Neural tube closure in *Xenopus laevis* involves medial migration, directed protrusive activity, cell intercalation and convergent extension

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SUMMARY

We have characterized the cell movements and prospective cell identities as neural folds fuse during neural tube formation in *Xenopus laevis*. A newly developed whole-mount, two-color fluorescent RNA in situ hybridization method, visualized with confocal microscopy, shows that the dorsal neural tube gene *xpax3* and the neural-crest-specific gene *xslug* are expressed far lateral to the medial site of neural fold fusion and that expression moves medially after fusion. To determine whether cell movements or dynamic changes in gene expression are responsible, we used low-light videomicroscopy followed by fluorescent in situ and confocal microscopy. These methods revealed that populations of prospective neural crest and dorsal neural tube cells near the lateral margin of the neural plate at the start of neurulation move to the dorsal midline using distinctive forms of motility. Before fold fusion, superficial neural cells apically contract, roll the neural plate into a trough and appear to pull the superficial epidermal cell sheet medially. After neural fold fusion, lateral deep neural cells move medially by radially intercalating between other neural cells using two types of motility. The neural crest cells migrate as individual cells toward the dorsal midline using medially directed monopolar protrusions. These movements combine the two lateral populations of neural crest into a single medial population that form the roof of the neural tube. The remaining cells of the dorsal neural tube extend protrusions both medially and laterally bringing about radial intercalation of deep and superficial cells to form a single-cell-layered, pseudostratified neural tube. While ours is the first description of medially directed cell migration during neural fold fusion and re-establishment of the neural tube, these complex cell behaviors may be involved during cavitation of the zebrafish neural keel and secondary neurulation in the posterior axis of chicken and mouse.

Time-lapse sequences online:
http://www.people.virginia.edu/~lad4x/tubeclosure.html and

Key words: Tyramide, Fluorescent in situ hybridization, Whole-mount confocal microscopy, *xk81*, Epidermal cytokeratin, *xslug*, Neural crest, *xpax3*, *n-tubulin*, neuron, *xash3*, Mediolateral intercalation, Radial intercalation, Directed protrusive activity, Convergent extension

INTRODUCTION

Neurulation is the process by which progenitors of the central nervous system are shaped, separated from and brought beneath the epidermis. The cellular basis of these movements have been investigated in amphibians (Jacobson, 1981; Jacobson and Gordon, 1976; Keller et al., 1992b), chicken (Schoenwolf and Smith, 1990b; Smith and Schoenwolf, 1997) and mice (Bush et al., 1990; Sausedo and Schoenwolf, 1994; Smith et al., 1994) but remains poorly understood. In the chick, which has been used extensively as a paradigm for vertebrate neurulation, a broad neural plate folds and the margins of the plate are raised and brought into apposition at the neural folds as cells take on stereotypical shapes. Analysis of serial sections and electron microscopy have identified cell movements and shape changes accompanying neural tube formation in chick (Schoenwolf and Alvarez, 1989; Schoenwolf and Smith, 1990a) where these cell behaviors (apical contraction and interkinetic nuclear migration) result in the formation of a ‘medial hinge’ overlying the notochord and a ‘dorsal lateral hinge’ near the prospective sulcus limitans, that together bring the neural folds into opposition. The epidermis then fuses, the neural ectoderm fuses and the neural crest is released from neural epithelium of the newly formed neural tube. In this manner, it is thought that the flat neural plate rolls into a tube with the lumenal face of the neural tube forming from the apical face of the neural plate.

However, additional processes appear to be involved in chick neurulation. By following the rapid events at the start of neurulation, van Straaten and coworkers (1996) found that the lateral face of the two neural folds ‘zip’ into apposition, starting near the floorplate and proceeding dorsally. They found that the lumen of the neural tube nearly disappears after apposition and then re-opens to form the lumen after the neural
vertebrates. Thus, while it is likely that cell shape changes and neural plate bending establishes the ventral and intermediate aspects of the neural tube, more complex and as yet undefined events are involved in forming the dorsal neural tube.

The revelations in chick inspired us to re-investigate the mechanisms of neurulation in the frog. Based on sectioned material and light and electron microscopy, Schroeder (1970, 1971) outlined four processes that he thought would 'constitute the ultimate mechanisms of neurulation'. (1) Superficial cells of the neural epithelium change from cuboidal to bottle-shaped initiating the formation of the neural groove. (2) Presomitic mesoderm and lateral deep neural plate cells elongate to help elevate the neural folds. (3) Both superficial and deep epidermis 'migrate' medially bringing the neural folds into apposition thus aiding closure of the neural tube. (4) Extension of the underlying notochord prevents an anteroposterior shortening of the neural plate that would result after apical contraction of superficial neuroepithelial cells.

However, the closure and formation of the neural tube also involves complex movements of radial intercalation of several layers of deep cells with the superficial layer of the multilayered neural anlagen of the frog (Schroeder, 1971). These cell movements and the identities of the cells involved are poorly understood, particularly at the lateral margin of the neural plate, which is the source of three distinct tissues, the epidermis, which will form the dorsal skin, the neural crest, and the dorsal neural tube (Schroeder, 1970). The role of these dorsolateral populations in neural tube formation are difficult to evaluate because of the low spatial resolution of current molecular marker methods. Correlation of cell identity with motility is difficult because fluorescence-based imaging of cell behaviors and tissue movements is largely incompatible with RNA in situ hybridization techniques.

To solve this problem, we have modified the RNA in situ hybridization protocol (Harland, 1991) using a recently synthesized fluorescent substrate of peroxidase (Kerstens et al., 1995) and have developed a streamlined whole-mount preparation for confocal microscopy. Using this method allows epidermal, neural crest and dorsal neural tube gene expression patterns to be visualized with the same high-resolution confocal techniques used for immunofluorescence and lineage analysis and is generally useful to analyze gene expression patterns in the context of cell and tissue shapes.

We show that the mediolateral organization of the early neural plate is thrown into disorder by the rapid rolling movements of the superficial neural ectoderm and fusion of the neural folds over the neural groove. Immediately after fusion, markers of the prospective dorsal neural tube cells are still found far lateral to the medial site of fusion, whereas at the midline, the expected site of the neural lumen is occupied by multiple layers of deep mesenchymal cells lying above the floorplate. The dorsal neural tube with a well-defined lumen and roof is reformed after a complex set of cell movements: medially directed migration and intercalation of neural crest, and radial intercalation of deep neural cells. These movements occur for several hours after neural fold fusion to create a single-cell-layered neural tube characteristic of vertebrates.

## MATERIALS AND METHODS

### Embryos

Embryos were obtained by standard methods (Kay and Peng, 1991) and staged according to Nieuwkoop and Faber (1967). Albino embryos were used for all in experiments. For fluorescence-based imaging, it is important not to use Nile blue to add contrast for staining. Nile blue contains the dye Neutral red, which fluoresces in the rhodamine channel and does not wash out of embryos (in contrast to the blue components of Nile blue) in organic solvents. For general histology of cell and tissue shapes, all the cells in the embryo were labeled by injection of 1.5 nL from a stock solution (25 mg/ml of water) of rhodamine-dextran amine (RDA; anionic lysine-fixable; Molecular Probes) at the 1-cell stage. Scattered, labeled cell populations were made by injecting single blastomeres at stages 6 or 7 when there are between 100 and 300 cells. Embryos used for both histology and in situ were fixed overnight at 4°C in MEMFA (0.1 M MOPS, pH 7.4, 2 mM EGTA, 1 mM MgSO4 and 3.7% formaldehyde) and stored at −20°C in 100% methanol. For high-resolution microscopy, embryos were mounted in custom chambers at stage 17 in 1/3× Modified Barth’s Solution with 0.1% bovine serum albumin. Single embryos were pierced transversely on a short piece of a thin plastic ‘speaker’ (1 mm long by 20 μm in diameter). The ends of this plastic spear were pressed into silicone grease (Dow Corning) by coverglass fragments producing an embryo solidly fixed in position whose dorsal surface is centered and slightly pressed against a coverslip. All embryos pierced in this manner develop normally.

### Videomicroscopy and morphometric analysis

Low-light, time-lapse recordings were collected using a Hamamatsu C2400-008 SIT camera, an Olympus IX-70 inverted microscope, a Uniblitz shutter and a Metamorph imaging system (Universal Imaging Corp.). From these time-lapse recordings, we quantified the angular protrusive activity of individual cells (see Elul et al., 1997). Briefly, the outline of a single cell was traced over the course of a time-lapse recording (from 40 to 100 frames). The pixel areas of cellular protrusions from subsequent frames were accumulated into each of twelve 30° sectors centered on the center of mass of the cell. The angular protrusive activity for a cell was calculated as the percentage of the total ‘protrusion’ area falling within each sector. The angular protrusive activity of cells within the same domain of gene expression was calculated as the mean of the percentages for all the cells within that domain. This analysis was carried out using macros written by the authors for NIH-Image (version 1.61; http://rsb.info.nih.gov/nih-image/).

### Variations on the Harland protocol for RNA in situ hybridization

We have made a number of modifications to the Harland protocol (Harland, 1991). Antisense RNA probes were transcribed (Ambion), labeled with either digoxigenin or fluorescein UTP (BMB) and used without being hydrolyzed. Embryos in 100% methanol were rehydrated through a series of 25:75, 50:50 and 75:25 PBS:methanol washes. Proteinase K digestion was not done. Hybridization was at 60°C overnight and the post-hybridization RNase treatment was skipped with little or no change in sensitivity. In several trials, the potential activity of endogenous peroxidases was reduced by incubating embryos for 1 hour with 1% H2O2 in PBS, but no change in background was observed. Embryos were then washed, blocked and incubated with peroxidase-coupled (POD) fab fragments (BMB) directed against digoxigenin or fluorescein in maleic acid buffer with 2% BMB blocker and 20% heat-inactivated goat serum. With the substitution of POD for alkaline phosphatase, this protocol was the same as that used in a recently revised protocol (Knecht and Harland, 1997).
Fluorescent-color POD reaction

Samples are equilibrated in POD reaction buffer (PBS, 0.1 M Imidazole pH 7.6, and 0.001% H2O2) for an hour and reacted with either tyramide-fluorescein or tyramide-rhodamine (TSA-Direct GreenFISH and RedFISH, NEN Lifesciences) for 30 to 60 minutes. Other tyramide-conjugated substrates are available commercially as kits or may be synthesized in the laboratory (Hopman et al., 1998; Jacobs et al., 1999). Fluorescence signal was increased with one to three rounds of POD-mediated deposition with fresh tyramide-fluorophore in buffer. After deposition of insoluble substrate, the embryos were washed extensively for up to 2 days in PBS to remove unreacted soluble tyramide-fluorophore revealing the expression pattern. A second fluorophore may be used by incubating the embryos for 45 minutes with 1% H2O2 in PBS to deactivate the first POD, followed by repeating the above protocol with an appropriate POD-conjugated fab fragment against the epitope carried by the second RNA probe. Prior to confocal sectioning, preliminary assessments of the outcome of the in situ reaction were made in whole embryos in PBS using an epifluorescence equipped stereoscope (Olympus, SZH10).

‘Half’-mount preparation for optical sectioning

Embryos from fixative or 100% methanol were ‘softened’ by incubating for 20 minutes in PBS and 0.01% Tween-20 (embryos processed for RNA in situ do not require softening) and then bisected transversely with a scalpel under a standard stereoscope. Tailbud-stage embryos could be cut into several pieces. These pieces were then dehydrated in 100% methanol and placed in disposable chambers constructed by stacking 10 to 15 clear plastic paper reinforcement rings (Avery cat. no. 05722) to form a well on a no. 1 plate and the prospective epidermis in sectional view. At late neural groove stages (stage 17), the neural folds rise above the neural groove part of the neural plate in contact with the notochord, forming a groove (Fig. 1B). These folds come into close apposition over the neural groove (stage 18; Fig. 1C) and fuse, enclosing a very small ventral neural lumen (stage 19; Fig. 1D). In some cases the entire lumen disappears after fold fusion (data not shown). Over the next several hours, the

dissolves the adhesive. For future confocal sessions, embryos can be retrieved immediately from these chambers, washed and stored in 100% methanol. Samples were optically sectioned immediately after mounting using a confocal scanning laser system attached to an inverted compound microscope (20x, 0.70 n.a. objective, Olympus or Nikon) at the W. M. Keck Center for Cellular Imaging (Biology Department, University of Virginia).

Probes

\( n\)-tubulin encodes a class II \( \beta \)-tubulin that is expressed in prospective motor neurons and prospective dorsal sensory neurons, including Rohon-Beard cells during midgastrulation in two to three mediolateral stripes (Chitnis et al., 1995). \( Xpax3 \) encodes a transcription factor that is expressed in prospective neural crest, dorsal neural tube, somitic and lateral plate mesoderm in chick, mouse and frog (see figure 2D in Bang et al., 1997; Goulding et al., 1991, 1993). \( xash3 \) encodes a transcription factor that is expressed in cells marking the prospective sulcus limitans (Ferreiro et al., 1994; Turner and Weintraub, 1994; Zimmerman et al., 1993). \( xk87 \) encodes a cytokeratin that is expressed in prospective epidermal cells (Fouquet et al., 1988; Jamrich et al., 1987; Jonas et al., 1985).

RESULTS

Cell morphology and arrangement during fusion of the neural folds over the neural groove

Shortly after the completion of gastrulation (stage 13), the neural plate in the prospective trunk region consists of two layers of cells (Fig. 1A). At this stage, there is no clear morphological boundary at between the prospective neural plate and the prospective epidermis in sectional view. At late neural groove stages (stage 17), the neural folds rise above the notoplate (see Jacobson and Gordon, 1976), that part of the neural plate in contact with the notochord, forming a groove (Fig. 1B). These folds come into close apposition over the neural groove (stage 18; Fig. 1C) and fuse, enclosing a very small ventral neural lumen (stage 19; Fig. 1D). In some cases the entire lumen disappears after fold fusion (data not shown). Over the next several hours, the

![Fig. 1.](image-url) Transverse confocal sections at the level of the trunk from late gastrula to early tailbud. Embryos labeled at the 1-cell stage with 10 kDa lysinated rhodamine dextran amine and fixed at various stages clearly show tissue morphology. (A) Late gastrula (stage 13) showing the three germ layers (en, endoderm; no, notochord; so, prospective somites; ne, neural ectoderm). (B) Late neural groove stage (stage 17) shows the medial groove has formed and the neural folds are rising. (C) Neural fold apposition (stage 18) shows the lips of the neural fold (arrows) nearly in contact. (D) Shortly after fusion of the neural folds, a slight groove remains in the ectoderm (arrow). Cells above the small incipient lumen (asterisk) are in disarray. Cells in the floorplate region have begun to radially intercalate. (E) The lumen is re-opened as radial intercalation proceeds from the ventral floorplate into more intermediate regions of the neural tube (stage 20/21). (F) Radial intercalation produces a single-cell-layered neural tube by the time the dorsal fin begins to form (stage 24/25). The deep layer of the dorsal epidermis has been re-established over the neural tube (arrowheads).
lumen is reconstructed from this rudiment, progressing from ventral to dorsal as radial intercalation (i.e. deep cells extending to the lumenal surface and superficial cells extending to the basal surface of the neural tube) brings the two layers of the early neural plate (Fig. 1E) into a single-layered neural tube (Fig. 1F).

**Cells participating in neural fold fusion over the neural groove are prospective epidermis**

Fusion of the neural folds over the neural groove creates a jumble of cells that can now be identified. Expression of xk81, an epidermal cytokeratin, shows that the cells at the fusing lips of the neural folds are epidermal (Fig. 2A) and that xk81 expression is limited to the outermost, single cell layer over the open neural plate and early neural tube. The deep layer of the epidermis is restored over the neural tube only later (see arrowheads in Fig. 1F). There are occasional, single-cell gaps in the expression of xk81 in the epidermis (data not shown) that may mark prospective epidermal ciliary cells (Chen and Grunz, 1997).

**Neural fold fusion occurs while the future dorsal cells of the neural tube are far lateral**

*xslug*, *xpax3*, *n-tubulin* and *xash3* gene expression patterns (Fig. 2C,E,G,I) were used to identify other dorsal cell types at neural fold fusion. Before fold fusion, genes such as *xslug* and *xpax3* are expressed far lateral to the site of fusion (Fig. 2C,E) but later are expressed in the dorsal aspect of the neural tube (Fig. 2D,F). The same is true of the dorsal limit of *n-tubulin* expression (Fig. 2G,H). In contrast, a mediolateral marker of the neural plate, *xash3*, is found much closer to its eventual position in the neural tube (Fig. 2I,J). Immediately at apposition or just after neural fold fusion, in each of these cases, the cells over the neural tube lumen do not express genes normally associated with the dorsal neural tube. Later, however, after the neural tube has formed a complete lumen, the dorsal aspect of the neural tube expresses its definitive pattern of gene expression. Thus, either gene expression is shifting from lateral to more dorsal cells or the cells themselves are moving.

**Medial shift in gene expression is due to medial migration of cells**

Time-lapse videorecordings of scattered, fluorescently labeled deep cells at the margin of the neural plate in the whole embryo revealed medially convergent movements of large groups of individual cells (Fig. 3A) just below the epidermis. The embryo

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**Fig. 2. Dorsal gene expression before and after neural fold fusion.** Transverse confocal sections in the trunk region (50-150 μm posterior of the hindbrain) of fluorescent RNA in situ showing gene expression in the forming neural tube. (A) Epidermal marker xk81 shows the cells at the contacting lips of the neural folds are epidermal. (B) xk81 expression over the neural tube as the lumen reforms. (C) Neural crest marker xslug shows the neural crest is far lateral as the neural folds come into apposition. (D) xslug expression is found medially in the dorsal neural tube after the tube has formed. (E) A dorsal neural tube marker, xpax3, is also found laterally as the folds come into apposition. (F) After the neural tube forms xpax3 is found in the dorsal aspect of the tube. (G) A marker for prospective neurons, n-tubulin, is expressed in two domains, a medial one marking prospective primary motorneurons at the borders of the floorplate and lateral one marking the dorsal primary sensory neurons. Again the prospective dorsal gene expression pattern is found far lateral to the site of apposition. (H) n-tubulin is expressed in a broad domain after the neural tube had formed. (I) A marker for an intermediate position in the neural tube, xash3 is found much closer to the midline. (J) xash3 is expressed in a narrow stripe midway between the floorplate and the roofplate in the single-cell-layered neural tube.
neural tube, a domain of protrusions in a bipolar manner directed mediolaterally (Fig. 4A). Cells within this domain exhibit monopolar protrusions directed toward the midline (Fig. 4B) as they migrate at the basal surface of the epidermis and more ventral neural tube cells. At the completion of neural fold fusion, these cells are isodiametrical (data not shown) but soon become mediolaterally elongated (i.e. transverse to the axis of the embryo). Once they approach the midline, they return to a more isodiametric shape (0:30 panel of Fig. 4A). These neural crest cells remain quiescent in this medial location until they start to emigrate from the tube (data not shown). Occasionally, neural crest cells overshoot their midline target and remain on the other side (Fig. 4C).

Medial migration of neural crest cells takes place by monopolar, mediolaterally directed protrusive activity
The thin epidermis allowed us to record the medial movements of several cells within the xslug-expressing neural crest domain (Fig. 4A). Cells within this domain exhibit monopolar protrusions directed toward the midline (Fig. 4B) as they migrate at the basal surface of the epidermis and more ventral neural tube cells. At the completion of neural fold fusion, these cells are isodiametrical (data not shown) but soon become mediolaterally elongated (i.e. transverse to the axis of the embryo). Once they approach the midline, they return to a more isodiametric shape (0:30 panel of Fig. 4A). These neural crest cells remain quiescent in this medial location until they start to emigrate from the tube (data not shown). Occasionally, neural crest cells overshoot their midline target and remain on the other side (Fig. 4C).

Medial migration and radial intercalation of dorsal neural cells takes place by bipolar, mediolaterally directed protrusive activity
In this period of medial migration and convergent extension, more medial (i.e. ventral) cells of the neural tube show bipolar protrusive activity. Prospective dorsal neurons (expressing n-tubulin) immediately beneath the neural crest, are also mediolaterally elongated even though their lateral face is bound to the outer surface of the neural tube (Fig. 5A). These two cells extend protrusions in a bipolar manner directed mediolaterally (Fig. 5B). These tracked cells represent the dorsalmost population of the neural tube, a domain of n-tubulin-expressing cells (Fig. 5C), with their lateral end on the outer surface of the neural tube and their medial end protruding beneath neural crest cells (Fig. 5D).

Relumenation involves simultaneous medial migration, radial intercalation and reconstruction of the lumen from ventral to dorsal
The medial movement of deep dorsal cells is paralleled by the radial intercalation of more ventral cells in the neural tube. Before neural fold fusion only the most ventral neural crest cells have interdigitated but have not yet intercalated radially between the more superficial cells surrounding the neural groove (Fig. 6A). Radial intercalation begins first among cells in the ventral tube (Fig. 6B) and progresses to more dorsal cells after fusion (Fig. 6C). This progression of radial intercalation coincides with the ventral-to-dorsal reconstruction of the neural lumen. The ventral floorplate is the first stable portion of the small round lumen immediately after the neural folds fuse (Fig. 6D). The lumen becomes flask shaped with new deep cells intercalating into the wall of the neural tube extending the dorsal aspect of the lumen (Fig. 6E). Finally, the dorsalmost surface of the lumen is completed and the single-cell-layered pseudostratified neural tube is formed (Fig. 6F).

Medial migration results in convergent extension of the dorsal neural tube
Time-lapse recordings of medial migration of dorsal cells of the neural tube and transverse confocal sections over the course of neural tube formation reveal that medial migration of dorsal neural tube cells contribute to the convergent extension of the dorsal neural tube. Convergent extension movements have previously been characterized in the neural plate by an anteroposterior dispersal of cells as local protrusive activity brings the cells medially (Elul et al., 1997; Keller et al., 1992a). These same features are seen in the time-lapse video(Fig. 3A) as an initially solidly labeled group of cells break up (unlabeled cells intercalate between labeled cells) as the group nears the midline. The results of convergent extension are also revealed in the decreasing number of cells in the neural tube visible in transverse section over the course of neural fold fusion and relumenation (compare the neural anlagen in Figs 1B,F, 6D,F).

Fig. 3. Neural crest cells migrate medially and do so as individuals. (A) A series of frames selected at 20 minute intervals from a low-light time-lapse videorecording begun during neural fold fusion. A patch of rhodamine-dextran-labeled cells is seen at the left of the frame (dark cells) against an otherwise unlabeled embryo (light). A dotted line in the :00 frame marks the midline. The solid line in the 1:20 frame marks the confocal section shown in B-D. An arrow in the 1:20 frame marks an individual cell that has migrated away from its labeled neighbors. (B) A confocal section transverse to the axis in the rhodamine channel at the line marked in the 1:20 frame of A. The labeled cells are shown in red and an outline of the tissue boundaries of the early neural tube, somites and notochord are shown in blue. (C) xslug expression in the same confocal section is shown in the fluorescein channel. (D) Dual image showing xslug (green) is expressed by the most medial group of cells whose edges were recorded in the low-light videorecording (red). Scale bar in (A) is 50 µm.
Analysis of neural tube closure, or its failure (Copp, 1994; Copp et al., 1990), has been hindered by poor definition of the cellular motility involved in this process. Here we use confocal microscopy, a newly developed whole-mount fluorescent in situ RNA hybridization method and correlated time-lapse recording of cell behaviors to characterize cell motility involved in neural tube closure in *Xenopus laevis*.

Formation of the dorsal neural tube involves previously unexpected types of cell motility: medial migration, polarized, directed protrusive activity, cell intercalation and convergent extension

Whole-mount RNA in situ showed prospective neural crest lies far lateral to the point of fusion of the neural folds and time-lapse recordings revealed the behaviors used by these cells to move to their definitive positions in the neural tube (Fig. 7). Immediately after fusion of the neural folds, the dorsal neural tube consists of mesenchymal cells derived largely from the deep cell layer of the neural plate. Then, the neural crest, arising from the lateral deep cell layer of the two-layered neural plate, begins migrating medially, using monopolar, medially directed protrusive activity. As the crest and other lateral cells migrate medially they intercalate between more medial cells, predominately along the anteroposterior axis, and thus elongate and narrow the future dorsal aspect of the neural tube. This pattern is reminiscent of the mediolateral cell intercalation seen earlier in the neural plate (Fig. 7A; Elul et al., 1997; T. M. Elul and R. E. K., unpublished data) and in the mesoderm (Keller and Winklbauer, 1992), which in each case produces convergent extension. At the outset of medial migration, the entire population of cells involved consist of a multilayered mesenchyme without a lumen. Through the process of relumenation, involving medial migration and radial intercalation (Fig. 7C; see below for discussion) the dorsal neural tube forms a single-cell-layered, pseudostratified epithelium surrounding a lumen (Fig. 7D).

Monopolar medially directed protrusive activity of neural crest cells drives convergent extension of the dorsal neural tube and formation of the roof plate

Without medial migration and narrowing in the mediolateral direction, the very wide anlagen at the time of neural fold fusion could not form a properly shaped neural tube. The monopolar, medially directed protrusive activity seen in the neural crest cells is consistent with the directed movement of these cells toward the dorsal midline and, indeed, such activity is always correlated with convergence movements. Thus, this...
specific type of directed motility is likely responsible for shaping the dorsal neural tube.

A medially directed, monopolar protrusive activity strongly resembling that seen here, is expressed earlier by deep cells in the open neural plate and is thought to underlie the mediolateral intercalation movements of cells that produce convergent extension (T. M. Elul and R. E. K., unpublished data). Whether the activity seen in the dorsal neural tube is a continuation of this earlier activity or a new and independently regulated behavior is not known. Although similar in appearance, the two behaviors may have different mechanisms of directionality; the earlier activity is directed toward the midline of the open neural plate, i.e. the notoplate, while the protrusive activity in the dorsal neural tube is directed toward the future roofplate. However, even that notion of directionality is not entirely accurate as we occasionally observe neural crest cells overshooting and crossing the midline. Such behavior is not consistent with a dorsal midline cue that attracts or directs protrusive activity medially. However, the few cells seen crossing the midline might represent the earliest stages of neural crest cell emigration from the neural tube, which is known to involve crossing the midline (Krotoski et al., 1988).

The potential cues for the monopolar, directed migration could come from the overlying epidermis, from the cells initially at the dorsal midline of the neural anlagen, or from cells still lateral to the neural tube. In the first case, one would envision the cue being a directional one on the undersurface of the single-cell-layered epidermis in this region. In the second case, one could envision a signal emanating, probably diffusing, from cells initially at the midline, or perhaps even from the neural cells last in contact with the epidermis. In the last case, one could envision an inhibitory signal, rather than a protrusion-stimulating signal, from tissues lying off the lateral margin of the neural plate, including the deep layer of the epidermis, or the lateral plate mesoderm, or at later stages, the somitic mesoderm.

Relumenation involves medial migration, and progressive radial interdigitation and radial intercalation

Surprisingly, immediately after the epidermis of the neural folds meet and fuse, the bulk of the neural anlagen consists of a large population of deep mesenchymal cells that do not immediately form a lumen. It is only after neural fold fusion that a lumen reforms. Relumenation involves two general forms of motility. First, the medially directed monopolar motility described above reforms the roofplate. Second, radial interdigitation and subsequent radial intercalation of the two cell layers of the neural anlagen occur during relumenation of the ventral aspect of the neural tube and result in formation of a single-layered tube.

Interestingly, this process of radial intercalation, as well as the radial interdigitation that precedes it, begins ventrally at the midline, and progresses dorsally. Whether it is coincidence that radial intercalation begins ventrally where the lumen of the neural anlagen persists or whether the lumen cues the process of radial intercalation is not known. It appears that radial intercalation occurs at the same time as relumenation. How and why these processes are linked is the subject of further investigation.
The progression of radial intercalation behavior from the ventral midline towards the dorsal was somewhat of a surprise and has implications for patterning. The directed cell protrusive activity driving convergent extension of the neural plate begins anteriorly and laterally near the lateral border of the hindbrain, and proceeds medially (future ventral) and posteriorly (Elul and R. E. K., unpublished data). This is, of course, the reverse of the progression seen in radial intercalation. If the progression of these behaviors reflects when the participating cells received the signals to execute the movements, then the earlier mediolateral cell intercalation behaviors are organized by signals emanating from lateral and anterior and progressing medially (ventrally with regard to the future tube) and posteriorly. In contrast, it could be that the radial intercalation described here is organized by a signal emanating from the ventral midline. Alternatively, it could simply be dependent on conditions that mature progressively in the neural anlagen from ventral to dorsal. Previous work by Hartenstein (1989) described a progression of both cell division and radial intercalation from dorsal (lateral) to ventral (medial).

**Bipolar protrusive activity may be involved in radial intercalation of the thick neural anlagen**

Time-lapse recordings of cell behavior in the thick dorsal region below the neural crest in a region undergoing radial intercalation shows bipolar protrusive activity directed both medially and laterally. Such behavior could be the principle form of motility underlying the radial intercalation of multiple layers of deep cells found in this region. Why the monopolar behavior is not used in this region is not known. Bipolar protrusive activity is expressed by deep neural cells as they mediolaterally intercalate during their convergent extension under conditions of planar induction (Elul et al., 1997); however, the bipolar form of intercalation may not be used during convergent extension under both planar and vertical inducing signals (T. M. Elul and R. E. K., unpublished data).

**Relumenation likely involves re-epithelialization**

The reformation of the lumen of the neural tube appears to involve a transition from a deep mesenchymal cell to an epithelial cell type. The dorsal neural anlagen is a jumble of interlaced deep cells after neural fold fusion but as relumenation occurs, the cells establish what appear to be stable surfaces at their luminal and basal faces, forming a single-layered array of cells. The behavioral and morphological evidence presented above suggests that a mesenchymal-to-epithelial transition is a major process in relumenation and thus closure of the *Xenopus* neural tube. We are examining this hypothesis with an ultrastructural and immunocytochemical analysis of junctional complexes during neural tube formation.

**What is the signal for radial intercalation and relumenation?**

We believe that radial intercalation and correlated relumenation depends on neural fold fusion. In previous work, ‘giant explants’ of the neural anlagen converged, extended and formed neural folds and grooves under both planar and vertical neural-inducing signals through the late midgastrula stage, but the neural folds produced by these explants would not fuse and radial intercalation did not occur (Poznanski et al., 1997). Here we show that radial intercalation does not occur until after neural fold fusion. In another study A. Edlund and R. E. K. (unpublished data) show that neural plates held open beyond the time of normal neural fold fusion likewise do not show radial intercalation of deep and superficial cells. These facts argue that there is some signal or condition associated with neural fold fusion that cues or enables radial intercalation. The fact that radial intercalation fails when folds are mechanically held open argues that radial intercalation depends directly on fold fusion and eliminates the possibility that both processes depend on yet a third signal.

**Medial migration, radial intercalation and relumenation appear to underlie the diverse mechanisms driving neurulation among other vertebrates**

Chordates neurulate using a variety of mechanisms. In the amphioxus, *Branchiostoma floridae*, the epidermal cells and neural ectoderm completely separate well before the neural tube forms and epidermal cells begin to crawl over the surface of the neural ectoderm (Holland et al., 1996). As the epidermis moves over the neural ectoderm, the neural plate itself begins to fold. Once the epidermis has fused, the neural plate bends at the midline and brings the edges of the plate into contact and fusion. In this case, the processes of fusion of the epidermis and fusion of the neural ectoderm have been uncoupled from each other. It is unclear whether cells in the neural plate begin directed medial migration of the type seen in *Xenopus*, or whether a mass tissue movement such as...
convergent extension or folding takes place under the already fused epidermis.

In zebrafish, neurulation involves transformation of the neural plate into the neural keel (Papan and Campos-Ortega, 1994). Keel formation appears to proceed in a ‘cell-by-cell’ manner as cells on opposite sides of the neural plate move medially, come into apposition and then move ventrally together, side-by-side to form the neural keel. Two apposing rows of cells lie on either side of the midline, but cells may divide with daughter cells crossing over to the other side of the keel (Kimmel et al., 1994). The keel appears to be a mesenchymal mass of cells, which subsequently cavitates to form the neural lumen. Relumenation, as in Xenopus, appears to start ventrally at the floorplate and proceed dorsally to the roofplate. It is unclear when the epidermis fuses over the top of the neural keel.

Both chick and mouse form their posterior neural tube (secondary neurulation) by cavitation (Costanzo et al., 1982; Schoenwolf, 1984; Schoenwolf and Delongo, 1980). The morphogenetic movements during this secondary neurulation in mouse and chick might bear some resemblance to the sorting out of mesenchymal cells in Xenopus. Moreover, the process of cavitation in these embryos may share mechanisms with the process of relumenation seen in Xenopus. Although, in the case of secondary neurulation, the lumen is reconstructed from the roofplate down to the floorplate.

Our work in Xenopus is the first instance in which the cell behaviors underlying neural tube closure have been described in detail. It is likely that, when broken down into their constituent cell behaviors, these other diverse forms of neurulation may begin to resemble neural tube closure in Xenopus. The separation and fusion of the epidermis in amphioxus might resemble the separation and fusion of the neural folds over the neural groove in Xenopus. The mesenchymal mass of neural keel cells in zebrafish may resemble the disorganized mass of mesenchymal cells in the dorsal aspect of the neural tube in Xenopus. The reformation in Xenopus from the ventral floorplate to the dorsal roofplate might also resemble cavitation in the zebrafish neural tube as well as cavitation in secondary neurulation in mouse and chick. Just as the various forms of neurulation are driven by similar cell behaviors, the cell behaviors themselves may be driven by similar molecular pathways. The identification of these molecular components and their relationship among the vertebrates is only just beginning.

**Fluorescent detection of gene expression and improvements in protein localization**

Further elaboration of these molecular components and their roles in driving neurulation require both sophisticated histology and fluorescence-based imaging techniques that allow characterization of cell behaviors, tissue movements and protein localization. Our fluorescent in situ RNA hybridization methods have allowed us to visualize epidermal, neural crest and dorsal neural tube gene expression patterns set in the context of cell and tissue shapes. However, in situ merely reflect gene expression and say little about the localization and function of their encoded proteins. The next steps that we take will involve expressing constructs encoding fluorescent chimeras of candidate molecules to dynamically image these molecules and explore their role in neurulation.

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