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Sonic hedgehog and the molecular regulation of mouse neural tube closure

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SUMMARY

Neural tube closure is a fundamental embryonic event whose molecular regulation is poorly understood. As mouse neurulation progresses along the spinal axis, there is a shift from midline neural plate bending to dorsolateral bending. Here, we show that midline bending is not essential for spinal closure since, in its absence, the neural tube can close by a 'default' mechanism involving dorsolateral bending, even at upper spinal levels. Midline and dorsolateral bending are regulated by mutually antagonistic signals from the notochord and surface ectoderm. Notochordal signaling induces midline bending and simultaneously inhibits dorsolateral bending. Sonic hedgehog is both necessary and sufficient to inhibit dorsolateral bending, but is neither necessary nor sufficient to induce midline bending, which seems likely to be regulated by another notochordal factor. Attachment of surface ectoderm cells to the neural plate is required for dorsolateral bending, which ensures neural tube closure in the absence of sonic hedgehog signaling.

Key words: Neurulation, Neural plate, Morphogenesis, Embryo culture, Notochord, *patched*

INTRODUCTION

Neurulation is the embryonic process in which the neural plate, a specialised region of ectoderm on the dorsal surface of the embryo, undergoes shaping and folding to form the neural tube. Failure of neural tube closure leads to clinically important birth defects, of which anencephaly and spina bifida are the commonest types (Copp and Bernfield, 1994).

Neurulation begins with differentiation of neurectoderm from lateral ectoderm. Concomitantly, the neural plate narrows mediolaterally and lengthens rostrocaudally by the process of convergent extension, in which neuroepithelial cells move towards the midline and intercalate along the rostrocaudal axis (Jacobson and Gordon, 1976; Schoenwolf and Smith, 1990; Keller et al., 1992). Subsequently, neural folds form at the edges of the neural plate, then elevate and fuse in the dorsal midline to create the neural tube.

In birds and mammals, bending of the neuroepithelium occurs at two specific sites in the neural plate (Schoenwolf and Smith, 1990; Shum and Copp, 1996): at the ventral midline (the median hinge point; MHP) and at paired lateral bending sites near the junction of the neural plate and surface ectoderm (the dorsolateral hinge points; DLHPs). MHP bending creates the 'neural groove', with a V-shaped cross section, while DLHP bending creates longitudinal furrows that bring the lateral aspects of the neural plate towards each other in the dorsal midline.

The mouse neural tube first fuses at the hindbrain/cervical boundary, at embryonic day (E) 8.5, creating two regions of active neural tube closure: rostrally within the hindbrain and caudally in the upper spinal region. Subsequent closure in the mouse brain involves two further sites of fusion initiation and two additional neuropores (Golden and Chernoff, 1993; Fleming and Copp, 2000). Closure in the spinal region involves the progressive 'zipping' of the neural tube down the spinal axis, until the posterior neuropore finally closes in the low spine at E10. We found that MHP and DLHPs play differing roles in closure of the neural tube at different levels of the mouse spinal axis (Shum and Copp, 1996; Ybot-Gonzalez and Copp, 1999). At E8.5, when the upper spine is forming, the closing neural folds exhibit an MHP but no DLHPs. By early E9.5, the neural plate begins to bend at DLHPs in addition to the MHP and, at the lowest spinal levels (at E10), the MHP disappears and the neural plate bends only at DLHPs.

These dorsoventral variations in morphogenetic activity of the closing neural plate overlap, temporally, with the onset of dorsoventral molecular interactions involving sonic hedgehog (Shh), bone morphogenetic proteins (BMPs) and Wnts. A concentration-dependent influence of Shh, originating from the notochord and floor plate, patterns the neuronal differentiation events along the dorsoventral axis of the neural tube (Jessell, 2000), while inhibiting the differentiation of dorsal neuronal cell types and neural crest cells. BMPs and Wnts, from the dorsal ectoderm, oppose Shh influence and promote dorsal patterning events (Lee and Jessell, 1999). It is not known whether these dorsoventral molecular interactions also regulate closure of the neural tube.

A possible involvement of dorsoventral signaling in mouse neural tube closure is suggested by the finding that formation of the MHP (the developmental precursor of the floor plate) requires an interaction between the ventral neural plate and the underlying notochord (Van Straaten et al., 1988; Smith and Schoenwolf, 1989; Placzek et al., 1990). Paradoxically, however, although the MHP is the only site of spinal neural

plate bending in the E8.5 mouse embryo (Shum and Copp, 1996), it seems not to be essential for neural tube closure. Mice that lack a floor plate, including null mutants for *HNF3β* (*Foxa2*), *Shh*, *Gli2* and *Gli1/2* (Ang and Rossant, 1994; Weinstein et al., 1994; Chiang et al., 1996; Ding et al., 1998; Matise et al., 1998; Park et al., 2000), do not exhibit spinal neural tube closure defects. Similarly, suppression of notochord development in both chick and mouse embryos fails to prevent neural tube closure, although MHP and floor plate formation are abolished (Smith and Schoenwolf, 1989; Davidson et al., 1999). These findings suggest the existence of a 'default mechanism' of neurulation that can ensure successful neural tube closure in the absence of a notochord and MHP.

Here, we demonstrate the nature of this default mechanism, which depends on the formation of DLHPs in the absence of Shh signaling. DLHP formation is normally suppressed by Shh signaling, at high levels of the spinal axis, although it is induced in cases of Shh deficiency. Notochordal Shh signaling is markedly weaker beneath the closing neural plate at the lowest spinal levels, so that formation of DLHP occurs unopposed, under the positive influence of the surface ectoderm.

MATERIALS AND METHODS

Mouse strains and embryo culture

Embryos were obtained from timed matings of inbred CBA/Ca mice (dbcAMP and bead implantation experiments) or random-bred CD1 mice (all other studies). Mice carrying the *cordon-bleu* gene trap insertion (Gasca et al., 1995), kindly provided by Janet Rossant, were bred to homozygosity and used in the node ablation studies. *Shh* gene-targeted mice, kindly provided by Chin Chiang and Gillian Morriss-Kay, were genotyped by polymerase chain reaction (Chiang et al., 1996). Whole embryo culture was performed as described previously (Copp et al., 1999) with addition of 1 mM dbcAMP dissolved in phosphate-buffered saline (10 μl of 0.1 M stock per ml of rat serum) or 0.05 μg/ml cytochalasin D dissolved in dimethyl sulfoxide (10 μl of 5 μg/ml stock per ml of rat serum).

Microsurgical procedures on embryos in vitro

Node ablation was performed by touching the node of an E7.5 (allantoic bud stage) embryo with the tip of a hot, hand-held tungsten needle, while viewing the embryo from its posterior surface, on the stage of a Zeiss SV6 stereomicroscope. Embryos (E9.5) for removal of surface ectoderm or paraxial mesoderm were prepared as described previously (Copp et al., 1999), with removal of the ectoplacental cone and opening of a window in the yolk sac and amnion over the posterior neuropore region. Hand-held tungsten needles and watchmakers' forceps were used to carry out the microsurgical procedures, after which the posterior neuropore region was replaced inside the extraembryonic membranes, for culture. AffiGel blue beads (BioRad) were soaked for at least 2 hours at 4°C in either PBS or 1 μg/μl Shh-N peptide (a gift from Sebastian Pons, Barcelona). A tungsten needle was used to make a slit in the surface ectoderm overlying the right-side neural fold of the E9.5 posterior neuropore (Modes 2 or 3). Beads, held by suction on the end of a mouth-controlled glass micropipette, were inserted singly or in pairs between the presomitic mesoderm and neural fold.

Analysis of DLHP morphology and bead position in normal and experimental embryos

Embryos were fixed in Bouin's fluid and serial 6 μm transverse sections through the posterior neuropore region were stained with Haematoxylin and Eosin (H&E). DLHP location was found to be approximately a quarter to a third of the distance from the lateral edge of the neural plate to the midline, depending on somite stage. In order to provide an objective definition of DLHPs, angles of dorsolateral neural plate bending were measured (see Fig. 1H) for normal and experimental embryos (Table 1). In bead-implanted embryos, bead position was classified as rostral (present in sections 1-10 from the rostral end of the neuropore), intermediate (in sections 11-20) or caudal (in sections 21 onwards). Each section containing a bead was assigned an 'asymmetry score' of 1 (symmetrical neural folds with similar DLHPs ipsilateral and contralateral to the bead), 2 (asymmetrical neural folds with ipsilateral DLHP less prominent than contralateral DLHP) or 3 (asymmetrical neural folds, with ipsilateral DLHP absent). Neural folds with score 3, did not differ significantly in 'DLHP angle' ($P>0.05$) from embryos in Mode 1 (absent DLHPs) (Table 1). Mean scores were compared, for each neuropore region, between PBS and Shh beads.

In situ hybridisation and histochemical analysis

Whole-mount in situ hybridisation, followed by preparation of 50 μm vibratome sections (Copp et al., 1999), and in situ hybridisation on paraffin-embedded sections (Breitschopf et al., 1992) were performed using digoxigenin-labelled cRNA probes for *Shh* (Greene et al., 1998), *Ptc1* (*Ptch*) (Goodrich et al., 1999), *Bmp2* (Furuta et al., 1997) and *HNF3β* (Sasaki and Hogan, 1993). Embryos in which the in situ hybridisation was to be compared were processed together in the same tube. β-galactosidase histochemistry was performed as described previously (Gasca et al., 1995).

RESULTS

Neural tube closure in the mouse initiates at the 6- to 7-somite stage (E8.5) and spreads in rostral and caudal directions from this site (Fig. 1A). With growth of the embryo caudally, the point of spinal neural tube closure (the posterior neuropore) moves to progressively lower axial levels, leaving a closed neural tube in its wake (Fig. 1A-C).

Shift from MHP to DLHP formation along the spinal axis

Comparison of histological sections through the rostral end of the posterior neuropore in embryos of increasing developmental stage reveals a striking shift in neural plate morphology (Modes 1-3; Fig. 1). At late E8.5, the neural plate bends sharply at the MHP, whereas its lateral regions are straight (Mode 1 neurulation; Fig. 1G). In more advanced embryos, at early E9.5, paired DLHPs are present as well as a sharp MHP (Mode 2; Fig. 1H). In E10 embryos, towards the end of primary neurulation, the neural plate no longer exhibits sharp bending at the MHP whereas bending at DLHPs is prominent (Mode 3; Fig. 1I). Comparison of the angle of bending of the neural plate at the site of DLHPs (approximately a quarter to a third of the distance between the lateral edge of the neural plate and the midline) reveals progressively greater bending as embryos progress from Mode 1 (almost no bending) to Mode 3 (extreme bending) (Table 1).

Strength of Shh signaling correlates with the pattern of neural plate bending

We found that disappearance of the MHP, and appearance of DLHPs, in progressively more advanced embryos correlates with decreased Shh signaling beneath the closing neural folds.

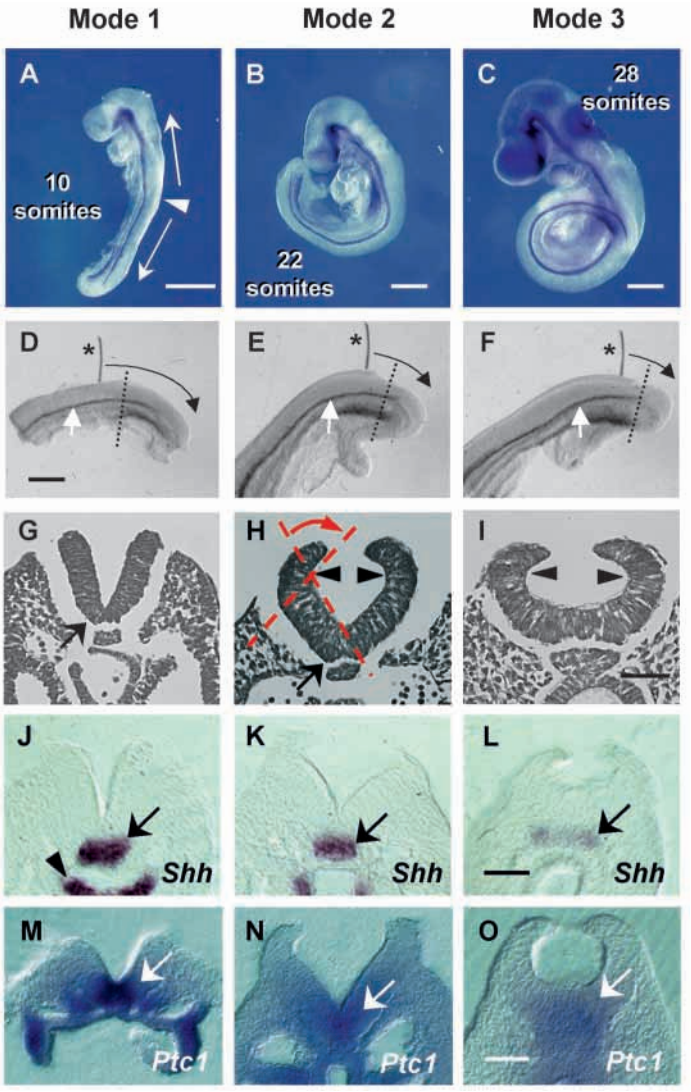

Fig. 1. Transition from midline to dorsolateral bending correlates with reduced strength of notochordal Shh signalling beneath the posterior neuropore. Modes 1-3: the three morphological patterns of neural plate bending. Mode 1 is characterised by an MHP only (E8.5; 7-15 somites; A,D,G,J,M); Mode 2 has both MHP and DLHPs (early E9.5; 16-24 somites; B,E,H,K,N); Mode 3 has only DLHPs (E10; 25-30 somites; C,F,I,L,O). (A-F) Whole-mount in situ hybridisation for *Shh* at Modes 1-3 (A-C: whole embryos; D-F: isolated caudal regions, rostral to left). Neural tube fusion begins at the hindbrain/cervical boundary (arrowhead in A) at the 6- to 7-somite stage, and then spreads in rostral and caudal directions (arrows in A). The notochord expresses *Shh* along its length (A-C), but the caudal termination point of the *Shh* domain varies with increasing developmental stage (D-F). Caudal regions in D-F are aligned against a vertical marker (asterisk): the posterior neuropore extends to the right of the marker (curved arrows). *Shh*-positive notochord (white arrows) extends far into the posterior neuropore in Mode 1 (D) whereas it barely reaches the rostral end of the relatively shorter neuropore in Mode 3 (F). Mode 2 shows an intermediate condition (E). **(**G-O) Transverse sections through rostral end of the posterior neuropore, at levels of equivalent neural fold elevation (dotted lines in D-F), in embryos of Modes 1-3. (G-I) H and E stained sections show the location of MHP (arrows in G,H) and DLHPs (arrowheads in H,I). Method of measuring DLHP angle (Table 1) is shown by red lines in H. (J-L) Sections of embryos hybridised as whole mounts for *Shh* expression: transcripts are present in the notochord (arrows) and ventral hindgut (arrowhead in J), but not in the presumptive floor plate, with a declining intensity of expression from Mode 1 to 3. (M-O) In situ hybridisation reveals *Ptc1* expression in the ventral midline neural plate (arrows), with expression declining in intensity from Mode 1 to Mode 3. Embryos for gene expression comparison hybridised under identical conditions, with at least 10 embryos studied at each stage, by each method. Scale bars: A-C, 0.5 mm; D-F, 0.25 mm; G-O, 50 μm.

Table 1. Angle of neural plate bending at the DLHP in normal and experimental embryos

		No. embryos (no. neural folds)	Mean angle of neural fold (± s.e.m.)*	Statistical comparison†
Normal embryos	Mode 1	2 (12)	15.0 (±3.5)	
	Mode 2	4 (26)	79.9 (±3.7)	$P<0.001$‡
	Mode 3	3 (18)	103.2 (± 6.7)	
dbcAMP	PBS	3 (22)	2.5 (±1.8)§	$P<0.001$
	1 mM dbcAMP	3 (26)	50.0 (±5.0)	
Shh knockout	+/+	3 (18)	12.6 (±3.3)§	$P<0.001$
	–/–	3 (20)	48.7 (±2.1)	
Bead implantation	PBS bead	3 (9)	83.5 (±3.1)§	
	Shh bead, score 1	3 (9)	81.7 (±3.9)	
	Shh bead, score 2	3 (9)	35.0 (±2.7)	$P<0.001$¶
	Shh bead, score 3	3 (16)	28.2 (±5.8)	
	No bead	12 (43)	79.8 (±2.2)	

*Angles of neural fold bending at the DLHP were measured as described in Materials and Methods and in Fig. 1. Angle data were pooled from bilateral neural folds in several sections of normal, dbcAMP-treated and *Shh* knockout embryos; DLHP angles were analysed separately for neural folds ipsilateral and contralateral (no bead) to implanted beads.

†Angle data from embryos of different treatment groups were compared, within each experiment, by one-way analysis of variance or Student's *t*-test.

‡Pairwise testing reveals significant differences ($P<0.005$) between all three closure Modes.

§PBS-treated embryos in the dbcAMP experiment, and $Shh^{+/+}$ embryos, exhibit small DLHP angles, as they were analysed at the stage of Mode 1, whereas embryos containing PBS beads were analysed at Mode 2 or 3, and so have large DLHP angles.

¶Pairwise testing reveals no significant differences between (i) PBS, Shh score 1 and no bead, nor between (ii) Shh score 2 and Shh score 3. In contrast, embryos in groups (i) and (ii) differ significantly from each other ($P<0.05$). Shh score 3 does not differ significantly from Mode 1 ($P>0.05$).

Whole-mount in situ hybridisation reveals *Shh* expression along the length of the notochord at all stages studied (Fig. 1A-C). However, the precise caudal limit of *Shh* transcription in the developing notochord varies with developmental stage (Fig. 1D-F). In Mode 1 embryos, the *Shh*-positive notochord extends far into the posterior neuropore region (Fig. 1D), whereas in Mode 3 it barely reaches the rostral end of the neuropore (Fig. 1F). Mode 2 embryos show an intermediate appearance (Fig. 1E). Sections through the site of active neural tube closure at the rostral end of the neuropore confirm these findings. *Shh* transcripts are most abundant in the notochord underlying the MHP of the posterior neuropore at Mode 1 (Fig. 1J), less abundant although detectable in the notochord beneath the MHP at Mode 2 (Fig. 1K), and barely detectable in the notochord at Mode 3, where an MHP is not present (Fig. 1L).

To determine whether Shh signaling, like *Shh* transcription, is reduced at low spinal levels, we studied transcription of *patched 1* (*Ptc1/Ptch*), a gene whose expression is under positive regulation by Shh signaling (Marigo and Tabin, 1996). *Ptc1* is expressed in the MHP region at all three stages studied, whereas the dorsal neural plate is negative for *Ptc1*, irrespective of whether DLHPs are present. In the MHP region, *Ptc1* expression is intense during Mode 1 neurulation (Fig. 1M), is weaker during Mode 2 (Fig. 1N) and is of relatively low intensity in the Mode 3 neural plate (Fig. 1O). Hence, the reduction in prominence of the MHP, and increasing prominence of DLHPs, correlates with diminishing notochordal Shh signaling as spinal neurulation progresses.

Notochord negatively regulates dorsolateral bending in the neural plate

Our observations on *Shh* and *Ptc1* expression raised the possibility that a notochordal factor, perhaps Shh, exerts a positive regulatory influence on MHP formation, while negatively regulating DLHP formation, during neurulation. To test this hypothesis, we suppressed notochordal development by ablating the node which, at the allantoic bud stage (E7.5; Fig. 2A,B), contains the precursors of the notochord (Sulik et al., 1994; Davidson et al., 1999). Using a gene-trap insertion, *cordon-bleu*, that is expressed specifically in the mouse node at E7.5 (Gasca et al., 1995), we verified that the node is ablated by our microsurgical technique (Fig. 2C,D). A proportion of embryos (5/11) cultured for up to 42 hours following node ablation failed to form a notochord in the caudal region, whereas the remaining embryos (6/11) developed a notochord despite the ablation procedure, perhaps suggesting a degree of developmental regulation, as has also been observed in the chick (Psychoyos and Stern, 1996; Yuan and Schoenwolf, 1999). Strikingly, all node-ablated embryos closed their caudal neural tubes, irrespective of whether a notochord was present or absent (Fig. 2E,F), confirming that a notochord is not required for mouse spinal neural tube closure. Analysis of node-ablated embryos after only 24 hours of culture, when neural tube closure was still in progress at the posterior neuropore, identified 24/39 cases

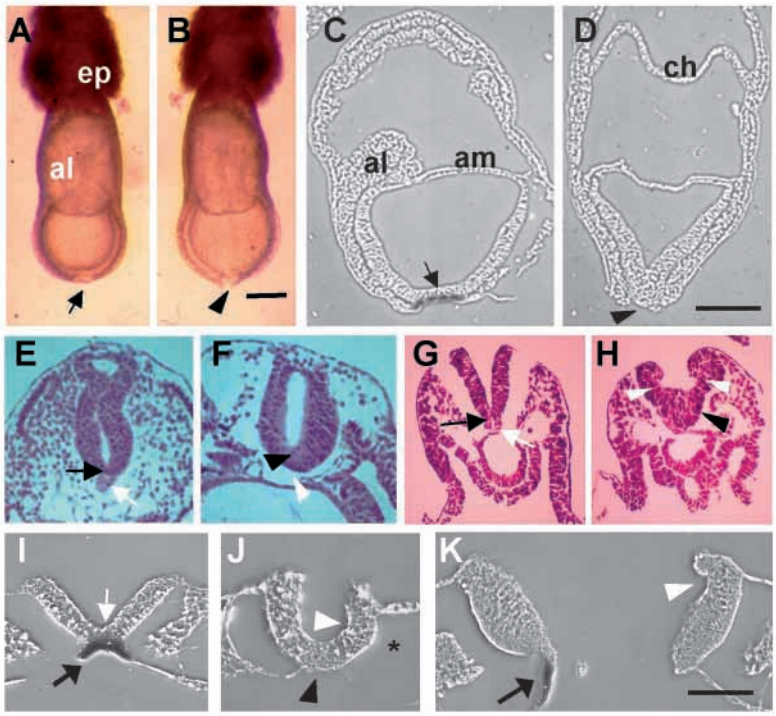

Fig. 2. Suppression of notochord development, by ablation of the node at E7.5, inhibits MHP development and induces premature DLHPs, permitting neural tube closure. (A,B) E7.5 (allantoic bud stage) conceptuses before (arrow in A) and immediately after (arrowhead in B) ablation of the node using a hot tungsten needle. (C,D) Transverse sections through E7.5 embryos homozygous for the *cordon-bleu* gene-trap insertion, in which the node and its derivatives express *lacZ* constitutively. The node is clearly indicated by *lacZ* expression in the ventral midline cells of an intact embryo (arrow in C) whereas these cells have been removed (arrowhead in D) in an embryo fixed immediately after ablation. (E,F) Transverse sections (H and E) of embryos cultured for 42 hours following node ablation. A notochord developed (white arrow in E), despite the ablation procedure in 6/11 cases, with induction of a floor plate in the neural tube (black arrow in E). In the remaining 5/11 ablated embryos, notochord development was suppressed (white arrowhead in F) with absence of a floor plate structure (black arrowhead in F). The neural tube always closed in these notochordless embryos. (G,H) Transverse sections (H and E) through the rostral end of the posterior neuropore of embryos cultured for 24 hours following node ablation. Non-operated control embryo exhibits Mode I neurulation, with MHP (arrow in G) but no DLHPs, whereas an ablated embryo lacking a notochord has a thickened ventral midline neural plate (black arrowhead in H), and DLHPs (white arrowheads in H; 5/7 cases studied), similar to Mode 3. (I-K) Transverse sections through the rostral end of the neuropore of embryos cultured for 24 hours following node ablation, followed by in situ hybridisation for *Shh*. Where a *Shh*-positive notochord develops despite the ablation procedure (black arrow in I), the neural folds are straight with a clear MHP (white arrow; Mode I neurulation; $n=14$). In contrast, complete suppression of the notochord results in a neural plate with Mode 3 morphology (8/17 cases studied), exhibiting DLHPs (white arrowhead in J) but no MHP (black arrowhead in J). Asterisk in J indicates artefactual absence of paraxial mesoderm. (K) Node-ablated embryo with a midline defect, in which the two halves of the neural plate are separated. The left hemi-plate has a *Shh*-positive notochordal fragment attached (black arrow), and exhibits Mode I neurulation (no DLHP), whereas the right hemi-plate has no notochord attached and has a DLHP (white arrowhead). al, allantois; am, amnion; ch, chorion; ep, ectoplacental cone. Scale bars: A,B, 0.2 mm; C,D 50 μm; E-K, 50 μm.

lacking a notochord, or exhibiting asymmetric notochordal development, as identified by expression of *Shh*. Neural plates in these embryos exhibited ectopic DLHPs, either unilaterally or bilaterally, in 13/24 cases (Fig. 2H,J). In contrast, node-ablated embryos in which a notochord developed normally (15/39), or non-operated control embryos, never exhibited DLHPs: their posterior neuropores were always in Mode 1 neurulation (Fig. 2G,I).

In some cases, node ablation produced a midline defect in the caudal embryonic region, yielding two hemi-neural plates not connected in the midline. In some of these embryos, a notochordal fragment was attached ventrally to one hemi-plate, but not to the other (Fig. 2K). Invariably, the neural fold with a notochordal fragment attached was straight, without evidence of a DLHP (left in Fig. 2K), whereas the neural fold lacking notochordal attachment exhibited a DLHP (right in Fig. 2K). Hence, embryos of this type exhibited Mode 1 neurulation on one side, and Mode 2 neurulation contralaterally.

Inhibition of Shh signaling induces premature development of DLHPs

In order to inhibit Shh signaling during neurulation, without suppression of the notochord, we cultured pre-somite stage embryos for 18-20 hours (ending at the 7- to 12-somite stage) in the presence of 1 mM dibutyryl cyclic AMP (dbcAMP). Activation of protein kinase A by dbcAMP inhibits Shh signaling by promoting a repressor form of Gli (Ruiz i Altaba, 1999). PBS-treated control embryos always had an MHP but no DLHPs (Mode 1 neurulation) prior to the 10-somite stage (Fig. 3A,B,I), whereas embryos treated with dbcAMP developed DLHPs prematurely, as well as exhibiting an MHP (Fig. 3C,D,I). Embryos treated with dbcAMP had significantly greater angle of DLHP bending than PBS-treated controls (Table 1), although the DLHP angle was lower than in Mode 2, reflecting the earlier somite stage of dbcAMP-treated embryos.

Expression of the Shh-regulated genes *Hnf3ß* and *Ptc1* was reduced in intensity in dbcAMP-treated embryos compared with PBS-treated controls (Fig. 3E-H), consistent with a reduction but not complete abolition of Shh signaling following dbcAMP treatment. Complete inhibition of Shh signaling using dbcAMP was unsuccessful, as higher concentrations proved toxic for embryos in culture. Hence, suppression of the notochord and exposure of embryos to dbcAMP both lead to the premature formation of DLHPs, supporting the idea that Shh signaling negatively regulates DLHP formation in the closing neural tube.

Shh null embryos close their neural tube by bending at DLHPs

To further investigate the role of Shh in regulating MHP and DLHP formation, we examined neural tube closure in E8.5 embryos homozygous for a null *Shh* mutation (Chiang et al., 1996). Transverse sections through the rostral end of the posterior neuropore of wild-type and heterozygous ($Shh^{+/-}$) littermates with 9-10 somites revealed Mode 1 neurulation, with an MHP but no DLHPs (Fig. 4A,B). In contrast, $Shh^{-/-}$ embryos of the same somite number exhibited DLHPs (Fig. 4C,D), with a significantly greater angle of neural plate bending (Table 1), consistent with a role for Shh in suppressing DLHP formation.

Although $Shh^{-/-}$ embryos are reported to lack a floor plate (Chiang et al., 1996), we detected MHP bending in some $Shh^{-/-}$ embryos, although others did not exhibit a sharp MHP

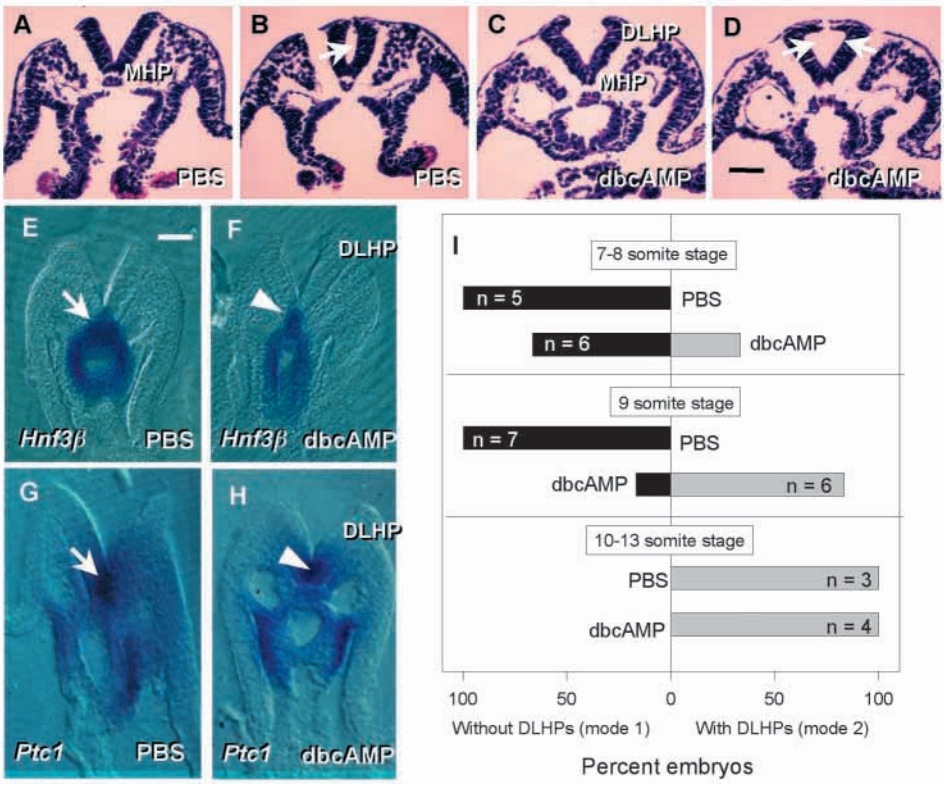

Fig. 3. Inhibition of Shh signaling by dbcAMP induces ectopic DLHPs. (A-D) Transverse sections through the rostral end of the posterior neuropore (A,C) or through recently closed neural tube (B,D) of embryos cultured for 18-20 hours in the presence of either PBS (A,B) or 1 mM dbcAMP (C,D). PBS-treated embryos following culture (7-9 somites) exhibit Mode I neurulation, with an MHP but no DLHPs (A). The recently closed neural tube has a slit-shaped lumen (arrow in B). In contrast, dbcAMP-treated embryos with the same somite number exhibit DLHPs as well as an MHP (i.e. Mode 2 morphology). The recently closed neural tube in these embryos has a diamond-shaped lumen (arrows in D). (E-H) Sections of embryos exposed in culture to PBS (E,G) or dbcAMP (F,H), and then processed for whole mount in situ hybridisation for the Shh-regulated genes *Hnf3β* (E,F; n=20) and *Ptc1* (G,H; n=10). Both genes are expressed with reduced intensity in dbcAMP-treated embryos (arrowheads in F,H) compared with PBS-treated embryos (arrows in E,G). (I) Bar chart showing the premature appearance of DLHPs (premature transition to Mode 2 neurulation) in embryos treated with dbcAMP, compared with PBS-treated controls. Scale bars: A-D, 50 μm; E-H, 50 μm.

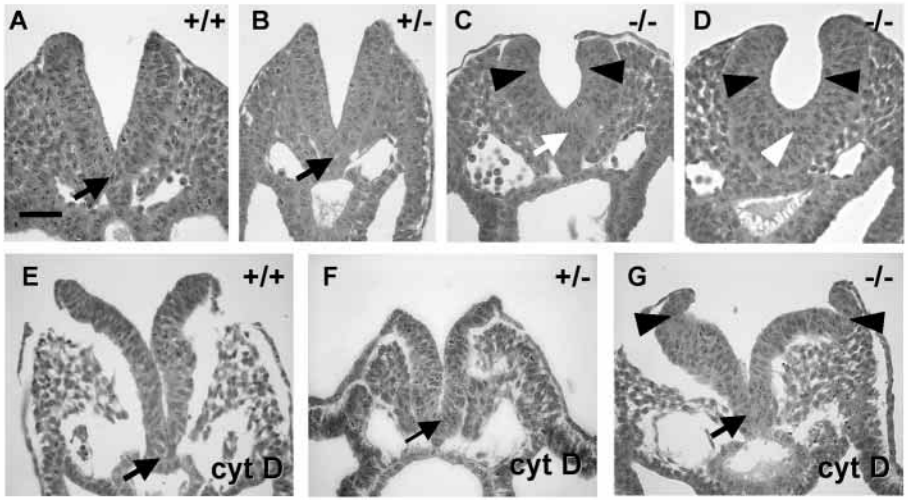

Fig. 4. Homozygosity for a null allele of *Shh* results in closure of the neural tube by bending at DLHPs, at E8.5 (9-10 somites) when wild-type and heterozygous embryos of this strain exhibit bending only at the MHP (Mode 1 neurulation). Transverse sections (H and E) through the rostral end of the posterior neuropore of embryos from $Shh^{+/-} \times Shh^{+/-}$ litters. (A-D) MHP (arrows in A,B) but no DLHPs are present in wild-type (A; n=10) and $Shh^{+/-}$ (B; n>20) embryos. In contrast, $Shh^{-/-}$ embryos (C,D; n=7) exhibit DLHPs (black arrowheads). MHP is present in some $Shh^{-/-}$ embryos (arrow in C) but not in others (white arrowhead in D). (E-G) E8.5 embryos from $Shh^{+/-} \times Shh^{+/-}$ litters, cultured for 6 hours in the presence of 0.05 μg/ml cytochalasin D, to reveal bending points in the neural plate, which are resistant to cytochalasin (Ybot-Gonzalez and Copp, 1999). Wild-type (E) and $Shh^{+/-}$ (F) embryos exhibit a clear MHP (arrows in E,F) but rarely show DLHPs (1/6 and 1/13 respectively). Cytochalasin treatment causes the normally straight neural folds of Mode 1 to flare outwards. In contrast, cytochalasin D-treated $Shh^{-/-}$ embryos exhibit DLHPs in 6/13 cases (arrowheads in G), and all have an MHP (arrow in G). Scale bar: 50 μm.

(compare Fig. 4C,D). To reveal more clearly the pattern of bending points in the $Shh^{-/-}$ neural plate, we cultured litters of E8.5 embryos from $Shh^{+/-} \times Shh^{+/-}$ matings for 6 hours in the presence of 0.05 μg/ml cytochalasin D, in order to disassemble the actin microfilaments. Cytochalasin D causes collapse of non-bending neuroepithelium, but leaves bending points unaffected, serving to highlight the hinge point structure of the neural plate (Ybot-Gonzalez and Copp, 1999). This analysis confirmed that DLHPs are present in the closing neural tube of $Shh^{-/-}$ embryos, whereas they are absent from somite stage-matched wild-type and heterozygous littermates (Fig. 4E-G). Moreover, we confirmed midline bending in all cytochalasin D-treated $Shh^{-/-}$ embryos, suggesting that genetic abrogation of Shh signaling does not prevent MHP formation.

Shh is sufficient to inhibit DLHPs but does not induce an MHP

The experiments in which Shh signaling was inhibited by dbcAMP or abolished in $Shh^{-/-}$ embryos suggest that Shh is necessary for DLHP inhibition but not essential for MHP formation. We next examined whether exogenously applied Shh is sufficient to inhibit DLHPs or to induce an MHP. Beads soaked in either Shh-N peptide or PBS were inserted adjacent to a neural fold in the posterior neuropore region of E9.5 embryos (Fig. 5A), a stage at which DLHPs are present (i.e. Mode 2 or 3). After only 3 hours culture, we detected suppression of ipsilateral DLHPs in a large proportion of neural folds adjacent to Shh-soaked beads (Fig. 5C,E,G,I; Table 1). In contrast, the great majority of embryos receiving PBS beads exhibited symmetrical neural folds with DLHPs present ipsilaterally and contralaterally to the bead (Fig. 5C,D,F,H). The suppressive effect of Shh-soaked beads was extremely local, since contralateral DLHPs were never affected and DLHP suppression was only very rarely observed in neuropore regions rostral or caudal to an implanted bead, even in embryos that received two beads rather than one. We also detected a differential effect of exogenous Shh along the length of the neuropore (Fig. 5B,C). DLHP suppression was most striking in rostral and intermediate neuropore regions (Fig. 5B,E,G), whereas more caudal regions were less severely affected by Shh beads (Fig. 5B,I). Initial DLHP formation may be relatively resistant to Shh inhibitory influence, whereas further development and maintenance of the DLHP as the neural folds elevate appears highly susceptible.

The great majority of beads (29/34 PBS, 38/45 Shh) were implanted unilaterally, adjacent to the right neural fold. In no case did either PBS- or Shh-soaked beads induce a bend in the lateral neural fold at the site of closest contact that might suggest induction of a ‘MHP’ (Fig. 5D-I). Moreover, a small number of beads were implanted at the caudal midline, in the position of the notochord underlying the median region of the neuropore. No induction of an MHP was observed in embryos with a Shh bead at this site (Fig. 5J,K).

Surface ectoderm is required for formation of DLHPs

Having demonstrated that DLHPs are suppressed by Shh signaling, we next investigated the interactions that promote DLHP formation. Neural folds in Modes 2 and 3 neurulation have a close physical association between the neuroepithelium and the surface ectoderm on the outer aspect of the DLHP (e.g. Fig. 6I). Hence, the surface ectoderm might be responsible for inducing bending at the DLHP. To test this idea, we cultured E9.5 embryos from which the surface ectoderm had been surgically removed unilaterally, in the posterior neuropore region (Fig. 6A,B). Embryos fixed immediately after surgery confirmed that the operation removes the surface ectoderm but leaves intact the neuroepithelium of the DLHP in the majority of cases (4/5 studied, Fig. 6C). Following 5 hours culture, 7/7 operated embryos that lacked dorsolateral surface ectoderm showed no ipsilateral DLHP, whereas a DLHP was present contralaterally in all cases (Fig. 6D). Even a small fragment of surface ectoderm left attached to the neural fold apex caused persistence of DLHP formation (data not shown). We verified the correlation between absence of surface ectoderm and

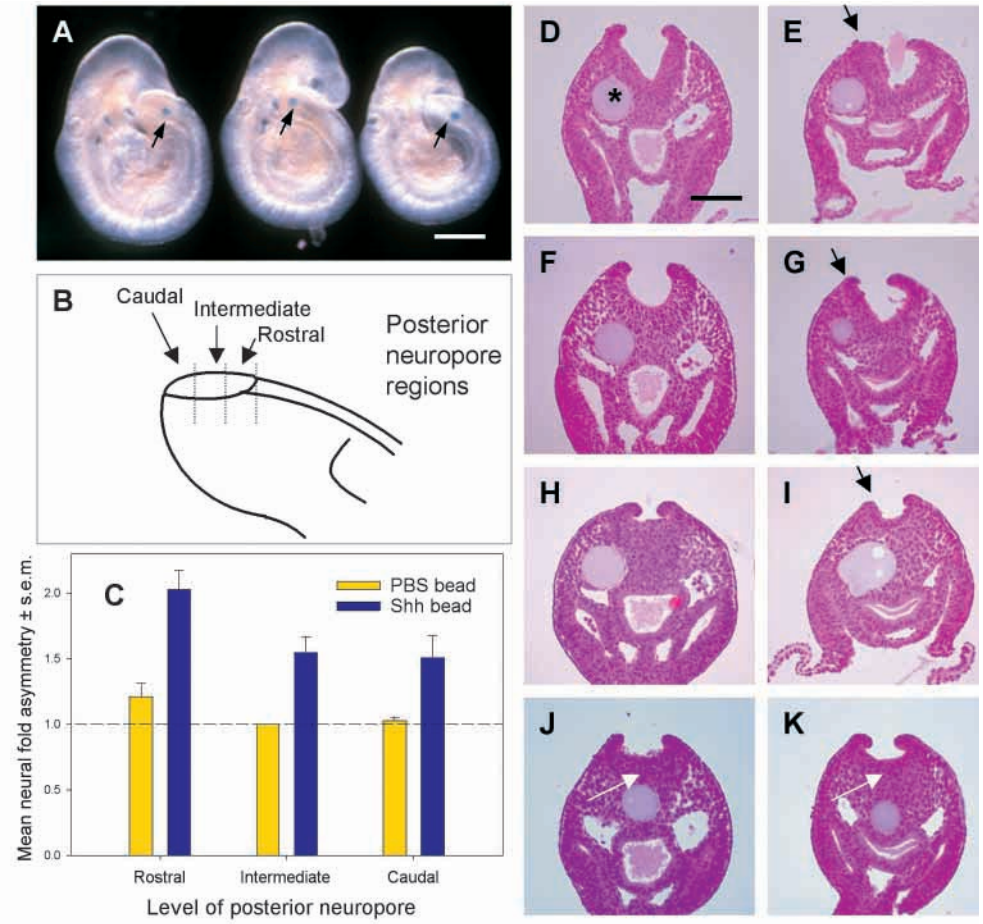

Fig. 5. Local release of Shh from implanted beads is sufficient to inhibit DLHP formation but does not induce an MHP. (A) E9.5 embryos after 3 hours culture following implantation with a single AffiGel blue bead (arrows), adjacent to the neural fold on the right side of the posterior neuropore. The middle embryo has a caudally located bead whereas other embryos have more rostral beads. (B) Sub-division of posterior neuropore into rostrocaudal regions for analysis. (C) Mean 'asymmetry scores' of sections containing PBS- or Shh-soaked beads located at each neuropore level. A mean score of 1 (dotted line) indicates symmetrical DHLPs. Scores of 2 and 3 indicate partial and complete suppression of the ipsilateral DLHP respectively. Mean angles of DLHP bending in neural plate with a score of 1, 2 or 3 are shown in Table 1. Mean scores for Shh beads significantly exceed values for PBS beads at rostral and intermediate levels ($P<0.001$) but not at the caudal position ($P>0.05$). (D-K) Transverse sections through the posterior neuropore of eight different cultured embryos containing beads (asterisk in D) soaked in PBS (D,F,H,J) or Shh (E,G,I,K). (D,F,H) PBS beads are associated with symmetrical DLHPs in all neuropore regions (27/29 beads in 23/25 embryos) whereas Shh beads regularly suppress formation/maintenance of the ipsilateral DLHP (19/38 beads in 16/31 embryos) in rostral and intermediate neuropore regions (E,G). (I) Complete suppression of DLHP by a caudally located Shh bead. (J,K) Caudal sections of embryos in which a bead has been implanted in the midventral region, beneath the Mode 3 neural plate. There is no sign of MHP formation in embryos containing either PBS ($n=2$) or Shh beads ($n=3$). Scale bars: A, 0.5 mm; D-K, 100 μm.

suppression of DLHP formation using *Bmp2*, a marker of surface ectoderm on the neural folds (Fig. 6E). Embryos in which DLHP formation was suppressed by removal of surface ectoderm consistently lacked *Bmp2* expression ipsilaterally (Fig. 6F). A very similar effect of surface ectoderm removal has been reported previously in amphibian and chick embryos (Jacobson and Moury, 1995; Moury and Schoenwolf, 1995). We conclude that attachment of surface ectoderm to the neuroepithelium is necessary for formation and maintenance of a DLHP, and that only a few surface ectoderm cells are sufficient for this effect.

No requirement for paraxial mesoderm in DLHP induction or neural tube closure

We assessed a possible role for the paraxial mesoderm in mouse spinal neural tube closure, by performing experimental ablation studies in cultured E9.5 mouse embryos. When the paraxial mesoderm was ablated bilaterally, leaving the dorsal-most portion of surface ectoderm intact on each side (Fig. 6G-I), DLHPs continued to form (Fig. 6J,K) and neural tube closure progressed at the same rate as in non-operated control embryos (data not shown). We conclude that paraxial mesoderm is not essential for DLHP formation, nor for neural tube closure in the spinal region of the mouse embryo, as has also been demonstrated for the chick embryo (Alvarez and Schoenwolf, 1992).

DISCUSSION

Fig. 7 summarises the model of mouse neural tube closure regulation that has emerged from the present study. Neural plate bending occurs at specific hinge points, MHP and DLHPs, with a temporal shift from midline to dorsolateral bending as neurulation progresses along the spinal region. Notochordal Shh inhibits formation of dorsolateral bending at upper spinal levels, whereas the appearance of DLHPs at lower spinal levels reflects a loss of Shh-mediated inhibition, allowing the unopposed action of inducing signals from the surface ectoderm. MHP formation operates conversely to DLHP regulation, with MHP induction by strong notochordal signaling in the upper spinal region, whereas the MHP is lost at low spinal levels, correlated with reduced notochordal signaling. Surprisingly, in view of the well established connection between Shh and floor plate induction, we find no evidence for a role of Shh in MHP induction.

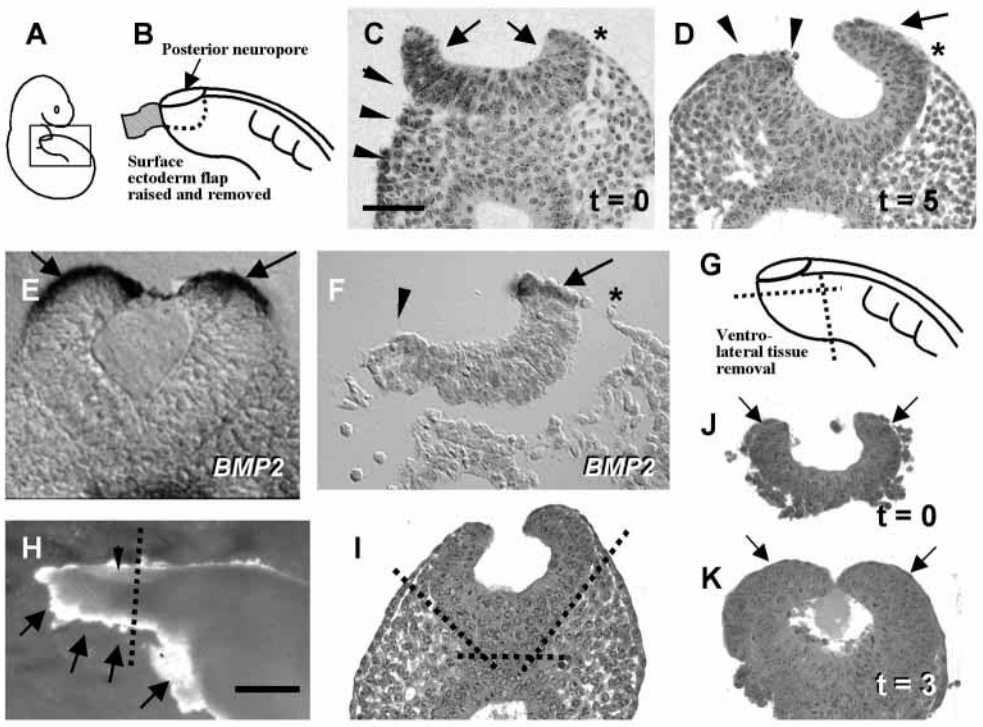

Fig. 6. DLHP formation requires the presence of surface ectoderm on the neural fold, whereas paraxial mesoderm is not required. (A,B) Diagrams of the surgical method used to remove surface ectoderm unilaterally from the E9.5 caudal region. The boxed region in A is shown enlarged in B,G. (C,D) Transverse sections (H and E) through the rostral end of the posterior neuropore of embryos fixed immediately (C; *n*=5) and 5 hours (D; *n*=7) after surface ectoderm removal. Immediately after surgery, surface ectoderm is absent from the operated side to the tip of the neural fold (arrowheads in C), but is present contralaterally (asterisk in C). Note the neuroepithelium of the neural fold is intact following surgery, with DLHPs on both sides of the neural plate (arrows in C). After 5 hours culture, the neuroepithelium on the operated side still lacks contact with surface ectoderm (between arrowheads in D) and does not form a DLHP. There is no evidence of cell death within the neural plate on the operated side, suggesting that absence of DLHP represents suppression of bending by surface ectoderm removal. A prominent DLHP is present contralaterally (arrow in D) where surface ectoderm contact is maintained (asterisk). (E,F) In situ hybridisation for *Bmp2*, a marker of dorsal surface ectoderm (arrows in E; *n*>10). Unilateral removal of the surface ectoderm abolishes *Bmp2* expression ipsilaterally (arrowhead in F; *n*=6), whereas expression is maintained contralaterally (arrow in F), where the surface ectoderm is intact (asterisk). (G-I) Surgical method for removing paraxial mesoderm from the caudal region of the E9.5 embryo, using cuts shown by dotted lines in G and I. Immediately after the operation, the open posterior neuropore is maintained dorsally (arrowhead in H) despite surgical removal of most of the ventrolateral tissue of the caudal region (arrows in H). (J,K) Transverse sections (H and E) through the rostral end of the posterior neuropore (plane of section shown by dotted line in H) of embryos following bilateral removal of the paraxial mesoderm. DLHPs are present in the neural plate immediately following removal of ventrolateral tissue (J; *n*=5). Note the presence of surface ectoderm on the outer aspects of the neural folds (arrows in J). After 3 hours culture, bending at the DLHPs has progressed with incipient closure of the isolated neural plate (arrows in K; *n*=5). The neural plate appears thickened after removal of paraxial mesoderm, perhaps indicating a slowing of axial elongation, with more cells appearing in transverse section. Scale bars: C-F and I-K, 50 μm; H, 0.2 mm.

Evidence from mouse gene knockouts and mutations

Neural tube closure defects occur following inactivation of more than 30 genes in the mouse (Juriloff and Harris, 2000). The great majority of these affect cranial neurulation, causing exencephaly, as seen for instance in mice lacking proteins with cytoskeletal functions including *shroom* (Hildebrand and Soriano, 1999), *MARCKS* (Stumpo et al., 1995), *Mena*/*profilin* (Lanier et al., 1999), and *Abl1* (*Arg*) (Koleske et al., 1998). The spinal neural tube closes normally in these mutant embryos. This finding is consistent with studies in chick, mouse and rat, which demonstrate that only cranial neural tube closure is sensitive to cytochalasins, drugs that disassemble microfilaments. Spinal neurulation is resistant, with continued formation of MHP and DLHPs in the absence of functional microfilaments (Morriss-Kay and Tuckett, 1985; Schoenwolf et al., 1988; Ybot-Gonzalez and Copp, 1999).

Neural tube closure defects of both cranial and spinal regions are seen in mice with loss of *Ptc1* function (Goodrich et al., 1997; Milenkovic et al., 1999). Over-expression of Shh-regulated genes in these mice (McMahon, 2000) may interfere with DLHP formation. In contrast, mutations that directly or indirectly reduce the strength of Shh signaling, as seen in knockouts of *Gli1/2* and *HNF3β*, fail to develop neural tube defects, presumably because of de-inhibition of DLHP formation, and successful closure, as we have shown for *Shh* null embryos. Loss of function of *Gli3*, a negative regulator of Shh signaling in some tissues (Ruiz i Altaba, 1999), leads to cranial neural tube defects (Hui and Joyner, 1993), suggesting a role for Shh signaling in cranial as well as spinal neurulation.

Mice with a null mutation in *Noggin*, which encodes a BMP antagonist, exhibit defects of both cranial and spinal neural tube closure (McMahon et al., 1998). Taken together with our finding of *Bmp2* expression in the surface ectoderm, at the site of DLHP formation, this may suggest an important role for precise regulation of BMP signaling in dorsolateral neural plate bending. Spinal neural tube defects are also seen in mice with mutations in the dorsally expressed genes *Zic2* (Nagai et al., 2000) and *splotch* (*Pax3*) (Auerbach, 1954), and in the *open brain* mutant (Günther et al., 1994). *Zic2*$^{-/-}$ and *open brain* (*Rab23/Rab23*) embryos lack dorsal neural cell types (Günther et al., 1994; Nagai et al., 2000; Eggenschwiler and Anderson, 2000), perhaps adversely affecting their ability to form DLHPs, whereas *splotch* embryos have intact DLHPs (A. J. C. unpublished data), suggesting that the *splotch* spinal neural tube closure fails owing to another, unidentified, defect.

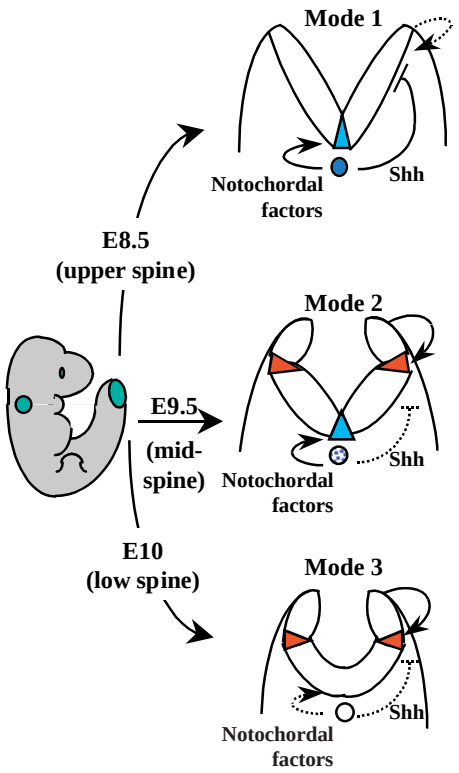

Fig. 7. Proposed interactions regulating neural tube closure at three progressive stages of mouse spinal neurulation. Solid arrows indicate active signaling influences, whereas dotted arrows show inactive/ineffective signals, as identified in the present study. See text for further explanation.

Role of extrinsic influences on neural tube closure

As in the mouse, bending at the MHP depends on an inductive interaction with ventral mesoderm in both amphibians and birds. MHP formation is not essential for closure in birds (Schoenwolf and Smith, 1990), although midline bending is required in *Xenopus*, in which the later events of neural fold bending and fusion can only be recapitulated in neural plate explants containing underlying mesoderm (Poznanski et al., 1997). The dependence of dorsolateral bending on an interaction between the neural plate and surface ectoderm has also been demonstrated in both urodele amphibians and birds (Jacobson and Moury, 1995; Moury and Schoenwolf, 1995).

The actual mechanisms whereby neural tube closure is accomplished in different animal groups is controversial. According to one view, the DLHP is a site of passive neural plate bending, while the motive force for bending is produced by the medially directed expansion of the surface ectoderm (Alvarez and Schoenwolf, 1992; Hackett et al., 1997). Isolated chick surface ectoderm fragments expand in a medial direction in the cranial region, although expansion is minimal at spinal levels (Moury and Schoenwolf, 1995) perhaps suggesting a cranial/spinal difference in mechanism. Our studies in the mouse, and previous work in chick (Hackett et al., 1997) and urodele amphibians (Jacobson and Moury, 1995), demonstrate that only a very narrow fragment of surface ectoderm needs to be attached to the neural plate to allow DLHP formation and neural tube closure. Such fragments of ectoderm have no lateral anchoring points against which to develop a push force, calling into question the mechanical role of medial ectoderm expansion in generating DLHPs.

Another view is that bending at the DLHP is an active neuroepithelial process, under the inductive influence of the surface ectoderm. Localised bending of the spinal neuroepithelium could result from the local accumulation of cells in S-phase. Nuclei are mainly in a basal location in S-phase, leading to the adoption of a wedged cell shape, as has been demonstrated for the MHP (Smith and Schoenwolf, 1988; Gerrelli and Copp, 1997). Alternatively, DLHPs could be the sites of locally increased cell proliferation, leading to cell accumulation and inward buckling of the neuroepithelium, as suggested for gut folding morphogenesis (Miller et al., 1999).

Mechanism of DLHP regulation by Shh

Shh influences gene expression within the neural tube through establishment of a ventrodorsal concentration gradient of biologically active Shh-N peptide (Jessell, 2000). Low Shh-N concentrations suffice to inhibit expression of dorsal genes such as *Pax7*, *Msx1/2* and *slug* (Lee and Jessell, 1999). In our study, DLHP formation was inhibited by Shh despite low or undetectable expression of *Ptc1*, a Shh-induced gene, in the dorsolateral neural plate. This finding may indicate that inhibition of DLHPs requires only a low concentration of Shh in the neuroepithelium, which is insufficient to markedly up-regulate *Ptc1*. We find that DLHP formation can be accentuated by inhibiting the sulfation of heparan sulfate proteoglycans (Yip et al., 2002), which may promote Shh signaling by providing binding sites for Shh-N peptide in the extracellular matrix (The et al., 1999). Thus, appropriate presentation of low concentrations of Shh to responding cells in the dorsolateral neural plate may be sufficient to inhibit DLHPs.

An alternative explanation for the lack of *Ptc1* expression in the dorsolateral neural plate is that the inhibitory action of Shh on DLHP formation may be indirect, perhaps mediated via another cell type in the vicinity of the neural fold. The surface ectoderm seems a likely target of Shh, since proliferation within the epidermis, a derivative of the surface ectoderm, is known to be highly Shh-dependent. Basal cell tumours occur in situations of pathologically enhanced Shh signaling in the epidermis, for instance as a result of loss of Ptc1 function (Gailani et al., 1996). Although we have demonstrated that paraxial mesoderm is not required for DLHP formation, we cannot rule out a role for this tissue in mediating the inhibitory effect Shh on DLHPs.

Mechanism of MHP induction

The floor plate of the neural tube is induced by high concentrations of Shh-N peptide, emanating from the notochord (Jessell, 2000). Moreover, release of Shh from COS cells can induce an ectopic floor plate (Roelink et al., 1994), whereas we were unable to induce MHP-like bending, in either lateral or midventral neural plate regions, by local application of exogenous Shh-N peptide. It is possible that our Shh-soaked beads delivered insufficient Shh to induce an MHP although it is striking that MHP formation is present in embryos lacking Shh, whereas it is completely abolished in embryos lacking a notochord. Our findings, and those of other authors who found MHP induction by a grafted notochord (Van Straaten et al.,

1988; Smith and Schoenwolf, 1989), suggest that Shh may not be the main factor emanating from the notochord with MHP-inducing properties. Several other molecules have been suggested to participate with Shh in ventralising the neural tube. For instance, *noggin* expression in the notochord (McMahon et al., 1998) and a proteolytic fragment of vitronectin in the ventral neural tube (Pons and Marti, 2000) each synergise with Shh in the induction of motor neurons. Interestingly, the medial floor plate cells of the zebrafish neural tube arise through a Shh-independent mechanism, which requires the function of the *nodal*-related *cyclops* gene (Hatta et al., 1991; Odenthal et al., 2000). Perhaps MHP formation is induced primarily by a notochordal factor other than Shh, explaining the persistent MHP formation we observe in $Shh^{-/-}$ embryos.

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