# The Morphogen Sonic Hedgehog Is an Axonal Chemoattractant that Collaborates with Netrin-1 in Midline Axon Guidance

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## Summary

**Developing axons are guided to their targets by attractive and repulsive guidance cues. In the embryonic spinal cord, the floor plate chemoattractant Netrin-1 is required to guide commissural neuron axons to the midline. However, genetic evidence suggests that other chemoattractant(s) are also involved. We show that the morphogen Sonic hedgehog (Shh) can mimic the additional chemoattractant activity of the floor plate in vitro and can act directly as a chemoattractant on isolated axons. Cyclopamine-mediated inhibition of the Shh signaling mediator Smoothened (Smo) or conditional inactivation of Smo in commissural neurons indicate that Smo activity is important for the additional chemoattractant activity of the floor plate in vitro and for the normal projection of commissural axons to the floor plate in vivo. These results provide evidence that Shh, acting via Smo, is a midline-derived chemoattractant for commissural axons and show that a morphogen can also act as an axonal chemoattractant.**

## Introduction

During nervous system development, axons respond to attractive and repulsive guidance cues to navigate to their targets (reviewed in Tessier-Lavigne and Goodman, 1996). Despite numerous efforts to identify the guidance cues involved in this process, only three sets of diffusible axonal chemoattractants have been identified so far: members of the Netrin, the scatter factor/hepatocyte growth factor (SF/HGF), and the neurotrophin families (Tessier-Lavigne and Goodman, 1996; Ebens et al., 1996; O'Connor and Tessier-Lavigne, 1999). Thus, the number of secreted attractive guidance cues identified seems small relative to the immense complexity of the nervous system, making it likely that additional chemoattractants remain to be discovered.

One promising system for the identification of chemoattractants is the spinal cord midline. During spinal cord development, commissural neurons, which differentiate in the dorsal neural tube, send axons that project toward

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and subsequently across the floor plate, forming axon commissures (Figure 1A and see also Colamarino and Tessier-Lavigne, 1995). These commissural axons project toward the midline in part because they are attracted by Netrin-1, a long-range chemoattractant secreted by the floor plate (Kennedy et al., 1994; Placzek et al., 1990; Serafini et al., 1996, 1994; Tessier-Lavigne et al., 1988). In mice mutant for *Netrin-1* or its receptor *DCC*, many commissural axon trajectories are foreshortened, fail to invade the ventral spinal cord, and are misguided (Fazeli et al., 1997; Serafini et al., 1996). However, some of them do reach the midline, indicating that other guidance cues apparently cooperate with Netrin-1 to guide these axons. Analysis of *Netrin-1* knockout mice suggested that the floor plate might actually express an additional diffusible attractant(s) for commissural axons (Serafini et al., 1996). This was shown using explants of rat dorsal spinal cord as a source of responding axons and testing whether floor plate cells from *Netrin-1* mutant embryos possessed the in vitro activities of wild-type floor plate cells (Serafini et al., 1996). Unlike floor plate tissue from wild-type or heterozygous embryos, floor plate from *Netrin-1* mutant embryos was ineffective in stimulating commissural axon outgrowth from rat dorsal spinal cord explants; thus, Netrin-1 appears to account for most or all of the outgrowth-promoting activity of floor plate cells. In contrast, floor plate explants from mutant embryos were as effective as those from wild-type embryos in eliciting turning of commissural axons, raising the possibility that floor plate cells secrete another attractant(s) for commissural neurons in addition to Netrin-1. The identity of this chemoattractant(s) has, however, remained elusive.

In addition to regulating axon guidance, the floor plate is also known for its role in morphogen secretion (reviewed in Jessell, 2000). Morphogens are signaling molecules that are produced in a restricted region of a tissue and move away from their source to form a long-range concentration gradient. Cells differentiate in response to morphogen signaling depending on their position within the gradient and thus on their distance from the morphogen source. In the spinal cord, Sonic hedgehog (Shh), a morphogen secreted by the floor plate, functions as a gradient signal for the generation of distinct classes of ventral neurons along the dorsoventral axis (reviewed in Jessell, 2000; Ingham and McMahon, 2001; Marti and Bovolenta, 2002); its inactivation, or inactivation of its signaling component *Smoothened* (*Smo*), severely perturbs the patterning of ventral neuronal progenitors without affecting the patterning of dorsal neuronal progenitors (Briscoe et al., 2001; Chiang et al., 1996; Wijgerde et al., 2002; Zhang et al., 2001).

Given its expression by the floor plate and its long-range effects in the spinal cord, Shh is an interesting candidate for a midline-derived axonal guidance cue, prompting us to test whether it is responsible for the Netrin-1-independent attractant activity of the floor plate. We show that Shh is indeed an axonal chemoattractant that can mimic the Netrin-1-independent chemoattractant activity of the floor plate. Conversely, loss-
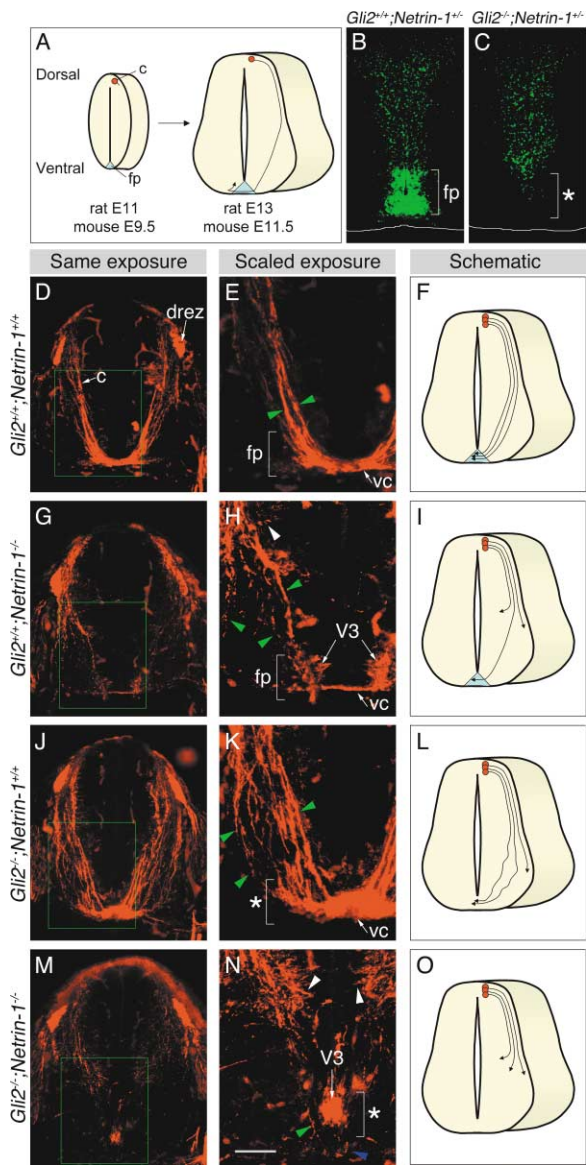
Figure 1. Genetic Ablation of the Floor Plate Enhances the Commissural Axon Guidance Phenotype of *Netrin-1* Mutant Embryos

(A) Diagram of the developing spinal cord and trajectory of TAG-1$^+$ commissural axons. c, commissural neurons; fp, floor plate.

(B and C) *Netrin-1* expression in *Gli2$^{+/+}$;Netrin-1$^{+/-}$* (B) and *Gli2$^{-/-}$;Netrin-1$^{+/-}$* (C) E11.5 mouse embryos. The *Netrin-1* mutant allele harbors a lacZ insertion (Serafini et al., 1996), allowing expression from the *Netrin-1* locus to be monitored with a β-Gal antibody. In *Gli2* mutant embryos, *Netrin-1* expression in the ventral-most portion of the spinal cord is absent because of absence of the floor plate (asterisk), but persists in the periventricular region.

(D–O) Commissural axon projections were visualized with a TAG-1 antibody in E11.5 mouse embryos of the following genotypes: *Gli2$^{+/+}$;Netrin-1$^{+/+}$* (D–E), *Gli2$^{+/+}$;Netrin-1$^{-/-}$* (G–H), *Gli2$^{-/-}$; Netrin-1$^{+/+}$* (J–K), and *Gli2$^{-/-}$;Netrin-1$^{-/-}$* (M–N).

In (D, G, J, and M), pictures were taken using the same exposure time to help reveal reduced axon growth into the ventral spinal cord in mice carrying the *Netrin-1* mutation.

(E, H, K, and N) show higher magnification views of the boxed regions in the adjacent images; the brightness of (H) and (N) was increased 5-fold to allow better visualization of the axons.

(D–F) TAG-1$^+$ commissural axons in wild-type embryos project

of-function experiments indicate that the Shh signaling mediator Smo is required for the Netrin-1-independent chemoattractant activity of the floor plate and for the normal projection of commissural axons to the floor plate in vivo.

## Results

### Evidence that a Netrin-1-Independent Attractant Activity of Floor Plate Guides Commissural Axons

Netrin-1 is a commissural axon chemoattractant expressed by the floor plate and the periventricular zone of the neural tube (Serafini et al., 1996 and see Figure 1B) that accumulates in an increasing dorsoventral gradient through the developing spinal cord (T. Kennedy and M.T.L., unpublished data), and is required for normal growth and guidance of these axons (Serafini et al., 1996 and see below). To test whether floor plate-derived activities other than Netrin-1 are important for commissural axon growth to the midline, we took advantage of the fact that *Gli2* mutant mice specifically lack floor plate cells yet still show normal patterning of neural progenitors, with the exception of a reduction in the V3 interneurons adjacent to the floor plate (Ding et al., 1998; Matise et al., 1998). We therefore introduced the *Gli2* mutation into a *Netrin-1* mutant background to examine the consequences of deleting the floor plate in animals lacking Netrin-1. We compared axon trajectories in these double mutants to those in *Netrin-1* or *Gli2* single mutants using immunohistochemistry with anti-TAG-1 antibodies to visualize a subset of commissural axons as they project to the floor plate. For simplicity, the phrase "commissural neurons" will refer here specifically to TAG-1$^+$ commissural neurons, whose cells bodies are located in the dorsal part of the developing spinal cord.

In wild-type embryos, these axons project ventrally near the edge of the spinal cord until they reach the level of the developing motor column (Figures 1D–1F). At embryonic day 11.5 (E11.5), most project through the motor column in a directed and highly fasciculated manner toward the ventral midline. As reported pre-

through the motor column in a directed and highly fasciculated manner toward the ventral midline (green arrowheads). drez, dorsal root entry zone; vc, ventral commissure; V3, V3 interneurons.

(G–I) In *Netrin-1* mutants, many TAG-1$^+$ axons are foreshortened and fail to invade the ventral spinal cord (green arrowheads), with some projecting medially toward the ventricle (white arrowhead).

(J–L) In *Gli2* mutants, TAG-1$^+$ axons are highly defasciculated and some project near the lateral edge of the spinal cord (green arrowheads), invading the ventral spinal cord with multiple projections all over the motor columns.

(M–O) In *Gli2$^{-/-}$;Netrin-1$^{-/-}$* double-mutant embryos, almost all TAG-1$^+$ axons are foreshortened and fail to invade the ventral spinal cord, with most projecting medially toward the ventricle (white arrowheads), resulting in a severe reduction in the number of TAG-1$^+$ axons reaching the midline (green arrowhead) and the absence of a ventral commissure (blue arrowhead). TAG-1$^+$ cells at the ventral midline are V3 interneurons. Each embryo section shown here is representative of multiple sections obtained from at least two embryos for each genotype. Scale bars are 150 μm in (D, G, J, and M) and 75 μm in (B, C, E, H, K, and N).

viously (Serafini et al., 1996), in *Netrin-1* mutants many TAG-1$^+$ commissural axons are foreshortened and fail to invade the ventral spinal cord (Figures 1G–1I). Some continue to extend, but in aberrant directions, with some projecting medially and others projecting in a defasciculated manner into the motor column. Nonetheless, some reach the floor plate and form a thin ventral commissure. Although the *Netrin-1* mutant allele is severely hypomorphic rather than a complete null (Serafini et al., 1996; see below), it is unlikely that the small number of TAG-1$^+$ axons reaching the floor plate is due entirely to low-level expression of Netrin-1, since a thin ventral commissure is also observed in mice carrying a complete null mutation of the Netrin-1 receptor DCC (Fazeli et al., 1997).

In *Gli2* mutants, *Netrin-1* expression is absent in the ventral-most portion of the spinal cord (because of the absence of the floor plate), but persists in the periventricular region (Matise et al., 1999; Figures 1B and 1C). In a previous study (Matise et al., 1999), it was reported that commissural axon trajectories to the ventral midline are largely normal in these mutants (abnormalities were only reported in the midline region, with axons remaining clustered and failing to project longitudinally). However, detailed analysis in fact revealed clear and consistent defects in trajectories to the ventral midline. At the forelimb level, we observed that commissural axons are highly defasciculated and some project near the edge of the spinal cord (Figures 1J–1L; see Figure 7M for quantification). These axons then invade the ventral spinal cord with multiple projections all over the motor columns. In agreement with Matise et al. (1999), however, we did observe that most commissural axons nonetheless still appear to reach the midline, forming a highly disorganized ventral commissure.

In contrast to *Netrin-1*$^{-/-}$ single mutants, in *Gli2*$^{-/-}$;*Netrin-1*$^{-/-}$ double-mutant embryos almost all commissural axons are foreshortened and fail to invade the ventral spinal cord, with most projecting medially toward the ventricle, resulting in a severe reduction in the number reaching the midline and the absence of a ventral commissure (Figure 1M–1O). These projection defects are unlikely to be due to defects in neural tube patterning, because we found that the expression of a battery of neural progenitor markers (the same as that used by Matise et al., 1998) is identical to that described previously for *Gli2* mutant embryos (Matise et al., 1998) (data not shown). Thus, removing the floor plate (with the *Gli2* mutation) enhances the guidance phenotype observed in *Netrin-1* single mutant animals, supporting the idea that another floor plate-derived chemoattractant(s) contributes to commissural axon guidance in vivo.

### Shh Causes Commissural Axon Turning within Spinal Cord Explants

In the spinal cord, Shh secreted by the floor plate functions as a gradient signal for the generation of distinct classes of ventral neurons along the dorsoventral axis (Jessell, 2000). To determine whether Shh can also reorient commissural axons, an aggregate of COS cells expressing a full-length Netrin-1 or Shh cDNA was positioned alongside rat E11 whole spinal cord explants and

cultured for 40 hr (Figure 2A). Whereas control COS cells had no effect, cells expressing Shh, like Netrin-1-expressing cells, caused reorientation of commissural axons within the spinal cord explant (Figures 2B–2E, 2H, 2I, and 3). If Shh is indeed a chemoattractant, we would predict that the notochord, another source of Shh (but not of Netrin-1), should also attract the axons, which was indeed observed (Figures 2L and 2M).

To examine whether the Shh turning activity is mediated by the canonical Shh signaling mediator Smo, we tested whether it is blocked by cyclopamine—a highly specific Smo inhibitor shown to act by direct binding to the heptahelical bundle of Smo without affecting Shh production or processing (Chen et al., 2002; Frank-Kamenetsky et al., 2002; Incardona et al., 1998). Although cyclopamine had no effect on attraction by Netrin-1 (Figures 2J and 2K), it completely inhibited the chemoattractant activity of COS cells expressing Shh (Figures 2F and 2G) or of the notochord (Figures 2N and 2O). These results indicate that Shh expression is sufficient to cause commissural axon turning within spinal cord explants and that this effect requires Smo activity.

### Inhibition of Smo Interferes with the Netrin-1-Independent Attractant Activity of the Floor Plate

We next examined whether Shh signaling is required for the Netrin-1-independent attractant activity of the floor plate, using cyclopamine to inhibit Smo. Although cyclopamine had no effect on attraction by wild-type floor plate (Figures 2P–2S)—emphasizing again its specificity—it almost completely blocked the attractant activity of *Netrin-1*$^{-/-}$ floor plate (Figures 2T–2W); reorientation was observed in only a small fraction of the explants and only over a very short distance (Figure 3). What is the cause of the residual turning activity? The *Netrin-1* allele we use is a severe hypomorph rather than a complete null mutation, and we previously showed that a very small amount of residual wild-type Netrin-1 mRNA and protein is apparently made by the mutant floor plate (Serafini et al., 1996), which might account for the residual turning activity. Alternatively, that activity could be due to a third chemoattractant(s) made by floor plate. Nonetheless, these results indicate that the Netrin-1-independent attractant activity of floor plate in this assay is largely dependent on Smo activity.

### Shh Does Not Induce Commissural Axon Outgrowth

Floor plate tissue from *Netrin-1* mutant embryos is ineffective in promoting commissural axon outgrowth (Serafini et al., 1996; see also Figures 4A and 4B), indicating that Netrin-1 accounts for most or all of the outgrowth-promoting activity of floor plate cells in vitro, and suggesting that the Netrin-1-independent attractant factor from floor plate should not promote outgrowth. Consistent with this, Shh-secreting COS cells and notochord explants, unlike Netrin-1-secreting COS cells, were unable to induce commissural axon outgrowth (Figures 4C–4F, 4I). Similarly, recombinant Netrin-1 protein added to the culture medium elicited axonal outgrowth, but recombinant Shh protein did not (Figure 4H and data not shown). Moreover, COS cells secreting Shh did not alter the outgrowth-promoting effect of Netrin-1 added
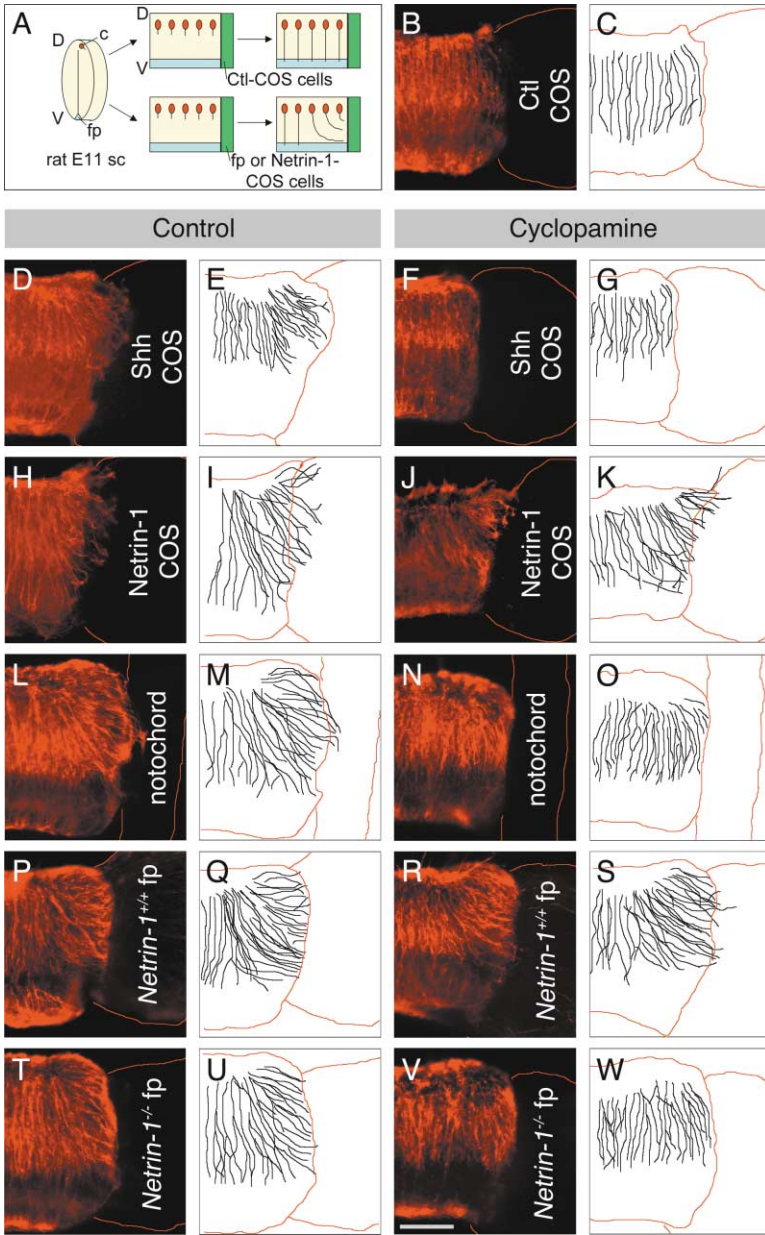
Figure 2. Commissural Axon Turning Induced by Shh-Expressing Cells or Tissues, and Its Cyclopamine-Dependence

(A) Schematic representation of the commissural axon turning assay. Explants of the entire E11 rat spinal cord were embedded in a three-dimensional collagen matrix and cocultured for 40 hr with various tissues or cells expressing Shh and/or Netrin-1.

(B, C) and left images (D, E, H, I, L, M, P, Q, T, and U): without cyclopamine; other right images (F, G, J, K, N, O, R, S, V, and W): with 10 μM cyclopamine. Commissural axon trajectories were detected by TAG-1 immunohistochemistry. D, dorsal; V, ventral; sc, spinal cord.

(B–O) Shh causes commissural axon turning within spinal cord explants in a cyclopamine-dependent manner. COS cells expressing Shh (D–E) or Netrin-1 (H–I) and notochord tissue (L–M), but not control COS cells (B–C), elicit commissural axon turning. Cyclopamine inhibits the attractant activity of COS cells expressing Shh (F–G) and of the notochord (N–O), but not of COS cells expressing Netrin-1 (J–K).

(P–W) Cyclopamine blocks the Netrin-1-independent attractant activity of the floor plate. Both wild-type (P–Q) and Netrin-1 mutant (T–U) floor plate tissue can elicit commissural axon turning, but cyclopamine blocks only the latter (V–W), not the former (R–S). Scale bars are 200 μm.

to the medium (Figure 4G). Thus, like *Netrin-1* mutant floor plate tissue, COS cells secreting Shh induce turning of commissural axons in dorsal spinal cord explants but not their outgrowth into collagen.

## Shh Chemoattractant Activity Is Not Apparently Due to Spinal Cord Repatterning

Although these results support a role for Shh and Smo in inducing the turning of commissural axons in vitro, they do not address how Shh elicits this effect. At least two models could account for its effects in axon guidance (Figure 5A). First, Shh could act directly as a chemoattractant, diffusing through the explants and acting directly on axons (Model 1). Alternatively, Shh could be acting indirectly on the axons by repatterning the explant, altering the expression of other guidance cues that then secondarily cause axon turning (Model 2). The

simplest version of Model 2 would involve the induction of expression of a distinct chemoattractant by cells adjacent to the Shh source, which then in turn diffuses and acts on growth cones to elicit turning. The reason it is necessary to consider Model 2 is that Shh is known to be able to repattern spinal cord tissue. However, this repatterning activity has been demonstrated at early stages of spinal cord development, namely at the neural plate stage in chick embryos (Ericson et al., 1996). It is not known whether older spinal cord tissue, like the E11 rat tissue used in our turning assays, would also be repatterned.

To assess whether repatterning was occurring, we performed E11 rat spinal cord turning assays using control COS cells, COS cells expressing Shh, or *Netrin-1*^{−/−} floor plate tissue, and performed immunohistochemistry to examine expression of the following markers:
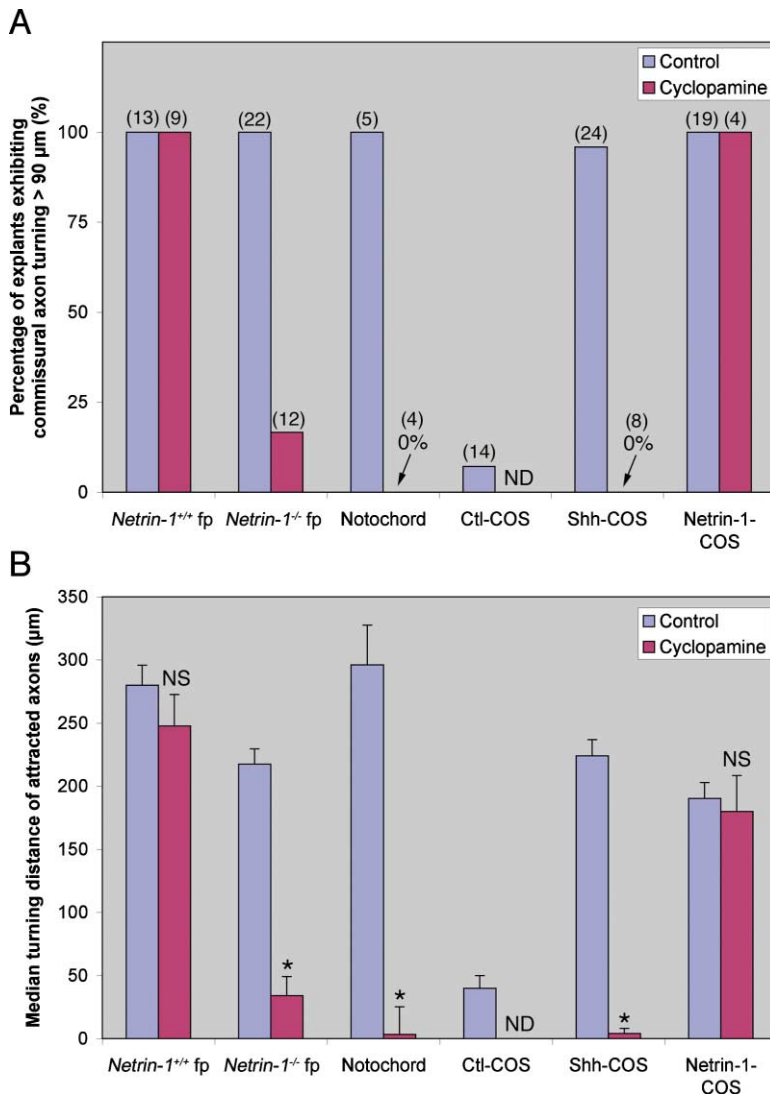
A



B



Figure 3. Cyclopamine Selectively Blocks Turning Induced by Shh-Expressing COS Cells and Tissues Expressing Shh but Not Netrin-1

This figure shows quantification of experiments in Figure 2 (see legend for conditions shown here).

(A) Percentage of explants exhibiting commissural axon turning over more than 90 μm. Numbers in parentheses indicate number of explants assayed.

(B) Quantification of the median turning distance of attracted commissural axons. For each explant, turning distance was measured from the edge of the explant to the most distant TAG-1$^+$ axon fascicle exhibiting turning. Error bars represent standard error of the mean. Asterisks (*): $p < 0.0002$ (Student's t test) compared to the same condition without cyclopamine. ND, not done; NS, not significant.

Note that in (A), explants were scored as positive only if turning was observed over more than 90 μm (when less turning was observed, this was usually seen for a very small number of axons in the roof plate region, where it was ambiguous whether the axons had actually turned or whether the edge of the explant has simply become distorted). Calculation of median turning distance in (B) was, however, calculated on all explants, explaining why non-zero median turning distances are seen for some conditions in which zero percent showed turning over more than 90 μm.

HNF-3β, a floor plate marker normally induced by high concentrations of Shh in neural plate explants; Nkx2-2, a marker of V3 interneurons, normally induced by high concentrations of Shh in neural plate explants; Isl-1 and HB9, two motoneuron markers normally induced by intermediate concentrations of Shh in neural plate explants; and Pax7, a dorsal spinal cord marker normally repressed by low concentrations of Shh in neural plate explants. Expression of HNF-3β, Isl-1, HB9, and Pax7 was unaffected by Shh or by the Netrin-1$^{-/-}$ floor plate tissue (Figures 5J, 5P, and 5V; 5L, 5R, and 5X; 5M, 5S, and 5Y; 5N, 5T, and 5Z). Importantly, the expression of Netrin-1 was also unaffected in these assays (Figure 5I, 5O, and 5U). Interestingly, the V3 interneuron marker Nkx2-2 was induced by both control COS cells and by COS cells expressing Shh (Figures 5K and 5Q), but it was not induced by Netrin-1 mutant floor plate (Figure 5W). Thus, induction of Nkx2-2 is caused by a factor made endogenously by COS cells, and does not correlate with turning activity since it was seen with control COS cells which do not induce turning but not with Netrin-1 mutant floor plate tissue which does. We con-

clude that the turning activity of Netrin-1 mutant floor plate tissue and Shh-expressing COS cells does not apparently reflect an ability of these tissues to repattern the E11 rat spinal cord, at least as assessed by these markers, presumably reflecting a loss of competence of spinal cord tissue to be repatterned by Shh as it ages. In control experiments, we tested the ability of Netrin-1$^{-/-}$ floor plate explants to repattern neural plate stage (E10) rat spinal cord explants. As expected, these floor plate explants were able to induce expression of Nkx2-2, Isl-1, and HB9, and to repress expression of Pax7 in younger spinal cord explants (Figures 5D–5G). Together, these results indicate that Shh is unable to repattern spinal cord explants at the stage used for the turning assays (E11).

## Shh Induces Turning of Growth Cones of Isolated Spinal Cord Neurons

To test directly whether Shh can act on growth cones to induce turning, we used an assay in which growth cones of individual embryonic Xenopus spinal axons in culture are exposed to gradients of soluble factors
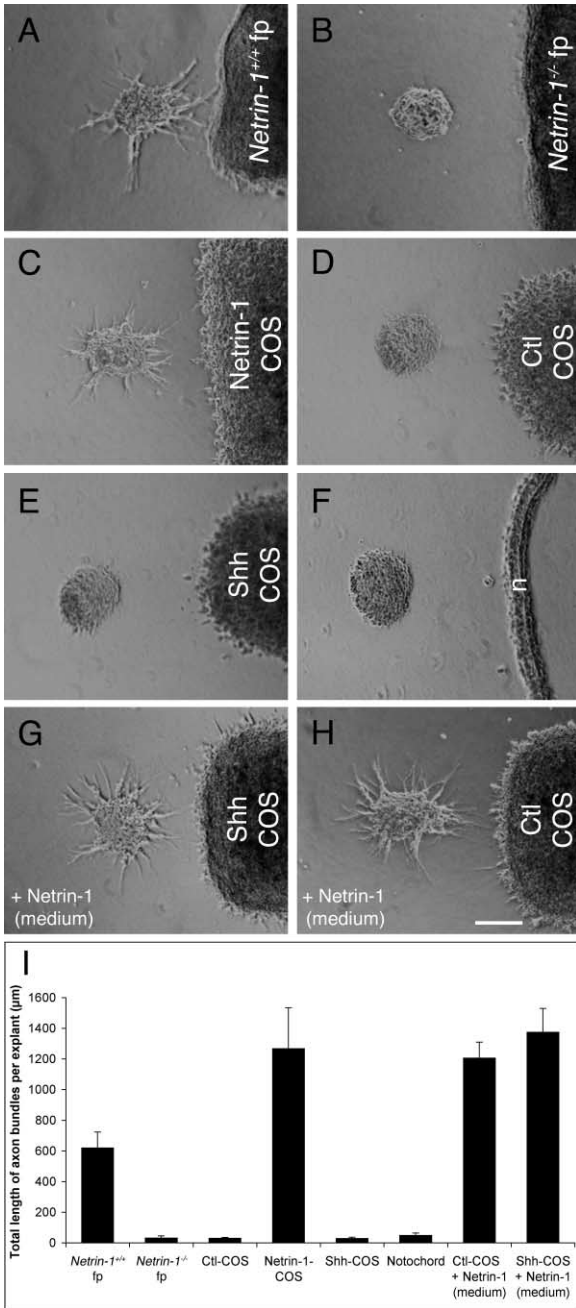
Figure 4. Shh Does Not Induce Commissural Axon Outgrowth into Collagen Gels

Rat E13 dorsal spinal cord explants were embedded in a three-dimensional collagen matrix and cocultured for 16 hr with (A) wild-type floor plate, (B) *Netrin-1* mutant floor plate, (C) COS cells expressing Netrin-1, (D and H) control (ctl) COS cells, (E and G) COS cells expressing Shh, and (F) notochord tissue.

In (G–H), Netrin-1 (100 ng/ml) was added in the culture media.

(I) Quantification of total length of axon bundles per explant. The data presented are the average of at least seven explants. Error bars represent the standard error of the mean. fp, floor plate; n, notochord. Scale bars are 110 μm.

established by repetitive pulsatile release from a micropipette (Zheng et al., 1994; de la Torre et al., 1997; Song et al., 1997). We tested the chemoattractant ability of N-Shh, a soluble amino-terminal fragment of Shh that

possesses the bioactivity of the full-length molecule in patterning assays (Ericson et al., 1996). Purified recombinant N-Shh was able to elicit axon attraction within 1 hr (Figure 6). As expected, this turning was almost totally inhibited by cyclopamine, whereas BDNF-induced turning (Song et al., 1997) was unaffected (Figure 6). Thus, Shh can act directly as a chemoattractant to induce growth cone turning in a Smo-dependent fashion.

## Conditional Inactivation of *Smo* Demonstrates a Cell-Autonomous Requirement for Hh Signaling in Commissural Axon Guidance In Vivo

These results are consistent with Shh functioning via Smo as a floor plate-derived chemoattractant for commissural axons. To determine the role of Shh in commissural axon guidance in vivo, we studied mice defective in Shh signaling. Unfortunately, *Shh*, *Ptc1*, and *Smo* null mice all exhibit defects in neural tube patterning that result in profound perturbation of the cellular environment where commissural axons normally migrate (Chiang et al., 1996; Goodrich et al., 1997; Zhang et al., 2001), precluding an examination of the specific role of Shh in chemoattraction. We therefore employed a more specific gene targeting technique. The fact that cyclopamine blocks Shh-induced turning indicates that Shh is acting through Smo to elicit chemoattraction. We therefore sought to inactivate *Smo* in commissural neurons without perturbing its function in the terrain traversed by their axons. Although *Smo* is expressed throughout the neural tube (Stone et al., 1996), we reasoned that *Smo* inactivation in commissural neurons would not affect their specification since Shh signaling is not required for dorsal spinal cord patterning (Briscoe et al., 2001; Wijgerde et al., 2002). Therefore, any commissural axon guidance defects observed after selective inactivation of Smo in these neurons would indicate that Smo signaling is required in vivo for commissural axon guidance and support a chemoattractant role for Shh.

To inactivate Smo selectively in commissural neurons, we used the Cre/loxP recombinase system. Since the *Wnt1* promoter drives expression in the dorsal spinal cord, we thought that *Wnt1-Cre* mice (Danielian et al., 1998) might be used to target Cre recombinase expression in dorsal commissural neurons. To test this possibility, we crossed *Wnt1-Cre* mice with ROSA26 Cre reporter mice (R26R; Soriano, 1999) and assessed β-Galactosidase (β-Gal) activity in the developing spinal cord. As shown in Figures 7A–7D, β-Gal activity was found in the dorsal spinal cord and neural crest cell derivatives, including the dorsal root ganglia. In addition, β-Gal activity was also detected in axons projecting to the floor plate and at the ventral commissure in E11.5 embryos (Figures 7C and 7D). Colocalization of TAG-1 and β-Gal proteins confirmed that these ventrally projecting axons are commissural axons (Figures 7E–7H), indicating that the *Wnt1-Cre* mice can be used to efficiently target Cre-mediated recombination in TAG-1^{+} commissural neurons. We cannot, however, be certain that all TAG-1^{+} commissural axons expressed β-Gal, i.e., that Cre induced recombination in all of them (see below). Importantly, no β-Gal activity was detected in the ventral spinal cord; thus, the *Wnt1-Cre* driver can be used to inactivate genes in many (and perhaps all)
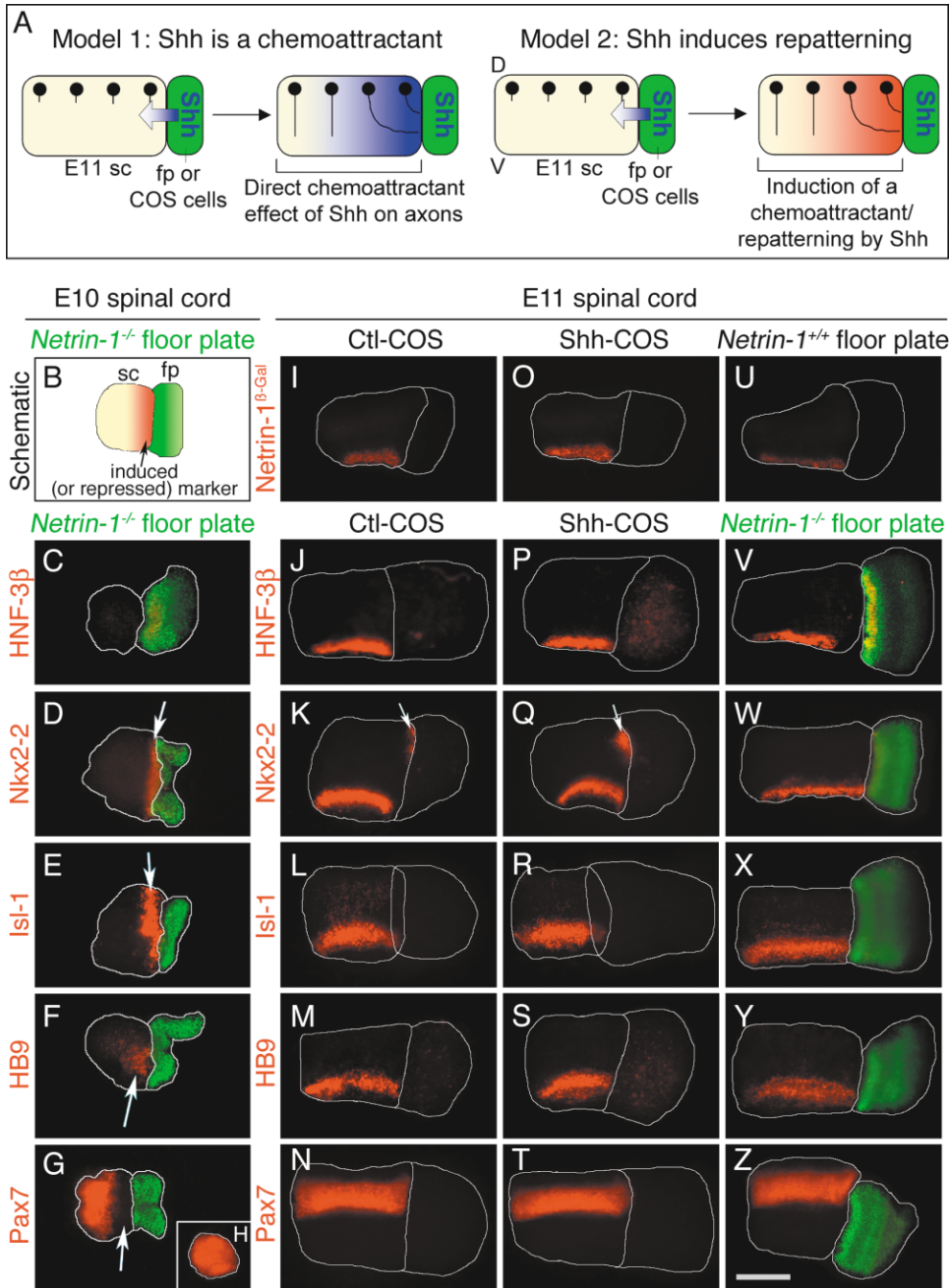
**Figure 5. Shh Chemoattractant Activity Is Not Apparently Due to Spinal Cord Repatterning**

(A) Two models could account for the effects of Shh in axon guidance. In Model 1, Shh acts directly as a chemoattractant. In Model 2, Shh acts by repatterning the spinal cord, altering the expression of other guidance cues that then secondarily reorient axon growth.

(B–H) *Netrin-1* mutant floor plate is able to repattern E10 rat spinal cord explants at the neural plate stage. As schematized in (B), E10 spinal cord explants obtained from the caudal-most (neural plate) level of the embryos were cultured under the same conditions as the E11 spinal cord explants used in the turning assays. After fixation, the explants were stained by immunohistochemistry for the following markers: HNF-3β, Nkx2-2, Isl-1, HB9, and Pax7 (red staining). *Netrin-1* expression is visualized using an antibody to β-Gal (expressed from the mutant *Netrin-1* allele) (green staining). Arrows indicate position where markers were induced or repressed. See text for details.

(H) (insert in G) is similar to (G), except that the E10 explant was cultured without floor plate to show lack of Pax7 repression.

(I, O, and U) wild-type floor plate or COS cells expressing Shh are not able to induce ectopic *Netrin-1* expression in whole mouse E9.5 *Netrin-1*^{+/−} spinal cord explants (oriented as diagrammed in A, i.e. with endogenous floor plate at bottom of each image). *Netrin-1* expression was detected by immunochemistry for β-Gal (red staining) driven from the mutant *Netrin-1* allele and is seen only in the normal floor plate region of the responding explants.

(J–N, P–T, and V–Z) *Netrin-1* mutant floor plate tissue (V–Z, stained in green by β-Gal immunohistochemistry) or COS cells expressing Shh (P–T), like control COS cells (J–N), are not able to repattern whole E11 rat spinal cord explants (oriented with endogenous floor plate at bottom in each image). Note that Nkx2-2 expression is induced dorsally by COS cells but not by floor plate.

Explants in (C–G) are representative of at least two explants and those in (I–Z) are representative of at least four explants. Scale bars are 200 μm in (C–G) and 320 μm in (I–Z).
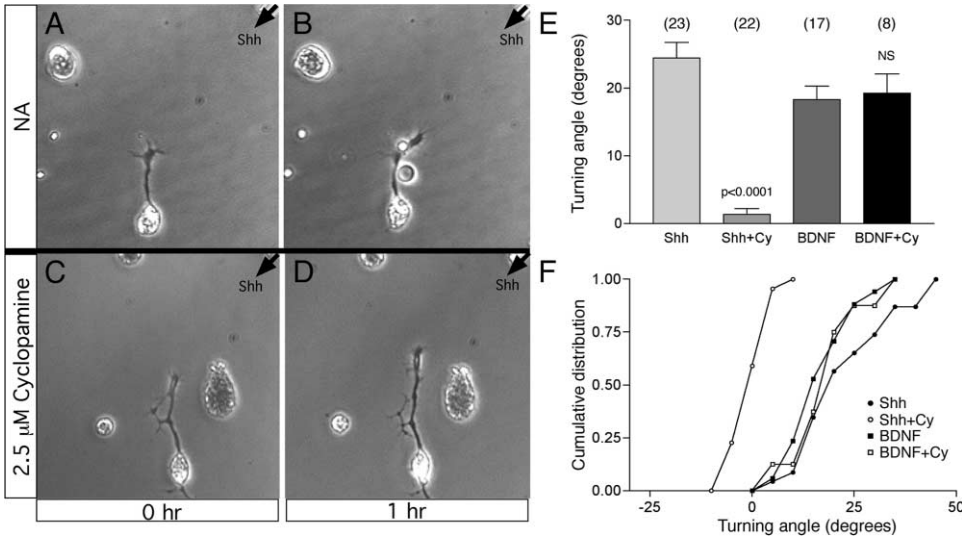
Figure 6. Shh Induces Turning of Growth Cones of Isolated *Xenopus* Spinal Cord Neurons

(A–D) A point source of purified recombinant N-Shh elicits growth cone attraction within one hour (A and B), an effect inhibited by cyclopamine (2.5 μM) (C and D).

(E) Average turning angle elicited by Shh and BDNF in presence and absence of cyclopamine. The numbers in parentheses indicate the number of neurons assayed. Error bars: standard error of the mean (p value determined using Student's t test). NS, not significant.

(F) Cumulative distribution of turning angles.

TAG-1^{+} commissural neurons without affecting the terrain through which their axons migrate.

We used, as a target for Cre, a conditional *Smo* allele (*Smo^c*) that acts as a wild-type allele but which, in presence of Cre, is converted to a null allele (Long et al., 2001). A constitutively null allele of *Smo* (*Smo^null*) has also been described (Zhang et al., 2001). To generate embryos with selective inactivation of *Smo* in commissural neurons, we crossed *Smo^c/c* mice with *Smo^null/wt*; *Wnt1-Cre* mice, and studied *Smo^c/null*;*Wnt1-Cre* progeny which should lack Smo activity in cells in which Cre has been active (and in their progeny), including in commissural neurons, but be heterozygous for Smo in other cells, including ventral spinal cord cells. As shown in Figures 7I–7L, immunohistochemical analysis of these embryos revealed abnormal projections of TAG-1^{+} commissural axons that resembled those in *Gli2* mutants lacking a floor plate: mutant commissural axons were highly defasciculated and some projected near the lateral edge of the spinal cord, forming a thick lateral bundle which then invaded the ventral spinal cord with multiple projections all over the motor columns (compare Figures 7J–7L with Figures 1J–1L). Although DCC expression in these embryos is observed at normal levels (data not shown), Netrin-1 chemoattraction was apparently not sufficient to fully compensate for the loss of Smo function in vivo. Quantification of the phenotype revealed a significant increase in the proportion of the spinal cord area occupied by TAG-1^{+} commissural axons, a phenotype also observed in *Gli2* mutant embryos, where it was slightly more severe (p < 0.05, Student's t test; Figure 7M). As mentioned above, we cannot be certain that Cre recombination occurred in all commissural neurons; if it did not, then some of the axons would be wild-type, and the phenotype we observed would be an underestimate of the true loss-of-Smo phenotype.

Despite these errors, most TAG-1^{+} commissural axons nonetheless ultimately appeared to reach the midline and form an apparently normal ventral commissure. As a control, the other progeny from this cross, i.e., *Smo^c/wt*, *Smo^c/null*, and *Smo^c/wt*;*Wnt1-Cre* animals, which possess either one or two functional copies of *Smo* in each cell, did not show any defects in commissural axon guidance (Figures 7I and 7M).

## Discussion

Floor plate cells stimulate outgrowth of commissural axons from explants of dorsal spinal cord and can attract commissural axons within the explants (Tessier-Lavigne et al., 1988; Placzek et al., 1990). Netrin-1 possesses both these in vitro outgrowth-promoting and chemoattractant activities (Serafini et al., 1994; Kennedy et al., 1994), and accounts for all the outgrowth-promoting activity of floor plate cells (Serafini et al., 1996). It does not, however, account for all their chemoattractant activity, since floor plate cells from *Netrin-1* knockout mice can still attract commissural axons (Serafini et al., 1996). Our results now identify Shh as a second chemoattractant made by floor plate cells that collaborates with Netrin-1 in commissural axon attraction in vitro, and which appears to be required for normal guidance of these axons to the floor plate in vivo.

### Shh Is a Chemoattractant for Commissural Axons Acting via Smo

Like floor plate cells and COS cells secreting Netrin-1, COS cells secreting Shh can reorient and attract the growth of commissural axons within explants of E11 rat spinal cord. The reorienting effect is blocked by cyclopamine, indicating a requirement for Smo in mediating this effect (Chen et al., 2002; Frank-Kamenetsky et al.,
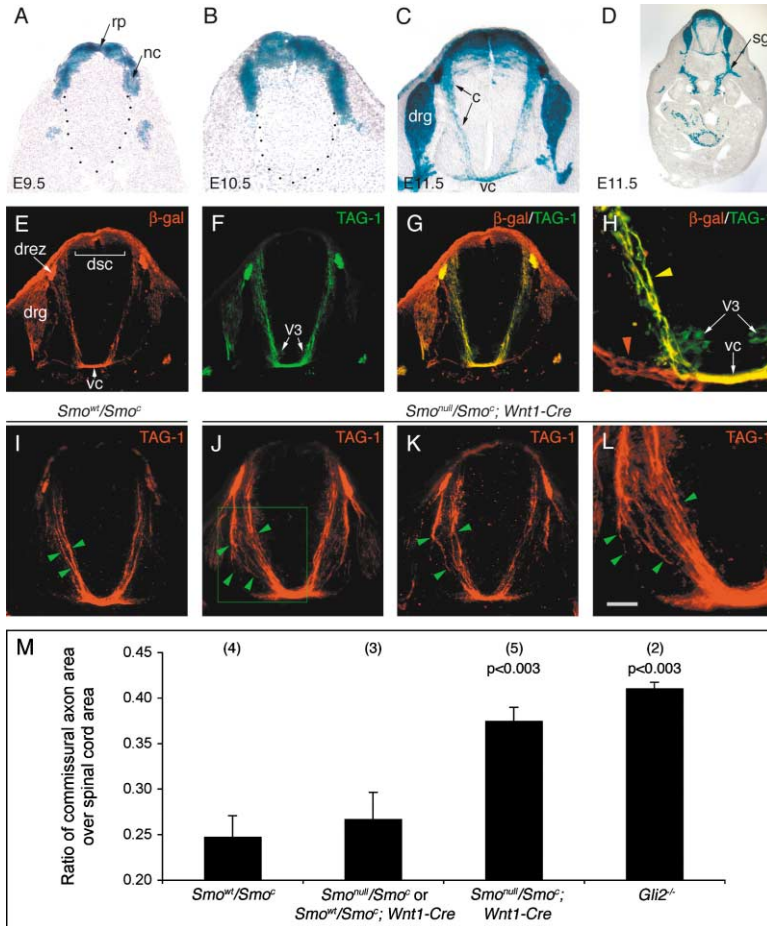
Figure 7. Conditional Inactivation of *Smo* Provides Evidence for a Cell-Autonomous Requirement for Shh Signaling in Commissural Axon Guidance In Vivo

(A–H) *Wnt1-Cre* mice can be used to efficiently target Cre-mediated recombination in commissural neurons, as assessed by analyzing sections of the progeny of crosses of *Wnt1-Cre* and R26R reporter mice for β-Gal activity. X-Gal staining of E9.5 (A), E10.5 (B), and E11.5 (C–D) mouse embryos shows β-Gal activity in the roof plate (rp), dorsal spinal cord (dsc), commissural axons (c), ventral commissure (vc), and neural crest cell derivatives (nc), including the dorsal root ganglia (drg) and the sympathetic ganglia (sg). Colocalization (G–H) of β-Gal (E) and TAG-1 (F) proteins confirms that these ventrally projecting axons are commissural axons (yellow arrowhead) whereas, as expected, V3 interneurons (V3) stained only for TAG-1, and postcrossing commissural axons stained only for β-Gal (since TAG-1 is downregulated on commissural axons after crossing) (red arrowhead).

(I–L) Embryos with selective inactivation of *Smo* in dorsal spinal cord were generated by crossing *Smo^{c/c}* mice with *Smo^{null/wt}*; *Wnt1-Cre* mice and analyzed by TAG-1 immunostaining. When compared to control (*Smo^{wt/c}*) embryos (I), commissural axons in *Smo^{c/null}*; *Wnt1-Cre* mutant embryos (J–L) were highly defasciculated and some projected near the lateral edge of the spinal cord (green arrowheads), forming a thick lateral bundle which then invaded the ventral spinal cord with multiple projections all over the motor columns, similar to what was observed in *Gli2* mutants. (J and K) are two different mutant embryos; (L) provides a close up view of (J).

(M) Quantification of the ratio of the area occupied by commissural axons to total spinal cord area (see Experimental Procedures). For each embryo, at least four sections were quantified and averaged. Each bar represents the mean of the averages obtained from different embryos (numbers analyzed indicated in parentheses). Error bars: standard error of the mean (p values determined using Student's t test). Scale bars are 50 μm in (A), 85 μm in (B), 90 μm in (C), 285 μm in (D), 140 μm in (E–G), 50 μm in (H), 120 μm in (I–K), and 60 μm in (L).

2002). Genetic, pharmacological, and biochemical studies have revealed an extreme specificity in the actions of cyclopamine (Chen et al., 2002; Frank-Kamenetsky et al., 2002; Incardona et al., 1998), which is evident here in the fact that cyclopamine specifically inhibits attraction mediated by Shh-secreting COS cells and by the notochord (an endogenous Shh source), but not by Netrin-1-secreting COS cells. Similarly, in the *Xenopus* spinal axon turning assay, cyclopamine blocked attraction by Shh but not by BDNF. These results support two conclusions. First, Shh-mediated chemoattraction appears to require Smo function—a result that was not necessarily anticipated, since no clear pathway from activated Smo to the cytoskeleton rearrangements required for growth cone turning has been described to date. Second, the finding that cyclopamine can almost entirely block the Netrin-1-independent activity of floor plate cells strongly implies that this activity is mediated by a member of the Hh family, which are the only known activators of Smo. Furthermore, of the three vertebrate Hh proteins, only Shh is made by floor plate cells (or indeed by any cells in the spinal cord); Desert Hedgehog (Dhh) and Indian Hedgehog (Ihh) are not expressed in the vicinity of the spinal cord (Zhang et al., 2001). Thus, in vitro, the cyclopamine-dependent chemoattractant activity of *Netrin-1* knockout floor plate is most likely mediated by Shh.

While the reorienting effect of Shh could be due to a direct chemoattractant effect, an alternative explanation is suggested by the fact that Shh is a potent morphogen. Since in our assays commissural axon turning occurs within the spinal cord tissue explant, it seemed possible that Shh was not acting directly on the axons but rather repatterning and altering the expression of guidance cues by cells within the explant, which then secondarily guided the axons to the Shh source. Arguing against this possibility is our finding that, whereas Shh is capable of repatterning newly formed spinal cord tissue in chick (Stage 10–12 embryos; Ericson et al., 1996) and in rat as well (E10; this study), we have found that the older E11 rat spinal cord explants used to assess chemoattractant activity have apparently lost the competence to be repatterned by Shh, as assessed using a battery of markers of dorsoventral patterning. Of course, such experiments can never rule out possible effects of Shh on genes that were not examined, even if this seems unlikely. To strengthen the conclusion that Shh is a chemoattractant, we therefore performed two other kinds of experiments.

First, we asked whether Shh can attract growth cones

in a dispersed cell assay, where potential indirect effects are ruled out. We found that Shh can indeed attract the growth cones of isolated *Xenopus* spinal axons in culture in a cyclopamine-dependent manner. This result proves that Shh, acting via Smo, can function as an axonal chemoattractant. It would, of course, be desirable to examine effects of Shh on isolated rodent commissural axons, but it has not proven so far possible to develop a turning assay using those axons. As a second way of confirming that Shh is acting directly on commissural axons, we sought to block Shh signaling selectively in commissural neurons without blocking it in the terrain through which the axons course. We achieved this by conditional inactivation of a floxed allele of *Smo* using the Cre recombinase expressed under the control of the *Wnt1* promoter. When Cre, driven by this promoter, is used to delete the floxed *Smo* allele in the dorsal spinal cord, commissural axon trajectories are defective in the ventral spinal cord, where Cre is not expressed. This result strongly implies that the axonal misrouting is not due to repatterning of the ventral spinal cord and must instead reflect a guidance defect arising from loss of Smo function in commissural neurons. The most parsimonious explanation, in light of our finding that Shh can attract isolated *Xenopus* spinal axons in a Smo-dependent fashion, is that the defects in commissural axon growth in the ventral spinal cord in the conditional *Smo* knockout mouse reflects loss of chemoattraction of those axons by Shh. The alternative possibility, that the defects are secondary to a defect in commissural neuron specification caused by a lack of Smo, is highly unlikely given a large body of evidence indicating that Shh is not accessible to the dorsal-most cells of the spinal cord and that specification of these cells, including commissural neurons (as assessed by Math1, LH2a/b, and Isl-1/2 expression), is normal in the absence of Shh signaling (Briscoe et al., 2001; Wijgerde et al., 2002). We also do not believe that the other two Hh proteins (Dhh and Ihh) are contributing to the in vivo guidance mediated by Smo, since they are not expressed in the vicinity of the spinal cord at these stages. These considerations together imply that the defect in commissural axon guidance observed in the Smo conditional animals most likely results from impairment of Shh-mediated chemoattraction.

Thus, taken together, our results strongly imply that Shh functions to guide commissural axons both in vitro and in vivo by acting directly as a chemoattractant on these axons through a Smo-dependent signaling mechanism.

### Roles of Netrin-1 and Shh in Commissural Axon Guidance

We have shown that in vitro both Shh and Netrin-1 can attract commissural axons within spinal cord explants. However, Shh, unlike Netrin-1, does not stimulate growth of commissural axons out of the explants into collagen gels, an observation consistent with the fact that Netrin-1 accounts for all of the outgrowth-promoting activity of floor plate cells in vitro (Serafini et al., 1996) and that Shh does not alter Netrin-1-stimulated outgrowth from such explants (Trousse et al., 2001; this study). We believe that the distinct actions of the two

proteins on commissural axons are reflected in distinct axon outgrowth and axon guidance phenotypes attributable to loss of Netrin-1 or Shh function in vivo, which we discuss in turn.

First, the outgrowth-promoting activity of Netrin-1 but not Shh seen in vitro appears to reflect a role for Netrin-1 in allowing invasion of the ventral spinal cord. In the Netrin-1 knockout mouse, two defects are observed: many commissural axons are misrouted, and many are foreshortened, failing to enter the ventral spinal cord (Serafini et al., 1996; this study). In contrast, in the *Smo* conditional knockouts (interpreted as being defective in Shh-mediated chemoattraction), the axons appear to be able to invade the ventral spinal cord normally, showing primarily a guidance, not a growth defect. Thus, it is tempting to propose that the ventral spinal cord is normally non-permissive to commissural axon growth in the absence of Netrin-1, and that Netrin-1 allows the axons to invade this non-permissive region, just as it can allow them to invade the non-permissive environment of a collagen gel in vitro. Shh does not, apparently, possess this permissive activity, since it cannot stimulate outgrowth into collagen in vitro and is not sufficient to allow normal growth of the axons in the ventral spinal cord in vivo in the absence of Netrin-1.

Second, the ability of both Shh and Netrin-1 to attract commissural axons in vitro appears to reflect a chemoattractant role for both of these proteins in vivo. In the presumed absence of Shh chemoattraction (in the *Smo* conditional knockout), many commissural axons fail to turn appropriately toward the ventral midline as they enter the ventral spinal cord, instead continuing inappropriately along a vertical dorsoventral trajectory. Interestingly, most of these misrouted axons appear to correct their errors, turning toward the ventral midline from a more ventral position. This error correction appears to be due at least in part to attraction by floor plate-derived Netrin-1, since in the *Gli2* mutants, which lack floor plate cells and hence both Shh and midline-derived Netrin-1, the defects are more severe: a greater proportion of axons head further ventrally than in the *Smo* conditional knockout, which is reflected in the fact that commissural axons occupy a slightly larger area in the spinal cord (Figure 7M). It should be noted that in the *Gli2*$^{-/-}$ animals, Netrin-1 mRNA expression is still present in the ventricular zone (Figure 1C), apparently providing enough Netrin-1 to overcome the non-permissive environment of the ventral spinal cord. Although the comparison of the *Smo* conditional knockout to the *Gli2* knockout thus implies that both Shh and Netrin-1 normally contribute to attracting the axons to the midline, it is complicated by the fact that we cannot be certain that Smo function is abrogated in all commissural axons. Determining the exact contribution of midline-derived Netrin-1 (independent of Shh) to attraction will thus require examining animals in which Netrin-1 expression is selectively deleted at the midline.

The most severe phenotype was observed in *Gli2*$^{-/-}$;*Netrin-1*$^{-/-}$ double mutants, which lack all Netrin-1, and also lack other midline-derived activities (including Shh) owing to the absence of floor plate in these animals. Because of the lack of Netrin-1, extension of the axons in the ventral spinal cord is profoundly impaired in these animals, and is more severely affected than in the *Netrin-*
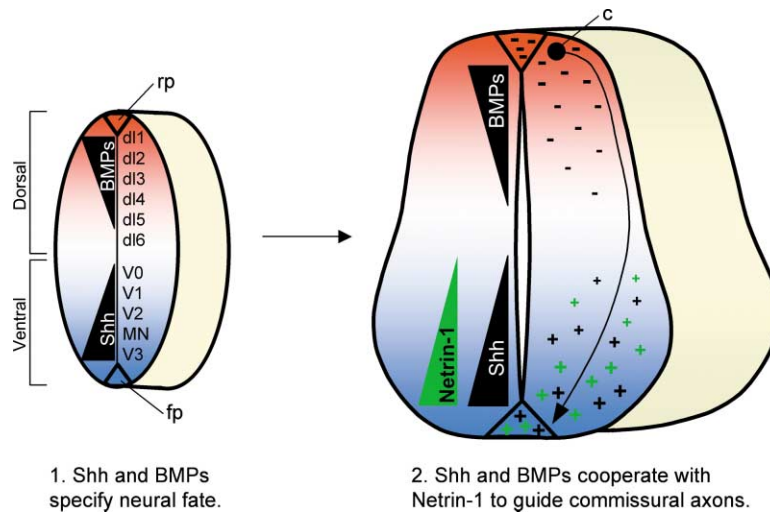
Figure 8. Guidance of Commissural Axons to the Midline by Netrin-1 and Morphogen Gradients

Two sets of morphogens, Shh and BMPs, are first used to pattern neural progenitors in the spinal cord, and then appear to be reused as guidance cues for commissural axons. In the early neural tube, Shh and BMP protein concentration gradients act to specify neural cell fate in the ventral and dorsal spinal cord, respectively. Later, the axons of differentiated commissural neurons are repelled from the dorsal midline by BMPs and attracted to the ventral midline by the combined chemoattractant effects of Netrin-1 and Shh. Netrin-1 also provides an essential permissive activity, allowing invasion of the otherwise non-permissive ventral spinal cord. rp, roof plate; fp, floor plate; c, commissural neurons.

1. Shh and BMPs specify neural fate.

2. Shh and BMPs cooperate with Netrin-1 to guide commissural axons.

$1^{-/-}$ single mutants, with essentially no axons reaching the ventral midline from the dorsal spinal cord—presumably reflecting the additional effect of loss of Shh attraction.

Although our results support a collaborative role for Shh and Netrin-1 in the guidance of commissural axons to the midline, they do not imply that Shh and Netrin-1 are the only two factors involved. First, our in vitro results show that there is a small amount of residual turning evoked by *Netrin-1* mutant floor plate in the presence of cyclopamine. This might be due to the fact that the mutant *Netrin-1* allele is not a complete null mutant (see Results; Serafini et al., 1996), so that residual Netrin-1 protein in the mutant might account for the residual turning. Alternatively, it is possible that this small turning activity is due to yet another chemoattractant(s) expressed by floor plate cells. Second, midline attractants are not necessarily the only factors guiding the axons. It is conceivable, for instance, that repellents in the ventrolateral regions of the spinal cord function to push commissural axons toward the midline. Examination of *Netrin-1/Smo* conditional double mutants should make it possible to define how much of the commissural axon guidance is accounted for by the combined actions of Netrin-1 and Shh.

**Reusing Morphogens to Guide Spinal Axons**
The specification of neuronal cell types along the dorsoventral axis of the spinal cord is known to be due to the antagonist effects of two sets of morphogens: Shh, derived from floor plate cells at the ventral midline, which specifies ventral cell types in a dose-dependent fashion, and BMP family members, derived from roof plate cells at the dorsal midline, to specify dorsal cell types (Jessell, 2000; Lee and Jessell, 1999). Our results imply that Shh is reused to attract commissural axons to the ventral midline. Importantly, Dodd and colleagues have provided evidence that BMPs also function to guide commissural axons, by repelling them away from the dorsal midline (Augsburger et al., 1999). Thus, together, these findings provide a pleasing result: Shh and BMPs, which initially cooperate to pattern cell types along the dorsoventral axis through antagonistic inductive effects, later

appear to cooperate to guide commissural axons ventrally through antagonistic guidance effects: one attracts and the other repels, but they cooperate rather than compete because the attractant is at the ventral midline, providing a pull, and the repellent is at the dorsal midline, providing a push (Figure 8).

Thus, it appears that the embryo, sensibly, recycles morphogen gradients for use in spinal axon guidance. From an evolutionary point of view, the use of Shh and BMPs for dorsoventral axon guidance appears to be a recent addition to Netrins, whose use for that purpose is more ancient. Indeed, Netrins are involved in attracting axons to the ventral midline in nematodes, insects, and vertebrates, although in all of these organisms loss of Netrin function only partially impairs growth to the midline (Hedgecock et al., 1990; Harris et al., 1996; Mitchell et al., 1996; Serafini et al., 1996). However, different cooperating guidance cues appear to be used in different species. Thus, *Hedgehog* genes are not present in the nematode genomes, and ventral guidance in *C. elegans* is instead due to attraction by the Netrin UNC-6 at the ventral midline and repulsion away from the dorsal midline by a Slit protein (Hao et al., 2001); in *Drosophila*, the identity of the cues that collaborate with Netrins in midline guidance are not known. It appears that during vertebrate evolution, establishment of Hh-BMP antagonistic gradients for dorsoventral patterning of the neural tube provided a useful set of molecular gradients that could be readily coopted for axon guidance, adding to the guidance already provided by Netrins.

**Morphogens as Guidance Molecules**
Our finding of a role for Shh in commissural axon guidance amplifies observations in other systems that have suggested other roles for Hh family members in the control of motility and migration (Marti and Bovolenta, 2002). In *Drosophila*, Hh has been proposed to serve as an attractive cue to guide germ cell migration through the embryo to form the primitive gonad (Deshpande et al., 2001)—a positive role that parallels the one we have suggested here in axon guidance. Conversely, negative effects of Hh family members on motility have been described in two sets of in vitro experiments in verte-

brates. Addition of Shh to neural tube explants was shown to inhibit neural crest cell migration, apparently due to decreased integrin-mediated cell adhesion (Testaz et al., 2001). Shh was also shown to suppress retinal ganglion axon outgrowth from retinal explants into collagen gels and to be capable of causing retinal growth cone collapse when added acutely in culture (Trousse et al., 2001). Our results, together with these other studies, suggest that Hh proteins might function widely in the control of cell motility and axon guidance and may, like other guidance molecules (Tessier-Lavigne and Goodman, 1996), be bifunctional, attracting some axons or cells, and repelling other axons or cells.

It will also be interesting to examine how extensively other morphogens are reused for axon guidance after they have performed their first job—patterning tissues. As mentioned, BMP family members have been implicated in spinal axon guidance (Augsburger et al., 1999), and in *C. elegans*, the divergent BMP family member UNC-129 is required for axon guidance (Colavita et al., 1998; although it is not yet known whether it is involved directly or indirectly in guidance). Furthermore, evidence is mounting that the other well-characterized morphogens, members of the Wnt family, also play roles in axon growth and remodeling (Hall et al., 2000; Wang et al., 2002). Future studies will reveal how widely morphogens are used in axon guidance, and whether they function not just in the embryo but also in axonal regeneration in the adult nervous system.

## Experimental Procedures

### Mouse Strains
*Netrin-1* mutant (Serafini et al., 1996), *Wnt1-Cre* (Danielian et al., 1998), and *Gli2* mutant mice (Mo et al., 1997) were maintained in a CD-1 background and genotyped as described. R26R (Soriano, 1999), *Smo^c/c^* (Long et al., 2001), and *Smo^null/wt^* (Zhang et al., 2001) mice were maintained and genotyped as described.

### Repatterning, Turning, and Outgrowth Assays
E11 whole rat spinal cord explants (for turning assays) and E13 rat dorsal spinal cord explants (for outgrowth assays) were dissected and embedded in three-dimensional collagen matrices as described (Tessier-Lavigne et al., 1988; Serafini et al., 1996) and cultured in 45% OptiMEM-1 (GIBCO BRL), 50% F12 (GIBCO BRL), 5% fetal bovine serum (FBS), 40 mM glucose, 2 mM GlutaMAX I (GIBCO BRL), 100 μg/ml streptomycin sulfate, and 100 U/ml penicillin G. Where indicated, cyclopamine (10 μM) was added to the culture media. For repatterning assays, E10 rat spinal cord explants were obtained from the caudal-most (neural plate) level of the embryo. Explants were cultured for 40 hr for turning and repatterning assays, and for 16 hr for outgrowth assays, either alone or with E13 rat notochord, E11.5 mouse floor plate (from wild-type or *Netrin-1* mutant mice), or COS cell aggregates transfected with a control, Netrin-1, or Shh-expression plasmid. When indicated, outgrowth was elicited by adding 100 ng/ml of purified Netrin-1 to the culture media.

### *Xenopus* Turning Assays
Cultures of *Xenopus* spinal neurons prepared from neural tube tissue of stage 22 embryos and growth cone turning assays were performed as described (Stein and Tessier-Lavigne, 2001) using BDNF (Calbiochem; 50 μg/ml) and N-Shh (R&D Systems; 2 μg/ml). Cyclopamine (2.5 μM) was added in the medium 30 min before presenting N-Shh or BDNF.

### Immunohistochemistry
Immunohistochemistry on sections of embryos and on explants was performed as described (Kennedy et al., 1994; Serafini et al., 1996).

The TAG-1 (clone 4D7, dilution 1:200), β-Gal (clone 40-1a, dilution 1:400), HNF-3β (clone 4C7, dilution 1:20), Nkx2-2 (clone 74.5A5, dilution 1:20), Isl-1 (clone 40.2D6, dilution 1:20), HB9 (clone 81.5C10, dilution 1:20), and Pax7 (dilution 1:20) monoclonal antibodies were obtained from the Developmental Studies Hybridoma Bank (University of Iowa). The rabbit polyclonal β-Gal antibody (dilution 1:200) was from Novus Biologicals.

### Quantification of the Area Occupied by Commissural Axons
To quantify the ratio of the area occupied by commissural axons to the total area of the spinal cord (Figure 7M), areas were measured by tracing their edges on TAG-1-stained E11.5 embryo cross-sections using NIH Image software. For each embryo, at least four sections were quantified and averaged. Each bar represents the mean of the averages obtained from different embryos.

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