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Neural plate morphogenesis during mouse neurulation is regulated by antagonism of Bmp signalling

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Dorsolateral bending of the neural plate, an undifferentiated pseudostratified epithelium, is essential for neural tube closure in the mouse spinal region. If dorsolateral bending fails, spina bifida results. In the present study, we investigated the molecular signals that regulate the formation of dorsolateral hinge points (DLHPs). We show that *Bmp2* expression correlates with upper spinal neurulation (in which DLHPs are absent); that *Bmp2*-null embryos exhibit premature, exaggerated DLHPs; and that the local release of Bmp2 inhibits neural fold bending. Therefore, Bmp signalling is necessary and sufficient to inhibit DLHPs. By contrast, the Bmp antagonist noggin is expressed dorsally in neural folds containing DLHPs, noggin-null embryos show markedly reduced dorsolateral bending and local release of noggin stimulates bending. Hence, Bmp antagonism is both necessary and sufficient to induce dorsolateral bending. The local release of Shh suppresses dorsal noggin expression, explaining the absence of DLHPs at high spinal levels, where notochordal expression of Shh is strong. DLHPs 'break through' at low spinal levels, where Shh expression is weaker. *Zic2* mutant embryos fail to express Bmp antagonists dorsally and lack DLHPs, developing severe spina bifida. Our findings reveal a molecular mechanism based on antagonism of Bmp signalling that underlies the regulation of DLHP formation during mouse spinal neural tube closure.

KEY WORDS: Neurulation, Morphogenesis, Neural tube defects, Noggin, Sonic hedgehog, Mouse, Zic genes

INTRODUCTION

Neurulation is the process in which the neural plate bends and fuses to form the neural tube – the developmental forerunner of the brain and spinal cord. Understanding the molecular regulation of neurulation is significant not only because of its pivotal importance in establishing the CNS primordium, but also because defects of neurulation result in clinically important congenital malformations, termed neural tube defects (NTDs). Failure of brain closure (anencephaly) and low spinal closure (open spina bifida) occur with a high prevalence in humans, around 1 per 1000 pregnancies (Mitchell, 2005), and form part of the phenotype of over 100 mutant mouse strains (Copp et al., 2003).

Mouse primary neurulation is characterised by a stereotypical pattern of neural plate bending along the spinal neuraxis (Shum and Copp, 1996). Neural tube closure begins at the level of the cervical/hindbrain boundary at embryonic day (E)8.5 (6-somite stage), and finishes at E10.5 (30-somite stage) when closure is completed at the upper sacral level. In the intervening 48-hour period, a wave of neural tube closure propagates in a cranio-caudal direction down the spine. Just caudal to the closure propagation front, a region of open, elevating neural folds comprises the 'posterior neuropore' (PNP). This represents the next axial region to undergo neural tube closure (Van Straaten et al., 1992). Previously, we divided the continuous process of spinal neurulation into three modes, according to the morphology of neural plate bending within the PNP (Shum and Copp, 1996). In mode 1 (E8.5-E9), the closing neural plate has a V-shaped cross section, with bending solely at the median hinge point (MHP), overlying the notochord. As neurulation progresses to lower spinal levels, mode 2 (E9-E9.75) becomes recognisable, in which the closing neural folds adopt a different morphology with paired dorsolateral hinge points (DLHPs) in addition to the MHP. At the most-caudal level of the spinal axis, just prior to completion of spinal neurulation, MHP bending disappears and the neural plate bends solely at the DLHPs (mode 3; E9.75-E10.5).

Although the cell shape changes that comprise bending of the neuroepithelium at MHP and DLHPs have been documented (Schoenwolf, 1985; Smith et al., 1994), the identity of the dorsoventral molecular signals that regulate these cell shape changes remains unknown. Previously, we and others showed that MHP bending requires the influence of the adjacent notochord: suppression of notochordal development results in the absence of midline bending (Davidson et al., 1999; Smith and Schoenwolf, 1989; Ybot-Gonzalez et al., 2002). In an analogous way, removal of the surface ectoderm, which normally covers the outer aspect of the dorsal neural fold, results in the absence of DLHPs, whereas just a small surface ectodermal remnant is capable of inducing a DLHP (Jacobson and Moury, 1995; Moury and Schoenwolf, 1995; Ybot-Gonzalez et al., 2002). Hence, signals from the notochord and surface ectoderm are required for MHP and DLHP formation, respectively. By contrast, the paraxial mesoderm can be removed without influencing MHP or DLHP formation (Ybot-Gonzalez et al., 2002). Further studies have demonstrated a negative influence of Shh, emanating from the notochord, on the presence of DLHPs. Shh is both necessary and sufficient to inhibit DLHP formation, as demonstrated by the occurrence of DLHPs at an abnormally rostral level in *Shh*$^{-/-}$ mice, and the inhibition of DLHP formation by beads releasing N-terminal Shh (Shh-N) peptide implanted adjacent to the dorsolateral neural plate at low spinal levels (Ybot-Gonzalez et al., 2002).

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Here, we identify the molecular interactions regulating dorsolateral bending during mouse neurulation. Analysis of embryos lacking function of *Bmp2* or noggin, and of wild-type embryos exposed to the local release of Bmp2 and noggin peptides, identifies Bmp2 as an inhibitor of DLHP formation. By contrast, noggin and probably another Bmp antagonist, neuralin (also called chordin-like 1), induce DLHP formation. DLHPs are restricted to low levels of the spinal neuraxis because, at upper levels, Shh concentrations are high and noggin expression is inhibited. DLHPs are absent from homozygous *Zic2*^{Ku} embryos, which later develop severe spina bifida owing to the absence of Bmp antagonists in the dorsal neural plate. Hence, this study reveals a molecular mechanism of regulation of neural tube closure, based on inhibition of Bmp signalling, in the spinal region of the mouse embryo.

MATERIALS AND METHODS

Mouse strains, embryo culture and insertion of beads

Non-mutant embryos were obtained from CD1 random-bred mice. Mice with mutations in *Bmp2* (Zhang and Bradley, 1996), noggin (McMahon et al., 1998), *Shh* (Chiang et al., 1996) and *Zic2* (Elms et al., 2003) were genotyped as described previously. Whole-embryo culture was performed as described (Copp et al., 1999), with opening of the yolk sac and amnion to a minimum extent compatible with access to the PNP region for bead insertion. AffiGel blue beads (BioRad, Cat. No. 153-7302) were soaked for at least 2 hours at 4°C in 0.5 μg/μl Bmp2 (R&D Systems), 1 μg/μl noggin (R&D Systems), 1 μg/μl Shh-N peptide (R&D Systems) or in PBS as a vehicle control. Beads were held by suction on the end of a mouth-controlled glass micropipette, inserted singly or in pairs through a slit in the surface ectoderm overlying the neural fold and positioned as closely as possible to the dorsolateral neural fold region. Following a variable period in culture, depending on the experiment, embryos were removed from their extraembryonic membranes, rinsed in PBS, and fixed in either Bouin's fluid for haematoxylin and eosin (H&E) staining, 4% paraformaldehyde (PFA) in PBS for in situ hybridisation, and either PFA or Sainte-Marie's fixative (95% ethanol, 1% acetic acid) for immunohistochemistry. Presence or absence of a DLHP was determined in transverse embryo sections using angle measurement criteria as described previously (Ybot-Gonzalez et al., 2002).

In situ hybridisation

Previously published probes were: *Bmp2*, *Bmp4* and *Bmp7* (Furuta et al., 1997), cadherin 6 (Henderson et al., 1997), chordin (Klingensmith et al., 1999), *Msx1* (Mackenzie et al., 1991), *Msx2* (Monaghan et al., 1991), neuralin (Coffinier et al., 2001) and *Zic2* (Gaston-Massuet et al., 2005). Additional *Msx1* and *Msx2* probes were as described (Catron et al., 1996). To prepare a cDNA probe for noggin, forward 5′-CCAGCACTAT-CTACACATCC-3′ and reverse 5′-ACTTGGATGGCTTACACACC-3′ primers were used to amplify a 518 bp fragment corresponding to nucleotides 327-845 of the noggin cDNA sequence (GenBank accession number u79163). Reverse transcriptase (RT)-PCR was performed on total RNA from E10.5 CBA/Ca embryos using TRIzol reagent (Gibco BRL). The amplified fragment was cloned into the pGEM-T vector (Promega, UK) and sequenced to confirm its identity. Whole-mount in situ hybridisation with preparation of 50 μm transverse vibratome sections was as described (Copp et al., 1999). In situ hybridisation on paraffin-embedded sections was performed using digoxigenin-labelled cRNA probes (Breitschopf et al., 1992). Sense-strand cRNA probes were tested for all genes with no specific hybridisation.

Immunohistochemistry, cell counting and statistical analysis

Fixed embryos were dehydrated, embedded in paraffin wax and sectioned at 7 μm. Sections were rehydrated and antigen retrieval was performed using Declere (Cell Marque). Antibodies to the phosphorylated forms of Smad1, Smad5 and Smad8 combined (phospho-Smad1,5,8; dilution 1:100; Cell Signalling Technology); caspase 3 (dilution 1:1000; Cell Signaling Technology); phospho-histone H3 (dilution 1:250; Upstate Biotechnology); noggin (dilution 1:7; R&D systems); and Bmp2 (dilution 1:10; R&D Systems) were diluted in 5% goat or rabbit serum, 0.15% glycine and 2 mg/ml BSA in Tris-buffered saline. Primary antibodies were detected with a biotinylated goat anti-rabbit or biotinylated rabbit anti-goat (both 1:250, DAKO), using a Vectastain ABC kit (Vector) and diaminobenzidine (peroxidase substrate kit DAB, Vector). Omission of primary antibody served as a negative control. Embryos for comparison of combined phospho-Smad1, -Smad5 and -Smad8 staining were processed together in the same paraffin block and on the same slide, to enable accurate comparison. Sections were counter-stained with methylene green. For analysis of apoptotic cell frequency, the number of cells positive for caspase 3 was determined in the dorsal and ventral halves of the neural plate in 7-12 sections through the PNP of three to four embryos at each of modes 1 and 3. The mean number of apoptotic cells per section was compared by two-way analysis of variance, using neurulation mode and neural plate region as variables. For analysis of cell proliferation, cells positive for phospho-histone H3 were counted in the dorsal two thirds of the neural plate in five sections of three embryos at each of modes 1 and 3. The mean number of mitotic cells per section was compared by *t*-test.

Scanning electron microscopy

Embryos were rinsed in PBS and fixed overnight in 2% glutaraldehyde, 2% PFA in PBS. Tissues were rinsed in phosphate buffer and post-fixed in 1% OsO_4 for 1 hour. Samples were dehydrated through an ascending alcohol series, using three changes of acetone to displace the alcohol, then CO_2 critical point dried, mounted on specimen stubs, gold sputter-coated and examined in a JEOL SEM 5410 LV scanning electron microscope.

RESULTS

In order to investigate the molecular regulation of DLHP formation, we first examined the expression of genes in the Bmp signalling pathway, because our previous studies had suggested a possible role for Bmp2 in this process (Ybot-Gonzalez et al., 2002).

Diminished Bmp signalling in association with DLHP formation

We compared mode 1, in which DLHPs are absent, with mode 3, in which DLHPs are present (Fig. 1A). *Bmp2* and *Bmp7* mRNA transcripts occurred with similar intensity in the surface ectoderm overlying the spinal neural folds of mode 1 (Fig. 1B,D) and mode 3 (Fig. 1C,E and data not shown) embryos. *Bmp2* was localised specifically to the dorsal-most ectoderm, whereas *Bmp7* was expressed throughout the surface ectoderm at this axial level. *Bmp4*, *Bmp5* and *Bmp6* were expressed in more-ventral or anterior embryonic regions, but not in the vicinity of the neural folds (data not shown). In the absence of any apparent difference in *Bmp2* or *Bmp7* expression between modes 1 and 3, we questioned whether the presence or absence of DLHPs might be associated with differences in the activity of downstream Bmp signalling. Cadherin 6, a gene regulated by Bmp signalling (Sela-Donenfeld and Kalcheim, 1999), was expressed intensely throughout the neural plate in mode 1 (Fig. 1F), but appeared downregulated at mode 3 (Fig. 1G and data not shown). By contrast, *Msx1*, which is also regulated by Bmps in some systems, was not detectable in the neural folds of mode 1 embryos (Fig. 1H), and was only weakly expressed at the neural fold tips in mode 3 (Fig. 1I and data not shown). *Msx2* was not detected in the PNP region (data not shown). Immunohistochemistry for phospho-Smad1,5,8, which are immediately downstream of Bmp receptor activation (Massague and Wotton, 2000), revealed markedly stronger expression in the dorsal neural folds at mode 1 than at mode 3 (Fig. 1J,K), consistent with the pattern of cadherin 6 expression. It seems, therefore, that, although Bmp gene expression per se does not differ along the neuraxis, downstream Bmp signalling is strongest in locations where DLHPs are absent. This suggests an inhibitory effect of Bmps on neural plate bending.

Next, we studied whether cell death or cell proliferation differ in the neural plate of mode 1 and mode 3 embryos. Immunohistochemistry for activated caspase 3 revealed considerable numbers of dying cells dorsally in the neural folds of mode 1, whereas cell death was only rarely observed in the mode 3 neural plate (Fig. 1L,M). The incidence of cell death was significantly increased in the dorsal region of the mode 1 neural plate (2.6±0.5 cells positive for caspase 3 per section) compared with the ventral region at mode 1 (0.3±0.1 cells per section; $P<0.05$), the dorsal region at mode 3 (0.4±0.01 cells per section; $P<0.05$) and the ventral region at mode 3 (0.2±0.1 cells per section; $P<0.05$). By contrast, phospho-histone H3 immunostaining showed no difference in the frequency of proliferating cells in the dorsal two thirds of the neural plate at mode 1 and 3 (Fig. 1N,O; mean number of H3-positive cells: 1.5±0.2 for mode 1; 1.2±0.1 for mode 3; $P>0.05$). Hence, programmed cell death, which is associated with active Bmp signalling in the early embryonic hindbrain (Graham et al., 1994; Yokouchi et al., 1996; Jernvall et al., 1998), also appears to correlate specifically with mode 1 neurulation in the mouse embryo, consistent with the idea that, at mode 1, Bmp signalling is strongest in the dorsal neural plate, in which DLHPs are absent.

Bmp2 is necessary and sufficient to inhibit dorsolateral neural plate bending

In order to examine the causal relationship between Bmp signalling and dorsolateral neural plate bending, we compared the spinal neural folds of wild-type embryos with those of embryos homozygous for a null mutation in *Bmp2*. Whereas $Bmp2^{-/-}$ embryos die at around mid-gestation (Zhang and Bradley, 1996), we found that neural fold morphology at the PNP was clearly identifiable in both E8.5 and E9.5 embryos. At the 7-somite stage, immediately after neural tube closure was initiated, $Bmp2^{-/-}$ embryos exhibited mode 1 neurulation ($n=5$), as did wild-type littermates (Fig. 2A,B). Just a few hours later, at the 9-somite stage, whereas wild-type embryos continued in mode 1 (Fig. 2C), all $Bmp2^{-/-}$ embryos studied ($n=7$) were found to exhibit striking DLHPs, indicating premature entry into mode 2/3 (Fig. 2D). This is despite the growth retardation that characterises null embryos at this stage (Zhang and Bradley, 1996). At 15 somites, the latest stage that could be examined owing to their imminent demise, $Bmp2^{-/-}$ embryos ($n=4$) exhibited an entirely closed spinal neural tube, consistent with the premature formation of DLHPs (Fig. 2F) (Yip et al., 2002). By contrast, wild-type littermates with 15 somites were in mode 2, with prominent DLHPs (Fig. 2E). We conclude that Bmp2 is necessary for the inhibition of DLHPs early in mouse spinal neurulation and that, in its absence, null embryos show premature, exaggerated formation of DLHPs.

To determine whether Bmp2 is sufficient for the inhibition of DLHP formation, we implanted AffiGel blue beads, soaked in either Bmp2 or PBS, within the presomitic mesoderm adjacent to one spinal neural fold, in wild-type embryos at either E8.5 (mode 1) or E9.5 (mode 2/3). After 5 hours culture, embryos were harvested and sectioned transversely to determine whether DLHPs had been induced (at mode 1, when DLHPs are normally absent) or inhibited (at mode 2/3, when DLHPs are normally present). Local release of

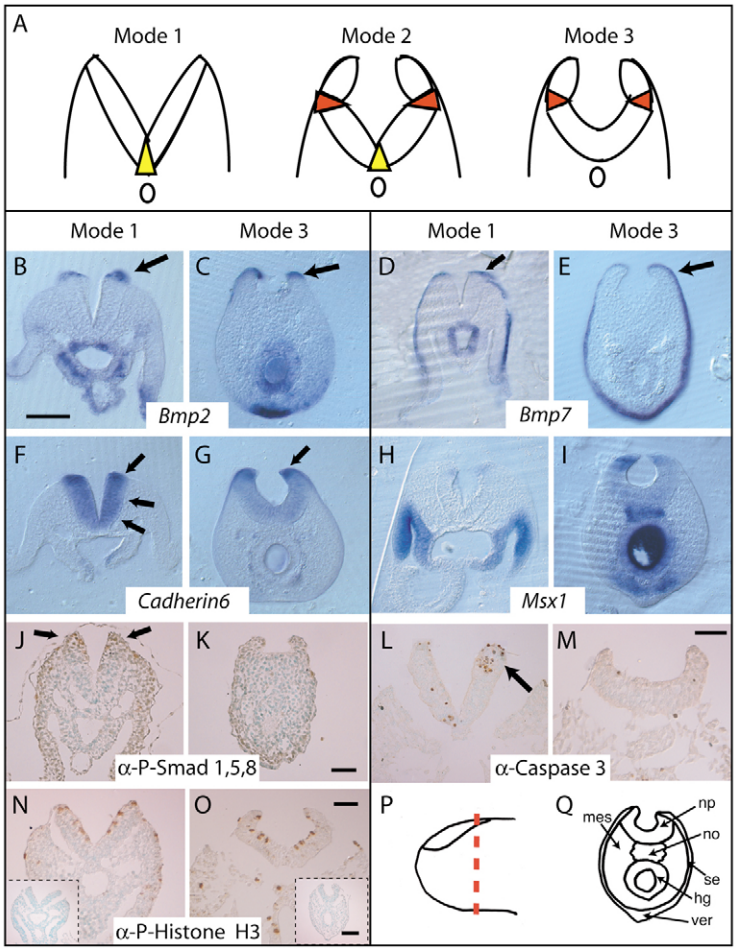


Fig. 1. Bmp signalling during mouse spinal neurulation. (**A**) Diagrammatic transverse sections through the PNP of embryos at modes 1 (E8.5-E9), 2 (E9-E9.75) and 3 (E9.75-E10.5) of spinal neurulation. Yellow triangles, median hinge point (MHP); red triangles, dorsolateral hinge points (DLHPs). (**B-I**) In situ hybridisation for *Bmp2* (B,C), *Bmp7* (D,E), cadherin 6 (F,G) and *Msx1* (H,I) during mode 1 (E8.5; B,D,F,H) and mode 3 (E9.5; C,E,G,I) neurulation as seen in transverse sections through the posterior neuropore (PNP) of whole-mount embryos. Arrows show intense in situ hybridisation signal. (**J-O**) Immunohistochemistry for phospho-Smad1, -Smad5 and -Smad 8 combined (phospho-Smad1,5,8; J,K); activated caspase 3 (L,M); and phospho-histone H3 (N,O) during mode 1 (J,L,N) and mode 3 (K,M,O) neurulation. (J) Arrows indicate high levels of phospho-Smad1,5,8 in dorsal neural folds. (L,M) Caspase 3-positive cells were accumulated over ten consecutive sections of the same embryo and projected onto a single section. (L) Arrow indicates intensive apoptosis in the dorsal neural fold at mode 1. (N,O; insets) Controls with the omission of the primary antibody. (**P**,**Q**) Diagrammatic right-sided view (P) of the PNP at mode 3. Transverse section (Q) at the level of the dotted line in P summarises the embryonic tissues that are visible in C,E,G,I,K,M,O: hg, hindgut; mes, mesoderm; np, neural plate; no, notochord; se, surface ectoderm; ver, ventral ectoderm ridge. Scale bars: 0.1 mm in B (also C-I), in K (also J), in M (also L), in O (also N) and in inset O (also inset N).

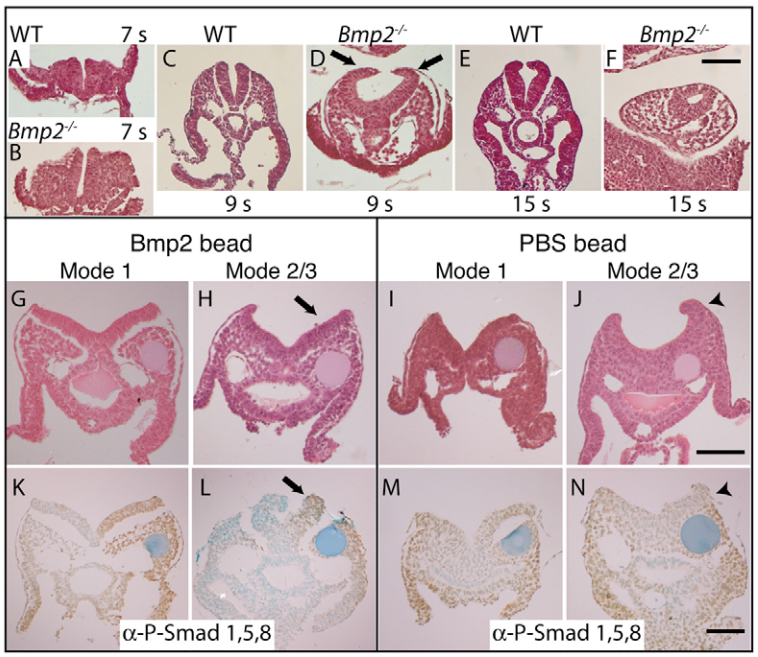


Fig. 2. Bmp2 is necessary and sufficient to inhibit DLHPs. (**A-F**) Transverse H&E-stained sections through the posterior neuropore (PNP) region of E8.5 (A-D) and E9 (E,F) embryos. At 7 somites (7 s), when closure is first initiated, dorsolateral hinge points (DLHPs) are absent from wild-type (WT, A) and *Bmp2*$^{-/-}$ (B) embryos. Whereas wild-type embryos at 9 somites continue in mode 1 neurulation (C), *Bmp2*$^{-/-}$ embryos show marked DLHPs, characteristic of modes 2/3 (arrows in D). At 15 somites, wild-type embryos have entered mode 2 neurulation with clear DLHPs (E), whereas 15-somite *Bmp2*$^{-/-}$ embryos have undergone premature closure of the neural tube (F). (**G-N**) Transverse sections through the PNP of mode 1 (G,I,K,M) and mode 2/3 (H,J,L,N) embryos stained with H&E (G-J) or after immunohistochemistry for phospho-Smad1, -Smad5 and -Smad8 combined (K-N). A bead soaked in Bmp2 (G,H,K,L) or PBS (I,J,M,N) has been implanted next to one neural fold. Exposure to Bmp2 inhibits DLHP formation in mode 2/3 (arrows in H,L), whereas DLHPs remain in PBS-treated control embryos (arrowheads in J,N). Note that the period of phospho-Smad staining was longer in PBS control embryos (M,N) than in those implanted with a Bmp2 bead (K,L) in order to reveal endogenous expression, as a positive control. Scale bars: 0.1 mm in F (also A-E); 0.1 mm in J (also G-I) and N (also K-M).

either Bmp2 or PBS had no discernible effect on the mode 1 neural fold (Fig. 2G,I,K,M), whereas, in the mode 2/3 neuropore, Bmp2 inhibited dorsolateral bending in 76% of cases (n=25; Fig. 3Q) compared with PBS beads, which had no effect (compare Fig. 2H,L with Fig. 2J,N). Immunohistochemistry on sections of embryos implanted with Bmp2 beads showed a striking upregulation of phospho-Smad1,5,8 in tissues on the same side as the bead, but not contralaterally (Fig. 2K,L). By contrast, embryos with implanted PBS beads demonstrated only background Smad staining (Fig. 2M,N). Hence, local release of Bmp2 stimulates downstream signalling and is sufficient to inhibit dorsolateral bending of the neural plate in mouse spinal neurulation.

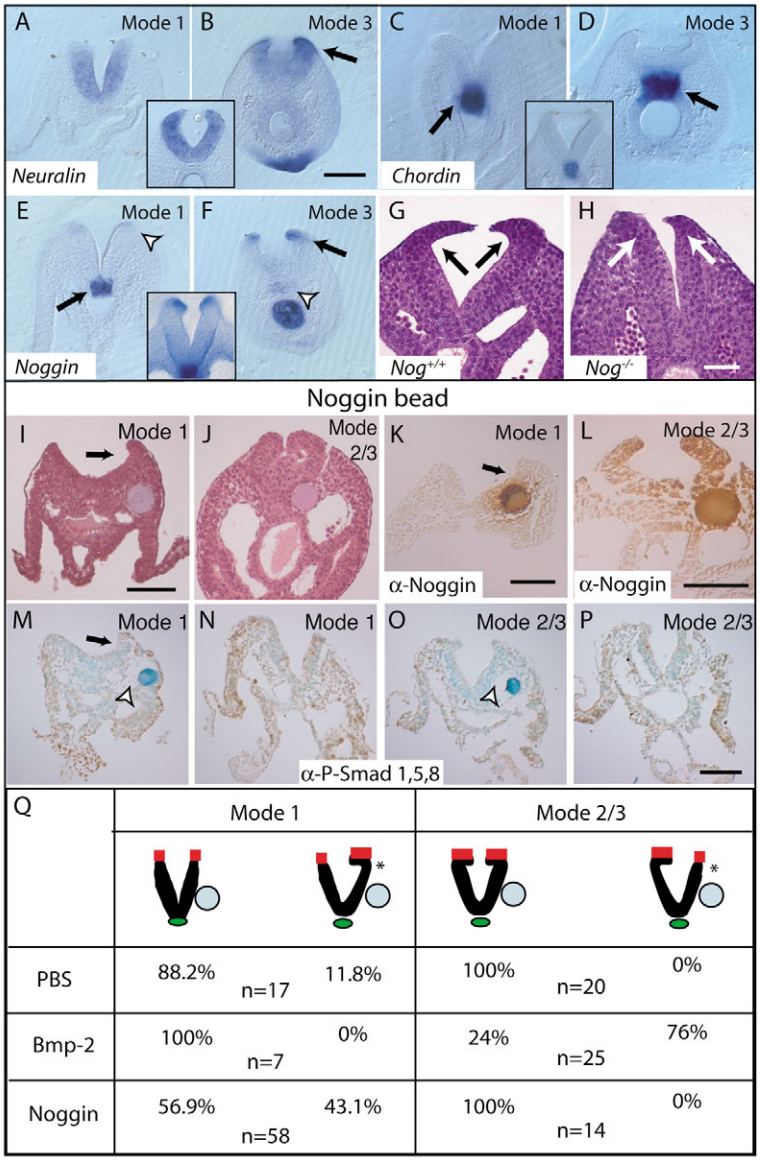
Noggin is necessary and sufficient for the induction of DLHPs

In view of the finding that both Bmp2 dorsally (Fig. 2), and Shh ventrally (Ybot-Gonzalez et al., 2002), are necessary and sufficient to inhibit DLHPs, we next considered the hypothesis that Bmp antagonists including noggin, neuralin (Coffinier et al., 2001) and chordin might be in vivo inducers of dorsolateral bending during mouse neurulation. In situ hybridisation showed that neuralin was expressed in the neural plate at modes 1, 2 and 3 of spinal neurulation (Fig. 3A,B and data not shown), whereas chordin was expressed solely in the notochord (Fig. 3C,D and data not shown). Noggin was expressed in the notochord, but also in the neural fold tips, immediately underlying the *Bmp2*- and *Bmp7*-positive surface ectoderm. Strikingly, in mode 1, noggin was expressed most-intensely in the notochord, but only weakly at the neural fold tips (Fig. 3E), whereas, in mode 3, the reverse was true: noggin transcripts were intense at the neural fold tips but weak in the notochord (Fig. 3F and data not shown). Mode 2 gave an intermediate result, in which noggin expression was strong in both neural folds tips and notochord (Fig. 3E,F, inset). Neuralin was also expressed more-intensely at the tips of the neural folds in mode 3 than it was in mode 1, and also had an intermediate appearance in mode 2 (Fig. 3A,B). Hence, the dorsal neural plate expression of noggin and neuralin correlates with the formation of DLHPs at mode 3.

To test whether Bmp antagonism is necessary for DLHP formation, we examined the PNP region of embryos homozygous for a loss-of-function allele of noggin, in the presence of which spina bifida is observed at high frequency (McMahon et al., 1998; Stottmann et al., 2006). On this genetic background, normal littermates displayed prominent DLHPs at mode 2 (17- to 18-somite stage), whereas stage-matched noggin$^{-/-}$ (*Nog*$^{-/-}$) embryos exhibited markedly reduced dorsolateral bending, with neural plate morphology closely resembling mode 1 (Fig. 3G,H). This finding is the opposite of that observed with *Bmp2*$^{-/-}$ embryos and is consistent with the idea that noggin is required for dorsolateral bending during PNP development.

To determine whether noggin is sufficient to induce DLHP formation, we inserted noggin-soaked beads adjacent to the neural fold in wild-type embryos. After 4-5 hours culture, in 43% of cases (n=58; Fig. 3Q), an ectopic DLHP was observed in the mode 1 neural plate (Fig. 3I,K,M), whereas noggin beads had no discernible effect on mode 2/3 neural folds (Fig. 3J,L,O). Immunohistochemistry for phospho-Smad1,5,8 confirmed that the local release of noggin markedly diminished downstream Bmp activation (Fig. 3M-P), although with variation between embryos. It is possible that this variable inhibition of phospho-Smad signalling by exogenous noggin can explain why noggin is not able to induct DLHPs in all bead-implanted embryos. We conclude that noggin is both necessary and sufficient to induce dorsolateral bending in mode 1 neural plate during mouse spinal neurulation.

Interestingly, exogenous noggin induced a contralateral DLHP in some embryos (13/58; Fig. 3Q), although it more frequently produced an ipsilateral DLHP (16/58; Fig. 3I,K,M). By contrast, the local release of Bmp2 inhibited DLHP formation and activated downstream Smad signalling solely on the same side as the implanted bead. Because the release of peptides from implanted beads is difficult to quantitate, we cannot rule out differential loading or diffusion of noggin and Bmp2 as an explanation for this observation. Alternatively, the strong expression of the Bmp antagonist chordin in the notochord (Fig. 3C,D) might serve to neutralise Bmp2 diffusing to the midline, whereas noggin is able to cross the midline unopposed.



Q	Mode 1		Mode 2/3	
PBS	88.2% n=17	11.8%	100% n=20	0%
Bmp-2	100% n=7	0%	24% n=25	76%
Noggin	56.9% n=58	43.1%	100% n=14	0%

Fig. 3. Antagonism of Bmps is necessary and sufficient to induce DLHPs. (**A-F**) In situ hybridisation for neuralin (A,B), chordin (C,D) and noggin (E,F) gene expression in mode 1 (A,C,E), mode 2 (insets in A-F) and mode 3 (B,D,F), as seen in transverse sections through the posterior neuropore (PNP) of whole-mount embryos. Arrows indicate detection of a strong signal in the dorsal neural plate or notochord (B-F); arrowheads indicate detection of a diminished signal in the dorsal neural plate or notochord (E,F). (**G**,**H**) Transverse sections, stained with H&E, through the PNP of E9.5 $Nog^{+/+}$ (G) and $Nog^{-/-}$ (H) embryos. Wild-type embryos show exaggerated dorsolateral hinge points (DLHPs; G, black arrows) whereas DLHPs in mutant embryos are absent (H, left white arrow) or markedly reduced (H, right white arrow). (**I-P**) Transverse sections through the PNP of mode 1 (I,K,M,N) and mode 2/3 (J,L,O,P) embryos after the implantation of beads soaked in noggin peptide. Sections were stained with H&E (I,J), or by immunohistochemistry for noggin (K,L) or phospho-Smad1,5,8 (M-P). Arrows, induction of DLHPs ipsilaterally (I,K,M) after exposure to exogenous noggin. Notice the reduced phospho-Smad staining around the noggin beads (arrowheads in M,O) compared with slightly more-rostral sections of the same embryos (N,P), which were not exposed to exogenous noggin and show normal endogenous levels of phospho-Smad1,5,8. (**Q**) Summary of experiments showing the induction of DLHPs by noggin (mode 1, second column, asterisk) and inhibition of DLHPs by Bmp2 (mode 2/3, second column, asterisk). PBS: implantation of saline control beads. Percentages of embryos with particular morphology are shown, together with total number (n) receiving bead implants. Out of 25 (43.1%) mode 1 embryos with noggin-induced DLHPs, 12 had an ipsilateral DLHP, nine had a contralateral DLHP and four had bilateral DLHPs. The DLHP was contralateral in both of the affected PBS-treated mode 1 embryos. The frequency of DLHP induction in mode 1 is significantly greater in noggin-treated embryos than in PBS controls (Fisher exact test; P=0.02). The frequency of DLHP inhibition in mode 2/3 is significantly greater in Bmp2-treated embryos than in PBS controls (P<0.001). Scale bars: 0.1 mm in B (also A,C-F and insets); 0.05 mm in H (also G); 0.1 mm in I (also J) in K, in L and in P (also M-O).

Regulation of noggin expression by Bmp2 and Shh during spinal neurulation

The analysis of *Bmp2*- and noggin-null embryos, and of wild-type embryos implanted with Bmp2 and noggin beads, suggests a mechanism in which Bmp signalling negatively regulates DLHP formation, with alleviation of this inhibition by noggin. At mode 1, noggin is expressed at low intensity in the dorsal neural plate so that Bmp signalling is dominant, preventing DLHP formation. By contrast, at mode 2/3, noggin expression is upregulated, preventing Bmp-mediated DLHP inhibition and enabling dorsolateral bending to occur. Two further questions arise: first, how is noggin expression regulated spatially, so that transcripts are found specifically at the tips of the neural folds? Second, how is noggin expression regulated temporally, so that noggin transcripts are more plentiful in the dorsal neural plate at mode 2/3 than at mode 1?

Our previous work demonstrated that surface ectoderm attachment is required for the formation and maintenance of DLHPs (Ybot-Gonzalez et al., 2002). Perhaps Bmp2 and/or Bmp7 in the surface ectoderm are responsible for inducing noggin expression, thereby explaining this dependence on surface ectoderm attachment. To investigate this possibility, we examined the expression of noggin mRNA in $Bmp2^{-/-}$ embryos compared with non-mutant littermates. At all stages prior to the completion of PNP closure, at around the 15-somite stage, we observed marked downregulation of noggin expression in $Bmp2^{-/-}$ embryos, with a total absence of transcripts from the dorsal neural plate (Fig. 4A-C). By contrast, notochordal expression of noggin continued to be detected in $Bmp2^{-/-}$ embryos, although its expression was less-intense caudally. We also implanted Bmp2 beads into wild-type embryos, and observed a massive upregulation of noggin transcripts ipsilateral to the bead (Fig. 4D,E). Hence, dorsal neuroepithelial expression of noggin is stimulated by Bmp2 from the overlying surface ectoderm, whereas notochordal noggin seems likely to be regulated by factors other than Bmp2.

The expression of neither *Bmp2* nor *Bmp7* varied along the spinal axis (Fig. 1B-E) suggesting that other factor(s) must be responsible for the temporal regulation of noggin expression from mode 1 to 3. We found previously that the strength of Shh signalling from the notochord diminishes as the wave of spinal neurulation passes down the body axis (Ybot-Gonzalez et al., 2002), raising the possibility that Shh might negatively regulate noggin expression in the dorsal neural plate. If confirmed, this would be an example of Shh-mediated dorsoventral regulation of neural tube gene expression at

a particularly early developmental stage. Most of the known Shh-regulated genes are expressed after spinal neural tube closure (Jessell, 2000). We examined *Shh*$^{-/-}$ embryos and found the spread of the dorsal domain of noggin expression to a more ventral level, both in the PNP and closed neural tube (Fig. 4H-K). Moreover, the implantation of beads soaked in Shh-N peptide led to marked inhibition of noggin expression at the tip of the ipsilateral neural fold in all embryos examined (n=6; Fig. 4F,G). Hence, it seems likely that noggin expression is confined to low spinal levels (modes 2 and 3) as a result of negative regulation by Shh during upper spinal neurulation. This finding provides a molecular explanation for the inhibitory effect of Shh on DLHP formation, demonstrated in our previous study (Ybot-Gonzalez et al., 2002).

Absence of DLHPs precedes the development of spina bifida in the *Zic2*^{Ku} mutant

Embryos homozygous for the *Kumba* (*Ku*) loss-of-function allele of *Zic2* (Elms et al., 2003) fail to close their neural tube in both cranial and caudal regions (Fig. 5A,B), with a 69% frequency of exencephaly and 100% penetrant spina bifida later in gestation (Elms et al., 2003). Scanning electron microscopy of the PNP at E9.5 showed that dorsolateral bending was present in wild-type littermates (Fig. 5C), whereas *Zic2*$^{Ku/Ku}$ embryos had completely straight neural folds throughout the enlarged PNP (Fig. 5D,E). Sections at the rostral end of the neuropore confirmed that DLHPs are present in E9.5 wild-type embryos, early in mode 2, but absent from stage-matched *Zic2*$^{Ku/Ku}$ mutants (Fig. 5F,G). To examine further the correlation between the absence of DLHPs and failure of spinal neural tube closure, we determined the rostral-most somite level at which the neural tube is persistently open in *Zic2*$^{Ku/Ku}$ embryos. Remarkably little inter-embryo variation was observed, with a mean somite level of 13.3±0.1 (range: 12-14; n=30). Wild-type embryos on the CBA/Ca and closely related C3H/He (*Zic2*^{Ku}) genetic backgrounds first exhibit DLHPs at the 14-somite stage (Shum and Copp, 1996). Hence, our findings support the idea that Zic2 function is required for the onset of mode 2 spinal neurulation, as marked by the first appearance of DLHPs, but is not necessary for mode 1 neurulation. The transition from mode 1 to mode 2 neurulation appears blocked in *Zic2*$^{Ku/Ku}$ embryos, and neural tube closure fails from this point onwards, leading to severe spina bifida.

Zic2$^{Ku/Ku}$ mutants lack dorsal expression of Bmp antagonists

The absence of DLHPs from *Zic2*$^{Ku/Ku}$ embryos indicates a requirement for *Zic2* specifically in modes 2 and 3 of neurulation. Consistent with this, we detected upregulation of *Zic2* expression in mode 3 compared with mode 1 (Fig. 6A,B). Because DLHP formation requires antagonism of the inhibitory influence of Bmp2, we considered two possible explanations for the absence of DLHPs in *Zic2*$^{Ku/Ku}$ embryos: overexpression of Bmp2 or a lack of Bmp antagonism. Elevated expression of phospho-Smad1,5,8 suggested supra-normal levels of Bmp signalling in *Zic2*$^{Ku/Ku}$ embryos compared with wild-type littermates (Fig. 6C,D). On the other hand, *Bmp2* expression in the surface ectoderm overlying the neural folds was similar in *Zic2*$^{Ku/Ku}$ and wild-type embryos (Fig. 6E,H). By contrast, the expression of noggin (Fig. 6F,I) and neuralin (Fig. 6G,J) was markedly downregulated in the dorsal neural tube of *Zic2*$^{Ku/Ku}$ embryos compared with wild-type littermates, strongly suggesting that Bmp antagonism is defective in the absence of functional Zic2. Taken together, these observations suggest that the lack of DLHPs in *Zic2*$^{Ku/Ku}$ embryos results from abnormally high levels of Bmp signalling owing to the failure of expression of the Bmp antagonists noggin and neuralin.

DISCUSSION

During avian and mammalian neurulation, the neural plate bends at the MHP overlying the notochord, and at paired DLHPs, in which the neural plate changes its basal contact from surface ectoderm to paraxial mesoderm (Schoenwolf, 1985; Shum and Copp, 1996). Whereas neural tube closure in the upper spine of mouse embryos is

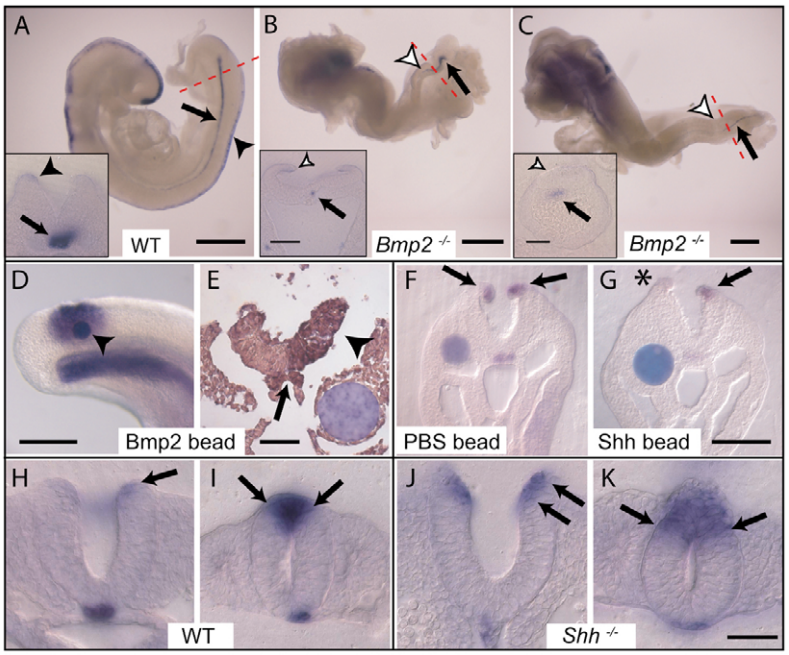


Fig. 4. Noggin expression is induced by Bmp2 and inhibited by Shh. (**A-C**) In situ hybridisation (lateral views) for noggin expression in wild-type (WT, A) and *Bmp2*$^{-/-}$ (B,C) embryos. Insets (A-C) correspond to transverse sections of the embryos at the level of the dashed red lines. Noggin is expressed in the notochord of all genotypes (arrows in A-C and in insets) but in the dorsal neural tube of only the wild-type embryo (black arrowheads in A and inset) and not in *Bmp2*$^{-/-}$ embryos (white arrowheads in B,C and insets). (**D**,**E**) In situ hybridisation for noggin in the posterior neuropore (PNP) region of an E9.5 wild-type embryo after the implantation of a bead soaked in Bmp2. Whole mount (right lateral view) shows intense expression of noggin around the bead (arrowhead in D), and transverse section (E) shows induction of noggin expression throughout the ipsilateral neural fold. Note the sharp boundary of expression at the midline (arrow in E) and the absence of dorsolateral hinge point (DLHP) ipsilaterally after exposure to Bmp2 (arrowhead in E). (**F**,**G**) In situ hybridisation for noggin in E9.5 embryos after the implantation of a bead soaked in PBS (F) or Shh (G). Noggin is expressed at the dorsolateral tips of both neural folds in the embryo with a PBS bead (arrows in F), but is downregulated on the neural fold ipsilateral to the Shh bead, with suppression of the DLHP (asterisk in G). (**H-K**) Transverse sections through wild-type (H,I) and *Shh*$^{-/-}$ (J,K) embryos, showing the ventralised expression domain of noggin in *Shh*$^{-/-}$ embryos compared with wild type (arrows), in both open PNP (H,J) and in closed neural tube (I,K). Scale bars: 0.4 mm in A-C; 0.1 mm in B inset (also A inset); 50 μm in C inset; 30 μm in D; 50 μm in E; 0.15 mm in G (also F); 50 μm in K (also H-J).

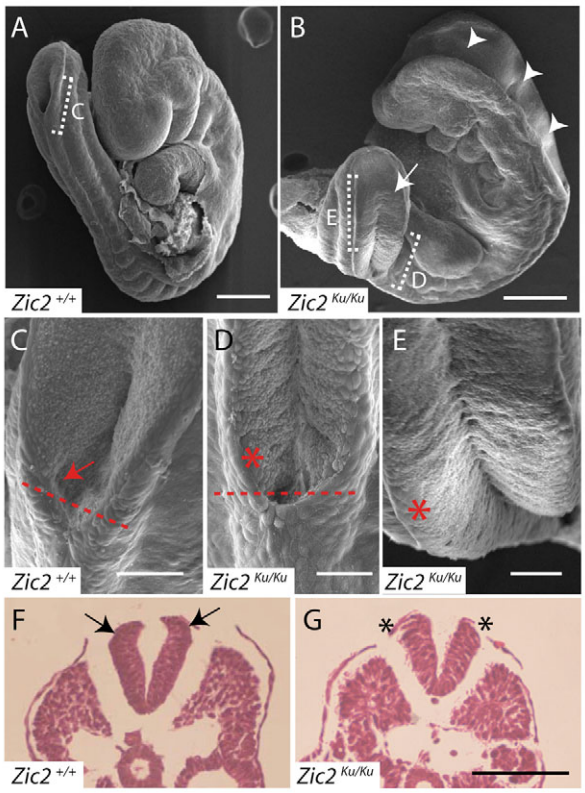


Fig. 5. DLHPs are absent from *Zic2*$^{Ku/Ku}$ embryos during the development of spina bifida. (**A-E**) Scanning electron micrographs of the E9.5 caudal region of wild-type (A,C) and *Zic2*$^{Ku/Ku}$ (B,D,E) embryos. (A) The wild-type embryo has a closed brain and an open posterior neuropore (PNP), as expected at this stage of development. (B) The *Zic2*$^{Ku/Ku}$ embryo has exencephaly (arrowheads) and a greatly enlarged PNP (arrow), indicative of incipient spina bifida. Higher-magnification views (C-E; see bracketed areas in A,B) show indentations, representing dorsolateral hinge points (DLHPs), in the dorso-medial region of wild-type neural folds (arrow in C), whereas these are absent from mutant neural folds (asterisks), both at rostral (D) and more caudal (E) levels of the PNP. (**F**,**G**) H&E-stained sections through the PNP show early DLHPs in a wild-type embryo (arrows in F) but entirely straight neural folds, with no DLHPs (asterisks in G), in a *Zic2*$^{Ku/Ku}$ embryo. The level of sections in F and G are indicated by dotted red lines in C and D, respectively. Scale bars: 0.2 mm (A,B); 50 μm (C,E); 25 μm (D); 0.2 mm (F,G).

achieved via bending solely at the MHP (mode 1), closure in the low spine also involves dorsolateral bending (modes 2 and 3). Our analysis of the *Zic2*^{Ku} mutant shows that the complete absence of DLHPs is incompatible with the progression of spinal neural tube closure beyond the level of the 14th somite. Hence, the formation of DLHPs is an obligatory part of low spinal neurulation and, in its absence, severe spina bifida results.

Molecular regulation of DLHP formation

We have identified an inhibitory influence of Bmp signalling on DLHP formation, with abrogation of this inhibitory Bmp effect by the action of co-existing Bmp antagonists, particularly noggin but also probably neuralin. As summarised in Fig. 6K, DLHPs are absent from mode 1 neurulation because of the unopposed inhibition of dorsolateral bending by Bmp2. Although the transcription of noggin is stimulated by Bmp2 at all levels of the body axis, Shh expression from the notochord is strong during mode 1 neurulation, inhibiting noggin expression. Hence, the inhibitory influence of Bmp2 on DLHP formation is not counteracted by noggin, which is expressed less-intensely in mode 1. By contrast, at lower levels of the neuraxis, in which mode 2 and 3 spinal neurulation occur, the influence of Shh is reduced because, as shown in our previous studies, the notochord is largely Shh-negative until after neural tube closure (Ybot-Gonzalez et al., 2002). Noggin expression is de-inhibited and antagonises the negative influence of Bmp2 on DLHPs, allowing bending to occur. *Shh*-null embryos exhibit mode 3-type neurulation along the entire body axis (Ybot-Gonzalez et al., 2002), demonstrating that DLHPs are the 'default' neural plate behaviour, in the absence of Shh influence. Hence, the transition from mode 1 to mode 2 to mode 3 in mouse spinal neurulation is regulated by Shh, with Bmp and its antagonists playing down-stream regulatory roles.

Evidence from other systems supports the existence of a negative-feedback loop in which Bmp antagonists are induced by Bmps, but then serve to limit the intensity of Bmp signalling. For example, Bmp4 induces noggin expression in chick muscle (Amthor et al., 1999) and somites (Sela-Donenfeld and Kalcheim, 2002), whereas, in the mouse embryo, Bmp2 from the ventral ectodermal ridge induces noggin expression in the adjacent ventral mesoderm (Goldman et al., 2000). Overexpression of noggin in the chick neural tube downregulates Bmp4 activity and delays neural crest induction (Sela-Donenfeld and Kalcheim, 2000; Sela-Donenfeld and Kalcheim, 1999; Liem et al., 1995; Liem, Jr et al., 1997), demonstrating the quantitative nature of the effect of noggin on Bmp signalling activity. Moreover, noggin behaves as a typical example of a dorsal neural tube gene that is repressed, in a quantitative manner, by the ventralising activity of Shh (Jessell, 2000).

Our findings provide a striking parallel to the well-established dorsoventral regulation of specific neuronal and glial cell types in the spinal cord, in which Shh ventralises and Bmps dorsalise the neural tube, promoting or inhibiting specific classes of downstream genes (Jessell, 2000; Rowitch, 2004). The neuroepithelium at the stage of neural tube closure is pseudostratified, with all cells remaining in the proliferative pool and no differentiated cells types being present. The MHP and DLHPs, although morphologically distinct, do not contain specific differentiated cell types. Therefore, the present study shows that diffusible factors, including Bmps and Shh, can act over a distance of several cell diameters to regulate the cell shape changes (Schoenwolf, 1985; Smith et al., 1994) that mediate neural plate bending, prior to the onset of differentiation of specific cell types.

Effect of loss of noggin function on spinal neurulation

Embryos homozygous for a null mutation of noggin show over-activation of Bmp signalling (McMahon et al., 1998), marked diminution of DLHPs (this study) and defects of neural tube closure, with failure of cranial neurulation and late-appearing spina bifida (McMahon et al., 1998; Stottmann et al., 2006). We observed residual DLHP activity in *Nog*$^{-/-}$ embryos, probably explaining the ability of the spinal neural tube to close in most homozygous embryos (Stottmann et al., 2006). Nevertheless, spinal neural tube closure in *Nog*$^{-/-}$ embryos is only temporary, with re-opening in all fetuses by E14 (Stottmann et al., 2006). Our findings suggest that *Nog*$^{-/-}$ embryos undergo 'pseudo-mode 1' closure, with minimal formation of DLHPs, even at low levels of the spinal neuraxis. This type of closure is probably unstable, in the highly curved lower body, and subject to a high risk of re-opening to yield spina bifida at later

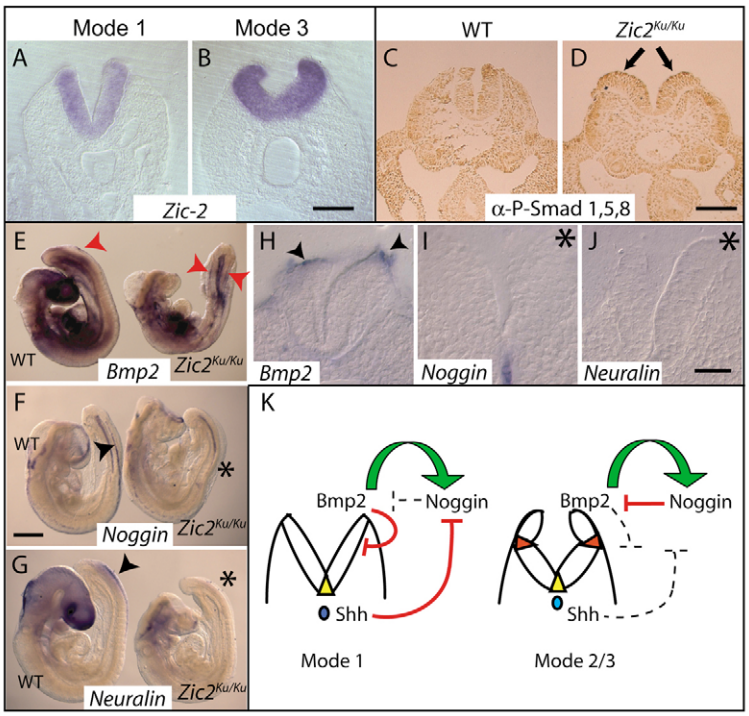


Fig. 6. *Zic2*$^{Ku/Ku}$ embryos show reduced expression of Bmp antagonists and over-stimulation of Bmp signalling. (**A**,**B**) In situ hybridisation for *Zic2* expression in transverse sections through the posterior neuropore (PNP) of mode 1 (E8.5) and mode 3 (E9.5) wild-type embryos. Note the more intense expression of *Zic2* in mode 3. (**C**,**D**) Immunohistochemistry for phospho-Smad1,5,8 in transverse sections of E9.5 wild-type (WT) and *Zic2*$^{Ku/Ku}$ embryos. The mutant neural plate shows more-intense phospho-Smad expression than wild type (arrows in D). (**E-G**) In situ hybridisation for *Bmp2*, noggin and neuralin in whole mounts of E9.5 wild-type and *Zic2*$^{Ku/Ku}$ embryos. Red arrowheads, *Bmp2* expression in the dorsal surface ectoderm of both genotypes; dorsal view of PNP in mutant shows *Bmp2* domains on both sides. Black arrowheads, noggin and neuralin expression in the dorsal neural plate of wild-type but not mutant (asterisks) embryos. (**H-J**) In situ hybridisation for *Bmp2*, noggin and neuralin in sections through the open neural tube of *Zic2*$^{Ku/Ku}$ embryos. Whereas *Bmp2* is expressed (arrowheads), expression of noggin and neuralin is absent (asterisks). (**K**) Summary of dorsolateral hinge point (DLHP) regulation by Bmp2, noggin and Shh in mouse spinal neurulation. Yellow triangles, median hinge point (MHP); red triangles, DLHPs; green arrows, stimulatory interactions; red lines, inhibitory interactions; dashed lines, inactive influences. Scale bars: 0.1 mm in B (also A); 0.15 mm in D (also C); 0.5 mm in F (also E,G); 0.05 mm in J (also H,I).

stages. *Nog*$^{-/-}$ embryos also exhibit defects of sclerotomal differentiation (Stottmann et al., 2006), which exacerbate the spinal malformations.

The residual DLHP formation in *Nog*$^{-/-}$ mutants might result from functional redundancy between noggin and neuralin, both of which are Bmp antagonists and expressed dorsally in the spinal neural folds. We predict, therefore, that embryos lacking both noggin and neuralin should exhibit more-severe neural tube defects than in embryos lacking noggin alone. Indeed, double-null embryos would be expected to mimic the severe spina bifida observed in *Zic2*$^{Ku/Ku}$ embryos, in which we observed the absence of both noggin and neuralin expression in the dorsal neural plate.

Role of Zic2 in spinal neurulation

Among the many mouse genetic models of spina bifida (Copp et al., 2003), *Zic2*^{Ku} is the first in which the absence of DLHPs has been described. Our analysis shows that, whereas *Bmp2* expression is similar in wild-type and mutant embryos, the expression of both noggin and neuralin is undetectable in the *Zic2*$^{Ku/Ku}$ dorsal neural plate. Significantly, we found that homozygotes for the *curly tail* mutation, which also develop spina bifida, exhibit normal expression of noggin and neuralin in the PNP region (data not shown), consistent with the presence of normal DLHP formation in these embryos (Shum and Copp, 1996). This finding argues that the absence of noggin and neuralin expression is unlikely to be a non-specific consequence of failed neural tube closure. Additional developmental defects, particularly enhanced ventral curvature of the caudal region, have been implicated in the failure of neural tube closure in the *curly tail* mutant (Brook et al., 1991).

Activation of the downstream Bmp signalling pathway appears enhanced in *Zic2*$^{Ku/Ku}$ embryos, providing an explanation for the complete absence of DLHPs, the formation of which requires Bmp antagonism. *Zic2* is expressed throughout the neural plate during neurulation, whereas, following neural tube closure, its expression becomes restricted to the dorsal neural tube and, eventually, to the roof plate (Elms et al., 2003; Gaston-Massuet et al., 2005). In the chick, the expression of Zic proteins is enhanced by the overexpression of Bmps, and is diminished by the overexpression of Shh (Aruga et al., 2002). The latter finding fits with the observations of the present study, in which *Zic2* expression was more-intense in mode 3, when Shh signalling is at its weakest. It remains to be determined precisely how Zic2 enables DLHP formation in the neural plate. The absence of noggin and neuralin expression from *Zic2*^{Ku} homozygotes suggests that Zic2 might positively regulate the expression of these Bmp antagonists in response to Bmp2. Alternatively, Zic2 might mediate a more downstream event in dorsolateral bending, with a secondary feedback effect on noggin and neuralin expression.

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