

THE GENETIC BASIS OF MAMMALIAN NEURULATION

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More than 80 mutant mouse genes disrupt neurulation and allow an in-depth analysis of the underlying developmental mechanisms. Although many of the genetic mutants have been studied in only rudimentary detail, several molecular pathways can already be identified as crucial for normal neurulation. These include the planar cell-polarity pathway, which is required for the initiation of neural tube closure, and the sonic hedgehog signalling pathway that regulates neural plate bending. Mutant mice also offer an opportunity to unravel the mechanisms by which folic acid prevents neural tube defects, and to develop new therapies for folate-resistant defects.

ECTODERM

The outer of the three embryonic (germ) layers that gives rise to the entire central nervous system, plus other organs and embryonic structures.

NEURAL CREST

A migratory cell population that arises from the midline of the neural tube, which gives rise to a range of cell types in the developing embryo.

Neurulation is a fundamental event of embryogenesis that culminates in the formation of the neural tube, which is the precursor of the brain and spinal cord. A region of specialized dorsal ECTODERM, the neural plate, develops bilateral neural folds at its junction with surface (non-neural) ectoderm. These folds elevate, come into contact (appose) in the midline and fuse to create the neural tube, which, thereafter, becomes covered by future epidermal ectoderm. As a model of embryonic morphogenesis, neurulation has long attracted the interest of developmental biologists¹. Epidemiologists and clinicians have also focused on neurulation, with the aim of understanding the origin of neural tube defects (NTDs), which are a group of severely disabling or life-threatening congenital malformations² (BOX 1). Recently, the prospect of using folic acid during early pregnancy to normalize neurulation and prevent the development of human NTDs³ has led to a renewed research focus on neurulation.

Notwithstanding the long history of neurulation studies, the fundamental developmental mechanisms of neural tube closure remain poorly understood. Although the cellular events of neurulation have been described in detail⁴, knowledge of its molecular regulation has lagged behind other areas of neural tube biology. For example, a great deal is known about the molecular regulation of the NEURAL CREST⁵ and the emergence of specialized neuronal populations at

distinct locations in the brain and spinal cord⁶. By contrast, the mechanisms that underlie the formation, elevation and fusion of the neural folds have remained elusive.

An opportunity has now arisen for an incisive analysis of neurulation mechanisms using the growing battery of genetically targeted and other mutant mouse strains in which NTDs form part of the mutant phenotype⁷. At least 80 mutant genes have been shown to affect neurulation (ONLINE TABLE 1). Moreover, the regional location of NTDs (brain versus spine) differs between mutants, which argues that there is a region-specific difference in neurulation-related gene expression.

In this review, we attempt a mechanistic analysis of the genetic influences on neurulation. We identify the main categories of genes that are required for each successive event of neurulation, and relate these functional gene groups to probable mechanisms. The initiation of neural tube closure, neural fold elevation and bending, and adhesion and fusion of the neural folds are each considered separately, as are several cranial neurulation-specific events and requirements. The prevention of NTDs by exogenous agents is reviewed briefly in relation to the possible underlying developmental mechanisms. Our aim is to create a framework in which future analysis of the genetic regulation of neurulation can proceed in a focused manner.

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Box 1 | Neural tube defects: common malformations of the central nervous system

Neural tube defects (NTDs) are a group of congenital malformations that arise when the neural tube fails to close during embryogenesis. NTDs occur at an average rate of 1 per 1,000 pregnancies worldwide, and are the second most prevalent malformations, after congenital heart defects, among human pregnancies. If closure fails in the developing brain, the result is **exencephaly**, in which the persistently open cranial neural folds have an everted appearance and seem transiently enlarged. As development proceeds, the exposed neural folds degenerate, which produces the defect **anencephaly** by late in gestation. In the anencephalic fetus, the interior of the brain is exposed to the outside and the skull vault is absent. Anencephaly can be associated with facial malformations, such as split face, which indicates that the most **ROSTRAL** part of the neural tube has failed to close. A related cranial defect is **encephalocele**, in which the neural tube closes normally but part of the brain herniates through a defect in the bony skull. In the spinal region, the failure of initiation of closure at the upper spinal level results in the severe defect **craniorachischisis**, in which most of the brain and the entire spinal cord remain open. The commonest defect of spinal closure, however, involves the lower spinal neural tube, which produces open **spina bifida** (also called **myelomeningocele** or **myelocele**). Unlike the cranial defects, which are usually lethal at or before birth, spina bifida is compatible with postnatal survival; however, affected individuals can suffer from motor and sensory defects in the legs, urinary and faecal incontinence, vertebral curvature defects and hydrocephalus (increased cerebrospinal-fluid pressure in the brain). A milder group of defects (**occult spina bifida** or **spinal dysraphism**) result from defective secondary neurulation, in which the spinal cord fails to separate from the adjacent tissues. Tethering of the spinal cord prevents its normal mobility in the vertebral canal and can cause leg weakness and difficulties in gaining urinary continence in young children.

The neurulation sequence and types of NTD

The targeted/mutant gene effects are best viewed in the context of the sequential steps of mammalian neural tube formation, as different mutant genes affect different neurulation events.

Primary neurulation. Neurulation is conventionally divided into primary and secondary phases. In primary neurulation, the neural tube forms by the shaping, folding and midline fusion of the neural plate. The neural tube then becomes covered by surface ectoderm that previously flanked the neural plate. Primary neurulation creates the brain and most of the spinal cord. A transition from primary to secondary neurulation occurs at the future upper sacral level⁸.

Secondary neurulation. At more **CAUDAL** levels, the neural tube is formed in the **TAIL BUD** (also called the caudal eminence) (FIG. 1) without neural folding. The tail bud comprises a stem-cell population that represents the remnant of the retreating **PRIMITIVE STREAK**. Mesenchymal cells in the dorsal part of the tail bud undergo condensation and epithelialization to form the secondary neural tube, the lumen of which is continuous with that of the primary neural tube⁹. Secondary neurulation creates the lowest portion of the spinal cord, including most of the sacral and all of the coccygeal regions.

Rostro-caudal events in primary neurulation. In the mouse, primary neural tube closure is initiated at the hindbrain/cervical boundary (closure 1), and then proceeds concurrently in both the future brain and spinal regions (FIG. 1). Brain closure comprises *de novo* events at the forebrain/midbrain boundary (closure 2) and at the extreme rostral end of the forebrain (closure 3). Closure between these initiation sites leads to completion of cranial neurulation at the anterior and hindbrain neuropores. Unidirectional closure along the spinal axis culminates in closure of the posterior neuropore, which marks the end of primary neurulation.

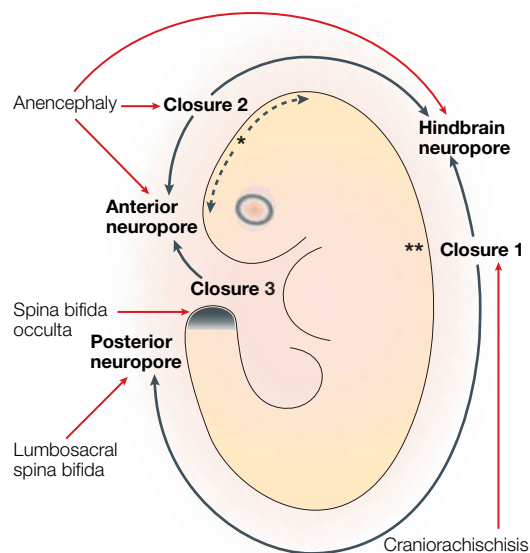


Figure 1 | The rostro-caudal sequence of neurulation events in the mouse embryo. The sequence of events¹¹⁰ begins with neural tube closure (closure 1), which is initiated at the hindbrain/cervical boundary (double asterisks) at the six to seven somite stage (embryonic day (E) 8.5). Neural tube closure spreads rostrally and caudally from this site. A second *de novo* closure event (closure 2) occurs at the forebrain/midbrain boundary (single asterisk) in most mouse strains, although more rostral and caudal locations of closure 2 occur in some strains (dashed lines and arrows). Closure also initiates separately at the rostral extremity of the forebrain (closure 3). Neurulation progresses caudally from closure 3 to meet the rostral spread of fusion from closure 2, with completion of closure at the anterior (or rostral) **NEUROPORE**. The spread of closure caudally from closure 2 meets the rostrally directed closure from closure 1 to complete closure at the hindbrain neuropore. The caudal spread of fusion from closure 1 progresses along the spinal region over a 36-hour period, with final closure at the posterior (or caudal) neuropore. Secondary neurulation proceeds from the level of the closed posterior neuropore, through canalization in the tail bud (shaded area). The main types of neural tube defect that arise from the failure of these closure events are indicated. Modified with permission from REF. 2 © (2002) Arnold, and REF. 111 © (1994) Lippincott, Williams and Wilkins.

ROSTRAL
The front end of the body axis of the developing embryo.

CAUDAL
The tail end of the body axis of the developing embryo.

TAIL BUD
The population of stem cells at the extreme caudal end of the embryo that contains the progenitor cells for formation of the lowest levels of the body axis.

NEUROPORE
A transient 'hole-like' opening in the neural tube at which neural tube closure is undergoing completion.

PRIMITIVE STREAK
The structure in the gastrulation-stage embryo at which ectoderm to mesoderm transformation occurs, with epithelium to mesenchyme transformation.

The positions of closures 1 and 3 seem to be invariant among mouse strains, and both certainly occur in human embryos¹⁰, whereas the position of closure 2 is polymorphic¹¹. In some mouse strains this closure is relatively caudal in the midbrain, whereas other strains undergo closure 2 more rostrally in the forebrain. Notably, strains with a caudal closure 2 are resistant to exencephaly, whereas those with a rostral closure 2 are highly predisposed¹². In the latter strains, closures 2 and 3 might be located extremely close together, making it difficult to distinguish between the two events. This variation in the morphology of mouse closure 2 resembles the human situation, in which a distinct closure 2 event has been inconsistently observed^{10,13}. So, the variable occurrence of closure 2 might represent an important variable risk factor for NTDs in humans and could explain some of the interstrain variation in NTD frequency that is observed in gene-targeted mouse models.

NTDs vary at different axial levels. A fundamental principle of neurulation is that only the levels of the body axis that undergo primary neurulation (that is, the brain and the cervical, thoracic, lumbar and upper sacral spine) lead to 'open' NTDs (for example, anencephaly, open spina bifida and craniorachischisis). The abnormality involves a pathological connection between the neural tube lumen and the outside environment. By contrast, defective secondary neurulation leads to 'closed' forms of spina bifida (also called 'dysraphic' conditions), which represent the failure of the emerging spinal cord to become separated from other tissue derivatives of the tail bud. Hence, spinal cord 'tethering' to adjacent tissues is a prominent feature of closed lesions that affect the lower part of the spine¹⁴. In contrast to the many mutant models of open NTDs, few mouse mutants (for example, *Gcm1*; see ONLINE TABLE 1) are known to have caudal closed NTDs, and this topic is not considered further in this review.

When closure 1 fails, almost the entire neural tube from the midbrain to the lower spine remains open, which is a condition known as craniorachischisis (FIG. 2a). Closures 2 and 3 are usually normal in such embryos, which have a closed and relatively well-developed forebrain and anterior midbrain. By contrast, embryos in which closure 2 fails or is disrupted at the anterior or midbrain–hindbrain neuropores, have exencephaly (FIG. 2b). Subsequent degeneration of the exposed neural

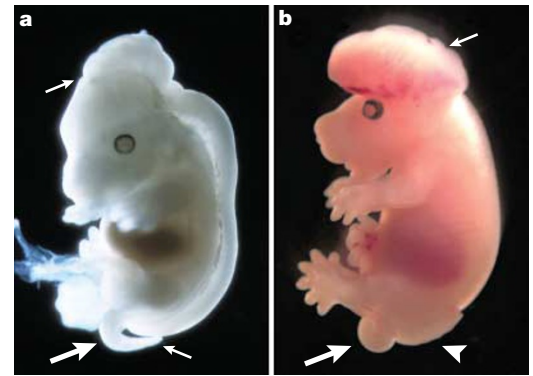


Figure 2 | Mouse fetuses with neural tube defects. Mouse fetuses at embryonic day (E) 15.5 illustrate the appearance of (a) craniorachischisis in a *Celsr1* mutant and (b) exencephaly and open spina bifida in a *curly tail* (*ct*) mutant. In craniorachischisis, the neural tube is open from the midbrain to the lower spine (between the two thin arrows in a). In the fetus shown in b, exencephaly is restricted to the midbrain (thin arrow in b), whereas spina bifida affects the lumbosacral region (arrowhead in b). Note the presence of a curled tail in both fetuses (thick arrows in a and b).

folds converts exencephaly, by late gestation, into anencephaly, in which the skull vault is missing and the brain tissue is destroyed. The specific failure of closure 3 leads to anencephaly that is confined to the forebrain region, often in association with a split-face malformation. If the spread of closure fails to be completed along the spinal region, the posterior neuropore remains open, which results in open spina bifida (also called myelocoele or myelomeningocele) (FIG. 2b).

When studying these mouse mutants, it should be noted that mid-gestation embryonic lethality can mimic NTDs, and care is needed to ensure that embryonic lethality and/or degenerative processes are not responsible for preventing normal development beyond neurulation (BOX 2).

Initiation of closure

Immediately preceding the onset of neural tube closure (closure 1), the embryo undergoes neural plate shaping (FIG. 3). The initially elliptical neural plate is converted to an elongated keyhole-shaped structure with broad cranial (rostral) and narrow spinal (caudal) regions. Described originally in amphibian and avian embryos^{15,16}, the

Box 2 | Mid-gestation embryonic lethality can mimic NTDs

Embryonic lethality around the stage of neural tube closure is often preceded by developmental retardation, so that dying embryos might seem to fail to close their neural tubes. However, if embryos die before the stage at which a particular aspect of neurulation would normally be completed, the finding of an open neural tube is not reliable evidence of a neurulation defect. For example, embryos that lack an active c-src tyrosine kinase (*Csk*) gene die after ~9 days of gestation, when their cranial neural tube has not yet closed, although prolonged survival might have been compatible with successful closure¹⁰⁸. Other examples of early embryonic lethality with apparent NTD formation are listed in ONLINE TABLE 1. Before concluding that a mouse mutant has NTDs, therefore, care must be taken to ensure that embryonic lethality and/or degenerative processes are not responsible for preventing normal development beyond neurulation. Mouse embryos do not die *in utero* because of NTDs¹⁰⁹ and, hence, lethal anomalies such as faulty maternal–embryonic connections or defective cardiovascular development are probably present. By altering the genetic background, or through the use of conditional gene-targeting approaches, it is increasingly possible to prolong the survival of embryos with lethal gene defects, which allows an evaluation of the role of the particular gene in neurulation.

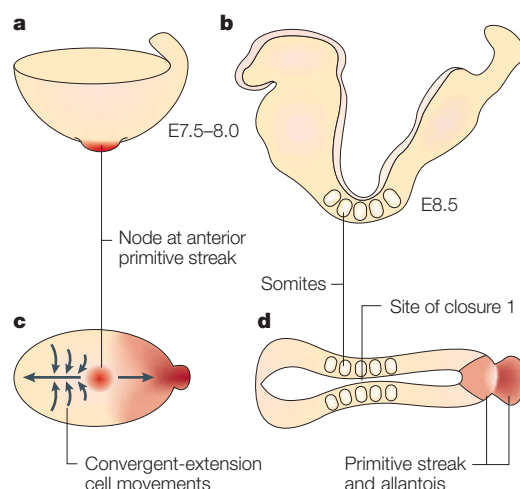


Figure 3 | Shaping of the neural plate at the onset of mouse neural tube closure. Views from the left side (**a,b**) and top (**c,d**) of embryonic day (E) 7.5–8.0 (**a,c**) and E8.5 (**b,d**) embryos. At E7.5–8.0, the retreating node, which is the site of origin of midline tissues including the NOTOCHORD and neural tube floor plate, is prominent at the anterior end of the primitive streak. Anterior to the node, cells move medially and intercalate in the midline — a process that is known as convergent extension — thereby increasing embryonic length relative to width. By E8.5, the primitive streak occupies only the caudal part of the embryo, with a well-defined anterior neural plate that is flanked by five pairs of SOMITES. The neural folds at the level of the third somite pair approach each other in the midline, to create the incipient closure 1 site.

main driving force for neural plate shaping seems to be convergent extension: a net medially directed movement of cells, with intercalation in the midline, which leads to narrowing and lengthening of the neural plate¹⁷. Similar cell movements occur simultaneously in both the neur ectoderm and the underlying mesoderm.

Recently, the requirement for convergent extension during the initiation of neural tube closure has become clear. To our knowledge, only a few mouse mutants — *loop-tail*, *crash*, *circletail* and *dishevelled-1;dishevelled-2* double mutants — fail to undergo closure 1, which subsequently leads to craniorachischisis. Positional cloning shows that each of the mutant genes in which craniorachischisis is observed encodes a protein that functions in non-canonical Wnt/frizzled signalling: the so-called planar cell-polarity pathway^{18–23}. *loop-tail* is a mutation of the mouse ORTHOLOGUE of the *Drosophila* gene *strabismus* — also called *Van Gogh* (*Vang*) — that interacts with *dishevelled* in planar cell-polarity signalling. *crash* mice harbour a mutation in *Celsr1*, which is a vertebrate orthologue of the *Drosophila* gene *flamingo* — also known as *starry night* (*stan*) — that encodes a binding partner of frizzled. *Scrib1*, which is the gene that is mutated in *circletail*, is also required for planar cell polarity²², although it is best known for regulation of apical–basal polarity in *Drosophila*. Not only do homozygotes for *loop-tail*, *circletail* and *crash* have severe NTDs, but the double mutants *loop-tail; circletail* and *loop-tail; crash* also have this phenotype²⁴, which is indicative of a shared developmental function.

The crucial role of planar cell polarity in regulating convergent extension and the onset of neurulation has been investigated experimentally in *Xenopus*. Misexpression of *dishevelled* or *strabismus* produces embryos with an abnormally short and broad neural plate in which the neural tube fails to close^{25–28}. A similar phenotype occurs in zebrafish after misexpression or mutation of the *strabismus* orthologue *trilobite* (*tri*)²⁹ and misexpression of Rho kinase 2, which lies downstream of Wnt11 in planar cell-polarity signalling³⁰. The *Xenopus* defects that result from *dishevelled* misexpression are similar to the NTDs in *loop-tail*, *circletail* and *crash* embryos. The neural plate is abnormally broad with a non-bending region intervening between the neural folds^{18,31}, in contrast to the well-defined MEDIAN HINGE POINT (MHP) in normal embryos (FIG. 4b). Although neural fold elevation (see below) occurs normally, the neural folds are located too far apart to achieve closure. Hence, normal convergent extension is required to establish a neural plate of a width that is compatible with the medial bending that is essential for closure 1.

It is notable that the *loop-tail*, *circletail*, *crash* and *dishevelled* group of functionally related mutants are the only known mouse models of craniorachischisis. All other mutants have exencephaly and/or spina bifida. This indicates that closure 1, although highly dependent on the establishment of planar cell polarity, is relatively independent of the later events that are crucial at other levels of the body axis.

Elevation and apposition of the neural folds

Bending of the mammalian neural plate occurs at two principal sites: the MHP that overlies the notochord (as at closure 1) (FIG. 4b), and the paired dorsolateral hinge points (DLHPs) that are situated at the point of attachment of the surface ectoderm to the outside of each neural fold³² (FIG. 4c,d). The MHP is induced by signals from the notochord and is the sole site of bending in the upper spinal neural plate. Experimental studies with cultured mouse embryos have shown that sonic hedgehog (Shh) that emanates from the notochord inhibits DLHP formation at this upper spinal level³³. As the wave of spinal neurulation passes down the NEURAXIS, the strength of Shh signalling from the underlying notochord lessens, which allows DLHP formation to ‘break through’ at lower spinal levels. DLHP formation in the lower spine can be inhibited by the local release of Shh peptide, which shows the negative regulation of dorso-lateral bending by Shh. A similar regulatory mechanism probably operates in the cranial region in which dorso-lateral bending is also prominent during neurulation, although this has not been examined in detail.

The idea that Shh negatively regulates DLHP formation is also supported by the phenotype of several gene-targeted mice. In the *Shh*-null mutant, DLHPs are observed at all levels of the body axis and the neural tube closes successfully³³. By contrast, *Shh* overexpression can produce NTDs³⁴, presumably by inhibiting dorso-lateral bending at levels of the body axis at which it is essential for closure. A similar mechanism probably underlies the cranial neurulation defects that are observed in the

NOTOCHORD

The rod-like mesodermal structure that extends the length of the body axis, beneath the neural tube of vertebrate embryos.

SOMITES

Segmented blocks of mesoderm on either side of the neural tube in vertebrate embryos.

ORTHOLOGUE

A gene that is the evolutionary counterpart of a similar gene in another species.

MEDIAN HINGE POINT

(MHP). A single midline bending point in the closing neural tube.

NEURAXIS

The developing central nervous system and its main subdivisions, both in the developing brain (forebrain, midbrain and hindbrain) and the spinal cord (cervical, thoracic, lumbar, sacral and caudal/coccygeal).

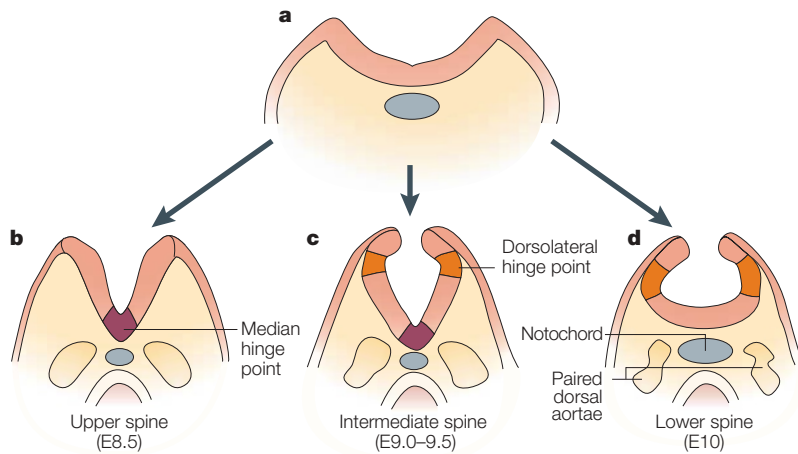


Figure 4 | Transition in the morphology of neurulation along the developing spine, as seen in schematic transverse section. The posterior neuropore translocates down the body axis, as development progresses from embryonic day (E) 8.5–10. After initial neural fold bending (a), neural fold elevation varies in morphology along the body axis, with bending solely at the median hinge point (MHP) at upper levels of the body axis (b), at both the MHP and the paired DORSOLATERAL HINGE POINTS (DLHPs) at intermediate spinal levels (c), and solely at DLHPs at the lowest spinal levels (d). Modified with permission from REF. 33 © (2002) The Company of Biologists.

DORSOLATERAL HINGE POINT (DLHP). Paired bending points in the dorsolateral region of the closing neural tube.

EPHRINS
A family of cell-surface ligands that interact with a family of cell-surface receptor tyrosine kinases (Eph receptors), which is implicated in the interaction of cell types and the mediation of chemorepulsion and cell adhesion.

GLYCOSYLPHOSPHATIDYL-INOSITOL (GPI) ANCHORS
Inositol-containing linkages that tether some proteins (for example, ephrins) to the cell surface.

homozygous *extra-toes* mouse, which is mutant for the negative regulator of Shh signalling *Gli3* (REF. 35). By contrast, *Gli1* and *Gli2* mediate the Shh signal, and mutation of these genes does not lead to NTDs^{36,37}. The Patched1 (*Ptc1*) receptor is responsible for maintaining Shh signalling in an 'off' state, in the absence of Shh ligand. Hence, mutation of *Ptc1* leads to ligand-independent constitutive Shh signalling and, as is seen for Shh overexpression, *Ptc1*-null mice have severe NTDs³⁸. Protein kinase A (PKA) is a heteromeric enzyme the functions of which include inhibition of Shh signalling. There is functional redundancy between the several PKA genes, but reducing the dose of PKA by 75% leads to a high frequency of spinal defects and partially penetrant exencephaly³⁹. Hence, over-stimulation of the Shh signalling pathway inhibits dorsolateral bending of the closing neural folds, which leads to both spina bifida and exencephaly in the various mutants. This indicates that the Shh regulation of neural fold bending is a mechanism that operates during neurulation at all levels of the body axis.

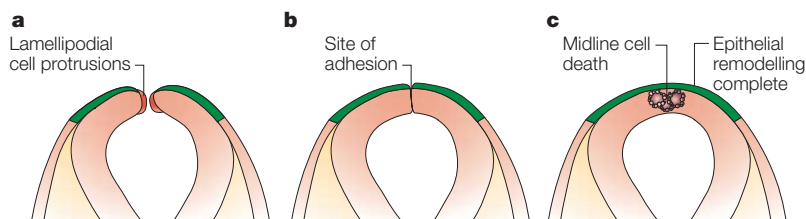


Figure 5 | Adhesion, fusion and remodelling of the midline at completion of neural tube closure. a | The site of neural fold fusion is characterized by lamellipodial cell protrusions from the apposing neural fold apices. b | The protrusions interdigitate, which leads to adhesion and fusion of the neural folds. c | Subsequent remodelling of the epithelia, which is accompanied by apoptotic cell death, leads to disruption of the connection between the surface (non-neural) ectoderm (green) and neuroepithelium on each side, and the establishment of separate surface ectodermal and neuroepithelial continuity across the midline.

Two further mutants, *open-brain* (*opb*) and *Zic2*, cause failure of neural tube closure in both the brain and lower spine^{40,41}. *opb* disrupts the *Rab23* gene, which encodes a negative regulator of Shh signalling⁴², whereas *Zic* genes, which encode a family of transcription factors that are named after zinc finger protein of the cerebellum, are reported to exert anti-Shh activity in *Xenopus*⁴³. In *opb* and *Zic2* homozygotes, dorsal and dorsolateral cell fates do not seem to be specified in the developing neural tube, as indicated by the lack of expression of genes such as *Mxsl* and *Wnt3a*^{41,44}. This might indicate that the action of Shh in negatively regulating DLHP formation at high spinal levels is to ventralize the closing neural tube and prevent dorsally located cells from differentiating in a direction that is necessary for DLHP participation. How such a mechanism relates to the regulation of morphogenetic cell behaviours such as cell-cycle progression⁴⁵ and cell-adhesion strength⁴⁶, the variation of which might underlie neural plate bending, is unclear at present.

Fusion of the neural folds

Any genetic disturbance that alters the adhesion of the neural fold apices should prevent neural tube closure. In practice, however, it has proven difficult to distinguish experimentally between the failure of neural fold elevation and neural fold fusion in mutant embryos. Unfused neural folds rapidly splay apart, which mimics defective neural fold elevation. As a consequence, few of the genetic NTD models are definitely known to disturb neural fold adhesion and/or fusion. Moreover, the molecules that mediate neural fold adhesion and fusion are poorly understood. Cellular protrusions extend from apical cells on the neural folds as they approach one another in the dorsal midline (FIG. 5a) and interdigitate as the folds come into contact⁴⁷. This might allow cell–cell recognition and provide an initial adhesion before more permanent cell contacts are established. The tips of the mouse neural folds are associated with a 'cell-surface coat' of carbohydrate-rich material^{48,49} the removal of which, by treatment with phospholipase C, prevents neural fold fusion⁵⁰. This finding has gained significance following a report of exencephaly that affected a proportion of embryos with a null mutation of *EPHRIN-A5*, which is a GLYCOSYLPHOSPHATIDYLINOSITOL (GPI)-LINKED cell-surface Eph ligand⁵¹. Inactivation of the EphA7 receptor also produces exencephaly⁵¹. In homozygotes, the neural folds seem to elevate and appose normally at the dorsal midline, but fusion is defective. Both ephrin-A5 and EphA7 are expressed in the cranial neuroepithelium before and during neural tube closure, but not in the spinal neuroepithelium, which accounts for the lack of spinal NTDs in these mice. These findings indicate a potentially crucial role of ephrin/Eph receptor interactions in neural fold fusion.

Traditionally, the fusion and subsequent separation of the neural tube and surface ectoderm have been thought to result from the differential adhesion of the two tissue types. The neural tube expresses the cell-adhesion proteins N-CAM (neural cell-adhesion molecule) and neural (N)-cadherin, whereas the surface

ectoderm expresses epithelial (E)-cadherin⁵². Indeed, *Xenopus* embryos in which the neural tube and surface ectoderm both express N-cadherin fail to separate their neural tube and surface ectoderm and undergo defective neural tube closure^{53,54}. Expression of a dominant negative form of N-cadherin in *Xenopus* embryos also causes defects in neural tube formation, although expression of a dominant negative E-cadherin molecule does not have this effect⁵⁵. However, ectopic expression of N-CAM in the surface ectoderm does not inhibit neural tube closure⁵³ and mice with null mutations in the *Ncam* or N-cadherin (also called *Cdh2*) genes undergo normal neural tube closure^{56,57}. So, it seems unlikely that these cell-adhesion molecules have an essential role in mammalian neurulation.

Neurulation events specific to the cranial region

Mouse cranial closure is more susceptible to disturbance than spinal closure, as evidenced by the more frequent occurrence of exencephaly than spina bifida in the knockouts and mutants (ONLINE TABLE 1). Similarly, many more teratogenic agents induce cranial rather than spinal NTDs⁵⁸. As well as the regulation of dorsolateral bending by Shh, which probably operates in both cranial and spinal regions, several other cellular events are essential for the successful elevation and apposition of the cranial neural folds (summarized in FIG. 6). These mechanisms seem to be solely relevant to the cranial region, as spinal defects are not observed in gene-targeted mice in which these putative mechanisms are faulty.

Expansion of the cranial mesenchyme. Elevation of the cranial neural folds in mammalian embryos begins with an initial phase in which the cranial mesenchyme undergoes marked expansion, with cell proliferation and a notable increase in the extracellular space⁵⁹. In the midbrain region, in particular, this phase of cranial neurulation produces biconvex neural folds that bulge outwards (FIG. 6a). In the second phase of cranial closure, the edges of the neural folds ‘flip around’ and approach the dorsal midline, with a biconcave morphology, until the tips of the neural folds meet in the midline⁶⁰ (FIG. 6b). This two-phase progression of cranial neurulation is unlike that seen in the spinal region, in which expansion of the PARAXIAL MESODERM does not accompany neural fold elevation. At upper spinal levels, the neural folds are flanked by epithelial somites, whereas at lower levels, compact presomitic mesoderm flanks the spinal neural folds. Moreover, experimental removal of the paraxial mesoderm fails to block closure of the spinal neural folds^{33,61}. By contrast, cranial neurulation seems to be highly dependent on proliferation and expansion of the cranial mesenchyme, as evidenced by the following knockout strains.

Twist and *Cart1* knockouts both have cranial NTDs, with the principal defect affecting proliferation and expansion of the cranial mesenchyme^{62,63}. Both genes are expressed in the mesenchyme, and chimeric analysis in the *Twist* mutant shows that a high proportion of *Twist*^{-/-} cells in the mesenchyme can lead to an exencephalic phenotype. By contrast, an abundance of

Twist^{-/-} cells in the neural plate does not compromise closure⁶². It seems likely, therefore, that the cranial mesenchyme has a vital supporting role in the first phase of cranial neurulation. This idea is supported by the finding that digestion of extracellular matrix HYALURONAN in cultured rat embryos causes the cranial mesenchyme to collapse and leads to a delay in cranial closure⁶⁴.

Actin cytoskeleton and cranial neurulation. The second phase of cranial neurulation, in which the neural folds become biconcave, seems highly dependent on the actin cytoskeleton. The targeting of genes that are required for normal cytoskeletal function leads to cranial NTDs in several instances. Exencephaly is observed in mice that lack the filamentous (F)-actin-associated protein shroom⁶⁵, the actin-binding protein vinculin⁶⁶, the protein kinase C target MARCKS (which crosslinks actin filaments)⁶⁷ and the RhoGAP p190 regulator of actin cytoskeleton⁶⁸. Cranial NTDs are also seen in embryos that are double mutants for the cytoskeleton-related genes *Mena* (also called *Enah*) and profilin (*Pfn1*)⁶⁹, and the non-receptor tyrosine kinases *Abl* and *Arg*, the functions of which are actin-related⁷⁰. It is notable that all of these knockout mice have defects only of cranial neurulation, whereas the spinal neural tube closes normally in the null mutant embryos.

These mouse mutant data are consistent with studies using cytochalasins, which are drugs that disassemble actin microfilaments. Cranial neural tube closure in chicken, mouse and rat embryos is highly sensitive to cytochalasins, whereas spinal neurulation is relatively resistant^{71,72}. Bending of the mouse neural plate at the MHP and DLHPs continues, with successful closure of the spinal neural tube, during the treatment of cultured mouse embryos with cytochalasin D⁷². These findings show that the ‘purse-string’ contraction of sub-apically arranged actin–myosin microfilaments in neuroepithelial cells is dispensable for spinal neurulation in which the actin cytoskeleton seems to have a primarily stabilizing role in the non-bending neural plate⁷². By contrast, this actin-mediated mechanism is crucial for closure of the cranial neural tube, probably by allowing dorsolateral bending of the cranial neuroepithelium.

Neural crest emigration and cranial neurulation. The relationship between neural crest migration and neural tube closure is a confused issue. The two processes are related spatially and temporally, and an interdependent relationship is often assumed. There is evidence in the cranial region that the transition from biconvex to biconcave neural plate morphology, which is necessary for neural tube closure, might depend on the initiation of cranial neural crest migration. In the midbrain and hindbrain, neural crest cells begin to detach from the apices of the neural folds and start migration well in advance of neural tube closure⁷³. Mice that overexpress the gap junction protein connexin 43 have marked cranial neural crest migration defects and also develop exencephaly⁷⁴. Similarly, neural crest emigration and dorsolateral bending of the cranial neural folds is delayed in cultured rat embryos that are treated with chondroitinase

PARAXIAL MESODERM

The unsegmented mesoderm of the caudal embryonic region that subsequently becomes segmented into somites.

HYALURONAN

A high molecular weight polysaccharide of repeating N-acetyl glucosamine and glucuronic-acid residues that is included among the proteoglycans (which are proteins that comprise a peptide backbone with abundant glycosaminoglycan side chains), although it does not have a protein backbone.

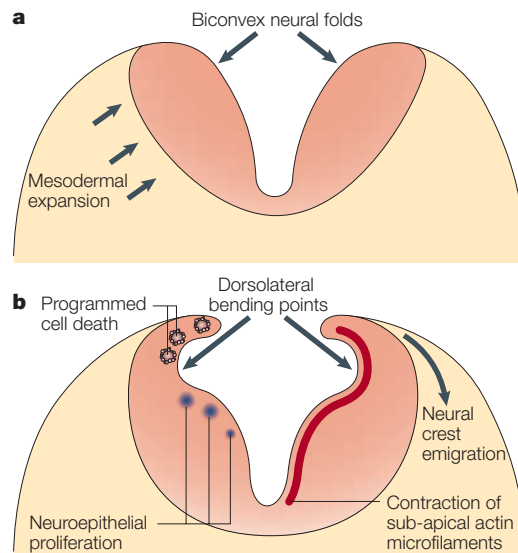


Figure 6 | Schematic summary of cranial neurulation as exemplified by neural tube closure in the midbrain region. The principal mechanisms that are required for the successful closure of the cranial neural tube, as shown by mouse mutant studies, are depicted unilaterally for the sake of clarity, although they operate bilaterally. **a** | The elevating neural folds initially adopt a biconvex morphology, which is assisted by the expansion of the cranial mesoderm. **b** | Subsequently, dorsolateral bending brings the apices of the neural folds into apposition in the dorsal midline. Contraction of sub-apical actin microfilaments, emigration of the neural crest, maintenance of a proliferative neuroepithelium and programmed cell death all have a role in dorsolateral bending. Also, Shh signalling probably participates in regulating dorsolateral bending, as in spinal neurulation. Apical apoptotic cell death is involved in epithelial remodelling following closure (FIG. 5).

ABC to digest pre-existing CHONDROITIN SULPHATE⁷⁵. These findings indicate that neural crest emigration is needed for the cranial neural tube to complete closure.

In the spinal region, the relationship between neural crest emigration and neural tube closure is different. Neural crest emigration begins several hours after spinal neural tube closure is complete⁷⁶. The defects that are most often associated with homozygous *spotch* (*Pax3*) mutants are neural crest defects — these are predominantly in the lower spine and the mutants also develop spina bifida. However, the defects of neural tube closure and neural crest migration have been functionally dissociated in this mutant. Failure of spinal neural tube closure can occur in the presence of apparently normal neural crest migration in *spotch* embryos⁷⁷. Similarly, the addition of a *spotch* mutant allele to mutant *curly tail* (*ct*) homozygotes exacerbates the spina-bifida phenotype without compromising neural crest migration⁷⁸. Hence, the neural crest and neural tube phenotypes of *spotch* mice seem to be independent consequences of the same underlying molecular defect in *Pax3* function. Precisely why the *spotch* neural tube fails to close is uncertain, although hypotheses that are based on excessive neuroepithelial apoptosis⁷⁹, reduced cell proliferation⁸⁰ and altered neuroepithelial cell associations⁸¹ have all been proposed.

Apoptosis in the cranial neural folds. Dying cells have long been observed in the neuroepithelium during neurulation⁸², and recent studies show that this cell death is apoptotic⁸³. Several knockout strains have neural tube closure defects that are associated with alterations in the extent of neuroepithelial cell death, with many of these knockouts involving apoptosis-related genes. Interestingly, it seems equally detrimental for the intensity of apoptosis to be decreased or increased. Hence, the *Apaf1*, caspase 9 (*Casp9*) and *p53* knockouts, and the double mutant combinations *Ikk1* (*Chuk*); *Ikk2* (*Ikkkb*) and *Jnk1* (*Mapk8*); *Jnk2* (*Mapk9*), all have reduced apoptotic cell death in association with the development of exencephaly, whereas the *AP-2*, *ApoB*, *Bcl10*, *Mdm4*, *Mtr*, *Tcofl* and *Tulp1* knockouts (ONLINE TABLE 1) show an increase in the number of apoptotic cells in the cranial neural folds or neural tube. It is noticeable that only cranial neurulation is disturbed in these mutants, with the single exception of *fog*, which is a spontaneous mutation of *Apaf1* in which spinal-closure defects have also been described⁸⁴.

Excessive cell death could disrupt cranial neurulation by producing an inadequate number of normally functioning cells for morphogenesis, as indicated for the teratogenic effect of ethanol in producing cranial NTDs⁸⁵. But the finding that reduced apoptosis can lead to exencephaly indicates a more specific physiological role for neuroepithelial cell death during neurulation. Apoptotic cells occur in the dorsolateral regions of the cranial neural plate and at the tips of the fusing neural folds (FIG. 6). Dorsolateral cell death might synergize with neural crest cell emigration to 'loosen' the cranial neuroepithelium, thereby allowing the conversion from a biconvex to biconcave neural fold morphology. Midbrain neurulation seems mechanically disadvantageous, owing to the cranial flexure in which the neuraxis undergoes a 90° ventral bend at midbrain level⁸⁶. Indeed, ventral curvature that is imposed experimentally on chicken and mouse embryos leads to delayed neural fold closure^{61,87}. Hence, any reduction of the ability of the midbrain neural folds to bend is likely to lead to exencephaly.

Apoptosis at the tips of the neural folds seems to have a different role. After the apposing neural folds have contacted and adhered, midline epithelial remodelling is needed to break the continuity between the neuroepithelium and surface ectoderm on each side, which allows separate neuroepithelial and ectodermal continuity across the midline (FIG. 5b,c). Inhibition of apoptosis using the peptide Zvad-fmk produces spinal NTDs in the chicken embryo, probably by preventing this dorsal-midline remodelling⁸⁸. Detailed studies of knockout mice with reduced apoptosis are needed to determine the precise neurulation mechanisms that are disrupted in exencephaly.

Disrupting the proliferation/differentiation balance. The neuroepithelium is entirely proliferative during neurulation. Cells begin to exit the cell cycle, with the onset of neuronal differentiation, only after neural tube closure is complete at each level of the body axis. In knockouts that

CHONDROITIN SULPHATE
A glycosaminoglycan of repeating N-acetyl-galactosamine and glucuronic-acid residues that forms part of some proteoglycan molecules.

affect the Notch and related signalling pathways, the failure of cranial neurulation is related to the premature onset of neuronal differentiation in the neural plate. The inactivation of *RBPJK*, *Hes1* and *Numb*^{89–91} all prevent cranial neural tube closure by de-repressing neuronal differentiation, which leads to the precocious appearance of markers of neuronal differentiation during neurulation. A more severe NTD phenotype is seen in *Hes1;Hes3* double mutants (REF. 92). The premature differentiation of the neuroepithelium in these mutants might render the neural plate mechanically inflexible and prevent dorso-lateral bending, interfere with the release of neural crest cells or inhibit the adhesion process that is necessary for neural fold fusion. At present, it is not possible to distinguish between these and other mechanisms of neurulation disruption by the Notch-pathway mutants.

Excessive cell proliferation is also implicated in some NTD mutants. The overexpression of *Notch3* produces increased numbers of cells that are positive for the neural progenitor marker nestin, in association with exencephaly⁹³. Moreover, the incorporation of bromodeoxyuridine (BrdU), which labels proliferating cells, is increased in the allelic *fog* and *Apaf1* mutants, along with reduced cell death⁹⁴. Homozygotes have ectopic masses of forebrain tissue that protrude from the forehead owing to the local absence of the skull vault, which is a defect that closely resembles human frontal encephalocele. Neuroepithelial ‘overgrowth’ is sometimes described in relation to the exencephalic brains of other mutants (FIG. 2b), but this is rarely more than a morphological impression and, when analysed in detail, a normal or even lengthened cell cycle has been observed in exencephalic brains^{80,95,96}.

Prevention of NTDs by exogenous agents

A proportion of human NTDs can be prevented by the administration of folic acid early in pregnancy³, which is an observation that is matched by the preventive action of folic acid on NTDs in the *spotch*, *Cart1*, *Crooked tail* and *Cited2* mouse models^{63,97–99}. Although a defect of folate metabolism can be detected in *spotch* mice, the same assay does not detect abnormal folate handling in *Cited2* embryos^{97,99}. Moreover, knockout of the important folate reductase enzyme 5,10-methylene tetrahydrofolate reductase¹⁰⁰ and pharmacological inactivation of methionine synthase¹⁰¹ do not lead to NTDs. Hence, the

mechanism that underlies folate-preventable NTDs might reside not in inherited abnormalities of folate metabolism, but in other pathways that are susceptible to correction by exogenous folic acid. One possibility is the enhancement of neuroepithelial cell survival by folic acid, through its effects in promoting DNA repair¹⁰².

In the Medical Research Council clinical trial of vitamin supplementation³, folic acid prevented 70% of human NTDs³, which raised the possibility of folate-resistance in the remaining cases. Interestingly, NTDs in several mouse mutants are folate resistant, including *curly tail*, *Axd* and *EphA7* (REFS 51,103,104). The vitamin-like molecule inositol can prevent the spinal NTDs that are observed in the folate-resistant *curly tail* mutant through a mechanism that requires the stimulation of protein kinase C in embryonic cells¹⁰⁵. The ultimate effect is stimulation of cell proliferation in the caudal embryonic region, which is known to be defective in the *curly tail* strain¹⁰⁶. The finding that inositol can prevent folate-resistant NTDs in one mouse model raises the possibility of its future use for the prevention of NTDs in human pregnancy, alongside folic acid. It remains to be determined whether other mutant models of NTDs will prove to be sensitive to inositol therapy.

Conclusions

Neurulation, similar to other morphogenetic events, has not attracted the attention of developmental biologists in the same measure as the regulation of patterning and differentiation events. The availability of many genetic mutants with neurulation phenotypes now provides an opportunity for a rigorous analysis of the molecular mechanisms of mammalian neurulation. Already, we can discern crucial roles for planar cell polarity in the initiation of closure and for Shh signalling in neural plate bending. Cytoskeletal function is crucial for cranial but not spinal neurulation. Areas for future work include the mechanisms of neural fold adhesion/fusion, and the role of precisely coordinated cell proliferation and cell death. An important unresolved issue is the role of endogenous retinoic acid (RA) in neurulation. NTDs result from the knockout of genes that either enhance or diminish RA signalling (ONLINE TABLE 1), but the precise action of RA in neurulation remains to be determined. Although we are far from ‘coming to closure’ on neurulation¹⁰⁷, at least the tube is now open for molecular business.

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