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Gli2 is required for induction of floor plate and adjacent cells, but not most ventral neurons in the mouse central nervous system

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SUMMARY

Induction of the floor plate at the ventral midline of the neural tube is one of the earliest events in the establishment of dorsoventral (d/v) polarity in the vertebrate central nervous system (CNS). The secreted molecule, Sonic hedgehog, has been shown to be both necessary and sufficient for this induction. In vertebrates, several downstream components of this signalling pathway have been identified, including members of the Gli transcription factor family. In this study, we have examined d/v patterning of the CNS in *Gli2* mouse mutants. We have found that the floor plate throughout the midbrain, hindbrain and spinal cord does not form in *Gli2* homozygotes. Despite this, motoneurons and ventral interneurons form in their normal d/v positions at 9.5 to 12.5 days postcoitum (dpc). However, cells that are generated in the region flanking the floor plate, including dopaminergic and serotonergic neurons, were greatly reduced in number or absent in *Gli2* homozygous embryos. These results suggest that early signals derived from the notochord can be sufficient for establishing the basic d/v domains of cell differentiation in the ventral spinal cord and hindbrain. Interestingly, the notochord in *Gli2* mutants does not regress ventrally after 10.5 dpc, as in normal embryos. Finally, the spinal cord of *Gli1*/*Gli2* zinc-finger-deletion double homozygous mutants appeared similar to *Gli2* homozygotes, indicating that neither gene is required downstream of Shh for the early development of ventral cell fates outside the ventral midline.

Key words: *Gli2*, *Gli1*, Floor plate, Dorsoventral patterning, Sonic hedgehog, Midbrain, Central nervous system, Neuron

INTRODUCTION

The vertebrate central nervous system (CNS) is a structure that is organized around an axis of bilateral symmetry. At the midline of the developing CNS lie two specialized structures, the floor plate ventrally and the roof plate dorsally, which are thought to play important roles in establishing and maintaining this bilaterality during embryogenesis. During the formation of the neural tube, the neural plate hinges at the ventral midline and closes dorsally. The induction of a floor plate at the ventral midline by the notochord is one of the earliest signs of CNS polarity along the dorsoventral (d/v) axis.

Both floor plate and ventral neuronal induction has been shown to be primarily directed by the secreted molecule, Sonic hedgehog (Shh; Echelard et al., 1993; Roelink et al., 1994; Marti et al., 1995a; Ericson et al., 1996; Chiang et al., 1996). *Shh* is expressed first in the notochord and then, shortly after its induction, in the floor plate. As the floor plate has been shown experimentally to possess similar signalling properties as the notochord (Yamada et al., 1991), it is thought that both the notochord and floor plate participate in establishing ventral cell fate through the action of a morphogenetic gradient of Shh that is secreted by these structures (Yamada et al., 1991).

In flies and vertebrates, members of the cubitus interruptus (*ci*)/ Gli zinc-finger-containing gene family have been implicated as transducers of the Hedgehog (Hh) signal (reviewed in Ruiz i Altaba, 1997; Kalderon, 1997). In *Drosophila*, there is evidence that *ci* activity may be regulated at the protein level by Hh signaling (Robbins et al., 1997; Aza-Blanc et al., 1997). In the absence of Hh signalling, ci undergoes proteolytic processing, which yields an N-terminal repressor form of the protein that acts in the nucleus (Aza-Blanc et al., 1997). When Hh binds to the Ptc/Smo receptor complex, this ci cleavage event is blocked, presumably generating a full-length ci protein that can activate Hh target genes in the nucleus (Aza-Blanc et al., 1997).

In mice, three *ci* homologues have been found, *Gli1*, *Gli2* and *Gli3* (Hui et al., 1994). The three Gli genes are expressed throughout the neural plate prior to floor plate induction, with the exception of *Gli3*, which is weak or absent from the midline (Hui et al., 1994; Sasaki et al., 1997; Lee et al., 1997). By 9.5 dpc, *Gli1*, *Gli2* and *Gli3* expression patterns become restricted

to overlapping but distinct domains along the d/v axis. *Gli1* is expressed in the ventralmost domain adjacent to the floor plate, *Gli2* is restricted to ventral and intermediate regions, while *Gli3* is only expressed dorsally in the neural tube (Hui et al., 1994; Sasaki et al., 1997; Platt et al., 1997; Lee et al., 1997).

In vivo, ectopic expression studies have shown that *Gli1*, but not *Gli3*, can activate downstream targets of *Shh*, such as *Hnf*-3β, in the CNS (Hynes et al., 1997; Lee et al., 1997). Consistent with this, tissue culture transfection assays have shown that *Gli1* activates and *Gli3* represses transcription of an *Hnf*-3β reporter gene (Sasaki et al., 1997). In addition, *Gli1*, but not *Gli3*, is activated by Shh when expressed ectopically in the CNS (Hynes et al., 1997). Analysis of mouse *Gli3*^{xt} mutants, which have multiple skeletal defects including polysyndactyly and dorsal neural tube defects (Hui and Joyner, 1993), shows that they express *Shh* ectopically in the anterior limb (Buscher et al., 1997). *Gli2* targeted mutants also have skeletal defects that are distinct from those in *Gli3*^{xt} mutants, but are exaggerated in *Gli2/Gli3* double homozygotes, suggesting some unique and redundant functions for these genes in skeletal development (Mo et al., 1997). Taken together, genetic, biochemical and expression studies suggest that the *Gli* genes are involved in some or all aspects of Shh signaling in many regions of the developing mouse, although the mechanisms by which each gene acts may differ. Moreover, the requirement of the *Gli* genes downstream of Shh signalling during CNS patterning has not been fully addressed.

In this study, we have analyzed CNS development in mice with a mutation in *Gli2* (Mo et al., 1997). We have found that, in homozygous mutant *Gli2* embryos, the floor plate does not form in the midbrain, hindbrain and spinal cord, but the notochord is present and expresses *Shh*. In addition, cells that form adjacent to the floor plate are greatly reduced in number or absent in *Gli2* homozygotes. Despite the absence of the floor plate and *Shh* expression in the ventral midline of the CNS, the pattern of cell differentiation along the d/v axis outside of the ventral midline appears largely normal in *Gli2* homozygotes. Interestingly, the notochord in *Gli2*$^{-/-}$ mutants does not regress after 10.5 dpc, as it does in normal embryos. Furthermore, *Gli1*/*Gli2* double homozygotes have a spinal cord phenotype similar to *Gli2*$^{-/-}$ mutants, while *Gli1*$^{-/-}$ mutants alone do not have any noticeable CNS defects. These results reveal that the induction of the floor plate by *Shh* is primarily dependent on *Gli2*. However, the specification of ventral cell fates outside of the ventral midline, a process also thought to be controlled by Shh, does not appear to require either normal *Gli1* or *Gli2* gene function or the presence of a floor plate in the mouse ventral CNS.

MATERIALS AND METHODS

Generation and genotyping of mice

Breeding and genotyping of *Gli2*$^{-/-}$ mutants was as described previously (Mo et al., 1997). To generate compound *Gli1*/*Gli2*$^{-/-}$ mutants, we bred *Gli2*$^{+/-}$ mice with *Gli1*$^{+/-}$ mice (H. L. P. et al., unpublished data) to produce double heterozygous *Gli1*$^{+/-}$/*Gli2*$^{+/-}$ mice, which were then intercrossed. Mice were kept on an outbred background. The targeting strategy for the *Gli1* gene was similar to that used for *Gli2* (Mo et al., 1997) and resulted in replacement of a 2.5 kb fragment containing 3 exons that encode zinc fingers 2-5, and ~43 amino acids downstream in the C terminus, with a PGK-Neo cassette. The production and analysis of *Gli1*$^{-/-}$ mutants will be described in detail elsewhere (H. L. P. et al., unpublished data). Genotyping of *Gli1*$^{-/-}$ mutants was performed by Southern blot hybridization using standard methods (Maniatis et al., 1982) with a 3′ *Gli1* cDNA probe after digesting yolk sac or tail DNA with *Xba*I, yielding a WT 9 kb band and 4.5 kb mutant band.

Shh reporter construction

To construct a *Shh*/lacZ reporter, genomic sequences upstream and downstream of the *Shh* promoter were tested for their ability to direct *lacZ* expression in a pattern that mimics normal *Shh* expression in transgenic mice (D. J. E., unpublished data). Using this approach, a region consisting of 7 kb downstream of the *Shh* start of transcription and a minimal *Shh* promoter was identified that was sufficient to drive *lacZ* expression in the floor plate, ventral midbrain and caudal diencephalon, but not in the notochord or other regions that normally express *Shh* (see Fig. 1J,K; D. J. E., unpublished data). Using this element, a line of mice was generated by injection of the reporter construct DNA into zygotes. Transgenic mice, identified by PCR to detect the *lacZ* sequence, were crossed with *Gli2* heterozygotes to obtain *Gli2*/*Shh-lacZ* double heterozygotes. These mice were then crossed with *Gli2*$^{+/-}$ mice to recover *Gli2*$^{-/-}$/*Shh-lacZ* embryos for analysis.

Immunohistochemistry and RNA in situ hybridization

Immunohistochemistry was performed as described for sections (Matise and Joyner, 1997) and whole mounts (Davis et al., 1991). Antibodies and dilutions were as follows: rabbit αShh, 1:100 (Marti et al., 1995b); αHnf-3β, 1:200 (Ruiz i Altaba et al., 1995); αEn, 1:500 (Davis et al., 1991); αChx-10, 1:50 (Liu et al., 1994); αIsl-1/2, 1:5000 (K5; Ericson et al., 1992); αTH, 1:250 (Pel Freeze, Inc.); α5HT, 1:500 (Incstar, Inc.); αparvalbumin, 1:1000 (Zhang et al., 1990); αS100, 1:200 (Sigma); mouse αNkx-2.2, 1:50 (Ericson et al., 1997); αPax6 (Ericson et al., 1997) and αPax7 (Ericson et al., 1996), 1:1000; αneurofilament-68, 1:400 (Sigma). Secondary antibodies, obtained from Jackson ImmunoResearch, were: Cy3- and fluorescein-conjugated goat and mouse anti-rabbit IgG, and HRP-conjugated goat anti-mouse IgG (used at 1:100-1:250). For qualitative comparisons, sections were processed at the same time and under the same conditions.

Detection of β-galactosidase activity was performed in whole mount as described (Matise and Joyner, 1997).

Whole-mount RNA in situ hybridization was performed as described (Parr et al., 1993) with modifications (Knecht et al., 1995). Probes used were: *Sim1* (Fan et al., 1996), *Shh* (Echelard et al., 1993), *Hnf*-3β (Sasaki and Hogan, 1993), *Ptc* (Goodrich et al., 1996) and *Gli1* (Hui et al., 1994). After whole-mount staining, some embryos were sectioned at 50-75 μm and counterstained as described (Matise and Joyner, 1997). For each genotype, stage or probe, between 2 and 18 embryos were examined. Image collection and processing was performed as described (Matise and Joyner, 1997).

Cell counts for En1- and Chx10-expressing cells were made at the forelimb level at 10.5 dpc. Counts were pooled from 4 embryos each of WT and *Gli2*$^{-/-}$ mutant phenotypes and are reported as mean±s.e.m.

RESULTS

Gli2 is required for floor plate induction

Previous studies have demonstrated neonatal lethality and skeletal defects in *Gli2*$^{-/-}$ homozygotes (Mo et al., 1997). In this study, we have focused on the development of ventral CNS cell fates in the absence of *Gli2* gene function. To determine if *Gli2* plays a role in the early development of the mouse CNS,

we studied the expression of markers of different cell types in the ventral CNS at stages shortly after neural tube closure at 9.5 or 10.5 dpc.

To begin, we examined the development of the floor plate at the ventral midline of the CNS by monitoring the expression of *Shh*, one of the earliest and most reliable markers of floor plate cell identity. We found that *Shh* expression was not detected in the ventral midline of *Gli2*$^{-/-}$ mutants in the midbrain, hindbrain and spinal cord at these early stages (*n*=12; Fig. 1A,B). To determine whether a floor plate had been induced at earlier stages but degenerated, we analyzed floor plate development by studying in detail the expression of *Shh* during the earliest stages of its appearance in the CNS.

Shh expression normally initiates in the CNS at the 6- to 7-somite stage in the ventral midline of the presumptive midbrain (Echelard et al., 1993). At this stage, *Shh* can also be detected in the underlying axial mesoderm (presumptive notochord) where its expression is detected approximately 24 hours earlier. By the 10-somite stage, *Shh* expression has proceeded rostrally into the forebrain (Echelard et al., 1993; Fig. 1C,E). Although in *Gli2*$^{-/-}$ embryos *Shh* expression is present in the axial mesoderm (Fig. 1D, arrowhead) at the 7- and 10-somite stage, it had failed to initiate anywhere in the CNS (Fig. 1D,F, and data not shown). CNS expression of *Shh* in *Gli2*$^{-/-}$ embryos was only clearly identified by the 12-somite stage, when it was detected in the ventral forebrain, albeit in a delayed manner (compare Fig. 1E and G, arrowheads).

To determine whether *Shh* expression was also initiated in a delayed fashion in other regions of the CNS in *Gli2*$^{-/-}$ embryos, we examined intermittent stages between 8.5 and 9.5 dpc when *Shh* expression can normally be found throughout the ventral neuraxis. We were unable to observe any expression of *Shh* in either the midbrain, hindbrain, or spinal cord of *Gli2*$^{-/-}$ embryos as assessed by RNA whole-mount in situ hybridization or antibody staining (Fig. 1I and data not shown). Notably, *Shh* expression was detected in the forebrain of *Gli2*$^{-/-}$ mutants (Fig. 1I), where it appeared similar to WT embryos (Fig. 1H). These data show that, although there is a short but consistent delay (of 1-2 somites) in *Shh* activation in the ventral forebrain of *Gli2*$^{-/-}$ mutants, by 9.5 dpc the expression in this region appears very similar to WT embryos, whereas *Shh* is never detected in more posterior regions of the CNS.

To further examine the loss of *Shh* expression in the ventral CNS, we generated a mouse line that expresses *lacZ* from a *Shh* enhancer that directs expression to the floor plate and ventral diencephalon (see Materials and Methods for details), and crossed this reporter line with *Gli2* mutants. Since β-gal protein is stable in cells (Echelard et al., 1994), its persistence allows one to follow the lineage or fate of β-gal-expressing cells for a short time, even after the transcription of the gene driving its expression has ceased. We were unable to detect β-gal expression in the ventral midline of the CNS or any other cell types outside of the forebrain in *Gli2*$^{-/-}$ mutants transgenic for the *Shh*/*lacZ* reporter (Fig. 1J,K and data not shown). Taken together, these observations demonstrate that floor plate cell fates, as judged by *Shh* reporter expression and endogenous gene expression, were not induced, even transiently, in *Gli2*$^{-/-}$ mutant embryos.

To further characterize the failure of floor plate formation in *Gli2*$^{-/-}$ mutants, we examined the expression of another marker of floor plate cells, *Hnf*-3β. In the ventral midline of the spinal cord, *Hnf*-3β expression precedes the expression of *Shh*, but unlike *Shh* is not only confined to floor plate cells but extends slightly dorsally in the ventral ventricular zone (Ruiz i Altaba et al., 1995; Fig. 2A). In the vast majority of *Gli2*$^{-/-}$ mutant embryos (*n*=8), the expression of *Hnf*-3β mRNA and protein in the ventral midline was completely absent in the spinal cord, hindbrain and midbrain at 9.5 to 10.5 dpc (Fig. 2B). However, expression was still detected in a patch of cells that mark the rostral extent of *Hnf*-3β expression in the caudal diencephalon (Fig. 2D,E, arrowheads). We also detected scattered Hnf-3β expression in posterior sections of some 9.5 dpc embryos (*n*=2; 3 out of 23 sections examined; Fig. 2C). Since we did not detect *Shh* in the ventral midline in *Gli2*$^{-/-}$ mutants at any stage examined, and since Hnf-3β is also expressed outside of the floor plate, it is possible that these few Hnf-3β cells detected in *Gli2*$^{-/-}$ mutants do not constitute definitive floor plate cells, but rather cells derived from adjacent regions where Hnf-3β is also expressed. In addition, this early, infrequently observed *Hnf*-3β expression was transient, since expression was never observed at 10.5 dpc or later. Together, these results suggest that, while *Gli2* function is not necessary for either *Hnf*-3β or *Shh* expression in the ventral forebrain, it is required for the expression of these genes and for the formation of the floor plate throughout the neuraxis up to the midbrain/forebrain junction.

Motoneurons occupy the ventral midline in Gli2$^{-/-}$ mutants

The failure of the floor plate to form in *Gli2*$^{-/-}$ mutants prompted us to examine the organization of cell differentiation in the ventral midline region of the spinal cord. We found that, whereas at 10.5 dpc in normal embryos the floor plate, and later the ventral median fissure, is located in the ventral midline, in *Gli2*$^{-/-}$ mutants this region was instead occupied by motoneurons expressing Islet-1/2 proteins (Fig. 2G). At early stages, motoneurons are normally located in two lateral clusters in the ventral spinal cord (Fig. 2F). This defect was observed at all rostrocaudal levels of the spinal cord and hindbrain of *Gli2*$^{-/-}$ mutants. As motoneuron induction has been shown to depend on Shh signalling (Chiang et al., 1996; Ericson et al., 1996), the appearance of these cells in *Gli2* mutants strongly suggests that at least some aspects of notochord signalling are intact in these embryos.

To further address this possibility, we examined two other markers of *Shh* signalling in the ventral CNS of *Gli2*$^{-/-}$ mutants: *Gli1* and *Ptc*. Expression of these two genes is normally seen in the ventral midline at neural plate stages, and then both are downregulated in the floor plate, but upregulated in ventricular zone cells immediately adjacent to the floor plate (Platt et al., 1997; Lee et al., 1997). When analyzed at 9.5 dpc, we found that both *Ptc* (*n*=2) and *Gli1* (*n*=4) expression were undetectable in the ventral spinal cord of *Gli2*$^{-/-}$ mutants, compared to WT embryos (data not shown). Thus, although the appearance of motoneurons suggests notochord Shh signalling is intact, the reduction in *Ptc* and *Gli1* expression indicates that some aspects of this pathway are perturbed in *Gli2* mutants. These results could be interpreted as suggesting that *Gli2* is required upstream of *Ptc* and *Gli1* in the ventral spinal cord; however, it is not clear whether *Gli2* acts directly on these genes to control their expression, or whether the loss or mis-

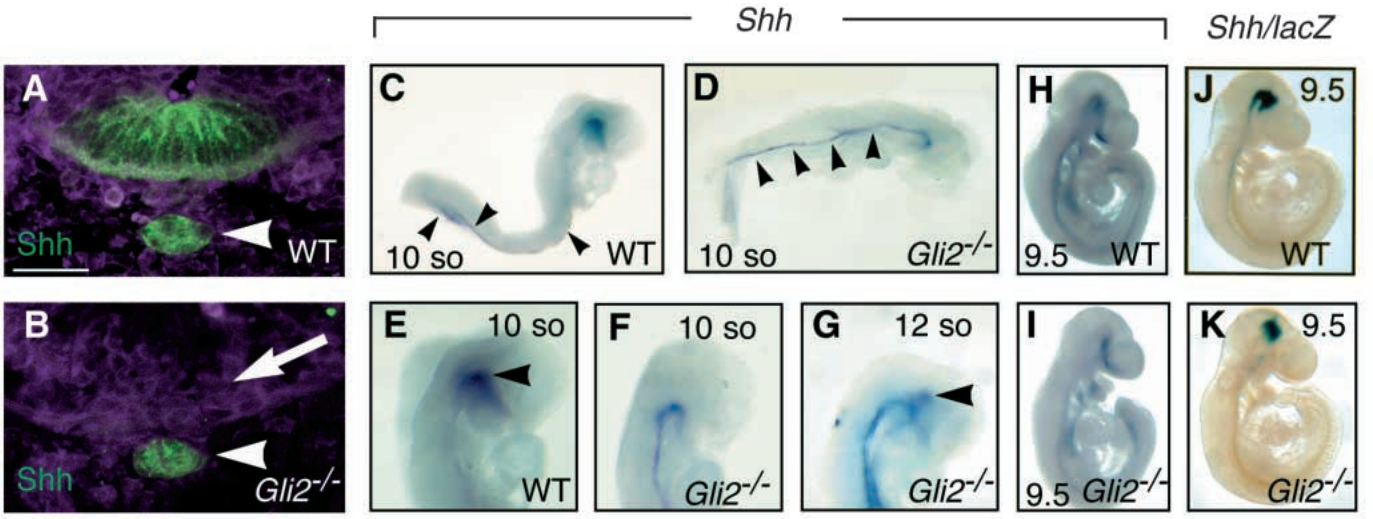

Fig. 1. The floor plate does not form in *Gli2*$^{-/-}$ mutants. (A,B) Transverse sections through thoracic spinal cords at 10.5 dpc stained with αShh antibodies. No Shh expression was detected in the ventral midline at any level in the midbrain, hindbrain and spinal cords of *Gli2*$^{-/-}$ mutants (arrow in B). (C-I) Whole-mount RNA in situ for *Shh* at the somites (so) stage indicated or 9.5 dpc (9.5) in wild type (WT) and *Gli2*$^{-/-}$ embryos. (C) WT *Shh* expression at 10 somites is detected in the ventral mesencephalon, and in the mesodermal precursor to the notochord (arrowheads) and prechordal plate. (D) In *Gli2*$^{-/-}$ embryos, no *Shh* is detected in the CNS. Expression is seen only in the midline mesoderm (arrowheads). (E) Enlargement of head of WT embryo showing *Shh* expression in the ventral diencephalon at 10 somites (arrowhead). (F) *Gli2*$^{-/-}$ mutants do not show *Shh* expression in the forebrain at 10 somites. (G) By 12 somites, *Shh* expression is detected in the ventral diencephalon of *Gli2*$^{-/-}$ embryos (arrowhead). (H,I) In WT embryos at 9.5 dpc (H), *Shh* is detected throughout the ventral CNS, including the forebrain. In *Gli2*$^{-/-}$ mutants (I), *Shh* is not detected in the spinal cord, hindbrain and midbrain, but expression in the forebrain is similar to WT embryos at this stage. (J,K) *LacZ* expression driven by a *Shh* enhancer element in WT embryos at 9.5 dpc is seen in the floor plate of the spinal cord extending to the level of the hindlimbs and rostrally into the ventral diencephalon. No β-gal expression is detected in the spinal cord, hindbrain or midbrain of *Gli2*$^{-/-}$ mutant embryos transgenic for this reporter. Rostral is to the right in C and D and to the top in E-K. Scale bar, 25 μm for A, B.

specification of tissues that normally express these genes results in their diminished expression.

Differentiation of cells adjacent to the floor plate is perturbed in *Gli2*$^{-/-}$ mutants

The finding that motoneurons differentiate in the ventral midline on *Gli2* mutants suggests that cells that normally flank the floor plate may be absent in these embyros. In addition, the loss of *Gli1* and *Ptc* expression may be partly explained by an absence of some cells that normally express these genes in *Gli2* mutants. To determine this, we examined a number of markers that are expressed in cells and their precursors located in the region immediately adjacent to the floor plate.

The homeobox gene Nkx-2.2 is expressed in ventricular zone cells, which flank the floor plate (Fig. 3A, and Ericson et al., 1997). In *Gli2*$^{-/-}$ mutants at 10.5 dpc, fewer Nkx-2.2-expressing cells were detected than in WT embryos, and these cells were found in a single domain, located in the ventral midline (Fig. 3B). In the rostral spinal cord and hindbrain of WT embryos, Nkx-2.2-expressing cells can be divided into two populations: those that lie between the floor plate and motor column in the region where *Hnf*-3β expression is also seen lateral to the floor plate (Fig. 3A, and data not shown) and those that lie more dorsally in motoneuron progenitors (Ericson et al., 1997). Most Nkx-2.2-expressing cells within this latter population can be distinguished by their transient co-expression of Islet-1/2 (Fig. 3A, arrows). In *Gli2*$^{-/-}$ mutants, we found that most midline Nkx-2.2-expressing cells also expressed Islet-1/2 (Fig. 3B). Thus, motoneurons that are found in the ventral midline of *Gli2* mutants possess the

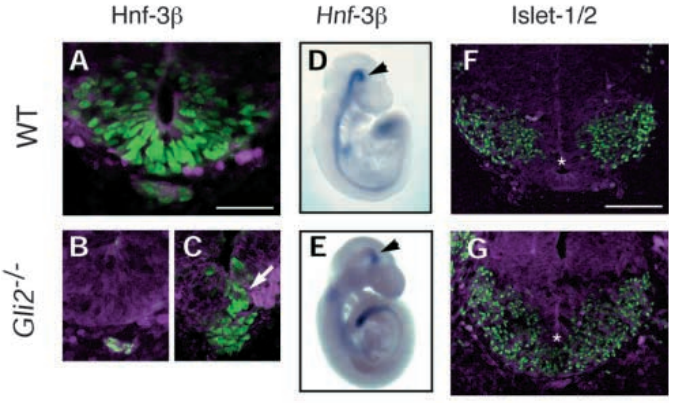

Fig. 2. *Hnf*-3β expression is absent and motoneurons are found in the floor plate region of *Gli2*$^{-/-}$ mutants. (A-C) Transverse sections through 9.5 dpc embryos stained with αHnf-3β. (A) WT expression at 9.5 dpc. While no expression was detected in the majority of *Gli2*$^{-/-}$ mutants (B), a minority of embryos showed a few Hnf-3β-expressing cells near the ventral midline of posterior regions (arrow in C). Sections in A-C are through midlumbar spinal cord regions. (D,E) Whole-mount staining for *Hnf*-3β mRNA at 9.5 dpc shows that expression in the forebrain of *Gli2*$^{-/-}$ mutants (arrowhead in E) is similar to normal embryos (D). Dorsal is to the top. (F,G) Motoneurons occupy the ventral midline in *Gli2*$^{-/-}$ mutants. Transverse sections through thoracic spinal cord at 10.5 dpc stained with αIslet-1/2 antibody reveals the abnormal presence of motoneurons in *Gli2*$^{-/-}$ mutants (G) in the region normally occupied by the floor plate (region below asterisks in F,G), instead of 2 lateral motor column clusters as in WT embryos (F). Scale bars, 25 μm for A, 20 μm for B, C (in A), 80 μm for F,G (in F).

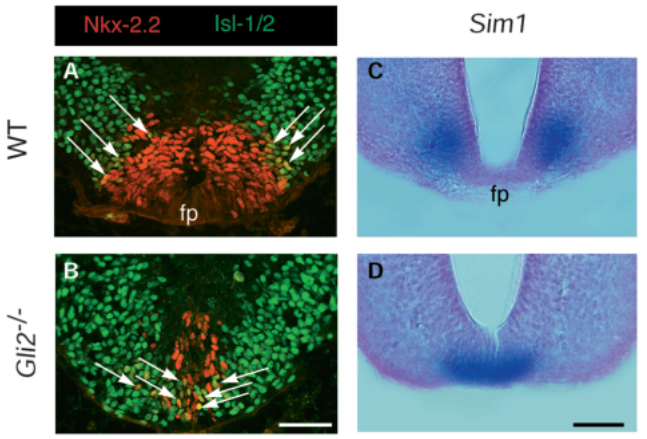

Fig. 3. Cell types that normally flank the floor plate are found in reduced numbers and in the ventral midline in *Gli2*$^{-/-}$ mutants. (A,B) Transverse sections through the rostral spinal cord at 10.5 dpc, double labeled with αNkx-2.2 (red) and αIslet-1/2 (green) antibodies. (A) In WT embryos, some ventrally located Islet-1/2-expressing motoneurons (green) transiently co-express Nkx-2.2 (red; double-labeled cells appear yellow, marked with arrows), while most Nkx-2.2 cells give rise to cells which lie between the floor plate (fp) and motoneurons. (B) In *Gli2*$^{-/-}$ mutants, Nkx-2.2 expression is found in the ventral midline, and most or all of these cells give rise to ectopically located motoneurons (yellow, arrows). (C,D) *Sim1* expression at 11.5 dpc. Normally, *Sim1* mRNA is detected in mantle zone cells flanking the floor plate (C). In *Gli2*$^{-/-}$ mutants, *Sim1* expression is detected in cell located at the ventral midline (D), similar to Nkx-2.2 and overlapping Islet-1/2 expression in this region. fp, floor plate. Scale bar in B, 40 μm (for A,B), and in D, 60 μm (for C,D).

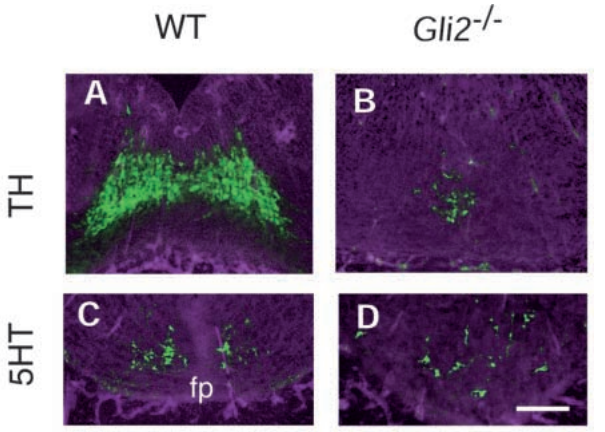

Fig. 4. Some later generated neurons which develop near the ventral midline in the rostral CNS are reduced in *Gli2*$^{-/-}$ mutants. (A, B) At 12.5 dpc, TH immunoreactivity (green) identifies immature dopaminergic neurons in the ventral part of the caudal midbrain in WT embryos, whereas in *Gli2*$^{-/-}$ mutants, the number of TH-expressing neurons in the midbrain is greatly reduced (~10% of WT). (C,D) 5HT immunoreactivity (green) at 12.5 dpc identifies serotonergic neurons that are generated adjacent to the floor plate (fp) in the rostral hindbrain. In *Gli2*$^{-/-}$ mutants, fewer 5HT-expressing neurons are detected (~50% of WT) and are abnormally located in the midline. Transverse sections through the midbrain (A,B), hindbrain (approx. rhombomere 3, C,D). Dorsal is to the top. Scale bar in D, 40 μm for A,B; 50 μm for C,D.

molecular identity of the most ventrally located population in WT embryos.

Sim1 expression is also detected in cells adjacent to the floor plate and outside of the ventricular zone at 10.5 dpc in WT mice (Fig. 3C; Fan et al., 1996). The precise domain and cell type that expresses *Sim1* has not been defined. In *Gli2*$^{-/-}$ mutants, *Sim1* expression was found in a single domain located in the ventral midline, in a similar location to Nkx-2.2 expression in these embryos (Fig. 3D). Since motoneurons, instead of the floor plate and flanking cells that express *Hnf-3β*, occupy this area in *Gli2*$^{-/-}$ mutants, it is possible that *Sim1*, like Nkx-2.2, is expressed in a subset of motoneurons in the *Gli2*$^{-/-}$ mutant spinal cord. Alternatively, *Sim1* may be inappropriately expressed in some motoneurons in *Gli2*$^{-/-}$ mutants.

To determine whether defects in ventral midline cell fates were confined to early generated cells, we examined cells that are born at later times (after 10.5 dpc) near the ventral midline of the CNS. Both dopaminergic (DA) and serotonergic (SER) neurons are generated adjacent to the floor plate in the midbrain and hindbrain, respectively (Yamada et al., 1991; Hynes et al., 1995). DA cells, identified by their expression of tyrosine hydroxylase (TH), first appear at 10.5-11 dpc in the ventral midbrain and are generated over the course of the next several days (Di Porzio et al., 1990). When examined at 12.5 dpc, we found a striking reduction in TH-expressing cells in the *Gli2*$^{-/-}$ mutant, compared to WT embryos (Fig. 4A,B; ~10% of normal number, *n*=21 sections from 3 mutant and WT embryos). We also examined SER neurons in the hindbrain using antibodies to 5-hydroxy-tryptophan (5HT). The expression of 5HT in WT embryos is first detected at 11.5 dpc in two small clusters flanking the floor plate in the rostral hindbrain. In *Gli2*$^{-/-}$ mutants, we found a 50% reduction in the number of 5HT-containing neurons, compared to WT, at 12.5 (Fig. 4C,D; ~50%, *n*=16 sections from 3 mutant and WT embryos). In addition, the 5HT-expressing cells were abnormally located in the ventral midline, instead of two bilateral clusters flanking the floor plate (Fig. 4D). Together, these results demonstrate that, in *Gli2*$^{-/-}$

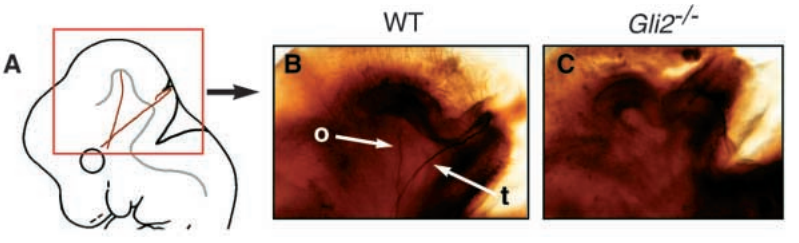

Fig. 5. Cranial nerves III and IV are absent in *Gli2*$^{-/-}$ mutants. (A) Schematic of lateral view of head of 11.5 dpc embryo. Boxed area is region depicted in B,C. (B,C) Whole-mount neurofilament staining (dark brown) at 11.5 dpc shows that the occulomotor (CN III) and trochlear (CN IV) nerves are absent in *Gli2*$^{-/-}$ mutants (C). No defects in CN V-XII were seen in *Gli2* mutants. o, occulomotor nerve, t, trochlear nerve. Rostral is to the left.

mutants, there is a striking reduction in the number and position of both early and late differentiating cells that form immediately adjacent to the floor plate.

We also performed whole-mount neurofilament (NF) staining on WT and *Gli2*$^{-/-}$ mutant embryos at 11.5 dpc to determine whether peripheral nerve organization was disrupted as a result of the abnormal midline location of motoneurons in *Gli2*$^{-/-}$ mutants. This analysis revealed that cranial nerve (CN) III and CN IV, normally present in the rostral and caudal mesencephalon, respectively, were absent in *Gli2*$^{-/-}$ mutants (Fig. 5B,C). Interestingly, motoneurons that contribute to these nerves are generated close to the floor plate. In contrast, CN V through XII, as well as spinal nerves throughout the hindbrain and spinal cord, appeared normal in *Gli2*$^{-/-}$ mutants (data not shown). This analysis shows an additional ventral CNS defect in *Gli2*$^{-/-}$ mutants as far rostrally as the mesencephalon, similar to the extent of loss of *Shh* and *Hnf*-3β expression in these mice. In addition, these findings indicate that the absence of a floor plate and adjacent cells, and the abnormal location of motoneurons in the ventral midline, does not disrupt the formation of spinal nerves or most cranial nerves in the periphery. Why midbrain motoneuron projections are affected whereas those in the spinal cord and hindbrain were not may reflect the proximity of CN III and IV cell bodies to the ventral midline and/or their special requirement for *Gli2* or the floor plate for their normal cell migration and axon projection patterns.

Early dorsoventral patterning is intact in *Gli2*$^{-/-}$ mutants

While ventral midline cells do not form in *Gli2* mutants, more dorsally located motoneurons are generated. This observation prompted us to ask whether the loss of *Gli2* affected the generation of other ventral cell types that differentiate outside of the ventral midline. To do this, we examined the expression of a number of genes that mark specific subsets of neurons and their precursors located at varying distances from the ventral midline.

Pax7 is initially expressed broadly in the neural plate, including the ventral midline (Jostes et al., 1990), but rapidly undergoes a *Shh*-dependent downregulation ventrally at neural plate stages (Ericson et al., 1996). This ventral *Pax7* repression has been shown to be a necessary prerequisite for the subsequent generation of ventral classes of neurons (Ericson et al., 1997). *Pax6* is also expressed widely in the early neural tube, but is not detected in the ventral midline (Fig. 6A; Goulding et al., 1993). By 9.5 dpc, Pax6 undergoes a Shh concentration-dependent change in its expression pattern, forming a dorsally decreasing gradient in the ventricular zone (VZ), dorsal to Nkx-2.2-expressing cells (Ericson et al., 1997).

To study the dynamics of Pax6 expression in *Gli2*$^{-/-}$ mutants, we analyzed expression in the spinal cord at 9.5-10.5 dpc. At 9.5 dpc, we found that Pax6 was expressed inappropriately in the ventral midline (Fig. 6A,B). By 10.5 dpc, however, Pax6 expression became restricted to more dorsal regions and was no longer detected in the ventral midline (Fig. 6D,E). As in WT embryos, Pax6 expression appeared to form a dorsally decreasing gradient in the VZ of *Gli2*$^{-/-}$ mutants (Fig. 6E). However, Pax6 expression also extends closer to the ventral limit of the VZ than in WT embryos, likely as a consequence of the floor plate and immediately adjacent cells being largely absent in *Gli2*$^{-/-}$ mutants. It is likely that Nkx-2.2 and Pax6 expression domains still abut one another in *Gli2*$^{-/-}$ mutants, however the overall pattern at the midline is reorganized as if a pie wedge was removed and adjacent/dorsal cells were brought together about the radial axis in the midline.

We found that Pax7 expression in *Gli2*$^{-/-}$ mutants at 9.5 dpc was restricted to the dorsal neural tube, similar to WT embryos (Fig. 6C; Jostes et al., 1990). At 10.5 dpc, the ventral boundary of Pax7-expressing cells was similar to WT embryos, as judged by its distance from the dorsal midline (data not shown), and by its location relative to En1-expressing ventral interneurons (Fig. 6F,G; see also below).

We next examined the differentiation of particular classes of ventral interneurons, which have been shown to be induced by specific Shh concentrations, marked by the expression of *En1* and *Chx10* (Ericson et al., 1997). Examination of these markers in *Gli2*$^{-/-}$ mutant embryos revealed that both *En1* (Fig. 6G) and *Chx10* (data not shown) were expressed in similar relative locations along the d/v axis, compared to WT embryos (Fig. 6F). Comparison of *En1* and Islet-1/2 expression in *Gli2*$^{-/-}$ mutants also revealed that cells marked by the expression of these genes differentiated in the appropriate, non-overlapping domains along the d/v axis (data not shown). In addition, the mean number of En1- and Chx10-expressing cells generated in *Gli2*$^{-/-}$ mutants (En1: $\bar{x}$=41.6±2.8, *n*=14 sections counted; Chx10: $\bar{x}$=7.9±0.8, *n*=21 sections counted) was similar to WT embryos (En1: $\bar{x}$=44.8±1.9; Chx10: $\bar{x}$=8.2±0.9).

We also examined the appearance of the Ca^{2+}-binding protein parvalbumin (PV) in the spinal cord and hindbrain at 12.5 dpc (Zhang et al., 1990). Normally, PV expression is initiated in ventral ventricular zone cells, dorsal to the floor plate, between 11.5 and 12.5 dpc (Fig. 6H). The pattern of PV staining in *Gli2*$^{-/-}$ mutants was similar to WT embryos at this stage, with the exception that PV expression was also found in the ventral midline (Fig. 6I). Thus, in *Gli2* mutants, both early and later appearing markers for cells that differentiate at defined positions along the d/v axis are arranged in a relative order similar to WT embryos, although the ventral-most cells are lost (floor plate and immediately adjacent cells) and more lateral cells (e.g., motoneurons) are situated in the midline.

The notochord does not regress in *Gli2*$^{-/-}$ mutants

In normal mice, the notochord is initially apposed to the ventral neural plate and neural tube at 8.5-9.5 dpc. After 10.5 dpc, the notochord begins to regress away from the spinal cord, eventually becoming surrounded by migrating sclerotomal cells and forming a small structure within the ventral vertebral bodies (Fig. 7A). In the mouse embryo during early ventral neurogenesis at 9.5 dpc, the notochord is still in contact with the spinal cord at all axial levels, including rostrally where the first postmitotic motoneurons are being generated (data not shown). By 10.5 dpc, the notochord has begun to regress ventrally in rostral regions, but at lumbar levels is still in contact with the spinal cord.

When we examined the notochord over this same period and at later stages (12.5 dpc) in *Gli2*$^{-/-}$ mutants, we found that the notochord remains closely apposed to the ventral spinal cord, and continues to express Shh at levels comparable to WT

embryos (Fig. 7B,C, and data not shown). This defect was also seen in the hindbrain of *Gli2*$^{-/-}$ mutants (data not shown). These findings suggest that the notochord may provide an extra source of Shh to the CNS in *Gli2* mutant embryos after 10.5 dpc. Thus, the persistent proximity of the notochord to the ventral spinal cord may account for the ventral midline location of some neuronal cell types in *Gli2*$^{-/-}$ mutants, especially those that differentiate after the notochord would normally have regressed in WT embryos (see Discussion).

It has been previously demonstrated that the formation of the ventral vertebrae is disrupted in *Gli2*$^{-/-}$ mutants (Mo et al., 1997). In WT embryos at 12.5 dpc, sclerotomal cells express S-100 as they condense around the notochord (which also expresses S-100 (Augustine et al., 1995); Fig. 7A). It is interesting to note that we also found S-100 staining in ventral sclerotomal cells in the *Gli2* mutant (Fig. 7C), but these cells were located ventral to the notochord rather than surrounding it as in WT embryos. Therefore, the abnormal location of the notochord in *Gli2*$^{-/-}$ mutants may partly explain the improper formation of the ventral vertebrae in these embryos (Mo et al., 1997). However, since *Gli2* is expressed in the sclerotome, it is not clear whether defects in the sclerotome cells themselves prevent the migration and intercalation of these cells between the spinal cord and notochord in *Gli2* mutants, or whether signals from the floor plate normally initiate notochord regression.

Normal *Gli1* and *Gli2* genes are not required for the generation of non-midline ventral spinal cord cell types

Since *Gli1* is expressed along with *Gli2* in the ventral spinal cord, one possible explanation for the finding that many ventral cell types could be detected in their normal d/v positions in *Gli2*$^{-/-}$ mutants is that *Gli1* also serves a role in the *Shh*-mediated specification of ventral neuronal fates. To examine this, we carried out a preliminary analysis of ventral gene expression in 12.5 dpc embryos carrying a mutation in *Gli1* (H. L. P., unpublished data) and in *Gli1/Gli2* double homozygotes. The *Gli1*^{zfd} mutant allele is similar to the *Gli2* mutation, with a deletion of the exons coding for zinc fingers 2 to 5. *Gli1*$^{zfd/zfd}$ embryos possess a floor plate and do not show any obvious abnormalities in the spinal cord or other regions of the embryo (Fig. 8A,B, and data not shown). Furthermore, we found that motoneurons were generated in *Gli1*/*Gli2* double homozygous mutants (n=3), in the ventral midline as well as laterally, similar to *Gli2*$^{-/-}$ mutants (Fig. 8C; compare to Fig. 2G). In addition, interneuron marker genes *En1* and *Chx10* marked distinct cell populations at similar positions along the d/v axis in *Gli1*/*Gli2* double mutants as in WT embryos (Fig. 8D, and data not shown). The similarity in phenotypes of *Gli2*$^{-/-}$ alone and *Gli1*/*Gli2* compound homozygotes indicates that neither *Gli1* nor *Gli2* normal genes are required to generate ventral neuron types outside of the ventral midline in the mouse spinal cord. Finally, we also examined *Gli3* expression in *Gli2* and *Gli1/Gli2* double mutants to determine whether it could compensate for the loss of *Gli1* and *Gli2* and mediate the Shh-dependent differentiation of ventral neurons. We found that *Gli3* expression was confined to the dorsal spinal cord and hindbrain in *Gli2*$^{-/-}$ mutants at 9.5 dpc (n=5; Fig. 8F) and 10.5 dpc (n=2; data not shown), and in *Gli1/Gli2* double mutants at 10.5 dpc (n=2; Fig. 8G), as in WT embryos (Fig. 8E). Thus, it does not appear that *Gli3* function compensates for the mutations in *Gli2* and/or *Gli1* to generate ventral cell types in *Gli2* and *Gli1/Gli2* mutants, suggesting that normal *Gli* genes are not required for the Shh-dependent induction of ventral cell types, outside of the ventral midline, in the mouse.

DISCUSSION

Gli2 is required for the development of the ventral midline of the mouse CNS

In this study, we have demonstrated that *Gli2* is required for the development of the floor plate. In addition, cells that flank the floor plate (hereafter referred to as ventral intermediate region, VIR, cells) were greatly reduced in number in *Gli2* mutants. We have shown that Shh expression, which identifies the floor plate, was not detected in the ventral CNS of *Gli2* homozygous mutants, except in the forebrain. Additionally, another floor plate marker, *Hnf*-3β, was only transiently detected at 9.5 dpc in a few cells, and was completely absent in all mutant embryos examined at 10.5 dpc, except in the forebrain. Two target genes of *Shh* signalling, *Gli1* and *Ptc*, were greatly downregulated in ventral regions of the spinal cord in 9.5 dpc *Gli2* homozygotes. These results, taken together with studies of Shh function, support the view that *Gli2* is a required activator of *Shh* target genes in the midline of the mouse ventral CNS. Preliminary analysis of *Gli1* homozygotes revealed that the spinal cord of these mice appeared normal. Furthermore, *Gli1/Gli2* double homozygotes had a similar defect to *Gli2* mutants alone, and *Gli3* was not expressed ventrally in these mutants. Thus, while *Gli1* is sufficient to activate *Shh* targets when expressed ectopically in the CNS (Hynes et al., 1997; Lee et al., 1997), *Gli2* appears to be the primary downstream activator of *Shh* targets in the presumptive floor plate region of the mouse CNS.

One explanation for the failure to induce a floor plate in *Gli2* mutants is that Shh expression in the axial mesoderm is reduced or delayed in its onset in these embryos. However, three observations argue against this interpretation. First, by analyzing the expression of genes located at a distance from the ventral midline that are influenced by Shh signalling, we found that their distance to the ventral midline was similar in *Gli2*$^{-/-}$ mutants compared to WT embryos. In particular, the state of expression of two markers shown to be responsive to the lowest Shh levels (Ericson et al., 1996, 1997), the ventral Pax7 boundary and appearance of *En1* expression, were similar in WT and *Gli2* mutant spinal cords. Second, using antibodies and RNA in situ hybridization to assay Shh expression, we were unable to detect any differences in Shh expression levels in the notochord of *Gli2*$^{-/-}$ mutants compared to WT embryos. We cannot, however, exclude the possibility that very small, but possibly significant, changes in Shh expression levels might not be detected by this qualitative assay. Third, we were able to detect *Shh* expression in *Gli2*$^{-/-}$ mutants in the axial mesoderm and node at the 7-somite stage. This time is within the competence period demonstrated in vitro for the induction of the floor plate by the notochord in rodent spinal cord (Placzek et al., 1993).

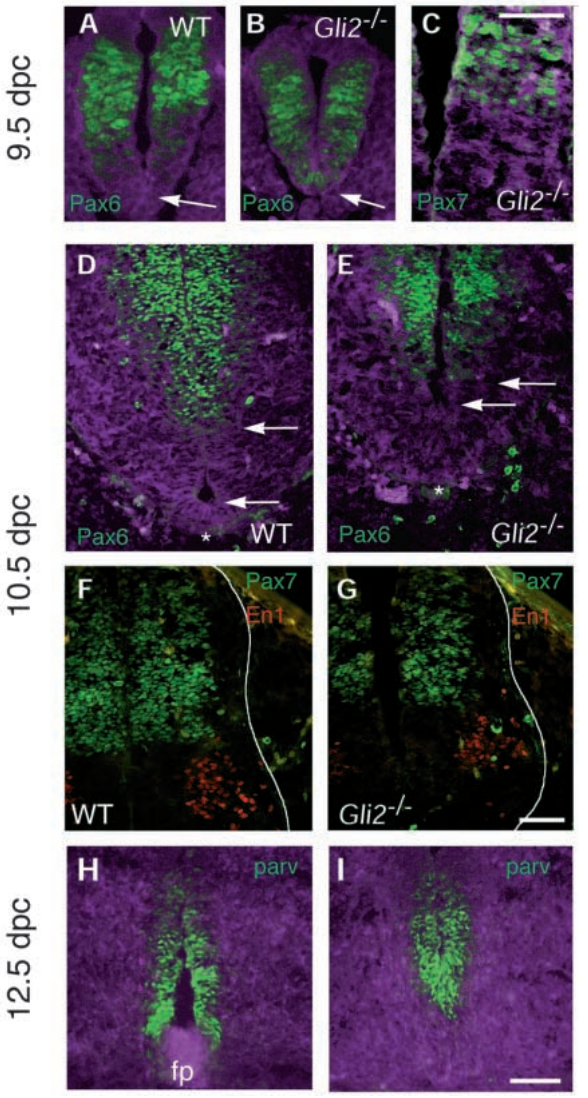

Fig. 6. Gene expression along the dorsoventral axis in *Gli2*$^{-/-}$ mutants. (A-C) 9.5 dpc sections through the lumbar region of the spinal cord stained with αPax6 (A,B) and αPax7 (C) antibodies. (A) In WT embryos, Pax6 is widely expressed in the ventricular zone of the spinal cord with the exception of the floor plate region (arrow). (B) In *Gli2*$^{-/-}$ mutants, Pax6 is detected in the ventral midline (arrow), in a region where the newly induced floor plate normally forms. (C) In *Gli2*$^{-/-}$ mutants, Pax7 expression (green) is only detected in dorsal regions of the ventricular zone, as in WT embryos at this stage (Jostes et al., 1990). Section shows the intermediate region of the lumbar spinal cord. (D,E) Pax6 expression at 10.5 dpc in WT and *Gli2*$^{-/-}$ mutants, showing that the abnormal ventral limit of Pax6 in the mutants is transient. Note the distance between the ventral limit of Pax6 expression appears similar in D and E, but the ventral boundary of Pax6 expression (top arrow) is closer to the ventral limit of the VZ (bottom arrow) in Gli2 mutants. (F,G) Pax7 (green) and En1 (red) expression at 10.5 dpc. Both proteins mark the position of distinct cell groups in both WT and *Gli2*$^{-/-}$ mutant spinal cords. (H,I) At 12.5 dpc, parvalbumin (parv) staining (green) detects ventrally located ventricular zone cells, which abut the floor plate (fp) in normal embryos. In *Gli2*$^{-/-}$ mutants, parvalbumin staining is detected in a similar region of the ventral VZ, but expression is also detected in the ventral midline. Section through mid-thoracic region. Spinal cord margins are outlined by white lines. Scale bar in C, 30 μm (for A), 50 μm for B,C; in G, 50 μm (for D-G); in I, 100 μm for H,I.

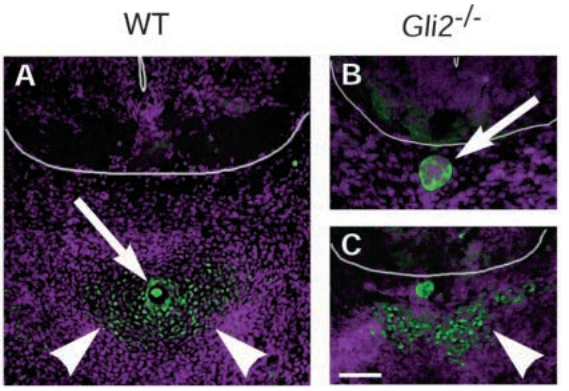

Fig. 7. The notochord does not regress in *Gli2*$^{-/-}$ mutants. (A-C) Transverse sections through lower thoracic spinal cords (at similar levels) stained with αS-100 antibodies. (A) At 12.5 dpc, the notochord (arrow) has moved ventrally away from the spinal cord and becomes surrounded by migrating sclerotomal cells (arrowheads). In *Gli2*$^{-/-}$ mutants (B), the notochord remains closely apposed to the ventral spinal cord at this stage (arrow). (C) In *Gli2*$^{-/-}$ mutants, sclerotomal cells (arrowhead), identified by their expression of S-100 at 12.5 dpc, can be seen migrating ventral to the abnormally positioned notochord. Outlines of the spinal cord and ventricular zone are shown by white lines. Dorsal is to the top. Scale bar, 50 μm for A, 20 μm for B, 25 μm for C.

One direct transcriptional target of Gli proteins is *Hnf*-3β, based on an enhancer analysis in mice (Sasaki et al., 1997). Our data are consistent with the suggestion that *Gli2* function is required for the *Shh*-dependent expression of *Hnf*-3β in the ventral midline of the mouse neural tube, which in turn may initiate *Shh* expression in the floor plate (Sasaki and Hogan, 1994). It has been demonstrated that *Hnf*-3β expression in the ventral midline of the CNS precedes, and is broader than, the expression of *Shh* and the appearance of the floor plate (Ruiz i Altaba et al., 1995). The failure to activate or sustain *Hnf*-3β expression in *Gli2* mutants may also provide an explanation for the reduction in VIR cells in *Gli2* mutants, which likely form within the *Hnf*-3β expression domain. Although the requirement for *Hnf*-3β in the generation of VIR cells has not been directly tested, the early loss of *Hnf*-3β correlates with a failure to induce these cells in *Gli2* mutants, suggesting that *Hnf*-3β may also be required for this process.

It has also been shown that Nkx-2.2 expression, and dopaminergic and serotonergic neuron differentiation, can be induced by Shh (Ericson et al., 1997; Yamada et al., 1991; Hynes et al., 1995). Therefore, the reduction in the number of these cells in *Gli2* mutants suggests either a direct requirement for *Gli2* in precursors to these cells, or alternatively that the floor plate is necessary for their normal development. In addition, the appearance of some cells expressing Nkx-2.2, 5HT and TH in *Gli2* mutants may be explained by the persistent proximity of the notochord in these embryos, which could provide enough Shh to induce their differentiation.

Our results are consistent with the suggestion that floor plate/VIR cell induction is distinct from motoneuron/interneuron induction, and are in agreement with previous in vitro experiments demonstrating the induction of motoneurons by *Shh* independent of floor plate formation (Tanabe et al., 1995; Marti et al., 1995a). Together, these data suggest two

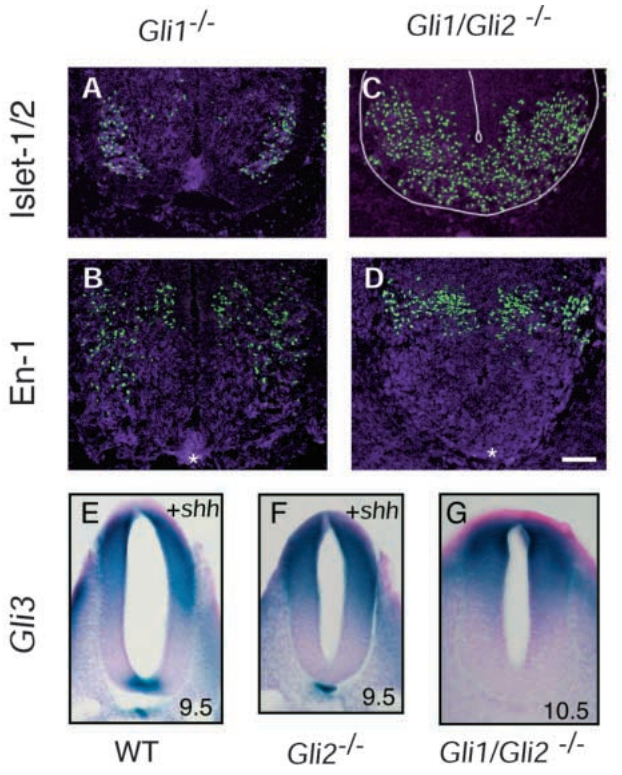

Fig. 8. *Gli1* mutants appear normal and *Gli1/Gli2* double mutants appear phenotypically similar to *Gli2* homozygotes. (A-D) Transverse sections through the lumbar spinal cord of a *Gli1*$^{-/-}$ (A,B) and *Gli1/Gli2* double homozygote (C,D) at 12.5 dpc stained with Islet-1/2 (A,C) or En (B,D) antibodies. The expression of Islet-1/2 and En1 in *Gli1*$^{-/-}$ mutants is similar to WT mice (compare with Figs 2F, 4F). *Gli1/Gli2* compound homozygotes show expression of Islet-1/2 in the ventral midline, similar to *Gli2*$^{-/-}$ embryos (compare with Fig. 2G). En1 interneurons differentiate in an appropriate d/v location in *Gli-1/Gli2* double homozygotes (compare with Figs 8B, 4F,G). (E-G) Transverse sections showing *Gli3* expression in *Gli2* (F) and *Gli1/Gli2* (G) double mutants. (E) In WT embryos at 9.5 dpc, *Gli3* expression is confined to the dorsal VZ. Embryo also stained with Shh. (F) In *Gli2* mutants at 9.5 dpc, *Gli3* expression is similarly confined to the dorsal VZ. Shh expression is only detected in the notochord of these embryos. (G) In *Gli1/Gli2* double-mutants, *Gli3* expression is similar to WT and *Gli2*$^{-/-}$ embryos. Outlines of the spinal cord and central canal are shown by white lines. Dorsal is to the top in all figures. Asterisks indicate ventral midline in B and D. Scale bar, 120 μm for A-D.

general explanations for the induction of ventral midline cell types by Shh (Fig. 9). In the first scenario, Shh signalling from the notochord can directly induce both floor plate and VIR cells in the midline, as well as motoneurons and ventral interneurons more dorsally/laterally. *Gli2* is required for floor plate/VIR induction but not for motoneurons/ventral interneurons. In the second scenario, Shh from the notochord first induces the floor plate as well as motoneurons/ventral interneurons, and then the floor plate induces VIR cells secondarily. In this latter case *Gli2* could be required only for floor plate induction but not floor-plate-dependent VIR cell induction. The role of *Gli2* in these two mechanisms is not necessarily different, since *Gli2* could be required for the induction of VIR cells in both scenarios, the only difference being the source of the Shh-inducing signal.

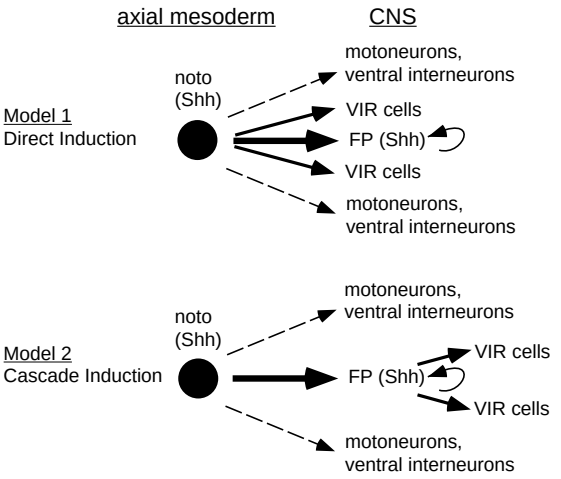

Fig. 9. Two possible mechanisms for the induction of ventral cell types by notochord-derived Shh. Model 1 proposes that the notochord coordinately induces floor plate, VIR and motoneuron/ventral interneuron cell differentiation. Arrow width indicates relative Shh levels required to induce these different cell classes, with the highest level (widest arrow) required for floor plate induction, and progressively lower amounts (thinner arrows) for VIR and motoneurons/ventral interneurons, respectively. VIR cells are defined by Nkx-2.2 expression in the absence of Islet-1/2, while some ventral motoneurons co-express these genes. In Model 2, the notochord induces floor plate and ventral motoneuron/interneuron cells, and the floor plate either on its own or in conjunction with the notochord induces VIR cells at intermediate Shh levels. In both models, solid arrows indicate *Gli2*-dependent (and *Gli1*-independent) pathways, while the broken arrows indicate *Gli*-independent pathways.

Normal *Gli1* and *Gli2* genes are required only for aspects of dorsoventral CNS patterning

There are a number of possible explanations for why the loss of *Gli2* leads to a failure to induce a floor plate and VIR cells in *Gli2*$^{-/-}$ homozygotes. It has been shown that Shh can induce distinct ventral cell fates at different concentration levels (Ericson et al., 1996). Thus, loss of *Gli2* could effectively lower the response to Shh signalling specifically at the midline, leading to the induction of cells that normally form at lower concentrations of Shh, such as motoneurons. However, we also found that the phenotype of compound *Gli1*/*Gli2* homozygous mutants was similar to *Gli2* homozygous mutants, at 12.5 dpc. These findings suggest that neither normal *Gli1* nor *Gli2* genes are necessary for patterning early cell differentiation along the d/v axis, dorsal to the ventral midline. Furthermore, the absence of detectable ventral *Gli3* expression in *Gli2* or *Gli1/Gli2* double homozygotes indicates that this Gli gene does not compensate for the loss of the more ventrally expressed *Gli1* and *Gli2* genes. As Shh has been shown to be both necessary and sufficient for the induction of most of the ventral cell types that we have studied (Chiang et al., 1996; Ericson et al., 1996), our results suggest the existence of a Gli-independent pathway downstream of Shh in the generation of ventral spinal cord neurons (represented by solid and broken arrows in Fig. 9).

Accumulating evidence suggests that there may be at least two distinct pathways downstream of Hh in both flies and

vertebrates, one acting through Ci/Gli and the other independent of these proteins. It has recently been reported that the activation of a motoneuron-specific enhancer element of the COUP-TFII gene by Shh can occur by a mechanism independent of *Gli* protein-binding activity (Krishnan et al., 1997). Similarly, in *Drosophila*, a *wingless (wg)* enhancer fragment has been isolated that drives wg expression in response to Hh in a pattern similar to normal gene expression, but this fragment does not possess Ci-binding sites (Lessing and Nusse, 1998). Our data does not, however, exclude the possibility that *Gli1* or *Gli2* mediate other, uncharacterized functions downstream of *Shh* in ventral CNS regions outside of the ventral midline. It is also possible that normally the two pathways downstream of Shh do not function independently, since *Ptc* and *Gli* are normally upregulated adjacent to the floor plate, but rather that they play redundant roles in some regions of the CNS.

It is also formally possible that *Gli* mRNA expression may not accurately reflect protein expression, since Gli protein expression has not yet been determined in higher vertebrates in vivo. It has been shown in *Drosophila* that ci activity is regulated post-translationally by a cytoplasmic complex that may be a target for Hh signalling (Aza-Blanc et al., 1997). It is therefore possible that regulation of Gli also occurs at the translational level, and that Gli1 and Gli2 proteins are not expressed dorsal to the ventral midline. For example, *Gli2* possesses DNA consensus sequences in its 3′ untranslated region that match those found in the closely related human *GLI* gene, which could mediate translational repression (Jan et al., 1997; B. B. and A. L. J., unpublished data). Thus Gli gene and protein function might be regulated at many different levels in the vertebrate CNS. Finally, it is possible that Gli1 and/or Gli2 proteins that do not contain a zinc finger domain are expressed in our Gli mutants, and that these abnormal proteins have some activity in response to Shh.

We have shown that while *Shh* and *Hnf*-3β expression are absent after 10.5 dpc throughout the spinal cord, hindbrain and midbrain of *Gli2*$^{-/-}$ mutants, their expression appears normal in the forebrain. This affected area corresponds to the region of the mouse CNS that overlies the notochord, but not anterior prechordal plate. In normal mouse embryos, *Shh* expression in the CNS extends from its initial point of induction in the midbrain, both caudally down the hindbrain/spinal cord and rostrally into the forebrain. It has recently been suggested that vertical signalling from the prechordal plate mesoderm to the overlying ventral forebrain involves cooperative signalling from both *Shh* and *BMP-7* to induce ventral differentiation (Dale et al., 1997). In contrast, in the midbrain, hindbrain and spinal cord, these two molecules are expressed in distinct d/v regions and are thought to act by competing mechanisms to establish d/v cell fates (Liem et al., 1995). Since we observed a delayed, but otherwise apparently normal, expression of *Shh* and *Hnf*-3β in the forebrain of *Gli2*$^{-/-}$ mutants, it is likely that different mechanisms downstream of axial mesoderm-derived Shh are involved in initiating *Shh* expression in the ventral forebrain versus midbrain/hindbrain/spinal cord.

Floor-plate-derived Shh signalling could have a limited role

While it has been shown in vitro that Shh acts in a concentration-dependent manner to induce distinct classes of ventral neurons in the spinal cord (Ericson et al., 1996), the relative contribution in vivo of notochord- and floor-plate-derived Shh to this process have not been distinguished. Our data suggests that *Shh* expressed in the floor plate may play a redundant role in providing d/v patterning signals during early ventral neurogenesis in the mouse. These results support the notion that early signals deriving from the notochord at neural plate and early neural tube stages are primarily responsible for setting up the basic early d/v pattern of cell differentiation in the developing spinal cord (Yamada et al., 1993).

A complicating factor in this interpretation is the persistent proximity of the notochord to the ventral spinal cord in *Gli2* mutants. This abnormal situation could allow for notochord-derived Shh to compensate for the loss of Shh from the floor plate in *Gli2* mutants after 10.5 dpc by providing an additional source of Shh. In addition, downregulation of *Ptc* mRNA in *Gli2* mutants indicates a perturbation of Shh signalling. This finding can be interpreted as indicating that Shh signalling is reduced, since activation of the Ptc receptor by Shh derepresses *Ptc* transcription (Ingham, 1994). Alternatively, lower *Ptc* mRNA levels in *Gli2* mutants would be expected to lead to a downregulation of the Ptc receptor. As Ptc function is inhibitory for Shh target genes, this could lead to a facilitation of the Shh signalling cascade in ventral cells. Since none of the *Gli* genes appear to be required for motoneuron/interneuron differentiation, this suggests any Shh signalling reflects Gli-independent elements of the pathway; i.e., motoneuron and ventral interneuron induction. Finally, we cannot rule out that, under normal circumstances, the floor plate provides an important source of Shh, which contributes to the ambient levels of Shh, and that a combination of factors in *Gli2* mutants allow notochord derived Shh to partially compensate for the loss of the floor plate and mask its required role in normal development.

Summary and conclusions

We have shown that *Gli2* is a required element in the *Shh*-dependent induction of the floor plate and most VIR cells, but not in the generation of motoneurons or ventral interneurons. Furthermore, neither *Gli1* nor *Gli3* expression appear to account for the development of ventrolateral neurons in *Gli2* mutants, suggesting a Gli-independent pathway downstream of Shh signalling in the ventral CNS of mice.

The failure to induce a floor plate in *Gli2* mutants appears to result in ventral midline cells differentiating into motoneurons. As floor plate cells are not terminally differentiated at early periods (9.5 to 12.5 dpc), it is possible that the induction of *Shh* expression in the floor plate normally serves an autocrine function to inhibit floor plate cell differentiation. In *Gli2* mutants, failure of this pathway appears to lead to ventral midline cells differentiating precociously into cell types appropriate for a more lateral and ventral location, motoneurons, instead of differentiating later, in a gliogenic environment. Thus, our results suggest that one function of *Shh* expression in the floor plate is to provide autocrine signalling to maintain its own expression and delay floor plate cell differentiation. These observations are consistent with recent data suggesting that diffusion of the N-terminal (signalling) component of Shh can be limited by its association with cholesterol on the surface of cells that secrete Shh (Porter et al., 1996). Finally, many later developmental processes, such

as axon guidance and cell migration, are likely to depend on the presence of ventral midline cells in the mouse CNS, and *Gli2* mutants provide a good opportunity to study their role in these events.

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