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REVIEWS

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Towards a Cellular and Molecular Understanding of Neurulation

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ABSTRACT **Neurulation occurs during the early embryogenesis of chordates, and it results in the formation of the neural tube, a dorsal hollow nerve cord that constitutes the rudiment of the entire adult central nervous system. The goal of studies on neurulation is to understand its tissue, cellular and molecular basis, as well as how neurulation is perturbed during the formation of neural tube defects. The tissue basis of neurulation consists of a series of coordinated morphogenetic movements within the primitive streak (e.g., regression of Hensen's node) and nascent primary germ layers formed during gastrulation. Signaling occurs between Hensen's node and the nascent ectoderm, initiating neurulation by inducing the neural plate (i.e., actually, by suppressing development of the epidermal ectoderm). Tissue movements subsequently result in shaping and bending of the neural plate and closure of the neural groove. The cellular basis of the tissue movements of neurulation consists of changes in the behavior of the constituent cells; namely, changes in cell number, position, shape, size and adhesion. Neurulation, like any morphogenetic event, occurs within the milieu of generic biophysical determinants of form present in all living tissues. Such forces govern and to some degree control morphogenesis in a tissue-autonomous manner. The molecular basis of neurulation remains largely unknown, but we suggest that neurulation genes have evolved to work in concert with such determinants, so that appropriate changes occur in the behaviors of the correct populations of cells at the correct time, maximizing the efficiency of neurulation and leading to heritable species- and axial-differences in this process. In this article, we review the tissue and cellular basis of neurulation and provide strategies to determine its molecular basis. We expect that such strategies will lead to the identification in the near future of critical neurulation genes, genes that when mutated perturb neurulation in a highly specific and predictable fashion and cause neurulation defects, thereby contributing to the formation of neural tube defects.**

Key words: biophysical determinants of form; canalization of development; cell behaviors; central nervous system; chick embryo; folic acid; genes; heredity; mouse knockouts; morphogenesis; neural folds; neural groove; neural plate; neural tube defects (NTDs); neurulation defects

INTRODUCTION

Neurulation is commonly described as the developmental process that results in the rolling up of a flat sheet of epithelial cells into an elongated tube. But how does neurulation actually occur? Does neurulation merely involve the readout of an evolved genetic program, or are other governing (i.e., epigenetic) principles involved? Are there key neurulation genes that have evolved specifically to regulate this process? What exact perturbations in neurulation result in the formation of neural tube defects?

In this article, we will address these questions by reviewing the tissue and cellular basis of neurulation and providing strategies for determining its molecular basis. Identifying the cause of morphogenesis will require a full understanding of the interaction between generic biophysical determinants of form present in all living tissues (e.g., viscoelastic characteristics, surface tensions) and genes that regulate morphogenesis (e.g., by controlling its timing and the direction and magnitude of the cell behaviors that drive morphogenetic movements) (Weiss, 1950; Newman and Comper, 1990; Drasdo and Forgacs, 2000; Hogeweg, 2000). We consider here the potential roles of such generic biophysi-

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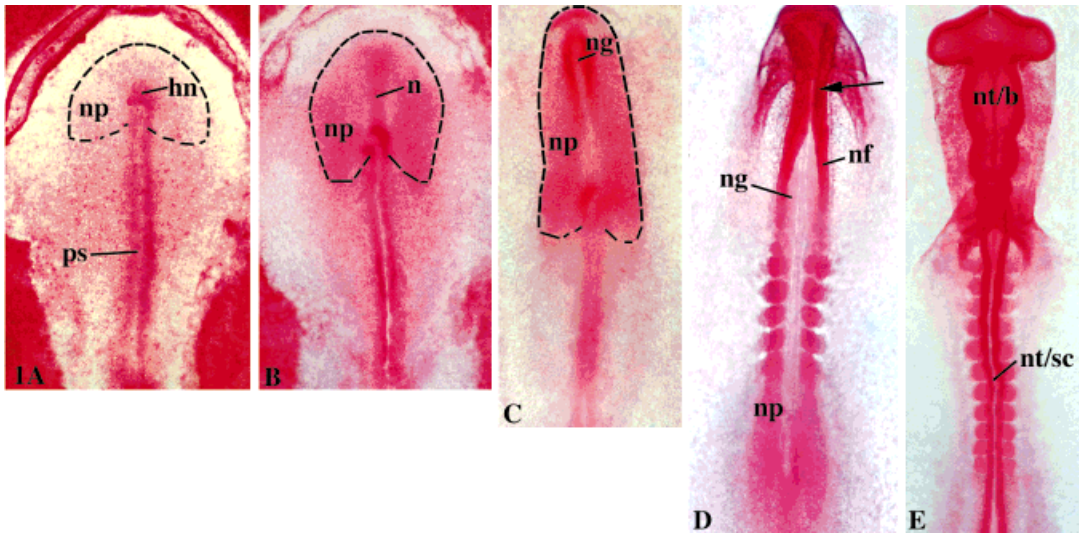

Fig. 1. Whole-mounts of chick embryos undergoing primary neurulation, viewed from the dorsal surface of the blastoderm. The range of stages shown (Hamburger and Hamilton, 1951, stages 4–11) represent about 24 hours of development (i.e., one day out of 21 in the life of a chick embryo), beginning at about 18 hours of incubation and ending at about 42 hours of incubation. **A,** The neural plate has just formed; its approximate borders are outlined. **B,** The neural plate is undergoing shaping; its approximate borders are outlined. **C,** The neural plate is initiating bending, establishing a neural groove, while still undergoing shaping; its approximate borders are outlined. **D,** The paired neural folds have come into contact at the level of the future mesencephalon region of the neural tube (arrow). **E,** A neural tube has formed throughout the length of the future brain and much of the length of the future spinal cord. hn, Hensen's node; n, notochord (seen through the neural plate); nf, neural fold; ng, neural groove; np, neural plate; nt/b, future brain level of the neural tube; nt/sc, future spinal cord level of the neural tube; ps, primitive streak. Modified from Smith and Schoenwolf (1997).

cal determinants of form in neurulation, to define better the influence of putative neurulation genes on the characteristic morphology of the resulting neural tube. We focus our discussion principally on the avian model, because this system has been the one most studied mechanistically. Additionally, we provide a classification scheme for identifying neurulation genes, and we use this scheme to classify those genes that have already been mutated in mouse embryos, leading to the formation of neural tube defects (NTDs), either specifically by directly altering critical events of neurulation, or more generally, by adversely affecting early embryonic growth and morphogenesis.

THE TISSUE BASIS OF NEURULATION: COORDINATED MORPHOGENETIC MOVEMENTS

At the tissue level, neurulation occurs in four stages: formation of the neural plate, shaping of the neural plate, bending of the neural plate and closure of the neural groove (Fig 1). The stages of neurulation are coordinated with movements of the primitive streak; namely, progression (i.e., rostrocaudal elongation) of the primitive streak during formation of the neural plate, and regression of the primitive streak (and especially of its rostral end, Hensen's node) during shaping and bending of the neural plate and closure of the neural groove. Neurulation begins with the formation of the neural plate, a process that is typically described as induction of the neural plate or neural induction. The details of neural induction are beyond the scope of this review (reviewed by Lemaire and Kodjabachian, 1996; Tanabe and Jessell, 1996; Gould and Grainger, 1997; Hemmati-Brivanlou and Melton, 1997; Sasai and Robertis, 1997; Weinstein and Hemmati-Brivanlou, 1999; Harland, 2000; Jessell and Sanes, 2000). However to summarize, recent studies have shown that neural induction actually involves suppression of an epidermal fate rather than induction of a neural fate, so that the default state of the naive ectoderm is neural, not epidermal as suggested by classical studies. The suppressive signal is generated by Hensen's node (Fig. 1A), the avian equivalent of Spemann's organizer, and it involves the binding of inhibitory molecules to secreted ligands, such as BMPs or Wnts, blocking their signaling.

Formation of the Neural Plate

Aside from the issue of neural induction, formation of the neural plate involves apicobasal thickening of the ectoderm, resulting in the formation of a placode, a flat but thickened epithelial rudiment. The neural plate can form in isolation from the surrounding epidermal ectoderm (Schoenwolf, 1988), demonstrating that once the ectoderm becomes committed to a neural fate, the process of formation of the neural plate is autonomous to the prospective neural plate and does not required the presence of non-neural ectodermal cells.

Shaping of the Neural Plate

At the time of its formation, the neural plate when viewed dorsally or ventrally is shaped like a spade shield, being relatively wide mediolaterally and short rostrocaudally (Fig. 1A). The caudal "wings" of the spade shield flank Hensen's node, the inducer of the neural plate. During shaping of the neural plate, the nascent neural plate continues to thicken apicobasally. Additionally, it undergoes a convergent extension movement; that is, it concomitantly narrows mediolaterally (i.e., transversely) and elongates rostrocaudally (Fig. 1B, C).

Tissue isolation experiments demonstrate that shaping of the neural plate is driven by changes in the behavior of its neuroepithelial cells; thus, the neural plate still undergoes shaping when it is separated from more lateral tissues (i.e., epidermal ectoderm, mesoderm and endoderm) or caudal tissues (the regressing primitive streak) and cultured (Schoenwolf, 1988; Schoenwolf et al., 1989; Moury and Schoenwolf, 1995). Although the processes of neurulation and gastrulation can be uncoupled experimentally by such isolation experiments, full rostrocaudal formation and extension of the neural plate requires normal gastrulation movements (and especially, regression of the primitive streak).

Bending of the Neural Plate

Bending of the neural plate is initiated as its shaping is underway (Fig. 1B–D). Bending involves the formation of the neural folds at the lateral extremes of the neural plate, and the subsequent elevation and convergence of these folds toward the dorsal midline. Elevation of the neural folds establishes a trough-like space called the neural groove, which becomes the lumen of the primitive neural tube after closure of the neural groove.

The rostrocaudal level at which bending is initiated differs among species. In the chick embryo, bending is initiated and completed first at the future mesencephalon region (i.e., midbrain level of the neuraxis; Fig. 1D) and progresses simultaneously both rostrally and caudally. In the human embryo, bending is completed first at the hindbrain/upper cervical region (Sadler, 2000). Regardless of the rostrocaudal level at which it is underway, bending occurs in two steps referred to as furrowing and folding (Fig. 2). The former occurs in three localized regions termed hinge points (a single median hinge point overlying the prechordal plate and notochord that extends along the entire rostrocaudal extent of the neuraxis; and paired dorsolateral hinge points, present within the neural folds principally at future brain levels (Schoenwolf and Franks, 1984; also see below and Fig. 5). Folding, in contrast to furrowing, involves the rotation of the neural plate around the hinge points, with folding around the median hinge point called elevation, and that around the dorsolateral hinge points called convergence. Therefore, by definition, convergence occurs principally at future brain levels (i.e., levels at which dorsolateral hinge points form).

Tissue isolation experiments demonstrate that bending of the neural plate is driven by changes in both neuroepithelial cells (driving furrowing) and the adjacent cells of the epidermal ectoderm (driving folding). Thus, in the prospective median hinge point, furrowing of the neural plate occurs when this region is separated from more lateral tissues, but elevation of the neural folds requires the presence of such lateral non-neuroepithelial tissues (Schoenwolf, 1988; Moury and Schoenwolf, 1995). Moreover, although furrowing is driven in the median hinge point by changes in the cells forming this structure, such furrowing requires an inductive signal from the underlying notochord (Smith and Schoenwolf, 1989). This signal, mediated by the secreted protein Sonic hedgehog, is involved not only in formation of the median hinge point but also in the formation of the floor plate of the neural tube, an important signaling center (for a review, see Jessell and Sanes, 2000). Whether the epidermal ectoderm induces furrowing in the dorsolateral hinge points has not been examined, owing to technical difficulties.

Refinements of the tissue isolation experiments show that the critical lateral, non-neuroepithelial tissue required for folding of the neural plate is the epidermal ectoderm. As just discussed, in the absence of *all* lateral tissues, the neural plate undergoes furrowing (especially within the median hinge point), but folding fails to occur. However, if the epidermal ectoderm is left intact and the other two of the three lateral tissues are removed, namely, the endoderm and mesoderm, folding, as well as furrowing, still occurs (Alvarez and Schoenwolf, 1992). If the converse experiment is done, that is, removal of the epidermal ectoderm only, leaving the mesoderm and endoderm intact, folding fails to occur (Hackett et al., 1997). Collectively, these experiments demonstrate that of the lateral non-neuroepithelial tissues required for folding of the neural plate during the stage of bending, the epidermal ectoderm is the only one of the three tissues that is both sufficient and necessary for folding.

In addition to the epidermal ectoderm, the neural folds play crucial roles during bending of the neural plate. Each neural fold is bilaminar, consisting of a layer of neuroepithelium capped by a layer of epidermal ectoderm. At the future brain levels, where neural folds are developed most extensively, formation and morphogenesis of the neural folds involves four key events termed epithelial ridging, kinking, delamination and apposition (Fig. 3; Lawson et al., 2001). The structure of the neural folds (and, in particular, the well developed interface that forms between the two layers and apparently binds them together through its contained extracellular matrix) would seem to be ideally suited to transduce forces generated by expansion of the more lateral epidermal ectoderm, aiding in bending of the neural plate. Additionally, expansion of the

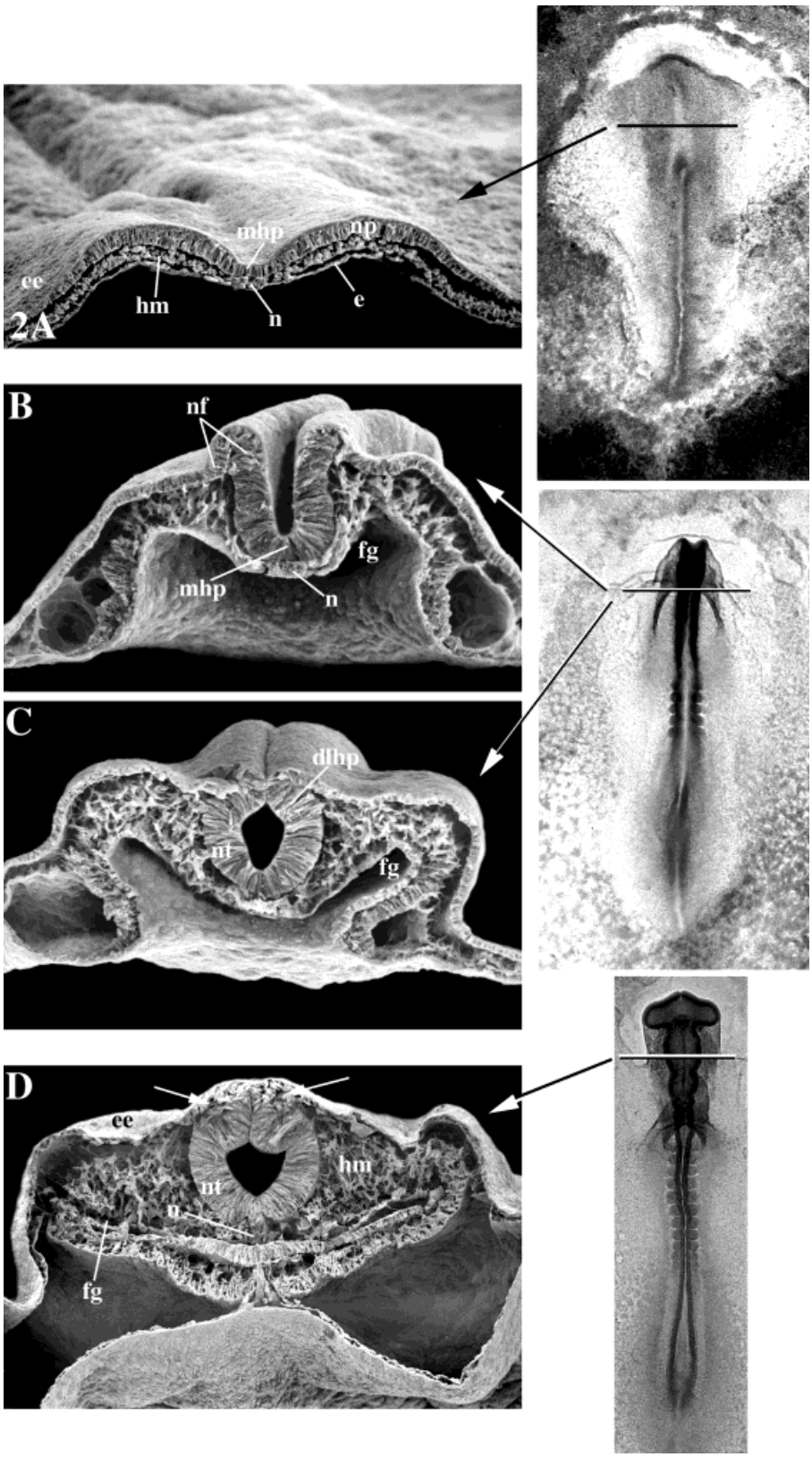

Fig. 2. Orientation whole-mounts (3 insets to right) and transverse sections (A–D) viewed with scanning electron microscopy at various periods during primary neurulation of chick embryos at approximately the future mesencephalon level (level of transverse lines in whole mounts; B and C are derived from the same level of two embryos, one at the neural groove stage (B) and the other at the incipient neural tube stage (C). **A:** Flat neural plate stage during shaping and early bending. Furrowing of the neural plate within the median hinge point has occurred. **B:** Neural groove stage. **C:** Incipient neural tube stage. Note that the neural folds are in contact with one another but not yet fused. **D:** Definitive neural tube stage. Note that the neural folds have largely fused forming the roof of the neural tube, neural crest (arrows) and mid-dorsal epidermal ectoderm. dlhp, dorsolateral hinge point; e, endoderm; ee, epidermal ectoderm; fg, foregut; hm, head mesoderm; mhp, median hinge point; n, notochord; nf, neural fold; np, neural plate. Modified from Schoenwolf (2001).

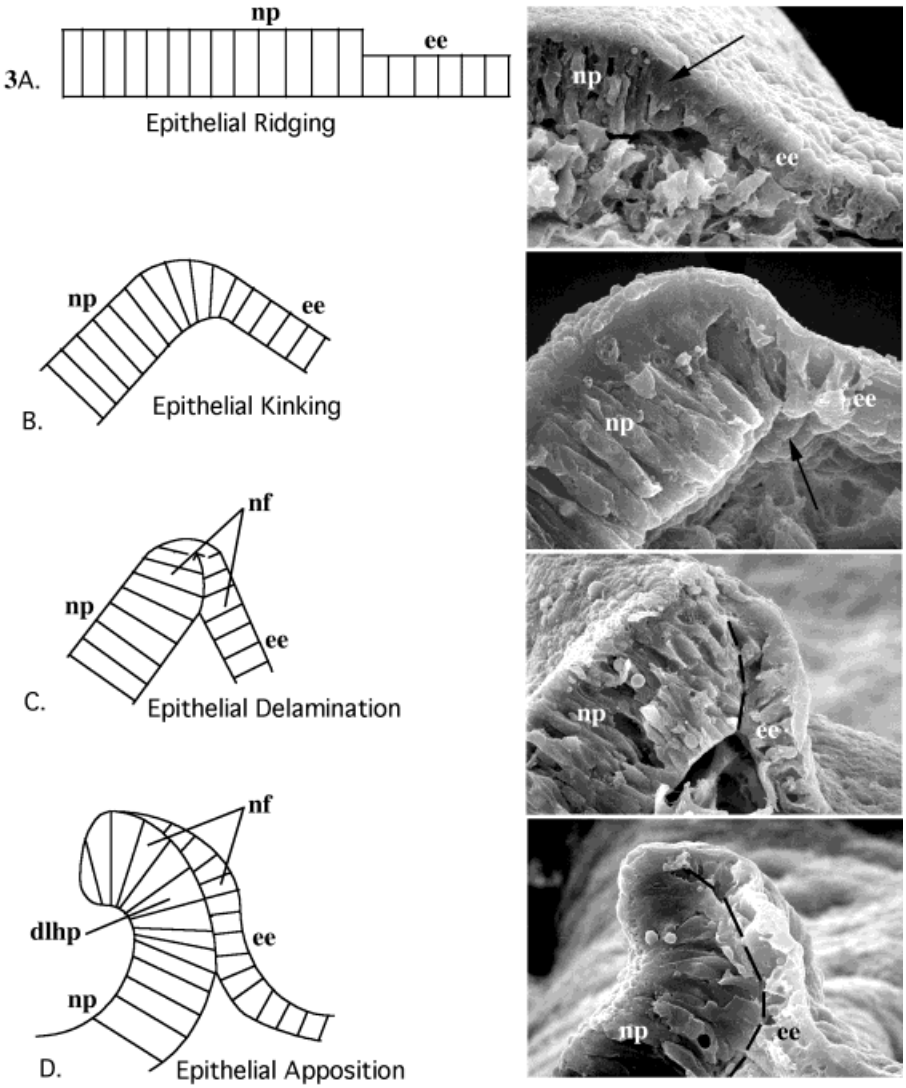

Fig. 3. Drawings and scanning electron micrographs showing the four key events of neural fold formation and morphogenesis at the future brain level in the chick embryo. Epithelial ridging (**A**), epithelial kinking (**B**), epithelial delamination (**C**) and epithelial apposition (**D**). dlhp, dorsolateral hinge point; ee, epidermal ectoderm; nf, neural fold; np, neural plate. Arrows on micrograph indicate the neural ridge (A) and point of kinking (B). Dashed lines on micrographs (C, D) indicate the neural fold interface. Based on the results of Lawson and coworkers (2001).

interface between the two layers of the neural folds at future brain levels (i.e., the process termed apposition) would seem to generate forces intrinsic to each neural fold, aiding in their convergence toward the dorsal midline. In fact, convergence of the neural folds often occurs when the more lateral epidermal ectoderm is removed, provided that the median epidermal ectoderm is left intact (Hackett et al., 1977), demonstrating the importance of the neural fold interface and epithelial apposition.

In contrast to the future brain levels, at most levels of the future spinal cord, the neural folds fail to undergo significant epithelial apposition; thus, formation and morphogenesis of the neural folds at these levels involves only three key events: epithelial ridging, kinking and delamination (Lawson et al., 2001). This difference in whether epithelial apposition occurs (along with the presence or absence of the dorsolateral hinge points; see below) contributes to the level-specific differences that appear in the cross-sectional shape of the neural tube and its contained lumen. Thus, at brain levels, the initial lumen is broadened transversely, but at spinal cord levels it is slit-like.

Surprisingly, only the neural folds and immediately lateral tissue (presumably, epidermal ectoderm) are required for the neurulation movements characteristic of folding of the neural plate during its bending. When virtually the entire neural plate is extirpated, including the median hinge point but leaving the most dorsal neuroepithelium and more lateral tissues intact, for-

mation and morphogenesis of the neural folds occurs normally, and the neural folds extend toward the dorsal midline where if they meet one another, they undergo fusion (Smith and Schoenwolf, 1991). Moreover in mouse embryos, neurulation does not require a functional median hinge point (e.g., a closed neural tube forms in the Sonic hedgehog null mouse, which lacks a normal median hinge point/floor plate; Chiang et al., 1996), although an abnormally large floor plate can apparently impede bending (e.g., homozygous *loop-tail* mutant mice have an enlarged floor plate and open NTDs; Greene et al., 1998).

Closure of the Neural Groove

Bending of the neural plate and formation and morphogenesis of the neural folds ultimately bring the paired neural folds into contact in the dorsal midline where they adhere to one another and fuse. Fusion establishes the roof of the neural tube and separates it from the overlying epidermal ectoderm, which will contribute to the skin of the back of the embryo. A third population of ectodermal cells, the neural crest, form either as the neural folds are elevating and converging (e.g., in the mouse) or during their fusion (e.g., in the chick). The neural crest is an important cell type that contributes extensively to the peripheral nervous system, among other structures (e.g., pigment cells, facial skeleton) reviewed by Anderson, 1999; Garcia-Castro and Bronner-Fraser, 1999; Groves and Bronner-Fraser, 1999; Hall, 1999; Le Douarin and Kalcheim, 1999).

THE CELLULAR BASIS OF NEURULATION: CHANGES IN CELL BEHAVIOR GENERATE MORPHOGENETIC MOVEMENTS

Formation of the Neural Plate

Formation of the neural plate in mammalian and avian embryos is driven through cell palisading; that is, apicobasal cell elongation rather than an increase in the number of layers of cells (in the amphibian embryo, the neural plate is a bilaminar structure; formation of the neural plate in the amphibian involves the apicobasal elongation of the deep cells only). Thus in "higher" vertebrates, the neural plate is a pseudostratified, columnar epithelium (i.e., each neuroepithelial cell extends from the apex to the base of the epithelium, but nuclei are positioned at different apicobasal levels, giving the false impression in histological section that the epithelium is stratified). Neuroepithelial cells divide throughout neurulation (Smith and Schoenwolf, 1987; Smith and Schoenwolf, 1988; and references therein). As they do so, they undergo interkinetic nuclear migration, synthesizing DNA while the nucleus of each cell moves toward the base of the epithelium, and rounding up for mitosis at the apex of the epithelium. Each daughter cell then extends a process toward the base of the epithelium, translocating its nucleus basally as it does so and re-entering the mitotic cycle (reviewed by Watterson, 1965).

Neuroepithelial cell elongation during formation of the neural plate presumably involves the activity of paraxial microtubules (i.e., microtubules elongated in the apicobasal plane of the cell), as well as other factors such as cell packing, changes in cell–cell adhesion and the elaboration of a variety of intercellular junctions (reviewed by Schoenwolf and Smith, 1990a). The exact role of paraxial microtubules remains controversial: paraxial microtubules could conceivably drive the process of cell elongation or they could stabilize the shapes of cells once elongation occurs.

In birds and mammals, the caudal part of the neural tube (future lumbar, sacral and tail levels) forms in a different manner than the more rostral part (Fig. 4). This process of caudal neural tube formation, called secondary neurulation (primary neurulation being the term used for the phase of neurulation we typically mean by "neurulation"), begins with the aggregation of tail bud cells into a solid epithelial cord called the medullary cord (Schoenwolf, 1979). Whether this process requires induction, similar to that occurring during formation of the neural plate (perhaps suppression of a mesodermal fate), remains unexplored.

Shaping of the Neural Plate

As described above, shaping of the neural plate involves three coordinated events: thickening, narrowing and lengthening. Thickening is driven by neuroepithelial cell elongation, as is formation of the neural plate. Assuming that the volume of neuroepithelial cells does not increase during their elongation and that cytoplasm would be distributed isotropically during this process, such elongation would be expected to decrease both the width and length of the neural plate concomitantly. When microtubules are disrupted using cold treatment, the thickness of the neuroepithelium decreases about 25% while the width of the neural plate increases roughly proportionately (a similar but smaller increase occurs in the length of the neural plate). This experiment provides direct support for the idea that thickening and narrowing of the neural plate are linked (but the lengthening of the neural plate is independent of its thickening, as discussed below; Schoenwolf and Powers, 1987). It also suggests that the elongated configuration of neuroepithelial cells is maintained only partially by microtubules, because although these cells decrease their heights following depolymerization of their microtubules, they remain substantially elongated. Thus the roles of other factors in maintaining cell elongation need to be tested in future studies.

Narrowing and lengthening of the neural plate are driven by three cell behaviors. As just discussed, apicobasal cell elongation contributes to narrowing. Additionally, neuroepithelial cells undergo intercalation in the mediolateral plane of the neural plate, thereby narrowing the neural plate and simultaneously lengthening it (Schoenwolf and Alvarez, 1989). Moreover, neuroepithelial cells divide about every 8–10 hours

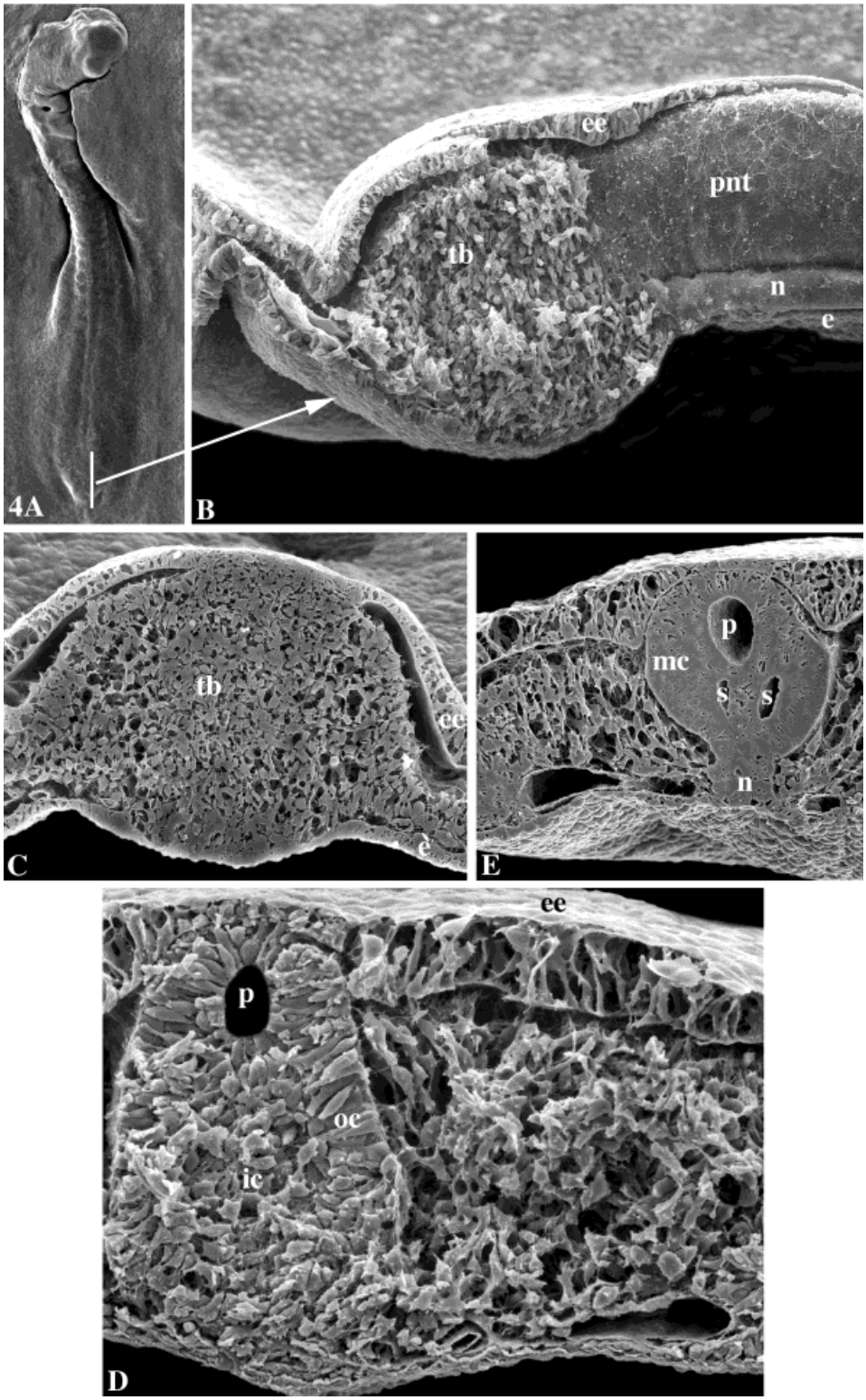

Fig. 4. Scanning electron micrographs of a whole mount (**A**), parasagittal slice (**B**), transverse cryofractures (**C, E**) and a transverse slice (**D**) illustrating secondary neurulation in the chick embryo. The three transverse images illustrate progressively later stages in development of the secondary neural tube. The line in (A) indicates the position of the parasagittal slice shown in (B). e, endoderm; ee, epidermal ectoderm; ic, inner cells of the medullary cord which will be removed during subsequent cavitation; mc, medullary cord undergoing cavitation; n, notochord; oc, outer cells of the medullary cord which will form the secondary neuroepithelium; p, primary lumen formed from the neural groove; pnt, caudal end of the primary neural tube; s, secondary lumen formed from cavitation of the medullary cord; tb, tail bud. Modified from Schoenwolf (2001).

during neurulation (Smith and Schoenwolf, 1987). About half of the cell division planes are oriented to place daughter cells into the length of the neural plate rather than into its width, thereby contributing further to neural plate lengthening (Sausedo et al., 1997).

During secondary neurulation, as the medullary cord is forming from cells of the tail bud, the outer cells of the cord undergo elongation to form a pseudostratified columnar epithelium reminiscent of that of the neural plate (Fig. 4; Schoenwolf and DeLongo, 1980). This process, like shaping of the neural plate during primary neurulation, likely involves both paraxial microtubules (Schoenwolf and Powers, 1987) and factors that remain to be tested, such as cell packing and changes in cell adhesion. Moreover, intercellular junctions develop to join lateral cell surfaces during formation of the medullary cord (Schoenwolf and Kelley, 1980), and epithelial cells become polarized, such that lumina develop between their apical surfaces and a central cluster of mesenchymal cells; the latter are "removed" during the process of cavitation (presumably, mainly by cell rearrangement and migration, with limited apoptosis), establishing a single, central lumen, which fuses with the lumen of the caudal end of the primary neural tube (Schoenwolf and DeLongo, 1980). Experiments have shown that secondary neurulation can occur in the absence of closure of the neural groove, suggesting that tissue morphogenesis during secondary neurulation occurs autonomously with respect to primary neurulation (Costanzo et al., 1982).

Bending of the Neural Plate

As described above, bending of the neural plate involves both furrowing and folding. The forces causing furrowing are generated by the wedging of neuroepithelial cells within the hinge points, a process driven by both apical narrowing and basal expansion (reviewed by Schoenwolf and Smith, 1990b). Apical narrowing involves the presence of circumferential apical bands of microfilaments (e.g., Lee and Nagele, 1985). However, like the role of paraxial microtubules in the elongation of neuroepithelial cells discussed above, the role of circumferential apical bands of microfilaments in apical narrowing is controversial. For example, neuroepithelial cells remain apically narrowed (and wedge shaped) following depolymerization of their microfilaments (Schoenwolf et al., 1988; Ybot-Gonzalez and Copp, 1999). Similarly, basal expansion involves a process that is independent of microfilaments (Schoenwolf et al., 1988), being mediated instead by the translocation and retention of the nucleus at the base of the cell, owing to prolongation of the cell cycle and resulting changes in interkinetic nuclear migration (Smith and Schoenwolf, 1987, 1988). It has been proposed that interkinetic nuclear migration, and particularly its outward phase (i.e., the migration of nuclei of newly formed daughter neuroepithelial cells from the apex of the neuroepithelium, where mitosis occurs to form them, toward the base of the neuroepithelium), is mediated by paraxial microtubules (Schoenwolf and Smith, 1990b), but this proposal has not yet been tested.

In contrast to furrowing of the neural plate, the forces driving folding of the neural plate are generated largely by changes in cell behaviors within lateral nonneuroepithelial tissues, especially the epidermal ectoderm. These changes in the epidermal ectoderm, which consist of cell flattening, intercalation and oriented mitosis (Schoenwolf and Alvarez, 1991; Sausedo et al., 1997), cause the epidermal ectoderm to expand medially (Lawson et al., 2001). Tissue isolation experiments show that such expansion is autonomous to the lateral tissues, occurring in lateral tissue isolates either containing (Smith and Schoenwolf, 1991) or lacking (Moury and Schoenwolf, 1995) neural folds and associated neuroepithelium. An intriguing idea is that the epidermal ectoderm signals the adjacent neuroepithelium (or vice versa) along the neural fold interface, coordinating the activities of the two tissues during folding of the neural plate, but this idea remains to be tested.

Formation and morphogenesis of the neural folds is driven by changes in multiple cell behaviors (Fig. 3; Martins-Green and Erickson, 1986; Martins-Green, 1988; Fernandez Caso et al., 1992; Moury and Schoenwolf, 1995; Lawson et al., 2001). Epithelial ridging, the initial event of neural fold formation, involves the apicobasal elongation of neuroepithelial cells, coupled with the apicobasal shortening of adjacent epidermal ectodermal cells. Epithelial kinking, the second event, involves change in cell shape within the incipient neural fold, so that both prospective neuroepithelial and epidermal ectodermal cells of the neural fold become inverted-wedge-shaped (i.e., constricted basally and expanded apically) and interconnected basally by extracellular matrix. Epithelial delamination, the third event, involves the deposition of extracellular matrix along the incipient neural fold interface and changes in the orientation of prospective neuroepithelial and epidermal ectodermal cells. In particular, an isolated extracellular space forms between the lateral surfaces of cells flanking the prospective neural fold interface. Cells then become reoriented along this incipient interface so that their basal surface abuts the interface and their apicobasal axis becomes oriented radially with respect to the interface. The final event, epithelial apposition, is restricted to future brain levels and it involves the further deposition of extracellular matrix along the expanding width (i.e., mediolateral extent) of the interface and the flattening of epidermal ectodermal cells, increasing their surface areas. Additionally, epithelial apposition involves intercalation within the epidermal ectoderm (Schoenwolf and Alvarez, 1991), as well as cell division, which is oriented to contribute to mediolateral expansion of the epidermal ectoderm (Sausedo et al., 1997).

Cell polarization, that is, the formation of distinct apical and basolateral cell surfaces, is an intrinsic property of epithelial sheets (e.g., Alberts et al., 1998).

The neuroepithelium, regardless of whether it forms from the neural plate during primary neurulation or the medullary cord during secondary neurulation, exhibits such polarization, forming intercellular junctional complexes and circumferential apical bands of microfilaments. Moreover, cells of both the primary and secondary neuroepithelia exhibit interkinetic nuclear migration, with mitosis being restricted to the nascent apical side of the epithelium. The fact that apical bands of microfilaments are present in neuroepithelial cells of both the primary and secondary neural tubes, and that these tubes form in strikingly different ways, supports the idea discussed above that circumferential bands of microfilaments likely serve principally to stabilize neuroepithelial cell shape, rather than causing these cells to change their shape (i.e., to undergo wedging).

Closure of the Neural Groove

The cellular basis of the final stage of neurulation, in contrast to that of the earlier stages, is poorly understood. Closure involves bringing the neural folds together, resulting in their contact in the dorsal midline, adhesion at points of contact and epithelial breakdown and fusion. This results in the formation of two separate epithelial layers, epidermal ectoderm and neuroepithelium, with intervening mesenchymal cells of the neural crest. The neural folds secrete a cell surface coat (e.g., Sadler, 1978), which presumably aids in these events, but its exact composition and function remain unknown. Recently, the importance of an ephrin in neural fold fusion has been demonstrated (Holmberg et al., 2000).

THE DEVELOPMENTAL DYNAMICS OF NEURULATION

Synergistic Roles of Intrinsic and Extrinsic Neurulation Forces in Bending of the Neural Plate: the Cooperative (Hinge Point) Model

As discussed above, bending of the neural plate is driven by both intrinsic and extrinsic forces, that is, forces generated by changes in cell behavior within the neural plate and (most notably) the epidermal ectoderm, respectively. Intrinsic and extrinsic forces are generated by essentially the same cell behaviors, namely, changes in cell shape, size, position, number and adhesion, but the magnitude of the changes, the type of change that occurs and the direction of tissue movements differ in the two tissues. For example, neuroepithelial cells undergo more divisions during neurulation than do epidermal ectodermal cells (Smith and Schoenwolf, 1987), and although cell shape changes occur in both tissues, different cell shapes are generated (neuroepithelial cells elongate apicobasally and those within the hinge points become wedge-shaped, whereas epidermal ectodermal cells flatten, and both neuroepithelial and epidermal ectodermal cells flanking the prospective neural fold interface become inverted-wedge-shaped). Moreover, changes in cell behaviors within the neural plate result in the movement of the neural plate caudally, whereas those in the epidermal ectoderm result principally in the movement of the epidermal ectoderm medially. Changes in cell size and number contribute to the growth of the neural plate, providing raw material for morphogenesis. Changes in cell shape and position likely occur both passively (through pushing and pulling forces generated by the extracellular matrix and neighboring cells) and actively (e.g., by alterations of cytoskeletal dynamics). The mechanical properties of the extracellular matrix and the cytoskeleton will need to be quantified to gain a full understanding of the dynamics of neurulation (Lane et al., 1993; Forgacs et al., 1998), but these factors are difficult to measure directly in small embryonic rudiments undergoing morphogenesis. Nevertheless, one can surmise the origin of mechanical forces in and around the neural plate (Schoenwolf and Franks, 1984; Nagele et al., 1989), and develop a model that interprets the force that would be generated by changing cell behaviors.

We have formulated such a model—the cooperative (or hinge point) model—that accounts for the roles of both intrinsic and extrinsic forces in bending of the avian neural plate (Fig. 5; Schoenwolf and Smith, 1990a; Moury and Schoenwolf, 1995). It is based on three main premises: (1) the neural plate is firmly anchored to adjacent tissues at the hinge points (to the notochord and prechordal plate for the median hinge point, and to the prospective epidermis of the neural folds for the dorsolateral hinge points), (2) neuroepithelial cell wedging within the hinge points generates furrowing and (3) forces for folding are generated lateral to the hinge points by the expanding epidermal ectoderm. Thus, the model predicts that each hinge point directs and facilitates bending, much like creasing a sheet of paper directs and facilitates its subsequent folding, and that each "hinge" acts like a standard door hinge rather than like a self-closing, spring hinge. Past experiments directly support these predictions (e.g., Smith and Schoenwolf, 1991). Consequently, the hinge point model has gained wide acceptance (e.g., Fleming et al., 1997; DeSesso et al., 1999; Harris and Juriloff, 1999; Gilbert, 2000; Kalthoff, 2001).

The Potential Role of Generic Biophysical Determinants of Form in the Developmental Dynamics of Neurulation

In addition to understanding the characteristic changes that occur in the intrinsic and extrinsic cell behaviors just described, to gain a more complete idea of how neurulation occurs it must be realized that at each stage of neurulation, the mechanical properties of the different tissues involved, and the distribution and timing of the forces acting on them, contribute to the course of neuroepithelial morphogenesis (Koehl, 1990; Moore et al., 1995). As it is being molded into a tube, the neuroepithelium owes its form to the laws of phys-

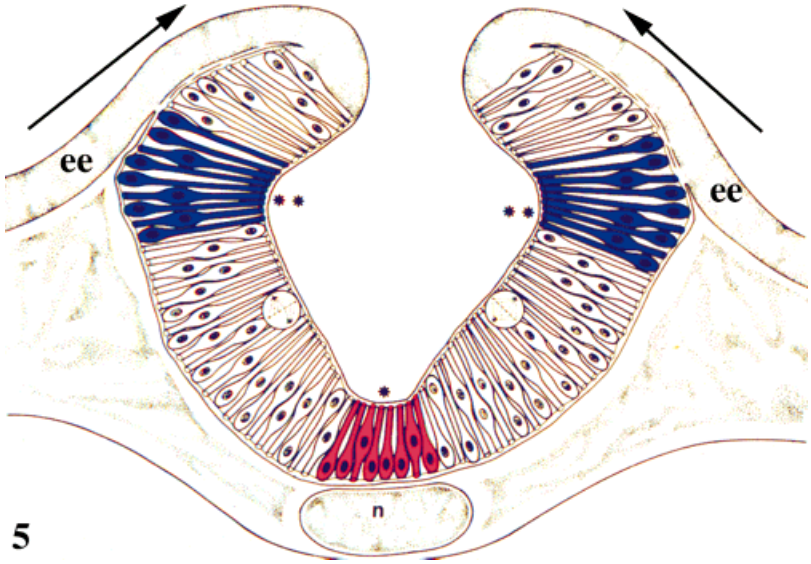

Fig. 5. Drawing illustrating the cooperative (hinge point) model of bending of the chick neural plate. Neuroepithelial cell wedging within the hinge points is indicated by red (median hinge point) and blue (dorsolateral hinge points). Arrows indicate mediolateral expansion of the epidermal ectoderm; single asterisk indicates furrowing associated with the median hinge point. Double asterisks indicate furrowing associated with the dorsolateral hinge points. ee, epidermal ectoderm; n, notochord. Modified from Schoenwolf and Smith(1990a).

ics like any other living tissues, or even inorganic matter. Realistic organic forms can be assumed by one fluid mass within another through ordinary inanimate surface-tension phenomena (Thompson, 1961). One of the fundamental differences between cells undergoing neurulation and many of those undergoing another morphogenetic event, gastrulation (e.g., cells ingressing through the primitive streak), is that the former cells are epithelial, being joined together in a sheet by intercellular junctions and cohesive interactions with the basal lamina and extracellular matrix, whereas the latter are mesenchymal, being more freely associated with one another. Because of its epithelial structure, the ectoderm will tend to bend rather than flow when forces are applied to it. Nevertheless, ectodermal cells exchange neighbors during neurulation through cell intercalation, allowing them to flow in the plane of the epithelium like a liquid. As for any morphogenetic event, the formation of the neural tube depends on recognizable generic biophysical determinants of form acting in epithelial rudiments, such as cell-adhesion–generated tissue surface tensions, gravitational effects, viscosity and elasticity. (Newman and Comper, 1990; Foty et al., 1996; Forgacs et al., 1998). According to the "differential adhesion hypothesis," morphogenesis is an inevitable outcome, specified by the second law of thermodynamics, of the ordinary liquid-like behavior of cells (i.e., by analogy with a multiphase system of immiscible liquids, cells segregate with their own type at every opportunity; Steinberg, 1998). The ability of cells to undergo sorting, based on differential adhesive properties, is likely a major factor involved in the formation and cavitation of the medullary cord during secondary neurulation, or in cell intercalation during convergent extension and formation of the neural folds in primary neurulation. Other generic determinants of form acting in epithelial rudiments likely include the tendency of epithelial rudiments to heal, restoring ionic balance across the epithelium (an explanation for the observation that isolated neural plates roll up into tubes, but sometimes in a direction opposite to that occurring during neurulation; reviewed by Schoenwolf and Smith, 1990a).

During primary neurulation, striking differences exist in the shape of the neural plate, folds and groove depending on its rostrocaudal level (Schoenwolf and Franks, 1984; Schoenwolf, 1985; Sakai, 1989; Shum and Copp, 1996; Juriloff and Harris, 2000; van Straaten et al., 2000; Lawson et al., 2001). These differences principally affect elevation and convergence of the neural folds, and the resulting overall diameter of the neural tube, as well as the shape of its lumen. Thus, for example, the transverse diameter of prospective brain level of the neural tube is considerably greater than that of the spinal cord, and the initial lumen of the former is broadly diamond shaped, whereas that of the latter is slit-like.

Even more striking is the difference that exists in the morphogenetic events underlying primary and secondary neurulation. As discussed above, the caudal end of the neural tube arises during secondary neurulation by the formation and subsequent cavitation of an initially solid, compact mass of cells, the medullary cord (Fig. 4; Schoenwolf and DeLongo, 1980). Cavitation of the medullary cord results principally from neighboring cells becoming polarized apicobasally and incorporating into

a primitive neuroepithelium (Schoenwolf, 1984). The columnar cells of this neuroepithelium have characteristics similar to those of the neural plate. Thus, both primary and secondary neurulation lead to essentially the same end-product, a hollow neural tube composed of a pseudostratified columnar epithelium, although the developmental events occurring in primary and secondary neurulation differ considerably. In both cases, neuroepithelial cells have an autonomous potential to polarize, elongate and organize into a tube. Moreover, the two tubes must have common adhesive properties, because at the transition zone (i.e., the neurulation overlap zone) between primary and secondary neurulation, where the caudal end of the closing neural groove overlaps the rostral end of the more ventral medullary cord (Fig. 4D), the two tubes ultimately join into a single tube containing one central lumen. The same inherent tendency to form a tube or a sphere has been observed with dissociated cells of the amphibian neural plate (Townes and Holtfreter, 1955) or in vitro, where neural tube-like structures called neural rosettes form from a teratocarcinoma-derived cell line, through a morphogenetic pathway involving extracellular matrix components (Kawata et al., 1991). The fact that similar structures arise from very different developmental pathways suggests that common generative rules are used that are based heavily on generic biophysical determinants of form (Beloussov and Lakirev, 1991).

To summarize, changes in cell behaviors played out in the context of generic biophysical determinants of form result in the process of neurulation. Although our understanding of neurulation is still far from complete, neurulation is one of the best understood examples at present of vertebrate morphogenesis leading to the formation of a major organ rudiment. Thus, establishing how genes influence this key example of morphogenesis will provide an enormous advance in our understanding of embryogenesis.

The Potential Role of Genes in the Developmental Dynamics of Neurulation

During tissue morphogenesis, form emerges from epigenetic processes; consequently, genes do not directly cause the development of form (Goodwin, 1985, 1988; Grobstein, 1988; Gilbert and Sarkar, 2000). Genetic mechanisms, such as the capacity of cells to modulate their adhesivity, acting in concert with the generic biophysical determinants of form, have been recruited through natural selection to stabilize and reinforce favorable forms (Newman and Comper, 1990; Newman, 1992). Regardless of the exact generic biophysical determinants of form contributing to neurulation, they alone cannot explain how cell behaviors are coordinated to produce characteristic forms within a biologically useful time frame, and the reproducible occurrence of rostrocaudal and species differences in the resulting shape of the neural tube. For example, by what mechanism is closure of the neural groove kept in register with the general growth of the embryo or with somite development? Or how are the "pulling" forces generated within the neural plate coordinated with the "pushing" forces generated by the lateral epidermis? Or how are primary and secondary neurulation coordinated? Or how is body folding and formation of the heart tube coordinated with neurulation? Certain gene products (i.e., catalyzers) could control the selection of different alternative (but equivalent) thermodynamics states, thereby facilitating the occurrence of a morphogenetic sequence in a biologically useful time frame (Goodwin, 1988; O'Shea, 1988; Nijhout, 1990; Oster and Weliky, 1990; Forgacs et al., 1998). The activation of these catalyzers could rely on previously formed embryonic structures to keep the sequence of gene expression in register with the course of morphogenesis (Edelman, 1992; Losick and Shapiro, 1993; Drasdo and Forgacs, 2000).

We submit that regional differences in neurulation result from the particular embryonic context in which they occur, presumably requiring that various neurulation events adapted during evolution to different mechanical constraints. For example, during primary neurulation, neuroepithelial cells are constrained by the neighboring lateral epidermal ectoderm; during secondary neurulation, neuroepithelial cells have to assemble from the mesenchymal cells of the tail bud. Moreover, primary neurulation at brain levels requires elevating and converging massive neural folds, but at spinal cord levels, neural folds are considerably smaller. The difference in embryonic context is itself, at least partially, a consequence of the difference in the timing at which the common set of generic and genetic factors are deployed.

The variable of time has profound effects on the development of form and, consequently, on the shape of the resulting neural tube formed during neurulation (Copp, 1985). A diversity of neural tube forms can be generated by combining the changes in cell behavior that drive neurulation in different ways over time. For example, uniform simultaneous wedging within the width of the neural plate during primary neurulation would generate a tube having a circular cross-sectional morphology. A lack of uniform wedging or regional changes in its timing would alter the cross-sectional morphology of the neural tube (Lewis, 1947; Schoenwolf, 1982; Nagele and Lee, 1987; Beloussov and Lakirev, 1991).

In summary, the common features of neural tube morphogenesis are largely dependent upon the thermodynamics of the self-organizing properties of the neuroepithelium. Regional (and species) differences in the morphogenesis of the neural tube are likely to arise from the activity of unique gene products. These molecules would directly establish the ultimate conformation of the neural tube by influencing the timing of morphogenetic events and regulating the type and magnitude of cell behaviors that occur (Beloussov and Lakirev, 1991; Steinberg, 1998).

GENETIC CONTROL OF NEURULATION INCREASES ITS EFFICIENCY AND ENSURES THE HERITABILITY OF NEURAL TUBE FORM

To understand how neurulation is inherited, we need to understand the role of genes in neurulation. Neurulation is driven by redundant mechanisms both at the tissue and cellular levels, as well as the molecular level of organization. This idea is evidenced by the observation that a particular experimental manipulation designed to remove a neurulation force (or forces) in amphibian or avian embryos rarely results in the disruption of neurulation (or a specific neurulation event) in all treated embryos. Similarly, teratogens rarely block neurulation in all treated embryos, and mouse embryos subjected to inactivation of a critical gene via homologous recombination infrequently form NTDs with complete penetrance. Besides illustrating the stochastic nature of developmental events, these observations suggest that a number of factors contribute to the formation of the neural tube, and that the putative genetic reinforcement of neurulation can be removed by mutation.

It is likely that such redundancy appeared during evolution to improve the fidelity of neurulation in concert with changes in the mechanical constraints of the embryonic environment (Brook et al., 1991; van Straaten et al., 1993). Nevertheless, even with both intrinsic and extrinsic cell-based forces acting redundantly, disruption of neurulation does occur, resulting in neural tube defects (NTDs). NTDs, such as anencephaly (e.g., encephalocele) and spina bifida (e.g., myelomeningocele), are associated with substantial human morbidity and mortality, and they result in significant fetal wastage. NTDs are among the most common birth defects present in newborn humans (as high as 1:500 births in USA; reviewed by Copp et al., 1990; DeSesso et al., 1999; Harris and Juriloff, 1999). It is clear from a wealth of genetic and clinical data that an individual's liability to form a neural tube defect has both a genetic and environmental cause. NTDs have familial distributions that cannot be accounted for by simple Mendelian models. A multifactorial threshold model has been proposed to explain the inheritance patterns and recurrence risks of such human congenital abnormalities in families. This model assumes the existence in the population of a continuous variable "liability" and of a developmental "threshold" value beyond which the individual is affected (Fraser, 1976). As for most congenital abnormalities, the actual nature of the numerous genetic and environmental factors causing the liability for NTDs in humans is largely unknown.

The incidence of NTDs can be reduced in human populations with folic acid supplementation during early pregnancy. With supplementation, one-half to two-thirds of the NTDs can be prevented (Czeizel and Dudas, 1992). The reasons for such prevention are unclear (Lucock and Daskalakis, 2000). One hypothesis is that folate supplementation neutralizes a genetic defect in folate homeostasis, such as a metabolic deficiency in its maternal supply (Fleming and Copp, 1998), or mutations in the embryo's gene for the enzyme methylenetetrahydrofolate reductase (Shields et al., 1999). However, these genes account for only a small percentage of the total genetic susceptibility to NTDs (Gibson and Bottiglieri, 2000). Interestingly, folate supplementation reduces the occurrence of several other congenital malformations (Rosenquist et al., 1996), questioning the specificity of its effect for NTDs. An alternative hypothesis is that folic acid is a requisite fuel for human gestation, necessary, for example, timed proliferative bursts (Antony and Hansen, 2000). Neurulation is susceptible to the perturbation of fundamental cell behaviors such as mitosis (Juriloff and Harris, 2000). Neurulation is also the developmental event that is the most sensitive to telomere loss and chromosomal instability (Herrera et al., 1999). Folic acid supplementation at the time of neurulation is very likely to galvanize an inherently fragile morphogenetic "machine," increasing the likelihood that neurulation will occur normally. Therefore, that an NTD can be prevented by folic acid supplementation does not necessarily mean that a genetic defect in folate homeostasis was neutralized. Because of this, and the fact that there are folic acid-resistant NTDs (Corcoran, 1998), we need to search for other genetic risk factors for NTDs, that is, for additional neurulation genes.

NTDs are fundamentally a problem of morphological heredity. As an example of this we consider the role played by serotonin during *Drosophila* gastrulation (Fig. 6). Gastrulation starts at the cellular blastoderm stage when the embryo consists of an epithelial monolayer enveloping the yolk. Ventrally, the mesoderm invaginates, owing to cell wedging, forming a tube that closes at the midline. Posteriorly, the endoderm invaginates also by cell wedging, and it forms a pocket containing the germ cells. Dorsally, the ectoderm undergoes a convergent extension driven by cell intercalation (Irvine and Wieschaus, 1994). This extension, called germ band extension, pushes the endodermal pocket dorsally and anteriorly, and it assists in the closure of the mesodermal tube (Fig. 6). Serotonin keeps the onset of germ band extension in register with the morphogenetic movements in the mesoderm and endoderm (Colas et al., 1999a). In a population of embryos genetically deprived of serotonin, germ band extension occasionally occurs normally in some embryos, that is, at the right time. The presence of serotonin in the ectoderm of wild-type embryos is required to increase the reproducibility of germ band extension, namely, its occurrence at the right time in virtually *all* individuals. Both maternal and zygotic genetic input are required to produce a peak of serotonin at the exact onset of germ band extension (Colas et al., 1999b). It takes the

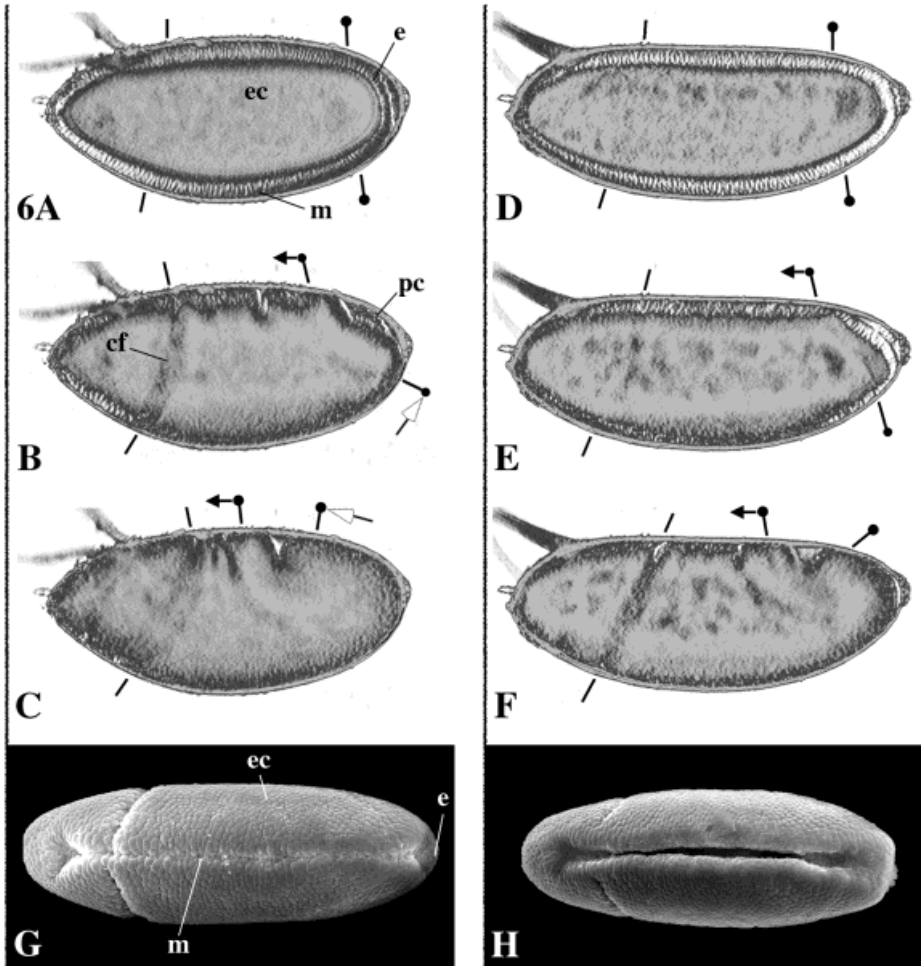

Fig. 6. Canalization of *Drosophila* gastrulation by serotonin. *Drosophila* embryos (A–C, G) wild-type (with the serotonin reinforcement) and (D–F, H) genetically depleted for serotonin signaling. (**A–F**) The most rapid phase of germ band extension (15 min) in one embryo of each genotype is illustrated by three frames in temporal sequence, selected at 7'30" intervals from time-lapse videos. The two embryos were filmed from their lateral side, with the cephalic pole at the left, the dorsal ectoderm at the top and the ventral mesoderm at the bottom. Before its extension, the germ band consists of a rectangularly shape area of the trunk region of the embryo, delimitated by bars at each corner. Its anterior border is where the cephalic furrow forms. Its posterior border is where the endoderm containing the pole cells invaginates. During the initial and most rapid phase of germ band extension, two independent forces act in concert: (1) the dorsal side of the embryo contracts as a result of a pulling force (of unknown origin, symbolized by the solid arrows in (B) and (C), and in E and F) under the posterior endodermal invagination, and (2) ectodermal cells push the endoderm primordium, in the same direction as the pulling that generates the dorsal contraction, by converging (through cell intercalation) from the dorsal towards the ventral side of the embryo and extending posteriorly (the pushing force is symbolized by an open arrow in B and C; note that this arrow is absent in E and F). The rapid phase of germ band extension occurs at reduced speed in the absence of serotonin signaling because the pushing force is missing. This may or may not lead to a complete morphogenetic block in gastrulation, depending of the level of desynchronization between germ band extension and mesodermal and endodermal invagination (that is, whether or not the pulling force succeeded in coping with the absence of the pushing force). **G** and **H:** Scanning electron micrographs (ventral views) showing the development of the mesodermal furrow at the ventral midline in (G) wild-type and (H) mutant embryos at the same stage of germ band extension as embryos shown in B and E, respectively. The presence of a non-closure phenotype in the mutant embryo suggests that the formation of the mesodermal tube requires the coordination between intrinsic wedging forces and extrinsic forces generated within the ectoderm. cf, cephalic furrow; e, endoderm; ec, ectoderm; m, mesoderm; pc, pole cells. Modified from Colas et al. (1999a).

upstream cascade of anteroposterior segmentation genes (more than 20 genes), and probably a few more in the dorsoventral axis cascade, to restrict the reception of the serotonin signal to seven stripes of ectodermal cells, a pattern matching the constraint of cell intercalation (Colas et al., 1995). When the striped pattern of expression of the serotonin receptor is slightly disorganized, germ band extension occurs normally, but it becomes less resistant to challenge by an environmental stress (e.g., mild heat shock; JFC, unpublished data), in which case the embryo either dies or hatches with overt abnormalities as a consequence of the desynchronization of gastrulation movements. It is unlikely that this complex genetic regulation would have appeared all at once during evolution, or that serotonin signaling would have initiated the morphogenetic

movements of the ectoderm in early ancestors. *Drosophila* ancestors most likely gastrulated without the serotonin reinforcement. Some of the laboratory-engineered serotonin-depleted embryos succeed in extending their germ band, probably because ectodermal cells are inherently, although less efficiently, capable of intercalating, and clearly because a second independent force, acting normally in concert with the ectodermal pushing, pulls the endoderm invagination. This is an obvious example of the so-called canalization of development (Waddington, 1942), that is, a genetic stabilization of development against mutational or environmental perturbations. Serotonin did not create a phenotypic novelty, but its recruitment by natural selection has made the expression of a phenotype more heritable (Newman, 1992).

Regarding human neurulation, it can be argued that in contrast to the highly evolved process of gastrulation in *Drosophila*, vertebrates have not yet inherited sufficient genetic mechanisms for a robust neurulation that is highly reproducible and insensitive to environmental perturbations. For example, neurulation seems to be far more susceptible to perturbation in the cranial region (future brain) than in the more caudal regions (future spinal cord). This is explained at least in part by the fact that elevation and convergence of the large cranial neural folds that give rise to the brain presumably requires more force than does elevation and closure of the much smaller caudal neural folds that give rise to the spinal cord. However, at least some genetic stabilizers seem to have already evolved to cope with the mechanical constraints encountered cranially. Mouse strains differ in their mode of cranial closure (Juriloff et al., 1991). Genetic polymorphisms have been identified in the control of the position of a closure site, which influences the embryo's susceptibility to a cranial NTD (Gunn et al., 1995; Fleming and Copp, 2000). The same type of genetic variation and problem of morphological heredity seems to occur in humans, as evidenced by the increased risks for NTDs associated with mutations in genes coding for the folate pathway (Shields et al., 1999; De Marco et al., 2000), the differences in prevalence of NTDs among different ethnic groups and the familial inheritance of NTDs (Van Allen et al., 1993; Copp and Bernfield, 1994; DeSesso et al., 1999; Nakatsu et al., 2000).

THE MOLECULAR BASIS OF NEURULATION: IN SEARCH OF NEURULATION GENES

What Is a Neurulation Gene?

In defining a neurulation gene, our underlying postulate is that there exists an inherent tendency, based on the generic biophysical determinants of form present in epithelial sheets and the changes that occur in fundamental cell behaviors, for the neural plate to form a neural tube by mechanisms not unique to neurulation (Ettensohn, 1985; Fristrom, 1988; Steinberg, 1998). However, this tendency has been reinforced by the natural selection of relevant genes expressed during neurulation. Thus, neurulation genes can be defined as genes that regulate and coordinate unique combinations of cell behaviors occurring in those tissues that generate neurulation forces. The products of neurulation genes may also have roles in other morphogenetic events in the embryo or adult, but what makes them specifically neurulation genes is that they are expressed in the context of neurulation. Therefore, this specificity resides even more in the regulatory sequences of these genes than in the gene products themselves.

Theoretical definition of a neurulation gene. The original propensity to neurulate appeared early in the evolution of the chordate lineage. The morphology of the neuroepithelium that was reinforced by genetic mutations was then perpetuated. For the morphology to survive, it had to fit the essential integrity of the organism, that is, to be favorable for further epigenetic interactions (Gilbert and Sarkar, 2000). As in other developing epithelial sheets, the dynamics of neural plate morphogenesis is constrained by the molecular properties of tactical genes products: the cytoskeletal and adhesive apparatus. What is unique to this morphogenesis (e.g., the cross sectional morphology of the neural groove; brain- and spinal cord-specific differences; etc.) is caused by the strategic genes expressed at the time of neurulation (Kerszberg and Changeux, 1998). Therefore, in theory, a neurulation gene is a context-dependent catalyzer, organizing the direction and magnitude of cell-based mechanical forces in or around the neural plate.

Practical definition of a neurulation gene. A practical definition of a neurulation gene is one whose loss of function (partial or total) at the time of neurulation weakens the identified cellular and tissue mechanisms responsible for transforming the flat neural plate into a neural tube; this weakening would not necessarily result in the presence of an NTD at the time of birth. Obviously, genes involved in establishing the early body plan or in regulating global growth of the embryo, and genes coding for the basic machinery of the cell, are necessary for normal neurulation, but they are not neurulation genes per se. Neurulation genes can be expressed within or outside the neural plate, but they must be expressed in tissues relevant to neurulation (i.e., epidermal ectoderm, neural ectoderm, head mesenchyme, neural folds, etc.). This also means that, for example, a neurulation gene specifically involved in stabilizing convergent extension of the epidermal ectoderm during elevation of the neural folds cannot be expressed in the same manner in the adjacent neural ectoderm, which behaves differently (the genetic difference may be qualitative or quantitative; lie at the transcriptional or post-transcriptional levels or both). The challenge is to observe these differences in vivo as normal neurulation is taking place and to determine their outcome, that is, their effects on localized cell behaviors and resulting neurulation forces. Neurulation genes must create the boundaries

necessary to nucleate and organize morphogenetic movements (Jacobson and Moury, 1995). The ectopic gain of function of a neurulation gene in a competent tissue might be expected to break these boundaries by changing cell behaviors.

Neurulation Defects and Neurulation Genes

Several genes, many of which would not be expected to be involved in neurulation (e.g., based on their temporospatial pattern of expression), when inactivated by homologous recombination in mouse result in NTDs. Thus, our current thinking about the molecular basis of neurulation is largely founded on the idea that a gene resulting in an NTD when mutated must be a neurulation gene. This idea is not necessarily true, and for most genes identified to date by this approach, we argue below that it is likely to be incorrect. For recent reviews of the mutations causing NTDs, see Table 1 of Harris and Juriloff (1999), Table 3 of Copp et al. (2000) and Table 1 of Juriloff and Harris (2000).

Here, we present a classification scheme designed to reveal the likelihood that a gene plays an important and direct role in neurulation (Appendix: Table 1). We used this scheme to classify mouse genes on the basis of the published neural tube phenotype in loss of function experiments, whether or not it demonstrates a specific defect in the actual process of neurulation; we also include a small number of other genes whose misexpression or pattern of expression suggests a role in neurulation. It must be emphasized that it remains unclear to what degree any of these genes fit the criteria for neurulation genes per se, mainly because detailed analyses of cell behavior in the mutated embryos as neurulation is occurring are often incomplete. Finally, the Appendix is not meant to be an exhaustive consideration of each gene; rather it serves principally as an entree into the primary literature.

From our classification of NTD-producing loss of function mutations in mouse (Appendix: Table 2), a total of 28 genes (16 of category XD++ and 12 of category D++) seem to be specifically required for normal neurulation (see Appendix for details), among which 16 (57%) have a restricted pattern of expression (i.e., XD++). These 28 genes are involved in cell adhesion or its regulation (Calr, Efna5, Fkbp1a, Itga3+Itga6, Lama5, Pig-a), regulation of cytoskeletal dynamics (Enah, Macs, Mlp, RhoGAP5, shrm, abl+arg, Vcl), transcription of downstream genes (jmj, Pax3, twist, Cart-1, RBP-J kappa, c-ski, Tcfap2a), nutrition of the embryo (apoB, Folbp1) and other diverse functions (Jnk1+Jnk2, terc, Csk, Bcl10, Ikk1+Ikk2, Psen1+Psen2).

The main strength of using mice with loss of function mutations is that it is at least theoretically possible to deconstruct the mouse embryo to determine the minimal requirements for generating a neural tube with regional-specific morphology. But there are numerous problems with using such mice as a sole model. As far as the human health is concerned, few of the mutant mice that have NTDs represent a good genetic model of human NTDs, whose commons forms are non-syndromic and survive to late gestation or birth (Seller, 1994; Harris and Juriloff, 1999; Juriloff and Harris, 2000). So far, with few exceptions (Nozaki et al., 1999; Nagai et al., 2000), most of the engineered mutations in mice that result in NTDs are null (total loss of function) and non-conditional. Such lesion experiments are subject to the reservation that during the developmental window preceding neurulation, some unknown function of the mutated gene may have been disrupted, or that compensatory mechanisms were activated. It is known that the same developmental end-point can be achieved by different courses of development (Waddington, 1952), with apparently normal individuals emerging from abnormal embryology. The very problem with existing research on mutated mice is that most of the time only the end products are examined, so that the cause of the NTDs is never unambiguously proven. Only a few of these mutants have been studied at the actual time when neurulation is taking place, and any alterations in tissue movements (and underlying cell behaviors) that might occur would be missed because only fixed tissue is used. This is worth noting because neurulation is a dynamic process and specific defects in its dynamics might go unnoticed if the resulting defect is not an overt NTD (Sim et al., 2000). As mentioned above, we know that folic acid supplementation can prevent one-half to two-thirds of the NTDs in the human population. We want to emphasize that it is not known how folic acid actually exerts this preventive effect, and whether the original defect was entirely rescued. We do not know whether the developmental history of each of the one-half to two-thirds of the "rescued" babies was normal, or whether the actual timing of neurulation occurred on schedule. Changes in timing of neurulation events might have dire consequences. For example, evidence suggests that the initiating developmental injury for autism occurs around the time of neural tube closure (Rodier et al., 1996). Yet, model animals for autism appear robust, with no external malformations being present at the time of birth.

It can be concluded that a majority (57%; 16/28) of the best candidate genes for neurulation discovered so far, using mice, could have been identified on the basis of a systematic study of their pattern of expression. Moreover, the use of mutated mice alone is not sufficient to reach a complete understanding of the genetic causation of normal and abnormal neurulation. There are likely other critical pathogenic factors to be found: for example, the target genes of the seven transcription factors listed above that result in neurulation defects when inactivated remain to be identified. Thus, we maintain that it is necessary to search for additional candidate neurulation genes using other approaches, and to examine the effects of controlled misexpression of such genes in real time (see below).

PERSPECTIVES: STRATEGIES FOR IDENTIFYING NEURULATION GENES

To gain insight in the interplay between generic and genetic mechanisms of neurulation, we need a systematic description of the molecules present during each stage of neurulation. It is hoped that the availability of such a complex molecular anatomy would help in designing biomechanical models at the level of the cell, tissue and embryo, providing a deeper understanding of neurulation and neural tube defects. New strategies are needed using reverse-genetic approaches, based on what is expected from neurulation genes, namely, their involvement in one of the well described cellular behaviors of neurulation. Regardless of the strategy used, determining the molecular basis of neurulation will not be an easy task. It will require detailed, painstaking approaches, much like those used over the last 25 years to discern the tissue and cellular basis of neurulation. Not only will it be necessary to identify those genes that seem to be viable candidate neurulation genes based on their temporospatial patterns of expression, such genes must be misexpressed (i.e., over and under) and studied in real time, analyzing changes in the behaviors of the populations of cells driving neurulation. Genes that are irrelevant for the process of neurulation will undoubtedly also be expressed in neurulating tissues. These genes will need to be discriminated from neurulation genes using misexpression and real-time analyses.

One approach, already under way in our laboratory, is to select the best candidate neurulation genes as identified above in mutated mouse models (Appendix: Table 2) and to clone chicken orthologs of these to study their role in neurulation in a more experimentally amenable system. This approach combines the serendipitous identification of candidate neurulation genes in a system well suited for molecular genetic analyses (i.e., the mouse) with the strengths of the chick system (low cost; availability of large numbers of embryos; ease of culture; ability to misexpress genes and to study the effects of misexpression in real time). Thus, this approach should considerably expand our understanding of those putative candidate neurulation genes identified to date, as well as revealing to what extent various neurulation events have been conserved or diverged among birds and mammals.

Below, we describe two additional strategies to begin to identify new neurulation genes. Both of these approaches (like the ones just described) take advantage of powerful molecular techniques that have been recently optimized for the avian system.

Subtractive Hybridization: A Strategy to Divide and Conquer the Molecular Complexity of Neurulation

During neurulation, local and global forces shape the neuroepithelium into its final form. A reasonable hypothesis is that the morphological differences that are obvious at the cellular and tissue level are underlain by differences in molecules. This molecular differentiation should match the characteristic morphology. We have, therefore, hypothesized that the expression of genes reinforcing the cell behaviors that generate many of the intrinsic forces for bending of the neural plate is restricted to the neuroepithelium of the neural folds (NF; and especially to the dorsolateral hinge points), and that those reinforcing the extrinsic cell-based forces are localized to the adjacent lateral tissues (referred to as the lateral plate, LP). To begin testing this hypothesis, we undertook two subtractions, in opposite directions, between the genes expressed in tissues explanted from Hamburger and Hamilton (1951) stage 8$^-$ chick embryos: LP minus NF and NF minus LP (Fig. 7). The two tissues were chosen owing to their importance in neurulation, and they are as similar as possible, yet they still display critical differences in relation to neurulation (e.g., differing in whether they exhibit behaviors that generate either intrinsic or extrinsic neurulation forces). Each tissue explant consisted of all three layers of the neurula (i.e., the ectoderm, mesoderm and endoderm), because experimental evidence supports a role for all three layers in providing extrinsic neurulation forces (reviewed by Smith and Schoenwolf, 1997). However, the epidermal ectoderm is the only tissue required to generate extrinsic forces (Hackett et al., 1997), suggesting that the mesodermal and endodermal tissues facilitate bending by assisting the epidermal ectoderm. This subtraction was conducted at only

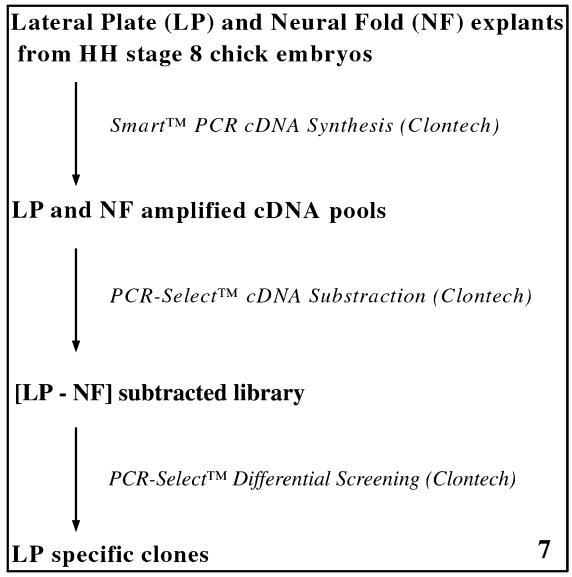

Fig. 7. Flow chart showing the PCR-based subtraction strategy used to identify candidate neurulation genes in the chick. The central procedure is the PCR-Select™ cDNA Subtraction (Clontech, Palo Alto, CA), which utilizes a method of selective amplification of differentially expressed sequences between the two tissues compared. The subtraction can be reversed to obtain NF specific clones. Based on the scheme used in Colas and Schoenwolf (2000).

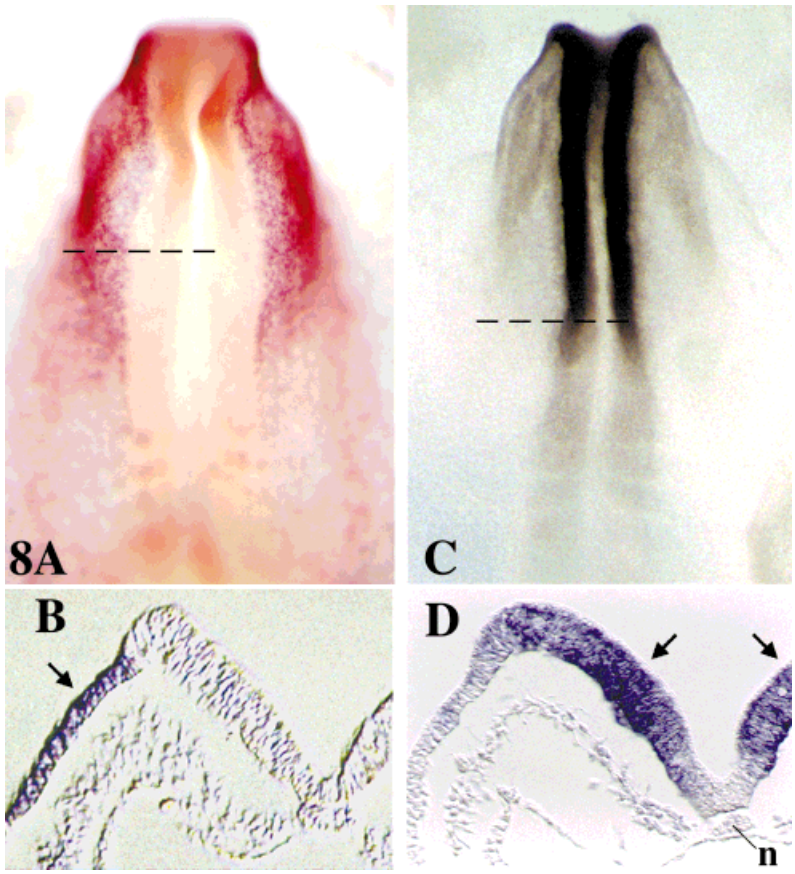

Fig. 8. Expression of two candidate neurulation genes identified by subtraction and assessed with in situ hybridization in Hamburger and Hamilton (1951) stage 8⁻ chick embryos. **A** and **B:** Whole-mount (rostral at the top) and section (level of transverse line in [A]; dorsal ectoderm at the top and ventral endoderm at the bottom), respectively, showing an embryo labeled with a riboprobe for the proteoglycan link protein (Colas and Schoenwolf, in preparation); only the epidermal ectoderm (arrow) is labeled. **C** and **D:** Whole mount (rostral at the top) and section (level of transverse line in (C); dorsal ectoderm at the top and ventral endoderm at the bottom), respectively, showing an embryo labeled with a riboprobe for a gene called Plato (Lawson et al., 2000). Plato labels the neural plate (arrows), with the exception of the midline incipient floor plate overlying the notochord (n).

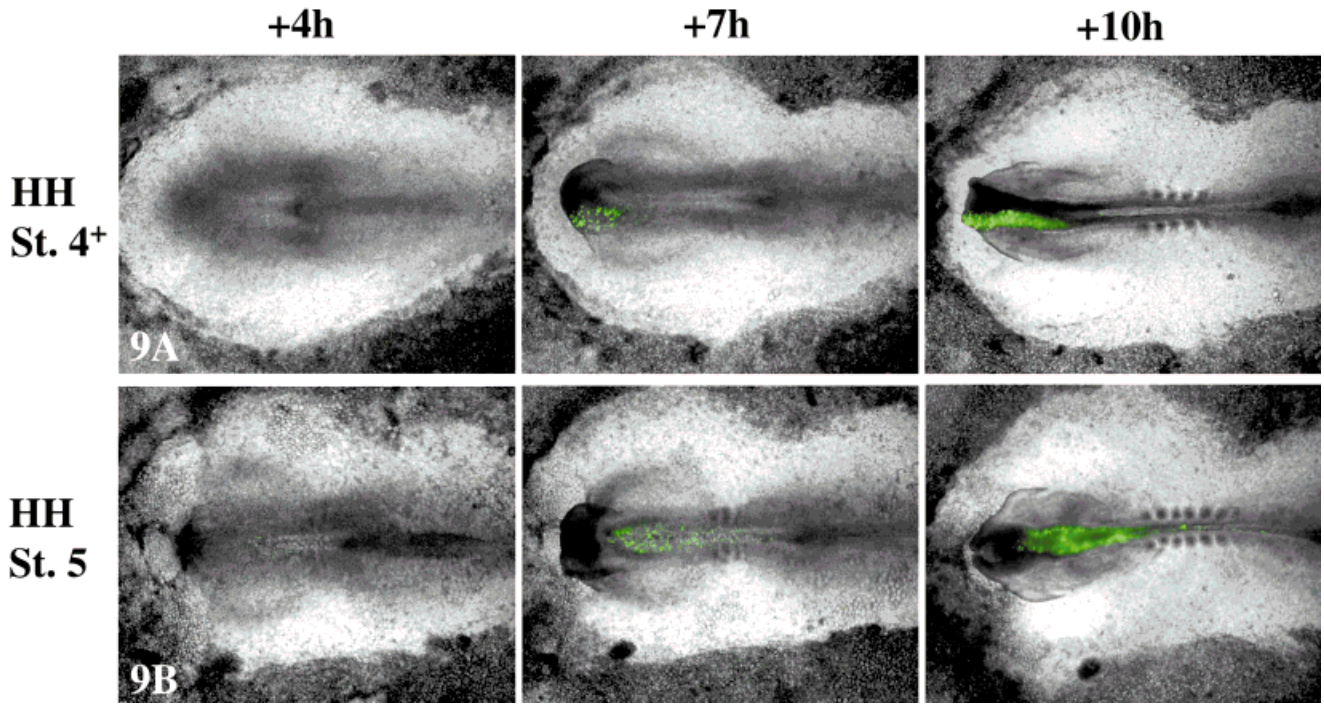

Fig. 9. Transgenic targeting of tissues undergoing neurulation in chick embryos using electroporation. An EGFP-expressing vector was electroporated into restricted regions of late gastrula-stage embryos, developing in culture. Pictures were taken at four hours after electroporation and at three-hour intervals during neurulation. Note that it is possible to target transgene expression to either (**A**) the neural fold or (**B**) the neural plate, as early as the beginning of bending of the neural plate. HH, Hamburger and Hamilton (1951) stages.

one level of the future neuraxis: the rostral hindbrain level. The future hindbrain level of the neuraxis was chosen because the dorsolateral hinge points are best developed at this level (reviewed by Schoenwolf and Smith, 1990a, b). As a result of these subtractions, both previously known and unknown genes have been identified as cDNAs fragments corresponding to differentially expressed LP- and NF-specific genes. Some were expressed only in the mesoderm and are more likely participating in heart development (Colas and Schoenwolf, 2000; Colas et al., 2000). Others are expressed in the ectoderm and represent good candidate neurulation genes (see Fig. 8 for one example from each subtraction).

Our preliminary studies demonstrated the feasibility and pertinence of comparing carefully chosen tissues to identify differentially expressed genes in embryonic tissues of limited quantity, which are involved in generating intrinsic and extrinsic neurulation forces. This approach can be applied to provide essential information on where and when genes are expressed in relation to other important neurulation events, such as the formation of the neural folds, formation of the dorsolateral hingepoints, elevation and convergence of the neural folds, fusion of the neural folds, etc. A more systematic approach would be to use DNA hybridization arrays to study, at the genomic scale, the transcriptional status of neurulating tissues (Freeman et al., 2000). Nevertheless, subtractive hybridization is still the best-suited technology for discovering unknown genes. The molecular basis of neurulation is also likely to involve differential translational and post-translational modifications. However, these await technological advances in proteomics, yet to be made.

Cloning Homologs of Genes Involved in Analogous Processes: "What's Good for One Is Good for the Other"

As a corollary to the above definition of a neurulation gene, that is, a gene that reinforces generic biophysical determinants of form in the context of neurulation, some neurulation genes might act in similar morphogenetic events occurring during the formation of other organ rudiments in the same organism or in different organisms. Thus, some neurulation genes might not be only neurulation genes per se. This would mean that (1) truly specific genes evolved for neurulation and were later reused (in chordates) for similar morphogenetic events; (2) the propensity to neurulate is rooted in an older mechanism, whose genetic specificity was reused in neurulation and other events (in invertebrates or vertebrates); (3) the same causes converge to the same effects, that is, an analogous morphogenetic context (in invertebrates or vertebrates) leads to the natural selection of homologous genes; or (4) what a gene product does in morphogenesis depends not only on its molecular nature but also on its morphogenetic context. In regards to the latter point, determining how the gene comes to be expressed in its particular context is equally important as is understanding its function. For example, the molecular responsiveness (and its evolution) to the morphogenetic context of neurulation may lie in the promoter sequence of key neurulation genes, as might the molecular basis of polymorphisms in the neural groove closure sites in humans.

If some neurulation genes have such a broader role in embryogenesis, then it will be possible to clone neurulation genes based on their homology with genes known to be involved in analogous morphogenetic processes, provided that one is able to identify such processes as analogous ones. Potentially helpful analogous processes, such as ventral furrowing or dorsal closure, have been well characterized at the molecular level in "genetic" models such as *Drosophila* (Grosshans and Wieschaus, 2000; Jacinto et al., 2000; Kiehart et al., 2000; McEwen et al., 2000). Examination of the potential role of such molecules in putative analogous events of neurulation will likely be fruitful.

CONCLUSIONS

One of the challenges for future research on neurulation will be to observe the products of neurulation genes during the morphogenetic movements of normal and abnormal neurulation. At the same time that we seek to find new neurulation genes and examine their pattern of expression, it will be necessary to precisely distinguish normal from abnormal neurulation *as the process is taking place,* rather than considerably after the fact, when anomalies secondary to defects in neurulation mechanics have developed.

To gain such information, we need noninvasive techniques to allow us to study changes in cell behaviors and gene expression in real time, thereby documenting the developmental history of the cells and molecules acting in neurulation. The chick embryo seems to be an almost ideal model to use such approaches because of the extensive knowledge that exists of the cell biology of neurulation, the ease at which the neurulating embryo can be viewed and manipulated in culture and the fact that the chick blastoderm is a flat, essentially two-dimensional structure. Early development of the chick from the flat blastoderm (rather than from the inverted blastoderm of rodent embryos) closely resembles that of humans. Because of the similarities in early development of the chick and human, results obtained in chick are likely to be clinically relevant. Moreover, data obtained from chick and mouse are more likely to be relevant to humans than those obtained form "lower" vertebrates, because in "higher" vertebrates (in contrast to lower vertebrates) true growth (i.e., increase in volume) accompanies morphogenesis. Data obtained on the cell biology of normal neurulation in chick from morphological and experimental studies could be enriched by a more refined molecular anatomy (gene/protein expression patterns) and by molecular perturbation (loss or gain of gene function). This is now possible in chick embryos developing either in ovo or in culture (Chapman et al., 2001)

using conditional electroporation (Fig. 9) of newly discovered genes (Momose *et al*., 1999). These new techniques could reveal large-scale coherent molecular processes, such as those transducing the biological response of a cell or collectives of cells to a mechanical signal (Ingber, 1993), allowing us to investigate how mechanical forces provide regulatory information for neurulation.

Another major challenge for understanding human neural tube defects is whether this problem of morphological heredity can be understood using animal models (George and Speer, 2000). Although the use of gene inactivation in mouse has shown that neurulation is susceptible to defects in some basic cellular processes, it is likely that subtle change of function in specialized neurulation genes better represent the cause of human NTDs. To identify such genes, we suggest that a systematic approach is necessary, especially based on the expression pattern of genes (i.e., the simple idea that critical neurulation genes should be expressed at the right time and at the right place), as well as an expansion of the effort of human geneticists to include genes in their studies other than those that are folate-related. Undoubtedly, more candidate NTD genes will be identified, with each having the potential to explain a particular familial recurrence of NTDs (Stumpo et al., 1998; Melvin et al., 2000; Joosten et al., 2001). Thus, the future will present new opportunities to build on our understanding of the tissue and cellular basis of neurulation, ultimately leading to the identification of neurulation genes and a molecular understanding of neurulation and neural tube defects.

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APPENDIX
Neurulation Defects and Neurulation Genes

Before detailing our classification scheme for candidate neurulation genes, four definitions are important to understand. A **neurulation defect** results in the formation of a neural tube defect (NTD) at the time of neurulation, and it occurs because the tissue and/or cellular basis of neurulation has been specifically disrupted within the tissues driving neurulation. Therefore, a **neurulation gene** is defined as one that causes a neurulation defect when mutated because it acts normally within the tissue(s) driving neurulation to generate neurulation-specific changes in cell behaviors. An **NTD** (see Inagaki et al., 2000) is defined as the abnormal development of the neural tube, occurring at any time prior to birth (i.e., at both neurulation and post-neurulation stages). A neurulation defect often results in an overt NTD at birth, but not necessarily so (restitution may occur during post-neurulation development). NTDs formed after normal closure of the neural groove are not neurulation defects by definition (i.e., they occur during post-neurulation development). An **NTD gene** is one that when inactivated results in an NTD. Neurulation genes are also NTD genes, but NTD genes are not necessarily neurulation genes.

The following classification of neurulation genes requires four levels of analysis. As a gene is assigned to progressively higher levels (in the + category), its candidacy as a neurulation gene strengthens. Genes excluded as neurulation genes at each level (assigned to the − category) are also interesting, because these genes may be NTD genes; they, like neurulation genes, are potential candidates for the causation of NTDs in humans, but they, unlike neurulation genes, do not normally influence the process of neurulation.

TABLE A1. Classification Scheme for Candidate Neurulation Genes

Level 1. Four criteria are used to identify initial candidate neurulation genes.

A gene product could have a role in neurulation if at least one of the following criteria is met:

- **X:** its expression at the time of neurulation is restricted (transcriptionally or posttranscriptionally) to the tissue(s) acting in neurulation (i.e., **X** defines genes that are differentially expressed).
- **D:** an NTD (resulting from a neurulation defect or not; see below) results as a consequence of the loss (partial or total) of its function. An NTD is defined as the abnormal development of the neural tube, occurring at any time prior to birth (i.e., at both neurulation and post-neurulation stages).
- **XD:** both **X** and **D** occur.
- **O:** other; that is, neither **X** nor **D** occur (for example, a gene associated with an uncharacterized mutation [such as a large deletion] causing an NTD, an NTD resulting from a gain of its function or a gene involved in a morphogenetic process analogous to neurulation).

Level 1 genes are classified into the following four categories.

Level 1 categories	**X**	**D**	**XD**	**O**
Level 1 genes: **Initial candidates**				

These four categories are designed to include the maximum number of potential candidate neurulation genes; they designate four ways in which genes have been chosen as candidates, or can be chosen in future studies. Genes may be reclassified when additional data are obtained, for example, from the O to the XD category.

Genes classified in level 1 are those for which level 2 criteria have not yet been addressed. For example, mouse genes classified in categories D and XD have been inactivated by homologous recombination, but the actual cellular defect causing the NTD is either not described or is unclear. Those in category X have not yet been inactivated, and those in category O require further analysis (including targeted mutation).

Level 2. Does the loss of gene function cause an NTD at the time of neurulation, that is, between the stages of formation of the neural plate and closure of the neural groove into a tube? This question is relevant for all level 1 genes.

- **+: yes**
- −: no

Level 2 genes are classified into the eight categories listed on the next page.

Level 2 categories	**X+**	X−	**D+**	D−	**XD+**	XD−	**O+**	O−
Level 2 genes: **Candidate neurulation genes** (+) Excluded as candidate neurulation genes (−)								

Genes classified in level 2 include those for which level 3 criteria have not yet been addressed (+ genes) or are NTD genes (D− and XD− genes) rather than neurulation genes. X− and O− genes are neither neurulation nor NTD genes and are, therefore, not considered further. From level 2 onward, O+ genes can be considered as D+ genes because they cause a NTD. Therefore, category O is not listed at subsequent levels. The same reasoning could be used to merge all + genes into one D+ category, but retaining categories X+ and XD+ is central to the definition of a neurulation gene. Moreover, D+ genes whose pattern of expression has not yet been analyzed can be reclassified as XD+ genes if they are shown to be restricted to the tissue(s) acting in neurulation.

Level 3. Is the NTD a neurulation defect per se? This question is only relevant for level 2 genes scored as + (excluding category O genes, which are no longer considered at this level).

At level 2, genes are classified based on the criterion of whether the NTD formed at the time of neurulation. An NTD formed at this time may or may not be a neurulation defect. To be a neurulation defect, the NTD must not only form at the time of neurulation, but also must occur because the tissue and/or cellular basis of neurulation has been specifically disrupted within the tissues driving neurulation. That is, the genetic cause of a neurulation defect is a mutation in a neurulation gene (in its coding or regulatory sequences).

Although neurulation genes are also NTD genes, the term NTD gene is used at this and subsequent levels to define the non-neurulation type of NTD genes (i.e., NTD genes that are not also neurulation genes). Such NTD genes could conceivably act at any time in development (i.e., prior to, during, and after neurulation). At the time of neurulation, NTD genes would be expected to act in tissues other than those that drive neurulation. These tissues although they do not generate normal neurulation force(s) can offer an abnormal resistance to the neurulation process in the mutant context. For example, a mutation in an NTD gene could affect development outside of the neural plate and place a constraint on neurulation, such as by tethering the neural plate to the underlying notochord. However, it is also possible that NTD genes could act within the neural plate and create an impediment to extrinsic forces for bending, for example, by affecting formation of the floor plate of the neural tube or by misregulating neuronal specification. To discriminate such NTD genes from neurulation genes per se, may require the analysis described for level 4 genes.

+: yes
−: no

Level 3 genes are classified into the following six categories.

Level 3 categories	**X++**	X+−	**D++**	**D+−**	**XD++**	**XD+−**
Level 3 genes: **Candidate neurulation genes (++)** or NTD genes (+−)						

Before progressing to level 4, the expression pattern of D++ genes needs to be assessed. As a result of their pattern of expression, they can be classified either in category XD++ or left in category D++. The latter are NTD genes that are non-differentially expressed, highlighting the inherent susceptibility of neurulation to defect in molecules shared with other tissues at the same time in development. To be tested in level 4, genes need to be differentially expressed. Therefore, category D is not listed at level 4.

Level 4. Does gain of gene function cause a change in cellular behavior that is typical for neurulation; that is, does the gain of a ++gene function in a naive, competent tissue or cell cause neurulation-like morphogenetic behaviors in vitro or in vivo, and does the ectopic gain of a ++gene function in vivo break the normal differential gene expression boundaries occurring during neurulation, resulting in a neurulation defect. This question is only relevant for level 3 genes scored as ++ (excluding category D genes).

Genes scored in level 4 as X+++ and XD+++ are defined as neurulation genes (as opposed to *candidate* neurulation genes). The difference between an X+++ and an XD+++ neurulation gene is slight and consists of the fact that mutation of the former will not necessarily result in an overt NTD at birth, which was the criterion initially used to identify the latter as candidates.

+: yes
−: no

Level 4 genes are classified into the following four categories.

Level 4 categories	**X+++**	X++−	**XD+++**	XD++−
Level 4 genes: **Neurulation genes (+++)** or NTD genes (++−)				

TABLE A2. Mouse Candidate Neurulation Genes Classified as in Table 1

Level 1 categories	**X**	**D**	**XD**	**O**
Level 1 genes: **Initial candidates**	**16s rRNA** **Gas5** **rnr-r1**	**Cbp** **Gadd45a** **Gap (+Nf1)** **p300** **Rara + Rarg** **Trp 53** **Tsc2** **Xpc (+Trp53)**	**Dlx5** **Dnmt3b** **Foxb1a** **Gli3** **Msx1 + Msx2**	**Dpp6** (and/or **Kit**) **Itgb1d** **Notch 3** **Zic3**

Level 2 categories	**X+**	X−	**D+**	D−	**XD+**	XD−	**O+**	O−
Level 2 genes: **Candidate neurulation genes (+)** or Excluded as candidate neurulation genes (−); D− and XD− genes are NTD genes			**Raldh2** **Sil**	Atoh4 Casp3 Casp9 Gata3 Hspg2 Pdgfra TREB5	**Fgfr1** **Nf1** **Nog** **Shh** **Traf6**	Apaf1 Nap1|2		Gja1 Pax1

Level 3 categories	**X++**	X+−	**D++**	**D+−**	**XD++**	**XD+−**
Level 3 genes: **Candidate neurulation genes (++)** or NTD genes (+−)			abl + arg Csk Fkbp1a Itga3 + Itga6 Lama5 Pig-a Vcl . . . apoB Bcl10 Folbp1 Ikk1 + Ikk2 Psen1 + Psen2	Arnt Axin1 Brca1 Fdft1 Madh5	**Calr** **Efna5** **Enah (+Pfn1)** **jmj** **Macs** **Mlp** **Pax3** **RhoGAP5** **shrm** **twist** **. . .** **Cart-1** **Jnk1 + Jnk2** **RBP-J kappa** **c-ski** **Tcfap2a** **terc**	Hes1 Hoxa1 Ptch Zic2

Level 4 categories	**X+++**	X++−	**XD+++**	XD++−
Level 4 genes: **Neurulation genes (+++)** or NTD genes (++−)				

In Table A2, we apply this classification scheme to those candidate genes already identified in mouse.

We list a total of 75 genes, with 20 genes implicated for the first time in a review as being involved in normal and abnormal neurulation (16s rRNA, Apaf1, Bcl10, Calr, Casp3, Casp9, Dpp6 (Kit), Efna5, Fdft1, Fkbp1a, Gas5, Ikk1+2, Jnk1+2, Nap1|2, Psen1+2, RhoGAP5, rnr-r1, Sil, Traf6, Zic2). From level 1 onward, we list the mouse genes whose loss of function (inactivated by targeted homologous recombination in mouse ES cells, by gene trap insertion or by classical mutations) is known, by the beginning of 2001, to cause neural tube defects, making them potential candidate neurulation genes. Note that some mutations only result in an NTD when they are combined with a loss-of-function mutation in another gene, either belonging to the same family (abl+arg, Jnk1+Jnk2, Itga3+Itga6, Ikk1+ Ikk2, Psen1+Psen2, Rara+Rarg) or to the same pathway (Enah+ Pfn1, Gap+Nf1, Xpc+Trp53). Other potential candidate neurulation genes are included because either they result in NTDs after other types of molecular perturbation (Dpp6, Gja1, Itgb1d, Notch3, Pax1, Zic3) or they have been identified on the basis of their expression in relevant tissues during neurulation (16s rRNA, Gas5, rnr-r1).

At level 2 and 3, we tried to determine, based on published phenotypes, whether the loss of gene function leads to a defect in the correct tissues at the time

that neurulation is taking place, that is, to a neurulation defect. We consider a neurulation defect to be a specific defect that occurs in the mechanics of neurulation, that is, in processes such as changes in cell behavior, and/or the underlying mechanistic events that mediate these changes (e.g., changes in cytoskeleton or cell adhesion). These types of defects have been documented for D++ and XD++ genes. Inactivated genes that lead to alterations in the rate of cell cycle progression, cell division and programmed cell death at the time of neural groove closure may alter specifically the course and outcome of neural tube formation; therefore categories D++ and XD++ also include this type of gene (i.e., apoB, Bcl10, Folbp1, Ikk1+Ikk2, Psen1+Psen2 and Cart-1, Jnk1+Jnk2, RBP-J kappa, c-ski, Tcfap2a, terc, respectively). Misregulation of cell number occurring after closure cannot be a neurulation defect (Casp3, Casp9, Apaf1, Nap1|2); the NTD in these mutations seems to result from neuroepithelial overgrowth/degeneration, subsequent to neurulation. Misregulation of neuronal specification, whatever its timing, is not considered as a neurulation defect because it is primarily a neurogenesis defect that causes an NTD (Atoh4, Hes1, Hoxa1, Ptch, Zic2). By definition, other non-neurulation defects include faulty neural tube development occurring *after* normal neurulation, such as the disruption or reopening of a previously normally closed tube (Hspg2), a spina bifida occulta (Pdgfra), late waviness in the neuroepithelium (TREB 5), etc. Some genes have been left in categories XD+ and D+ because it is unclear whether they affect neurulation specifically. This is especially true for the midline mutants Sil and Shh, which are known to cause a certain type of NTD, holoprosencephaly, which originated at the time of neurulation and at least in part in tissue involved in neurulation. Whether this phenotype is a neurulation defect and it demonstrates an instrumental role of the normal gene product in the morphogenesis of the neural plate into a tube is open to interpretation. A careful examination of level 4 criteria might help to make a definitive choice. Genes in category D+− have been shown, when mutated, to impede neurulation by an indirect defect (i.e., subsequent to another perturbation in early development that is necessary for normal neurulation, such as the growth or the axis formation of the embryo).

The best currently known candidate neurulation genes are those 16 level 3 XD++ mouse genes that are expressed at the right time and at the right place and when inactivated, result in a specific neurulation defect. A specific neurulation defect also results for 12 level 3 D++ genes when they are inactivated, although their pattern of expression is not consistent with a specific role in neurulation, revealing the susceptibility of this developmental process to defects in ubiquitous cellular processes, such as mitosis (Juriloff and Harris, 2000), or to conditions of placental insufficiency leading to malnutrition (apoB, Folbp1). It must be emphasized that the effect on neurulation of over expressing (gain of function; level 4) any of these 28 genes has not yet been studied.

Analysis of the Jnk1+Jnk2 double mutant provides the only well documented example of the physiological role of programmed cell death in neurulation. In contrast, transcriptional control of cytoskeletal dynamics specific to the neural plate is well documented (e.g., Enah, Macs, Mlp, RhoGAP5, shrm). The data from mutated mice are also beginning to reveal an integrin-dependent mechanism of neurulation, which is not surprising given the role of these molecules in epithelial morphogenesis and tissue integrity (De Arcangelis and Georges-Labouesse, 2000). The ephrin-A5 (Efna5) mutation results in the formation of anencephaly, presumably owing to the failure of the neural folds to fuse in the dorsal midline (Holmberg et al., 2000). Activation of ephrin-A5, induces changes in cell adhesion and cell morphology in an integrin-dependent manner (Davy and Robbins, 2000). Class A ephrins are tethered to the plasma membrane by a GPI anchor, giving at least one good reason why embryos mutant for the Pig-a gene (a gene involved in phosphatidylinositol glycan synthesis) have NTDs as well (Nozaki et al., 1999). Calr (possibly like Fkbp1a) is essential for the integrin-mediated flux of extracellular calcium (Shou et al., 1998; Rauch et al., 2000). Upon activation of neural adhesion molecules (especially integrin-dependent adhesion signaling), the action of PKC and the adhesion signaling molecule RhoGAP5 (Brouns et al., 2000) lead to a modulation of Rho GTPase activity, directing several actin-dependent morphogenetic processes within the neuroepithelium that are required for normal neurulation. Itga3 and Itga6 are prominent receptors for lama5 (i.e., laminin alpha5 chain; De Arcangelis et al., 1999). The Lama 5 mutation reveals the mechanical stress borne by the cranial neural folds (Miner et al., 1998). Lama 5 is the only neurulation gene documented to date that demonstrates an involvement in lateral extrinsic forces (i.e., in mutated embryos, there is a weakening of the lateral strip of epidermal ectoderm that decreases the amount of mediolateral force the ectoderm can generate on the neural fold).

Obviously, some candidate genes may need to be reclassified as additional data are obtained. With future better descriptions of the actual defects in neurulation caused by genetic modification, a better gene classification could be based on the type of tissue or cell behaviors affected. At present, such a classification is impossible.

Further Details on Genes Listed in Table A2

Gene symbol (synonym or mutation symbol); Gene name (mutation name); Reference(s); GenBank accession number (when available).

Mus musculus gene nomenclature as in Entrez, Nucleotide Sequence Search: http://www.ncbi.nlm.nih.gov/Entrez/nucleotide.html

LEVEL 3 GENES:

XD++ genes:

Calr (=CRT); calreticulin; Rauch et al. (2000); NM_007591.

Efna5; ephrin A5; Holmberg et al. (2000); NM_010109.

Enah (+Profilin 1 [Pfn1]−/+); enabled, *Drosophila,* homolog of, also known as Mammalian enabled (Mena); Lanier et al. (1999); NM_010135.

jmj; jumonji (gene trap insertion); Takeuchi et al. (1995, 1999); Toyoda et al. (2000); NM_021878.

Macs; gene encoding MARCKS (Myristoylated, Alanine-Rich C-Kinase Substrate) protein; Stumpo et al. (1995); NM_008538.

Mlp; gene encoding MARCKS-like protein (MLP), also known as F52 (Wu et al., 1996), or MacMARCKS (Chen et al., 1996); NM_010807 and X61399.

Pax3 (Sp); paired box gene 3 (locus corresponding to the mutation splotch); Epstein et al. (1991); Li et al. (1999); NM_008781.

RhoGAP5 (=Arhgap5 = p190 RhoGAP); rho GTPase activating protein 5 (=p190 RhoGTPase Activating Protein); Brouns et al. (2000); NM_009706.

shrm; shroom PDZ domain-containing actin-binding protein (gene trap insertion); Hildebrand and Soriano (1999); NM_015756.

twist; Twist, *Drosophila,* homolog of; Chen and Behringer (1995); NM_011658.

. . .

Cart-1; Cartilage Homeoprotein 1; Zhao et al. (1996); AA388194.

Jnk1 + Jnk2; c-jun NH2-terminal kinase 1 and 2; Kuan et al. (1999); Sabapathy et al. (1999).

RBP-J kappa; recombination signal-binding protein 1 for J-Kappa, also, known as recombining binding protein suppressor of hairless-like (*Drosophila*) (Rbpsuhl); Oka et al. (1995); NM_009035 and NM_009036.

c-ski; v-ski avian sarcoma viral oncogene homolog; Lyons et al. (1994); Berk et al. (1997); U14173.

Tcfap2a (=AP2); transcription factor activating enhancer-binding protein 2, alpha; Schorle et al. (1996); Zhang et al. (1996); NM_011547.

terc; telomerase RNA component; Herrera et al. (1999); AF047387.

D++ genes:

abl + arg; tyrosine kinases; Koleske et al. (1998); L10656 + U40827.

Csk; c-src tyrosine kinase; Imamoto and Soriano (1993); NM_007783.

Fkbp1a; FK506 binding protein 1a (12 kDa); Shou et al. (1998); NM_008019.

Itga3 + Itga6; integrin alpha3 + alpha6; De Arcangelis et al. (1999) NM_013565 + NM_008397.

Lama5; laminin alpha 5 chain; Miner et al. (1998); U37501.

Pig-a; phosphatidylinositol glycan, class A; Nozaki et al. (1999); S78188.

Vcl; vinculin; Xu et al. (1998); NM_009502.

. . .

apoB; apolipoprotein B; Homanics et al. (1995); Huang et al. (1995); M35186.

Bcl10; B-cell leukemia/lymphoma 10; Ruland et al. (2001); NM_009740.

Folbp1; folate binding protein 1; Piedrahita et al. (1999); NM_008034.

Ikk1 + Ikk2; I-kappa-B kinase 1 and 2; Hu et al. (1999); Li et al. (2000).

Psen1 + Psen2; presenilin 1 and presenilin 2; Lee et al. (1996); Donoviel et al. (1999); NM_008943 + NM_011183.

XD+− genes:

Hes1; hairy and enhancer of split homolog 1; Sasai et al. (1992); Ishibashi et al. (1995); NM_008235.

Hoxa1; homeo box A1 (or 1F or −1.6) gene; Lufkin et al. (1991); NM_010449.

Ptch; patched, Drosophila, homolog of; Goodrich et al. (1997); NM_008957.

Zic2; zinc finger protein of the cerebellum 2 (knockdown); Nagai et al. (2000); NM_009574.

D+− genes:

Arnt; aryl hydrocarbon receptor nuclear translocator; Kozak et al. (1997); Maltepe et al. (1997); NM_009709.

Axin1 (Fused locus); also known as Axis inhibitor 1; Zeng et al. (1997); AF009011.

Brca1; murine homologue of the human breast and ovarian cancer susceptibility gene, type 1; Gowen et al. (1996); Hakem et al. (1996).

Fdft1 (=SS); farnesyl diphosphate farnesyl transferase 1, also known as squalene synthase; Tozawa et al. (1999); NM_010191.

Madh5 (=Smad5); Mothers Against Decapentaplegic, homolog 5; Chang et al. (1999); NM_008541.

LEVEL 2 GENES:

XD+ genes:

Fgfr1; fibroblast growth factor receptor 1; Deng et al. (1997); Xu et al. (1999); NM_010206.

Nf1; Neurofibromatosis type 1, also known as Neurofibromin; Lakkis et al. (1999); X54924.

Nog; Noggin; McMahon et al. (1998); NM_008711.

Shh; sonic hedgehog, *Drosophila,* homolog of; Chiang et al. (1996); X76290.

Traf6; Tumor necrosis factor receptor-associated factor 6; Lomaga et al. (2000); NM_009424.

D+ genes:

Raldh2; retinaldehyde dehydrogenase 2; Niederreither et al. (1997); Niederreither et al. (1999); NM_009022.

Sil; SCL-interrupting locus, also known as Tall interrupting locus; Izraeli et al. (1999); NM_009185.

XD− genes:

Apaf1; apoptotic protease activating factor1; Yoshida et al. (1998); NM_009684.

Nap1|2; nucleosome assembly protein 1-like 2; Rogner et al. (2000); NM_008671.

D− genes:

Atoh4 (=Ngn2); atonal homolog 4, also known as neurogenin2; Fode et al. (1998); NM_009718.

Casp3; Caspase 3 (=CPP32), apoptosis related cysteine protease 3; Kuida et al. (1996); NM_009810.

Casp9; Caspase 9; Kuida et al. (1998); NM_015733.

Gata3; GATA-binding protein 3; Pandolfi et al. (1995); NM_008091.

Hspg2 (=Plc); heparan sulfate proteoglycan of basement membrane 2, also known as perlecan; Costell et al. (1999); NM_008305.

Pdgfra; platelet derived growth factor receptor, alpha polypeptide (deleted in Patch mutation); Payne et al. (1997); NM_011058.

TREB5; tax-responsive element-binding protein 5, also known as CRE-binding factor; Masaki et al. (1999); AB036745. *O− genes:*

Gja1 (=Cx43); gap junction membrane channel protein alpha 1, also known as gap junction polylpeptide connexin 43-kD or alpha 1 connexin; Reaume et al. (1995); NM_010288.

Pax1; paired box gene 1; Wilm et al. (1998); NM_008780.

LEVEL 1 GENES:

XD genes:

Dlx5; distal-less homeobox 5; Acampora et al. (1999); Depew et al. (1999); NM_010056.

Dnmt3b; DNA methyltransferase 3B; Okano et al. (1999); NM_010068.

Foxb1a (=Mf3); forkhead box B1a, also known as Fkh5, TWH or Hfh-e5.1; Labosky et al. (1997); NM_010793.

Gli3 (Xt); GLI-Kruppel family member GLI3 (Extratoes mutation); Schimmang et al. (1992); Hui et al. (1994); NM_008130.

Msx1 + Msx2; muscle segment homeobox (msh), homolog 1 and 2; Foerst-Potts et al. (1997); NM_010835 + NM_013601.

X genes:

16s rRNA; mitochondrial 165 ribosomal RNA; Ibrahim et al. (1998); AF089815.

Gas5; growth arrest specific 5; Vacha et al. (1997); NM_013525.

rnr-r1; ribonucleotide reductase R1 subunit; Craig et al. (2000); X72306.

D genes:

Cbp; CREB-binding protein; Yao et al. (1998); S66385.

Gadd45a; growth arrest and DNA-damage-inducible, alpha; Hollander et al. (1999); NM_007836.

Gap (+Nf1); encoding p120 rasGTPase-Activating Protein; Henkemeyer et al. (1995).

p300; E1A binding protein, 300 kD; Yao et al. (1998).

Rara + Rarg; retinoic acid receptor, alpha and retinoic acid receptor, gamma; Lohnes et al. (1994); NM_009024 + NM_011244.

Trp 53; transformation related protein 53, also known as Tumor Protein p53; Armstrong et al. (1995); Sah et al. (1995); Ibrahim et al. (1998); AJ297973.

Tsc2; tuberous sclerosis 2 gene, protein product designated tuberin; Kobayashi et al. (1999) NM_011647.

Xpc; (+Trp53); xeroderma pigmentosum, complementation group C; Cheo et al. (1996); NM_009531.

O genes:

Dpp6 (and/or **Kit**); dipeptidyl aminopeptidase-like protein 6; Hough et al. (1998); AF092505.

Itgb1d; integrin_1D Baudoin et al. (1998); U37029.

Notch 3; Notch, homolog 3; Lardelli et al. (1996); NM_008716.

Zic3; zinc finger protein of the cerebellum 3; Klootwijk et al. (2000) NM_009575.

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