BMP type I receptor complexes have distinct activities mediating cell fate and axon guidance decisions

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The finding that morphogens, signalling molecules that specify cell identity, also act as axon guidance molecules has raised the possibility that the mechanisms that establish neural cell fate are also used to assemble neuronal circuits. It remains unresolved, however, how cells differentially transduce the cell fate specification and guidance activities of morphogens. To address this question, we have examined the mechanism by which the Bone morphogenetic proteins (BMPs) guide commissural axons in the developing spinal cord. In contrast to studies that have suggested that morphogens direct axon guidance decisions using non-canonical signal transduction factors, our results indicate that canonical components of the BMP signalling pathway, the type I BMP receptors (BMPRs), are both necessary and sufficient to specify the fate of commissural neurons and guide their axonal projections. However, whereas the induction of cell fate is a shared property of both type I BMPRs, axon guidance is chiefly mediated by only one of the type I BMPRs, BMPRIB. Taken together, these results indicate that the diverse activities of BMP morphogens can be accounted for by the differential use of distinct components of the canonical BMPR complex.

KEY WORDS: Axon guidance, Bone morphogenetic proteins, Commissural neurons, Morphogen, Spinal cord

INTRODUCTION

Organisms develop using a remarkably small number of growth factor families to specify a multitude of distinct cellular responses. This biological economy is often achieved by feed-forward mechanisms, where cells respond differentially to the same signal received reiteratively over time. A striking example of this phenomenon is the recent finding that molecules that act as morphogens to induce the formation of diverse cell types in developing tissues, also function as axon guidance cues in the establishment of neuronal circuits (reviewed by Charron and Tessier-Lavigne, 2005; Salie et al., 2005). The ability of morphogens to act as axon guidance cues was first shown for the commissural neurons (Augsburger et al., 1999), a class of dorsal sensory interneurons in the developing spinal cord (Holley, 1982; Dodd et al., 1988). Commissural neurons differentiate adjacent to the dorsal midline in response to inductive signals from Bone Morphogenetic Proteins (BMPs) present in the roof plate (RP) (Liem et al., 1997; Lee et al., 1998). They then extend axons away from the RP, in a ventral and circumferential route through the dorsal spinal cord (Holley, 1982; Oppenheim et al., 1988). Our previous studies have shown that this initial trajectory of commissural axons is also directed by the activity of BMPs, exerting their function as a heterodimer of BMP7 and Growth/Differentiation Factor 7 (GDF7) (Augsburger et al., 1999; Butler and Dodd, 2003). Thus, BMPs act as a classic feed-forward signal, directing different outcomes at different stages of commissural neuronal development: BMPs first act as morphogens to specify commissural cell fate and then as axon guidance cues to direct commissural axons away from the RP.

The ability of the BMPs to act as axon guidance molecules appears to be an activity common to other morphogens. Sonic Hedgehog (Shh) and members of the Wnt and Fibroblast Growth Factor (FGF) families also act as axon guidance molecules (Irving et al., 2002; Charron et al., 2003; Lyuksyutova et al., 2003), suggesting a model in which the same factors pattern the diversity of both cell fate and axonal connectivity within the nervous system. These studies suggest that a single factor can specify unexpectedly diverse activities for developing neurons, but they do not resolve how this process is achieved mechanistically. Morphogens specify cell fate over many hours by initiating global changes in the transcriptional status of the cell (Tabata and Takei, 2004). By contrast, axon guidance cues locally activate signal transduction pathways in the axonal growth cone (Dickson, 2002), resulting in the rapid reorganization of the cytoskeleton (Dodd and Jessell, 1988; Tessier-Lavigne and Goodman, 1996) in a process that is independent of the nucleus (Campbell and Holt, 2001). It remains unclear how cells distinguish between the cell fate specification and axon guidance activities of morphogens. One possibility is that the diverse activities of morphogens are transduced by signalling through distinct signal transduction pathways. Alternatively, morphogens could signal through the same receptor and signalling components, with the outcome being determined by the context in which the signal is perceived. Studies addressing this question have suggested that morphogens employ both of these strategies to mediate their diverse activities. For example, distinct receptor complexes appear to translate the guidance and inductive activities of Shh. Shh has recently been shown to direct commissural axon guidance decisions by activating either the non-canonical Boc (Okada et al., 2006) or Hip (Bourikas et al., 2005) receptors, whereas Patched and Smoothened (Smo) mediate the inductive activities of Shh (Nybakken and Perrimon, 2002). By contrast, the canonical FGF receptors appear to be important in retinal axon guidance (Brittis et al., 1996; McFarlane et al., 1996). For the Wnts, a combinatorial model is emerging in which the canonical Wnt receptor Frizzled interprets the attractive axonal responses to Wnts, whereas repulsive Wnt guidance cues are transduced by the atypical receptor Ryk (reviewed by Bovolenta et al., 2006).

Here, we assess the mechanism by which the activity of the BMP-mediated RP chemorepellent is transduced. The canonical BMP receptor (BMPR) complex consists of type I and II serine/threonine kinases (Ebendal et al., 1998) that, upon ligand binding,
phosphorylate the receptor-regulated Smads (R-Smads), a class of intracellular signalling effectors (Heldin et al., 1997). The specification of cell fate by the BMPs is thought to be mediated by the ability of the Smad complex to regulate the transcription of target genes, following its translocation to the nucleus (Kretzschmar and Massague, 1998). However, it is unlikely that BMPs guide axons by altering the transcriptional status of the cell; the direct application of BMPs to commissural growth cones rapidly causes their collapse, suggesting that the BMP guidance signal is transduced locally in the growth cone by a non-transcriptionally based mechanism (Augsburger et al., 1999). Additionally, BMP ligands may have differential activities. BMP homodimers important for inductive activity are not the principal mediators of guidance activity; rather, this function is carried out by a BMP7-GDF7 heterodimer (Butler and Dodd, 2003).

These results prompted us to determine whether the different activities of the BMPs can be mediated solely through the canonical BMPR complex by examining the contribution of the type I BMPRs, BMPRIA and BMPRIB, to commissural axon guidance. Biochemical studies have suggested that the type I BMPRs may determine the specificity of BMP ligand binding (ten Dijke et al., 1994); however, the type I BMPRs have been largely shown to function redundantly in the specification of cell fate (Murati et al., 2005; Yoon et al., 2005). In particular, the dorsal-most neurons in the mouse spinal cord are only lost in Bmpr1a-/--;Bmpr1b-/--; double mutants (Wine-Lee et al., 2004) and the constitutive activation of either BMPRIA or BMPRIB in the chick spinal cord leads to the increased production of dorsal neurons (Timmer et al., 2002). Here, we use both gain- and loss-of-function approaches to show that the type I BMPRs also mediate commissural axon outgrowth and guidance. However, whereas both type I BMPRs contribute to the assignment of dorsal cell fates in the spinal cord, axon guidance activity is principally mediated by BMPRIB, as only BMPRIB is both necessary and sufficient to mediate the known activities of the BMP component of the RP chemorepellent. These results suggest that the differential activation of particular BMPR complexes distinguishes between the inductive and guidance activities of the BMP morphogen.

MATERIALS AND METHODS

Expression constructs and in ovo DNA electroporation

Expression constructs encoding constitutively active (ca) haemagglutinin (HA)-tagged forms of both BMPRIA (human) and BMPRIB (mouse) were described by Wieser et al. (Wieser et al., 1995) and Akiyama et al. (Akiyama et al., 1997). Expression constructs containing either the caBMPRs or farnesylated EGFP (fGFP, Invitrogen) fused to the Math1 enhancer were expressed specifically in the dorsal and intermediate VZ, and in the dorsal-most population of postmitotic neurons in the mantle layer (dashed lines, Fig. 1B). These neurons are absent from the mantle layer, outlined in A and B. (A) Bmprla is expressed throughout the VZ and is absent from the mantle layer, outlined in A and B. (B, C) Bmprlb is expressed specifically in the dorsal and intermediate VZ, and in the dorsal-most neurons in the mantle layer (open arrowhead, C). The yellow box in B is shown at higher magnification in C. (D) Bmprlb is expressed in an overlapping population of commissural progenitor cells and neurons with that labeled by antibodies against Math1 (open arrowheads, C, D). Scale bars: in A, 50 μm for A,B; in C, 25 μm for C,D.
Either CMV::caBMPRIA or CMV::caBMPRIB at stage 11/12 or 14/15. For both receptors, there was a significant increase in the number of dI1 electroporated side following electroporation of neurons on the electroporated side versus the non-electroporated sides of the spinal cord (electroporated side marked with +). (I-L) The activation status of the BMP-specific Smad (Smad1/5/8) second messenger intermediates was assessed using antibodies against the phosphorylated (phos) forms of Smad1/5/8, which endogenously labels the progenitor domain of dI1 neurons (open arrowheads, I,K). Both constructs can activate Smad1/5/8/8, which temporally restricted. The CMV enhancer was used to direct the constitutively active (ca) type I BMPRs on dorsal neural identity is suggested that the type I BMPRs transduce the RP-derived BMP morphogen signal. Thus, it was crucial in our studies to determine whether the effect of modulating BMPR activity on the trajectory of commissural axons was due to a primary defect in axon guidance or was a secondary consequence of altered inductive signalling. To address this question, we determined whether the effect of constitutively active (ca) type I BMPRs on dorsal neural identity is temporally restricted. The CMV enhancer was used to direct the expression of EGFP (CMV::GFP) in combination with either haemagglutinin (HA)-tagged caBMPRIA or caBMPRIB (CMV::caBMPRIA, CMV::caBMPRIB). These constructs were introduced into the chick spinal cord at different developmental stages by in ovo electroporation (Swarz et al., 2001). The status of cellular identity in the chick spinal cord following electroporation was determined in Hamilton Hamburger (HH) stage 22/23 embryos using a panel of markers for spinal neuronal progenitors and early differentiated neurons (Fig. 2M). These markers included antibodies against pLh2, which labels postmitotic commissural (dI1) neurons (Liem et al., 1997), and pIsl, which labels association (dI3) neurons and motoneurons (MNs) (Tsuchida et al., 1994).

Consistent with previous reports (Timmer et al., 2002), the misexpression of either caBMPR in HH stage 11/12 chick embryos resulted in an alteration in the cellular identity of the spinal cord (see Fig. S1 in the supplementary material), presumably because the constitutive activation of either type I BMPR leads to both the induction of cells with dorsal fates and the suppression of the ventral cell fates. By contrast, when the expression constructs were electroporated into HH stage 14/15 chick embryos, the fate of the dorsal spinal cord was unaffected (Fig. 2A-H). Ventral cellular identity was, nevertheless, partially affected; the number of pIsl+ MNs appeared to be slightly decreased on the electroporated side (Fig. 2E-H), although no loss of Nkx2.2+ V3 interneurons was observed (data not shown). Both type I caBMPR constructs were functional: at all stages tested, the BMP-specific R-Smads, Smad1/5/8/8, were activated to the same extent after the electroporation of either caBMPR (Fig. 2I-M; see also Fig. S1I-L in the supplementary material).
To quantify these results further, we compared the number of dI1 and dI3 interneurons on the electroporated and non-electroporated sides of the spinal cord (Fig. 2N,O). The electroporation of either CMV::caBMPRIA or CMV::caBMPRIB into stage 11/12 embryos resulted in a significant increase in the number of either dI1 or dI3 neurons (see Fig. S1 in the supplementary material; Fig. 2O). By contrast, when these constructs were electroporated into stage 14/15 embryos, similar numbers of dI1 and dI3 neurons were seen on both sides of the spinal cord. These results suggest that only spinal tissue at the earliest stages of chick neural tube development is competent to respond to the type I caBMPRs and to adopt a dorsal cellular fate. This competence is lost by stage 14, when commissural axiogenesis is beginning. Taken together, these results suggest that the type I BMPRs no longer mediate the inductive activities of the BMPs by this point in development.

The constitutive activation of only BMPRIB results in commissural axon guidance defects

To determine whether the type I BMPRs also transduce the BMP axon guidance signal, we assessed the effect of constitutively activating BMPRIA or BMPRIB (Akiyama et al., 1997) on the trajectory of chick commissural axons. Chick commissural axons have an indistinguishable trajectory from rodent commissural axons in the transverse plane of the spinal cord and are responsive to the same axon guidance cues (Holley, 1982; Kennedy et al., 1994; Serafini et al., 1996). If local activation of the type I BMPRs in commissural neurons translates the BMP gradient from the RP into directed axonal growth away from the dorsal midline, then a neuron expressing a caBMPR should perceive an altered gradient of BMPs making it possible that the axon guidance defects seen resulted from non-autonomous alterations in the properties of the tissue surrounding the commissural neurons. Thus, it was crucial to examine the effect of expressing the type I caBMPRs solely in postmitotic commissural neurons. Towards this end, we generated constructs in which either farnesylated (f) GFP, caBMPRIA or caBMPRIB were expressed under the control of the Math1 enhancer, which drives gene expression specifically in early-born commissural neurons in the developing spinal cord (Helms et al., 2000; Lumpkin et al., 2003). Both the Math1::caBMPRIA and Math1::caBMPRIB constructs contained an IRES-fGFP reporter to visualise the trajectories of the electroporated commissural axons.

The electroporation of either the control Math1::fGFP vector or the Math1::caBMPRIA-IRES-fGFP construct had no effect on the trajectory of either Axonin1+ or GFP+ axons; the GFP+ axons projected normally, either exiting from the spinal cord or crossing the FP (arrowhead, Fig. 3B). Similarly, Axonin1+ axons on the electroporated side of the spinal cord behaved identically to those on the non-electroporated side, projecting ventrally, away from the RP (Fig. 3C,D). By contrast, the trajectories of both GFP+ and Axonin1+ axons were severely compromised after misexpression of caBMPRIB (Fig. 3E-H). The electroporated axons exhibited two behaviours: some Axonin1+ axons were misoriented, extending medially into the VZ (arrows, Fig. 3G,H); others appeared to stall as they approached the ventral midline (open arrowhead, Fig. 3G). Supporting this latter observation, no dorsally derived GFP+ axons made the ventral contralateral projection across the FP (arrowhead, Fig. 3F), although the ventrally derived GFP+ motor axons exited the spinal cord normally.

These results suggest that the type I BMPRs differ in their abilities to mediate commissural axon guidance. This divergence in function was unexpected, given that BMPRIA and BMPRIB have significantly overlapping functions in other systems, in particular, the specification of dorsal cell fate in the developing spinal cord (Wine-Lee et al., 2004). However, a caveat in these experiments is the ubiquitous expression of caBMPRIA and GFP from the CMV enhancer, the (B) GFP+ (blue) and (C) Axonin1+ (green) cross the spinal cord normally at the FP (arrowhead, B). (D) The electroporated Axonin1+ axons also project around the circumference of the spinal cord similar to control axons. (E-H) By contrast, after misexpression of caBMPRIA and GFP from the CMV enhancer, (F) no GFP+ axons cross the FP (arrowhead), and (G,H) Axonin1+ axons are both mispolarized medially towards the lumen of the spinal cord (arrows, G,H) and stalled (open arrowhead) above the ventral midline. + indicates the electroporated side. Scale bar in A: 100 μm for A-H.

![Fig. 3. BMPRIB specifically mediates commissural axon outgrowth and guidance.](image)
axons either misprojected medially into the VZ (arrow, Fig. 4F) or they stalled above the developing MN column (arrowheads, Fig. 4F,H), phenotypes consistent with BMPRIB transducing both directional and outgrowth information for commissural axons. The extent of the axon outgrowth defect was quantified by determining the percentage of electroporated commissural neurons that had extended axons to the midpoint of the dorsal (MD), intermediate (INT) or ventral (MV) regions of the spinal cord or to the FP (Fig. 4J). In Math1::fGFP embryos, 62.2%±1.5 of GFP+ neurons had extended axons to the MD line by stage 22/23 (Fig. 4I). Commissural axiogenesis is still ongoing at this stage, thus axon outgrowth evenly decreased with distance from the RP, with the majority (55%±1.4) of GFP+ axons having reached the MV region. In embryos electroporated with Math1::caBMPRIA-IRES-fGFP at the same stage, 48.7%±1.7 of GFP+ axons that crossed lines drawn (J) in the mid-dorsal (MD), intermediate (INT) and mid-ventral (MV) spinal cord, and the FP. Of the control commissural neurons extending axons to the MD line, over 55% of these axons subsequently project to the MV line (n=158 sections from 10 embryos). By contrast, less than 23% of the Math1::caBMPRIA-IRES-fGFP commissural axons that extend to the MD line subsequently reach the MV line (n=145 sections, 15 embryos), a figure significantly different from control (P<2.4×10^-5). Scale bar in F: 100 μm for A-F.

BMPRIB is required for commissural axon reorientation

We also assessed the requirement for both BMPRIA and BMPRIB by determining the consequence of functionally inactivating type I BMPRs in mouse embryos. Single mutations in either of the type I BMPRs have no effect on the fate of dorsal spinal neurons (Wine-Lee et al., 2004). Rather, these neurons were lost only in BmprIa; BmprIb double mutant embryos (Wine-Lee et al., 2004), suggesting that any defects in axon guidance observed in the absence of either BMPRIA or BMPRIB do not result from a failure of dorsal neural differentiation. Mice mutant for BmprIb are viable (Yi et al., 2000; Yi et al., 2001); however, the BmprIa mutation is lethal (Mishina et al., 1995), necessitating the use of a conditional allele of BmprIa (BmprIafllox) (Mishina et al., 2002). Tissue-specific recombination of BmprIa was achieved by mating the BmprIafllox line to transgenic mice expressing cre recombinase under the control of the Math1 enhancer (Matei et al., 2005). The Math1 enhancer drives the expression of cre in postmitotic commissural neurons (Fig. 5E,F), resulting in Cre-mediated recombination by stage E10 (Matei et al., 2005), the stage after cell fate specification, but before the onset of commissural axiogenesis. Thus, it was possible to determine the requirement for each of the type I BMPRs in commissural axon guidance without the complicating effects from disruptions in cell fate.

The trajectory of commissural axons was first assessed in transverse sections of wild-type (Fig. 5A), Math1::cre;BmprIafllox (Fig. 5B) and BmprIb-/- (Fig. 5C) E11.5 embryos. In all three cases, commissural axons extended in a highly polarized manner away from the RP. However, in BmprIb-/- embryos, a small population of commissural axons was mislocalized medially towards the lumen (arrowhead, Fig. 5C). To quantify this phenotype, the number of pLh2+ postmitotic commissural neurons that extend a Tag1+ axon medially was determined in sections of wild-type and BmprIb-/- spinal cords taken from the same axial levels. In wild-type embryos, 0.1%±0.1 of commissural axons extended aberrantly. By contrast, 1.7%±0.4 of commissural axons were mislocalized in BmprIb-/- embryos, a figure comparable to the number of mislocalized commissural axons in Bmp7 mutant embryos (Butler and Dodd,
This result suggests that the loss of BMPRIB, but not BMPRIA, results in a perturbation of the commissural axon trajectory in vivo.

To examine the response of wild-type, BMPRIA- and BMPRIB-deficient commissural axons to the repellent activity of the RP, whole-mount fillet preparations of the spinal cord were taken from E11.5 mouse embryos. In fillet preparations, the spinal cord is opened ventrally like a book making it possible to examine the trajectory of the commissural axons immediately adjacent to the RP. Both wild-type (open arrowhead, Fig. 6A) (Butler and Dodd, 2003) and Math1::cre; BmprIaflox/flox (Fig. 6B) commissural axons very rarely project into the RP and never cross the RP. However, the polarity of the commissural axon trajectory was perturbed in BmprIb–/– fillet preparations (Fig. 6C,D). A significantly higher (P<0.001) percentage of commissural axons are mispolarized medially in BmprIb–/– embryos compared with their wild-type littermates. (E,F) The Math1::cre line drives expression of Cre recombinase (green) specifically in the phl2+ (red) population of commissural neurons. Scale bar in A: 75 μm for A-C, E,F.

In other fillet preparations, many Tag1+ axons extend into the RP (open arrowheads, C,F), with commissural axons (closed arrowheads, D’,F’) now observed to cross the RP (outlined in D,F). (G) There is no significant difference (P>0.27) between the percentage of mispolarized axons in BmprIaflox/flox control fillets (0.75%±0.19 s.e.m., n=8213 phl2+ neurons from 9 embryos) and the BMPRIA-deficient (Math1::cre;BmprIaflox/flox) fillets (0.95%±0.20 s.e.m., n=9262 phl2+ neurons, 9 embryos). By contrast, a significant increase (P<0.002) is observed in BmprIb–/– mutants (1.32%±0.14 s.e.m., n=7940 phl2+ neurons from 10 embryos) compared with wild-type litter-mates (0.73%±0.10 s.e.m., n=10379 phl2+ neurons, 12 embryos). The percentage of mispolarized commissural axons seen in fillets from the Math1::cre;BmprIaflox/flox; BmprIb–/– double mutant embryos (3.5%±0.35 s.e.m., n=2114 phl2+ neurons, 2 embryos) is statistically identical (P>0.4) to that seen in fillets from Gdf7–/– embryos (Butler and Dodd, 2003). Scale bar in B: 10 μm for A-F.

In these fillets, the extent of commissural axon mispolarization was now found to be comparable to that seen in either Bmp7 or Gdf7 mutants (see Fig. S2 in the supplementary material).
type I BMPRs are required to transduce the BMP component of the RP chemorepellent in vivo. BMPRIB appears to be the principal type I receptor that mediates the axon guidance activity of the BMPs, with BMPRIA necessary for commissural axon orientation only in the absence of BMPRIB.

The phenotype of the Bmpr1a; Bmpr1b double mutants suggests that BMPRIA might have weak activity as an axon guidance receptor. However, Bmpr1a is not present in postmitotic commissural neurons (Fig. 1) and our gain-of-function studies (Figs 3, 4) suggest that the misexpression of BMPRIB, but not BMPRIA, affects commissural axon outgrowth and guidance. These observations are more consistent with a model in which BMPRIA redundantly contributes to the establishment of neuronal polarity through an earlier role in the specification of commissural cell fate, rather than BMPRIA acting directly to mediate axon guidance. Since it is difficult to separate a polarizing activity from a guidance activity in vivo, we used the in vitro reorientation assay to further assess the response of wild-type, BMPRIA and BMPRIB-deficient commissural axons to the RP chemorepellent. The reorientation assay is a robust and sensitive measure of guidance activity (Augburger et al., 1999; Butler and Dodd, 2003) that can be used to measure the extent to which commissural axons respond to the RP chemorepellent. Explants of the dorsal spinal cord were dissected from E10.5 wild-type, Math1::cre;Bmpr1aflx/flx and Bmpr1b–/– mouse embryos. The commissural axon trajectory was then challenged by placing a RP explant, taken from E11 rat embryos, in contact with one of the lateral edges of the dorsal spinal explant (Fig. 7D). Commisural growth cones extending adjacent to the appended RP grow under both its influence and that of the endogenous RP, and the extent to which they are reoriented under these circumstances can be quantified. Consistent with previous observations (Butler and Dodd, 2003), E10.5 wild-type mouse commissural axons were reoriented by a rat RP explant (Fig. 7A,A′), with an average reorientation angle of 21.8°±2.0 (Fig. 7E). Math1::cre;Bmpr1aflx/flx commissural axons were deflected to a similar extent (Fig. 7B,B′), with an average reorientation angle of 20.4°±2.0 (Fig. 7E). By contrast, Bmpr1b–/– commissural axons were severely compromised in their ability to reorient away from the RP explant (Fig. 7C,C′).

The average angle by which Bmpr1b–/– commissural axons are reoriented is reduced to 9.55°±1.8 (Fig. 7E), which is statistically identical to the reorientation angles seen when RP explants taken from Bmp7–/– (9.26°±1.9) or Gdf7–/– (8.23°±1.4) mutant mice were used to challenge wild-type rat commissural axons (see Fig. S3 in the supplementary material) (Butler and Dodd, 2003). Thus, removing BMPRIB, but not BMPRIA, from commissural neurons has the same biological consequence as removing the BMPs from the RP, an observation that strongly suggests that BMPRIB is the sole type I receptor that mediates the ability of the BMP ligand to deflect commissural axons in the reorientation assay.

**DISCUSSION**

The discovery that inductive growth factors, such as the BMPs, have dual activities at different times in development, acting as both morphogens and axon guidance cues, has suggested a model in which the signals that initially establish the cellular fate of neurons are subsequently reused to specify the pattern of axonal trajectories. However, it remains unclear how these growth factors result in such different cellular outcomes during development. To address this question for the BMPs, we have determined that one of the canonical type I receptors, BMPRIB, is both necessary and sufficient to mediate the known guidance activities of the RP chemorepellent. Thus, the feed-forward mechanism that underlies the ability of the BMPs to signal different activities to developing commissural neurons does not depend on divergent receptor signalling, as had been seen for other morphogens, rather it requires the sequential use of the canonical BMPR complex. However, the type I BMPRs do not function interchangeably in this process, rather the exact composition of the canonical BMPR complex crucially determines the nature of the response of commissural neurons to the BMP signal (Fig. 8).

**Only BMPRIB is sufficient to disrupt commissural axon guidance**

Our gain-of-function studies have demonstrated that the activities of the type I BMPRs can be temporally separated. Thus, neural progenitors appear to have a limited period during early spinal
development in which they are competent to distinguish the BMPs as morphogens. It remains unclear how dorsal neural progenitors modulate their ability to respond to the BMP signal. The downregulation of Bmpr1a in postmitotic commissural neurons (Fig. 1) suggests that the competence to respond to the BMPs as morphogens could depend on the presence of BMPRIA. However, this model cannot be the case, because misexpressing Bmpr1a later in spinal development has no effect on dorsal cell induction (Fig. 2). The presence of Bmpr1a in the ventral spinal cord is intriguing, given that BMP signalling has been shown to antagonize Shh signalling in the specification of ventral cell fates (Liem et al., 2000). However, it remains unclear whether BMPRIA can modulate ventral cell identity.

Of the type I BMPRs, BMPRIB is primarily responsible for translating the gradient of BMPs from the RP into axon guidance cues for commissural neurons. Bmpr1b is specifically expressed in postmitotic dorsal neurons and introducing constitutively active forms of BMPRIB, but not BMPRIA, into the developing spinal cord results in the misprojection of axons into the VZ. The extent to which the direction of outgrowth was randomized remains unclear, since it was not possible to assess whether electroporated axons were mispolarized dorsally. Farnesylated GFP fills the entire neuronal process making it difficult to distinguish dorsally projecting axons from trailing processes. Additionally, more severe axon guidance defects might have been observed had it been possible to use a form of caBMPRIB that was completely independent of ligand activation. For both type I caBMPRs, although the activation of the receptor no longer requires ligand binding, the activity of the caBMPRs can be further enhanced by ligand binding (Akiyama et al., 1997). Thus, the electroporated commissural growth cones presumably perceive a foreshortened gradient of BMPs, rather than the uniform distribution of BMPs.

We also observed an unexpected defect in axon outgrowth following in ovo electroporation with caBMPRIB: commissural axons stalled upon reaching the ventral spinal cord. This defect does not appear to be a general delay in axon outgrowth because commissural axons did not grow uniformly more slowly as they projected ventrally around the spinal cord. Rather, there was a sharp decline in the number of caBMPRIB+ axons projecting beyond the dorsal spinal cord, suggesting that these axons stalled upon reaching the ventral spinal cord. The basis for stalled axon outgrowth remains unclear. The RP-derived BMPs may signal outgrowth information to commissural neurons. Alternatively, caBMPRIB+ axons may be compromised in their ability to respond to Shh and/or other attractive signals emanating from the FP. The elevated levels of BMP signalling achieved in caBMPRIB+ axons may antagonize Shh signalling, as has been shown for cell fate decisions (Liem et al., 2000), thus affecting the ability of commissural axons to respond to signals from the FP.

**Differential requirements for the type I BMPRs in commissural axon guidance**

The results from the gain-of-function studies suggest that commissural axons are guided away from the dorsal midline by the asymmetric activation of BMPRIB within the commissural growth cone. This model predicts that commissural growth cones will be similarly misguided by the uniform presence of BMPs, i.e. after either the constitutive activation of BMPRIB, or the loss of graded BMP signalling in the absence of either the ligand or relevant receptor. Supporting this prediction, commissural axons in Bmpr1b single mutants and Bmpr1a; Bmpr1b double mutants showed mispolarization defects similar to those observed in the gain-of-function studies.

A further prediction of the loss-of-function studies is that the loss of the receptor that mediates the BMP component of the RP repellent will result in comparable phenotypes to those seen in Bmp7 and Gdf7 mutants. In our previous work, we showed that the BMPs are required for the ability of the RP to reorient commissural axons in vitro, and to establish the polarized growth of commissural axons away from the RP in vivo (Augsburger et al., 1999; Butler and Dodd, 2003). In this study, the absence of the type I BMPRs from commissural neurons phenocopies the loss of either BMP gene from the RP. The in vitro reorienting activity of the RP is transduced solely by BMPRIB, and BMPRIB appears to be the principal receptor that mediates the BMP component of the RP in vivo, with BMPRIA supplying a compensatory activity in the absence of BMPRIB. Only small effects on commissural axon guidance were seen in our analysis of loss-of-function mutations in either the BMP genes (Butler and Dodd, 2003) or the type I BMP receptor genes (this study). However, it is not unusual that the loss of key axon guidance signals in vivo results in guidance defects that are either weak or transient, presumably because of the presence of other redundant signals. Thus, the activities revealed in in vitro assays, where such compensatory signals are not present, may be a more accurate indication of the role of an axon guidance cue or receptor than is revealed by loss-of-function genetic studies. Taking the in vivo and in vitro studies together, these data strongly suggest that BMPRIB is the crucial guidance receptor that translates BMP chemorepellent signals into the directed movement of commissural axons away from the dorsal midline (Fig. 8B).

The nature of the compensatory activity from BMPRIA remains unclear. BMPRIA alone is neither necessary nor sufficient as a guidance receptor for commissural axons. Thus, BMPRIA has either a very weak activity as a guidance receptor, or the compensatory activity of BMPRIA is a secondary effect of the role of dorsal cell fate specification in the assignment of neuronal polarity. Supporting this latter idea, Bmpr1a is not expressed in postmitotic commissural neurons and BMPRIA is required only in the absence of BMPRIB, consistent with the specification of cell fate being a redundant shared activity of BMPRIA and BMPRIB. Preliminary analysis has suggested that the distribution of Bmpr1a is not altered in Bmpr1b.
mutants (K.Y. and S.J.B., unpublished). Thus, the phenotypes seen in either the BmprIa; BmprIb double mutants or the BMP single mutants may be the result of defects both in neuronal polarity and axon guidance.

**Differential roles of BMPRIA and BMPRIB in cell fate specification and axon guidance**

In summary, our studies have suggested that the known activities of the BMP guidance cue in the RP can be accounted for by signalling through the canonical BMP signal transduction pathway. However, the type I BMPRs diverge functionally in their ability to translate the inductive and guidance activities of the Bmps. The specification of cell fate by the Bmps is a shared activity of both type I BMPRs, whereas commissural axon guidance is predominantly mediated by only one of the type I BMPRs, BMPRIB (Fig. 8). The extent to which BMPRIA mediates guidance decisions elsewhere in the developing nervous system remains to be determined. However, studies showing that BMPRIA is required for axon targeting in the developing retina (Liu et al., 2003) suggests BMPRIA may have a widespread role transducing BMP guidance signals.

How does BMP signalling result in two such different outcomes during development? One possibility is the type I BMPRs are differentially activated by particular BMP ligands. Thus, BMP homodimers direct cell fate decisions by activating the shared property of the type I BMPRs, whereas the Bmp7:GDF7 heterodimer reorients commissural axons by signalling through a unique property of BMPRIB (Fig. 8). Such differential signalling is then translated into a particular outcome by the activation of the relevant second messenger intermediate. The morphogenic activity of the Bmps is thought to be transduced by the Smad complex acting as transcriptional regulators (Massague et al., 2005). Additionally, BMP signalling has been shown to control the activation status of Lim kinase 1 (Limk1), a direct regulator of cofilin (Foletta et al., 2003; Lee-Hoeflich et al., 2004). Recent studies in vitro have shown that a gradient of BMP7 can regulate actin dynamics in Xenopus laevis growth cones by controlling the activity of cofilin (Wen et al., 2007). However, it remains to be determined which second messenger is relevant for commissural axon guidance in vivo. BMPRIA could activate a different second messenger to locally reorganize the cytoskeleton, such as Limk1, or the Smad complex could have a novel role outside of the nucleus. The Smad complex has not been previously shown to be active in the cytoplasm, although it is intriguing that a neomorphic mutation in Smad1 can result in the remodeling of the actin cytoskeleton (Aubin et al., 2004). Thus, through the sequential use of overlapping subsets of BMP ligands and receptors in a feed-forward mechanism, BMP signalling could direct multiple stages in the development of a single class of neurons.

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**Supplementary material**

Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/135/6/1119/DC1

References


