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 Mouri, 1995; Mouri 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).
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, neurulin (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 KO 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 extramembranous membranes, rinsed in PBS, and fixed in either Bouin’s fluid for haematoxylin and cosin (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), caderhin 6 (Henderson et al., 1997), chordin (Klingensmith et al., 1999), Msx1 (Mackenzie et al., 1991), Msx2 (Monaghan et al., 1991), neurulin (Coffinier et al., 2001) and Zic2 (Gaston-Massuet et al., 2005). Additional Msx1 and Msx2 probes were as described (Caton et al., 1996). To prepare a cDNA probe for noggin, forward 5′-CCAGCAGCTAT-CCTACATCC-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 Decle (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 Signalling 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% glycerine 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 2-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% OsO4 for 1 hour. Samples were dehydrated through an ascending alcohol series, using three changes of acetone to displace the alcohol, then CO2 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* null 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* null 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* null 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* null 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 neural development 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

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**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-F) In situ hybridisation for *Bmp2* (B,C), *Bmp7* (D,E), cadherin 6 (F,G) and *Mx1* (H,I) during mode 1 (E8.5; B,D,F,H) and mode 3 (E9.5; C,E,G,J) 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,J,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).
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 neurupore, 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 neurulin was expressed in the neural fold 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 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 induce 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.
To investigate this possibility, we examined the expression of noggin thereby explaining this dependence on surface ectoderm attachment.

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 ipsilaterally 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 Zic2Ku 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 littersmates (Fig. 5C), whereas Zic2Ku/Ku embryos had completely straight neural folds throughout the enlarged PNP (Fig. 5D,E). Sections at the rostral end of the neurepore confirmed that DLHPs are present in E9.5 wild-type embryos, early in mode 2, but absent from stage-matched Zic2Ku/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 Zic2Ku/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 (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 Zic2Ku/Ku embryos, and neural tube closure fails from this point onwards, leading to severe spina bifida.

**Zic2Ku/Ku mutants lack dorsal expression of Bmp antagonists**

The absence of DLHPs from Zic2Ku/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 Zic2Ku/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 Zic2Ku/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 Zic2Ku/Ku and wild-type embryos (Fig. 6E,H). By contrast, the expression of noggin (Fig. 6E,I) and neuralin (Fig. 6G,H) was markedly downregulated in the dorsal neural tube of Zic2Ku/Ku embryos compared with wild-type littersmates, strongly suggesting that Bmp antagonism is defective in the absence of functional Zic2.

Taken together, these observations suggest that the lack of DLHPs in Zic2Ku/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 MHB overlying the notochord, and at paired DLHPs, in which the neural plate changes its basolateral 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
Development in a wild-type embryo (arrows in F) but entirely straight neural folds, expression from the notochord is strong during mode 1 neurulation, noggin is stimulated by Bmp2 at all levels of the body axis, Shh also probably neuralin. As summarised in Fig. 6K, DLHPs are the action of co-existing Bmp antagonists, particularly noggin but residual DLHP activity in Nog–/– embryos (Stottmann et al., 2006). Nevertheless, spinal neural tube closure in Nog<sup>−/−</sup> embryos is only temporary, with re-opening in all fetuses by E14 (Stottmann et al., 2006). Our findings suggest that Nog<sup>−/−</sup> 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 stages.

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 cell 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<sup>−/−</sup> embryos, probably explaining the ability of the spinal neural tube to close in most homozygous embryos (McMahon et al., 1998; Stottmann et al., 2006). Nevertheless, spinal neural tube closure in Nog<sup>−/−</sup> embryos is only temporary, with re-opening in all fetuses by E14 (Stottmann et al., 2006). Our findings suggest that Nog<sup>−/−</sup> 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 stages.

**Fig. 5. DLHPs are absent from Zic<sup>2Ku/Ku</sup> embryos during the development of spina bifida.** (A–E) Scanning electron micrographs of the E9.5 caudal region of wild-type (A,C) and Zic<sup>2Ku/Ku</sup> (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 Zic<sup>2Ku/Ku</sup> 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 (asterisks), whereas these are absent from mutant neural folds (asterisks), both at rostral (D) and more caudal (E) levels of the PNP. (F,G) Higher-magnification sections through the PNP show early DLHPs in wild-type embryos (Zic<sup>2+/+</sup>) and dorsal neural tube 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 cell 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.
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 Zic2Ku/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), Zic2Ku/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 Zic2Ku/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 Zic2Ku/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 Zic2Ku 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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**References**


Bmp signalling and neural tube closure


