Novel Repellent Activity of Motor Neurons in Commissural Axon Guidance

BISC 494 Honors Thesis
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Abstract

During embryonic development, commissural axons, originating in the dorsal spinal cord, send axons ventrally towards the floor plate. The trajectory of these axons is mediated by both repulsive cues secreted from the roof plate, as well as attractive cues secreted from the floor plate. To investigate the guidance activity of the floor plate, we first attempted to examine the role of Sonic Hedgehog (Shh), a proposed chemoattractant in commissural axon guidance, by misexpressing Shh in chick embryos. While our misexpression experiments showed strong misexpression, we were unable to evaluate Shh’s role as a guidance cue due to drastic changes in spinal cord patterning. We then took a different approach and examined commissural axon trajectory in the absence of guidance cues from the floor plate. By analyzing the Gli2−/− mouse, which lacks a floor plate, we found that commissural axon trajectories become highly disorganized as they project through the medially and ventrally shifted population of motor neurons. Further investigation of the interaction between commissural axons and motor neurons revealed that commissural axons avoid areas rich in motor neurons and favor regions with few motor neurons as they navigate towards the ventral midline. Thus we propose motor neurons possess a repellent activity that facilitates the guidance of commissural axons in the ventral spinal cord.
Introduction

During embryonic development, neurons send out axons, which project toward their targets. As axons grow, they receive and interpret chemical signals in the environment known as guidance cues, which dictate exactly how to reach their target cells. Depending on the situation, these guidance cues can be either attractive or repulsive; for example, they can direct axons towards their target cells or away from an intermediate target. While several guidance cues have been identified, many of the specifics of how they operate have not been resolved.

One of the more intriguing guidance cues found in the embryonic spinal cord is the morphogen Sonic hedgehog (Shh). Shh has recently been discovered to play an important role in the guidance of the axons of a specific group of neurons called commissural neurons (Charron et al., 2003). These neurons originate in the dorsal spinal cord and send out axons that project ventrally towards the floor plate, then, after crossing the midline, turn to project rostrally towards the developing brain (Fig. 1). Before crossing the midline, commissural axons respond sequentially first to repulsive cues secreted by the roof plate in the dorsal spinal cord and then to attractive cues secreted by the floor plate in the ventral spinal cord, which results in dorsal to ventral axonal projection. After crossing the midline, commissural axons then respond to repulsive cues secreted by the floor plate, and then towards the brain.

Within this model system, Shh may function as both an attractive and repulsive guidance cue. In the dorsal-ventral axis, Shh is believed to be secreted in a concentration gradient from the floor plate. It has been shown that the dorsal-ventral gradient of Shh attracts commissural axons towards the floor plate (Charron et al., 2003). Another Shh gradient may also exist along the anterior-posterior axis. *In ovo* RNAi studies have shown that once the axons have crossed the midline, the effect of Shh on the axons reverses as the axons are repelled away from high levels of Shh and are thus directed towards the brain (Bourikas et al., 2005). However, this proposed dual nature of Shh as both a positive and a negative cue remains controversial, as it is not yet fully understood how to reconcile these two roles.

To help resolve this controversy, we first examined the effect of misexpressing Shh throughout the spinal cord of developing chick embryos so as to remove its normal graded presence. By electroporating chick embryos with a DNA construct that expresses Shh at various stages of development, we attempted to observe the effect on commissural axons when they encounter high concentrations of Shh. Also, by labeling various populations of cells within the spinal cord, we were able to examine the morphogenic effects of misexpressing Shh.
Another method of examining the effects of Shh on the guidance of commissural axons is through the analysis of \textit{Gli2}\(^{-/-}\) mutants. \textit{Gli2}, one of the three Gli genes that are expressed in the developing vertebrate spinal cord, is a zinc-finger containing transcription factor that is a downstream target of the Shh signaling pathway (Ruiz I Altaba, 1998). The \textit{Gli2}\(^{-/-}\) mutant provides an interesting system in which to study the effects of chemoattractants secreted by the floor plate because \textit{Gli2}\(^{-/-}\) embryos lack a floor plate and the adjacent, ventral-most interneurons (Matise et al., 1998). Surprisingly, it was found that although \textit{Gli2}\(^{-/-}\) mutants lack a floor plate, commissural axons are still able to reach the ventral midline (Matise et al., 1999). These results suggested that the floor plate, and subsequently the chemoattractants it secretes, is not required for the guidance of commissural axons to the midline. Further analysis of the \textit{Gli2}\(^{-/-}\) mutant showed that although commissural axons reach the ventral midline, their trajectories are defasiculated and perturbed (Charron, et al., 2003).

To further investigate these previous findings, we also analyzed \textit{Gli2}\(^{-/-}\) mice. By labeling the commissural axons in spinal cords of E11.5 and E12.5 mice, we were able to analyze the trajectories of commissural axons. Our analysis of the \textit{Gli2}\(^{-/-}\) mutant revealed that although commissural axons reach the ventral midline, their trajectories become disrupted as they project through the medially and ventrally shifted population of motor neurons. Further investigation of the interaction between commissural axons and motor neurons revealed that commissural axons avoid areas rich in motor neurons and favor regions with few motor neurons as they navigate towards the ventral midline. Thus we propose that motor neurons possess a repellent activity that facilitates the guidance of commissural axons in the ventral spinal cord.

\textbf{Results}

\textit{Shh misexpression alters spinal cord patterning \textit{in vivo}}

To examine whether Shh misexpression has an effect on commissural axons during spinal cord development, we electroporated chick embryos at stages ranging from Stage 13 to Stage 17 after they were injected with the Shh expressing DNA construct Vhh PMT21 tagged with the CMV-GFP viral enhancer. Nineteen embryos were successfully electroporated, indicated by the expression of GFP in one half of the spinal cord, but only one embryo showed visible Shh misexpression, which was electroporated at Stage 13 and dissected at Stage 22 (Fig. 3). It is unclear why we saw strong misexpression in only one embryo.

To visualize Shh expression, we stained spinal cord sections with an antibody against the Shh protein. In the spinal cord, Shh is secreted by the floor plate, and then diffuses dorsally to produce a concentration gradient. Staining for the Shh protein in wild type embryos reflects this expression pattern, as Shh is highly concentrated at the floor plate and then diminishes in concentration at more
dorsal levels (Fig. 2A). In the electroporated embryo however, Shh is expressed in a high concentration along the entire dorsal-ventral axis on the electroporated half of the spinal cord (Fig. 3A, 3K).

To assess whether this dramatic Shh misexpression has an effect on the trajectory of commissural axons, we performed immunohistochemistry to examine the expression of Axonin1, which is a marker of commissural axons. In wild type embryos, axons of commissural neurons in the dorsal spinal cord project towards the floor plate at the ventral midline (Fig. 2B). Whereas normal commissural axon trajectory is present in the non-electroporated half of the electroporated embryo, there are no visible Axonin1 positive axons in the electroporated half (Fig. 3B, 3L).

To investigate why Axonin-1 positive axons are absent in the electroporated half of the spinal cord, we performed immunohistochemistry to examine the expression of pLh2, a marker of post-mitotic commissural neurons. In wild type embryos, pLh2 expression is localized to the small population of post-mitotic commissural neurons in the dorsal spinal cord (Fig. 2C). In the electroporated half, there are no pLh2 positive cells (Fig. 3C, 3M). The absence of Axonin-1 positive axons can therefore be attributed to the absence of commissural neurons. This result suggests that the dramatic Shh misexpression has altered the patterning of the electroporated half of the spinal cord.

To further investigate the morphogenic effects of Shh misexpression, we performed immunohistochemistry to examine the expression of pIsl, a marker of motor neurons, and Nkx2.2, a marker of V3 interneurons. In wild type embryos, pIsl expression is localized to a small population of sensory neurons in the dorsal spinal cord and a larger cluster of motor neurons in the ventral spinal cord (Fig. 2D), and Nkx2.2 expression is localized to the interneurons that flank the floor plate (Fig. 2E). In the electroporated embryo, there is a dramatic expansion of both of these populations of neurons, as expression for both markers is present throughout the entire dorsal-ventral axis of the spinal cord (Fig. 3D, 3N, 3E, 3O).

**Gli2**^{-} mutants lack V3 interneurons

To examine spinal cord patterning of **Gli2**^{-} mutants, we first performed immunohistochemistry to examine the expression of pLh2, a marker for post-mitotic commissural neurons, and Nkx2.2, a marker for V3 interneurons, in spinal cord sections from E11.5 mouse embryos. As in chick, pLh2 expression in wild-type mouse is localized to the population of post-mitotic commissural neurons in the dorsal spinal cord (Fig. 4A, 4C). We found a similar pLh2 expression in **Gli2**^{-} mutant embryos (Fig. 4E, 4G). In wild type embryos, Nkx2.2 expression is localized to the interneurons that flank the floor plate (Fig. 4A, 4D). However in **Gli2**^{-} mutants, the V3 interneurons are absent (Fig. 4E, 4H). This result shows that in the absence of **Gli2**, ventral patterning is altered as previously described ((Matise et al., 1998).
Rostral-caudal analysis of perturbed commissural axon trajectory in Gli2\(^{-/-}\) mutants

To analyze the trajectory of commissural axons in Gli2\(^{-/-}\) mutants, we performed a series of immunohistochemistry experiments to examine the expression of Tag1, a marker for commissural axons. In wild-type embryos, Tag1 positive axons project from the dorsal spinal cord along the lateral edges of the spinal cord and once they reach the ventral spinal cord, they become highly fasiculated as they project medially towards the floor plate (Fig. 5M-5O). Furthermore, this pattern of trajectory is conserved along the rostral-caudal axis. In Gli2\(^{-/-}\) mutants, the trajectory of commissural axons is perturbed. While the trajectory of commissural axons of Gli2\(^{-/-}\) mutants is similar to wild-type in the dorsal spinal cord, once they reach the ventral region of the spinal cord and begin to turn towards the ventral midline, they become highly defasiculated and disorganized (Fig. 5P-5R, and at higher magnification, Fig. 5S-5U). Furthermore, while some axons appear to reach the ventral midline, many seem to be reaching the ventral most boundary of the spinal cord at positions lateral to the ventral midline. Rostral-caudal analysis of this phenotype revealed that the trajectory of commissural axons is most perturbed at more rostral levels. This result suggests that the phenotype becomes more pronounced as the embryo develops.

Commissural axons trajectory becomes disturbed as axons project through motor neuron population in Gli2\(^{-/-}\) mutants

To further examine ventral spinal cord patterning in both E11.5 and E12.5 Gli2\(^{-/-}\) mutants, we also examined the expression of pIsl, a marker for motor neurons. As in chick, pIsl expression in wild-type mouse is localized to a small population of sensory neurons in the dorsal spinal cord, and a larger population of motor neurons in the lateral regions of the ventral spinal cord (Fig. 6C, 6I). In Gli2\(^{-/-}\) mutants however, the ventral population of motor neurons is shifted ventrally and medially (Fig. 6F, 6L).

To visualize how the commissural axon trajectory in Gli2\(^{-/-}\) mutants correlates with this spatially shifted population of motor neurons, we again labeled axons with Tag1. In wild type embryos, once commissural axons reach the ventral spinal cord, they turn in towards the ventral midline and project in a highly fasiculated manner towards the floor plate, while cleanly circumventing the ventral population of motor neurons (Fig. 6A, 6G). In Gli2\(^{-/-}\) mutants, Tag1 positive axons remain fasiculated in the dorsal spinal cord. However, Tag1 positive axons become highly disorganized commissural axons once they reach the level of the shifted population of motor neurons (Fig. 6D, 6J). This result suggests there is an interaction between commissural axons and motor neurons in Gli2\(^{-/-}\) mutants.

Commissural axons avoid motor neurons while projecting to the ventral midline in Gli2\(^{-/-}\) mutants
To further determine the spatial relationship between commissural axons and motor neurons in \( \text{Gli}2^{-/-} \) mutants, we performed open book preparations, in which we dissected out the spinal cords of E 10.5 \( \text{Gli}2^{-/-} \) mutants and cut along the roof plate so that the spinal cord could be opened up and flattened out. In wild type explants, the population of motor neurons, visualized by pIsl positive cells, is localized to areas dorsal to the ventral midline (Fig. 7E). Commissural axon trajectory towards the floor plate is very organized, with all axons are more or less perpendicular to the horizontal axis (Fig. 7C). However, in \( \text{Gli}2^{-/-} \) mutants, the population of motor neurons is shifted towards the ventral midline and forms one continuous population (Fig. 7F). The commissural axon trajectory towards the ventral midline is much less perpendicular to the ventral midline. Moreover, as they navigate their way through the ventral spinal cord, \( \text{Gli}2^{-/-} \) commissural axons project to and cross the midline in areas with the fewest motor neurons (Fig. 7D). This result shows commissural axons avoid motor neuron-rich areas, suggesting motor neurons possess a repellent activity.

**Discussion**

**Challenges of the electroporation assay**

During development, secreted diffusible molecules such as Shh are involved in the patterning of the embryonic spinal cord. These molecules, known as morphogens, are present in a gradient and specify spatial patterning by inducing different cell fates at different concentrations. It has been shown that Shh is responsible for patterning the ventral spinal cord, for cells exposed to high concentrations of Shh adopt more ventral fates, and cells exposed to lower concentrations of Shh adopt more dorsal fates (Marti et al., 1995).

Our results confirm the morphogenic role of Shh in the developing spinal cord. The large expansion of motor neurons and V3 interneurons in the electroporated half of the spinal cord show that the misexpression of Shh throughout the dorsal-ventral axis was sufficient to induce ventral cell fates in even the most dorsal regions. Due to the absence of commissural neurons, and subsequent absence of commissural axons, in the electroporated half of the embryo, we were unable to examine the effect of Shh misexpression on commissural axon trajectory.

This assay proved to be very challenging as a means to evaluate Shh’s role as an axon guidance cue. Since Shh has a role in patterning the spinal cord as well as guiding commissural axons, inducing Shh misexpression though electroporation must be performed at a temporally opportune period in development so as to induce Shh misexpression early enough to evaluate Shh’s role as a chemoattractant, yet late enough to avoid inducing morphogenic effects. Exactly when that window of opportunity during development is, provided
it exists, remains elusive. However, since other successfully electroporated stage 13 embryos did not show Shh misexpression, this suggests that there may be other complications with this assay as well.

Re-examination of the \textit{Gli2}⁻/⁻ mutant commissural axon phenotype

In regard to spinal cord patterning, our results are consistent the previous description of \textit{Gli2}⁻/⁻ mutant Matise et al. (1998), with the results of our immunohistochemistry experiments show that \textit{Gli2}⁻/⁻ mutants have normal dorsal spinal cord patterning but possess abnormal ventral spinal cord patterning. By staining for Nkx2.2 and pIs1, we show that \textit{Gli2}⁻/⁻ mutants lack V3 interneurons and have a ventrally and medially shifted population of ventral motor neurons.

In regard to commissural axon trajectory, our results suggest the \textit{Gli2}⁻/⁻ mutant phenotype is more dramatic than what was described by Charron et al. (2005.) By labeling commissural axons of E 11.5 and E 12.5 embryos with Tag1, we observed that commissural axons become highly defasiculated and disorganized once they reach the ventral spinal cord. While some axons appear to reach the ventral midline, many seem to reach the ventral most boundary of the spinal cord at positions lateral to the midline. This observation raises interesting questions regarding the nature of the signals that mediate commissural axon crossing at the ventral spinal cord in \textit{Gli2}⁻/⁻ mutants. Further experiments examining post-commissural axons in the \textit{Gli2}⁻/⁻ mutant would provide insight as to how commissural axons project once they reach the ventral spinal cord.

Furthermore, our analysis of the commissural axon trajectory in \textit{Gli2}⁻/⁻ mutants along the rostral-caudal axis shows that the phenotype is more pronounced at rostral levels than at caudal levels. Since caudal regions of the spinal cord develop later than more rostral regions, examining the phenotype along the rostral-caudal axis provides us with a measure for how the phenotype progresses during development. Our results show that the phenotype is less severe in more caudal regions, with most of the axons reaching the ventral midline. However, at more rostral levels, the phenotype is more dramatic, as axons are highly defasiculated and many do not appear to reach the ventral midline. This suggests the phenotype becomes more pronounced as the embryo develops.

In the absence of diffusible chemoattractants secreted by the floor plate, commissural axons are still able to reach the ventral midline in \textit{Gli2}⁻/⁻ mutants. However, their trajectories are highly defasiculated and disorganized in the ventral spinal cord. \textit{Gli2}⁻/⁻ mutant commissural axons maintain a projection pathway similar to wild type commissural axons in the spinal cord region dorsal to the motor neuron population. However, it is once commissural axons of \textit{Gli2}⁻/⁻ mutants reach the population of medially and ventrally shifted motor neurons that they become highly defasiculated and disorganized. This observation spurred us to further investigate the spatial relationship between commissural axons and motor neurons. From our explant studies, we found that as commissural axons
of \(Gli2^{-/-}\) mutants navigate their way through the ventral spinal cord, they project to and cross the midline in areas with the fewest motor neurons.

The results from our \(Gli2^{-/-}\) mutant mouse studies suggest that motor neurons in the ventral spinal cord provide a novel source of guidance information for commissural axons. In wild type embryos, motor neurons are localized to two lateral clusters in the ventral spinal cord. As wild type commissural axons enter the ventral spinal cord, they turn away from the lateral edges of the spinal cord and project more medially towards the floor plate. Taking into consideration the wild type commissural axon pathway and wild type motor neuron patterning, the repellent activity of motor neurons, as suggested by our \(Gli2^{-/-}\) mutant studies, could be guiding commissural axons to turn as they enter the ventral spinal cord. Thus we propose a model in which the attractive activity of the floor plate and the repellent activity of the motor neurons both mediate commissural axon guidance in the ventral spinal cord (Fig. 8).

Methods

Electroporation

Fertilized chick embryos were incubated at 37°C for 48-60 hours to yield embryos at stages ranging from stage 13 to stage 17. A solution of 4 µl Vhh PMT21 DNA construct, 1 µl CMV-GFP, and 1 µl Fast Green was injected into the spinal cord using a mouth pipette. Embryos were electroporated at 30 volts with the electrodes placed on either side of the spinal cord. A few drops of an antibiotic solution (1% PSG in PBS) was added to each embryo before being covered with Parafilm and incubated at 37°C for an additional 48 hours.

Dissection of Chick Embryos and Preparation of Slides

After incubation, the healthy embryos were dissected out and fixed in 5 ml 4% PFA for 3 hours at 4°C. Embryos were then washed three times in 5 ml 1X PBS for 30 min each and the placed in 5 ml 30% sucrose overnight at 4°C. Successfully electroporated embryos were then embedded in OCT medium and kept at -80°C. Transverse, serial, 30 µm thick sections were made using a Cryostat and the sections were mounted onto glass slides.

Immunohistochemistry for Chick

Slides were hydrated with 1 ml PBTN for 20min. Primary antibodies and dilutions used were as follows: \(\alpha\)-Shh at 1:100, \(\alpha\)-Axonin-1 at 1:2000, \(\alpha\)-pIsl at 1:2000, \(\alpha\)-pLh2 at 1:2000, and \(\alpha\)-Nkx2.2 at 1:100. Slides were incubated with primary antibodies overnight at 4°C. Slides were then washed three times with 1 ml
PBTN for 5 min each at room temperature. The secondary antibodies and dilutions used were as follows: for α-Shh, α-Axonin-1, α-pIsl, and α-pLh2, GαRbCy3 was used at a 1:500 dilution and for α-Nkx2.2, GαMCy3 was used at a 1:500. Slides were incubated with secondary antibodies for two hours at room temperature, and then were washed three times with 1 ml PBTN for 5 min each. Slides were then coverslipped using Vectashield or Prolactin Gold and kept at -20°C.

**Mice, Dissection of Mouse Embryos and Preparation of Slides**

Gli2+/- mice were crossed to produce Gli2-/− mutants. Embryos were dissected out at either E 11.5 or E 12.5 and fixed in 5 ml 4% PFA for 3 hours at 4°C. Embryos were then washed three times in 5 ml 1X PBS for 30 min each and then placed in 5ml 30% sucrose overnight at 4°C. Embryos were then embedded in OCT medium and kept at -80°C. Transverse, serial, 30 µm thick sections were made using a Cryostat and the sections were mounted onto glass slides.

**Genotyping**

Embryonic head tissue was used for genotyping. 500 µl Tail Buffer Solution and 5 µl Proteinase K were added to each tissue sample and samples were then incubated overnight in a Thermomix set at 55°C and max agitation. After 500 µl phenol/ chloroform was added to each sample, then samples were vortexed and centrifuged at 14,000 rmp for 2 min. The supernatant from each sample was then transferred to a new tube and 400 µl chloroform was added, followed by vortexing and centrifuging. The supernatant from each sample was then transferred to a new tube and 600 µl 100% ethanol was added. The samples were gently shaken and then the precipitated DNA was scooped out and placed into a new tube containing 100 µl TE solution. Samples were then prepared for PCR by creating the following mixture per 1 µl DNA sample: 1 µl Taq Polymerase Buffer, 0.5 µl dNTPs, 0.2 µl Gli2 Neo primer, 0.2 µl Gli2 S primer, 0.2 µl Gli AS primer, and 8.3 µl dd water. PCR for the samples was carried out using the Hot start Gli2 Program. After first denaturizing step, the following Taq enzyme mixture was added to each sample: 1 µl Taq Polymerase Buffer, 0.2 µl Taq enzyme and 7.4 µl dd water. The resulting PCR products were then run on a 2% agarose gel at 90 volts for 40 min. The result bands were compared to wt control and heterozygous control.

**Immunohistochemistry for Mouse**

Slides were hydrated with 1 ml PBTN for 20min. Primary antibodies and dilutions used were as follows: α-Tag1 at 1:6, α-pLh2 at 1:2000, α-pIsl at 1:2000, and α-Nkx2.2 at 1:100. Slides were incubated with primary antibodies overnight at 4°C. Slides were then washed three times with 1 ml PBTN for 5 min each at
room temperature. The secondary antibodies and dilutions used were as follows: for α-Tag1, GdMFitc was used at 1:100, for α-pLh2, GdRbCy3 was used at 1:500, for α-pIsl, GdRbCy3 was used at 1:500, and for α-Nkx2.2, GdMCy5 was used at 1:400. Slides were incubated with secondary antibodies for two hours at room temperature, and then were washed three times with 1 ml PBTN for 5 min each. Slides were then coverslipped using Vectashield or Prolactin Gold and kept at -20°C.

Open Book Preparations

Embryos from Gli2+/- crosses were dissected out at E10.5. For each embryo, the spinal cord was further dissected out. The spinal cord was then bisected at the roof plate so that the spinal cord was flattened out with the ventral most region being at the center of the explant. Explants were then embedded in collagen, fixed and prepared for immunohistochemical analysis. Embryonic head tissue was used for genotyping as described above.
References


Commissural (C) neurons differentiate adjacent to the roof plate (RP) in the dorsal spinal cord. Post-mitotic commissural neurons can be visualized by staining for pLh2. During spinal cord development, commissural neurons project axons, which can be visualized by staining for Tag1. Commissural axons are directed into a dorsal to ventral circumferential route through the spinal cord by diffusible chemorepellent cues secreted by the roof plate and by chemoattractants secreted by the floor plate (FP). The repellent activity is mediated by two Bone Morphogenic Proteins (BMPs), BMP7 and Growth Differentiation Factor (GDF) 7 while Netrin and Sonic Hedgehog (Shh) attract commissural axons toward the floor plate. Once commissural axons cross the ventral midline at the floor plate, they turn to project rostrally towards the brain.
Figure 2 Shh is secreted by the floor plate and diffuses dorsally.

(A) Shh (Shh) is secreted by the floor plate and diffuses dorsally in chick spinal cords.
(B) Commissural axons (axonin) project from the dorsal spinal cord to the ventral midline.
(C) Post-mitotic commissural neurons (pLH2) originate in the dorsal spinal cord.
(D) A Small population of sensory neurons (pIsl) resides in the dorsal spinal cord and large population of motor neurons (pIsl) resides in the ventral spinal cord.
(E) V3 interneurons (Nkx2.2) flank the floor plate.
**Figure 3** Shh misexpression causes dramatic changes to spinal cord patterning.

(A-E) Overlay of GFP with other spinal cord markers.

(F-J) Strong electroporation (GFP) is visible in one half of the spinal cord.

(K) Shh (Shh) is misexpressed along the entire dorsal-ventral axis of the electroporated half of the spinal cord.

(L) Commissural axons (axonin) are also absent in the electroporated half of the spinal cord.

(M) Post-mitotic commissural neurons (pLH2) are absent in the electroporated half of the spinal cord.

(N) Motor neuron population (pIsL) expands the entire dorsal-ventral axis of the electroporated half of the spinal cord.

(E) V3 interneurons (Nkx2.2) also expands the entire dorsal-ventral axis of the electroporated half of the spinal cord.
Figure 4 *Gli2*<sup>−/−</sup> mutants have altered commissural axon trajectory and spinal cord patterning.

(A and E) Overlay of Tag1, pLh2 Nkx2.2 in wild type (A) and E11.5 *Gli2*<sup>−/−</sup> mutant spinal cords (E).

(B and F) Commissural axons (Tag1) are follow stereotyped trajectory in wild type (B) and show disrupted trajectory in *Gli2*<sup>−/−</sup> mutants (F).

(C and G) Post-mitotic commissural neurons (pLh2) originate in the dorsal spinal cord and migrate ventrally in wild type (C) and *Gli2*<sup>−/−</sup> mutants (G).

(D and H) V3 interneurons (Nkx2.2) flank the floor plate in wild type (D) and are absent in *Gli2*<sup>−/−</sup> mutants (H).
**Figure 5** *Gli2*<sup>−/−</sup> axon phenotype is more pronounced at rostral levels.

(A-C) Overlay of Tag1 and pLh2 in E11.5 wild type spinal cords.
(G-I) Post-mitotic commissural neurons (pLh2) originate in the dorsal spinal cord and migrate ventrally E11.5 wild type spinal cords.
(M-N) Commissural axons (Tag1) remain fasciculated as they project from the dorsal spinal cord to the floor plate all along the rostral-caudal axis E11.5 wild type spinal cords.
(D-F) Overlay of Tag1 and pLh2 in E11.5 *Gli2*<sup>−/−</sup> mutant spinal cords.
(J-L) (G-I) Post-mitotic commissural neurons (pLh2) originate in the dorsal spinal cord and migrate ventrally in E11.5 *Gli2*<sup>−/−</sup> mutant spinal cords.
(P-R) Commissural axons (Tag1) become defasciculated as they project into the ventral spinal cord and the phenotype is more pronounced rostrally in E11.5 *Gli2*<sup>−/−</sup> mutant spinal cords.
Figure 6 Commissural axon trajectory becomes disturbed as axons project through motor neuron population in Gli2−/− mutants.

(A-J) Overlay of Tag1 and pIsL in wild type and Gli2−/− mutant spinal cords.

(B and H) Commissural axons (Tag1) remain fasiculated in wild type E11.5 (B) and E12.5 (H) spinal cords.

(C and I) Ventral motor neurons (pLh2) are localized to the lateral edges of the ventral region in wild type E11.5 (C) and E12.5 (I) spinal cords.

(E and K) Commissural axons (Tag1) become defasiculated as they project into the ventral region of E11.5 Gli2−/− (B) and E12.5 Gli2−/− (H) spinal cords.

(F and L) Motor neuron population is shifted medially and ventrally in E11.5 Gli2−/− (F) and E12.5 Gli2−/− (B) spinal cords.
**Figure 7** Commissural axons avoid motor neurons while projecting to the ventral midline in *Gli2* mutant mutants.

(A and B) Overlay of Tag1 and plsl in E10.5 wild type (A) and E 10.5 *Gli2* mutant (B) spinal cord open book preps.

(C) Commissural axons (Tag1) project perpendicularly to the ventral midline in E10.5 wild type explants.

(E) Motor neurons (plsl) are localized to region dorsal to the ventral midline in E10.5 wild type explants.

(D) Commissural axons (Tag1) project non-linearly to areas with fewest motor neurons (plsl in F) at the ventral midline in E 10.5 *Gli2* explants.

(F) Motor neurons (plsl) are shifted ventrally towards the ventral midline in E 10.5 *Gli2* explants.
Figure 8 Motor neurons repel commissural axons in the ventral spinal cord.

Commissural axons (C) are directed into a dorsal to ventral circumferential route through the spinal cord by diffusible chemorepellent cues secreted by the roof plate and by chemoattractants secreted by the floor plate (FP). In addition, we propose that repellent cues from the motor neurons (MNs) cause commissural axons to turn away from the lateral edges and towards the ventral midline of the spinal cord.