

Comparative Analysis of Neurulation: First Impressions Do Not Count

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SUMMARY

The central nervous system of vertebrate embryos originates from the neural tube (NT), a simple epithelium surrounding a central lumen. The mechanisms underlying the shaping of the NT, a process otherwise known as neurulation, have been the focus of numerous studies, using a variety of model systems. Yet, it remains unclear to what extent neurulation is conserved across vertebrates. This review provides a comparison between modes of neurulation, with a focus on cellular mechanisms. An emerging concept is that cell behaviors reveal similarities between modes of neurulation that cannot be predicted from morphological comparisons.

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INTRODUCTION

Many organs are shaped as hollow tubes, including the lung, kidney, and vascular system. Despite their common basic architecture, these biological tubes are shaped by a variety of morphogenetic mechanisms (Hogan and Kolodziej, 2002; Lubarsky and Krasnow, 2003; Nelson, 2003). The neural tube (NT), the precursor of the brain and spinal chord, is no exception. In amniotes, the NT is formed by a process known as primary neurulation in the head and trunk regions and by secondary neurulation in the posterior region of the embryo. Moreover, modes of neurulation appear to vary across vertebrate species, although, as discussed in this review, cellular comparisons reveal conserved mechanisms.

While one might argue that “a tube is a tube,” regardless of the manner in which it is formed, the process matters

perhaps even more than the end result to those interested in evolutionary mechanisms and the etiology of NT birth defects (NTDs). The latter are very frequent in human populations, amounting to one in every thousand live births (Bower et al., 1993; Shaw et al., 1994; Lary and Edmonds, 1996). The aim of this review is to compare and contrast mechanisms of neurulation in different model organisms, with a particular emphasis on the cell behaviors that drive these morphogenetic movements. Comparative analyses of this sort not only enable a better understanding of how the pathways regulating neurulation might have evolved, but also demonstrate the use of model organisms such as *Xenopus* and zebrafish to understand the molecular underpinnings of human NTDs. Neurulation has been studied most extensively in mouse (*Mus musculus*), chick (*Gallus gallus*), frog (*Xenopus laevis*), and more recently, zebrafish (*Danio rerio*). However, it is important to keep in mind that

these model systems may not be representative of the families to which they belong and caution should be used when making/assessing generalizations based on these organisms. Light and scanning electron microscopy combined with the use of molecular techniques for imaging gene and protein expression in chick and mouse embryos have provided a basic understanding of mechanisms underlying primary and secondary neurulation. Real-time imaging techniques in *Xenopus* and zebrafish embryo have more recently added a temporal dimension to the study of neurulation, by documenting the cellular dynamics that take place during this process. The next challenge is to determine the extent of conservation of mechanisms of neurulation. We propose here that this can be best accomplished by focusing on cellular behaviors rather than on morphology.

PRIMARY VERSUS SECONDARY NEURULATION IN AMNIOTES

Defining Primary Versus Secondary Neurulation

Since the main emphasis of this review is on comparisons of modes of neurulation across different vertebrate model organisms, only the broad thematic of primary and secondary neurulation will be provided here. A more detailed discussion of the cellular events of neurulation can be obtained in several excellent reviews (Colas and Schoenwolf, 2001; Lowery and Sive, 2004; Wallingford, 2005; Clarke, 2009). At a morphological level, primary neurulation

can be described as the bending and folding of the neural plate, a flat and thickened epithelial layer on the dorsal surface of the embryo, to form a hollowed NT. In amniotes, this process is brought about by the elevation of the lateral regions of the neural plate, the neural folds, the narrowing of the neural plate, the bending of the neural plate to form a neural groove, and the medial movement of the neural folds toward the midline, where they meet and fuse to seal the NT (Fig. 1A; Colas and Schoenwolf, 2001). Several studies suggest that the nonneural ectoderm adjacent to the neural plate (the prospective epidermis) generates a pushing force that aids in the medial movement of the neural folds and the closure of the NT. Thus, primary neurulation is thought to be driven by a combination of internal forces (within the neural plate) that shape the neural plate and groove (discussed in further detail below) and external forces, originating in the nonneural ectoderm (Smith and Schoenwolf, 1997; Colas and Schoenwolf, 2001).

In contrast to this mode of neurulation, secondary neurulation is mediated by the condensation of a cluster of mesenchymal cells in the tailbud region, below the surface ectoderm, to form a cord-like structure. The NT is shaped by the formation of a central lumen inside the initially compact cord, a process known as cavitation (Fig. 1B; Schoenwolf, 1979; Schoenwolf and Delongo, 1980; Nakao and Ishizawa, 1984; Griffith et al., 1992; Lowery and Sive, 2004).

Thus, at a morphological level, primary and secondary neurulation can be distinguished based on several features.

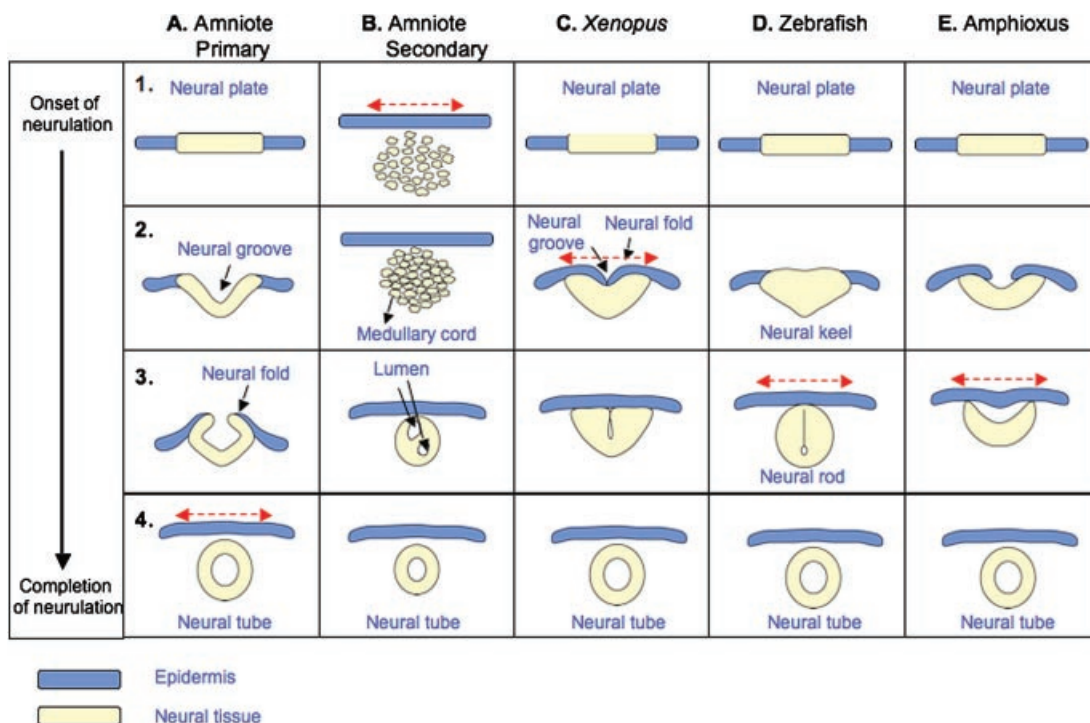


Figure 1. Variations of neurulation at a morphological level. Primary neurulation (A) and secondary neurulation (B) in amniotes, neurulation in *Xenopus* (C), zebrafish (D), and amphioxus (E). Blue represents the epidermis, and yellow, the neural tissue. Red double arrowheads indicate epidermal fusion. Numbers 1–4 indicate different stages of neurulation.

A recent review by Lowery and Sive (2004) suggested that one of the defining characteristics of primary neurulation is the presence or absence of an epithelial neural plate (Lowery and Sive, 2004). Another difference between the modes of primary and secondary neurulation is the relation between the neural and the nonneural ectoderm. Fusion of the nonneural ectoderm (epidermis) at the dorsal midline and NT closure is coupled during primary neurulation, but the two tissues are independent of one another during secondary neurulation (Colas and Schoenwolf, 2001). A third important difference between primary and secondary neurulation is the cytoarchitecture of the cells.

Cells Undergoing Primary and Secondary Neurulation Have Distinct Cytoarchitectures

At a cellular level, an important difference between primary and secondary neurulation is the epithelial versus mesenchymal nature of cells. It is therefore worthwhile to clearly define this terminology. Epithelial cells form layers that are held together by junctional complexes such as tight junctions, adherens junctions, desmosomes, and gap junctions. In addition, epithelial cells exhibit apico-basal polarity, which is manifested by the localized distribution of adhesion molecules, the apical position of the centrosome/basal body, the organization of the microtubule and actin cytoskeleton, the transport of cellular components and solutes across the epithelium, and the presence of basal lamina at the basal surface. Mesenchymal cells generally do not form an organized layer nor do they have apico-basal polarity. Rather, these cells are typically migratory and exhibit a front-to-back polarity. They contact neighboring mesenchymal cells only transiently and are not usually associated with a basal lamina (Hay, 2005; Lee et al., 2006; Thiery and Sleeman, 2006). However, some mesenchymal cells do not fit these definitions, such as the presomitic mesoderm in vertebrates, which forms organized layers and is surrounded by extracellular matrix (ECM) (Ostrovsky et al., 1983; Lash et al., 1987; Rhee et al., 2003; Yin et al., 2008). Knowledge of a tissue's cytoarchitecture is key to understanding morphogenesis, as the degree of epithelialization directly impacts cell behavior. For example, epithelial cells tend to be stationary, as they are held together tightly by a number of junctional complexes, whereas mesenchymal cells, which lack rigid cell–cell contacts, are often migratory.

Cells that undergo primary neurulation exhibit epithelial characteristics throughout the duration of NT formation (Fig. 2A). The neural plate is a monolayer of pseudostratified cells whose apical poles face the amniotic cavity and basal surfaces are in contact with a basal lamina. As expected, neural plate cells express the apical tight junction markers occludin and ZO-1 (Aaku-Saraste et al., 1996). Despite these epithelial characteristics, a recent study of the chick neural plate has revealed that these neural cells are surprisingly migratory when grown in cell culture, suggesting the existence of *in vivo* regulatory mechanisms to restrict cell motility (Duband et al., 2009). Upon NT closure, apical markers are retained (Fig. 2A), although occludin is down-regulated and replaced by higher levels of ZO-1 and the adherens junction marker N-cadherin (N-Cad; Aaku-Sar-

aste et al., 1996). This shift in junctional marker expression is thought to reflect a decrease in the epithelial nature of neural progenitor cells as they begin to differentiate into neurons (Aaku-Saraste et al., 1996, 1997).

In contrast to the neural plate, cells in the posterior (tailbud) region appear mesenchymal but rapidly transition into an epithelial configuration as cavitation begins (Fig. 2B). These observations are primarily based on light and scanning electron microscopy studies (Schoenwolf, 1979; Schoenwolf and Delongo, 1980; Catala et al., 1995; Yang et al., 2003). However, analysis of ECM protein expression in mice provides further evidence that cells in the tailbud are initially unpolarized. Indeed, fibronectin and heparan sulfate proteoglycan fully surround cells in the loose mesenchyme. These proteins are gradually lost from the middle of the aggregate following coalescence and re-expressed as components of the basement membrane in regions surrounding the secondary NT (O'Shea, 1987), consistent with the establishment of apico-basal polarity (Fig. 2B). It has been proposed that the deposition of ECM proteins at the basal surface of cells may play a role in the reorganization of the cytoskeleton as the cells elongate and polarize (O'Shea, 1987). Analysis of junctional complexes using electron microscopy and freeze fracture also suggests a gradual establishment of apico-basal polarity, as apical junctional complexes (either gap junctions or tight junctions) are observed in mice and chick embryos following cavitation (Schoenwolf and Delongo, 1980; Schoenwolf and Kelley, 1980; Schoenwolf, 1984).

Cell Behaviors During Primary and Secondary Neurulation

Prior to the onset of primary neurulation, the neural plate narrows, a process that involves cell elongation and oriented cell division (Colas and Schoenwolf, 2001) and is regulated in part by the noncanonical Wnt-signaling pathway (also known as the planar cell polarity/PCP pathway; Ueno and Greene, 2003; Zohn et al., 2003; Copp, 2005; Doudney and Stanier, 2005; Wang and Nathans, 2007). Following narrowing, the neural plate bends into a tube, while maintaining rigid cell–cell contacts. Bending is accomplished by furrowing (the formation of hinge points; a single median hinge point above the prechordal plate and notochord; and paired dorso-lateral hinge points present principally at future brain levels) and folding (the rotation of the neural plate around hinge points; Fig. 3A,A.1; Shum and Copp, 1996; Smith and Schoenwolf, 1997; Colas and Schoenwolf, 2001; Lowery and Sive, 2004; Wallingford, 2005). The rostrocaudal level at which bending is initiated varies amongst species. In the chick embryo, bending is initiated and completed first in the midbrain region (Colas and Schoenwolf, 2001). The mechanisms underlying hinge point formation are not fully understood, but apical constriction, which changes the cell shape from columnar to wedged, is likely to play a major role (compare Fig. 3A.1 and A.2). Apical constriction is thought to involve a contractile ring at the apical pole of neuroepithelial cells, although other models have also been proposed (Wallingford, 2005). Genes implicated in hinge point formation include *p190RhoGAP* and *shroom*, which encode a

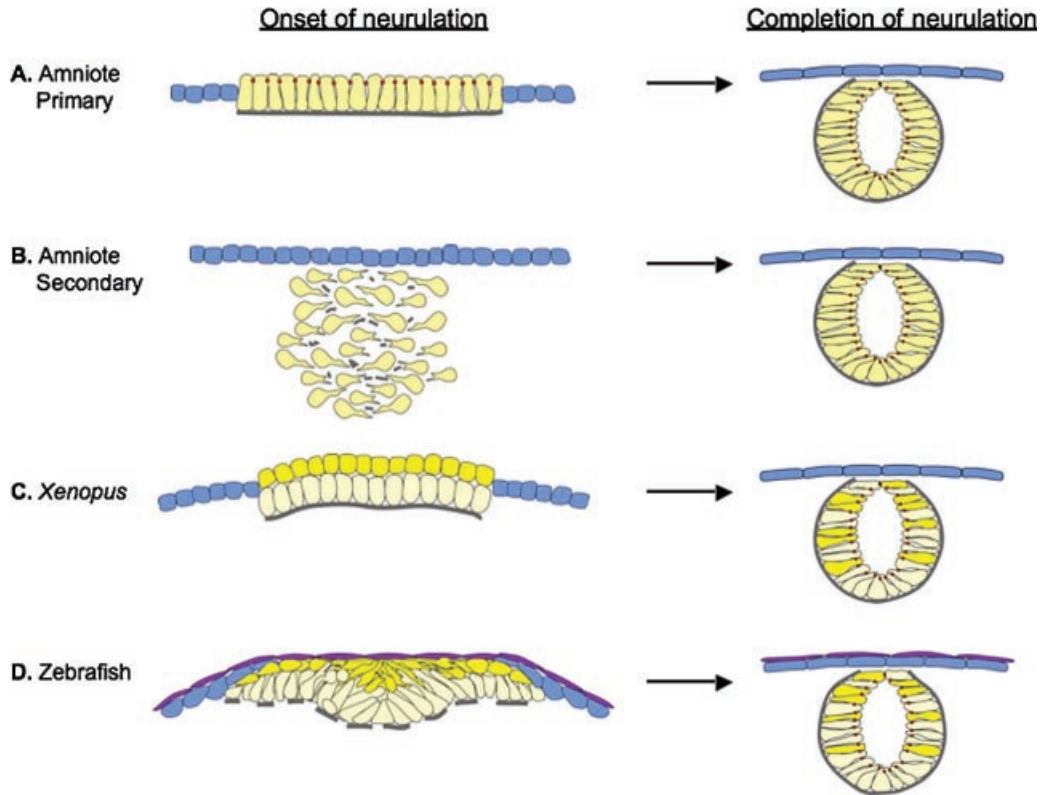


Figure 2. Variations in the organization of neural cells at the onset and completion of neurulation. Organization of cells at the onset (**left**) and completion (**right**) of neurulation in amniotes (**A,B**), *Xenopus* (**C**) and zebrafish (**D**). (**A**) Primary neurulation (anterior regions) and (**B**) secondary neurulation (posterior regions). Yellow indicates neural cells. Dark yellow cells represent superficial cells and light yellow cells are deep cells in (**C,D**). Blue cells are epidermis and purple cells in (**D**) represent the enveloping layer (EVL) in zebrafish. Gray lines show the basal lamina. Gray dotted line in (**D**) indicates the possible presence of the basal lamina in zebrafish. Red circles show junctional complexes.

negative regulator of Rho GTPase and an actin-binding protein, respectively (Brouns et al., 2000; Martin, 2004). Interestingly, loss of p190RhoGAP (Brouns et al., 2000) and Shroom (Hildebrand and Soriano, 1999; Hildebrand, 2005) function prevents proper bending of the neural plate and results in exencephaly (defective cranial neurulation) in mice. The narrowing of the neural plate prior to the onset of neurulation and the formation of hinge points provide internal forces that, in combination with the aforementioned external forces from the nonneural ectoderm, drive NT closure. It is thought that closure itself involves adhesion at points of contact and epithelial breakdown and fusion, resulting in the formation of two separate epithelial layers (epidermis and neuroepithelium), with mesenchymal neural crest cells in between (Colas and Schoenwolf, 2001).

At the time of anterior (cranial) neural closure, there is regulated proliferation along the dorso-ventral axis of the NT, with more proliferation in the dorsal versus the ventral half (Copp et al., 2003). Following NT closure, proliferation becomes more uniform, however, a gradient of cell differentiation is now observed, with a greater number of differentiated cells in the ventral NT. Disruption of the proliferation/differentiation balance in mouse mutants in which cell division is either up- or downregulated causes exencephaly

(Ishibashi et al., 1995; Sah et al., 1995; Gowen et al., 1996; Zhong et al., 2000; Kim et al., 2007; Lee et al., 2007). While the underlying cause for these NT defects is not understood, it has been speculated that the premature differentiation of the neuroepithelium in some mutants might render the neural plate mechanically inflexible and prevent dorsolateral bending or inhibit the adhesion process that is necessary for neural fold fusion (Copp et al., 2003).

In contrast to the well-documented morphogenetic movements that take place during primary neurulation, less is known about the cellular basis of secondary neurulation. What can be inferred from light and electron microscopy studies in avian embryos (Schoenwolf, 1979; Schoenwolf and Delongo, 1980) indicates that the spaces between mesenchymal cells in the tailbud region of the embryo gradually collapse, forming a solid structure, called the medullary cord. Following coalescence, cells in the medullary cord separate into two populations, outer elongated cells that exhibit apico-basal polarity and inner mesenchymal cells (Fig. 3B). Cavitation initially occurs at the boundary between the outer and inner cells and is thought to involve the recruitment of mesenchymal cells into the outer epithelial layer, possibly via cell intercalation (Fig. 3B', B'.1). Although not explicitly discussed in Schoenwolf (1979)

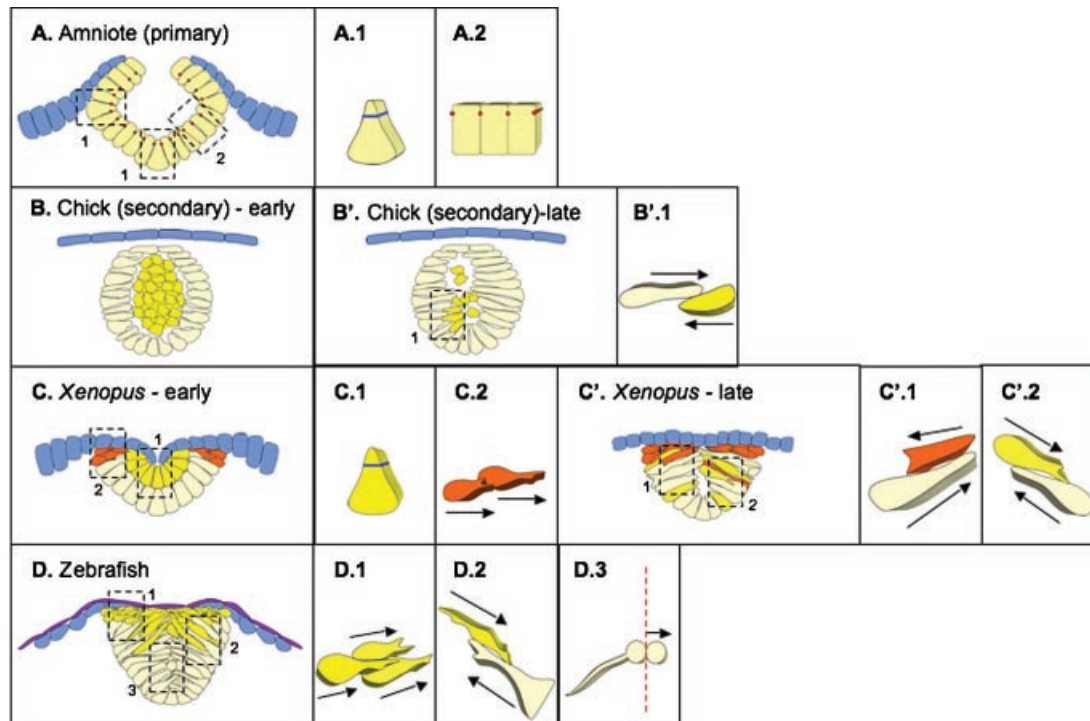


Figure 3. Cell behaviors that drive neurulation. Cell behaviors observed during neurulation in anterior regions (A–A.2) and posterior regions (B–B'.1) in amniotes, in *Xenopus* (C–C'.2), and in zebrafish (D–D.3). Ventral and lateral cells undergo apical constriction to form hinge points (A,A.1) while other cells in the neural tissue remain columnar (A,A.2). In the chick medullary cord, peripheral cells have an elongated epithelial morphology while central cells are mesenchymal (B). During cavitation (B') inner cells are thought to intercalate between outer cells (B'.1). At the onset of neurulation in *Xenopus*, superficial cells undergo apical constriction (C,C.1). Neural crest cells extend protrusions and converge toward the midline (C,C.2). Later, the superficial and deep cells undergo radial intercalation (C' - C'.2). In zebrafish, superficial cells converge medially (D,D.1) and undergo radial intercalation with deep cells (D,D.2). Cells divide apically and one daughter crosses the midline (D,D.3). Yellow represents neural cells; orange, neural crest; and blue, the epidermis. Arrows show the direction of movement. Red dotted line represents the midline. Red dots in (A–A.2) are junctional complexes. Blue line in (A.1,C.1) represents the contractile ring which is thought to enable apical constriction. Boxed regions in A,B',C,C',D are shown in magnified views.

and Schoenwolf and Delongo (1980), another possible mechanism of cavitation is apical membrane biogenesis, whereby internal vacuoles (known as vacuolar apical compartments—VACs) move to regions of cell–cell contact, creating an apical surface and lumen (Lubarsky and Krasnow, 2003). In addition, midline-crossing divisions, as occur in the zebrafish embryo, may facilitate the formation of a lumen (see below; Clarke, 2009). It is noteworthy that necrosis of inner cells is not observed, ruling out cell death as a mechanism of cavitation (Schoenwolf, 1979; Schoenwolf and Kelley, 1980). At a later stage of chick development, a second wave of epithelialization results in the formation of secondary lumina within the central mass. Eventually, primary and secondary lumina merge to form one central cavity. Secondary neurulation in mouse embryos differs from avian embryos in the pattern of recruitment of mesenchymal cells (as inner cells are not observed), however, it does involve cavitation (Schoenwolf, 1984). Collectively, the cellular events that transform loosely aggregated, often migratory mesenchymal cells into an epithelial sheet, are known as mesenchymal-to-epithelial transition (MET) and are likely to apply to secondary neurulation (Lee et al., 2006; Thiery and Sleeman, 2006; Baum et al., 2008).

MECHANISMS OF NEURULATION IN ANAMNIOTES

All amniotes studied thus far, including humans (O'Rahilly and Muller, 1994; Saitsu et al., 2004), appear to undergo primary and secondary neurulation. To be deemed useful for the study of human NTDs, the onus has therefore been on other model organisms such as *Xenopus* and zebrafish to conform to the pre-ascribed terminology of “primary” versus “secondary.” This has fostered a focus on morphological similarities between modes of neurulation in these model organisms, whereas obvious differences have sometimes been brushed aside. However, we argue that morphological similarities may be misleading, as they are poor predictors of underlying cellular behaviors. A closer examination of the latter reveals different and perhaps more relevant commonalities in mechanisms of neurulation across vertebrates. In the sections below neurulation in amniotes is compared with neurulation in anterior regions of *Xenopus* and zebrafish. The cellular basis of posterior NT morphogenesis has not been described for either *Xenopus* or zebrafish (although a manuscript is currently in preparation on this topic for zebrafish, by Harrington and Brewster).

Comparisons at the Morphological Level

Modes of neurulation in different vertebrates have traditionally been compared at the anatomical level, as morphological criteria provide a convenient common denominator. Based on morphology, *Xenopus* embryos are generally thought to undergo primary neurulation, involving the formation of a neural groove from a pre-existing neural plate and the juxtaposition of neural folds at the dorsal midline (Fig. 1C). The mode of neurulation in zebrafish (Fig. 1D) has remained a controversial topic. Even though the NT forms from a neural plate, a characteristic of primary neurulation (Lowery and Sive, 2004), it has also been argued that zebrafish undergo secondary neurulation (Papan and Campos-Ortega, 1994; Kimmel et al., 1995; Geldmacher-Voss et al., 2003; Handrigan, 2003). The controversy stems in part from the absence of a neural groove and neural folds in this organism. Rather, the neural plate transitions into a solid mass, the neural keel (Fig. 1D.2) which upon medial convergence of cells becomes a neural rod (Fig. 1D.3). The latter eventually cavitates to form a NT (Fig. 1D.4).

An often overlooked difference between primary and secondary neurulation is the relation between the neural and nonneural ectoderm. As previously mentioned, during primary neurulation, the neural folds fuse at the dorsal midline, completing NT closure (Fig. 1A.3, A.4). In contrast, at the onset of secondary neurulation, neural cells underlie the epidermis and complete neurulation independently from this tissue (Fig. 1B.1). Closer examination of neurulation in amniotes reveals deviations from these two scenarios. In *Xenopus*, the neural folds were shown to fuse at the midline prior to completion of neurulation (Fig. 1C.2; Davidson and Keller, 1999). The timing of fusion of the neural and non-neural ectoderm in zebrafish has not been investigated in detail and we present here some data shedding light on this topic (Fig. 4). In order to visualize neural and epidermal cells, embryos were immunolabeled at different stages of neurulation with α -Sox3C (a marker for neural progenitor cells) and α -p63 (an epidermal cell marker). Analysis of double-labeled embryos in cross sections revealed that the epidermis remains far lateral to the neural tissue at the neural keel (Fig. 4B) and neural rod (Fig. 4C) stages, suggesting that the formation of the NT occurs independently from epidermal fusion. To further test this hypothesis, we analyzed the expression of Sox3C and p63 in *N-cad* mutants, in which neurulation is blocked, resulting in a T-shaped NT (Lele et al., 2002). In these mutants, we observe that the epidermis seals over the T-shaped NT (Fig. 4E), indicating that epidermal fusion and NT formation are uncoupled. Interestingly, amphioxus (*Branchiostoma floridae*), a cephalochordate, presents an extreme version of the situation in zebrafish, as the neural and nonneural ectoderm appear to fully separate prior to the onset of neurulation and epidermal cells crawl over the surface of the neural plate (Fig. 1E; Holland et al., 1996). Uncoupling of fusion of the epidermis and neural ectoderm in zebrafish, *Xenopus*, and amphioxus has some important implications on cellular mechanisms of neurulation, since the nonneural ectoderm is unlikely to provide an external force driving the medial movement of the edges of the neural plate and closure of the

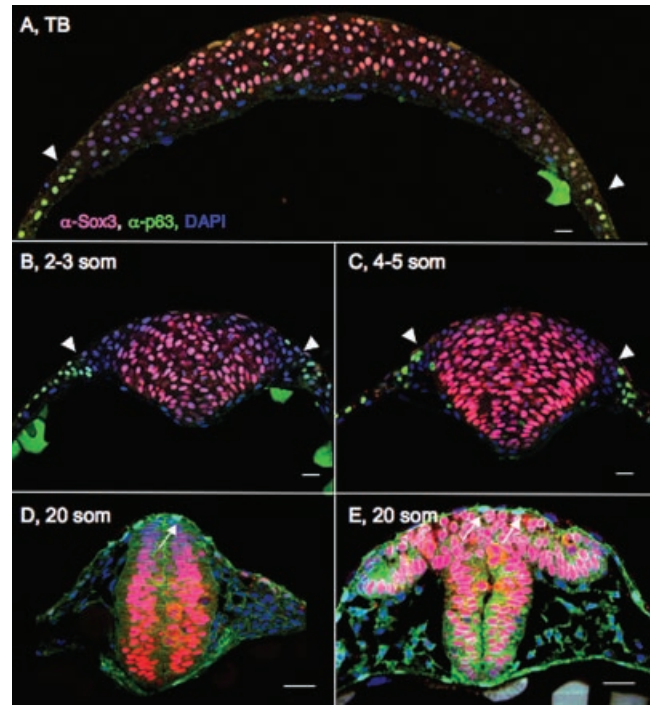


Figure 4. NT closure and epidermal fusion are uncoupled in zebrafish. Transverse sections through the hindbrain of WT (A–D) and *N-cad*^{p79emcf} mutant (E) embryos at the TB (A), 2–3 som (B), 4–5 som (C), and 20 som (D,E) stage. Embryos were labeled with α -Sox3 (pink, 1:1,000 dilution), α -p63 (green, 1:200 dilution; Santa Cruz Biotech, Santa Cruz, CA), and DAPI (blue, Invitrogen, Carlsbad, CA). White arrowheads indicate the medial edge of the epidermis. Arrows point to the nuclei of individual epidermal cells. Scale bar, 20 μ m.

NT, as takes place during primary neurulation. Rather, neuroepithelial cells in these organisms must devise other mechanisms to converge toward the midline (discussed below).

In summary, neurulation in *Xenopus* and zebrafish presents some similarities to primary neurulation at a morphological level, although there are also some clear differences. We argue that, ironically, some of the cell behaviors in both of these organisms more closely resemble the events that are thought to take place during secondary neurulation.

Comparisons at the Cellular Level

The cellular basis of neurulation in *Xenopus* has been investigated in some detail (Schroeder, 1970, 1971; Jacobson and Gordon, 1976; Keller et al., 1992; Elul et al., 1997; Davidson and Keller, 1999; Elul and Keller, 2000; Ezin et al., 2003, 2006). In contrast to amniotes, the neural plate of *Xenopus* starts out as a bilayered structure, composed of deep and superficial cells (Fig. 2C; Jacobson and Gordon, 1976). Prior to the onset of neurulation, the neural plate narrows and elongates via a mechanism known as convergent extension (CE), involving the medial migration of cells (Elul et al., 1997). CE is essential for proper neurulation, as

narrowing of the neural plate brings the lateral neural folds in closer proximity, facilitating the closure of the NT (Wallingford and Harland, 2002). These polarized cell movements, controlled by the PCP pathway, have been the focus of several recent articles and reviews and will not be further discussed here (Keller et al., 2000; Zohn et al., 2003; Copp, 2005; Doudney and Stanier, 2005). Following narrowing of the neural plate, cells in the superficial layer change from cuboidal to wedge-shaped, resulting in the formation of a neural groove (Fig. 3C,C.1; Schroeder, 1971; Haigo et al., 2003). As in mice, this process is mediated by the actin-binding protein Shroom and disruption of Shroom function results in a specific failure of hinge point formation and brain NTDs (Haigo et al., 2003; Martin, 2004; Wallingford, 2005; Lee et al., 2007). Further shaping and closure of the NT involves a number of complex cell movements. Mesenchymal cells in the deep layer intercalate between cells in the superficial layer, a process known as radial intercalation (Fig. 3C'–C'.2) to form a single-layered NT (Davidson and Keller, 1999). In addition, owing to the fact that the fusion of neural folds occurs while the edges of the neural plate are still in a far lateral position (Fig. 1C.2), NT closure in this organism involves the medial migration of lateral (neural crest) cell populations (Fig. 3C.2; Davidson and Keller, 1999). Relumination of the NT is thought to be intimately coupled with the process of cell intercalation and epithelialization (Davidson and Keller, 1999). The end result of neurulation in *Xenopus* is the formation of an epithelial NT that expresses apical junctional markers (Fig. 2C; N. Papalopulu, personal communication).

Neurulation has been studied at a cellular level in zebrafish using a combination of fate mapping tools, time-course sectioning and histology, and real-time imaging (Schmitz et al., 1993; Kimmel et al., 1994; Papan and Campos-Ortega, 1994, 1999; Geldmacher-Voss et al., 2003; Lowery and Sive, 2004; Ciruna et al., 2006; Hong and Brewster, 2006; Tawk et al., 2007; Clarke, 2009). These studies reveal remarkable similarities to the mode of neurulation in *Xenopus*. The zebrafish neural plate is also composed of a layer of deep and superficial cells (Fig. 2D), although this bilayered organization is very transient (Hong and Brewster, 2006). Cells in the deep layer are columnar and remain in contact with the basal lamina throughout the duration of neurulation (Papan and Campos-Ortega, 1994). Single cell labeling has revealed that deep cells appear to change their angular orientation from vertical in the neural plate to horizontal in the neural rod (Papan and Campos-Ortega, 1994; Hong and Brewster, 2006), which is suggestive of an epithelial infolding process. In contrast, cells in the superficial layer are elongated along the medio-lateral axis and migrate individually toward the midline (Fig. 3D.1; Hong and Brewster, 2006). Thus, the mechanisms shaping the zebrafish NT involve both infolding (of deep cells) and medial migration (of superficial cells) and are collectively referred to as neural convergence. Moreover, these events occur concomitantly with radial intercalation, as superficial cells which have converged medially insert themselves between deep cells (Fig. 3D.2), establishing contact with the basal surface of the neuroepithelium and creating a single cell layered neural rod (Fig. 2D). Both convergence and intercalation appear to be

very active processes, involving the generation of polarized membrane protrusions (Hong and Brewster, 2006). A feature apparently unique to zebrafish is the ability of dividing cells to cross the midline at the neural keel and neural rod stage (Fig. 3D.3), a process that involves a 90° rotation of the mitotic spindle (Schmitz et al., 1993; Kimmel et al., 1994; Papan and Campos-Ortega, 1994, 1999; Concha and Adams, 1998; Geldmacher-Voss et al., 2003; Clarke, 2009). These midline-crossing divisions (also known as C Divisions) generate daughters with mirror-image apico-basal polarity and are important for transforming the midline of the neural rod into a lumen (Tawk et al., 2007; Clarke, 2009). Interestingly, and in contrast to what is observed in the mouse, cell division is not required for neurulation per se, as the NT forms properly in absence of cell proliferation (Lowery and Sive, 2005; Ciruna et al., 2006; Tawk et al., 2007; Nyholm et al., 2009). By the neural rod stage, the apico-basal axis is established, as determined by the apical localization of tight junction and adherens junction markers (Geldmacher-Voss et al., 2003; Hong and Brewster, 2006) and protrusive activity ceases (Hong and Brewster, 2006). Continued cavitation (lumen formation) is thought to involve apical membrane biogenesis (Munson et al., 2008), to establish an epithelial seam that divides the left and right halves of the neural rod, lumen inflation, and localized cell proliferation (Lowery and Sive, 2005). While hinge points are not observed during neurulation in the zebrafish, they are involved in shaping the lumen after the neural rod stage (Gutzman et al., 2008; Nyholm et al., 2009) and may be controlled by the same molecular mechanisms that regulate hinge point formation in amniotes (Lowery et al., 2009; Nyholm et al., 2009).

The cell behaviors in both *Xenopus* and zebrafish are consistent with early mesenchymal properties of neuroepithelial cells. These include medially oriented cell migration, the ability of deep and superficial cells to intercalate radially among one another, and the lack of apically localized markers for tight junctions and adherens junctions. Despite these mesenchymal characteristics, neuroepithelial cells also exhibit some epithelial features during neurulation. For example, the neural plate in zebrafish appears to overlie a basement membrane, as seen by light microscopy (Papan and Campos-Ortega, 1994). Moreover, cells in the deep layer have a columnar (elongated) shape and maintain their relative medio-lateral position throughout neurulation (Papan and Campos-Ortega, 1994; Hong and Brewster, 2006). In *Xenopus*, cells in the superficial layer undergo apical constriction (Haigo et al., 2003; Wallingford, 2005), an epithelial cell property. Thus, both early zebrafish and *Xenopus* neuroepithelial cells appear hybrid in terms of their properties. However, as neurulation proceeds, these cells become progressively more epithelial, exhibiting a clearly defined apico-basal axis by the time the neural rod is formed (Fig. 2C,D; Geldmacher-Voss et al., 2003; Hong and Brewster, 2006). The non-epithelial organization of the *Xenopus* and zebrafish neural plate and the lack of a patterned bending and folding mechanism may explain why these organisms are less sensitive to perturbations in cell proliferation (Harris and Hartenstein, 1991; Lowery and Sive, 2005; Ciruna et al., 2006; Tawk et al., 2007; Nyholm

et al., 2009) than are mouse embryos (Ishibashi et al., 1995; Sah et al., 1995; Gowen et al., 1996; Zhong et al., 2000; Copp et al., 2003; Kim et al., 2007).

How similar are these cell behaviors to those observed during primary and secondary neurulation? The answer to this question depends on what criteria are used. Apical constriction of superficial cells in *Xenopus* is akin to hinge point formation during primary neurulation. However, the balance may tilt in favor of secondary neurulation, as this morphogenetic process is thought to involve cell intercalation and a MET. These events are also observed during neurulation in *Xenopus* and zebrafish, although it is unlikely that these organisms undergo a full MET (given that their neural plate cells start out with some epithelial properties). Clearly, if one is to better understand the level of conservation between secondary neurulation and the mode of neurulation in *Xenopus* and zebrafish, there is a need to document cellular dynamics in amniotes using current technologies, including time lapse recording. In addition, neurulation should be studied at the subcellular level in all these organisms, to increase our knowledge of the events that take place during epithelialization.

Interestingly, mechanisms of secondary neurulation/neurulation in *Xenopus* and zebrafish bear some resemblance to modes of tubulogenesis reported in other organs. Lubarsky and Krasnow (2003) propose that there are five basic mechanisms to form biological tubes (Lubarsky and Krasnow, 2003). Of these, the process of “hollowing” appears most similar to secondary neurulation, as it is brought about by the creation of a lumen between cells of a thin cylindrical cord, without cell loss. Examples of hollowing include development of the *Caenorhabditis elegans* gut and the *Drosophila* heart and lumen formation in Madin–Darby canine kidney (MDCK) cultured cells. Hollowing is best understood in the latter and involves trafficking of vesicles carrying apical membranes, their fusion to create pockets of lumen at the apical surface, and merging of these pockets to form a complete lumen (Lubarsky and Krasnow, 2003). A similar process of apical membrane biogenesis is thought to be implicated in lumen formation in the zebrafish (Munson et al., 2008), suggesting a conservation of cellular mechanisms.

PERSPECTIVES

While all amniotes appear to undergo both primary and secondary neurulation, NT formation in other vertebrates such as *Xenopus* and zebrafish does not clearly correspond to either mode. Indeed, at a morphological level it has been argued that these organisms undergo a form of primary neurulation, while at a cellular level, we and others propose that the “early mesenchymal” and “late epithelial” properties of neuroepithelial cells are more similar to the MET that takes place during secondary neurulation. Thus, for these and probably other anamniotes, it makes sense to avoid the “primary versus secondary” terminology and to rather focus on more relevant similarities in cytoarchitectures and cell behavior. The latter are central to understanding the molecular underpinnings of neurulation.

Why Use Different Ways to Make a NT?

Why do vertebrates present such morphological diversity in ways to make the NT? Diversity is observed within single organisms (primary versus secondary neurulation) and across vertebrates. There is no single or clear answer to this question but it lends itself well to rampant speculation. One intriguing possibility is that modes of neurulation closely correlate with methods of reproduction. Both *Xenopus* and zebrafish embryos are produced by external fertilization or egg laying. Embryos of these organisms are exposed to the outside environment from the earliest stages of development, despite the protection of the chorion. In contrast, embryos of amniotes are protected by an amniotic membrane and the lumen of their anterior NT is exposed to amniotic fluid during primary neurulation. Egg layers may therefore have developed alternative strategies to form a NT, minimizing contact between the apical (future lumen) side of the neural plate and the outside environment. *Xenopus* could have achieved this by sealing off neural tissue prematurely (Fig. 1C.2). Zebrafish embryos have an outer protective enveloping layer (EVL) that does not participate in neurulation (Fig. 2D; Sagerstrom et al., 2005). The presence of this outer layer may explain how the non-epithelial characteristics of the zebrafish neural plate and the dynamic cell behaviors observed during neurulation might have evolved, in absence of any constraint to perform a protective role (Clarke, 2009).

If this hypothesis is well founded, one would expect to observe a diversity of modes of neurulation in fish, as there are multiple mechanisms of reproduction and early development. Egg layers (including zebrafish) deposit and fertilize their eggs externally. Live bearers retain eggs within their body and give birth to live, free-swimming young. Mouthbrooders take care of their young by holding them in their mouths for extended periods of time. Focusing on egg layers, a consensus is hard to reach, as published reports for two teleosts, *Oryzias latipes* (medaka) and *Cichlasoma nigrofasciatum*, describe the presence of a neural groove, typically associated with primary neurulation (Miyayama and Fujimoto, 1977; Reichenbach et al., 1990; Papan and Campos-Ortega, 1994). However, one should keep in mind that a detailed morphological and cellular analysis of neurulation in these species is lacking. In particular, the relation between the neural and nonneural ectoderm was not investigated. The complex mode of neurulation in *Xenopus* is an important reminder that the formation of a neural groove alone is not a hallmark of primary neurulation.

HOW CONSERVED ARE THE MOLECULAR PATHWAYS REGULATING NEURULATION?

With a basic understanding of cellular mechanisms in place, we can begin grappling with the molecular pathways that control the cell behaviors driving neurulation and address the extent to which they are conserved. Clearly, the PCP pathway is involved in neurulation across vertebrates, including humans (Kibar et al., 2007). This pathway is generally thought to narrow and elongate the neural plate prior to the onset of neurulation. In amphibians, PCP

signaling controls polarized cell movements in the neural plate, prior to the onset of neurulation (Keller et al., 2000; Ueno and Greene, 2003; Zohn et al., 2003; Wang and Nathans, 2007). In amniotes, in which neural plate cells do not exhibit mesenchymal characteristics (and are thus unlikely to be migratory), this pathway may regulate other processes, such as the orientation of cell division (Sausedo et al., 1997; Wallingford, 2005; Wang et al., 2006; Wang and Nathans, 2007). The molecular pathways controlling apical constriction are also likely to be conserved across vertebrates (Martin, 2004; Wallingford, 2005; Nyholm et al., 2009).

The cell–cell adhesion molecule N-cad is an interesting case study. N-cad belongs to the subfamily of classical cadherins, characterized by five extracellular cadherin-binding domains and an intracellular region that interacts dynamically with the actin cytoskeleton via its association with α - and β -catenin (Tepass et al., 2000; Derycke and Bracke, 2004). Interestingly, *N-cad* is broadly expressed in neural tissue in both anterior and posterior regions of all vertebrates in which it has been analyzed, immediately following neural induction (Hatta and Takeichi, 1986; Detrick et al., 1990; Radice et al., 1997; Harrington et al., 2007). In epithelial cells, classical cadherins cluster in adherens junctions and participate in the establishment and maintenance of apico-basal polarity and cell adhesion (Tepass et al., 2000). In mesenchymal cells, cadherins are broadly distributed throughout the plasma membrane and are generally thought to mediate cell traction during migration and cell intercalation (Keller, 2002). Given the different cytoarchitectures of cells undergoing neurulation, these observations raise the intriguing possibility that N-cad may function as a versatile protein to promote different modes of neurulation. In amniotes, N-cad may maintain the epithelial organization of the neural ectoderm and restrict cell motility (Duband et al., 2009) during primary neurulation. While mouse knockouts do not have any overt NT defects, possibly due to functional redundancy (Radice et al., 1997), injection of function-blocking antibodies in the brain ventricles of chick embryos disrupts the integrity of the neuroepithelium due to loss of cell–cell adhesion (Bronner-Fraser et al., 1992; Ganzler-Odenthal and Redies, 1998). In zebrafish, loss of *N-cad* blocks neurulation, as lateral neural plate cells are unable to converge medially and intercalate (Lele et al., 2002; Hong and Brewster, 2006). At later stages of development, these mutants exhibit loss of cell–cell adhesion in the neuroepithelium (Lele et al., 2002), similar to that reported in chick embryos. Thus, in zebrafish, N-cad may have the dual role of promoting neural convergence at an early stage and epithelialization at a later stage of NT development. The versatility of N-cad function across vertebrates is most likely explained by differential posttranslational modifications of the protein or/and alternative binding partners (Derycke and Bracke, 2004). These observations indicate that while the conserved expression of N-cad is informative, the molecular pathways regulating N-cad function may be more relevant to understanding the evolution of mechanisms of neurulation across vertebrates.

Analysis of cell behaviors during neurulation can also drive the search for underlying molecular mechanisms. For

example, secondary neurulation in amniotes and NT formation in *Xenopus* and zebrafish involve an epithelialization process or MET. Molecular pathways regulating MET have been well described for several tissues (Dressler, 2002; Hogan and Kolodziej, 2002; Lubarsky and Krasnow, 2003; Nelson, 2003; Hay, 2005; Lee et al., 2006; Thiery and Sleeman, 2006; Baum et al., 2008) and some components of these pathways could also be implicated in neurulation. Moreover, epithelialization of the NT is also likely to involve genes expressed in the NT that are not known components of these pathways. Among these, *Pax3* is an interesting candidate, since it is known to promote epithelialization when overexpressed and depletion of *pax3* can lead to loss of cell adhesion (Wiggan et al., 2002, 2006).

In conclusion, while we are still far from fully understanding how the NT forms in vertebrates, combined cellular and molecular approaches already hint that there is a common thread between different modes of neurulation.

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