Neural tube closure requires Dishevelled-dependent convergent extension of the midline

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SUMMARY

In *Xenopus*, Dishevelled (Xdsh) signaling is required for both neural tube closure and neural convergent extension, but the connection between these two morphogenetic processes remains unclear. Indeed normal neurulation requires several different cell polarity decisions, any of which may require Xdsh signaling. In this paper we address two issues: (1) which aspects of normal neurulation require Xdsh function; and (2) what role convergent extension plays in the closure of the neural tube. We show that Xdsh signaling is not required for neural fold elevation, medial movement or fusion. Disruption of Xdsh signaling therefore provides a specific tool for uncoupling convergent extension from other processes of neurulation. Using disruption of Xdsh signaling, we demonstrate that convergent extension is crucial to tube closure. Targeted injection revealed that Xdsh function was required specifically in the midline for

normal neural tube closure. We suggest that the inherent movement of the neural folds can accomplish only a finite amount of medial progress and that convergent extension of the midline is necessary to reduce the distance between the nascent neural folds, allowing them to meet and fuse. Similar results with *Xenopus* strabismus implicate the planar cell polarity (PCP) signaling cascade in neural convergent extension and tube closure. Together, these data demonstrate that PCP-mediated convergent extension movements are crucial to proper vertebrate neurulation.

Movies available on-line

Key words: Dishevelled, Strabismus, Planar cell polarity, Convergent extension, Neural tube defect, Neurulation, Midline, Floorplate, Craniorachischisis, Looptail, *Xenopus*

INTRODUCTION

Vertebrate neurulation (Fig. 1) involves a precisely orchestrated set of morphogenetic movements within the neural plate itself (intrinsic processes) and also within neighboring tissues (extrinsic processes) (Smith and Schoenwolf, 1997). Intrinsic processes include elongation and shaping of the neural plate by cell rearrangement, cell shape-change, and cell division; elevation and apposition of the neural folds; and fusion of the folds at the dorsal midline (Davidson and Keller, 1999; Jacobson and Gordon, 1976; Schoenwolf and Alvarez, 1989; Schroeder, 1970; van Straaten et al., 1993). Extrinsic processes include medial pushing on the neural folds by the adjacent epidermis, as well as inductive and mechanical contributions from the underlying mesoderm (Alvarez and Schoenwolf, 1992; Brun and Garson, 1983; Jacobson and Jacobson, 1973; Moury and Schoenwolf, 1995; Poznanski et al., 1997; Schroeder, 1970; van Straaten et al., 1996).

Mammalian neural tube defects (NTDs) stem from a failure of one or more of these morphogenetic processes (Harris and Juriloff, 1999), and recent genetic experiments in mice have identified individual genes that control specifically the many individual behaviors that comprise neurulation. For example, loss of p190RhoGAP inhibits neural fold closure by disrupting apical constriction of neuroepithelial cells (Brouns et al.,

2000). Mutation of ephrin A5 does not affect the elevation or apposition of neural folds, but instead precludes their normal fusion at the dorsal midline (Holmberg et al., 2000). Genes controlling extrinsic factors in neurulation have also been identified, such as AP-2, which is expressed in the non-neural ectoderm but is essential for tube closure (Zhang et al., 1996).

One morphogenetic process that has not been mechanistically related to NTDs is convergent extension, in which a tissue narrows in one axis and elongates in a perpendicular axis (reviewed by Wallingford et al., 2002). Though it has been described during neurulation in a variety of vertebrates, the contribution of convergent extension to tube closure has been difficult to assess without tools for uncoupling it from the many other morphogenetic processes involved (Burnside and Jacobson, 1968; Elul and Keller, 2000; Jacobson and Gordon, 1976; Keller et al., 1992; Lawson et al., 2001; Schoenwolf and Alvarez, 1989; van Straaten et al., 1996). Indeed, defects in axial elongation have been postulated to contribute to NTD in the classical mouse mutant *looptail* (Kibar et al., 2001; Smith and Stein, 1962; Wilson and Wyatt, 1992), though other studies have challenged that view (Gerrelli and Copp, 1997; Murdoch et al., 2001).

Convergent extension is driven by the polarized rearrangement of cells within the tissue (Elul and Keller, 2000; Keller et al., 2000; Shih and Keller, 1992), and *Xenopus*

Dishevelled (Xdsh) controls this polarity via a vertebrate cognate of the planar cell polarity (PCP) cascade (Tada and Smith, 2000; Wallingford et al., 2000). In our previous work, we showed that disruption of Xdsh signaling results in a failure of both neural convergent extension and neural tube closure (Wallingford and Harland, 2001).

These results suggest the possibility that PCP-mediated convergent extension is directly required for neural tube closure. But without a mechanistic analysis, it remains equally possible that Xdsh controls other important movements during neurulation. Indeed, each of the many processes comprising neurulation requires coordinated cell polarity (Fig. 1), and PCP
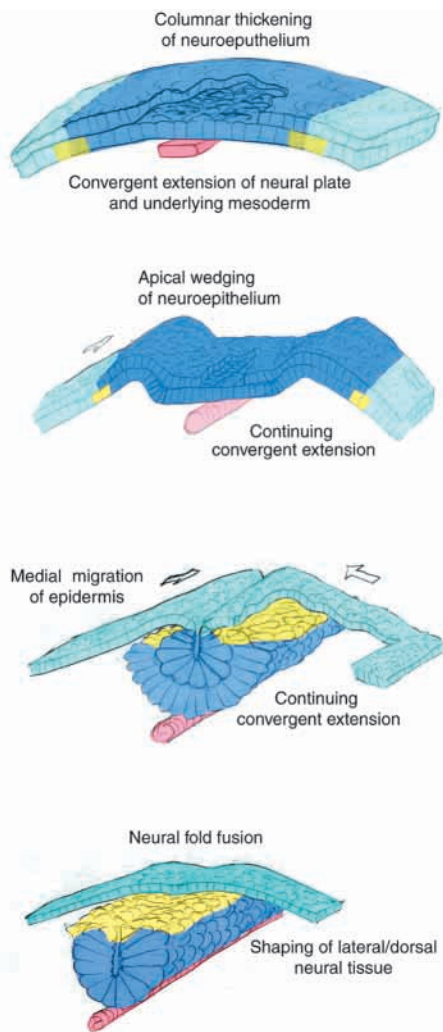
Fig. 1. The morphogenetic processes involved in *Xenopus* neurulation. All of the behaviors shown require proper establishment of either apicobasal or mediolateral cell polarity. During early neurulation, the neuroepithelium thickens apicobasally while convergent extension movements shape the neural plate. At mid-neurulation, convergent extension continues, while apical wedging initiates in the neuroepithelium, elevating the folds. Medially directed movement of the epidermis also begins. At late neurula stages, the neural fold fuse dorsally and convergent extension of the dorsal neural tissues shapes the neural tube. Neuroepithelium, blue; dorsal neural tissue and neural crest, yellow; epidermis, light blue; notochord, red. Adapted from Davidson and Keller (Davidson and Keller, 1999).

signaling components have been implicated in establishing both planar and apicobasal cell polarity in a wide variety of tissues and animals (Bellaiche et al., 2001; Marsden and DeSimone, 2001; Sun et al., 2001; Theisen et al., 1994; Wiggan and Hamel, 2002).

In light of the previous work, two questions need to be addressed. First, which processes of neurulation require Xdsh signaling? Second, does convergent extension directly contribute to neural tube closure? In this paper, we show that Xdsh signaling is dispensable for the elevation, medial movement and fusion of the neural folds, but time-lapse analysis revealed a strict correlation between convergent extension and neural tube closure. The results here demonstrate that midline convergent extension is a crucial component of normal vertebrate neurulation, narrowing the distance between the neural folds, allowing them to meet and fuse. In light of the severe NTDs of Dishevelled mutant mice (Hamblet et al., 2002), our results may provide important insights into the pathology of specific human neural tube defects.

MATERIALS AND METHODS

Embryos, in situ hybridization, microinjection and constructs

Female adult *Xenopus laevis* were ovulated by injection of human chorionic gonadotropin and eggs were fertilized in vitro and dejellied in 3% cysteine (pH 7.9) and subsequently reared in 1/3×MMR. Embryos were staged according to Nieuwkoop and Faber (Nieuwkoop and Faber, 1994).

For microinjections, embryos were placed in a solution of 2.5% Ficoll in 1/3×MMR, and injected using forceps and an Oxford universal micromanipulator, then reared in Ficoll + 1/3×MMR to stage 9 then washed and reared in 1/3×MMR alone. Xdd1 and Xdsh-D2 were injected at 1 ng per blastomere. XStbm was injected at 1.25 ng per blastomere.

Embryo culture, solutions, in vitro transcription, and in situ hybridization were as previously described; some embryos were dehydrated and cleared (Sive et al., 2000).

Constructs used were: Xdsh-ΔPDZ/D2 (Xdsh-D2) (Rothbächer et al., 2000), Xdd1 (Sokol, 1996), XStbm (Darken et al., 2002), Xash-3 (Zimmerman et al., 1993), Xpax-3 (Bang et al., 1999), Xnetrin (de la Torre et al., 1997), Sox-2 (Mizuseki et al., 1998) and Xfd12 (Solter et al., 1999).

Time-lapse microscopy and morphometric analysis

For time-lapse, embryos were devitellinated with forceps and placed in wells of modeling clay in 1/3×MMR. Images were collected on a Zeiss SV6 stereoscope using a Zeiss Axiocam. Images were collected every 2 minutes, and embryos were manipulated manually with a hairloop to maintain the proper viewing angle. Time-lapse stacks were assembled and viewed in NIH Image. LWR (length/width ratio) measurements (Fig. 5) were made at various stages by tracing embryos in NIH Image and calculating major and minor axes of the best-fitting ellipse of the trace. Rates of neural tube closure (Fig. 4) were estimated by tracing the edges of the neural folds in time-lapse movies and calculating the minor axis of the best-fitting ellipse of that trace. To confirm that this estimation was accurate, the distance between the apices of the neural folds from fixed specimens was measured directly from confocal sections at stages 16 and 17. Rates of fold apposition obtained from the fixed material paralleled that obtained from the time-lapse (not shown).

Confocal microscopy

Embryos were fixed in MEMFA overnight, washed in PBS, dehydrated overnight in methanol at 4°C, and rehydrated in PBS + 0.1% Tween-20. Embryos were then bisected transversely using forceps and a fragment of razor blade. These 'half-mounts' were then redehydrated in an ethanol series and cleared for confocal viewing in 2:1 benzyl benzoate:benzyl alcohol. Images were collected on an inverted Zeiss 410 confocal microscope using an HQ long-pass 580 emission filter (Chroma).

RESULTS

Inhibition of Xdsh function results in neural tube (NT) closure defects

Deletion mutants of Xdsh allow the signaling properties of the protein to be uncoupled. For example, the Xdd1 mutant disrupts both canonical Wnt and PCP signaling pathways in *Xenopus*, while Xdsh-D2 disrupts only PCP signaling and is fully functional for canonical Wnt signaling (Rothbächer et al., 2000; Sokol, 1996; Wallingford et al., 2000). Injection of 1 ng of either of Xdd1 or Xdsh-D2 mRNA elicited an open neural tube phenotype in roughly 50% of embryos (Fig. 2), while the remainder had normally closed neural tubes. In most cases, only a region of the neural tube remained open, with properly closed regions of neural tube anterior and/or posterior to the defect (Fig. 2C-E). In severe cases, the entire neural tube remained open (Fig. 2F). Increasing doses of mutant Xdsh mRNA had more severe effects at a higher frequency (not shown). Disruptions of other PCP genes, such as *Strabismus*, elicit similar neural tube closure defects (Darken et al., 2002; Goto and Keller, 2002).
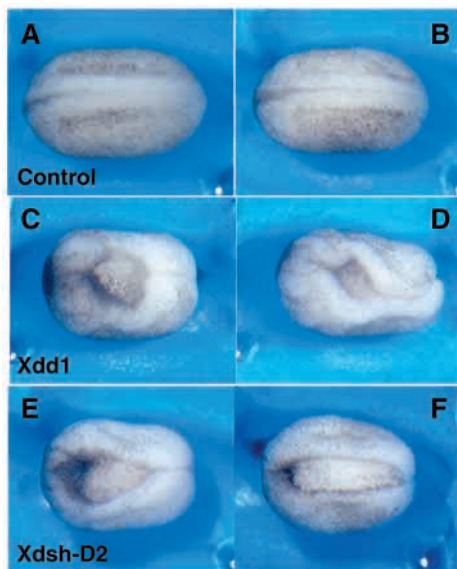
Fig. 2. Expression of mutant Xdsh elicits NT closure defects. (A,B) Control embryos at stage 22 have closed neural tubes. (C) An embryo expressing Xdd1 with an open neural tube. (D) Another embryo expressing Xdd1 with a milder open NT defect. NT is closed normally anterior and posterior to the NT defect. (E) An embryo expressing Xdsh-D2 displaying an NT defect. (E) Another Xdsh-D2 expressing embryo with a severe NT defect in which the entire length of the NT has failed to close.

Mutant Xdsh does not inhibit neural fold elevation

Confocal optical sections revealed the subtle nascent neural folds of stage 16 embryos (Fig. 3A, arrows). As neurulation proceeded, the folds became more pronounced and moved medially (Fig. 3D). In embryos expressing Xdd1, subtle neural folds were also apparent by stage 16, but these folds were displaced laterally when compared with controls (Fig. 3B,C). As in controls, neural folds in Xdd1-expressing embryos were more pronounced at stage 17 (Fig. 3E,F).

In many Xdd1-expressing embryos, the midline tissues appeared somewhat disorganized at stage 16 with less clearly defined boundaries between notochord, somites and neural plate when compared with controls. At stage 17, the tissues were more distinct (Fig. 3B,C). In some embryos, the open neural tube was associated with a severely broadened notochord (Fig. 3F). However, in other embryos, open neural tubes were associated with a normal notochord, suggesting that the defect in neural tube closure lies within the neural tissue (Fig. 3E).

Xdsh function is not required for medial movement of the neural folds

To better understand the nature of the neural tube defects elicited by mutant Xdsh, we made time-lapse movies of neurulating embryos (see movies at http://dev.biologists.org/supplemental). At the doses used here, expression of Xdd1 inhibits neural tube closure in approximately 50% of embryos. We refer to Xdd1-injected embryos which will eventually exhibit an open neural tube defect as 'open-NT' embryos, and embryos which will eventually close their neural tube as 'closed-NT' embryos. Xdd1 closed-NT embryos serve as a useful control group for identifying aspects of morphogenesis which are associated with successful versus failed NT closure.

In control uninjected embryos, neural folds were evident by stage 16 and the folds moved steadily toward the midline, meeting and fusing at stage 20 (Movie 1 at http://dev.biologists.org/supplemental; Fig. 4A, part a′, 4E). The folds of closed-NT embryos were also evident at stage 16 and like the controls, closed-NT embryos displayed a steady movement of the neural folds toward the midline followed by fold fusion (Fig. 4B, part b′, 4E). There was often a brief delay in the timing of neural tube closure in closed-NT embryos (Movie 1 at http://dev.biologists.org/supplemental; Fig. 4E).

Surprisingly, Xdd1-injected open-NT embryos also formed pronounced neural folds at stage 16 and these folds moved steadily toward the dorsal midline as neurulation proceeded (Movie 1 at http://dev.biologists.org/supplemental; Fig. 4C,D,F). Similar results were obtained from movies of embryos expressing Xdsh-D2 (Movie 2 at http://dev.biologists.org/supplemental). The distance traveled by the folds of open-NT embryos between stage 16 and 20 was comparable with the distance traveled in the same period by folds of closed-NT or control embryos (Fig. 4E,F), suggesting that Xdsh function is not required for the movement of neural folds toward the midline. In fact, the rate at which neural folds approached one another during mid- and late-neurulation was marginally faster in the open-NT embryos than in wild-type or closed-NT embryos (Fig. 4G).

The crucial difference observed between open-NT embryos and control embryos was the initial distance separating the forming folds at stage 16. The width between the nascent
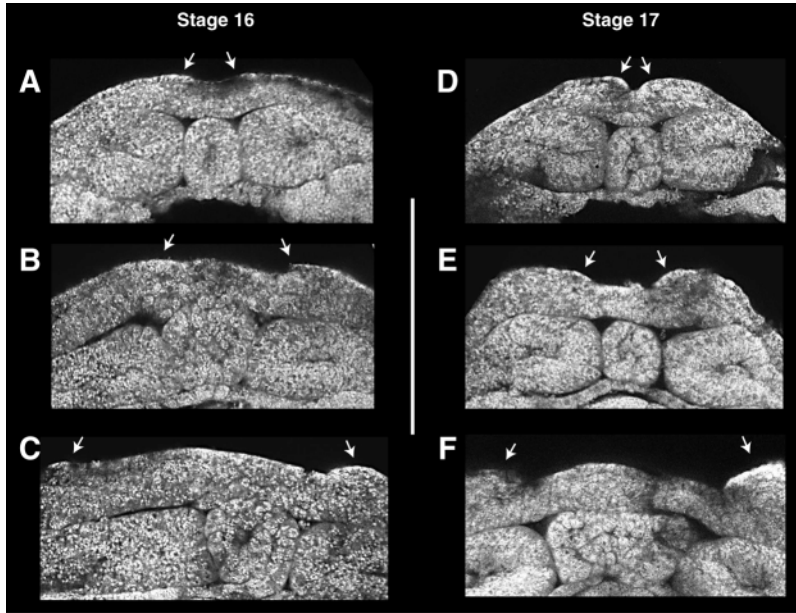
Fig. 3. Transverse optical sections of NT defects. (A) Control embryo at stage 16. (B,C) Xdd1-injected embryos at stage 16. (D) Control embryo at stage 17. (E,F) Xdd1-injected embryos at stage 17. Arrows indicate neural folds in all panels. Note that at each stage, neural folds in Xdd1-injected embryos are wider than controls at the same stage. Midline tissues are less organized in Xdd1-injected embryos at stage 16, though organization is better at stage 17. In many embryos, a prominent bulge was observed in the midline of the open neural plate (Fig. 3F). Although thinning of the neural plate has been suggested to contribute to tube closure (Poznanski et al., 1997), because it was not consistently observed (Fig. 3E), this midline bulge of the neuroepithelium cannot be the primary cause of the open NT phenotype in Xdd1-injected embryos.

neural folds was considerably greater in open-NT embryos than in control embryos (Fig. 4A-D), and throughout neurulation, folds of open-NT embryos remained substantially farther apart than folds of control embryos (Fig. 4F).

Finally, the dorsal epidermis has been implicated in generating force for the apposition of the neural folds. Our time-lapse analysis revealed no defect in the medial movement of the dorsal epidermis in Xdd1-injected embryos. Dorsal superficial cells can be seen actively and robustly moving medially in both control embryos (Movie 3 at http://dev.biologists.org/supplemental) and open-NT embryos (Movie 4 at http://dev.biologists.org/supplemental).

Failure of neural tube closure accompanies failure of convergent extension

In our time-lapse movies of neurulation, we noticed a strong correlation between the degree of axis elongation and the severity of the neural tube defect. For example, Xdd1 closed-NT embryos elongated substantially between stage 16 and stage 20 (Fig. 4B, part b′), while embryos with severely open NTs elongated negligibly during the same period (Fig. 4D, part d′). Interestingly, embryos with smaller regions of open neural tube elongated to an intermediate degree (Fig. 4C, part c′). Because there is no increase in mass in early amphibian development, elongation of the AP axis is directly linked to mediolateral narrowing. We therefore quantified overall embryonic convergent extension by measuring the length-to-width ratio (LWR) of embryos, and this analysis confirmed a correlation between LWR and neural tube closure (Fig. 4H).

To further investigate this correlation, we made time-lapse movies of wild-type and Xdd1-injected embryos from early gastrulation until the end of neurulation and compiled the LWR data from several movies (Movie 5 at http://dev.biologists.org/supplemental; Fig. 5). In normal embryos, a very dramatic increase in LWR was observed at the onset of neurulation (Fig. 5B). Control embryos then underwent steady convergent extension, increasing their LWR consistently throughout the neurula stages (Fig. 5B).

In Xdd1-injected embryos which would eventually close their neural tubes, a very similar pattern was observed except that by mid- to late-neurula stages, a mild defect in the overall elongation of the axis became apparent in closed-NT embryos (Fig. 5B). This mild defect in elongation probably accounts for the delay in closure observed in these embryos (Movies 1, 5 at http://dev.biologists.org/supplemental). A delay in tube closure is also observed in mice lacking one copy of the PCP gene *Strabismus* (Copp et al., 1994).

In Xdd1-injected open-NT embryos, a severe deficiency in LWR was apparent by the early neurula stages (Movie 5 at http://dev.biologists.org/supplemental; Fig. 5C). This defect became progressively more severe during neurulation. Xdd1-injected open-NT embryos failed to elongate steadily, and periods with no elongation were often observed at mid-neurulation and again at the end of neurulation. Similar defects in axis elongation were observed in movies of Xdsh-D2 expressing embryos (Movie 2 at http://dev.biologists.org/supplemental).

Finally, we observed an overall acceleration in the rate of elongation during neurula stages in control embryos (Fig. 5D). Closed-NT embryos also displayed this continuing increase in rate of elongation, while open-NT embryos did not (Fig. 5D).

Neural tube closure requires Xdsh function in the midline, but not in the lateral/dorsal neural tube

The neural plate is patterned along its mediolateral axis, and as a result of neurulation this mediolateral pattern becomes the dorsoventral pattern within the neural tube; the medial neural plate becomes the ventral neural tube, while the lateral neural plate becomes the dorsal neural tube (Bronner-Fraser and Fraser, 1997). To define the regions of the neural plate in which Xdsh is required for tube closure, we performed targeted injections of Xdsh mutants (Fig. 6A,B). At the 16-cell stage, injection into medial animal blastomeres targets expression to the ventral/medial neural tissue and to the notochord, while injection into the lateral animal blastomeres targets expression
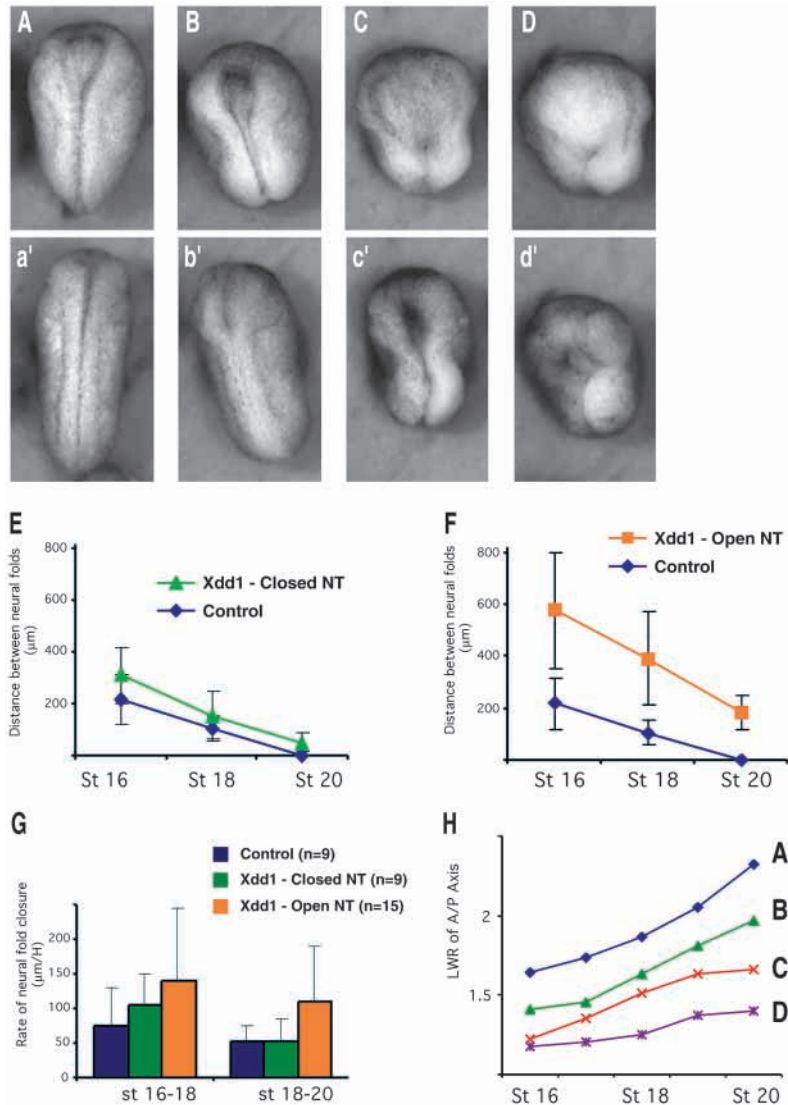
Fig. 4. Neural fold apposition in Xdd1 expressing embryos. See also Movies 1-4 at http://dev.biologists.org/supplemental/ (A) Control embryo at stage 16. (B) Xdd1-injected closed-NT embryo at stage 16. (C,D) Xdd1-injected open-NT embryos at stage 16. (a′-d′). Same embryos as in A-D shown at stage 23. (E) Plot of average distance between neural folds for control and Xdd1 closed-NT embryos. (F) Plot of average distance between neural folds for control and Xdd1 open-NT embryos. (G) Plot of rate of neural fold apposition (change in average distance between neural folds/time) for control, closed-NT, and open-NT embryos at mid- and late-neurula stages. Data shown in E-G are mean±s.d. from six different movies on different days (control *n*=9; open-NT *n*=15; closed NT *n*=9). (H) Plot of LWR of the AP axis of each embryo in A-D; there is a strong correlation between axis elongation and success of NT closure.

to the dorsolateral neural tissue and to the dorsal epidermis (Hirose and Jacobson, 1979).

Because Xdsh-D2 is fused to GFP, the location of cells expressing the construct can be followed. To confirm the proper targeting of injections, embryos were examined at the early neural plate stage. In medially injected embryos, GFP-positive cells were present in a single stripe in the dorsal midline (Fig. 6C). In laterally injected embryos, two distinct domains of GFP-positive cells were observed at the lateral borders of the neural plate, where the neural folds are forming (Fig. 6D).

Open neural tubes were only observed in medially injected embryos (Fig. 6E) where GFP-positive cells could be seen at the floor of the open neural tube (Fig. 6e′). In laterally injected embryos, neural tubes were closed (Fig. 6F) and GFP-positive cells could be observed in the dorsal neural tube and epidermis (Fig. 6f′). Like Xdsh-D2, Xdd1 was also found to elicit neural tube closure defects when targeted medially, but not when targeted laterally (not shown). Consistent with our time-lapse data, this result indicates that Xdsh function is not required in the forming or fusing neural folds or in the medially moving dorsal epidermis. These data suggest that for neural tube closure, Xdsh function and convergent extension are required only in the midline.

Defective midline neural convergent extension is evident at gastrula stages in Xdd1-injected embryos

To further characterize the convergent extension defects in the neural midline of Xdd1-injected embryos, we examined the morphology of the ventral/medial neural tissue with molecular markers. During gastrula stages, Xfd12 is expressed in ventral/medial neural precursors (Solter et al., 1999). During early gastrulation, robust convergent extension reorganizes the neural plate (Keller et al., 1992), and these movements are very closely paralleled by the expression pattern of Xfd12 (Fetka et al., 2000). At early gastrula, both control and Xdd1-injected embryos express similar levels of Xfd12 in a broad, dorsal arc. No difference in the Xfd12 expression pattern was observed between control and Xdd1-injected embryos at this stage (Fig. 7A,B). By mid-gastrulation, the Xfd12 expression pattern in control embryos had undergone dramatic convergent extension (Fig. 7C). However, in Xdd1-expressing embryos Xfd12 still marked a short, wide arc reminiscent of its earlier expression pattern (Fig. 7D), indicating a failure of convergent extension in the midline.

We examined the medial/ventral limit of the neural plate at neurula stages by in situ hybridization for Xnetrin, which marked a thin line along the length of the neuraxis of control embryos at mid- and late neurula stages (Fig. 7E,G). In Xdd1-injected embryos, the Xnetrin staining pattern was extremely broad and significantly shortened (Fig. 7F,H). In many cases, regions in the center of the Xnetrin domain failed to stain (Fig. 7F, green arrow), suggesting some disruption of ventral neural cell fates in these regions. This disruption was confirmed by expression of the midline marker Sonic Hedgehog (SHH; Fig. 7I). In Xdd1-injected embryos, SHH expression was abnormally broad, and in some embryos SHH expression was lost in certain regions (Fig. 7J, green arrow).

Although failure of floorplate convergent extension always accompanied the open-NT phenotype, disorganization of floorplate marker expression was inconsistently observed in open-NT embryos (Fig. 7, green arrows). Likewise, the
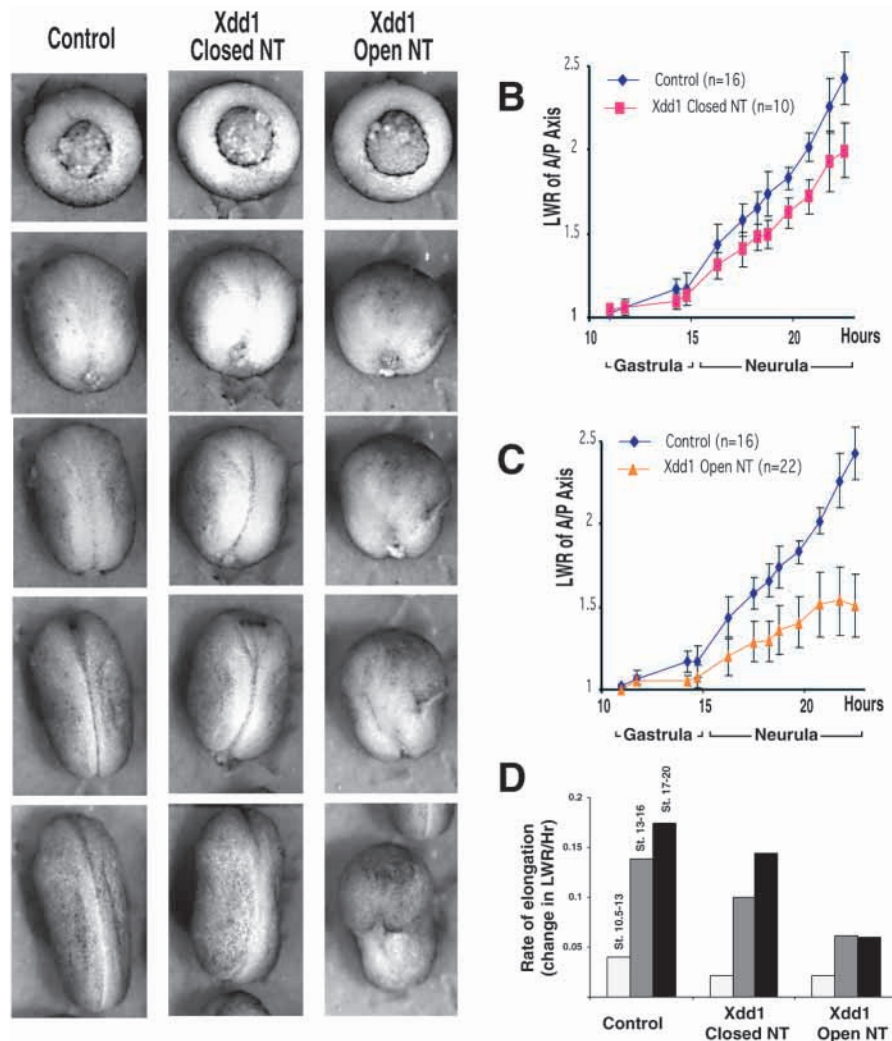
Fig. 5. Failure of NT closure correlates with defective convergent extension of the embryo. (A) Individual images from time-lapse movies of neurulation showing control, closed-NT and open-NT embryos at stages 11.5, 14, 16, 18 and 22 (first panel shows vegetal view; all subsequent panels show dorsal view). (B) Plot of LWR of AP axis for control and Xdd1 closed-NT embryos. (C) Plot of LWR of AP axis for control and Xdd1 open-NT embryos. Data for B,C are mean LWR±s.d. (control $n=16$ embryos, eight movies; closed-NT $n=10$ embryos, six movies; open-NT $n=22$ embryos, seven movies; data points are for stages 10.5, 11, 12.5 and 13-20). (D) Rate of axis elongation (change in LWR/time) for control, closed-NT and open-NT embryos during early (white bars), middle (gray bars) and late neurulation (black bars). See also Movie 5 at http://dev.biologists.org/supplemental/

expanded floorplate did not necessarily occupy the entire open-NT region (Fig. 7, red arrows).

Xdd1 inhibits the elongation and narrowing of the neural plate at all dorsoventral levels

For normal neural tube closure, Xdsh signaling is not required in dorsolateral neural tissue (Fig. 6). However, convergent extension does occur in the lateral regions of the neural plate (Keller et al., 1992), and we examined the requirement for Xdsh in convergent extension movements of these regions of the neural plate.

We examined morphology of the lateral/dorsal neural plate by in situ hybridization to Xpax3. At mid-neurula stages, Xpax3 marks two elongate domains in the lateral neural plate

(Fig. 8A). In Xdd1-injected embryos, each of these domains was shorter and wider and the two were separated by a considerable distance (Fig. 8B), consistent with the widened floorplate (Fig. 7).

We then examined the effect of Xdd1 on the intermediate neural plate. In normal embryos at the late neurula stages, Xash3 is expressed in two elongate stripes at the borders between the presumptive alar and basal regions on either side of the neural plate (Fig. 8C). Each Xash3 expression domain was foreshortened in Xdd1-injected embryos, and like the Xpax3 domains these Xash3 domains were more widely spaced (Fig. 8D).

Finally, we examined the shape of the entire neural plate at the onset of neurulation (stage 14) as revealed by the pan-neural marker Sox-2 (Fig. 8E). Embryos injected with Xdd1 uniformly stain positive for Sox2, indicating that Xdd1 does not affect neural induction. In Xdd1-injected embryos, the Sox2 expression domain was shorter and much broader than in uninjected embryos (Fig. 8F). Together with the Xfd12 data (above, Fig. 6), this result indicates that defective convergent extension of the neural tissue prior to the onset of neurulation results in an abnormally broad neural plate and subsequently in abnormally wide spacing of the neural folds.

Overexpression of wild-type Xenopus Stbm phenocopies the effects of mutant Xdsh expression

The similarity between the Xdd1 and the Xdsh-D2 phenotypes (compare Movie 1 with Movie 2 at http://dev.biologists.org/supplemental) indicate that the NT defects result from disruption of PCP signaling and not the canonical Wnt pathway (see Wallingford and Harland, 2001). To explore this idea further, we made use of the fact that overexpression of wild-type components of the PCP pathway specifically disrupt cell polarity but do not affect Wnt signaling (Krasnow and Adler, 1994). Indeed, overexpression of wild-type *Xenopus* Stbm disrupts mediolateral cell polarity, convergent extension and NT closure in *Xenopus* (Darken et al., 2002; Goto and Keller, 2002).

Like the Xdd1 phenotype, XStbm-induced NT defects (Fig. 9A,B) were accompanied by defective convergent extension of the midline during gastrula stages, as indicated by the abnormally broad expression domain of Xfd12 (Fig. 9C,D). In addition, XStbm disrupted convergent extension throughout the neural plate, as evidenced by expression patterns of XPax3, Xash-3 and Sox2 (Fig. 9E-J).
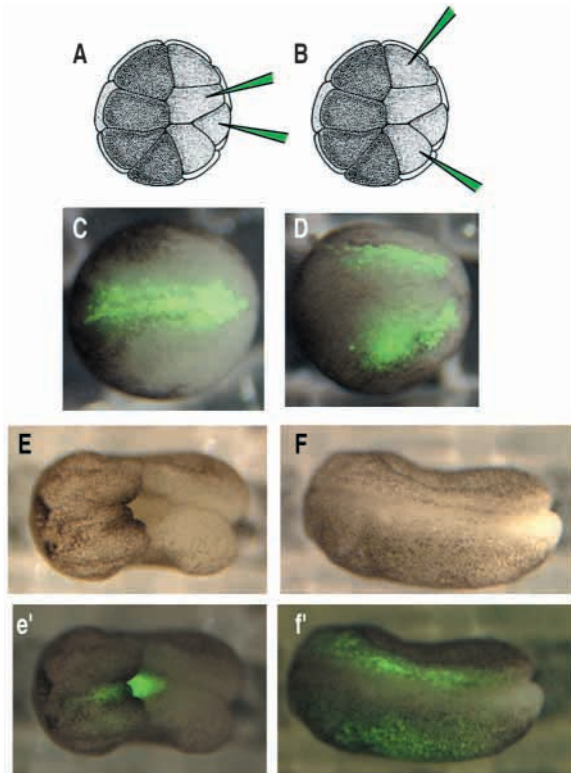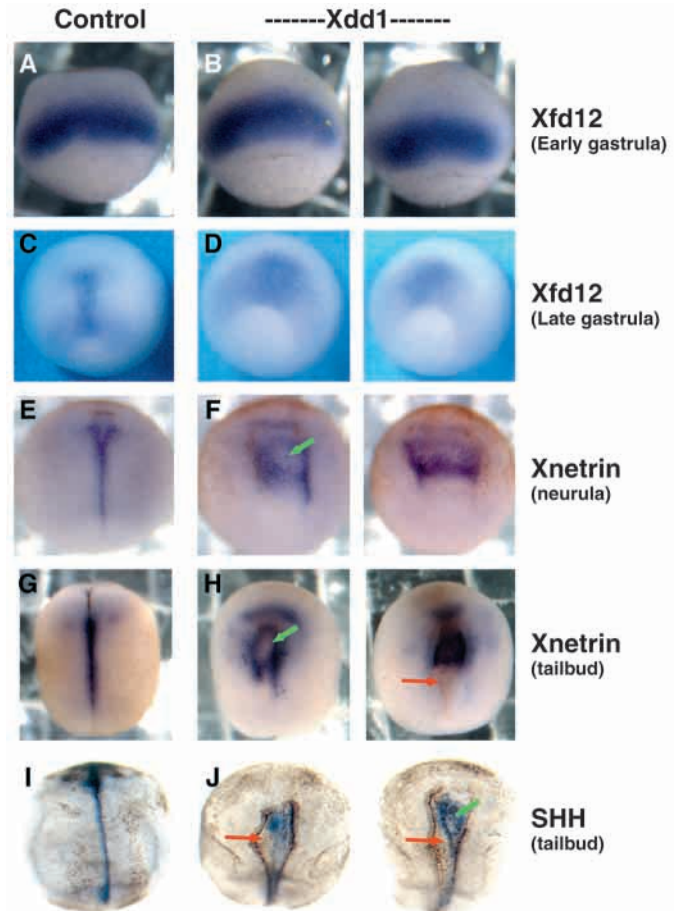
Fig. 6. Neural tube closure requires Xdsh function in the midline. (A) Targeted injection into dorsal medial animal blastomeres at the 16-cell stage. (B) Targeted injection into the dorsal lateral animal blastomeres. (C) GFP lineage tracing of medial injection at stage 14 reveals delivery to midline. (D) GFP lineage tracing of lateral injection at stage 14 reveals delivery to lateral neural plate. (E) Medial injection results in NT closure defects, and GFP labels floor of open NT (e′). (F) Lateral injection does not elicit NT closure defects and GFP labels dorsal NT and epidermis (f′).

Fig. 7. Persistent failure of midline neural convergent extension in Xdd1 injected embryos. (A) Control embryos stained for Xfd12 expression at stage 10.5. (B) Xdd1-injected embryos stained for Xfd12 at stage 10.5. (C) Control embryos stained for Xfd12 expression at stage 11.5. (D) Xdd1-injected embryos stained for Xfd12 at stage 11.5. (E) Expression of Xnetrin in control embryo at mid-neurulation. (F) Expression of Xnetrin in two Xdd1-injected embryos at mid-neurulation; green arrow indicates gap in Xnetrin expression domain. (G) Expression of Xnetrin in control embryo at late neurulation. (H) Expression of Xnetrin in two Xdd1-injected embryos at late neurulation; green arrow indicates gap in Xnetrin expression domain; red arrow indicates area at posterior floor of the open NT, which does not express Xnetrin. (I) Expression of SHH in control embryo at late neurulation (embryo has been cleared). (J) Expression of SHH in two Xdd1-injected embryos at late neurulation; green arrow indicates gap in SHH expression domain; red arrow indicates area in the floor of the open NT, which does not express SHH.

DISCUSSION

We have previously demonstrated that Xdsh signaling serves an important function in neural convergent extension (Wallingford and Harland, 2001). Indeed, our finding that Xdsh controls the stability and polarity of lamellipodia provides a cell biological basis for Xdsh function during convergent extension (Wallingford et al., 2000). However, disruption of Xdsh signaling also impairs neural tube closure, and our previous work did not address whether other polarized morphogenetic processes of neurulation (Fig. 1) were also affected by Xdsh mutants.

In this report, time-lapse analysis, cross-sections, and targeted injections revealed that Xdsh mutants do not disrupt elevation, medial movement or fusion of neural folds, and do not disrupt the medial movement of the epidermis (Figs 2, 3, 4, 9; Movies 1, 3, 4 at http://dev.biologists.org/supplemental). Although we have not ruled out a role for Xdsh signaling in establishing apicobasal polarity in the neural epithelium, the results presented here do demonstrate that mutant Xdsh specifically disrupts neural convergent extension movements and does not affect other polarized processes of neurulation.

Convergent extension movements in the neural plate and tube have been documented in a variety of animals, particularly amphibians and chicks (Burnside and Jacobson, 1968; Jacobson and Gordon, 1976; Keller et al., 1992; Lawson et al., 2001; Schoenwolf and Alvarez, 1989; van Straaten et al., 1996). However, without tools for uncoupling the various morphogenetic process, very limited experimental analysis of its role in tube closure has been possible. For example, failure of NT closure is correlated with reduced neuraxis elongation in UV-irradiated chick embryos, but other aspects of neurulation were not described (Jacobson, 1984). The specificity of Xdsh mutants as inhibitors of convergent
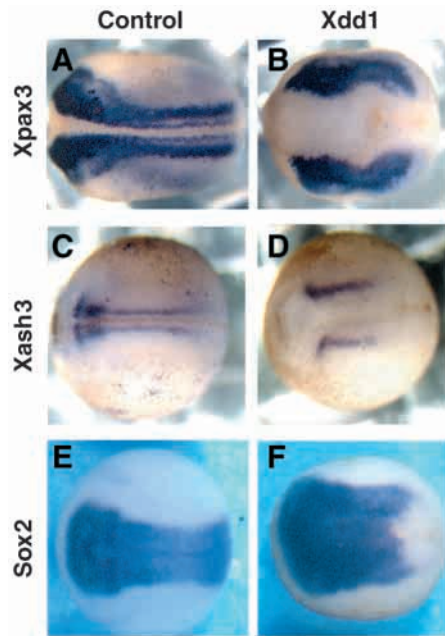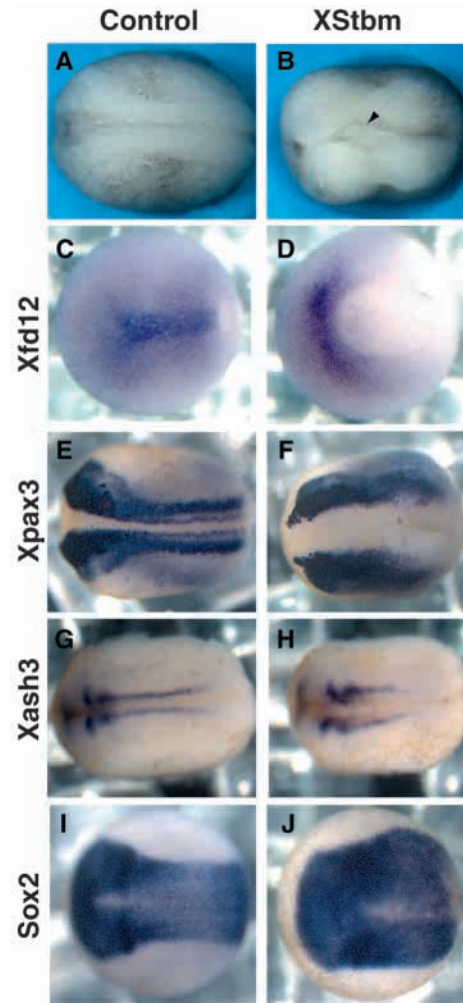
Fig. 8. Xdd1 disrupts convergent extension of dorsolateral neural tissue. (A) Dorsal view Xpax3 expression (blue staining) in a control embryo at mid-neurula stage. (B) Xpax3 expression at mid-neurulation in an embryo injected with Xdd1. It should be noted that embryological and fate-mapping experiments have identified an additional convergent extension event which shapes the dorsal neural tube following overt tube closure (Davidson and Keller, 1999). This later convergent extension event was also disrupted by expression of Xdd1 (not shown). (C) Expression of Xash3 in a control embryo. (D) Expression of Xash3 in an Xdd1-injected embryo; the expression domains are foreshortened. (E) Expression of Sox2 in a control embryo at stage 14. (F) Expression pattern of Sox2 in Xdd1-injected embryos. As a control, we targeted Xdd1 injections to the dorsal, vegetal blastomeres, where expression inhibits predominantly mesodermal convergent extension and does not inhibit NT closure (Wallingford and Harland, 2001); in those embryos, Sox2 expression resembled wild type (not shown).

Fig. 9. Expression of wild-type Stbm elicits neural tube closure defects and failure of neural convergent extension. (A) Control embryo at mid-neurulation with closing NT. (B) Stbm-injected embryo with defective NT closure. (C) Xfd12 expression in control embryo at stage 14. (D) Xfd12 expression reveals defective neural convergent extension in a stage 14 Stbm-injected embryo. (E) Expression of Xpax3 in a control embryo. (F) Expression of Xpax3 in an XStbm injected embryo. (G) Expression of Xash3 in a control embryo. (H) Expression of Xash3 in an XStbm-injected embryos. (I) Expression of Sox2 in a control embryo. (J) Expression of Sox2 in an XStbm-injected embryos.

extension movements in the neural plate allows us to test directly the contribution of this morphogenetic movement to neural tube closure. While convergent extension of both dorsal and ventral neural tissue was inhibited by mutant Xdsh (Fig. 8A-E), targeted injections reveal that only midline convergent extension is required for tube closure (Fig. 6).

Convergent extension contributes directly to neural tube closure by narrowing the distance between the nascent neural folds

What biomechanical roles does convergent extension play in neural tube closure? It has been shown that proper signaling from underlying mesoderm is required for the fusion of neural folds in *Xenopus* (Poznanski et al., 1997), so it is possible that the NT defect arises from disruption of involution due to defective convergent extension in the mesoderm. However, this possibility is unlikely, as Xdd1 open-NT embryos successfully complete gastrulation (not shown). Moreover, disruption of convergent extension by targeted injection of mutant Xdsh into the mesoderm has little effect on tube closure (Wallingford and Harland, 2001).

Another possibility is that elongation of the neuroepithelium may be involved directly in generating force for neural fold apposition. It has been proposed that anteroposterior elongation of the neural plate results in transverse buckling of the neuroepithelium, promoting the elevation of the folds (Jacobson, 1978). Interestingly, detailed analysis of the rate of elongation in *Xenopus* revealed that elongation occurs episodically, with the rate fluctuating even as it accelerates overall (not shown). Similar episodic elongation is observed during chick and newt neurulation (Jacobson, 1978; van Straaten et al., 1996). Medially directed force generated by amphibian neural folds has also been demonstrated to be episodic (Selman, 1958), suggesting that

it may arise in part from the episodic convergence and extension.

It has also been suggested that convergent extension may compensate for the anteroposterior shortening of the neuroepithelium, which results from apical constriction (Jacobson and Gordon, 1976; Schoenwolf and Alvarez, 1989). The constriction of the apical surfaces of neuroepithelial cells is a crucial component of neural fold elevation, and this constriction is radial; buckling the neural plate in all directions. Indeed, computer modeling predicts that apical wedging in the absence of neural plate elongation will produce a cup or bowl, rather than a tube (Jacobson and Gordon, 1976). Our movies bear out this prediction (see, in particular, Movie 4 at http://dev.biologists.org/supplemental). Such a cup-shape appears to mechanically disallow the neural folds reaching one another and fusing.

Finally, our morphometric and molecular marker data both indicate that the crucial difference between open-NT embryos and control embryos was the width of the early neural plate (Figs 7, 8) and the distance between the forming neural folds (Fig. 4). In addition, our targeted injections demonstrate a requirement for convergent extension in the midline. We conclude that the mechanism which advances the elevated neural folds produces only a finite amount of medial movement. In order for tube closure to be successful, midline convergent extension is required to decrease the width between the folds, reducing the distance folds must travel in order to meet and fuse (Fig. 10).

Convergent extension and mammalian caudal neural tube closure

The similarity between the phenotypes of *Xenopus* and mouse embryos in which PCP signals are disrupted is striking and suggests a conservation not only of molecular controls but also of morphogenetic mechanisms. As is the case in *Xenopus*, disruption of Dishevelled or Stbm signaling in mice results in severe defects in both elongation of the AP axis and neural tube closure (Hamblet et al., 2002; Kibar et al., 2001; Murdoch et al., 2001). Several other results also suggest that convergent extension movements may be crucial to mammalian spinal neurulation. For example, a spontaneous mutation that results in severe spinal NTD in the rat also elicits a severe foreshortening of the AP axis (Layton and Smith, 1977). Likewise, mice lacking retinaldehyde dehydrogenase 2 display specific defects in spinal NT closure and fail to elongate the posterior region of the embryo (Niederreither et al., 1999). Indeed, a connection between defective axial elongation and defective neural tube closure has long been suggested for the *looptail* mouse, which lacks PCP signaling (Kibar et al., 2001; Smith and Stein, 1962; Wilson and Wyatt, 1992). It will now be very interesting to assess directly the convergent extension of the neural plate at early stages in the *looptail* and *Dishevelled* mutant mice.

Convergent extension, PCP signaling, and human neural tube defects

Neural tube defects are among the most common and most debilitating human birth defects, affecting close to 1 in 1000 pregnancies (Botto et al., 1999; Manning et al., 2000). While folate supplements have dramatically reduced the frequency of neural tube defects, studies indicate an important genetic component to their etiology (George and Speer, 2000; Harris, 2001). It is therefore crucial that we understand all of the genetic and embryological mechanisms that govern normal and abnormal neural tube closure in vertebrates.

Craniorachischisis (CRS), in which the entire caudal NT fails to close, accounts for ~10% of human NTDs (Kirillova
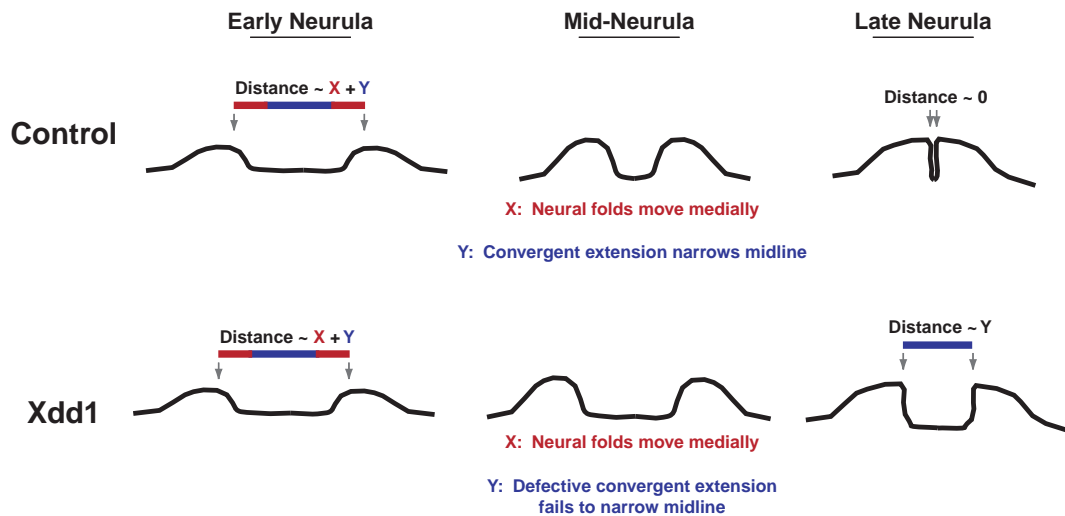
Fig. 10. Biomechanical contribution of convergent extension to neural tube closure. During normal neurulation, mechanisms that advance the elevated neural folds toward the midline (i.e. apical wedging, pushing by the epidermis, etc.) produce a finite amount of medial movement, arbitrarily defined as 'X' in this figure (red bars). Convergent extension narrows the midline by a finite amount, defined as 'Y' in this figure (blue bar). So, the distance between the forming neural folds can be defined as X+Y; at the end of normal neurulation this distance is reduced to zero, and the folds can meet and fuse. In embryos that lack PCP function, the distance X is covered by the normally functioning mechanisms (see Fig. 4). However, in the absence of convergent extension, the midline fails to narrow and at the end of neurulation, the folds cannot fuse as they remain separated by a distance roughly equal to 'Y.'

et al., 2000; Nakatsu et al., 2000). Strikingly, human embryos with CRS consistently display shortened anteroposterior axes and widened mediolateral axes; in particular, the neural arches of the vertebrae in these embryos are displaced laterally (Kirillova et al., 2000; Marin-Padilla, 1966). In addition to defective convergent extension and failed neural tube closure, human CRS is associated with bifurcated notochords, disorganized expression of SHH in the floorplate and bulging of the neural plate midline (Kirillova et al., 2000; Marin-Padilla, 1966; Saraga-Babic et al., 1993). All of these abnormalities are observed in frog or mouse embryos in which PCP function is disrupted (this study) (Greene et al., 1998; Wallingford and Harland, 2001). While the etiology of CRS remains poorly understood, these data suggest that PCP signaling and convergent extension may provide an effective starting point for directed studies into the molecular and biomechanical underpinnings of this neural tube defect.

PCP signaling and floorplate specification

Previous studies have suggested that in embryos with disrupted PCP signaling, the open-NT phenotype results from an abnormally wide floorplate caused by a failure to restrict floorplate cell fates (Murdoch et al., 2001). Our data are not consistent with that hypothesis, but rather indicate that the widening of the floorplate, like the widening of all levels of the neural plate and tube, results from a persistent defect in convergent extension beginning during gastrulation and continuing throughout neurulation (Figs 5-10).

Nonetheless, the disorganization of SHH and Xnetrin expression and the indistinct tissue boundaries at the midline of some Xdd1-injected *Xenopus* embryos (Figs 3, 7) are consistent with the disruptions of midline structures and cell fates observed in *looptail* mice (Greene et al., 1998; Murdoch et al., 2001). It is possible that the defects in floorplate patterning reflect a secondary effect on cell fate resulting from improper morphogenetic movements. For example, convergent extension movements enable short-range signaling events that are crucial to the differentiation of posterior notochord and somite (Domingo and Keller, 1995). A similar mechanism may be required in the floorplate, as the posterior, medial neural tissue of open-NT embryos often lack Xnetrin expression (Fig. 7F, red arrow). However, it has also been suggested that PCP signals influence floorplate cell fates directly (Murdoch et al., 2001). Interestingly, PCP signals interact with the Notch pathway to modulate cell fate in *Drosophila* (Cooper and Bray, 1999; Fanto and Mlodzik, 1999), and the Notch pathway has been implicated in controlling cell fates in the vertebrate midline (Appel et al., 1999). Together, the data from mice and from frogs indicate that PCP signaling may play a dual role, integrating cell fate and morphogenetic cell movement in the vertebrate midline.

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