Zic2-Dependent Axon Midline Avoidance Controls the Formation of Major Ipsilateral Tracts in the CNS

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SUMMARY

In bilaterally symmetric organisms, interhemispheric communication is essential for sensory processing and motor coordination. The mechanisms that govern axon midline crossing during development have been well studied, particularly at the spinal cord. However, the molecular program that determines axonal ipsilaterality remains poorly understood. Here, we demonstrate that ipsilateral neurons whose axons grow in close proximity to the midline, such as the ascending dorsospinal tracts and the rostromedial thalamocortical projection, avoid midline crossing because they transiently activate the transcription factor Zic2. In contrast, uncrossed neurons whose axons never approach the midline control axonal laterality by Zic2-independent mechanisms. Zic2 induces EphA4 expression in dorsospinal neurons to prevent midline crossing while Robo3 is downregulated to ensure that axons enter the dorsal tracts instead of growing ventrally. Together with previous reports, our data reveal a critical role for Zic2 as a determinant of axon midline avoidance in the CNS across species and pathways.

INTRODUCTION

The bilaterally symmetrical organization of the nervous system in vertebrates requires routes of communication between the two brain hemispheres. Contralateral (commissural or crossed) and ipsilateral (or uncrossed) tracts distribute sensory information coming from both sides of the body to integrate it in the main processing centers of the brain and generate coordinated motor responses (Burgess et al., 2009; Jen et al., 2004; Kullander et al., 2003; Sperry, 1982).

The mammalian visual system is a classic and particularly well-understood example of bilateral wiring. Optic nerve fibers from each eye segregate at the optic chiasm to either cross or avoid the midline, and this pattern of axonal divergence is critical for allowing binocular vision. Guidance molecules that partici-

pate in axonal navigation at the chiasmatic midline include Slit proteins and their receptors, the Robos (Erskine et al., 2000; Fricke et al., 2001; Hutson and Chien, 2002; Plump et al., 2002), vascular endothelial growth factor that interacts with the Neuropilin1 receptor (Erskine et al., 2011), and the tripartite molecular complex formed by Sema6D/NrCam and Plexin-A1 (Kuwajima et al., 2012; Williams et al., 2006). The repulsive response of the ipsilateral axons to the chiasm midline is mediated, at least in part, by the guidance receptor EphB1 and its ligand EphrinB2 (García-Frigola et al., 2008; Petros et al., 2009; Williams et al., 2003). In the spinal cord, another well-studied midline-crossing model, the signaling mediated by these molecules also modulates the responsiveness of commissural axons to midline cells in the floor plate (Brose et al., 1999; Chen et al., 2008; Nawabi et al., 2010; Ruiz de Almodovar et al., 2011; Wang et al., 1999), demonstrating that many of the guidance cues involved in axon midline navigation play analogous functions in different parts of the CNS.

Regarding the regulatory mechanisms that specify the crossed versus the uncrossed subpopulations, the LIM homeodomain transcription factor Islet2 (Isl2) has been associated with contralateral features in the retina (Pak et al., 2004), while the zinc finger transcription factor Zic2 is the major determinant of ipsilateral identity (Herrera et al., 2003). In the spinal cord, the generation, distribution, and specification of ipsilateral and contralateral-projecting neurons is not as obvious as in the retina. Spinal dorsal horns neurons are generated in two phases. In the early phase, six types of neurons are born, including relay neurons (dl1–dl3) that project to either ipsilateral or contralateral targets (Gross et al., 2002; Jessell, 2000; Müller et al., 2002). Late-born cells (dIL neurons), generated in a second round of differentiation, comprise inhibitory (dIL$_A$) and excitatory (dIL$_B$) cell types and will populate the superficial dorsal horns (Altman, 1984; Gross et al., 2002; Müller et al., 2002). dIL$_B$ neurons extend axons ipsilaterally within the dorsal fascicle (DF) and the dorsal-lateral fasciculus (DLF) to ascend longitudinally to the brain (Gross et al., 2001, 2002; Szentagothai, 1964). So far, only the Lim homeodomain transcription factors Lhx2/9, through the control of the Robo3 receptor, have been directly involved in axon midline crossing during spinal cord development (Ding et al., 2012; Wilson et al., 2008), and it is unclear whether Isl2 or Zic2, the factors determining axonal laterality in the visual system, are implicated in the bilateral spinal cord wiring.

Our knowledge of the regulatory mechanisms specifying the axonal laterality of thalamic neurons is equally limited. Thalamic nuclei symmetrically distributed at both sides of the midline receive sensory and motor information that is subsequently relayed to the ipsilateral cortex through the thalamocortical (TC) projections. However, the mechanism that directs midline avoidance and consequently the projection to the ipsilateral cortical hemisphere of TC neurons is still unknown.

In an attempt to clarify to what extent different bilateral pathways share regulatory mechanisms that govern axon midline decisions, we investigated the function of Zic2 in the formation of major ipsilateral pathways: the ascending spinofugal tracts and the thalamocortical projection. We demonstrate that in both systems, Zic2 is transiently and postmitotically expressed in neurons whose axons approach the midline but then turn to project ipsilaterally. Downregulation of Zic2 in the dorsal spinal cord and in thalamic neurons elicits aberrant midline crossing, whereas ectopic expression of Zic2 in spinal commissural interneurons switches axonal laterality and represses the expression of genes that promote crossing (Lhx2 and Robo3). Functional experiments in vivo demonstrate that Zic2 controls the expression of the guidance receptor EphA4 in a subset of dorsal spinal neurons. Furthermore, we show that Zic2 activity is dispensable in other ipsilaterally projecting neurons whose axons never approach the midline such as the ipsilateral interneurons from the dI1 spinal domain (dI1i) or the caudolateral TC (cITC) neurons. Together, our results reveal a Zic2-dependent mechanism, shared by different neural populations, essential to prevent midline crossing during the development of the nervous system and highlight the generality of regulatory programs controlling bilateral wiring in the CNS.

RESULTS

Zic2 Is Transiently Expressed in Postmitotic Dorsal Horn Neurons during Development

To investigate a possible role of Zic2 in axon guidance in the spinal cord, we first analyzed its spatiotemporal expression pattern in the developing spinal cord of wild-type mice. As previously reported (Nagai et al., 1997), we detected high levels of Zic2 expression in the ventricular zone of the dorsal neural tube from embryonic day 10 (E10) to E12 (Figures 1A and 1B). In addition, we found that from E13 to birth, Zic2 is expressed in two populations of cells at both sides of the dorsal midline (Figures 1C–1F). This pattern was maintained until early postnatal stages when the spinal expression of Zic2 was downregulated.

Using different markers for progenitors (Sox2), postmitotic cells (HuCD), and differentiated neurons (NeuN), we observed that Zic2 is first expressed in the dorsal progenitors and then becomes restricted to a subpopulation of postmitotic neurons (Figures 1G–1J). Bromodeoxyuridine (BrdU) uptake experiments showed that the vast majority of Zic2 postmitotic spinal cord neurons are born at E12 (Figures 1K and 1L; Figure S1 available online).

Zic2+ Postmitotic Spinal Neurons Become dIL$_B$ Projection Neurons

To characterize the origin, fate, and projection pattern of Zic2+ neurons, we used a Zic2 reporter mouse line referred to as Tg(Zic2^{EGFP}). We confirmed that enhanced green fluorescent protein (EGFP) expression faithfully reproduced the endogenous expression of Zic2 in different tissues of these mice, including the spinal cord (Figures 2A–2D; Figure S2). Hence, EGFP expression can be used as a transient fate-map reporter for Zic2 expression. Zic2/EGFP double-labeled cells were located in the proximity of the dorsal midline, whereas Zic2-derived dorsal horn neurons, which no longer expressed Zic2 but maintained EGFP expression (Zic2$^-$/EGFP$^+$), populated more lateral areas in the dorsal horns (Figures 2A–2D). We also found that Zic2/EGFP expression is preserved across the rostrocaudal extent of the spinal cord (data not shown).

dIL neurons are specified by the expression of Lbx1 (Gross et al., 2002; Müller et al., 2002). Immunostaining for Lbx1 in transverse spinal cord sections of Tg(Zic2^{EGFP}) embryos confirmed that postmitotic Zic2/EGFP neurons are dIL neurons (Figure 2E), an expected result based on their late birthdate. The dIL$_A$ neurons are GABAergic interneurons that express the transcription factor Pax2, whereas the dIL$_B$ cells are glutamatergic/projection neurons defined by the expression of Tlx3 and Lmx1b (Cheng et al., 2005; Gross et al., 2002; Mizuguchi et al., 2006). From E13 to postnatal day 0 (P0), most Zic2/EGFP cells were positive for Tlx3 and Lmx1b but negative for Pax2 (Figures 2F–2H), indicating that glutamatergic, but not GABAergic, neurons expressed Zic2 (Figure 2I). In fact, 91.15% ± 0.85% of the Lmx1b neurons were also positive for EGFP, suggesting that almost the entire population of dIL$_B$ neurons expressed Zic2 at some point.

To further confirm the relationship between Zic2 and glutamatergic fate, we artificially increased the number of GABAergic neurons in the chick spinal cord and tested the expression of Zic2. We first corroborated that expression of Zic2 in the chick spinal cord was similar to mouse Zic2 expression (Figures S3A–S3E). Next, E2 chick embryos were electroporated with plasmids encoding Ptf1a, a transcription factor that determines GABAergic fate during the formation of the dorsal horns (Glasgow et al., 2005; Mizuguchi et al., 2006). As expected, both glutamatergic markers and Zic2 expression were downregulated (Figures S3F–S3H). Moreover, Ptf1a-electroporated spinal cords analyzed at later stages demonstrated that, as previously suggested (Petkó and Antal, 2012), GABAergic neurons project locally and/or contralaterally (Figures S3I–S3K). Altogether, these observations suggest that dIL$_B$ glutamatergic neurons are positive for Zic2, whereas GABAergic neurons do not express Zic2.

Spinal Neurons that Express Zic2 Postmitotically Project Ipsilaterally

Next, we analyzed the projections of spinal Zic2 neurons by visualizing EGFP axons in the Tg(Zic2^{EGFP}) mice. Between E13 and E14, two populations of EGFP axons were detected. One set of axons extended toward the DF (Figures 3A and 3A′), and another group of fibers grew into the DLF (Figures 3A″ and 3B). By birth, many EGFP axons were observed in both the DF and the DLF (Figure 3B). In open-book preparations from the spinal cord of E16 Tg(Zic2^{EGFP}) embryos, EGFP axons ascended ipsilaterally into the DLF (Figure 3C). Neurofilament staining in longitudinal sections at different levels of the dorsal cord
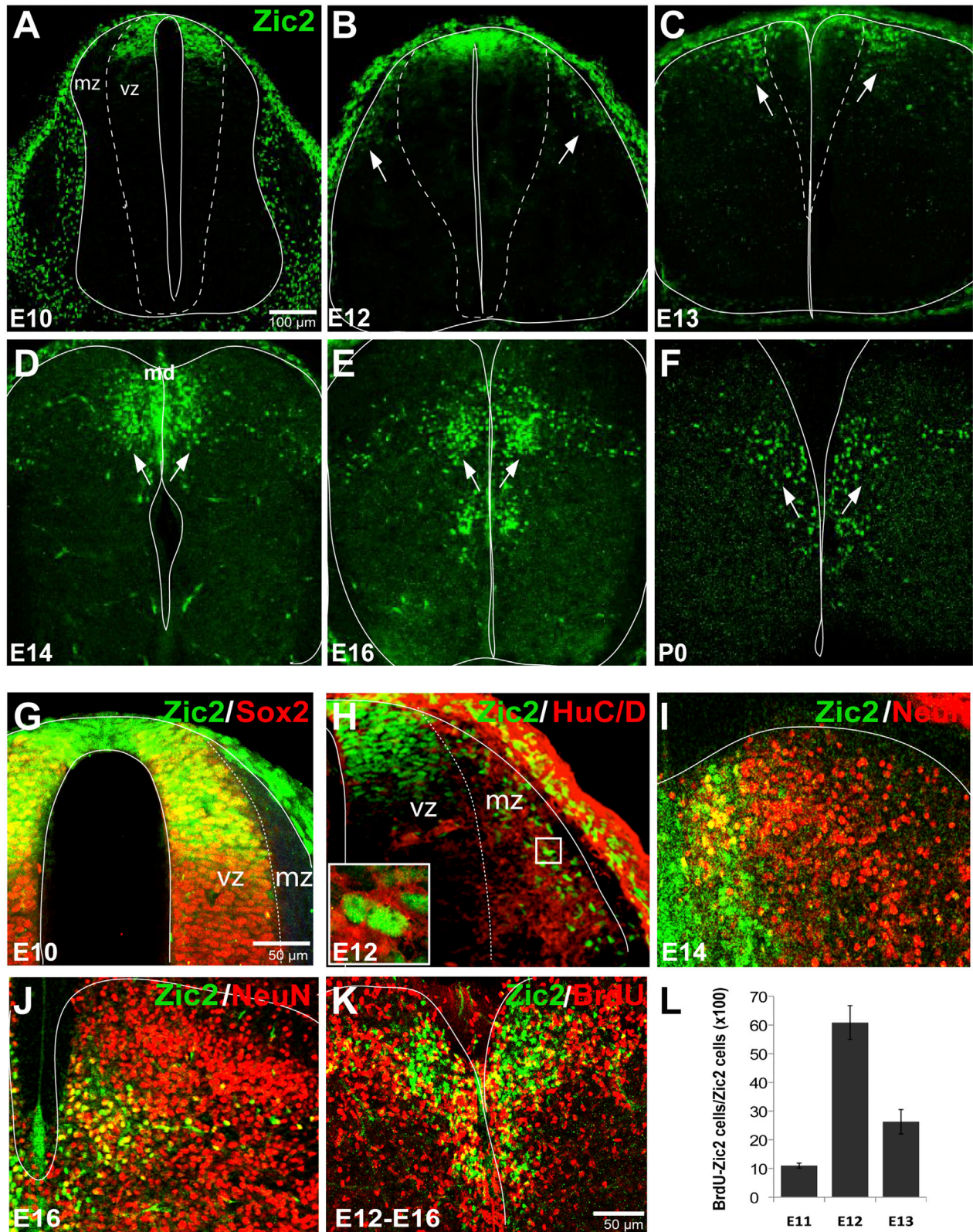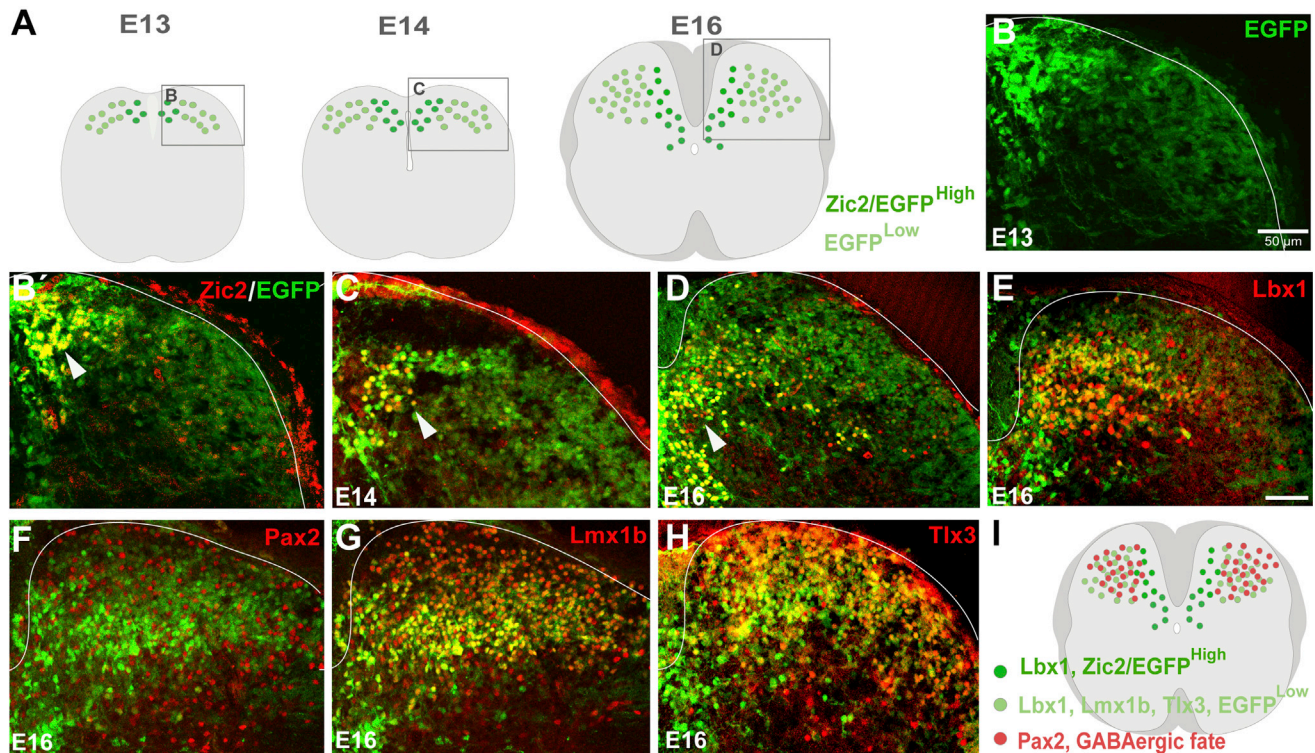
(legend on next page)

Figure 2. The Spinal Neurons that Transiently Express Zic2 Are dIL$_B$ Projection Neurons

(A) The scheme summarizes the postmitotic spatiotemporal expression of Zic2 and EGFP in Tg(Zic2^{EGFP}) embryos. Zic2-expressing neurons located in dorsocentral regions of the spinal cord express high levels of EGFP (dark green). As these Zic2/EGFP cells move to more dorsal and lateral locations to populate the dorsal horns, they downregulate Zic2, and the levels of EGFP decrease (light green).

(B) Transverse spinal cord section from an E13 Tg(Zic2^{EGFP}) embryo. EGFP is highly expressed in cells located close to the midline and faintly in dorsal horn cells.

(B′) The same image combined with Zic2 staining shows that Zic2 cells are highly positive for EGFP.

(C and D) Zic2 staining in transverse spinal cord sections from Tg(Zic2^{EGFP}) embryos at the indicated stages. White arrows point to the regions with greater concentrations of double-labeled Zic2/EGFP cells.

(E–H) Transverse spinal cord sections from Tg(Zic2^{EGFP}) E16 embryos stained with the indicated markers.

(I) Schematic representation summarizing the results. Zic2 neurons (EGFP^{high}) express Lbx1. The Zic2 fate-mapped neurons (EGFP^{low}) express glutamatergic markers, such as Lmx1b and Tlx3, but not GABAergic markers, such as Pax2.

confirmed that EGFP axons run into both the DF and the DLF (Figures 3D and 3E). To further test whether EGFP axons that extend to the DF are ipsilateral axons, we unilaterally electroporated plasmids encoding the red fluorescent protein (pCAG-DsRed2) in the spinal cord of E12 Tg(Zic2^{EGFP}) embryos. Axons expressing DsRed and EGFP projected ipsilaterally into the DF or the DLF, not crossing the midline (Figure 3F).

These observations, together with previous report demonstrating that dIL$_B$s are association neurons (Gross et al., 2002), suggest that Zic2 is expressed in dorsal horn neurons that form the two main ascending ipsilateral tracks in the dorsal spinal cord: (1) association neurons whose axons run into the DLF and connect intraspinal segments, and (2) projection neurons

with axons ascending to supraspinal targets through the DL or the DLF.

Zic2 Expression Prevents Midline Crossing in the Dorsal Spinal Cord

The spinal cord of Zic2 mutant mice is highly distorted due to failures in the closure of the neural tube that result in anencephaly and spina bifida (Brown et al., 1998; Elms et al., 2003; Nagai et al., 2000). This early and strong phenotype makes the assessment of Zic2 function in more mature stages difficult. Therefore, to investigate the role of Zic2 in late-born spinal neurons in vivo, small hairpin RNAs (shRNAs) against Zic2 (Zic2RNAi) were injected and electroporated into the spinal cord of E12 embryos,

Figure 1. Spatiotemporal Expression of Zic2 in the Developing Spinal Cord

(A–F) Zic2 staining in transverse spinal cord sections from wild-type mouse embryos at the indicated stages.

(G–J) Double immunostaining of Sox2, HuC/D, or NeuN and Zic2 in transverse sections from the spinal cord of wild-type embryos at the indicated stages.

(K) Double staining for BrdU and Zic2 in transverse spinal cord sections from E16 embryos injected with BrdU at E12.

(L) Quantification of the number of double-labeled Zic2/BrdU cells in E16 embryos injected with BrdU at E11, E12, and E13. The error bars indicate ±SEM (**p < 0.01, Student's unpaired t test). Quantifications were performed in at least four sections per embryo and three embryos per condition.
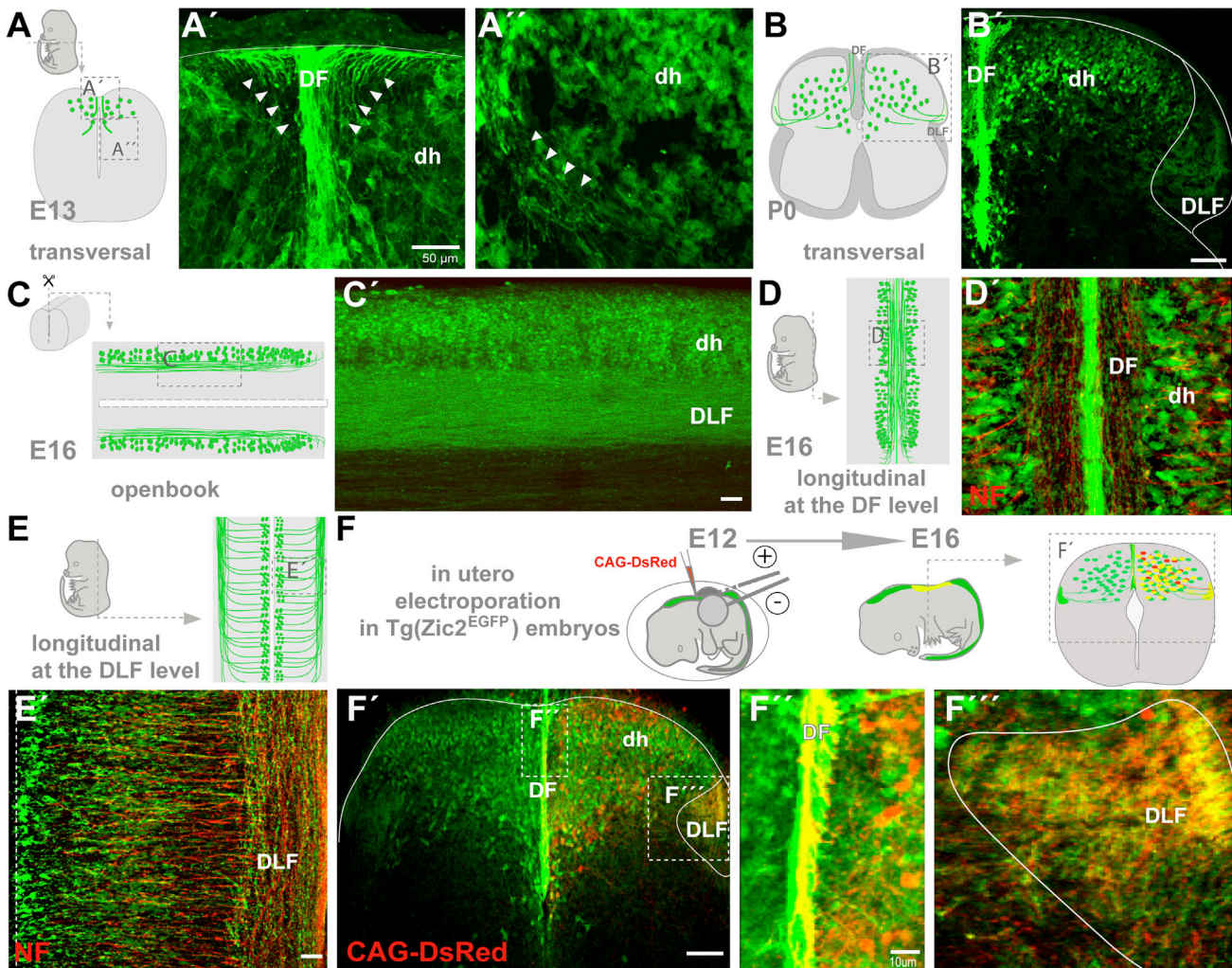
Figure 3. Zic2 Spinal Neurons Project Ipsilaterally

(A–E) The schematics illustrate the location of Zic2 neurons (green) at E13 (A), P0 (B), and E16 (C–F) in transverse (A and B) and longitudinal (D and E) sections as well as in open-book preparations (C). (A′–E′) Pictures from Tg(Zic2^{EGFP}) mice despite the squared areas represented in (A–E). Longitudinal sections in (D and E) were stained with neurofilament (NF). (A′) Axons from Zic2 neurons (white arrows) exit the spinal cord throughout the future dorsal fascicle (DF). (A′′) In the same section, a number of EGFP axons (white arrows) extend turning away from the midline. (B′) Transverse spinal sections of newborn Tg(Zic2^{EGFP}) mice show EGFP axons running into both the DLF and the DF. (C′) In open-book preparations, EGFP axons project ipsilaterally into the DLF. EGFP axons running into the DF (D′) or into the DLF (E′) are positive for neurofilament.

(F) Schematic representation of the experimental procedures. Plasmids encoding for DsRed were unilaterally injected in the spinal cords of E12 Tg(Zic2^{EGFP}) embryos by in utero electroporation, and transverse spinal cord sections from electroporated embryos were analyzed 4 days later. (F′) Transverse spinal cord sections of E16 Tg(Zic2^{EGFP}) embryos unilaterally electroporated with the DsRed-encoding plasmids show that DsRed/Zic2^{EGFP} axons project ipsilaterally into the DF and into the DLF. (F′′ and F′′′) Higher magnifications of the squared areas in (F).

when most Zic2$^+$ postmitotic neurons are generated in the mouse (Figure 4A). Four days after Zic2RNAi electroporation, Zic2 expression was reduced by approximately 10% in the targeted side compared to the endogenous expression in the nonelectroporated side (nonelectroporated side: 100% ± 2.71%, electroporated side: 90% ± 2.91%; n = 9 embryos) (Figure 4B). The downregulation of Zic2 in the dorsal cord led to a significant increase in the number of targeted axons that aberrantly crossed the dorsal midline compared to the controls (Figures 4C–4E). We also noticed a modest but significant number of Zic2RNAi/EGFP-targeted cell bodies located in the nonelectroporated

side (Figures 4C–4E) and a number of neurons that were mislocated into the superficial layers compared to the embryos electroporated with EGFP alone (data not shown). Together, these results indicate that Zic2 expression is essential in dorsal horn neurons to avoid midline crossing.

Zic2 Is Sufficient to Switch Axonal Laterality in the Spinal Cord

To investigate whether Zic2 is also sufficient to switch the laterality of spinal cord axons, we performed in vivo gain-of-function experiments. Considering that electroporation of E12 mouse
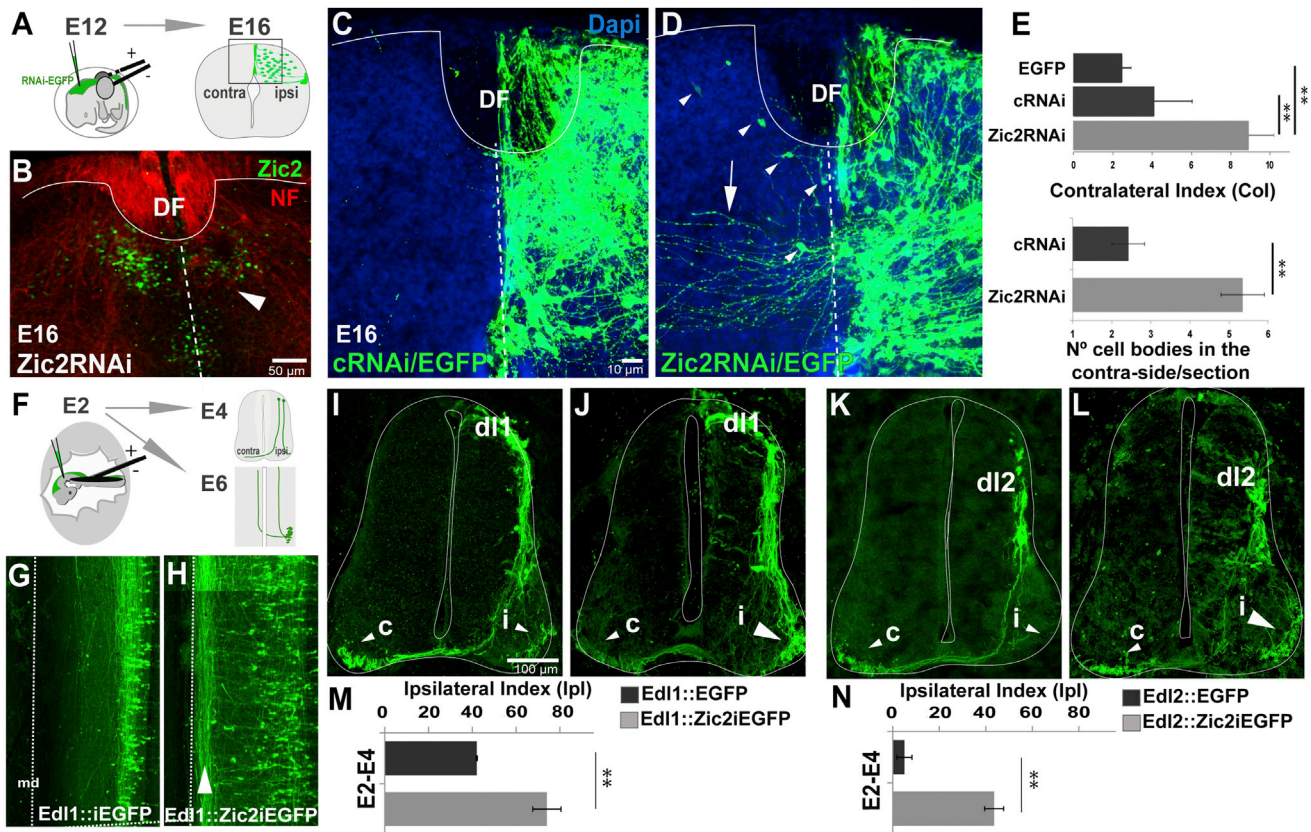
Figure 4. Zic2 Is Necessary and Sufficient to Determine Axonal Ipsilaterality in the Spinal Cord

(A) Schematic representation of the experimental procedures. E12 embryos were electroporated with Zic2RNAi and harvested at E16 for analysis of the contralateral index (Col). The squared area marks the analyzed region.

(B) A transverse spinal cord section of an E16 mouse embryo electroporated unilaterally with Zic2RNAi shows the effective downregulation of Zic2 protein (green) in the electroporated side (white arrow). Dashed line marks the midline. Red is neurofilament (NF) staining.

(C and D) Transverse spinal cord sections from E16 mouse embryos electroporated at E12 with control RNAi/EGFP (cRNAi/EGFP) or Zic2RNAi/EGFP-encoding plasmids. Zic2 downregulation causes massive aberrant crossing (white arrow) at the dorsal midline (dashed white line). The arrowheads point to the cell bodies of mislocated neurons on the side contralateral to the electroporation.

(E) Quantification of the contralateral index (Col) in embryos electroporated with control plasmids (CAG-EGFP or cRNAi) or plasmids encoding Zic2RNAi (top). A significant increase of axons crossing the dorsal midline was observed after downregulation of Zic2. Average number of cell bodies per section counted in the side contralateral to electroporation (bottom). Error bars indicate ±SEM (**p < 0.01, Student's unpaired t test). Quantification was performed in at least four sections per embryo and three embryos per condition.

(F) Schematic representation of the experimental procedures. E2 embryos were electroporated and harvested at E4 or E6 for analysis and quantification of the ipsilateral index.

(G and H) Open-book preparations of E6 chick embryos electroporated at E2 with plasmids driving EGFP or Zic2EGFP expression to the dl1 domain. The dotted line delineates the ipsilateral side of the midline.

(I–L) Transverse spinal cord sections from E4 chick embryos electroporated at E2 with plasmids driving EGFP expression to the dl1 domain (Edl1) or to the dl2 domain (Edl2) show an increase in ipsilateral axons after Zic2 induction compared to the controls. C, contralateral side; I, ipsilateral side.

(M and N) Quantification of the ipsilateral index (Ipl) in E4 chick embryos electroporated at E2 with Edl1:EGFP or Edl2:EGFP plasmids revealed an increase in the number of axons projecting ipsilaterally after the induction of Zic2. Error bars indicate ±SEM (**p < 0.01, Student's unpaired t test). Quantification was performed in at least four sections per embryo and three embryos per condition.

embryos rarely targeted contralateral interneurons, we turned to the chick spinal cord because in ovo electroporations provide access to earlier stages of development, when most of the generated neurons are contralateral (Altman and Bayer, 2001; Nandi et al., 1991; Wentworth, 1984). Vectors driving the expression of EGFP alone (pCAG-EGFP) or Zic2 plus EGFP (pCAG-Zic2iresEGFP) were unilaterally introduced into the spinal cords of chick embryos at E2.5, when most commissural interneurons are generated. At E4, embryos were sacrificed and the expres-

sion of cell-fate markers was studied. Colabeling with markers for the different cell types that populate the dorsal cord (Lhx2/9, Lhx1/5, Isl1, Brn3a, Tlx3, Lbx1, Lmx1b, and Pax2) demonstrated no effect on cell differentiation or fate acquisition after alteration of Zic2 expression (Figure S4). To better visualize and quantify axonal trajectories after ectopic expression of Zic2, we used vectors that drive gene expression specifically in postmitotic dl1 interneurons (pEdl1::EGFP). As expected from previous reports (Avraham et al., 2009), most dl1 axons

projected contralaterally at E4. However, following the electroporation of pEdI1::Zic2iresEGFP, approximately 50% of the targeted commissural axons switched their projection and became ipsilateral (Figures 4F, 4I, 4J, and 4M). This result was confirmed in open-book preparations from embryos electroporated with pEdI1::EGFP or pEdI1::Zic2iresEGFP (Figures 4F–4H).

To address whether Zic2 was also sufficient to switch axonal laterality in spinal domains that contain only commissural neurons, we used enhancers to drive gene expression in the dI2 population (pEdI2::EGFP) (Avraham et al., 2009). As in previous reports (Avraham et al., 2009), only commissural axons were observed in embryos electroporated with pEdI2::EGFP. In contrast, dI2 neurons expressing Zic2iresEGFP showed a significant increase in the number of ipsilateral axons, both in transverse spinal cord sections (Figures 4K, 4L, and 4N) and in open-book preparations (data not shown). These results demonstrate that postmitotic expression of Zic2 is sufficient to switch the axonal laterality of commissural spinal neurons.

Zic2 Is Not Expressed in Ipsilateral Neurons whose Axons Never Approach the Midline

Apart from dIL$_B$ neurons, the dorsal spinal cord contains other populations that project ipsilaterally such as the ipsilateral interneurons from the dI1 domain (dI1i) (Bermingham et al., 2001; Helms and Johnson, 1998; Wilson et al., 2008) and the dI3 neurons (Avraham et al., 2010). The LIM homeodomain transcription factors Lhx2 and Lhx9 are expressed in dI1 interneurons and distinguish between commissural (dI1c) and ipsilateral (dI1i) pools. At early stages, when postmitotic neurons migrate out of the ventricular zone, Lhx2 and Lhx9 are strongly expressed by both dI1c and dI1i neurons. Later, Lhx2, but not Lhx9, disappears from the dI1i. Because dI1i neurons project ipsilaterally, we considered the possibility that Zic2 expression was responsible for their axonal laterality. To address this question, we performed Zic2 immunostaining in EdI1::EGFP-electroporated embryos and found that dI1 targeted neurons never expressed Zic2 (Figure 5A). In addition, double immunolabeling for Lhx2/9 and Zic2 at different stages showed that by the time that Lhx2/9 neurons are differentiating, Zic2 is expressed only in the ventricular layer (Figures 5B and 5C). At E12.5, when the first postmitotic Zic2 neurons leave the cell cycle, most Lhx2/9 neurons were already located in the medial deep dorsal horn (Figure 5D), and although a few Lhx2$^+$ cells were still in dorsal areas, Zic2 and Lhx2 were expressed in different cells (Figure 5D'). Moreover, electroporation of control RNAi or Zic2RNAi in the chick spinal cord at E2 produced similar ratios of ipsilateral-projecting axons contrasting to a decrease in the number of ipsilateral axons in Zic2RNAi- versus cRNAi-expressing embryos that were electroporated 2 days later (Figures 5E and 5F). These observations demonstrate that Zic2 is not required to determine the ipsilateral trajectory of dI1i neurons and suggest that Zic2-dependent mechanisms are only needed in those ipsilaterally projecting neurons that actively avoid midline crossing.

Zic2 Expression Is Sufficient to Repress the Commissural Program

To understand the mechanisms underlying the ability of Zic2 to instruct an ipsilateral phenotype, we analyzed its relationship with *Lhx* genes that are reported to promote midline crossing. Ectopic induction of Zic2 into the spinal cord of chick embryos did not affect *Lhx9* mRNA expression but produced a strong reduction of *Lhx2* mRNA levels (Figures 5G and 5H).

Robo3 is a guidance receptor induced by Lhx2/9 transcription factors and essential for midline crossing in dI1 commissural interneurons (Wilson et al., 2008). A significant decrease in the expression of this guidance receptor (both mRNA and protein) was also detected after ectopic expression of Zic2 (Figures 5I–5K). At later stages, we detected a population of Robo3$^+$ neurons located very close to the Zic2 neurons (Figure 5L), and this late expression of Robo3 at E14 was shut down after ectopic expression of Zic2 (Figure 5M). These results led us to postulate that deactivation of Robo3 may be a requisite for midline avoidance of dorsal horn neurons. To explore this possibility, we ectopically expressed Robo3.1 (the Robo3 isoform expressed in precrossing axons; Chen et al., 2008) in dorsal cord cells by in utero electroporation. Strikingly, after ectopic expression of Robo3.1 in the dorsal cord, axons from dorsal horn neurons were aberrantly guided toward ventral regions instead of following their normal path to the dorsal fascicles. A significant number of these Robo3-misrouted axons crossed the ventral midline compared to the controls (Figures 5N–5P). These results support the notion that the downregulation of Robo3 is necessary to prevent axon extension into ventral regions of the spinal cord.

Zic2 Effectors in the Dorsal Spinal Cord

Our results suggest that Robo3 must be downregulated to maintain axonal extension of dorsal horn neurons into the dorsal fascicles. However, although Zic2 downregulation is sufficient to provoke axon crossing at the dorsal midline, ectopic expression of Robo3 alone is not. This suggests that Zic2 regulates other guidance molecules to promote dorsal midline avoidance. The tyrosine kinase receptor EphB1 has been described as the main effector of Zic2 in ipsilateral visual fibers (García-Frigola et al., 2008). Therefore, EphB1 was our first candidate as a possible effector of Zic2 in the dorsal spinal cord. We found, however, that this receptor is not expressed in the dorsal spinal cord at the time that Zic2 is postmitotically expressed in this region (Figure S5). Thus, we analyzed the expression of other guidance receptors known to mediate axon repulsion in different contexts such as EphB2, EphA4, EphA5, EphA6, Robo1, Robo2, Unc5C, PlexinA1, PlexinA2, and Neuropilin1 (Figure S5). None of these molecules matched Zic2 expression in the developing dorsal cord with the exception of EphA4, which appeared to colocalize, at least partially, with Zic2 (Figures 6A and 6B). We also observed that the ligands for EphA4 (ephrinB1 [North et al., 2009], ephrinB2 [Gale et al., 1996], and ephrinB3 [Bergemann et al., 1998]) were highly expressed in the dorsal spinal cord midline at the same stages than Zic2 and EphA4 (Figures 6C–6E). Therefore, EphA4/ephrinB signaling looked like a good candidate to mediate the midline avoidance controlled by Zic2 in dIL$_B$ spinal neurons. To evaluate this hypothesis, we analyzed EphA4 expression levels after induction of Zic2 expression in the dorsal cord and found a significant increase of *EphA4* mRNA levels in the electroporated side compared to the nonelectroporated side (Figures 6F, 6G, and 6I). Conversely, *EphA4* mRNA levels were downregulated after electroporation of Zic2RNAi
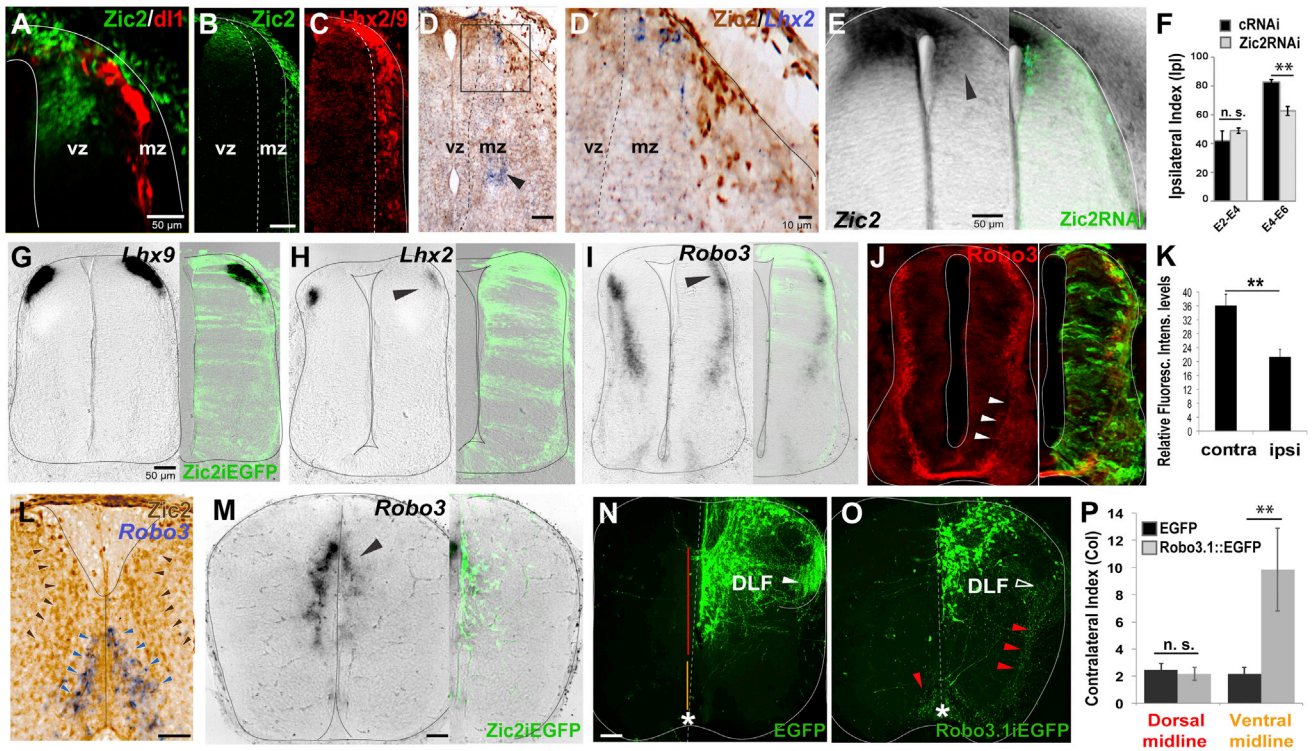
Figure 5. Zic2 Is Able to Repress the Commissural Program

(A) Zic2 immunostaining (green) in transverse spinal sections from a E4 chick embryos electroporated with EdI1:EGFP plasmids (red) show that Zic2 is expressed in progenitors while dl1 neurons are already differentiated and extending their axons ventrally.

(B and C) Zic2 and Lhx2/9 stainings in consecutive transverse spinal cord sections from E11 wild-type embryos show that Lhx2/9 and Zic2 do not localize at this stage.

(D) Zic2 immunostaining (brown) combined with in situ hybridization for Lhx2 mRNA (blue) in transverse spinal cord sections from E12 wild-type mouse embryos show that at this stage, most Lhx2 neurons are located in the deep horn while very few are still in dorsal areas. Neither of these two Lhx2 populations expresses Zic2, which is expressed in more laterally located cells. (D′) High magnification of the squared area in (D) confirms no colocalization of Zic2 and Lhx2.

(E) In situ hybridization for Zic2 in transverse spinal cord sections from E4 chick embryos unilaterally electroporated with Zic2RNAi at E2 demonstrates that Zic2 is downregulated in the electroporated side (black arrow).

(F) Quantification of the number of axons projecting ipsilaterally versus total number of targeted axons (Ipl) in chick embryos electroporated at E2 or at E4 with control RNAi (cRNAi) or Zic2RNAi analyzed 2 days later. Downregulation of Zic2 reduced the number of ipsilateral axons in embryos electroporated at late, but not at early, stages. Quantification was performed in at least four sections per embryo and three embryos per condition. Error bars indicate ±SEM (**p < 0.01, Student's unpaired t test).

(G–I) In situ hybridization against Lhx9, Lhx2, and Robo3 in transverse spinal sections from E4 chick embryos unilaterally electroporated with CAG-Zic2iEGFP plasmids showed no alteration of *Lhx9 mRNA* levels. However *Lhx2* and *Robo3 mRNA* levels decreased after Zic2 induction (black arrows). The electroporated side is shown at the right of each section.

(J) Robo3.1 immunostaining (red) in transverse spinal sections from E4 chick embryos unilaterally electroporated with Zic2-encoding plasmids. Robo3 levels decreased in the commissural axons of the electroporated side (white arrows).

(K) Quantification (measured by fluorescence intensity levels) of Robo3.1 levels on the side electroporated with Zic2-encoding plasmids compared to the nonelectroporated side. Error bars indicate ±SEM (**p < 0.01, Student's unpaired t test). Quantification was performed in at least four sections per embryo and three embryos per condition.

(L) In situ hybridization against Robo3.1 (blue) combined with Zic2 immunostaining (brown) in a transverse spinal section from an E14 embryo. Brown arrows point to the location of Zic2 neurons while blue arrows point to *Robo3 mRNA* expression.

(M) In situ hybridization in transverse spinal sections from an E14 mouse embryo unilaterally electroporated with Zic2 or EGFP-encoding plasmids reveal that the levels of *Robo3.1 mRNA* were reduced (black arrow) in the electroporated compared to the nonelectroporated side.

(N and O) Transverse spinal sections of E16 embryos electroporated at E12 with Robo3.1- or EGFP-encoding plasmids show that axons ectopically expressing Robo3.1 grow ventrally (red arrows) and cross the ventral midline instead of projecting to the dorsal fascicles. Note that after Robo3.1 electroporation, the DLF is nearly empty (empty arrow), whereas in the control many axons are observed in the DLF (white arrow). Red and yellow lines indicate the dorsal and ventral midlines, respectively. Asterisks mark the ventral midline.

(P) Quantification of the number of axons that cross the dorsal and the ventral midline in E16 embryos electroporated at E12 with Robo3.1-encoding plasmids. Quantification was performed in at least four sections per embryo and three embryos per condition. Error bars indicate ±SEM (n. s., nonsignificant; **p < 0.01, Student's unpaired t test). Vz, ventricular zone; mz, mantle zone; DLF, dorsolateral fascicle.
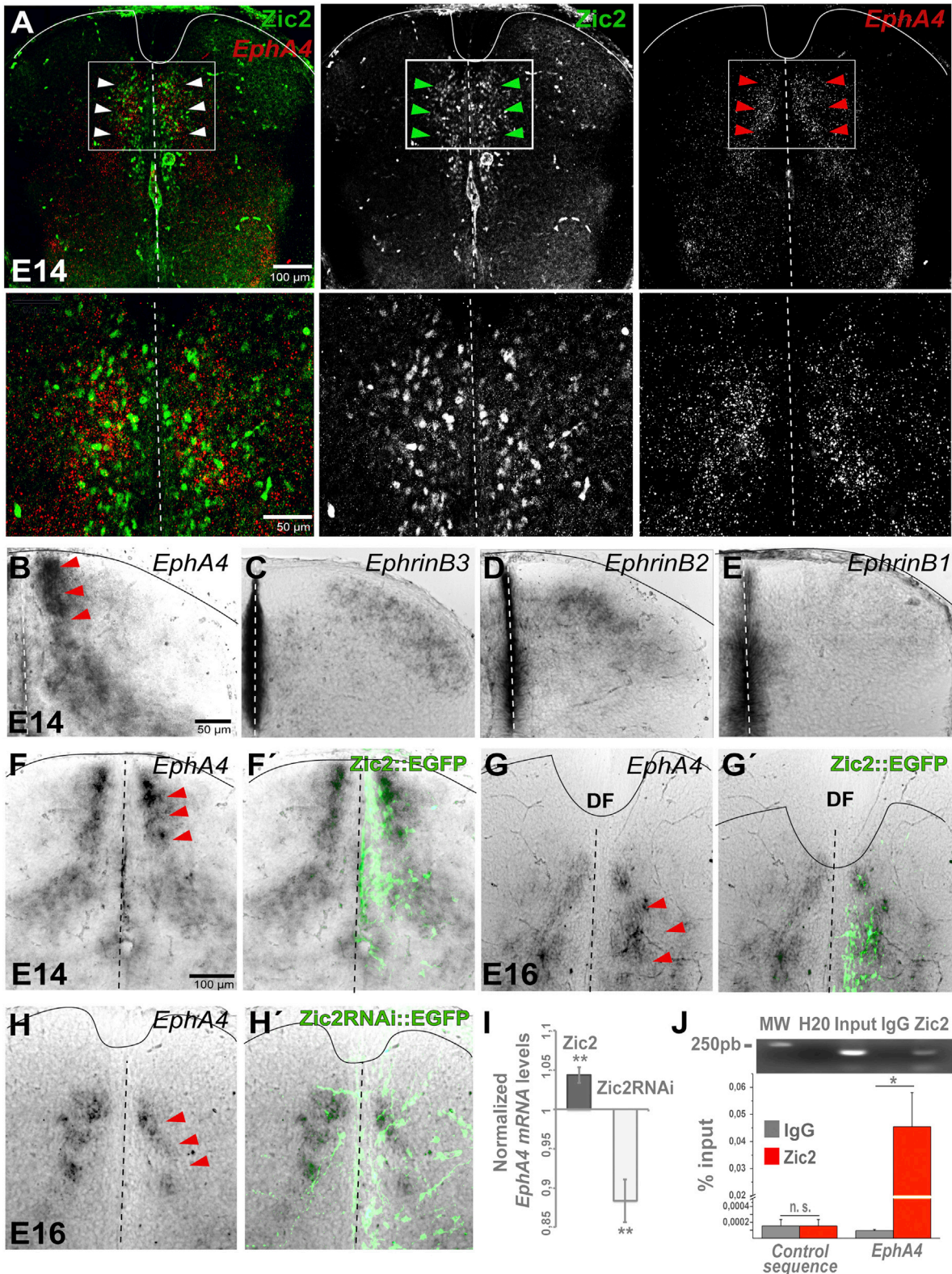
Figure 6. Zic2 Regulates the Expression of EphA4 in the Dorsal Cord

(A) Zic2 immunostaining (green arrows) in transverse spinal cord sections from E16 wild-type embryos combined with in situ hybridization against EphA4 (red). Lower panels show high-magnification images of the squared areas.

(legend continued on next page)

(Figures 6H and 6I). We also investigated whether Zic2 binds to the *EphA4* promoter region by chromatin immunoprecipitation (ChIP) assays using E16.5 wild-type spinal cords and anti-Zic2 antibodies. We found specific binding of Zic2 to the sequence located immediately upstream of the *EphA4* transcription starting site (Figure 6J). Together, these results indicate that Zic2 regulates EphA4 and suggest that EphA4/ephrinB signaling mediates midline avoidance in at least a subpopulation of dorsal horn neurons, which is consistent with results by Paixão et al. (2013) (in this issue of *Neuron*) demonstrating that in the absence of EphA4 a subpopulation of dorsal horn neurons aberrantly cross the dorsal midline.

Zic2 Is Also Essential to Prevent Midline Crossing in the Thalamus

To further explore whether Zic2 is a general determinant of axonal laterality in the CNS, we focused on another major ipsilateral pathway, the thalamocortical (TC) projection. For this, we first examined Zic2 expression in the thalamus of embryonic and newborn mice. Zic2 is symmetrically expressed at high levels in rostromedial thalamic areas from E13 to E16 (Figures 7A–7C) and downregulated by P0. BrdU uptake experiments, similar to those performed in the spinal cord, demonstrated that the thalamic neurons that express high levels of Zic2 were late-born thalamic neurons that differentiate predominantly at E13 (Figures 7D–7F).

To visualize the projection phenotype of Zic2 thalamic neurons, we examined the TC axons of Tg(Zic2^{EGFP}) embryos at different stages. At E14, Zic2^{EGFP} TC axons extended ventrally and semiparallel to the midline (Figure 7L). By E16, Zic2^{EGFP} axons had already passed the internal capsule (Figure 7G), and by E18 they reached the cortical layers (Figure 7H). Immunostaining with calretinin and L1 (two markers of TC axons) in thalamic sections of the Tg(Zic2^{EGFP}) mice revealed that Zic2 TC axons are a fraction of the total population of TC axons. In particular, those that ran into the medial area of the internal capsule and project to the cingulate cortex (Figures 7I–7K).

The thalamus of Zic2 hypomorphic mice (Zic2$^{kd/kd}$) is not as grossly affected as the spinal cord and, therefore, it was possible to analyze the projection phenotypes of TC axons in these mice. Zic2^{EGFP} TC axons aberrantly crossed the midline in E14 Zic2 mutant mice (Tg(Zic2^{EGFP}); Zic2$^{kd/kd}$) compared to their control littermates (Tg(Zic2^{EGFP}); Zic2$^{+/+}$) (Figures 7L and 7M). This phenotype was even more evident at E16. In seven

out of ten Zic2 mutants, all the EGFP axons crossed the midline (Figures 7N–7P), whereas the three remaining mutants showed a milder phenotype. In contrast, the wild-type littermate embryos (nine out of nine) did not show any defect. By labeling TC axons with calretinin antibodies, we also analyzed single Zic2$^{+/+}$ and Zic2$^{kd/kd}$ hypomorphic mutant mice that do not express EGFP. A significant number of calretinin-positive TC axons were also visualized crossing the midline at E16 in this background (four out of four), contrasting with wild-type littermates in which no midline crossing defects were observed (five out of five) (data not shown). These experiments indicate that Zic2 expression is also required to impede midline crossing in the population of TC neurons rostromedially located in the thalamus (i.e., the rmTC neurons).

DISCUSSION

Our findings contribute to understand how axons acquire their laterality to reach the final targets in the correct side of the CNS in bilaterally symmetrical organisms. Here, we show that the transcription factor Zic2 is necessary in spinal dIL$_B$ and thalamic rmTC neurons to keep their axons away from the midline. However, Zic2 expression is not required in ipsilateral neurons that extend axons laterally and never confront short-range midline cues, such as the spinal dI1i or the caudolateral TC neurons. These results, together with previous observations in the visual system, demonstrate that Zic2 plays a general role in the establishment of bilateral circuits in the CNS by preventing the crossing of ipsilateral axons that approach the midline along their trajectory.

Zic2 Prevents Midline Crossing in the Dorsal Spinal Cord

In addition to the described function of Zic2 in neural progenitors, this transcription factor is expressed in a restricted population of postmitotic spinal neurons located dorsally and near the midline that project to both the ipsilateral DF and the DLF. Mature dorsal horn neurons do not express Zic2. However, because dIL$_B$ neurons in Tg(Zic2^{EGFP}) mice express EGFP, we assume that they arise from the Zic2 postmitotic population generated proximal to the dorsal midline. This observation suggests a rapid downregulation of Zic2 once axons are repelled from the midline and before these neurons reach their final location in the dorsal horns.

Loss-of-function experiments demonstrate that the expression of Zic2 in differentiated neurons is essential to avoid

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(B–E) In situ hybridization for EphA4, ephrinB3, ephrinB2, and ephrinB1 mRNAs in transverse spinal cord sections from E14 embryos. All three ephrinBs were expressed at the dorsal midline. In addition, ephrinB3 and ephrinB2 were expressed in the prospective dorsal horns.

(F and G) In situ hybridization in transverse spinal cord sections from E14 and E16 embryos electroporated at E12 with Zic2-encoding plasmids show that *EphA4 mRNA* levels increased on the electroporated (red arrows) versus the nonelectroporated side. (F′ and G′) Electroporated side of the same section.

(H) In situ hybridization in transverse spinal cord sections of E16 embryos electroporated with Zic2RNAi at E12 demonstrate that *EphA4 mRNA* levels decreased in the electroporated (red arrows) versus the nonelectroporated side. (H′) Electroporated side of the same section.

(I) Quantification of *EphA4 mRNA* levels in transverse spinal cord sections from E16 embryos electroporated at E12. *EphA4 mRNA* levels increased after ectopic expression of Zic2 and decreased after Zic2RNAi electroporation. *EphA4 mRNA* levels in the electroporated side were normalized to the levels in the respective nonelectroporated side. Quantification was performed in at least four sections per embryo and three embryos per condition. Error bars indicate ±SEM (**p < 0.01, Student's unpaired t test).

(J) ChIP assay from E16 mouse embryos (n = 7) demonstrated that Zic2 binds to a region upstream of the transcription starting site (TSS) of EphA4, but not to the negative control sequence (genomic region with no annotated genes). As an example, the PCR products resulting from one ChIP experiment were loaded in a gel of agarose. Quantitative PCR samples were analyzed by two dilutions and in duplicated (n = 3). Error bars indicate ±SEM (*p < 0.05, Mann-Whitney test).
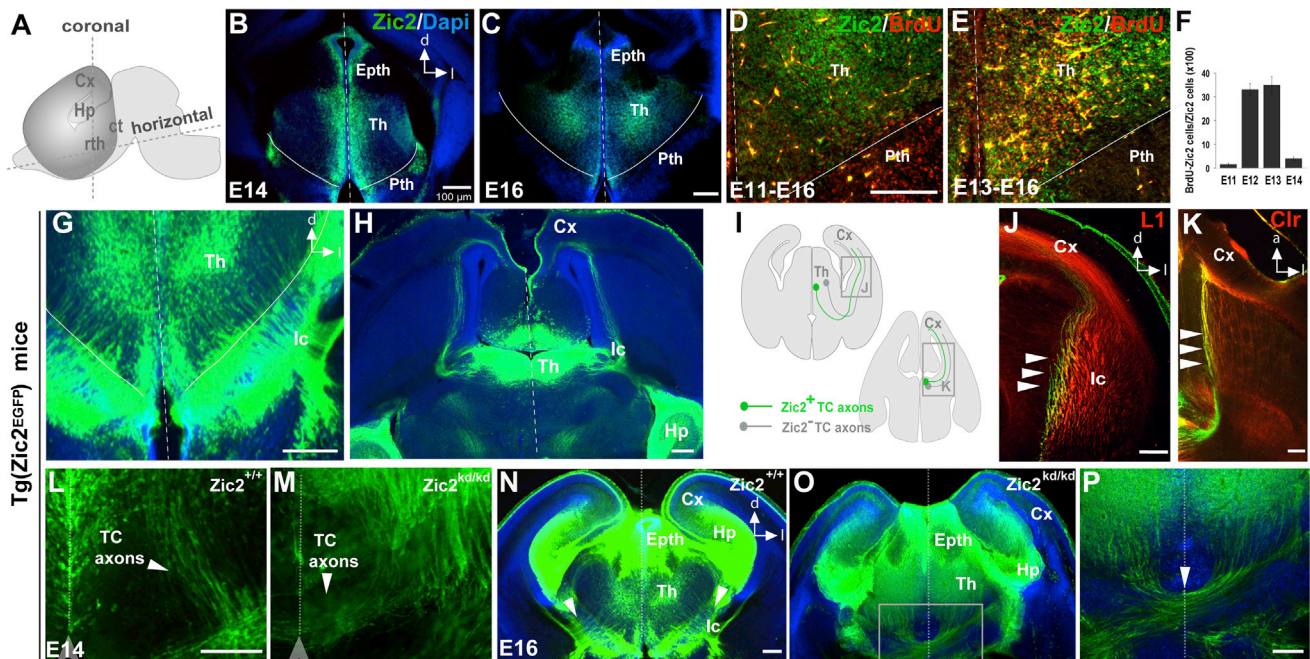
Figure 7. Zic2 Prevents Midline Crossing in Rostromedial TC Axons

(A) Sagittal view of a developing mouse brain. The dashed lines indicate the level of the sections shown in this figure.

(B and C) Zic2 immunostaining in coronal thalamic sections from E14 and E16 embryos.

(D and E) BrdU (red) and Zic2 (green) double immunostaining in coronal thalamic sections from E16 embryos injected with BrdU at E11 or E13 shows extensive Zic2/BrdU colocalization in embryos injected at E13.

(F) Quantification of the number of double-labeled Zic2/BrdU neurons compared to the total number of Zic2 neurons in E16 embryos injected at E11, E12, and E13 with BrdU. Error bars indicate ±SEM (**p < 0.01, Student's unpaired t test). Quantification was performed in at least four sections per embryo and three embryos per condition.

(G and H) Coronal and horizontal sections through the brains of E16 and E18 Tg(Zic2^{EGFP}) embryos show that the Zic2-rostral thalamic neurons project to the ipsilateral cingulated cortex.

(I) Schematic representation of coronal (top) and horizontal (bottom) brain sections summarizes the results in (J and K).

(J and K) L1 and calretinin (Clr; red) stainings in coronal and sagittal sections through the brains of E16 Tg(Zic2^{EGFP}) embryos show that axons from Zic2 neurons (green) extend into the internal aspect of the internal capsule, whereas TC axons negative for Zic2 run laterally.

(L–O) Coronal sections through the thalamus of E14 and E16 embryos show that EGFP TC axons grow away from the midline to enter the ipsilateral internal capsule in the (Tg(Zic2^{EGFP}); Zic2$^{+/+}$) embryos while they aberrantly cross the midline in the (Tg(Zic2^{EGFP}); Zic2$^{kd/kd}$) embryos.

(P) High magnification of the squared area in (O).

Cx, cortex; Hp, hippocampus; cTh, caudal thalamus; Epth, epithalamus; Th, thalamus; m, medial; l, lateral; Pth, prethalamus; md, midline; lc, internal capsule; Ob, olfactory bulb.

crossing at the dorsal midline. In contrast, gain-of-function experiments, in the dI1 and the dI2 populations, indicate that the ectopic expression of Zic2 in differentiated neurons is sufficient to switch axonal laterality. Interestingly, after downregulation of Zic2, in addition to aberrant axon midline crossing, we noticed a number of cell bodies located in the opposite side of the cord (Figures 4D and 4E). This finding is in agreement with the spinal phenotype of EphA4-deficient mice because, as discussed in Paixão et al. (2013), it is known that cell bodies of migrating interneurons follow their axons and it is possible that Zic2/EphA4 neurons that fail to recognize ephrinBs at the midline relocate their cell bodies to more medial positions. In general, these results support the notion that the primary function of Zic2 in postmitotic dorsal cord neurons is to trigger a gene program that impedes dorsal midline crossing.

Our findings address, at least in part, the current uncertainty regarding the regulatory mechanisms that govern axonal lateral-

ity in the spinal cord. Ectopic expression of Zic2 in the spinal cord downregulates Lhx2, but not Lhx9, supporting the previously suggested hypothesis that Lhx2 is implicated in midline crossing while Lhx9 is not (Ding et al., 2012). The function of Lhx9 as a mediator of axonal laterality in dI1i neurons remains unclear and deserves further exploration.

How does Zic2 exert its function in the dorsal cord? Robo3 is an essential guidance molecule that promotes axon crossing at the mouse ventral midline by preventing Robo1 insertion on the growth cone surface (reviewed in Chédotal, 2011). Therefore, ipsilateral neurons should not express this receptor. Accordingly, Robo3 is not expressed in Zic2 neurons and the delivery of Zic2 in contralateral neurons represses Robo3 expression. However, whereas downregulation of Zic2 promotes axon crossing at the dorsal midline, ectopic expression of Robo3.1 in dorsal neurons was not sufficient to induce roof plate crossing. Instead, Robo3.1 gain of function in dorsal horn

neurons provoked axonal misrouting to the ventral cord (Figure 5), suggesting that deactivation of Robo3 is required to become an ipsilateral/dorsal horn neuron. Our results on the ectopic expression of Robo3.1 suggest that Robo3.1 may mediate attraction to the floor plate, as it has been already proposed (Chen et al., 2008; Jaworski et al., 2010; Sabatier et al., 2004). This idea is also supported by Robo3 loss-of-function experiments, including both downregulation of Robo3 by RNAi injections in chick (Philipp et al., 2012) and Robo3 null mutant mice (Sabatier et al., 2004), in which it seems that aberrant ipsilateral axons turn away before reaching the ventral midline to project to the lateral fascicles.

On the other hand, the dramatic axonal turning of Zic2 neurons observed at the dorsal midline (Figure 3) suggested that repulsive signaling leads this process (Figure 3A). EphB2 and PlexinA1 receptors are ubiquitously expressed in the developing cord, and although we cannot completely discard a role for these proteins in midline crossing prevention, this is not a likely possibility because their expression does not coincide with Zic2. ChIP assays, together with functional and anatomical experiments in vivo, demonstrated that Zic2 is necessary and sufficient to trigger EphA4 expression in the dorsal cord and that the three main EphA4 ligands are highly expressed in the dorsal midline. Both EphA4 and EphB1 bind to ephrinB2 (Kullander and Klein, 2002; Murai and Pasquale, 2003), and it is conceivable that the Eph regulatory sequences that respond to Zic2 activity were evolutionarily selected. Therefore, it is not surprising that instead of EphB1, the Eph receptor regulated by Zic2 in the cord is EphA4.

Zic2 Regulates Midline Avoidance, but Not Ipsilaterality Per Se

During development, different distances separate early-born (dl1–dl6) and late-born (dlLB$_{A-B}$) neurons from the midline. At early stages, the dorsal ventricular zone occupies more than half of the thickness of the neural tube wall. By the time that dl1–dl6 interneurons start axonogenesis, they are located laterally in the mantle and separated from the midline by the ventricular zone. dl1–dl3 neurons extend their axons circumferentially along the basal membrane to finally settle their cell bodies in the deep dorsal horn, never encountering short-range midline cues along their trajectory (Avraham et al., 2010; Bermingham et al., 2001; Helms and Johnson, 1998; Wilson et al., 2008). Under these circumstances, establishing an ipsilateral projection may depend on fasciculation with the contralateral axons coming from the other side in the white matter, a process that could depend on Lhx9 function in the case of the dl1 neurons. In contrast, during the differentiation of dIL neurons, the progenitors layer is dramatically reduced in size and, as a consequence, dIL neurons are born in close proximity to the midline. We propose that dlLB neurons, due to its proximal location to the midline at the time of axon extension, express Zic2 to actively avoid the midline. Intriguingly, some spinothalamic neurons located in the lamina I of the dorsal cord have been described as contralaterally projecting neurons (Todd, 2010). The origin of these dorsal horns commissural neurons and their genetic characterization are currently unknown. We predict they are Zic2 negative neurons, but it will be important to confirm this.

Zic2 is not expressed in the ventral cord, but a number of ventral interneurons are known to project ipsilaterally (V0c, V1, V2a, V2b) (reviewed in Alaynick et al., 2011). This apparent inconsistency may be explained as for the trajectories of the ipsilateral dl1 neurons. The generation of ventral spinal neurons (between E10 and E12) occurs in a period when the ventricle physically separates the layer of postmitotic neurons from the midline. Pioneering axons at this time extend the axon laterally to contact with motor neurons or project into the ventrolateral bundles, never approaching the midline. In contrast to the development of the dorsal spinal cord, in the ventral neural tube a second round of neural differentiation proximal to the midline never takes place and, therefore, there would be no need for Zic2 expression. A similar reasoning may apply to the developing thalamus. Early-born neurons would never have the option of crossing the midline due to the existence of the third ventricle and progenitors located in the medial thalamic regions. At later stages, the ventricle shrinks, progenitors become postmitotic neurons, and the two thalamic leaves fuse in a caudorostral manner. At this time, the late-born population of TC cells (rmTC neurons) located nearby the midline extend their axons, and it is at this time that Zic2 expression starts to impede midline crossing.

In summary, our results show that Zic2 determines midline avoidance in at least three different systems that are crucial for the formation of bilateral circuitry in the CNS (Figure 8). Further investigations comparing the gene profiles of postmitotic Zic2 cells from the retina, dorsal spinal cord, and thalamus will clarify the similarities and differences between the mechanisms governing Zic2-dependent axon guidance laterality in different areas of the CNS.

EXPERIMENTAL PROCEDURES

Mouse Lines
The Tg(Zic2^{EGFP})HT146Gsat line was obtained from the Mutant Mouse Regional Resource Center. The Zic2^{kd} mouse line was obtained from the RIKEN repository. The transgenic line Tg(Zic2^{EGFP}) was crossed with the Zic2$^{kd/+}$ mouse line, and the resulting F1 progeny was crossed to generate (Tg(Zic2^{EGFP}); Zic2$^{kd/kd}$) and (Tg(Zic2^{EGFP}); Zic2$^{+/+}$) littermate embryos. More information is provided in Supplemental Experimental Procedures.

In Ovo and In Utero Electroporation, In Situ Hybridization, Immunohistochemistry, BrdU Experiments, and ChIP Assays
Protocols describing these methods are detailed in Supplemental Experimental Procedures. The sources of plasmids, antibodies, and probes for in situ hybridization used in this study are described in Tables S1–S3.

Quantification of Zic2 Downregulation, Cell Bodies Mislocation, and Axonal Misprojections
For quantification of functional experiments in chicks, ImageJ software was used to select two mirror regions of interest (ROIs) (ipsilateral [iROI] and contralateral [cROI]) per image, taken from the white matter (where longitudinal axons were labeled with L1 antibodies). The ipsilateral Index (IpI) was obtained as a measure of EGFP levels in the iROIs and cROIs and expressed as a function of (iROI/iROI + cROI) × 100. To quantify Zic2 downregulation in mouse Zic2, we assessed protein levels by fluorescence intensity after immunohistochemistry in sections of E16 embryos (electroporated at E12). Fluorescence intensities from the electroporated and the nonelectroporated sides were measured and compared. Cell bodies expressing EGFP were also counted in each section and represented in a different graph in Figure 4E. For quantification of functional experiments in mice,
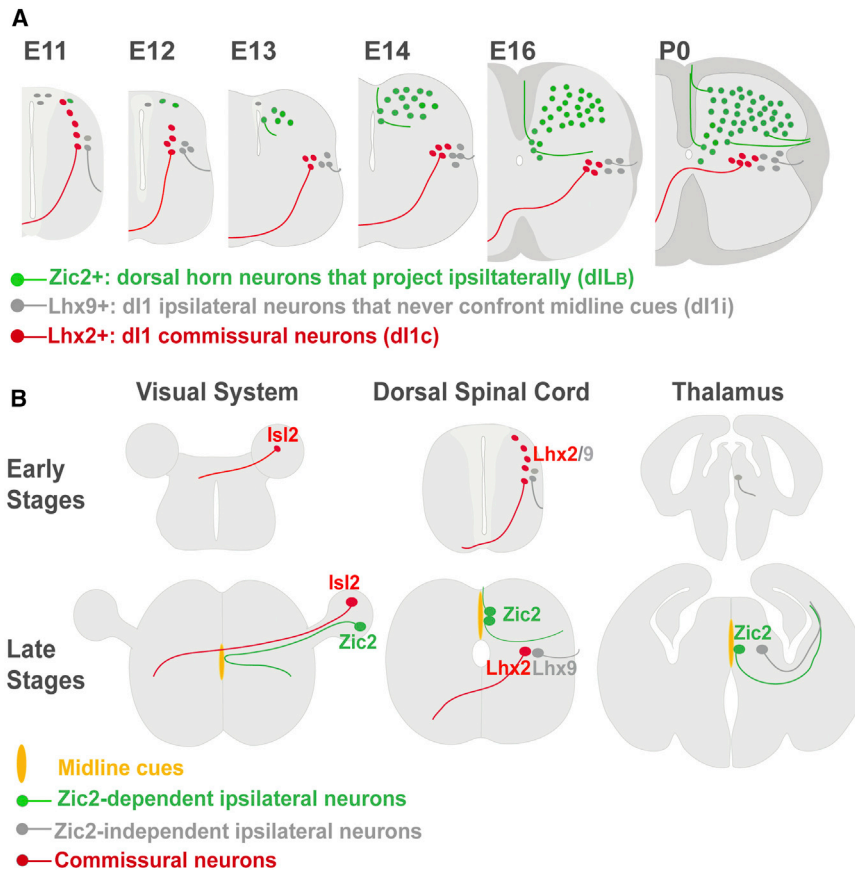
Figure 8. Schematic Summary of the Results

(A) dI1 interneurons become postmitotic as soon as E10 and migrate extending axons ventrally, in the case of Lhx2 neurons (red), or laterally, in the case of Lhx9 neurons (gray). The first postmitotic Zic2 neurons (green) appear around E11, at the time that some dI1 neurons are still being generated. Zic2 postmitotic expression peaks from E13 to E16 and during this period Zic2 neurons extend their axons ipsilaterally to the DF or to the DLF. In contrast to the ipsilateral dI1 neurons whose axons never approach the midline, late-born neurons that express Zic2 and differentiate in the proximity of the dorsal midline are exposed to midline cues soon after differentiation.

(B) In the visual system, Isl2 is expressed in contralateral retinal ganglion cells (RGCs) and Zic2 in the ipsilaterally projecting RGCs. In the early spinal cord, Lhx2/9 are both expressed in dI1 interneurons. Ipsilateral dI1 interneurons migrate ventrally, and their axons navigate laterally and never confront midline cues. In contrast, later-born interneurons (dIL_B) express Zic2, which triggers the expression of a set of molecules that are essential to respond to dorsal midline cues. In the thalamus, axons from early-born neurons extend ventrally at the time that the thalamic halves are physically separated by the third ventricle. Later, as the thalamic halves fuse, later-born neurons located in medial positions express Zic2 to respond to midline cues and avoid crossing.

two ROIs per section were selected in the nonelectroporated side adjacent to the midline. A ROI was created from the central canal to the dorsal fascicle (dorsal crossing ROI [dcROI]) and another from the central canal to the floor plate (ventral crossing ROI [vcROI]). The dcROI and vcROI-EGFP signals were normalized to the EGFP levels in the gray matter of the electroporated side ($_{EGFP}$ROI). Contralateral indices (CoIs) of the dorsal and ventral cords were obtained as (dcROI/$_{EGFPROI}$) × 1,000 and (vcROI/$_{EGFPROI}$) × 1,000 function, respectively.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, five figures, and three tables and can be found with this article online at http://dx.doi.org/10.1016/j.neuron.2013.10.007.

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