Novel brain wiring functions for classical morphogens: a role as graded positional cues in axon guidance

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Summary

During embryonic development, morphogens act as graded positional cues to dictate cell fate specification and tissue patterning. Recent findings indicate that morphogen gradients also serve to guide axonal pathfinding during development of the nervous system. These findings challenge our previous notions about morphogens and axon guidance molecules, and suggest that these proteins, rather than having sharply divergent functions, act more globally to provide graded positional information that can be interpreted by responding cells either to specify cell fate or to direct axonal pathfinding. This review presents the roles identified for members of three prominent morphogen families – the Hedgehog, Wnt and TGFβ/BMP families – in axon guidance, and discusses potential implications for the molecular mechanisms underlying their guidance functions.

Introduction

Neuronal connections form during embryonic development when neurons send out axons, tipped at their leading edge by the growth cone, which migrate through the embryonic environment to their synaptic targets. Studies of developing axonal projections have revealed that axons extend to the vicinity of their appropriate target regions in a highly stereotyped and directed manner by detecting a variety of attractive and repulsive molecular guidance cues presented by cells in the environment (Dickson, 2002; Tessier-Lavigne and Goodman, 1996).

In the 1990s, genetic, biochemical and molecular approaches together identified four major conserved families of guidance cues with prominent developmental effects: the Netrins, Slits, Semaphorins and Ephrins (Dickson, 2002; Tessier-Lavigne and Goodman, 1996). Netrins, Slits and some Semaphorins are secreted molecules that associate with cells or the extracellular matrix, whereas Ephrins and other Semaphorins are anchored to the cell surface. Netrins can act as attractants or repellents; Slits, Semaphorins and Ephrins act primarily as repellents but can be attractive in some contexts. For each of these sets of cues, one or more families of transmembrane receptors have been identified: DCC and UNC-5 receptors for Netrins, Roundabout (Robo) receptors for Slits, Neuropilin and Plexin receptors for Semaphorins, and Eph receptors for Ephrins. In addition to these 'classic' axon guidance molecules, some growth factors, including Neurotrophins and Scatter Factor/Hepatocyte Growth Factor (SF/HGF), have been implicated in axon guidance (Ebens et al., 1996; O'Connor and Tessier-Lavigne, 1999; Tucker et al., 2001).

Although the identification of these major guidance cues has increased our understanding of how the nervous system is wired, many guidance events observed during development do not appear to be accounted for by these molecules. Moreover, the number of guidance cues and receptors identified seem small relative to the immense complexity of nervous system wiring; thus, additional guidance cues and receptors probably remain to be discovered. Remarkably, over the last few years, members from three other families of secreted signaling molecules have been shown to act as guidance cues: the Wingless/Wnt, Hedgehog (Hh) and Decapentaplegic/Bone Morphogenic Protein/Transforming Growth Factor β (Dpp/BMP/TGFβ) families. In addition to their axon guidance properties, these molecules share a common characteristic of having been previously identified as morphogens controlling cell fate and tissue patterning. This discovery has facilitated the study of an entirely new set of axon guidance cues and changed our current notions about morphogenic and axon guidance molecules. Additionally, it suggests that these proteins can be thought of more generally as providing graded positional information, which can be interpreted by responding cells as either a cell-fate specification signal or one for axonal pathfinding.

Here, we focus on the emerging evidence that these three morphogen families are reused later in development to guide axons, and compare the similarities and differences in how they provide positional information that can be interpreted for axon guidance versus cell fate specification. Additionally, we also briefly discuss the increasingly appreciated role for morphogens in cell migration (Box 1).

Morphogens, cell fate specification and tissue patterning

Morphogens are signaling molecules produced in a restricted region of a tissue that provide positional information by

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diffusing from their source to form a long-range concentration gradient. A cell's program of differentiation in response to a morphogen is dictated by its position within the gradient and thus on its distance from the morphogen source. Two criteria determine whether a secreted signaling protein acts as a morphogen: it must have a concentration-dependent effect on its target cells and it must exert a direct action at a distance. To date, only three protein families have members that fulfill these criteria: the Wingless/Wnt, Hh and Dpp/BMP/TGFβ families (Teleman et al., 2001). Although there is abundant evidence for concentration-dependent activity of signaling proteins during development (reviewed by Gurdon et al., 1998), evidence for direct action at a distance has only been provided recently in some vertebrate systems (Chen and Schier, 2001; Briscoe et al., 2001). In the following section, we summarize briefly some of the biological processes that involve members from each morphogen family, with a special emphasis on vertebrate neural tube development, which provides a convenient system in which to compare and contrast roles of classic guidance molecules and morphogens in axon guidance.

In vertebrate embryos, one of the first steps in nervous system development is the specification of the diverse neural cell fates. Members of each of the three morphogen families are expressed in the developing neural tube and are implicated in its patterning, as summarized below.

Box 1. Morphogens and cell migration

In addition to their role in cell fate specification and axon guidance, a role for morphogens in cell migration is also becoming increasingly appreciated. 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). In vertebrates, a negative effect of Shh on cell migration has been described: the addition of Shh to neural tube explants has been shown to inhibit neural crest cell migration in vitro (Jarov et al., 2003; Testaz et al., 2001). Although the negative effect of Shh on neural crest cells was independent of the canonical Ptc/Smo signaling pathway, and was apparently due to decreased integrin-mediated cell adhesion, the positive effect of Hh on germ cells involved the canonical Ptc, Smo and Fu signaling components.

Several TGFβ family members, including BMP7, have been shown to elicit chemotaxis by peripheral blood monocytes and polymorphonuclear leukocytes (Cunningham et al., 1992; Postlethwaite et al., 1994). Although the cytoskeletal mediators of BMPs in chemotaxis are not known, it was found that LIM kinase, a key regulator of actin dynamics, directly interacts with the type II BMP receptor and is activated by BMP stimulation, suggesting a direct link between BMPs and the cytoskeleton (Foletta et al., 2003).

In *Drosophila*, Wnt4 is required for cell movement during ovarian morphogenesis (Cohen et al., 2002). However, these data are inconsistent with a role for Wnt4 in providing a polarizing cue; rather they indicate that Wnt4 promotes the motility of apical cells through the regulation of focal adhesions. As this effect requires Fz2, Dsh and PKC, the Wnt4 cell motility pathway identified appears to be distinct from the canonical Wnt pathway and the PCP pathway. Wnt signaling has also been implicated in the control of cell movement in vertebrates (Strutt, 2003), but the mechanism through which Wnt proteins influence motility in these experiments is unclear.

Together, these studies suggest that morphogens might function widely in the control of cell motility and axon guidance, a finding reminiscent of the growing role that classical axon guidance molecules play in cell migration (Hinck, 2004).

The Hedgehog family

Hedgehog proteins are found in insects and vertebrates, but not nematodes. There is a single Hedgehog gene in flies, and three in mammals: Sonic hedgehog (Shh), Indian hedgehog (Ihh) and Desert hedgehog (Dhh). Shh is secreted by the notochord and by floor-plate cells at the ventral midline of the neural tube, and functions as a graded signal for the generation of distinct classes of ventral neurons along the dorsoventral (DV) axis of the neural tube (Fig. 1A) (reviewed by Jessell, 2000; Ingham and McMahon, 2001; Marti and Bovolenta, 2002). In agreement with its role as a morphogen, Shh is able to induce a range of ventral spinal cord cell fates in a concentration-dependent manner (Roelink et al., 1995) and has been shown to exert a direct action at a distance to specify neural tube cell fate (Briscoe et al., 2001).

Much evidence indicates that these cell-fate-specification and tissue-patterning activities of Hhs are mediated by members of the Ci/Gli transcription factor family, but the signaling mechanisms that lead to the activation of these transcription factors are not fully elucidated (Ingham and McMahon, 2001). Genetic and biochemical experiments have shown that Hhs activate signaling by binding to their receptor Patched (Ptc), which leads to the relief of Ptc-mediated inhibition of Smoothened (Smo), also a transmembrane protein, which can then activate downstream signaling (Fig. 2A). Smo associates directly with a Ci-containing complex, which contains the atypical kinesin Costal 2 (Cos2) and the protein kinase Fused (Fu) (Lum and Beachy, 2004). This complex constitutively suppresses pathway activity. Activation of Hh signaling reverses this regulatory effect and allows Ci to activate transcription of Hh target genes, thus specifying cell fate.

The Decapentaplegic/Bone Morphogenic Protein/Transforming Growth Factor-β family

The roof plate at the dorsal midline of the neural tube is the major source of inductive signals controlling the generation of dorsal cell types (Lee and Jessell, 1999). Around the time when dorsal neurons are generated, the roof plate expresses many members of the Dpp/BMP/TGFβ family, and some of them are required for the normal specification of dorsal neurons (Fig. 1A) (Lee et al., 1998). Whether they function specifically as morphogens in this system remains to be determined (Jessell, 2000).

Members of the Dpp/BMP/TGFβ family regulate cell fate by inducing the dimerization of type I and type II TGFβ receptors, resulting in phosphorylation and activation of the intracellular kinase domain of the type I receptor (Fig. 2B). Targets of the type I receptor are the receptor-regulated Smads (R-Smads) which, upon phosphorylation, associate with co-Smads and translocate to the nucleus where they activate transcription.

The Wingless/Wnt family

Roof-plate cells also express several members of the Wnt family (reviewed by Lee and Jessell, 1999). Although Wnt1

and Wnt3a are required for normal specification of dorsal neurons (Muroyama et al., 2002), it also remains an open question whether they function specifically as morphogens in this system.

Wnt ligands can activate several different signal transduction pathways. The most extensively studied is the canonical Wnt pathway, which controls gene expression by stabilizing β-Catenin (Fig. 2C). This pathway involves evolutionarily conserved cellular components (reviewed by Nelson and Nusse, 2004; Strutt, 2003). Frizzled (Fz) proteins are seven-transmembrane-domain molecules that function as Wnt receptors. When Wnts are absent, β-Catenin is phosphorylated by GSK3β, leading to its degradation. Binding of Wnts to their receptors results in Dishevelled (Dsh) activation and suppression of GSK3β activity, thus stabilizing β-Catenin. Accumulated β-Catenin converts the lymphoid enhancer factor (Lef)/Tcf from a transcriptional repressor to an activator.

Recently, much attention has been given to two β-Catenin-independent non-canonical Wnt pathways, the Wnt/Ca^{2+} pathway and the planar cell polarity (PCP) pathway (Fig. 2C). The PCP pathway involves a non-canonical, β-Catenin-independent, Wnt/Fz pathway that requires Dsh. The Wnt/Ca^{2+} pathway is thought to signal via heterotrimeric G-proteins to mobilize intracellular Ca^{2+} and, in some contexts, to stimulate protein kinase C (PKC).

Below, we discuss the functions of these morphogen families in axon guidance.

Morphogens in axon guidance

The Hedgehog family

Shh is a chemoattractant for commissural axons

During spinal cord development, commissural neurons, which differentiate in the dorsal neural tube, send axons that project toward and subsequently across the floor plate, forming axon commissures (Fig. 1B) (see also Colamarino and Tessier-Lavigne, 1995). These axons project toward the midline in part because they are attracted by Netrin 1 (Ntn1), a long-range chemoattractant secreted by the floor plate (Kennedy et al., 1994; Placzek et al., 1990; Serafini et al., 1996; Serafini et al., 1994; Tessier-Lavigne et al., 1988). In mice mutant for *Ntn1* 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 cooperate with Ntn1 to guide these axons. Further analyses of *Ntn1* knock-out mice have suggested that the floor plate might actually express an additional diffusible

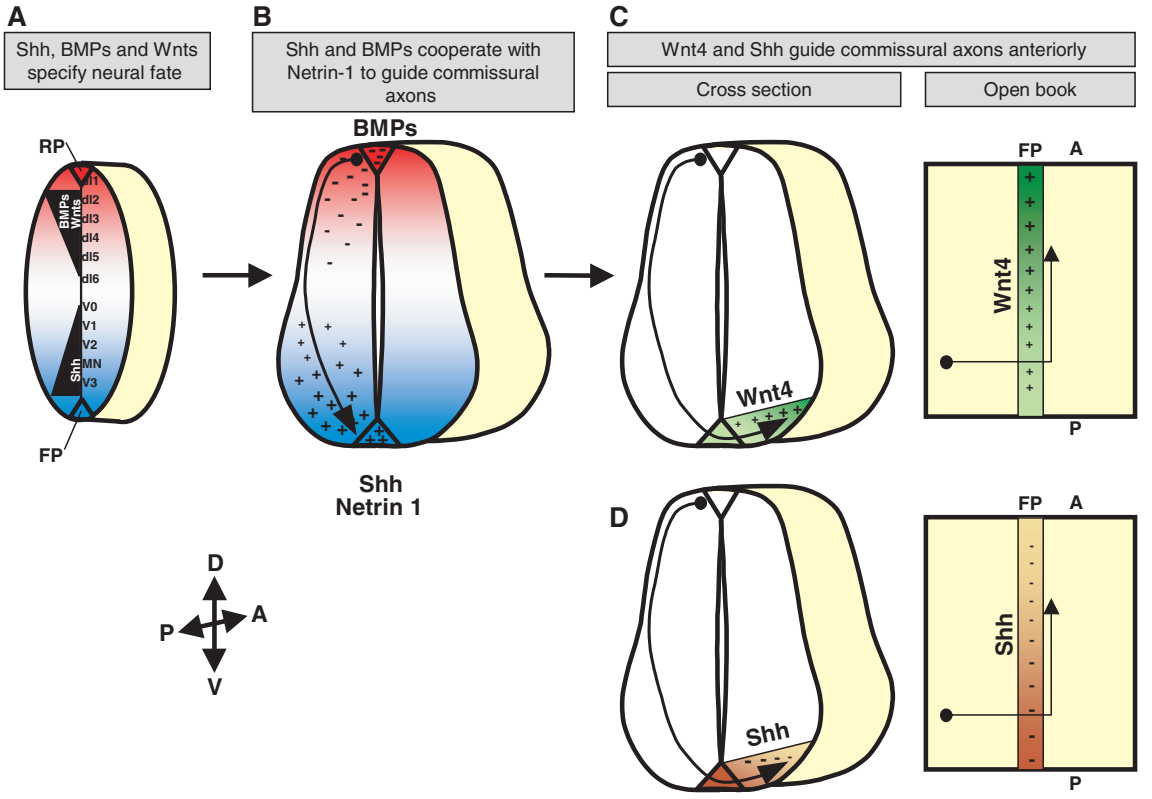

Fig. 1. Neuronal cell fate specification and guidance of commissural axons by morphogens and Netrin 1. Three sets of morphogens, Shh, BMPs and Wnts, are first used to pattern neural progenitors in the spinal cord, and then appear to be reused as guidance cues for commissural axons. (A) In the early neural tube, Shh, BMP and Wnt protein concentration gradients act to specify neural cell fate in the ventral and dorsal spinal cord. (B) Later, the axons of differentiated commissural neurons are repelled from the dorsal midline by BMPs (red) and attracted to the ventral midline by the combined chemoattractant effects of Netrin 1 and Shh (blue). (C,D) After crossing the floor plate, commissural axons are attracted anteriorly by a Wnt4 gradient (C, green) and repelled from the posterior pole by a Shh gradient (D, orange). A and B, and left panels in C and D, are cross section representations of the developing spinal cord; right panels in C and D are open book representations. V0-3, ventral interneuron sub-populations; dI1-6, dorsal interneuron sub-populations; MN, motoneurons; RP, roof plate; FP, floor plate; D, dorsal; V, ventral; P, posterior; A, anterior.

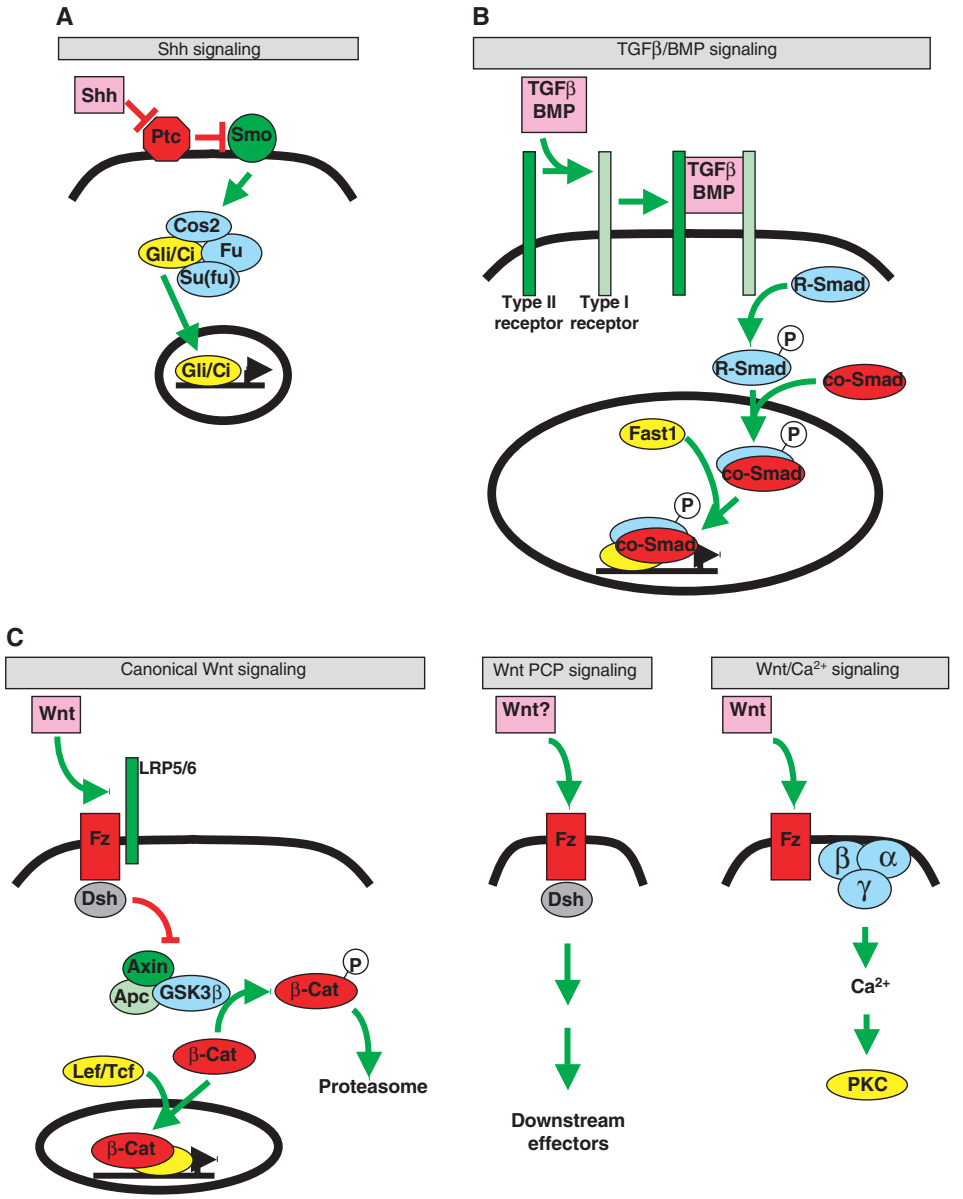

Fig. 2. Overview of the Shh, TGFβ/BMP and Wnt morphogenic signaling pathways. (A) Shh signaling pathway. Genetic and biochemical experiments have shown that Hhs activate signaling by binding to their receptor Patched (Ptc; a 12-pass transmembrane protein), which leads to the relief of Ptc-mediated inhibition of Smoothened (Smo), a seven-pass transmembrane (7TM) protein, which can then activate downstream signaling. Smo associates directly with a Ci-containing complex, which contains the atypical kinesin Costal 2 (Cos2), the protein kinase Fused (Fu) and the Suppressor of Fused [Su(fu)]. This complex constitutively suppresses pathway activity by leading to the proteolytic cleavage of Ci, which acts as a transcriptional repressor. Activation of Hh signaling reverses this regulatory effect and leads to the production of full-length Ci, which activates transcription of Hh target genes. (B) TGFβ/BMP signaling pathway. Members of the Dpp/BMP/TGFβ family regulate cell fate by inducing the dimerization of type I and type II TGFβ receptors, resulting in phosphorylation and activation of the intracellular kinase domain of the type I receptor. Targets of the type I receptor are the receptor-regulated Smads (R-Smads) which, upon phosphorylation, associate with co-Smads and translocate to the nucleus where, together with DNA-binding partners such as Fast1, they activate transcription. (C) Wnt signaling pathway. Wnt ligands can activate several different signal transduction pathways. The canonical Wnt pathway controls gene expression by stabilizing β-Catenin (β-Cat). Frizzled (Fz) proteins are 7TM molecules that together with the low-density lipoprotein (LDL) receptor-related protein 5 and 6 (LRP5/6; Arrow in Drosophila) family of co-receptors, function as Wnt receptors. When Wnts are absent, β-Catenin is phosphorylated by GSK3β, leading to its proteasomal degradation. This process requires the formation of a complex scaffolded by Axin and adenomatous polyposis coli (Apc). Binding of Wnts to their receptors results in Dishevelled (Dsh) activation and suppression of GSK3β activity, thus protecting β-Catenin from degradation. Accumulated β-Catenin converts the lymphoid enhancer factor (Lef)/Tcf from a transcriptional repressor to an activator. Two non-canonical Wnt pathways are: the Wnt/Ca^{2+} pathway and the planar cell polarity (PCP) pathway. The PCP pathway involves a non-canonical, β-Catenin-independent, Wnt/Fz pathway that requires Dsh. A Wnt ligand for this pathway has yet to be identified in *Drosophila*, but Wnt ligands can activate an analogous pathway in vertebrates. The Wnt/Ca^{2+} pathway probably signals via heterotrimeric G-proteins (α, β and γ subunits) to mobilize intracellular Ca^{2+} and, in some contexts, to stimulate protein kinase C (PKC). In vertebrates, Wnt/Ca^{2+} signaling is activated by the same ligands as the PCP pathway, suggesting that these pathways might overlap.

attractant(s) for commissural axons (Serafini et al., 1996; Charron et al., 2003).

Given its expression by the floor plate and its long-range effects in the spinal cord, Shh was a candidate for a midline-derived axonal guidance cue. Shh was indeed shown to function as an axonal chemoattractant that can mimic the Ntn1-independent chemoattractant activity of the floor plate in in vitro assays (Charron et al., 2003). The chemoattractant activity of Shh, like the chemoattractant activity of floor plate derived from *Ntn1* mutants, can be blocked by cyclopamine, which blocks the actions of Shh in cell fate determination by inhibiting the Shh signaling mediator Smo. This shows that Smo is required for Shh-mediated axon attraction and, importantly, that the Netrin 1-independent chemoattractant activity of the floor plate also requires Hh signaling. As Shh is the only Hh family member expressed in the spinal cord at this stage, these results suggest that Shh is functioning as a floor plate-derived chemoattractant for commissural axons.

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. Because in these assays commissural axon turning occurs within the spinal cord tissue explant, it seemed possible that Shh was not acting directly on the axons but was 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 the finding that the spinal cord explants used to assess chemoattractant activity are at a developmental stage at which they have apparently lost the competence to be repatterned by Shh, as assessed using a battery of markers of DV patterning (Charron et al., 2003).

A direct action of Shh in attracting the axons was supported further by two sets of experiments (Charron et al., 2003). First, Shh was shown to attract the growth cones of isolated *Xenopus* spinal axons in dispersed cell culture in a cyclopamine-dependent manner, proving that Shh, acting via Smo, can function as a chemoattractant at least for these *Xenopus* axons. A direct demonstration that it can attract rodent commissural axons remains, however, to be obtained. A second way of providing evidence that Shh can act directly on commissural axons to guide them relied on blocking Shh signaling selectively in commissural neurons without blocking it in the terrain through which their axons course. This was achieved by the conditional inactivation of a floxed allele of *Smo* using Cre recombinase expressed under the control of the Wnt1 promoter, which drives Cre expression in the dorsal spinal cord (and in neural crest progenitors). When Cre, driven by this promoter, was used to delete a floxed *Smo* allele in the dorsal spinal cord, commissural axon trajectories were defective in the ventral spinal cord, where Cre is not expressed (Table 1). 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 the 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* knock-out mouse reflect loss of chemoattraction of those axons by Shh. Taken together, these results therefore 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.

Remarkably, the guidance of commissural axons to the floor plate is not, apparently, the only effect of Shh on these axons: later in this review, we summarize recent evidence that Shh may also guide post-crossing commissural axons along the longitudinal axis of the spinal cord.

Shh is a negative regulator of retinal ganglion cell axon growth

Retinal ganglion cell (RGC) axons growing towards the diencephalic ventral midline are faced with the decision to project either contralaterally or ipsilaterally in response to guidance cues at the optic chiasm (Fig. 3). Homozygous inactivation of the mouse *Pax2* gene alters the development of the optic chiasm and RGC axons never cross the midline in these mice. Interestingly, whereas in wild-type mice Shh expression is downregulated in the chiasm as RGC axons are migrating towards this region, Shh expression is ectopically maintained along the ventral midline in *Pax2*$^{-/-}$ mice (Torres et al., 1996). These observations raised the possibility that the continuous expression of Shh at the ventral midline might contribute to preventing RGC axon crossing. In agreement with this idea, Trousse et al. (Trousse et al., 2001) found that ectopic expression of Shh in the midline region interferes with RGC axon growth and prevents them from crossing the midline (Fig. 3) (Trousse et al., 2001). Consistent with the idea that Shh might be acting directly on RGC axons, it was shown that these manipulations do not affect patterning and neural differentiation in the eye. Further experiments will be required to determine whether the chiasm region is repatterned in these experiments, but results from in vitro experiments support the idea that Shh acts directly to control RGC axon migration. These studies show that the addition of exogenous recombinant Shh to retinal explants decreases the number and length of growing axons, without interfering with the rate of proliferation and differentiation of cells in the explant, and time-lapse analysis shows that addition of Shh to retinal explants rapidly causes growth cone arrest and subsequent retraction of RGC axons (Trousse et al., 2001). As the response of the growth cone to many extracellular guidance cues appears to be modulated, and in some cases perhaps even mediated, by intracellular cyclic nucleotide levels (cAMP and cGMP) (Song et al., 1997; Song et al., 1998), the possibility was explored that the effect of Shh on retinal axons in vitro might be due to a change in cAMP levels. In agreement with this, the addition of Shh to retinal growth cones was shown to decrease intracellular levels of cAMP, a finding consistent with the observation that lowering cAMP levels favors growth inhibition (Song and Poo, 1999).

Taken together, these results provide evidence that Shh expression at the chiasm border helps define a barrier within the ventral midline that serves to guide RGC axons, and suggest that Shh may be acting on the axons directly, rather than indirectly by repatterning the chiasm. However, proving that the effect in vivo is direct will require additional studies, such as identifying the mechanism that mediates retinal growth cone responses to Shh and showing that cell-autonomous inhibition of this signaling pathway in the neurons results in guidance defects in vivo.

The opposite effects of Shh on commissural and retinal axons (attraction and repulsion) might be due to an intrinsic or

Table 1. In vivo experiments supporting a role for morphogens in axon guidance

Morphogen pathways	Genes	Species	Experiments	Phenotypes	References
Hedgehog	*Smo*	Mouse	Conditional inactivation of *Smo* in commissural neurons	Commissural axons invade the motor columns	Charron et al., 2003
	Shh	Chick	Ectopic expression of Shh in the optic chiasm	Retinal axons are prevented from crossing the chiasm	Trousse et al., 2001
	Shh	Chick	Silencing of *Shh* by RNA interference	Commissural axons stall at the contralateral floor plate border, with some axons randomly turning caudally or rostrally	Bourikas et al., 2005
TGFβ/BMP	*Bmp7* and *Gdf7*	Mouse	*Bmp7* and *Gdf7* gene inactivation	Initial trajectory of commissural axons aberrant, axons invade the roof plate	Augsburger et al., 1999; Butler and Dodd, 2003
	unc-129	*C. elegans*	*unc-129* mutation	Defects in the dorsally oriented trajectories of motor axons	Colavita and Culotti, 1998; Colavita et al., 1998
Wnt	*drl*	*Drosophila*	*drl* mutation	Axons that normally project into the anterior commissure now project into the posterior commissure	Bonkowsky et al., 1999
	drl	*Drosophila*	Ectopic expression of Drl in posterior commissure neurons	Forces posterior commissure neurons to cross in the anterior commissure	Bonkowsky et al., 1999
	wnt5	*Drosophila*	*wnt5* mutation	Axons that normally project into the anterior commissure project into the posterior commissure	Yoshikawa et al., 2003
	wnt5	*Drosophila*	Ectopic expression of Wnt5 throughout the CNS midline	Prevents the anterior commissure from forming	Yoshikawa et al., 2003
	Fz3	Mouse	*Fz3* gene inactivation	Defects in anteroposterior guidance of commissural axons after midline crossing	Lyuksyutova et al., 2003

extrinsic factor that modulates cyclic nucleotide levels, much as extrinsic factors can convert Netrin attraction to repulsion by modulating cyclic nucleotide levels (Hopker et al., 1999). Alternatively, as the molecular mechanisms underlying the effects of Shh on commissural and retinal axons are poorly understood, it is also possible that these two effects are mediated by distinct signaling pathways that result in opposite guidance effects – a possibility that also has a precedent in the case of Netrins, which can attract axons by activating DCC family receptors, and repel them by activating UNC5 family receptors (Tessier-Lavigne and Goodman, 1996; Dickson, 2002).

The Dpp/BMP/TGF-β family

BMPs are chemorepellents for commissural axons

In *Ntn1* and *Dcc* mutants, commissural axons initially migrate ventrally for approximately the first third of their normal trajectory before becoming misrouted (Serafini et al., 1996; Fazeli et al., 1997), suggesting that an additional guidance cue might be acting to control their dorsal migration.

The proximity of commissural neurons to the roof plate and their initial growth away from the dorsal midline indicated that the roof plate might repel commissural axons away. A direct test of this possibility showed that the roof plate expresses a diffusible activity that repels commissural axons in vitro (Fig. 1B) (Augsburger et al., 1999). By testing a battery of candidate diffusible molecules that might act as repellent signals, it was found that BMP7 and BMP6, two BMP family members expressed by the roof plate, can each mimic the chemorepellent activity of the roof plate in vitro without causing changes in spinal cord cell fate at the doses used for chemorepulsion. The inhibition of BMP7 activity using soluble inhibitors of BMP activity, BMP7-blocking antibodies and genetic inactivation of *Bmp7* showed that BMP7 contributes to the chemorepellent activity of the roof plate for commissural axons (Augsburger et al., 1999). Moreover, BMP7 was shown to induce the collapse of commissural axon growth cones, providing evidence that it can act directly on growth cones to elicit a rapid change in cytoskeletal organization (Augsburger et al., 1999). Further evidence indicated that the roof plate chemorepellent BMP complex consists of a BMP7 and GDF7 heterodimer, as genetic inactivation studies showed that expression of both *Bmp7* and *Gdf7* by roof-plate cells is required for the fidelity of commissural axon growth in vivo (Butler and Dodd, 2003). Together, these results support a model in which a GDF7:BMP7 heterodimer mediates the roof plate chemorepellent activity that guides the initial trajectory of commissural axons in the developing spinal cord.

Although GDF7 and BMP7 are essential for the roof plate chemorepellent activity that guides the initial trajectory of commissural axons, it is interesting to note that the initial high incidence of mispolarization of commissural axons in the mutants is later restored and they recover their correct projection pattern for their entire trajectory. Although the mechanisms underlying this ‘rescue’ are not known, it is possible that the chemoattractant activity of Netrin 1 and/or Shh contributes to the correction of the ventral trajectory.

The molecular mechanisms underlying the effect of BMPs on growth cones are not known. Although BMPs typically activate signaling through type I and II receptors, it is not known whether these receptors also transduce the BMP chemorepellent activity. The activation of BMP receptors normally leads to the transcriptional activation of BMP target genes by Smads, but the timecourse of commissural growth

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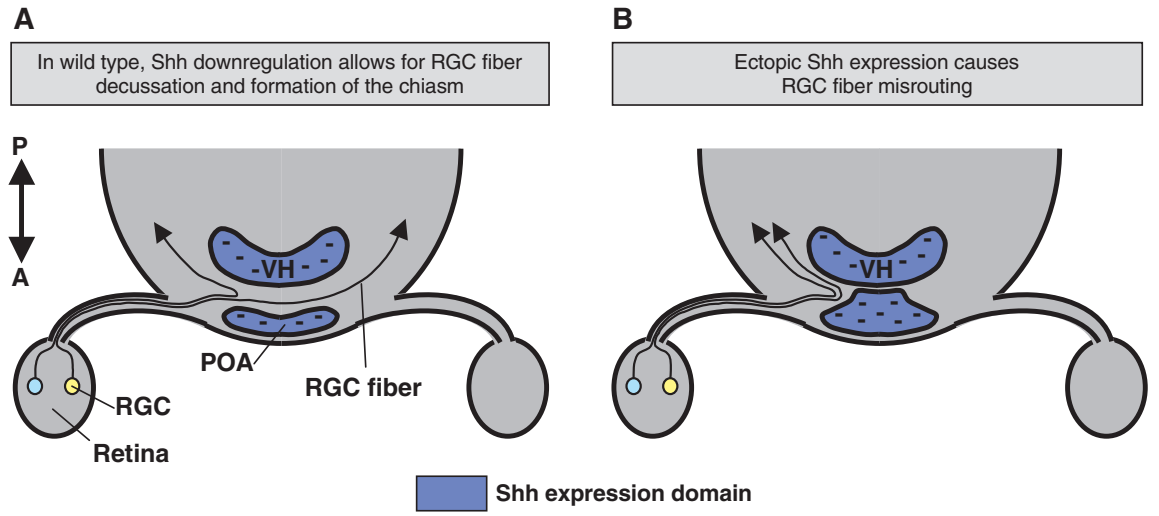

Fig. 3. Shh expression at the chiasm border defines a barrier within the ventral midline that is implicated in guiding RGC axons. Retinal ganglion cell (RGC) axons growing towards the diencephalic ventral midline project either contralaterally or ipsilaterally in response to guidance cues at the optic chiasm. (A) In wild-type animals, downregulation of Shh expression allows RGC axon decussation and formation of the chiasm. (B) Ectopic expression of Shh in the ventral midline region interferes with RGC axon growth and prevents them from crossing the midline. The Shh expression domain is shown in blue. Shh can inhibit retinal axons in vitro, suggesting that in vivo it might act on axons directly rather than by altering the expression of distinct guidance cues in the chiasm, although conclusive evidence for this guidance function in vivo remains to be obtained. A, anterior; P, posterior; POA, pre-optic area; VH, ventral hypothalamus.

cone collapse is difficult to reconcile with the idea that a transcriptional effect mediates the chemorepellent effects of BMPs. Thus, the guidance effect of BMPs could be mediated independently of Smads or may involve non-transcriptional effects. Indeed, some of the cytoplasmic components implicated in guidance responses to other pathfinding cues are activated in response to TGFβ/BMP family members. For example, BMPs activate PKA and LIM kinase (Lee and Chuong, 1997; Foletta et al., 2003), and other TGFβ family members stimulate phospholipase C, PKC and Rho GTPase (Halstead et al., 1995; Choi et al., 1999).

It is interesting to note that gradients of BMPs and Shh appear to cooperate at least twice during neural tube development: first to specify cell fate, and later to guide commissural axons to the ventral midline (see Fig. 1). In the case of Shh, the same molecule plays both roles. In the case of BMPs, it remains to be determined whether the same family member can play both roles or whether different BMPs independently perform each role.

The TGFβ family member unc-129 is required for motor axon guidance

Intriguing evidence for a role of TGFβ family members in axon guidance has come from studies on the *C. elegans* gene *unc-129*, a TGFβ family member that is required for proper guidance of pioneer motor axons along the DV axis (Colavita and Culotti, 1998; Colavita et al., 1998). Mutations in *unc-129* cause defects in the dorsally oriented trajectories of motor axons that resemble those present in *unc-5*, *unc-6/Netrin* and *unc-40/Dcc* mutants, without causing other overt patterning or morphological defects (Colavita and Culotti, 1998; Colavita et al., 1998; Hedgecock et al., 1990). The dorsal expression of *unc-129* suggests that it might be acting as a chemoattractant for motor axons; however, whether UNC-129 acts directly on growth cones remains to be established. Interestingly, UNC-129 function does not require DAF-4, the only known type II TGFβ receptor in *C. elegans*, suggesting that UNC-129 may act through a novel receptor mechanism. Identification of the UNC-129 receptor will help determine whether UNC-129 functions directly to guide motor axons, and will help identify the mechanisms of UNC-129 signaling.

Interestingly, *unc-129* appears to act in parallel to the *unc-6/Netrin* pathway (Colavita et al., 1998; Nash et al., 2000). Thus, if UNC-129 acts directly as a guidance cue, it would provide another example of a BMP collaborating with a Netrin to guide axons; in this case, however, the sign of the guidance would be inverted compared with the spinal cord, with the BMP attracting and the Netrin repelling. As in the spinal cord, the presence of the two cooperative pathways providing ‘a push from behind’ and ‘a pull from afar’ would then help to ensure the necessary fidelity in axon guidance required for invariant and robust development.

The Wg/Wnt family and commissural axon guidance

Wnt5 repels commissural axons from the posterior commissure

The ability of Wnt proteins to stimulate a reorganization of the cytoskeleton during axonal growth and growth cone extension (Hall et al., 2000) suggested that Wnt proteins might also be involved in guiding axons to their targets. The first direct demonstration of a guidance role was obtained in studies of commissural neurons in the fly central nervous system (CNS). During *Drosophila* development, the array of axons composing the CNS has a ladder-like structure: each body segment comprises an anterior and a posterior commissural tract that cross the midline and join one of the two lateral longitudinal tracts that extend the length of the embryo (Fig. 4). The attractive and repulsive signals regulating the decision of commissural axons to cross or not have been well characterized; however, how axons choose between the two

major subdivisions of the crossing pathways – the anterior or posterior commissure – was only recently elucidated.

Based on the initial observation that the expression of the Derailed (Drl) receptor tyrosine kinase, a relative of the vertebrate RYK family, is restricted to the growth cone and axons of neurons that project in the anterior commissure (Callahan et al., 1995), the role of Drl in guiding these neurons through the anterior commissure was explored (Bonkowsky et al., 1999). In the absence of Drl, the neurons that normally project into the anterior commissure were found often to project into the posterior commissure. Conversely, misexpression of Drl in posterior commissure neurons forced them to cross in the anterior commissure. Remarkably, Drl can apparently redirect any crossing axon into the anterior commissure, as misexpression of Drl in neurons that do not normally cross the midline but that have been genetically engineered to do so directs them to the anterior commissure. Thus, Drl appears to be both necessary and sufficient for guiding axons in the anterior commissure.

To explore the mechanism underlying Drl function, a soluble, labeled version of the extracellular domain of Drl was used to detect potential cell-surface ligands for Drl in the fly ventral nerve cord (Bonkowsky et al., 1999; Yoshikawa et al., 2003). Drl-binding sites were observed specifically in the posterior commissure, suggesting that Drl functions to guide axons into the anterior commissure by repelling them away from the region of ligand expression in the posterior commissure. The fact that Drl, like other Ryk family members, possesses a so-called Wnt inhibitory factor (WIF) domain, which in other proteins functions to bind Wnt proteins (Patthy, 2000), suggested that a Wnt might be the repellent in the posterior commissure that repels the axons by binding Drl. Indeed loss of *wnt5* function resulted in commissural axon defects similar to those in *drl* mutants, and decreased the ability of misexpressed Drl to force axons into the anterior commissure (Fig. 4) (Yoshikawa et al., 2003). Moreover, overexpressing Wnt5 throughout the midline prevented the anterior commissure from forming, whereas the overexpression of Wnt5 in *drl* mutants did not. Taken together, these results imply that Wnt5 repels Drl-expressing axons and suggest that Drl might function as a receptor for Wnt5. In a direct test of this possibility, the soluble Drl extracellular domain was shown to bind to the endogenous Wnt5 protein from fly extracts, and its binding to the fly ventral nerve cord was found to disappear in *wnt5* mutants (Yoshikawa et al., 2003). Thus, biochemical and genetic data indicate that Wnt5 is a Drl ligand responsible for repelling axons from the posterior commissure.

Importantly, this work is the first to identify a ligand for the Drl family of receptors and suggests that the other member of the family, Drl2, might also act as a Wnt receptor. This receptor-ligand interaction appears to be specific for Wnt5 as Drl does not interact with the two other Wnt family members tested, Wingless and Wnt4, a finding consistent with the lack of genetic interaction between *drl* and either *wg* or *wnt4* (Yoshikawa et al., 2003). It remains to be determined whether Drl acts to transduce the repulsive Wnt signal directly, or functions to prevent, or to reverse, attraction through an alternative receptor, possibly of the Fz family.

Wnt4 controls the anteroposterior guidance of commissural axons

After commissural axons have reached and crossed the floor plate, they make a sharp anterior turn toward the brain (Fig. 1C). The molecules involved in the DV projection of commissural axons to and at the floor plate have been well described, but it is only recently that a cue controlling anteroposterior (AP) guidance has been identified. Using a novel in vitro assay, evidence was obtained that the activity responsible for the anterior guidance of post-crossing commissural axons is an increasing posterior to anterior gradient of a diffusible attractant (Lyuksyutova et al., 2003). Several members of the Wnt family were then shown to be able to affect the growth of post-crossing commissural axons. Among them, Wnt4 was found to be expressed in an increasing posterior to anterior gradient, at least at the RNA level. Importantly, an ectopic posterior source of Wnt4 was found to redirect post-crossing axons posteriorly in vitro, whereas the Wnt inhibitors sFRP1, sFRP2, and sFRP3 (secreted frizzled-related proteins; soluble proteins that block the interaction of Wnts with their receptor) made post-crossing commissural axons stall and turn randomly along the AP axis. In the presence of Wnt4, the growth cones of post-crossing commissural axons were enlarged and more complex; addition of sFRP2 reduced this effect within one hour, suggesting that Wnt4 might be acting directly on the growth cone. These results indicate that Wnt activity is essential for the normal guidance of post-crossing commissural axons, and that Wnt4 can act as an instructive post-crossing commissural axon attractant (Fig. 1C).

In agreement with a role for Wnt factors in the control of post-crossing guidance of commissural axons, it was found that

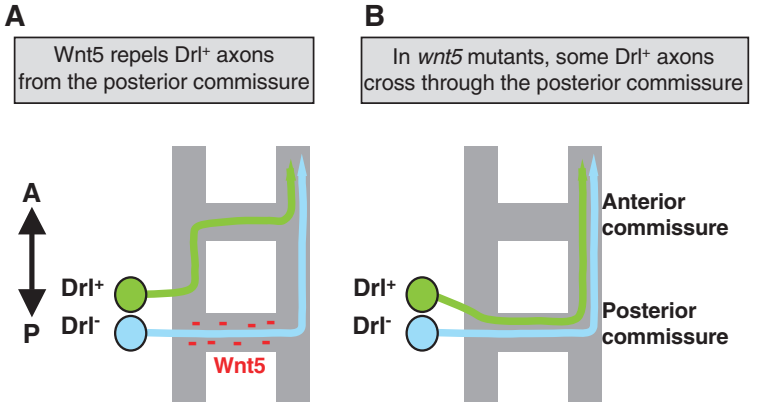

Fig. 4. Wnt5 repels commissural axons from the posterior commissure. During *Drosophila* development, the axons composing the CNS have a ladder-like structure: each segment comprises an anterior and a posterior commissural tract that cross the midline and join the longitudinal tracts. (A) In wild-type animals, the Wnt5 ligand, which is restricted to the posterior commissure (red), repels axons expressing Derailed (Drl$^+$; light green) from the posterior commissure into the anterior commissure. (B) In *wnt5* mutants (as well as in *drl* mutants, not shown), the Drl$^+$ neurons that normally project into the anterior commissure project into the posterior commissure. A, anterior; P, posterior.

mice lacking the Wnt receptor Frizzled 3 (Fz3) have normal pre-crossing commissural axon behavior but display defects in AP guidance of commissural axons after midline crossing (Lyuksyutova et al., 2003). It will, of course, be important to determine whether Fz3 is required specifically in commissural neurons for this effect; however, a lack of apparent patterning defects in the neural tube of *fz3* mutants (Lyuksyutova et al., 2003), combined with the in vitro experiments described above, already provide strong evidence that Wnt-Frizzled signaling directly guides commissural axons along the AP axis of the spinal cord.

It is noteworthy that the inactivation of *Fz3* in mice also results in other axonal abnormalities (Wang et al., 2002). These animals display severe defects in many major axon tracts within the forebrain, including complete loss of the thalamocortical, corticothalamic and nigrostriatal tracts and of the anterior commissure, and a variable loss of the corpus callosum. These results suggest that, in addition to guiding commissural axons at the spinal cord midline, Wnt-Frizzled signaling might also play a much broader role in axonal development, although in each case it will again be important to determine whether Wnt-Frizzled signaling mediates axon guidance directly, or only indirectly as a secondary consequence of cell fate changes.

An interesting question is how commissural neurons, which apparently express Fz3 early before reaching the floor plate (Lyuksyutova et al., 2003), do not respond to the Wnt gradient until after they have crossed the floor plate. A crucial step in our understanding of how guidance cues accomplish their pathfinding role and then 'pass the baton' to subsequent cues is to determine how axonal responsiveness is regulated during an axon's migratory journey. One mechanism is to have the receptor or an essential signaling component of the pathway expressed and/or transported at the right time and right place only when an axon is required to respond. Such a mechanism has been shown to be responsible for the induction of Slit responsiveness in post-crossing commissural axons in *Drosophila*, which is explained by the upregulation of Robo protein on the axons after crossing (Kidd et al., 1998). Alternatively, an inhibitory molecule could block the function of a receptor and/or its signaling components when it is inappropriate for the axon to respond to a particular cue (Sabatier et al., 2004; Stein and Tessier-Lavigne, 2001). Thus, mechanisms inhibiting premature (in this case, pre-crossing) sensing of Wnts (e.g. through active inhibition of the receptor or a signaling component) or, alternatively, allowing the sensing of Wnts only post-crossing (e.g. through induction of a required receptor or signaling component) might also act to confer post-crossing-specific responsiveness to Wnts.

LRP6 is a Fz co-receptor required for the canonical Wnt/β-catenin signaling pathway (He et al., 2004). The axon guidance signaling pathway downstream of Fz3 has not been investigated but, interestingly, the pathfinding of commissural axons is reported to be normal in *LRP6* mutant embryos, suggesting that the canonical Wnt signaling pathway is not required for Wnt-mediated commissural axon guidance (Lyuksyutova et al., 2003).

The requirement of a Fz receptor to guide commissural axons along the AP axis contrasts with the finding that, in flies, Wnt5 acts through Drl to mediate axonal repulsion from the posterior commissure (Yoshikawa et al., 2003). It is possible that the Wnt/Fz pathway signals attraction and the Wnt/Drl pathway repulsion. Alternatively, as discussed above, it may be that the presence of Drl modulates signaling through Fz receptors. In this case, Drl would not directly transduce a repulsive signal, but would reverse an attractive signal transduced by Fz receptors, similar to the repellent effect that the expression of the UNC-5 receptor has on UNC-40/Frazzled/DCC-mediated attraction by UNC-6/Netrin (Hamelin et al., 1993; Hong et al., 1999).

Shh guides commissural axons along the longitudinal axis of the spinal cord

In addition to Wnt4, a recent publication has reported that Shh also guides commissural axons in the rostral direction along the longitudinal axis of the spinal cord (Bourikas et al., 2005). Using a subtractive hybridization approach to identify guidance cues responsible for the rostral turn of post-crossing commissural axons in chick embryos, Bourikas and colleagues identified differentially expressed candidates, the function of which they investigated by RNA interference (RNAi)-mediated in ovo gene silencing. Unexpectedly, one of their candidates turned out to be Shh. In agreement with these results, silencing of the *Shh* gene by a different RNAi construct, or by injecting a hybridoma producing a function-blocking Shh antibody, led to axon stalling at the contralateral floor plate border, with some axons turning caudally or rostrally, apparently in a random manner. Importantly, marker analysis revealed that the patterning of the spinal cord was not apparently affected by these manipulations, suggesting that these experiments were done after neural cell fate specification by Shh had occurred. Finally, post-crossing commissural axons were shown to avoid ectopic Shh in vivo. Together, these results provide strong evidence that Shh is essential for the normal guidance of commissural axons along the longitudinal axis of the spinal cord.

A Shh gradient could be guiding commissural axons directly, or could alternatively be acting only indirectly by controlling a graded distribution of a distinct guidance cue. For example, Shh could be involved in repressing Wnt4 expression along the AP axis (contributing to a Wnt gradient) or inducing a sFRP gradient. Two lines of evidence, however, were provided for a direct role of Shh (Bourikas et al., 2005). The first came from an investigation of the receptor mechanism for this guidance. Interestingly, neither cyclopamine nor Smo RNAi interfered with the rostral turn of commissural axons along the longitudinal axis, suggesting that Smo might not be involved in this process. Instead, RNAi-mediated silencing of *Hip1*, a gene encoding a Shh-binding membrane protein transiently expressed in commissural neurons at the time when they cross the floor plate (as well as in the peri-ventricular region), resulted in the same post-crossing phenotype as Shh RNAi. These results, which contrast with the essential role of Smo in Shh-mediated attraction of commissural axons to the floor plate (Charron et al., 2003), suggest that Hip1 might be involved in transducing a Shh guidance signal in post-crossing commissural neurons. The relatively restricted expression of *Hip1* mRNA to commissural neurons would be consistent with a direct action of Shh on these axons. A second line of evidence that supports a direct role for Shh was obtained in vitro, in experiments that showed that post-crossing commissural axons from spinal cord explants could be repelled by Shh beads in

vitro. Together, these results suggest a model in which Shh could be functioning directly through Hip1 as a chemorepellent for post-crossing commissural axons (Fig. 1D).

Although it is not yet known whether Shh guides post-crossing commissural axons in rodents nor whether Wnt4 guides post-crossing commissural axons in chicks, it is nonetheless interesting to note that the complementary Wnt4 and Shh gradients might act cooperatively in the rostral guidance of commissural axons.

Interpreting positional information: signaling components in axon guidance and cell fate specification

In the studies summarized above, members of all three morphogen families were shown to act rapidly (in an hour or less) to affect growth cone morphology. Although these results appear to be inconsistent with these proteins mediating their axon guidance effects through their canonical, transcriptional signaling pathways, this needs to be formally proven, as none of the above studies has addressed this issue directly. Nonetheless, even if a transcriptional response is found to be necessary, additional local signaling would still be required in the growth cone to generate a polarized response that leads to growth cone turning in a specific direction. Evidently, a purely transcriptional response consisting of a retrograde signal to the nucleus followed by an anterograde signal back to the growth cone cannot account for the polarized turning effect of a guidance cue. Studies aimed at understanding the molecular mechanisms underlying growth cone turning by morphogens will be necessary to identify the molecules that link morphogen signaling to localized growth cone effects.

In this regard, at least three possible models may account for the effects of morphogens in axon guidance. The first is based on the fact that the Wnt, BMP and Hh signaling pathways are only beginning to be understood; many of their intermediate signaling molecules remain to be identified and characterized. Thus, it might be that the signaling proteins that elicit the growth cone effects are also components of the signaling pathways that are required for cell fate specification.

A second model is that the same cue might be acting through entirely different signaling pathways, including the use of a different receptor. In the case of BMP/TGFβ family members, no known receptor has so far been implicated in the commissural axon guidance activity of these proteins in vertebrates. In worms, UNC-129 does not appear to require the classical TGFβ receptors, suggesting that it may be functioning through an alternative receptor family; it will be exciting to determine whether the classical BMP receptors are required for the guidance activity of the BMPs on vertebrate commissural axons or whether they signal through non-classical BMP receptors. In the case of Shh, although Smo is required for Shh-mediated commissural axon guidance to the floor plate, it is not known whether Ptc, the Shh-binding component of the Shh receptor, is involved. This finding contrasts with chick post-crossing commissural axon guidance, where Smo does not appear to be required for the rostral turn away from the Shh gradient (Bourikas et al., 2005). Additional experiments on commissural and retinal axons are required to determine the receptor components mediating Shh effects on axon guidance. Finally, for Wnt-mediated axon guidance, where the identity of the receptors involved has been more thoroughly investigated, an unexpected situation was uncovered: the non-classical Wnt receptor Drl is required for repulsion from the posterior commissure in *Drosophila*, and the classical receptor Fz3 is required for attraction towards the anterior pole of the spinal cord in mouse.

The third model for how morphogens guide axons is a combination of the first two, and postulates that a morphogen might use the upstream part of the classical cell fate signaling cascade but then diverge and use a non-classical pathway to elicit its effects on the growth cone. In this model, the divergence – or branching – from the classical pathway might occur directly downstream of the receptor or further down the signaling cascade. AP commissural axon guidance by Wnt4 through Fz3 might use such a mechanism: although this effect requires a Fz receptor, the fact that the Fz co-receptor LRP6 is not required suggests that Wnt4 may regulate commissural axon guidance through a non-canonical pathway. In this case, the non-canonical – and potentially overlapping – Wnt/Ca^{2+} and PCP pathways are potential candidate signaling cascades to mediate the axon guidance effects of Wnt4. Indeed, in *Xenopus*, Wnt4 and its related family members Wnt5a and Wnt11 were found to regulate various morphological events by activating signaling by heterotrimeric G-protein, Ca^{2+} and PKC pathways (Strutt, 2003), and Fz3 was shown to activate PKC (Kuhl et al., 2000). Together, these results raise the possibility that axon guidance mediated by Wnt4 might be operating through a non-canonical, PKC-dependent Wnt/Fz signaling pathway and, more importantly, that axon guidance and PCP might overlap not only conceptually – by controlling the polarity of a growth cone or an entire cell, respectively – but also mechanistically. Indirect evidence supporting mechanistic links between PCP and axon guidance is provided by the recent finding that the receptor tyrosine kinase-like protein PTK7 regulates planar cell polarity in vertebrates (Lu et al., 2004), whereas its *Drosophila* homolog Off-track (Otk) participates in Semaphorin signaling in axon guidance (Winberg et al., 2001).

Concluding remarks

The discovery that morphogens can be reused to guide axons has generated considerable excitement in the field. It remains an open question as to how widespread these guidance effects are. At one extreme, the examples of guidance by morphogens may be isolated instances. At the other, morphogens may prove to be as important as the classic axon guidance molecules (Netrins, Slits, Semaphorins, Ephrins and growth factors) in guiding axons. Elucidating the precise contribution of morphogens will, however, continue to be difficult for some time, given the significant difficulty in determining in any particular situation whether a morphogen is functioning directly or indirectly to regulate axonal guidance. In any gain- or loss-of-function experiment in vivo, the morphogen may be altering the expression of guidance cues in the environment where the guidance effects are observed, or the fate of cells (and hence their responses to guidance cues) that are showing guidance responses. Thus, several tests are required to prove that altered guidance in such experiments reflect direct guidance effects of the morphogen: (1) evidence against a change in the fate of cells or expression of other guidance cues in the environment; (2) evidence that cell autonomous manipulation of the morphogen's signal transduction pathway

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in the responsive neuron results in similar guidance deficits; and (3) evidence that growth cones of responsive axons can respond directly to the morphogen – which may most frequently be obtained in vitro in growth cone collapse or turning assays. It can be expected that the level of proof that is obtained will increase over time as the signaling pathways linking the morphogens to the cytoskeleton for growth cone turning are elucidated. These findings should provide entry points with which to interfere selectively with the guidance effects of the morphogens in the responsive neurons, without altering their transcriptional effects either in those neurons or in the environment. Nonetheless, the collective weight of the experiments summarized above, many of which attempted and succeeded, at least partly, in distinguishing between direct and indirect effects of the morphogens, already provide strong evidence that morphogens have widespread roles in axon guidance, no doubt with more to come.

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