Neural Tube Defects

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Abstract

Neural tube defects (NTDs), including spina bifida and anencephaly, are severe birth defects of the central nervous system that originate during embryonic development when the neural tube fails to close completely. Human NTDs are multifactorial, with contributions from both genetic and environmental factors. The genetic basis is not yet well understood, but several nongenetic risk factors have been identified as have possibilities for prevention by maternal folic acid supplementation. Mechanisms underlying neural tube closure and NTDs may be informed by experimental models, which have revealed numerous genes whose abnormal function causes NTDs and have provided details of critical cellular and morphological events whose regulation is essential for closure. Such models also provide an opportunity to investigate potential risk factors and to develop novel preventive therapies.

Contents

INTRODUCTION

Neural tube defects (NTDs) are severe birth defects of the central nervous system that originate during embryogenesis and result from failure of the morphogenetic process of neural tube closure (see sidebar). In higher vertebrates, the neural tube is generated by the processes that shape, bend, and fuse the neural plate, and fusion in the dorsal midline progressively seals the neural tube as it forms. If closure is not completed, the neuroepithelium remains exposed to the environment and consequently subject to degeneration and neuronal deficit. The type and severity of these open NTDs vary with the level of the body axis affected. Thus, failure of closure in the prospective brain and spinal cord results in anencephaly and open spina bifida (myelomeningocele), respectively.

NTDs: Neural tube defects

NEURAL TUBE CLOSURE

Neural tube closure is discontinuous: Closure initiates de novo at specific sites along the body axis, and progression of fusion in the dorsal midline extends the closed region to close the neuropores in the brain (anterior and hindbrain neuropores) and spine (posterior neuropore).

Although the unifying feature of open NTDs is incomplete neural tube closure, evidence points to many different possible causes, both genetic and environmental. In humans, it appears that most NTDs are multifactorial, resulting from an additive contribution of several risk factors, which are each individually insufficient to disrupt neural tube closure (the multifactorial threshold model) (Harris & Juriloff 2007). The challenge of identifying the primary cause of NTDs in individual patients is highlighted by the numerous candidate genes and environmental factors indicated by epidemiologic studies and experimental models. Moreover, the potential for gene-gene and gene-environment interactions introduces further potential complexity.

Craniorachischisis: a condition where the neural tube remains open throughout the hindbrain and entire spinal region

Neuropores: open regions of neural folds, which gradually shorten and close, forming the sealed primary neural tube

Neural plate: a thickened region of ectoderm, induced on the dorsal surface of the postgastrulation embryo

UNDERSTANDING THE EMBRYONIC BASIS OF NTDs: NEURAL TUBE CLOSURE

Determining the specific causes of NTDs is best achieved in the context of an understanding of the mechanisms underlying neural tube closure (reviewed by Copp & Greene 2013, Greene & Copp 2009). Given the inaccessibility of the neurulation-stage human embryo, our knowledge of the key principles of neural tube closure comes mainly from analysis of experimental models, particularly other mammals, amphibians, and birds, in which primary neural tube closure is achieved through folding and fusion of the neuroepithelium.

Primary Neurulation: Subtypes of NTDs Relate to Stages of Closure

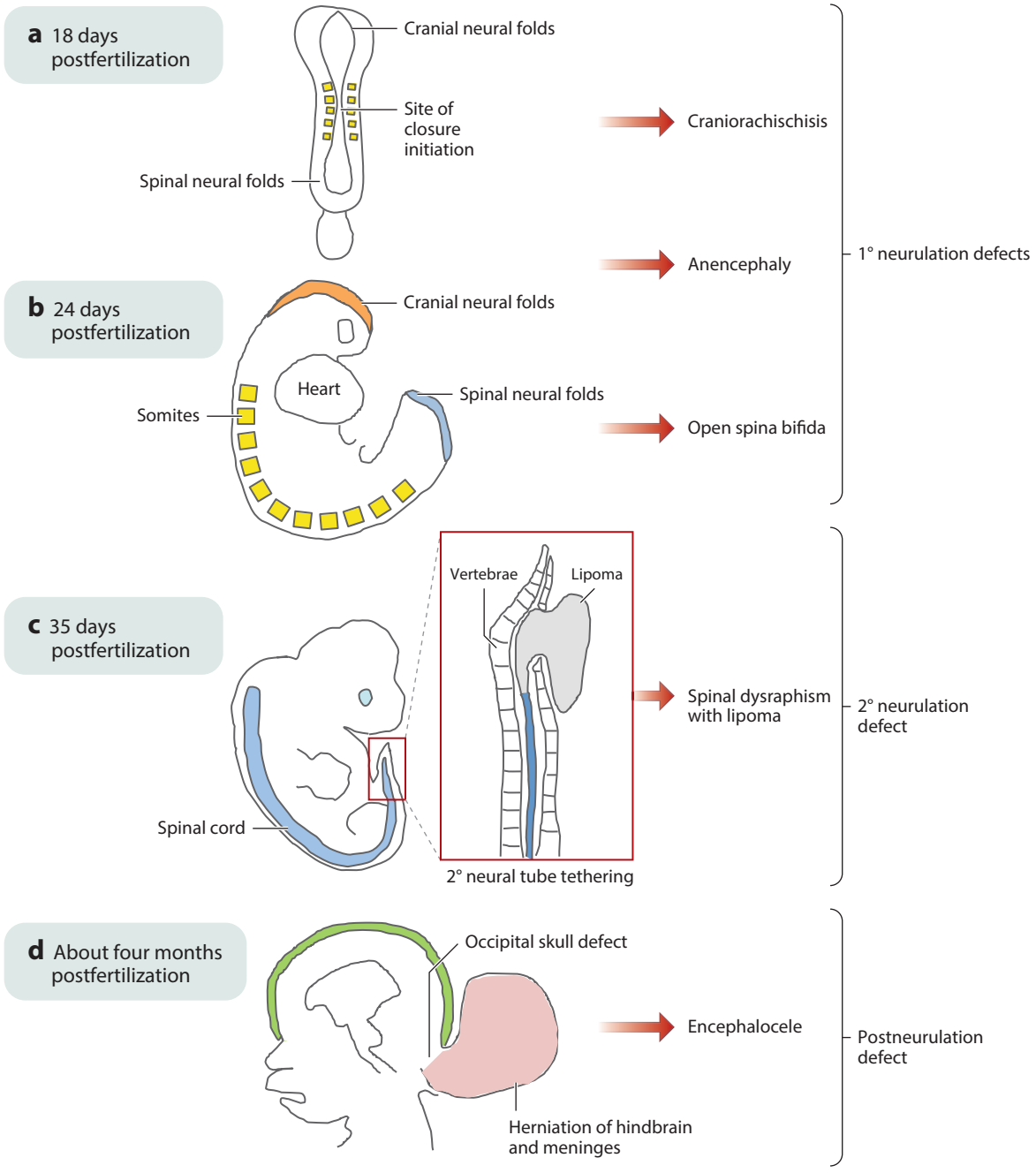
In the prospective brain and most of the spinal cord, neural tube formation essentially involves the bending of the neuroepithelium at the midline to generate neural folds that elevate, meet, and fuse in the dorsal midline (primary neurulation). Rather than simultaneously rolling up along the extent of the rostrocaudal axis, neural tube closure is discontinuous with distinct sites of initiation located at characteristic axial levels. Moreover, the morphological and molecular requirements for closure vary along the body axis, such that an individual NTD usually affects only a portion of the neural tube. NTDs can thus be attributed to failure of particular initiation events or disruption of the progression of closure between these sites (**Figure 1**).

In mice, closure is first achieved on embryonic day 8.5 at the level of the hindbrain/cervical boundary (closure 1) (**Figure 1*a***), and failure of this event leads to craniorachischisis (Copp et al. 2003). Closure initiates at a second site on embryonic day 9, closure 2, in the caudal forebrain or forebrain/midbrain boundary. Once initial contact and fusion have been established between the tips of the neural folds, closure spreads bidirectionally from the sites of closures 1 and 2 and in a caudal direction from the rostral end of the neural tube (closure 3). The open regions of neural folds, termed neuropores, gradually shorten, leading to complete closure of the anterior neuropore (between closures 2 and 3) on embryonic day 9 and the hindbrain neuropore (between closures 1 and 2) a few hours later. Cranial NTDs (anencephaly, see sidebar) result from failure of closure 2, or incomplete "zippering" between closures 1 and 2, which closes the midbrain and hindbrain. If fusion does not progress from the anterior end of the neural plate (closure 3), the resultant phenotype is a split face usually accompanied by forebrain anencephaly.

ANENCEPHALY

Cranial NTDs result from failed closure of the neural folds in the mid/hindbrain. This leads to exencephaly in which exposed neuroepithelium bulges from the brain. Subsequent degeneration of this tissue results in anencephaly.

Unlike the cranial region where closure proceeds bidirectionally, spinal neurulation is entirely caudally directed as the embryo continues to grow. Primary neurulation completes with final closure of the posterior neuropore on embryonic day 10. Impaired progression of closure, and consequently the presence of a persistently open posterior neuropore, results in spina bifida, and the size of the ensuing lesion relates directly to the axial level at which closure stops.



Primary Neurulation in Humans

Examination of human embryos suggests that initiation of closure is discontinuous, as in the mouse (Nakatsu et al. 2000, O'Rahilly & Müller 2002). Bending of the neural plate begins at approximately 18 days after fertilization, with an event equivalent to closure 1 at approximately 21 days and completion of closure at the posterior neuropore by 26–28 days postfertilization (**Figure 1*a*,*b***). It appears that closure of the forebrain and midbrain in human embryos may be achieved by progression between the site of closure 1 and the rostral end of the neural plate without an intervening initiation site analogous to closure 2 (O'Rahilly & Müller 2002, Sulik et al. 1998).

PCP: planar cell polarity

Secondary Neurulation

In mice and humans, the neural tube caudal to the midsacral region is continuous with the caudal end of the primary neural tube but forms by a distinct process, termed secondary neurulation (Copp & Brook 1989, Schoenwolf 1984). This process involves condensation of a population of tail bud–derived cells to form an epithelial rod that undergoes canalization to form the lumen of the tube in the lower sacral and coccygeal regions. Malformations resulting from disturbance of secondary neurulation are closed (skin covered) and often involve tethering of the spinal cord, with associated ectopic lipomatous material (**Figure 1*c***) (Lew & Kothbauer 2007).

MECHANISMS UNDERLYING NEURAL TUBE CLOSURE

Studies of neurulation-stage embryos, both normal and developing NTDs, provide insights into key molecular and cellular pathways underlying the morphological tissue movements of neural tube closure (Copp & Greene 2010). The occurrence of isolated NTDs at cranial or caudal levels in humans and different mouse models suggests the likely involvement of region-specific mechanisms, dependent on different gene products, in addition to ubiquitous requirements that are essential at all levels.

Shaping of the Neural Plate: Convergent Extension Is Required to Initiate Closure

Concomitant with the onset of neural tube closure, the neural plate undergoes narrowing in the mediolateral axis (convergence) and elongation in the rostrocaudal axis (extension), owing to intercalation of cells at the midline (Keller 2002). Convergent extension depends on activity of a noncanonical Wnt signaling pathway, homologous to the planar cell polarity (PCP) pathway first described in *Drosophila* as regulating cell polarity in the plane of epithelia (Goodrich & Strutt 2011).

Figure 1

Diagrammatic representation of the developmental origin of malformations broadly classified as neural tube defects in humans. (*a*,*b*) Disorders of primary neurulation include craniorachischisis (*a*) in which the neural tube fails to initiate closure, leaving most of the brain and the entire spine open. If closure initiates successfully, then the cranial and/or spinal neural folds may fail to close (*b*), generating exencephaly/anencephaly and open spina bifida (myelomeningocele), respectively. (*c*) Disorders of secondary neurulation comprise failure of the neural tube to separate completely from adjacent tissues, resulting in tethering and diminished mobility. The spinal cord is covered by skin and often associated with fatty tissue accumulation (lipoma) through as-yet-unknown mechanisms. (*d*) Postneurulation defects can arise when the bony structure of the skeleton fails to develop fully. Herniation of the meninges, with or without brain tissue, through a skull defect (shown here as occipital but sometimes parietal or fronto-ethmodial) generates encephalocele, while an analogous defect in the spinal region produces meningocele.

Signaling occurs via Frizzled (Fzd) membrane receptors and cytoplasmic Dishevelled (Dvl) but without stabilization of β-catenin.

Median hinge point (MHP): the bend in the midline of the neuroepithelium

Dorsolateral hinge points (DLHPs): paired bends that arise in the lateral neural folds

Functional disruption of PCP mediators prevents convergent extension, and the neural plate remains broad in *Xenopus* (Wallingford & Harland 2001, 2002) and mouse embryos (Greene et al. 1998, Ybot-Gonzalez et al. 2007b). Hence, closure 1 fails, leading to craniorachischisis in mice homozygous for mutations in core PCP genes including *Vangl2* and *Celsr1*, or double mutants for *Dvl-1* and *-2*, or *Fzd-3* and *-6* (Juriloff & Harris 2012a). Craniorachischisis also results from mutation of the PCP-related genes *Scrb1* (Murdoch et al. 2001) and *Ptk7* (Lu et al. 2004) or genes encoding accessory proteins, such as *Sec24b*, which affects Vangl2 transport (Merte et al. 2010). Ultimately, failure of closure initiation in PCP-mutant embryos is thought to result from insufficient proximity of the neural folds, owing to the broadened midline.

Failure of closure 1 in most of the core PCP mutant embryos precludes analysis of a requirement for convergent extension at later stages of neurulation. However, spina bifida occurs in some *loop-tail* heterozygotes ($Vangl2^{Lp/+}$) (Copp et al. 1994) and in compound heterozygotes of $Vangl2^{Lp/+}$ with mutations of *Ptk7*, *Sec24b*, or *Sdc4* (Escobedo et al. 2013, Lu et al. 2004, Merte et al. 2010). Moreover, non-canonical Wnt signaling is compromised in *Lrp6* null embryos that develop spina bifida (Gray et al. 2013). These observations suggest that PCP signaling may continue to be required as spinal neurulation proceeds.

Despite the entirely open spinal neural tube in $Vangl2^{Lp/Lp}$ embryos with craniorachischisis, closure does occur in the forebrain and much of the midbrain, implying that PCP-dependent convergent extension is not required throughout the cranial region. Nonetheless, exencephaly is observed in digenic combinations of $Vangl2^{Lp/+}$ with some Wnt pathway genes (e.g., $Dvl3^{+/-}$, $Fzd1^{+/-}$, and $Fzd2^{+/-}$) (Etheridge et al. 2008, Yu et al. 2010). Exencephaly also develops in mutants for the PCP effector genes *Fuz* or *Intu*, but the role of these genes in cilium-dependent hedgehog signaling seems more likely to explain their loss-of-function effect on cranial neural tube closure than does a role in regulating convergent extension (Gray et al. 2009, Heydeck & Liu 2011, Zeng et al. 2010) (see Bending of the Neural Folds: Regulation by Shh and BMP Signaling, below). Thus, components of PCP signaling potentially affect neural tube closure via multiple cellular mechanisms.

Bending of the Neural Folds: Regulation by Shh and BMP Signaling

To achieve closure, the neuroepithelium must bend to bring the tips of the neural folds into apposition. Bending occurs in a stereotypical manner at hinge points: a median hinge point (MHP) in the midline and paired dorsolateral hinge points (DLHPs) that arise laterally (Shum & Copp 1996). The morphology varies along the body axis with differing modes in the upper (MHP only), midspine (MHP and DLHPs), and caudal (DLHPs only) regions of the primary spinal neural tube.

The mechanisms underlying neuroepithelial bending are not fully understood, but one notable feature of the MHP is the predominance of wedge-shaped cells (wider basally than apically) compared with nonbending regions (Schoenwolf & Smith 1990). At neural plate stages, the neuroepithelium is a pseudostratified epithelium in which nuclei move to the basal pole during S-phase, owing to interkinetic nuclear migration (see sidebar). Prolongation of S-phase at the MHP provides a possible means by which regulation of the cell cycle may contribute to cell wedging and hence MHP formation (Schoenwolf & Smith 1990).

Bending is regulated by signals emanating from nonneural tissues dorsal and ventral to the neural folds (reviewed by Greene & Copp 2009). The MHP is induced by signals from the notochord, located immediately ventral to the midline of the neuroepithelium (Smith & Schoenwolf 1989, Ybot-Gonzalez et al. 2002). At the molecular level, notochord-derived Shh induces the floor plate of the neural tube at the MHP (Chiang et al. 1996, Placzek & Briscoe 2005). However, this action is

INTERKINETIC NUCLEAR MIGRATION

Interkinetic nuclear migration occurs in the neuroepithelium and describes the apico-basal migration of the nucleus according to the phase of the cell cycle such that S-phase occurs at the basal aspect and mitosis at the apical aspect.

not essential for spinal neural tube closure, which completes in the absence of a floor plate in mouse embryos lacking Shh or Fox A2 (Ang & Rossant 1994, Chiang et al. 1996). Thus, the MHP may be functionally important in floor plate development but is not essential for neural tube closure.

In contrast to the MHP, DLHPs appear essential for the neural tube to close in the low spinal region. For example, *Zic2* mutant embryos, in which DLHPs are absent, develop severe spina bifida (Ybot-Gonzalez et al. 2007a). The formation of DLHPs is actively regulated; the interplay of inhibitory and inductive signals determines their appearance at different axial levels (Copp & Greene 2013). These signals include inhibitory effects of Shh from the notochord and BMP signaling from the surface ectoderm at the dorsal tips of the neural folds. These signals are opposed by the BMP antagonist noggin, whose expression in the dorsal neural folds is sufficient to induce DLHPs (Ybot-Gonzalez et al. 2002, 2007a).

In contrast to the effects of an absence of Shh signaling, NTDs do result from mutations that enhance Shh signaling, for example, through deficient function of inhibitory or cilia-related genes such as *Gli3*, *Rab23*, *Fkbp8*, *Tulp3*, and *Ift40* (Miller et al. 2013, Murdoch & Copp 2010). Mutants involving increased Shh signaling display NTDs at cranial and/or spinal levels. Although spina bifida in some of these models appears to be associated with suppression of dorsolateral bending of the neural folds (Murdoch & Copp 2010), the mechanism underlying cranial NTDs is not clear.

Cranial Neurulation: Additional Complexity and Sensitivity to Disruption

The neural folds in the cranial region bend in the midline and dorsolaterally as in the midspinal region, but the closure process appears morphologically more complex. The folds are initially biconvex, with the tips facing away from the midline, and then switch to a biconcave shape allowing the tips to approach in the midline. The additional complexity of cranial compared with spinal neurulation appears to be reflected in a more extensive genetic underpinning and a greater sensitivity to disruption, at least in rodents. Exencephaly occurs in approximately three times as many knockout mouse models as does spina bifida and is the NTD type most commonly induced by teratogens (Copp et al. 1990, Harris & Juriloff 2010).

Cranial neurulation may rely on specific contributory factors that are not involved in the spinal region such as expansion of the mesenchyme underlying the neural folds (Greene & Copp 2009, Zohn & Sarkar 2012). Moreover, disruption of the actin cytoskeleton prevents closure in the cranial but not the spinal region (Morriss-Kay & Tuckett 1985, Ybot-Gonzalez & Copp 1999). Similarly, exencephaly is observed, but spinal neurulation completes successfully in null mutants for several cytoskeletal components (e.g., *n-cofilin*, *vinculin*) (Gurniak et al. 2005, Xu et al. 1998). Nevertheless, apically located actin microfilaments are present throughout the neuroepithelium (Sadler et al. 1982), and functional disruption of the cytoskeleton-associated proteins *MARCKS-related protein* or *Shroom3* causes both spinal and cranial NTDs (Hildebrand & Soriano 1999, Xu et al. 1998), suggesting that regulation of the actomyosin cytoskeleton plays a role in closure in both regions. Shroom proteins appear to play a key role: Expression of Shroom in *Xenopus* is sufficient to induce apical constriction of epithelial cells, whereas functional disruption inhibits neural fold bending and suppresses closure (Haigo et al. 2003).

Adhesion and Fusion of the Neural Folds

Once the neural folds meet at the dorsal midline, processes of adhesion, fusion, and remodeling give rise to two discrete epithelial layers, with the nascent neural tube overlain by an intact surface ectoderm (Pai et al. 2012). At the closure site, the neural fold tips are composed of neuroepithelium continuous with the nonneural surface ectoderm. The cell type that adheres first may differ at varying axial levels (Geelen & Langman 1979, Ray & Niswander 2012). Nevertheless, at all levels initial contact appears to involve subcellular protrusions, resembling lamellipodia and filopodia, observed by electron microscopy (Geelen & Langman 1979) and in live embryos (Pyrgaki et al. 2010). The molecular basis of adhesion is not well characterized, perhaps owing to functional redundancy among the proteins involved. However, a role for the interaction of cell surface ephrin receptors with Eph ligands is suggested by the occurrence of cranial NTDs in mice lacking ephrin-A5 or EphA7 (Holmberg et al. 2000) and by delayed spinal closure in embryos exposed to peptides that block ephrin-A/EphA interactions (Abdul-Aziz et al. 2009).

Knockout of protease-activated receptors (PAR1 and PAR2) in the surface ectoderm also causes cranial NTDs, implicating a role for signaling via these G protein–coupled receptors in closure (Camerer et al. 2010). Further evidence for the function of the nonneural ectoderm is provided by *Grhl2* null mutants, which fail in closure throughout the cranial region and exhibit spina bifida (Brouns et al. 2011, Rifat et al. 2010, Werth et al. 2010). *Grhl2* is expressed in the surface ectoderm overlying the neural folds and regulates expression of several components of the apical adhesion junction complex, including E-cadherin (Pyrgaki et al. 2011, Werth et al. 2010).

Regulation of Cell Proliferation and Cell Death

During neurulation the embryo grows rapidly. Cell cycle exit and neuronal differentiation begin in the neuroepithelium shortly after closure, and maintenance of adequate proliferation in the neuroepithelium appears crucial for closure, particularly in the cranial region. Thus, in mice, NTDs can be caused by exposure to antimitotic agents (Copp et al. 1990) or mutation of genes encoding proteins associated with cell-cycle progression (e.g., *neurofibromin 1*, *nucleoporin*) or prevention of neuronal differentiation (e.g., Notch pathway genes *Hes1*, *Hes3*, *RBP-Jκ*) (Harris & Juriloff 2007, 2010). Conversely, excessive cell proliferation is also associated with NTDs in several mouse models, such as *Phactr4* mutants (Kim et al. 2007).

Characteristic patterns of apoptotic cell death occur in the neural folds and the midline of the closed neural tube (Geelen & Langman 1979, Massa et al. 2009, Yamaguchi et al. 2011). Increased cell death could inhibit closure by compromising the functional and/or mechanical integrity of the neuroepithelium. It is associated with NTDs in several teratogen-induced and genetic models, although only rarely has a direct causal link been definitively established (Copp & Greene 2013, Fukuda et al. 2011). The occurrence of exencephaly in mice lacking apoptosis-related genes such as *caspase3* or *Apaf1* suggests a requirement for apoptosis in closure (Harris & Juriloff 2010). However, forebrain and spinal closure occurs normally in these models and pharmacological suppression of apoptosis does not cause NTDs, suggesting that it is dispensable to complete closure (Massa et al. 2009).

CLINICAL FEATURES OF NEURAL TUBE DEFECTS

Owing to their multifactorial causation, NTDs represent a group of disorders. However, after failure of neural fold closure has occurred, defects originating from various primary causes may share similar pathogenic features.

Open NTDs and Associated Conditions

Open NTDs can result from failure of closure at a de novo initiation site or incomplete progression of closure following successful initiation (**Figure 1**). Where embryos are available for examination, as in experimental models, NTDs can be recognized during or immediately after neurulation stages owing to the persistently open neural folds. However, at later embryonic and fetal stages, the morphological appearance changes considerably owing to secondary changes and degeneration.

In cranial NTDs, the open neural folds undergo growth and differentiation and typically appear to bulge from the developing brain, termed exencephaly. Inability to form the skull vault over the open region causes the exposed neural tissue to degenerate, leading to the characteristic appearance of anencephaly, observed later in human or rodent pregnancy (Wood & Smith 1984, Seller 1995). Both anencephaly and craniorachischisis (~10% of NTDs) are lethal conditions at or shortly after birth.

Open neural folds in the spinal region prevent the sclerotome-derived vertebral arches from covering the neuroepithelium, the consequent opening in the vertebral column giving rise to the term spina bifida (Copp et al. 2013). The neural tissues may be contained within a meninges-covered sac that protrudes through the open vertebrae (myelomeningocele; spina bifida cystica) or exposed directly to the amniotic fluid (myelocele). Babies born with open spina bifida usually survive with appropriate medical care but suffer neurological impairment, the severity of which depends on the level of the lesion. Associated conditions include hydrocephalus, Chiari malformation type II, and vertebral abnormalities as well as genitourinary and gastrointestinal disorders.

Diagnosis, Treatment, and Maternal-Fetal Surgery

NTDs can be diagnosed prenatally by ultrasound (Cameron & Moran 2009). However, where prenatal diagnosis is not routinely available and/or therapeutic abortion is not an option, many babies with NTDs are born. Postnatal medical care for babies born with open spina bifida usually involves surgery to close and cover the lesion. Multiple subsequent surgeries are commonly required to alleviate tethering of the spinal cord, treat hydrocephalus, and/or address orthopedic and urological problems.

As open NTDs arise early during pregnancy, there is a prolonged period during which secondary neurological damage may occur owing to exposure of nervous tissue to the amniotic fluid environment. These considerations provided impetus for the development of in utero fetal surgery for spina bifida, which may improve neurological outcomes compared with postnatal repair, although with fetal and maternal risks (Adzick et al. 1998, 2011). Experimental models of spina bifida are being used to investigate the possible combination of surgical intervention with additional therapy, intended to remediate neural damage. Examples include the implantation of biodegradable scaffolds to promote neural regeneration and/or neural stem cells to populate the damaged spinal cord (Saadai et al. 2011, 2013).

Disorders of the Closed Neural Tube

This review focuses on open NTDs, characterized by failure of neural tube closure. Various other conditions are also associated with abnormalities of the closed brain or spinal cord and are often categorized as NTDs under a broader definition (**Figure 1**). There is also a less well-defined group of closed spinal NTDs in which the vertebral arches are malformed but covered by skin. These conditions, including spina bifida occulta and spinal dysraphisms, vary widely in clinical presentation. The more severe subtypes are associated with various abnormalities of the spinal

cord, lipoma (**Figure 1*c***), and/or anorectal abnormalities. The embryonic origin of closed spina bifida is not well defined but is hypothesized to involve abnormalities of secondary neurulation (Copp et al. 2013).

Abnormal development of the skull or vertebrae may also allow herniation of the closed neural tube through the affected bony region, as in encephalocele (**Figure 1*d***) or meningocele, respectively.

CAUSES OF NTDs

NTDs are among the most common birth defects worldwide with a prevalence that varies from 0.5 to more than 10 per 1,000 pregnancies. This variance likely reflects differing contributions from risk factors such as nutritional status, prevalence of obesity and diabetes, usage of folic acid supplementation and/or fortification, the presence of environmental toxicants, and differing genetic predisposition among ethnic groups. In most populations, there is also a striking gender bias: Anencephaly is more prevalent among females than males. Many NTD mouse strains also show a female preponderance among cranial NTDs, apparently reflecting a fundamental higher sensitivity of cranial neural tube closure to disturbance in female embryos (Juriloff & Harris 2012b). Overall, although studies have identified numerous risk factors, these may account for less than half of NTDs, suggesting that additional genetic and nongenetic factors remain to be identified (Agopian et al. 2013).

Environment Factors

Various teratogenic agents induce NTDs in rodent models (Copp et al. 1990, Copp & Greene 2010). In humans, teratogens that have been associated with NTDs include the anticonvulsant drug valproic acid (Wlodarczyk et al. 2012) and the fungal product fumonisin (Missmer et al. 2006). Other nongenetic risk factors include maternal fever and excessive use of hot tubs (Moretti et al. 2005), consistent with the induction of NTDs by hypothermia in rodent models.

Maternal obesity and diabetes are well-recognized risk factors for NTDs (Correa et al. 2003). Determining the cause of diabetes-related NTDs is hampered by the complexity of the diabetic milieu, although hyperglycemia alone is sufficient to cause NTDs in cultured rodent embryos. It has been proposed that NTDs may result from increased oxidative stress, altered expression of genes such as *Pax3*, and neuroepithelial cell apoptosis (Fine et al. 1999, Reece 2012). Recent findings suggest that activation of apoptosis signal-regulating kinase 1 (ASK1) in hyperglycemic conditions leads to activation of the apoptosis mediator caspase 8 by stimulating the FoxO3a transcription factor (Yang et al. 2013).

Nutritional factors and folate. The historical link between lower socioeconomic status and higher risk of birth defects led to examination of the possible involvement of nutritional factors in NTDs. Lower blood levels of the B-vitamin folate were observed in mothers of NTD fetuses (Smithells et al. 1976), prompting an intervention trial of a folic acid–containing multivitamin supplement to prevent NTD recurrence (Schorah 2008, Smithells et al. 1981). A multicenter randomized controlled trial confirmed that maternal folic acid supplementation (at 4 mg per day) significantly reduces the recurrence risk (Wald et al. 1991). Additional clinical trials provided evidence for reduction of occurrence risk (Berry et al. 1999, Czeizel et al. 2011, Czeizel & Dudás 1992).

Questions remain concerning the mechanism by which folic acid prevents NTDs (Blom et al. 2006, Copp et al. 2013). Although maternal folate status is a risk factor, in most cases, maternal

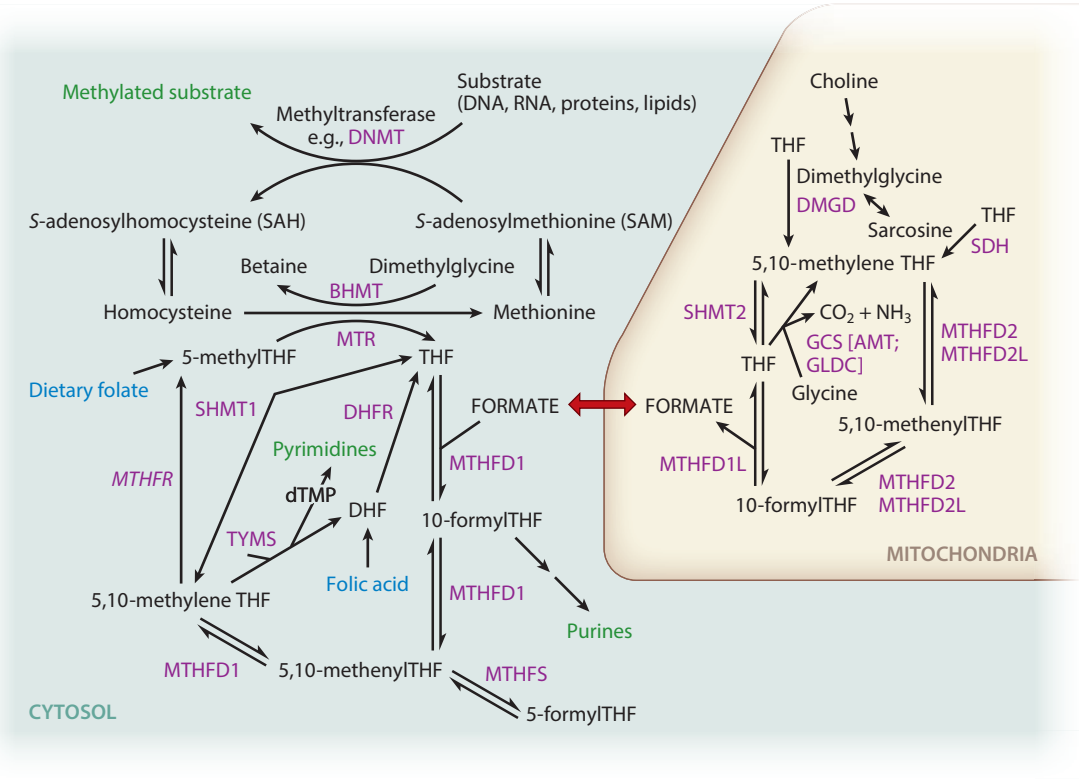


Figure 2

Overview of folate one-carbon metabolism. Folates provide a backbone for the transfer of one-carbon units. Key outputs (*green*) include nucleotide biosynthesis and methylation. Among methylation cycle intermediates, homocysteine may also be converted to cystathionine in the transulfuration pathway and *S*-adenosyl methionine is involved in polyamine biosynthesis. Folate-mediated one-carbon metabolism (FOCM) is compartmentalized: one-carbon units from the mitochondria enter cytoplasmic FOCM as formate while reactions of thymidylate biosynthesis also operate in the nucleus (catalyzed by SHMT1, TYMS, and DHFR). In loss-of-function mouse models, neural tube defects (NTDs) arise in mutants for Mthfd1l and genes encoding the glycine cleavage system (GCS). Shmt1 and Mthfr null mice are viable to birth but may develop NTDs under folate-deficient conditions. Entry points of folate, from dietary sources or folic acid, are indicated in blue. Enzymes that catalyze steps of one-carbon metabolism and methylation reactions are indicated in purple: BHMT, betaine-homocysteine S-methyltransferase; DNMT, DNA methyltransferase; DHFR, dihydrofolate reductase; DMGD, dimethylglycine dehydrogenase; GCS, glycine cleavage system (including AMT, aminomethyltransferase; GLDC, glycine decarboxylase); MTHFD (1, 1L, 2, 2L), methylenetetrahydrofolate dehydrogenase; MTHFR, methylenetetrahydrofolate reductase; MTHFS, methenyltetrahydrofolate synthetase; SDH, sarcosine dehydrogenase; SHMT, serine hydroxymethyltransferase.

folate levels are within the normal range and rarely clinically deficient. Nonetheless, data have shown an inverse relationship between blood folate concentration and risk of an affected pregnancy (Daly et al. 1995). Suboptimal folate levels may contribute to NTD development in individuals who are genetically susceptible. Such a gene-environment interaction has been demonstrated in mice, where folate deficiency does not cause NTDs unless deficiency is present in combination with a mutation of a predisposing gene, such as *Pax3* (Burren et al. 2008).

Folate one-carbon metabolism (**Figure 2**) comprises a complex network of interlinked reactions that mediate transfer of one-carbon groups for several biosynthetic processes (Stover 2009). Among these, attention has focused particularly on the requirement for nucleotide biosynthesis and methylation reactions in neural tube closure. Abnormal thymidylate and purine biosynthesis

have been identified in mouse NTD models (Beaudin et al. 2011, Fleming & Copp 1998) and in a proportion of NTD cases (Dunlevy et al. 2007), whereas deficient methylation may also be implicated in NTDs (see Gene-Regulatory Mechanisms and NTDs, below).

Genetics of NTDs

Most NTDs occur sporadically, with a relative scarcity of multigenerational families. Nevertheless, there is strong evidence for a genetic component in the etiology of NTDs, and the pattern of inheritance favors a multifactorial polygenic or oligogenic model, as opposed to an effect of single genes with partial penetrance (Harris & Juriloff 2007). Most studies of NTD genetics have focused on one or more candidate genes (reviewed by Boyles et al. 2005, Greene et al. 2009, Harris & Juriloff 2010). In general, candidates have been (*a*) human orthologs of genes whose mutation causes NTDs in mice, of which there are more than 200 examples; or (*b*) genes related to environmental risk factors, particularly folate metabolism.

Case-control association studies have implicated several genes, whereas mutation screening by sequencing has identified putative pathogenic mutations. However, the definitive assignment of a gene variant as causative is complicated by the apparent multigenic nature of NTDs and by the large number of possible candidate genes, modifier genes, epigenetic factors, and environmental influences. Moreover, where putative mutations have been identified in specific genes, each has been involved in only a small proportion of NTD patients, suggesting that there is considerable heterogeneity underlying the genetic basis of NTDs. Thus, although the morphological and cellular bases of neural tube closure have become increasingly well understood, the genetic basis of NTDs in individual cases remains largely unclear.

Gene-gene interactions and effect of modifier genes. Mouse studies suggest three broad mechanisms by which genetic interactions may result in NTDs. First, in some instances functional redundancy makes it necessary for two orthologous genes to be mutated [e.g., *Dvl1-Dvl2* (Hamblet et al. 2002), *Cdx1-Cdx2* double knockouts (Savory et al. 2011)], in order to reveal a requirement in neural tube closure. Second, additive effects of heterozygous mutations may result in NTDs that resemble those of individual homozygotes [e.g., *Dvl3* with *Vangl2*^{Lp} (Etheridge et al. 2008)]. Third, variation in the penetrance and expressivity of NTD phenotypes between inbred mouse strains is widely reported to reflect variants in modifier genes. For example, the rate of exencephaly resulting from *Cecr1* mutation is strongly affected by strain background (Davidson et al. 2007). Whereas the identity of modifier genes for NTDs has rarely been determined, a variant in *Lmnb1* is present in some mouse strains and significantly increases the frequency of NTDs in *curly tail* (*Grhl3*^{ct}) embryos (De Castro et al. 2012).

Genes implicated through experimental models. In mice, mutation of genes encoding components of the PCP pathway causes NTDs (see Shaping of the Neural Plate, above). Sequencing of PCP genes in humans has identified putative mutations in *CELSR1*, *VANGL1*, *VANGL2*, *FZD6*, *SCRIB1*, and *DVL2* in some patients with craniorachischisis, spina bifida, anencephaly, or closed forms of spina bifida (Chandler et al. 2012; De Marco et al. 2013; Kibar et al. 2007; Lei et al. 2010, 2013; Robinson et al. 2012; and reviewed by Juriloff & Harris 2012a). As in mice, heterozygous human PCP mutations could hypothetically interact with other genetic NTD risk factors in a digenic or polygenic fashion to cause a range of NTD types. This interaction could involve summation of multiple variants in PCP genes. For example, a putative mutation in *DVL2* was identified in a spina bifida patient in combination with a second, previously identified missense variant in *VANGL2* (De Marco et al. 2013).

Among other genes implicated in NTDs from mouse models, association studies have not provided evidence for a major contribution to risk, and few positive results have emerged from sequencing-based mutation screens. As data begin to emerge from large-scale exome sequencing studies of NTD patients, it will become possible to evaluate the contribution of multiple genes in the same patient cohorts and the mutational load associated with individual risk.

Analysis of genes related to environmental risk factors. The identification of environmental factors such as maternal diabetes and folate status as risk factors for NTDs provides impetus for analysis of related genes in affected families. Risk could be associated with maternal genotype if genetic variation alters maternal metabolism and secondarily affects the developing embryo. However, the inheritance of maternal alleles by the embryo complicates interpretation of such effects. Alternatively, a genetically determined abnormality in the embryo itself could influence risk of NTDs, potentially through interaction with a predisposing environmental factor. For example, it may be informative to analyze genetic data on folate-related genes in the context of maternal folate status (Etheredge et al. 2012).

Association with risk of spina bifida has been reported for several genes implicated in diabetes, obesity, glucose metabolism, and oxidative stress. These potential "risk" genes include *GLUT1*, *SOD1*, and *SOD2* (Davidson et al. 2008, Kase et al. 2012). Maternal variants in the obesity-related genes *FTO*, *LEP*, and *TCF7L2* are also associated with NTDs, consistent with maternal obesity as a risk factor (Lupo et al. 2012).

Genes related to folate one-carbon metabolism have been perhaps the most intensively studied group of candidates for NTDs (reviewed by Blom et al. 2006, Greene et al. 2009, Shaw et al. 2009). The C677T polymorphism of *MTHFR*, which encodes an alanine-to-valine substitution, has been associated with NTDs. The TT genotype is found at higher frequency among NTD cases than in controls in some populations (e.g., Irish) but not others (e.g., Hispanics) (Botto & Yang 2000). Several studies indicate positive associations with other folate-related genes, including *MTRR*, although these have generally not been observed in all study populations.

In mice, mutations in folate-metabolizing enzymes (e.g., *Mthfd1*) are sometimes lethal before the stage of neural tube closure (e.g., Christensen et al. 2013, MacFarlane et al. 2009), whereas others do not disrupt closure (e.g., Chen et al. 2001, Di Pietro et al. 2002). Null embryos for the folate receptor, *Folr1*, die preneurulation but develop NTDs when supplemented with sufficient folic acid to prevent early lethality (Piedrahita et al. 1999). NTDs are also observed in *Shmt1* knockouts, under folate-deficient conditions (Beaudin et al. 2011). In contrast, NTDs occur spontaneously in mice carrying loss-of-function alleles of *Amt* (Narisawa et al. 2012) or *Mthfd1L* (Momb et al. 2013), both of which encode enzymes of mitochondrial folate metabolism (see sidebar and **Figure 2**) (Tibbetts & Appling 2010). The homologous genes in humans have also been linked to NTDs. Missense mutations have been identified in NTD patients in *AMT* as well as in *GLDC*, which encodes its partner enzyme in the glycine cleavage system (Narisawa et al. 2012). Genetic associations with NTDs have been reported for *MTHFD1L* (Parle-McDermott et al. 2009) and

MITOCHONDRIAL FOLATE METABOLISM

Mitochondrial folate metabolism comprises a set of reactions of folate one-carbon metabolism that are compartmentalized in mitochondria and generate formate that is transferred to the cytoplasm where it acts as a one-carbon donor. AMT, GLDC (components of the glycine cleavage system), and MTHFD1L are specific to mitochondrial folate metabolism.

SLC23A32 (*MFTC*), encoding a mitochondrial folate transporter (Pangilinan et al. 2012). Altogether, these findings suggest that NTD risk is influenced by the function of mitochondrial folate metabolism, a major source of one-carbon units to the cytoplasm.

Gene-Regulatory Mechanisms and NTDs

Identification of causative genes may be complicated, in addition to the potential multigenic nature of NTDs, by the potential involvement of aberrant gene expression, perhaps resulting from mutations in regulatory elements. For example, mutations resulting in insufficient expression of *Grhl3* or excess expression of *Grhl2* cause NTDs in mice in the absence of coding mutations (Brouns et al. 2011, Gustavsson et al. 2007). Further complexity may be added by the potential for regulation by epigenetic modifications such as DNA methylation, histone modification, or chromatin remodeling, each of which has been associated with NTDs in mice and in some cases in humans (reviewed by Greene et al. 2011, Harris & Juriloff 2010). For example, methylation of LINE-1 genomic elements was lower than normal in DNA of anencephalic but not spina bifida fetuses (Wang et al. 2010).

A simple model predicts a positive correlation between folate status and methylation. However, data from human pregnancy suggest that the relationship is not straightforward (Crider et al. 2012). A recent study found an inverse correlation of LINE-1 methylation with maternal and cord blood folate, whereas different imprinted genes showed positive or negative associations (Haggarty et al. 2013). Somewhat counterintuitively, use of folic acid supplements was associated with reduced LINE-1 methylation.

A requirement for DNA methylation in mouse neural tube closure is suggested by the occurrence of NTDs in knockouts of *Dnmt3b*, encoding a DNA methyltransferase, and in embryos cultured with 5-azacytidine (Matsuda & Yasutomi 1992, Okano et al. 1999). Similarly, inhibition of the methylation cycle reduces DNA methylation and causes NTDs in cultured mouse embryos (Burren et al. 2008, Dunlevy et al. 2006). However, *Mthfr* null embryos do not develop NTDs despite a significant reduction in global DNA methylation (Chen et al. 2001), nor is there an exacerbating effect of *Mthfr* loss-of-function on *Pax3* or *curly tail* mutants, although both show increased rates of NTDs under folate-deficient conditions (Burren et al. 2008, De Castro et al. 2010, Pickell et al. 2009). Thus, questions remain about the relationships among folate status, DNA methylation, and risk of NTDs.

Other epigenetic mechanisms include various modifications of histone proteins, which potentially misregulate genes that influence neurulation. NTDs occur in mice carrying mutations in the histone demethylases *Jarid2* (Takeuchi et al. 1999) and *Fbxl10* (Fukuda et al. 2011). Similarly, histone acetylases and deacetylases, which regulate the equilibrium of histone acetylation, are implicated in NTDs. An acetylase-specific knockin mutation of *Gcn5* causes cranial NTDs (Bu et al. 2007), as does loss-of-function of another histone acetylase, p300 (Yao et al. 1998). Increased acetylation is also associated with NTDs. For example, cranial NTDs occur in mice carrying mutations in the histone deacetylases *Sirt1* or *Hdac4* (Cheng et al. 2003, Vega et al. 2004). The teratogenic effects of valproic acid and trichostin A may also be mediated through their inhibition of histone deacetylases (Finnell et al. 2002).

PRIMARY PREVENTION OF NTDs

Once the neural tube has failed to close, ensuing damage to the exposed neural tissue is irreversible, despite possible palliative benefit of in utero surgery (see Diagnosis, Treatment, and

Maternal-Fetal Surgery). Therefore, primary prevention is the optimal approach for reducing the burden of NTDs.

Folic Acid Supplementation and Fortification

The reduction in risk of NTDs following maternal folic acid supplementation led to public health recommendations that women who may become pregnant should consume 0.4 mg of folic acid daily or 4 mg daily following a previous affected pregnancy (Czeizel et al. 2011). To ensure that additional folate was received, food fortification programs were introduced in many countries. This approach has raised blood folate levels and has been associated with lower NTD frequency (Crider et al. 2011). The magnitude of effect varies, with the greatest reduction found where preexisting rates were highest (Blencowe et al. 2010, Rosenthal et al. 2013). Some countries have delayed a decision on fortification owing to safety concerns (e.g., possible enhancement of bowel cancer), but a recent meta-analysis found no evidence for increased cancer rates following folic acid supplementation (Vollset et al. 2013).

Folate-Resistant NTDs

Folic acid supplementation in clinical trials has not approached 100% NTD prevention, and an estimated one-third of NTDs may be folic acid resistant (Blencowe et al. 2010). A study in the United States, where folate fortification of food is mandatory, found no apparent protective effect of folic acid supplements (Mosley et al. 2009), suggesting that increased dosage would not necessarily provide additional preventive effects.

Given the multifactorial causation of NTDs it seems reasonable to suppose that optimal prevention will require a combination of multiple interventions. Possible approaches may relate to folate one-carbon metabolism. For example, as with folate, evidence shows a graded relationship between lower levels of circulating vitamin B_{12} and increased risk of an NTD-affected pregnancy (Molloy et al. 2009). Perhaps use of B_{12} supplements would further reduce NTD frequency, although this approach remains to be tested.

Another possibility is that folic acid cannot ameliorate some defects that result from abnormal folate metabolism, owing to defects in the intervening enzymes required to transfer one-carbon units to key downstream metabolites. In this case, supplementation with alternative folates, such as 5-methylTHF (Czeizel et al. 2011), or key downstream molecules may be advantageous. For example, supplementation with formate prevented NTDs in *Mthfd1L* null mice (Momb et al. 2013), whereas combinations of thymidine and purine precursors prevented NTDs in *curly tail* mice, in which folic acid is not protective (Leung et al. 2013).

In addition to low levels of folate and vitamin B_{12}, lower maternal levels of other vitamins, including vitamin C, have been reported in NTDs (Smithells et al. 1976). Conversely, intake of several vitamins and maternal diet are associated with lower risk of NTDs, which suggests that nutrients other than folic acid may be beneficial (Chandler et al. 2012, Sotres-Alvarez et al. 2013). Experimental analysis of individual vitamins found that *myo*-inositol deficiency caused NTDs in cultured rodent embryos (Cockroft 1988). Inositol supplementation significantly reduced NTD frequency in *curly tail* mice (Greene & Copp 1997) and in rodent models of diabetes (Reece et al. 1997), and inositol is in clinical testing for prevention of NTD recurrence.

SUMMARY

Experimental models provide systems for analysis of the developmental events of neural tube closure, and fundamental cellular and morphological processes continue to be defined in more

detail. In principle, NTDs may result from insufficiency of one or more of the key driving forces (e.g., cellular properties and/or morphogenetic movements) that are necessary to achieve closure, for example, through mutation of a PCP gene. Alternatively, a genetic lesion or environmental insult may disrupt the closure process even where the underlying machinery is intact, for example through induction of aberrant cellular behaviors such as excess apoptosis. Experimental models require careful analysis to disentangle these possibilities. A key challenge will be to understand how the molecular and cellular determinants of neurulation relate to the biomechanical forces required to fold the neuroepithelium to achieve closure.

Advances in exome and whole-genome sequencing may help researchers begin to understand the genetic basis of NTDs in humans. The multifactorial complexity of NTDs means that analysis of data from such studies will present a major challenge. Moreover, investigators will need to integrate genetic data with information on epigenetic and environmental factors to obtain a more complete understanding of the cause of individual NTDs.

Folic acid supplementation provides a means to reduce NTD risk and represents a major public health advance. Nevertheless, the heterogeneity of NTDs suggests that primary prevention may be achieved best by multiple interventions, and use of additional micronutrients alongside folic acid may provide additional opportunities to further reduce risk.

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