embryos, both Nr-CAM and TAG-1 are expressed in a specific manner on C-axons. In Gli2−/− mutants, the expression of these molecules is abnormal, and the localization of Nr-CAM and TAG-1 on C-axons is disorganized and difficult to interpret. These results suggest that interactions with the floor plate are necessary for the normal localization of these molecules on C-axons.

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 dorsal 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 E1J. 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.
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 VR cells in the mouse.

Normal C-neuron differentiation in Gli2−/− mutants embryos

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

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<td>Wild type</td>
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<td>Gli2−/−</td>
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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 DI tracing. CNIII can be labeled anterogradely by DI 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, DI 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 (p1/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 trajectory 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 chemorepellent 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...
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. 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.

### 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.
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,
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 repellusive molecules whose expression is not confined to the floor plate in the mouse CNS and that these putative chemorepellusive 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 Nkx2.2 (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 Nks2.2 (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. Kaprielian, 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.

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