WILEY
Interdisciplinary Reviews
DEVELOPMENTAL BIOLOGY

In partnership with Society for Developmental Biology

## Article type: Advanced Review

## Article title: Neural tube defects – disorders of neurulation and related embryonic processes

## Authors:

| |
|---|
| **First author**<br>Andrew J. Copp *, Neural Development Unit, Institute of Child Health, University College London, UK. Email: a.copp@ucl.ac.uk. No conflict of interest. |
| **Second author**<br>Nicholas D. E. Greene, Neural Development Unit, Institute of Child Health, University College London, UK. No conflict of interest. |

### Abstract

Neural tube defects (NTDs) are severe congenital malformations affecting 1 in every 1000 pregnancies. ***'Open' NTDs*** result from failure of primary neurulation as seen in anencephaly, myelomeningocele (open spina bifida) and craniorachischisis. Degeneration of the persistently open neural tube *in utero* leads to loss of neurological function below the lesion level. ***'Closed' NTDs*** are skin-covered disorders of spinal cord structure, ranging from asymptomatic spina bifida occulta to severe spinal cord tethering, and usually traceable to disruption of secondary neurulation. ***'Herniation' NTDs*** are those in which meninges, with or without brain or spinal cord tissue, become exteriorised through a pathological opening in the skull or vertebral column (e.g. encephalocele and meningocele). NTDs have multifactorial etiology, with genes and environmental factors interacting to determine individual risk of malformation. While over 200 mutant genes cause open NTDs in mice, much less is known about the genetic causation of human NTDs. Recent evidence has implicated genes of the planar cell polarity signalling pathway in a proportion of cases. The embryonic development of NTDs is complex, with diverse cellular and molecular mechanisms operating at different levels of the body axis. Molecular regulatory events include the BMP and Sonic

hedgehog pathways which have been implicated in control of neural plate bending. Primary prevention of NTDs has been implemented clinically following the demonstration that folic acid, when taken as a peri-conceptional supplement, can prevent many cases. Not all NTDs respond to folic acid, however, and adjunct therapies are required for prevention of this folic acid-resistant category.

## Introduction

Congenital malformations (i.e. birth defects) are important causes of infant morbidity and mortality in developed nations, for example affecting one in every 40 pregnancies in Europe [1]. As perinatal and infant mortality has declined progressively, with improvements in prenatal, intra-partum and neonatal care, so congenital defects have come to represent an ever more significant proportion of the life-threatening conditions of infancy. While around 20% of individuals with birth defects die *in utero*, as stillbirths or as therapeutic abortions, the remainder survive beyond the first week of life [1]. Such infants have a 15-fold increased risk of death during the first year, with 9-10% dying during this period [2]. Those who live beyond one year of age are often destined for a life-time of ill-health, with repeated medical and surgical interventions.

NTDs affect 0.5-2 per 1000 established pregnancies, world wide [3] and are the second commonest group of birth defects, after congenital heart defects. There is a huge range of clinical severity amongst the NTDs. At the severe end of the spectrum, open lesions affecting the brain (anencephaly, craniorachischisis) are invariably lethal before or at birth, and encephalocele can be lethal depending on the extent of brain damage during herniation. While open spina bifida is generally compatible with postnatal survival, it nevertheless results in neurological impairment below the level of the lesion which can lead to lack of sensation, inability to walk and incontinence. Associated conditions include hydrocephalus, which often requires cerebrospinal fluid shunting, vertebral deformities, and genitourinary and gastrointestinal disorders. Closed spinal lesions are less severe and can be asymptomatic, as with spina bifida occulta, although lumbosacral spinal cord tethering can be associated with lower limb motor and sensory deficits and a neuropathic bladder. The severity of symptoms increases with age and surgical untethering of the cord may provide some relief from disability.

## TYPES OF NTDs

A variety of malformations are included under the overall description of NTD. However, it is important to examine carefully whether these are abnormalities of the embryonic neurulation process itself, or alternatively result from some later developmental disturbance that secondarily affects the neural tube.

**Exencephaly and anencephaly**

Failure of cranial neural tube closure results in NTDs in which the brain neural folds remain open (Figure 1A) and exposed to the environment. With continued growth and differentiation, the neuroepithelium characteristically appears to protrude from the developing brain, termed exencephaly (Figure 1B). The skull vault does not form over the open region and subsequent degeneration of the exposed neural tissue gives rise to the typical appearance of anencephaly, as observed in later human pregnancy or at birth in rodents (Figure 1C)[4]. Human anencephaly can be subdivided into those cases mainly affecting the rostral brain and skull (meroacrania) and those also affecting posterior brain and skull (holoacrania) [5]. Mouse exencephaly can similarly affect the forebrain, midbrain or hindbrain, or a combination of adjacent brain regions, depending on the precise event(s) of cranial neurulation that are disturbed.

**Myelomeningocele (open spina bifida; spina bifida aperta)**

If the progression of spinal neurulation along the body axis is severely delayed or halted, then open spina bifida results. As with cranial NTDs, the morphological appearance varies considerably depending on the developmental stage at which the embryo or fetus is examined (Figure 1D-F). In humans, the major forms are myelomeningocele (spina bifida cystica), in which the neural tissue is contained within a meninges-covered sac, and myelocele, in which neural tissue is exposed directly to the amniotic fluid. In normal embryos, the vertebral arches develop from the sclerotomal component of the axial mesoderm, which migrates dorsally to surround the closed neural tube before undergoing cartilaginous and bony differentiation. When the neural folds remain open, the sclerotome is unable to cover the neuroepithelium and a bifid vertebral column is the secondary result.

**Craniorachischisis**

In addition to lesions that separately affect the cranial or spinal neural tube, around 10% of NTDs

comprise a more extensive lesion, termed craniorachischisis, in which the entire neural tube remains open from midbrain to low spine. Such individuals show a characteristically short rostro-caudal body axis, a phenomenon that results from a disruption of the embryonic process of convergent extension. This morphogenetic event, which depends on signalling via the planar cell polarity (PCP) pathway, is specifically defective in craniorachischisis (see below).

**Closed spinal lesions**

This is the least severe, and least well-defined, group of NTDs often referred to as 'occult spina bifida' or 'dysraphic disorders'. Defects of skeletal development, particularly absent neural arches or a midline bony spur, are associated with abnormalities of the spinal cord including over-distension of the central canal (*hydromyelia*), longitudinal duplication or splitting (*diplomyelia*, *diastematomyelia*) and tethering of the cord's lower end. Disorders are often associated with lipoma (as in *lipomyelomeningocele*), and anorectal abnormalities such as anal stenosis or atresia. Embryologically, there is little experimental evidence (e.g. from animal models) to identify the developmental origin of closed spinal lesions, although the neurosurgical literature abounds with theoretical speculation. It seems most likely that abnormalities of secondary neurulation (see Section below) are responsible for closed spina bifida, as: (i) most defects are sacro-coccygeal (i.e. at the level of secondary neurulation); (ii) they do not open to the external environment; (iii) the spinal cord is characteristically 'tethered' of adjacent tissues, as expected of faulty tissue separation during secondary neurulation; (iv) cell types of multiple 'germ layers' are often present, representing the multi-potential nature of the tail bud.

**Encephalocele and meningocele**

In contrast to anencephaly, which results from failure of cranial neural tube closure, encephalocele appears to be primarily a defect of cranial mesoderm development. A persistent opening in the skull, at occipital, parietal or fronto-ethmoidal locations, allows the meninges to herniate creating an extra-cranial mass. In severe cases, brain tissue can also herniate, in which case pathological analysis reveals relatively normal morphology with no evidence of failed neural tube closure [6]. In recent years, Meckel-Gruber syndrome, in which occipital encephalocele is a cardinal feature, has been found to be a 'ciliopathy'; that is, several of the causative genes have key functions in determining structure and function of primary cilia [7]. While many other inherited human disorders

also result from disturbance of 'cilia' genes, this has not generally been reported for open NTDs. Hence, exencephaly/anencephaly appear developmentally and genetically distinct from encephalocele which should, therefore, not be considered an NTD in the strict sense of a defect that has arisen specifically from disturbance of neurulation. Spinal meningocele resembles encephalocele in comprising herniation of meningeal tissue through a skeletal opening (i.e. in the vertebral column). However, much less is known about the etiology or pathogenesis of meningocele, and animal models are required to advance understanding of this defect.

**Iniencephaly**

Often included within the NTD category of malformations, iniencephaly is a severe defect of the cervical spine, including bifid neural arches, with backward flexion of the skull and an extremely short neck. An occipital encephalocele often co-exists [8] and there is a strong female preponderance, as in anencephaly. Too little is known of the pathogenesis of this disorder to determine whether it arises during neurulation, or later during cervical skeletogenesis.

**Hydrocephalus**

While not classified as an NTD *per se*, hydrocephalus is very commonly associated with open spina bifida. The link is the presence of the Chiari type II malformation, in which the brain stem descends into the foramen magnum, blocking circulation of cerebro-spinal fluid (CSF) and leading to hydrocephalus. The origin of Chiari II malformation and its association with open spina bifida is controversial. The persistently open neural tube is physically tethered to adjacent skin [9] and cannot ride upwards within the vertebral canal, as occurs with the normal, mobile spinal cord. This may generate a downward pulling force on the upper, closed cord causing the brain stem to descend. However, other explanations have been suggested, including a primary defect of CSF production (as can occur in the absence of NTDs), or the local effect of a relatively small posterior skull fossa that may lead directly to the Chiari II malformation.

## CAUSATION OF NTDs

**Evidence for a significant genetic etiology**

Both genetic and non-genetic factors are involved in the etiology of NTDs, with up to 70% of the variance in NTD prevalence due to genetic factors [10]. Evidence for genetic causation includes the high recurrence risk for siblings of index cases (2-5%), approximately 50-fold more than in the general population, together with a gradually decreasing risk in more distant relatives. Women with two or more affected pregnancies have a very high risk (~ 10%) of further recurrence [11]. NTD prevalence is greater in like-sex twins (which are assumed to include all monozygotic cases) compared with unlike-sex pairs, consistent with a significant genetic component. It is accepted, therefore, that genetic factors contribute importantly to NTD risk, although the precise nature of this genetic contribution remains unclear. NTDs occur at high frequency across the world, but with a sporadic pattern that rarely involves multi-generational families. This evidence is consistent with a multifactorial polygenic or oligogenic pattern of inheritance, rather than a model based on single dominant or recessive genes with partial penetrance.

**Search for genes causing human NTDs**

The analysis of candidate genes in cohorts of patients with NTDs [12,13] has focused particularly on genes participating in folate one-carbon metabolism, in view of the preventive action of folic acid (FA), and those involved in glucose metabolism, owing to the predisposition of diabetic pregnancy to NTDs. The most robust finding to emerge from this analysis has been the C677T and A1298C polymorphisms of methylenetetrahydrofolate reductase (*MTHFR*), which encodes a key enzyme of folate metabolism responsible for homocysteine remethylation. These polymorphisms are associated with approximately 1.8-fold increased risk of NTDs, although the predisposition is detectable only in non-Hispanic populations [14]. To date, no positive associations with NTD risk have been found for genes regulating glucose metabolism.

**Assessment of human orthologues of mouse NTD genes**

More than 200 genetic models of NTD have been described in mice, which include examples all the main open NTD phenotypes: anencephaly, open spina bifida and craniorachischisis [15]. These mouse models have provided invaluable information on the role of molecular signalling pathways and cell biological processes in neurulation (see Section 3). In addition, the human orthologues of some of the mouse genes have been examined as candidates for human NTD causation, using either case-control association studies or direct sequencing in mutation screens. Apart from recent studies that

have identified putative human mutations in the planar cell polarity pathway (see Section 3), there have been few other positive findings [13].

**Modifier gene functions**

Many studies have demonstrated inbred strain variation in the penetrance and expressivity of NTD phenotypes in mice, providing evidence of significant modifier gene function during neurulation. This likely reflects the action of loci that are polymorphic between strains. For example, the *Cecr2* mutation that causes exencephaly in mice is strongly affected in its expression by one or more modifier genes on Chromosome 19. These vary between the BALB/c strain, where NTDs occur in 75% of homozygotes, and the FVB/N strain where 0% NTDs are observed [16]. Strain differences have also been described for non-genetic causes of NTD including hypoglycaemia, hyperthermia, valproic acid and cytochalasins [17]. While few modifier genes have been definitively identified, a morphogenetic variation in the pattern of cranial neural tube closure was demonstrated to underlie the propensity of some strains (e.g. NZW) but not others (e.g. DBA/2) to promote exencephaly. While most strains exhibit Closure 2 at the midbrain-forebrain boundary (Figure 2A), the DBA/2 strain undergoes Closure 2 more caudally within the midbrain, whereas the NZW strain exhibits Closure 2 more rostrally within the forebrain. When the *splotch* (*Pax3*) mutation was bred onto the DBA/2 background, midbrain closure was enhanced and the frequency of exencephaly amongst homozygous *splotch* embryos was reduced from 80% to around 40%. Conversely, breeding *splotch* onto the NZW strain maintained a forebrain closure point and the high exencephaly rate persisted [18]. Hence, modifier genes may regulate the expression of 'major' NTD genes by altering the developmental substrate on which the latter act.

**Environmental factors**

Neural tube closure in mice and rats is adversely affected by a wide variety of teratogenic agents [17], whereas a much smaller number of non-genetic factors have been definitively associated with human NTDs (Table 1). Valproic acid (VPA), a widely used anticonvulsant agent, increases the risk of NTDs by approximately 10-fold, when taken during early in pregnancy [19]. The potent histone deacetylase (HDAC) inhibitory activity of VPA may disturb the balance of protein acetylation versus deacetylation, leading to failure of neural tube closure. This parallels the action of the HDAC inhibitor, trichostatin-A, which causes NTDs in mice [20]. The fungal product fumonisin is a potent

NTD-causing teratogen, with marked effects on spingolipid metabolism, that likely disturbs downstream embryonic gene expression [21]. Other teratogens with a role in human NTDs are agents that diminish folate uptake/metabolism (e.g. the anticonvulsant carbamazepine and the antibiotic trimethoprim) or availability (conditions of metabolic folate or vitamin B12 deficiency). In mice, diminished access to folate metabolically exacerbates genetic risk of NTDs in a converse way to prevention by exogenous folic acid [22]. Other critical factors for neural tube closure include inositol (see below), diabetes mellitus which predisposes to a range of birth defects including NTDs, maternal obesity, which may act via glycaemic dysregulation, and hyperthermia, with reports of NTDs following high maternal fever or extreme sauna usage in early pregnancy.

### Gene-gene and gene-environmental interactions

Gene-gene and gene-environment interactions are well documented in mouse models of NTDs. While most predisposing mouse mutations are recessive, with NTDs occurring only in homozygotes [15], NTDs also occur in compound heterozygotes for two predisposing genes, or in single heterozygotes exposed to an adverse environmental influence. For example, NTDs in *splotch* mice result from homozygosity for *Pax3* mutations but can also occur in *Pax3* heterozygotes as a result of interaction with mutations in *Nf1* or *Grhl3* [13]. Environmental factors including folate deficiency and arsenic can also exacerbate NTDs in *Pax3* homozygotes, or induce NTDs in the usually unaffected *splotch* heterozygotes [23]. It seems likely that the majority of sporadic human NTDs will also ultimately prove to result from two or more heterozygous genetic risk factors co-existing in individuals who are also exposed to adverse environmental influences, such as sub-optimal folate intake.

## DEVELOPMENT OF NTDs

### Primary neurulation events in mice

In mammals and birds, unlike amphibia, primary neural tube closure is initiated at several discrete points along the rostro-caudal axis; closure is therefore a discontinuous process (Figure 2A)[17,24]. In the mouse, closure is initiated at the hindbrain/cervical boundary (termed Closure 1) on embryonic day (E) 8.5. Closure then progresses in a rostral direction to form the neural tube in the future brain, and simultaneously in a caudal direction along the future spinal cord [25]. Closure initiates separately

around 12 hours later at the forebrain/midbrain boundary (Closure 2) and also at the rostral end of the future forebrain (Closure 3)[26]. The open regions of neural folds between the sites of initial closure are termed 'neuropores', and these close progressively as the neural tube 'zips up' bi-directionally from the sites of Closures 1 and 2, and in a caudal direction from the site of Closure 3. The anterior neuropore completes closure a few hours after Closures 2 and 3, and the hindbrain neuropore finishes closing around the 16 somite stage. From this stage onwards the cranial neural tube is entirely closed, whereas spinal neurulation continues by zipping caudally along the spine until the posterior neuropore closes at the 30 somite stage, on embryonic day 10. This marks the end of primary neurulation.

**Variations in cranial neural tube closure in humans and mice**

In human embryos, neural tube closure begins at 17-18 days post-fertilisation and is discontinuous as in the mouse (Figure 2B). The site of closure initiation resembles Closure 1 in mice, and the onset of closure from the extreme rostral end of the neural plate ('adjacent to the chiasmatic plate') appears comparable to mouse Closure 3 [27]. However, the existence of a Closure 2-like event in human embryos is controversial: some authors describe a similar event as in the mouse [28] while others report the lack of an initiation event between Closures 1 and 3 [27,29]. If human embryos indeed lack a Closure 2 event, then brain formation must be achieved by neurulation progressing directly between Closures 1 and 3, with completion of a single cranial (rostral) neuropore. Human populations could be polymorphic for Closure 2, an idea supported by the finding in mice of variable positioning of Closure 2 between inbred strains [18] (see above). In the SELH/Bc strain, Closure 2 is entirely absent [30] leading to exencephaly in 17% of embryos. Strikingly, however, the remaining 83% of SELH/Bc embryos manage to complete cranial neurulation between Closures 1 and 3, demonstrating that Closure 2 is not needed for brain formation even in the mouse. The human embryo, with its rather smaller midbrain than in the mouse (Figure 2C), may have dispensed with Closure 2 as an evolutionarily unnecessary process.

**Sex and predisposition to exencephaly/anencephaly**

Females are disproportionately represented amongst fetuses with exencephaly/anencephaly both in humans and mice, with a male:female sex ratio often approaching 1:3 [31,32]. In contrast, open spina bifida exhibits a nearly equal sex ratio, or even a male preponderance. While anencephaly could be

a more severe condition in males than females, leading to early pregnancy loss of affected males, the analysis of mouse strains has provided strong evidence against such a 'differential survival' hypothesis. Moreover, male and female mouse embryos progress through the stages of neurulation at identical rates [33], even though females are at a slightly earlier mean developmental stage than male litter mates. This suggests that females become developmentally retarded earlier in pregnancy, an effect that appears to result from the possession of two X chromosomes: presence or absence of a Y chromosome had no effect on frequency of exencephaly in the *Trp53* (formerly known as *p53*) knockout mouse [34]. The developmental mechanism of this interesting sex difference remains to be determined.

**Secondary neurulation**

Following completion of primary neurulation, the neural tube in the lower sacral and coccygeal regions is formed by the process of secondary neurulation, a well recognised feature of both mouse and human development [35,36]. At the lowest spinal levels, the tail bud represents the remnant of the primitive streak and is the sole source of all non-epidermal tissues including the neural tube and the vertebrae. The tail bud contains a self-renewing stem cell population whose derivatives proliferate rapidly leading to longitudinal growth of the body axis. As cells are left in the wake of the 'retreating' tail bud, they condense into cell masses that subsequently differentiate to form the main structures of the post-lumbar region: the neural tube, notochord, somites and hindgut. The neural tube is formed when this cellular condensation undergoes 'canalisation', converting the solid neural precursor into a hollow secondary neural tube. Cell lineage analysis shows that tissues of the low body axis arise from the same stem cell population [37], in contrast to the corresponding tissues at higher levels of the body axis which arise from different 'germ layers' of the gastrulation stage embryo. It is probably for this reason that malformations and tumours (e.g. teratomas) of the sacral and coccygeal regions are often found to embrace several tissue types.

**Assigning NTDs to particular events of neurulation (Figure 2)**

Analysis of mouse mutants such as *loop-tail* (*Vangl2*) has demonstrated that failure of Closure 1 is the fundamental neurulation defect leading to craniorachischisis, the most severe NTD [38]. If Closure 1 is completed successfully, then later arising NTDs present as separate open lesions of the cranial and/or spinal regions: i.e. anencephaly and open spina bifida. In mice, incomplete closure of the

cranial neural tube may result from failure of one of the initiation events (Closures 2 or 3) or from a defect in the subsequent 'zippering' and closure of the anterior or hindbrain neuropores [26]. The commonest defect is failure of zippering through the midbrain, between Closures 1 and 2, owing to the mechanical difficulty of achieving closure on the outer (convex) side of the cranial flexure which is located at midbrain level. Nevertheless, forebrain and hindbrain defects are also observed in different mouse strains, as is failure of Closure 3 which produces the specific phenotype of split face with forebrain anencephaly. In the spinal region, the wave of zippering down the body axis can be arrested at any stage, yielding an open spina bifida of varying length depending on the time of closure cessation. For example, spinal neurulation in the *Kumba* (*Zic2*) mutant fails at the 14-16 somite stage, when dorsolateral hinge point formation first becomes required [39], yielding a large spina bifida from thoracic level downwards. In contrast, spinal closure in the *curly tail* (*Grhl3*) mutant fails after the 25 somite stage, owing to enhanced axial curvature late in neurulation [40], producing a small sacral, or at most lumbo-sacral, spina bifida.

**Key signalling pathways in neurulation and NTDs**

*PCP signalling*

At the onset of neurulation, lengthening and narrowing of the initially disc-shaped neural plate, termed convergent extension, is required to ensure the neural folds are spaced sufficiently closely for closure initiation [41]. First described in amphibia, convergent extension comprises the lateral to medial displacement of cells in the presumptive mesoderm and neural plate. Cell intercalation in the midline leads to medial-lateral narrowing (convergence) and rostro-caudal lengthening (extension) of the body axis [42]. At the molecular level, convergent extension depends on a non-canonical WNT signalling cascade, the planar cell polarity (PCP) pathway which signals via frizzled membrane receptors and cytoplasmic dishevelled, but does not involve downstream stabilisation of beta-catenin [43]. Specific inhibition of PCP signalling in *Xenopus laevis*, by functional disruption of the key signalling molecule dishevelled (DVL), resulted in inhibition of convergent extension and gave rise to short, broad embryos whose neural folds failed to close [44]. At the cellular level, PCP signalling is thought to control polarized cellular motility, in particular by regulating formation of stable mediolaterally oriented actin-rich lamellipodia, which provide cell-cell and cell-matrix traction [42].

In mice, loss of function of the core PCP pathway genes *Vangl2* (the homologue of *Drosophila*

*strabismus/Van gogh*) in *loop-tail* mutant, *Celsr1* (the homologue of *Drosophila flamingo/starry night*) in *Crash* mice, or double mutants for *Fzd3* and *Fzd6*, or two of the three *disheveled* genes (*Dvl1* and *Dvl2*) all suppress convergent extension cell movements. Loss of function of other PCP-related genes also cause craniorachischisis including *Scirb* (in the *Circletail* mouse) and the tyrosine kinase *Ptk7* [45]. A broad neural plate results in which Closure 1 fails, leading to craniorachischisis [46]. Hence, there is a specific relationship between PCP signalling, convergent extension, and initiation of neural tube closure.

*SHH signalling*

Sonic hedgehog (SHH), one of three mammalian homologues of *Drosophila* hedgehog, initiates an intracellular signalling pathway by binding to its transmembrane receptor, Patched1 (PTCH1). In the absence of SHH ligand, PTCH1 interacts with an associated membrane protein, Smoothened (SMO), to inhibit its activity. SHH binding to PTCH1 removes the inhibitory effect on SMO, allowing members of the GLI family of proteins to be processed as transcriptional activators. In addition to this core signal transduction machinery, a variety of other proteins have been identified that exert either positive or negative influences on SHH signalling. Strikingly, however, it is primarily genetic changes in proteins with a negative influence on the SHH pathway that lead to NTDs in mice [47]. For example, mutations in *PTCH1* that relieve the inhibition of SMO activity, and abolition of inhibitory phosphorylation sites for protein kinase A in GLI2, both lead to NTDs. Loss of function of other SHH inhibitory genes, including *Fkbp8*, *Gli3*, *Rab23* and *Tulp3*, also produce NTDs. In contrast, loss of function of proteins that activate signalling, including SMO and SHH itself, does not produce NTDs [48,49], whereas over-expression of such proteins can compromise neural tube closure. These findings argue for an overall negative influence of SHH signalling on neural tube closure, an effect that appears to be mediated via the inhibition of dorsolateral neural plate bending [50], which is essential for closure in both the midbrain and lower spinal region (see next section).

*BMP signalling*

Bone morphogenetic proteins (BMPs) are members of the TGFβ superfamily of proteins that signal via specific BMP receptors to regulate transcription via Smad proteins in a canonical pathway and via several tyrosine kinases in non-canonical signalling. BMPs specify dorsal identity in the early embryo, acting reciprocally to Shh which is the main ventralising factor of the early nervous system.

Strikingly, however, both BMP and Shh signalling have been found to inhibit dorsolateral hinge point (DLHP) formation during mouse neurulation. Loss of activity in either pathway yields precocious DLHP formation, whereas gain of function inhibits bending [39,50]. Factors that induce DLHP formation include the BMP antagonists noggin and neuralin. These proteins are secreted by the dorsal neural plate and enable DHLP formation via antagonism of the BMP inhibitory influence. Their production is under negative regulatory control by SHH so that, at upper spinal levels where strong SHH expression occurs in the notochord, noggin and neuralin production is inhibited and DLHP formation is blocked. Upper spinal neurulation therefore exhibits only midline bending, with a V shaped neural groove. Lower in the spinal region, however, SHH expression in the notochord declines and noggin/neuralin production is able to occur. This enables DLHP formation to 'break through', facilitating closure under circumstances of convex body axis curvature, which characterises the embryo after axial rotation is complete. Failure to develop DLHPs, as in the *Kumba* (*Zic2*) mutant, leads to failure of closure in the lower spinal region, and severe spina bifida results [39].

*Grainyhead-like* (*GRHL*) genes

*Curly tail* (*ct*) is one of the best understood mouse models of NTDs [40]. Neural tube closure is delayed in the low spinal region owing to a growth imbalance between the slowly-proliferating hindgut and the normally-proliferating neural plate. This growth imbalance enhances curvature of the caudal region, which mechanically opposes neural fold elevation and fusion [51]. The molecular basis of NTDs in *curly tail* was elucidated after mice null for *Grhl3*, a gene within the *ct* critical region on Chromosome 4, were found to display spina bifida closely resembling *curly tail* [52]. A *Grhl3*-containing bacterial artificial chromosome (BAC) completely rescued spinal NTDs in transgenic *curly tail* embryos, demonstrating that *Grhl3* is the main defective gene in the *ct* mutant [53]. Indeed, at the stage of low spinal closure, *Grhl3* transcripts localize specifically to the hindgut, the site of the cell proliferation defect. Another family member, *Grhl2*, also causes spina bifida in mice. *Axial defects* (*Axd*) mutants, which exhibit spina bifida and tail flexion defects, over-express *Grhl2*, and their phenotype can be ameliorated by introducing a null *Grhl2* allele [54]. Moreover, mice homozygous for loss of *Grhl2* function also exhibit NTDs, demonstrating that both loss and gain of function of *GRHL* genes is a potent cause of neurulation disturbance [54].

*Retinoid signalling*

Retinoic acid (RA) has long been studied as a potent teratogen in rodent systems, with NTDs among the malformations most often observed. It is now realised that endogenous synthesis of retinoids forms an integral part of early development, and that any disturbance in the balance between production and turnover of retinoids can adversely affect developmental events including neural tube closure. Hence, NTDs are observed in mice with loss of function of the genes encoding *Aldh1a2* (formerly known as *Raldh2*), a principal enzyme of RA synthesis, *Cyp26a1*, a key RA metabolising enzyme and RA receptor genes *Rara* and *Rarg* through which RA signalling is mediated [55]. While the existence of signalling pathways downstream of the RA receptors is well known, it remains unclear precisely how RA signalling participates in neural tube closure, at the cellular and tissue levels.

**Key cellular functions in neurulation and NTDs**

*Cytoskeleton*

A long standing question relates to the role of the cytoskeleton in neurulation. Actin microfilaments are localised circumferentially in the apices of all neuroepithelial cells [56]. Acto-myosin contraction could therefore reduce the apical surface area of the neuroepithelium and contribute to bending and closure of the neural folds. Consistent with this, experimental disruption of the cytoskeleton by actin-disassembling drugs such as cytochalasins causes exencephaly in cultured rodent embryos indicating a role in cranial neural tube closure [57]. Higher doses of cytochalasin D also inhibit closure 1, but spinal neurulation is resistant to cytochalasin D, with the stereotypical midline and dorsolateral bending points maintained in the absence of apical microfilaments [58]. These observations using cytoskeletal inhibitors are mirrored in studies of mice with null mutations in genes encoding cytoskeletal proteins [45]. Whereas cranial NTDs are seen in several mice with compromised cytoskeletal function (e.g. *Cfl1*, *Palld*, *Vcl* mutants), spinal neurulation is completed successfully in such embryos. Only mice null for the cytoskeleton-associated proteins *Shroom3* and MARCKS-like 1 (formerly known as MARCKS-related protein) exhibit both exencephaly and spina bifida in a proportion of homozygotes. Hence, while regulation of the acto-myosin cytoskeleton appears essential for cranial neural tube closure, its role in spinal neurulation remains unclear.

*Apoptosis*

Excessive cell death has been implicated in the NTDs resulting from many genetic and environmental insults. It is crucial, however, to demonstrate that increased apoptosis precedes failed neural tube closure, as later-appearing abnormalities could be a secondary consequence of the failed morphogenesis. Genetic defects in which excessive cell death occurs prior to or during neural tube closure include the anti-apoptotic *Bcl10* gene, and inhibitors of kappa B kinase (formerly known as IKK factors) that activate NFκB in an anti-apoptotic pathway, and the transcription factors TFAP2A (formerly known as AP2) and BRCA1, which are required for cell survival. Conversely, NTDs also occur in mice lacking gene functions including *Casp3* and *Casp9* which are required for apoptosis [45]. In these cases, diminished or abolished cell death is observed and NTDs are localised to the midbrain and hindbrain, whereas neurulation in forebrain and spinal regions is completed normally. Similarly, in the *Apaf1* mutant, apoptosis is diminished and yet NTDs only affect the cranial region. Chemical blockade of apoptosis in mouse embryo culture, by inhibition of effector caspases with Z-vad-fmk or by inhibition of TRP53 with pifithrin-α, produced no defects of neural tube closure [59], suggesting that *Apaf-1* or *Casp 3/9* mutants are unlikely to develop exencephaly solely due to diminution of apoptosis. It remains to be determined what is the cause of the plentiful cell death observed in the dorsal midline of the closing neural tube, although anoikis owing to transient remodelling of epithelial layers appears a possibility.

*Cell proliferation*

The neurulation stage embryo is a rapidly proliferating system, with neuroepithelial cell cycle times as short as 4-6 hours. On the other hand, neuroepithelial cells begin to exit the cell cycle and embark upon neuronal differentiation soon after neural tube closure, suggesting that the balance between continued proliferation and onset of neuronal differentiation may be critical for closure. Indeed, several genes whose mutations lead to NTDs are essential for cell proliferation and/or prevention of premature neurogenesis [45]. These include *Jarid2* (formerly known as *jumonji*), which has homology to cell cycle-associated retinoblastoma (RB1)-binding proteins, NEUROFIBROMATOSIS 1 and NUCLEOPORIN 50, which are negative regulators of the cell cycle inhibitors CDKN1A and CDKN1B (formerly known as p21 and p27) respectively, and *PAX3* which promotes rapid dorsal neuroepithelial cell proliferation. NTDs also result from defects in genes encoding negative regulators of the Notch signalling pathway including HES1, HES3 and RBPJ which are required to postpone neuronal differentiation until neural tube closure is complete. Conversely, a number of genetically-induced NTDs have been associated with excessive cell proliferation, the best

characterised example being loss of function of *Phactr4*, which is required to maintain a high-dorsal, low-ventral gradient of cell proliferation in the neuroepithelium. Phactr4 regulates a cascade of protein phosphorylation, involving the phosphatase PPP1CC so that, in its absence, RB1 becomes hyperphosphorylated and can no longer regulate E2F protens and their targets to limit cell-cycle progression. Excessive ventral cell proliferation ensues which causes exencephaly in *Phactr4* mutants [60].

## CLINICAL DIAGNOSIS AND MANAGEMENT OF NTDs

### Historical trends in management

Prior to the 1970s, management of NTDs consisted solely of palliative surgical and medical support. While children with open spina bifida generally survive if their lesion is closed surgically, thereby avoiding ascending infection, neurological outcome varies markedly with the vertebral level of lesion (i.e. higher defects have greater neurological handicap). This led to suggestions that surgery should be offered only in cases with a better prognosis [61]. An ethical debate ensued, around whether surgical treatment should be withheld, but this was superseded in the 1970s when methods for prenatal diagnosis of open NTDs were developed. Initially, diagnosis was based on measurement of alphafetoprotein (AFP) concentration in the amniotic fluid and maternal blood, but later technological improvements enabled ultrasound to replace AFP measurement as the mainstay of prenatal diagnosis [62]. Today, most fetuses with NTDs are diagnosed prenatally in developed countries, and many are aborted therapeutically. In contrast, large numbers of babies with NTDs continue to be born in developing countries where prenatal diagnosis is not routine, as well as in countries where therapeutic abortion is not available.

### In utero surgery

In both humans and mice, the open neural tube undergoes relatively normal neuronal differentiation, with development of spinal motor and sensory function even below the lesion level. As gestation progresses, however, neurons die within the exposed spinal cord, axonal connections are interrupted, and function is lost [9]. Hence, neurological disability in open spina bifida is a 'two-hit' process: failed neural tube closure followed by neurodegeneration *in utero*. This has encouraged attempts to cover the persistently open neural tube during fetal development, in order to arrest or

prevent the neurodegeneration. Surgical repair *in utero* for early open spina bifida is practised in several centres in the USA and the success of this procedure has recently been evaluated in a randomised controlled trial [63]. This showed significant benefits for the child postnatally, including a 50% reduction in shunting for hydrocephalus and a significant improvement in spinal neurological function. Against this was a significantly higher rate of premature birth and maternal complications such as uterine dehiscence at the operation site. Clearly, surgery *in utero* for open spina bifida has both benefits and risks.

**Economic effect of NTDs and cost-benefit analysis of primary prevention**

The life-time medical and non-medical costs of a person with spina bifida are estimated at $560,000 [64], without taking into account the lost earnings of the individual. On the other hand, public health measures to prevent NTDs, which include education programmes to promote voluntary FA supplementation and mandatory food fortification, are themselves costly undertakings. A systematic review of cost-benefit analyses has concluded that both voluntary FA supplementation and food fortification with FA are cost-effective in a range of countries [65]. Benefit-cost ratios for food fortification were 4.3 to 1 in USA, 11.8 to 1 in Chile and 30 to 1 in South Africa, emphasising the desirability of extending such primary preventive measures for NTDs.

## PRIMARY PREVENTION OF NTDs

### Folic acid and history of prevention strategies

Among the most significant advances in prevention of birth defects has been the finding, through clinical trials, that use of maternal folic acid supplements can substantially reduce the risk of a pregnancy affected by NTD [66,67]. These results prompted the recommendation that all women planning pregnancy should consume 0.4 mg folic acid per day, and that women at high risk of NTD should receive 4-5 mg per day. In order to increase the population intake of folic acid, mandatory food fortification was introduced in the USA in 1999, and later in other countries. Subsequent studies suggest this has contributed to a decline in NTD incidence [68]. Nevertheless, it is apparent that a subset of NTDs are not prevented by current therapeutic strategies such that approximately 0.7-0.8 per 1,000 pregnancies persist despite folic acid usage [69].

**Folate one carbon metabolism and NTDs**

It is often assumed that NTDs are a vitamin-deficiency condition, but in fact the great majority of human NTD-affected pregnancies are not clinically folate-deficient [70]. Moreover, severe folate deficiency in mouse models does not cause NTDs in the absence of a genetic predisposition [22]. It seems more likely that exogenous folic acid is able to stimulate a cellular response, enabling the developing embryo to overcome the adverse effects of genetic and/or environmental disturbances that would otherwise lead to NTDs. These disturbances could involve abnormalities in folate-related pathways, but might also affect systems unrelated to folate metabolism. Folates are integral to intracellular one-carbon metabolism which produces pyrimidines and purines for DNA synthesis and s-adenosyl methionine, the universal methyl group donor for all macromolecules. Hence, cell proliferation and/or cell survival, which depend on DNA synthesis, are likely effects of folate supplementation although, to date, no well characterised example has been reported of folic acid preventing NTDs in a model system via stimulation of cell proliferation, or suppression of apoptosis. Similarly, stimulation of DNA, protein and/or lipid methylation are possible outcomes of folate supplementation, although no specific examples of increased methylation linked to NTD prevention exist as yet. Interestingly, during mouse pregnancy, severe folate deficiency was not found to decrease embryonic global DNA methylation [22] and conversely NTDs do not arise in models such as *Mrhfr* knock-outs in which overall DNA methylation is significantly diminished.

Clearly, a priority for future research is to improve our knowledge of the cellular mechanism(s) underlying the preventive action of folate supplementation. This could indicate why some NTDs are folate-responsive and others folate-resistant, in turn focusing on the different aetiologies in each case. This might lead to better prediction of the likely importance of folate supplementation in pregnancy, or the need for additional treatments such as inositol.

**Folate-resistant NTDs and inositol**

Estimates vary as to the effect of FA supplementation on the prevalence of NTDs. Even after high dose (4 mg) therapy in the MRC trial, 1% of pregnancies still had recurrent NTDs [67], a 10-fold excess over the general population frequency. It appears, therefore, that not all cases of NTDs are preventable by folic acid, a conclusion supported by reports of NTD pregnancies recurring in families despite high dose folate intake [71]. Novel therapies are needed, therefore, to improve NTD prevention particularly for folate-resistant cases which currently cannot be prevented.

Among mouse NTD models, some show prevention by FA whereas others appear resistant. For example, NTDs in the *splotch* (*Pax3*) and *Cited2* mutant mice are reduced in frequency and severity by FA, whereas NTDs in the *curly tail* mutant mouse are resistant [72]. Inositol is the only vitamin-like molecule to be required for the normal rodent neural tube to close [73], and both *myo*-inositol and D-*chiro*-inositol can prevent NTDs in the FA-resistant *curly tail* NTD model [72]. Direct treatment of *curly tail* embryos in culture normalises low spinal neural tube closure, demonstrating that inositol's action is independent of the maternal environment. Moreover, inositol is also able to prevent diabetes-induced NTDs in mice and rats [72], arguing for a general effect of inositol in enhancing neural tube closure. Indeed, targeted mutations in the *Itpk1*, *Pip5kIc* and *Inpp5e* genes of mice produce NTDs via direct disturbance of inositol metabolism [45]. The observations in mouse models together with the finding that some human NTD pregnancies have lower maternal inositol concentrations than unaffected pregnancies [74] has prompted an ongoing clinical trial to evaluate inositol as a preventive agent for NTDs, alongside FA (www.pontistudy.ich.ucl.ac.uk).

## Conclusion

NTDs represent arguably the best understood category of human birth defects. Many decades of research by developmental biologists, epidemiologists and clinicians has led to significant advances in our knowledge of the embryonic process of neurulation and its disorders, the patterns of variation of NTDs in human populations, and the role of folic acid in primary prevention. Clinical methods have been developed and refined for the prenatal diagnosis and *in utero* surgical repair of NTDs. Much less well understood is the area of NTD genetics and, while there appears no doubt that genetic factors play a key (perhaps predominant) role in etiology, we are yet to define the principal genes that determine risk of human NTDs. Advances in NTD genetics should herald a new era in which the complex sub-types of these disorders will become discernible enabling, for example, much more precise targeting of preventive therapies. Hence, some genetic sub-types may require only low dose (e.g. 400 µg) FA for prevention, others may require higher doses (e.g. 4 mg), and others may be resistant to FA, necessitating the use of alternative preventive agents. In few other areas of human biology is there a greater need for a multi-disciplinary approach to such a complex developmental disorder.

## References


1. Dolk H, Loane M, Garne E. The prevalence of congenital anomalies in Europe. Adv Exp Med Biol 2010, 686:349-364.

2. Malcoe LH, Shaw GM, Lammer EJ, Herman AA. The effect of congenital anomalies on mortality risk in white and black infants. Am J Public Health 1999, 89:887-892.

3. Mitchell LE. Epidemiology of neural tube defects. Am J Med Genet C Semin Med Genet 2005, 135:88-94.

4. Wood LR, Smith MT. Generation of anencephaly: 1. Aberrant neurulation and 2. Conversion of exencephaly to anencephaly. J Neuropath exp Neurol 1984, 43:620-633.

5. Seller MJ. Sex, neural tube defects, and multisite closure of the human neural tube. Am J Med Genet 1995, 58:332-336.

6. Harding BN, Copp AJ. Malformations. In Love S, Louis DN, Ellison DW, eds., Greenfield's Neuropathology. Hodder Arnold, London; 2008, 335-479.

7. Logan CV, Abdel-Hamed Z, Johnson CA. Molecular genetics and pathogenic mechanisms for the severe ciliopathies: Insights into neurodevelopment and pathogenesis of neural tube defects. Mol Neurobiol 2010, 43:12-26.

8. Erdincler P, Kaynar MY, Canbaz B, Kocer N, Kuday C, Ciplak N. Iniencephaly: neuroradiological and surgical features. Case report and review of the literature. J Neurosurg 1998, 89:317-320.

9. Stiefel D, Shibata T, Meuli M, Duffy P, Copp AJ. Tethering of the spinal cord in mouse fetuses and neonates with spina bifida. J Neurosurg (Spine) 2003, 99:206-213.

10. Leck I. Causation of neural tube defects: clues from epidemiology. Br Med Bull 1974, 30:158-163.

11. Rampersaud E, Melvin EC, Speer MC. Nonsyndromic neural tube defects: genetic basis and genetic investigations. In Wyszynski DF, ed., Neural Tube Defects: From Origin to Treatment. Oxford University Press, Oxford; 2006, 165-175.

12. Boyles AL, Hammock P, Speer MC. Candidate gene analysis in human neural tube defects. Am J Med Genet C Semin Med Genet 2005, 135:9-23.

13. Greene NDE, Stanier P, Copp AJ. Genetics of human neural tube defects. Hum Mol Genet 2009, 18:R113-R129.

14. Amorim MR, Lima MA, Castilla EE, Orioli IM. Non-Latin European descent could be a requirement for association of NTDs and MTHFR variant 677C > T: a meta-analysis. Am J Med Genet A 2007, 143A:1726-1732.

15. Harris MJ, Juriloff DM. An update to the list of mouse mutants with neural tube closure defects and advances toward a complete genetic perspective of neural tube closure. Birth Defects Res A Clin Mol Teratol 2010, 88:653-669.

16. Davidson CE, Li Q, Churchill GA, Osborne LR, McDermid HE. Modifier locus for exencephaly in Cecr2 mutant mice is syntenic to the 10q25.3 region associated with neural tube defects in humans. Physiol Genomics 2007, 31:244-251.

17. Copp AJ, Brook FA, Estibeiro JP, Shum ASW, Cockroft DL. The embryonic development of mammalian neural tube defects. Prog Neurobiol 1990, 35:363-403.

18. Fleming A, Copp AJ. A genetic risk factor for mouse neural tube defects: defining the embryonic basis. Hum Mol Genet 2000, 9:575-581.

19. Lammer EJ, Sever LE, Oakley GP. Teratogen update: valproic acid. Teratology 1987, 35:465-473.

20. Menegola E, Di RF, Broccia ML, Prudenziati M, Minucci S, Massa V, Giavini E. Inhibition of histone deacetylase activity on specific embryonic tissues as a new mechanism for teratogenicity. Birth Defects Res B Dev Reprod Toxicol 2005, 74:392-398.

21. Gelineau-van WJ, Voss KA, Stevens VL, Speer MC, Riley RT. Maternal fumonisin exposure as a risk factor for neural tube defects. Adv Food Nutr Res 2009, 56:145-181.

22. Burren KA, Savery D, Massa V, Kok RM, Scott JM, Blom HJ, Copp AJ, Greene NDE. Gene-environment interactions in the causation of neural tube defects: folate deficiency increases susceptibility conferred by loss of *Pax3* function. Hum Mol Genet 2008, 17:3675-3685.

23. Greene ND, Massa V, Copp AJ. Understanding the causes and prevention of neural tube defects: Insights from the splotch mouse model. Birth Defects Res A Clin Mol Teratol 2009, 85:322-330.

24. Van Straaten HWM, Jansen HCJP, Peeters MCE, Copp AJ, Hekking JWM. Neural tube closure in the chick embryo is multiphasic. Dev Dyn 1996, 207:309-318.

25. Copp AJ, Greene NDE, Murdoch JN. The genetic basis of mammalian neurulation. Nat Rev Genet 2003, 4:784-793.

26. Copp AJ. Neurulation in the cranial region - normal and abnormal. J Anat 2005, 207:623-635.

27. O'Rahilly R, Müller F. The two sites of fusion of the neural folds and the two neuropores in the human embryo. Teratology 2002, 65:162-170.

28. Nakatsu T, Uwabe C, Shiota K. Neural tube closure in humans initiates at multiple sites: evidence from human embryos and implications for the pathogenesis of neural tube defects. Anat Embryol 2000, 201:455-466.

29. Sulik KK, Zuker RM, Dehart DB, Cessot F, Delezoide AL, Attié T, Vekemans M. Normal patterns of neural tube closure differ in the human and the mouse. Proceedings of the Greenwood Genetic Center 1998, 18:129-130.

30. MacDonald KB, Juriloff DM, Harris MJ. Developmental study of neural tube closure in a mouse stock with a high incidence of exencephaly. Teratology 1989, 39:195-213.

31. Seller MJ. Neural tube defects and sex ratios. Am J Med Genet 1987, 26:699-707.

32. Harris MJ, Juriloff DM. Mouse mutants with neural tube closure defects and their role in understanding human neural tube defects. Birth Defects Res A Clin Mol Teratol 2007, 79:187-210.

33. Brook FA, Estibeiro JP, Copp AJ. Female predisposition to cranial neural tube defects is not because of a difference between the sexes in rate of embryonic growth or development during neurulation. J Med Genet 1994, 31:383-387.

34. Chen X, Watkins R, Delot E, Reliene R, Schiestl RH, Burgoyne PS, Arnold AP. Sex difference in neural tube defects in p53-null mice is caused by differences in the complement of X not Y genes. Dev Neurobiol 2008, 68:265-273.

35. Schoenwolf GC. Histological and ultrastructural studies of secondary neurulation of mouse embryos. Am J Anat 1984, 169:361-374.

36. Lemire RJ. Variations in development of the caudal neural tube in human embryos (Horizons XIV-XXI). Teratology 1969, 2:361-370.

37. Cambray N, Wilson V. Axial progenitors with extensive potency are localised to the mouse chordoneural hinge. Development 2002, 129:4855-4866.

38. Copp AJ, Checiu I, Henson JN. Developmental basis of severe neural tube defects in the *loop-tail* (*Lp*) mutant mouse: Use of microsatellite DNA markers to identify embryonic genotype. Dev Biol 1994, 165:20-29.

39. Ybot-Gonzalez P, Gaston-Massuet C, Girdler G, Klingensmith J, Arkell R, Greene ND, Copp AJ. Neural plate morphogenesis during mouse neurulation is regulated by antagonism of BMP signalling. Development 2007, 134:3203-3211.

40. Van Straaten HWM, Copp AJ. Curly tail: a 50-year history of the mouse spina bifida model. Anat Embryol 2001, 203:225-237.

41. Ybot-Gonzalez P, Savery D, Gerrelli D, Signore M, Mitchell CE, Faux CH, Greene NDE, Copp AJ. Convergent extension, planar-cell-polarity signalling and initiation of mouse neural tube closure. Development 2007, 134:789-799.

42. Keller R, Shook D, Skoglund P. The forces that shape embryos: physical aspects of convergent extension by cell intercalation. Phys Biol 2008, 5:15007.

43. Montcouquiol M, Crenshaw EB, III, Kelley MW. Noncanonical Wnt signaling and neural polarity. Annu Rev Neurosci 2006, 29:363-386.

44. Wallingford JB, Harland RM. Neural tube closure requires Dishevelled-dependent convergent extension of the midline. Development 2002, 129:5815-5825.

45. Copp AJ, Greene NDE. Genetics and development of neural tube defects. J Pathol 2010, 220:217-230.

46. Greene NDE, Gerrelli D, Van Straaten HWM, Copp AJ. Abnormalities of floor plate, notochord and somite differentiation in the *loop-tail* (*Lp*) mouse: a model of severe neural tube defects. Mech Dev 1998, 73:59-72.

47. Murdoch JN, Copp AJ. The relationship between Hedgehog signalling, cilia and neural tube defects. Birth Defects Res A Clin Mol Teratol 2010, 88:633-652.

48. Chiang C, Litingtung Y, Lee E, Young KE, Corden JL, Westphal H, Beachy PA. Cyclopia and defective axial patterning in mice lacking *Sonic hedgehog* gene function. Nature 1996, 383:407-413.

49. Zhang XM, Ramalho-Santos M, McMahon AP. *Smoothened* mutants reveal redundant roles for Shh and Ihh signaling including regulation of L/R asymmetry by the mouse node. Cell 2001, 105:781-792.

50. Ybot-Gonzalez P, Cogram P, Gerrelli D, Copp AJ. Sonic hedgehog and the molecular regulation of neural tube closure. Development 2002, 129:2507-2517.

51. Brook FA, Shum ASW, Van Straaten HWM, Copp AJ. Curvature of the caudal region is responsible for failure of neural tube closure in the curly tail (ct) mouse embryo. Development 1991, 113:671-678.

52. Ting SB, Wilanowski T, Auden A, Hall M, Voss AK, Thomas T, Parekh V, Cunningham JM, Jane SM. Inositol- and folate-resistant neural tube defects in mice lacking the epithelial-specific factor Grhl-3. Nature Med 2003, 9:1513-1519.

53. Gustavsson P, Greene ND, Lad D, Pauws E, de Castro SC, Stanier P, Copp AJ. Increased expression of Grainyhead-like-3 rescues spina bifida in a folate-resistant mouse model. Hum Mol Genet 2007, 16:2640-2646.

54. Brouns MR, de Castro SC, Terwindt-Rouwenhorst EA, Massa V, Hekking JW, HIrst CS, Savery D, Munts C, Partridge D, Lamers W, et al. Over-expression of Grhl2 causes spina bifida in the Axial defects mutant mouse. Hum Mol Genet 2011, 20:1536-1546.

55. Pennimpede T, Cameron DA, MacLean GA, Li H, Abu-Abed S, Petkovich M. The role of CYP26 enzymes in defining appropriate retinoic acid exposure during embryogenesis. Birth Defects Res A Clin Mol Teratol 2010, 88:883-894.

56. Sadler TW, Greenberg D, Coughlin P, Lessard JL. Actin distribution patterns in the mouse neural tube during neurulation. Science 1982, 215:172-174.

57. Morriss-Kay GM, Tuckett F. The role of microfilaments in cranial neurulation in rat embryos: effects of short-term exposure to cytochalasin D. J Embryol Exp Morphol 1985, 88:333-348.

58. Ybot-Gonzalez P, Copp AJ. Bending of the neural plate during mouse spinal neurulation is independent of actin microfilaments. Dev Dyn 1999, 215:273-283.

59. Massa V, Savery D, Ybot-Gonzalez P, Ferraro E, Rongvaux A, Cecconi F, Flavell RA, Greene NDE, Copp AJ. Apoptosis is not required for mammalian neural tube closure. Proc Natl Acad Sci U S A 2009, 106:8233-8238.

60. Kim TH, Goodman J, Anderson KV, Niswander L. Phactr4 regulates neural tube and optic fissure closure by controlling PP1-, Rb-, and E2F1-regulated cell-cycle progression. Dev Cell 2007, 13:87-102.

61. Lorber J. Results of treatment of myelomeningocele. An analysis of 524 unselected cases with special reference to possible selection for treatment. Dev Med Child Neurol 1971, 13:279-303.

62. Cameron M, Moran P. Prenatal screening and diagnosis of neural tube defects. Prenatal Diag 2009, 29:402-411.

63. Adzick NS, Thom EA, Spong CY, Brock JW, III, Burrows PK, Johnson MP, Howell LJ, Farrell JA, Dabrowiak ME, Sutton LN, et al. A randomized trial of prenatal versus postnatal repair of myelomeningocele. N Engl J Med 2011, 364:993-1004.

64. Grosse SD, Ouyang L, Collins JS, Green D, Dean JH, Stevenson RE. Economic evaluation of a neural tube defect recurrence-prevention program. Am J Prev Med 2008, 35:572-577.

65. Yi Y, Lindemann M, Colligs A, Snowball C. Economic burden of neural tube defects and impact of prevention with folic acid: a literature review. Eur J Pediatr 2011, 170:1391-1400.

66. Smithells RW, Sheppard S, Schorah CJ, Seller MJ, Nevin NC, Harris R, Read AP, Fielding DW. Possible prevention of neural-tube defects by periconceptional vitamin supplementation. Lancet 1980, 1:339-340.

67. Wald N, Sneddon J, Densem J, Frost C, Stone R, MRC Vitamin Study Res Group. Prevention of neural tube defects: Results of the Medical Research Council Vitamin Study. Lancet 1991, 338:131-137.

68. Eichholzer M, Tonz O, Zimmermann R. Folic acid: a public-health challenge. Lancet 2006, 367:1352-1361.

69. Heseker HB, Mason JB, Selhub J, Rosenberg IH, Jacques PF. Not all cases of neural-tube defect can be prevented by increasing the intake of folic acid. Br J Nutr 2008, 16:1-8.

70. Kirke PN, Molloy AM, Daly LE, Burke H, Weir DG, Scott JM. Maternal plasma folate and vitamin $B_{12}$ are independent risk factors for neural tube defects. Q J Med 1993, 86:703-708.

71. Cavalli P, Tedoldi S, Riboli B. Inositol supplementation in pregnancies at risk of apparently folate-resistant NTDs. Birth Defects Res A Clin Mol Teratol 2008, 82:540-542.

72. Greene ND, Copp AJ. Mouse models of neural tube defects: investigating preventive mechanisms. Am J Med Genet C Semin Med Genet 2005, 135:31-41.

73. Cockroft DL. Changes with gestational age in the nutritional requirements of postimplantation rat embryos in culture. Teratology 1988, 38:281-290.

74. Groenen PM, Peer PG, Wevers RA, Swinkels DW, Franke B, Mariman EC, Steegers-Theunissen RP. Maternal myo-inositol, glucose, and zinc status is associated with the risk of offspring with spina bifida. Am J Obstet Gynecol 2003, 189:1713-1719.

## Figure captions

**Figure 1**

Natural history of open cranial (A-C) and spinal (D-F) NTDs in mice. After an initial failure of neural tube closure in either the midbrain (A) or low spine (D), the neuroepithelium continues to proliferate and undergoes neuronal differentiation, appearing to protrude from the surface of the embryo (B,E). This is termed 'exencephaly' in cranial lesions. With continued gestation, the exposed neuroepithelium becomes damaged by continuous exposure to the amniotic fluid. Apoptosis and necrosis intervene so that, by the time of birth, the neuroepithelium has degenerated, yielding the phenotype of anencephaly (C) or myelocele (D). Developmental stages indicated as E (embryonic day) or P (postnatal day). Figures modified from: Dunlevy et al, 2006, *FEBS Letters* 580, 2803-7 (A); Copp et al, 2003, *Nature Reviews Genetics* 4, 784–793 (B); Copp, 2005, *Journal of Anatomy* 207, 623-35 (C); Van Straaten & Copp, 2001, *Anatomy and Embryology*, 203, 225-237 (D,E); Stiefel et al, 2003, *Journal of Neurosurgery (Spine)* 99, 206–213 (F).

**Figure 2**

Diagrammatic representation of the main events of neural tube closure in mouse (A) and human (B) embryos. The main types of NTD resulting from failure of closure at different levels of the body axis are indicated. The red shading on the tail bud indicates the site of secondary neurulation in both species. Disturbance of this process leads to closed spina bifida. In each species the initial *de novo* closure event (Closure 1) occurs at the hindbrain/cervical boundary and closure spreads bidirectionally from this site. In the mouse, a second *de novo* closure site (Closure 2) occurs at the forebrain/midbrain boundary with closure also spreading rostrally and caudally. Closure 2 does not appear to occur in human embryos (B). A third *de novo* initiation event (Closure 3) occurs in both species at the rostral extremity of the neural plate, with closure spreading caudally from here. Hence, in mice, closure is completed sequentially at the anterior neuropore, hindbrain neuropore and posterior neuropore. In humans, owing to the lack of Closure 2, there are likely to be only two neuropores: anterior and posterior. (C) Human embryo aged 35 days post-fertilisation from the Human Developmental Biology Resource (www.hdbr.org). Neurulation has recently been completed in the low spinal region. The positions of Closures 1 and 3, and the directions of closure are marked. Note the relatively small size of the midbrain (red asterisk) in this human embryo compared with the mouse embryo (Figure 1D,E) which, in evolution, may have rendered Closure 2 an unnecessary

intermediate step in achieving cranial neural tube closure. Part B modified from: Copp, 2005, *Journal of Anatomy* 207, 623-35.

## Tables

**Table 1. Environmental factors linked to the causation of NTDs in human pregnancy**

| Category | Teratogenic agent | Proposed teratogenic mechanism |
|---|---|---|
| Folate antagonists | Carbamazepine | Inhibition of cellular folate uptake |
| | Trimethoprim | Disturbance of folate-related metabolism |
| Gene expression dysregulation | Fumonisin | Disturbance of sphingolipid biosynthesis and metabolism with effects on signalling pathways in neurulation |
| | Valproic acid | Histone deacetylase inhibition leading to disruption of key signalling pathways in neurulation |
| Glycaemic dysregulation | Hyperglycaemia (in poorly controlled maternal diabetes mellitus) | Increased cell death in neuroepithelium |
| | Maternal obesity | Unknown |
| Micronutrient deficiencies | Folate | Disturbance of folate-related metabolism |
| | Inositol | Disturbance of phosphorylation events downstream of protein kinase C |
| | Vitamin B12 | Disturbance of folate-related metabolism |
| | Zinc | Unknown |
| Thermal dysregulation | Hyperthermia (e.g. maternal fever in weeks 3-4 of pregnancy) | Unknown |

For references to original literature, see: Copp & Greene, 2010, *Journal of Pathology* 220, 217-30.

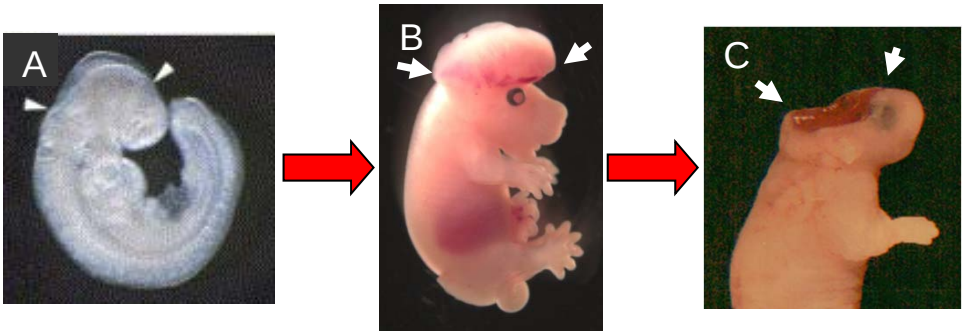
## Further Reading/Resources

Wyszynski DF, ed. Neural Tube Defects: From Origin to Treatment. Oxford University Press, Oxford, 2006, 15-28.

## Related Articles

| Subtopic | Article title |
|---|---|
| Nervous system development | Morphogenetic movements in the neural plate and tube, floor plate: Xenopus |
| Nervous system development | Morphogenetic movements in the neural plate and tube, floor plate: Zebrafish |
| Nervous system development | Morphogenetic movements in the neural plate and tube, floor plate: Mouse |

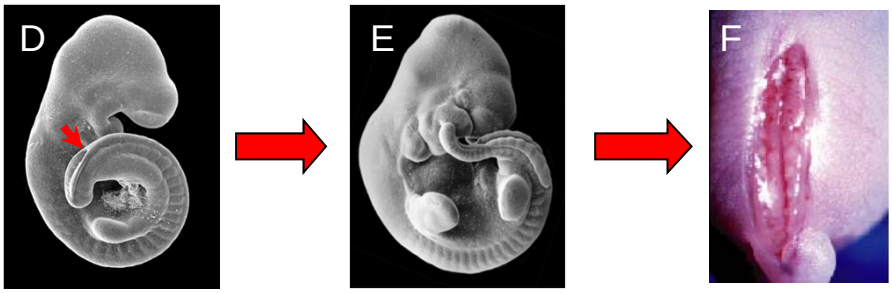
Figure 1




Failed cranial neurulation

Exencephaly

Anencephaly

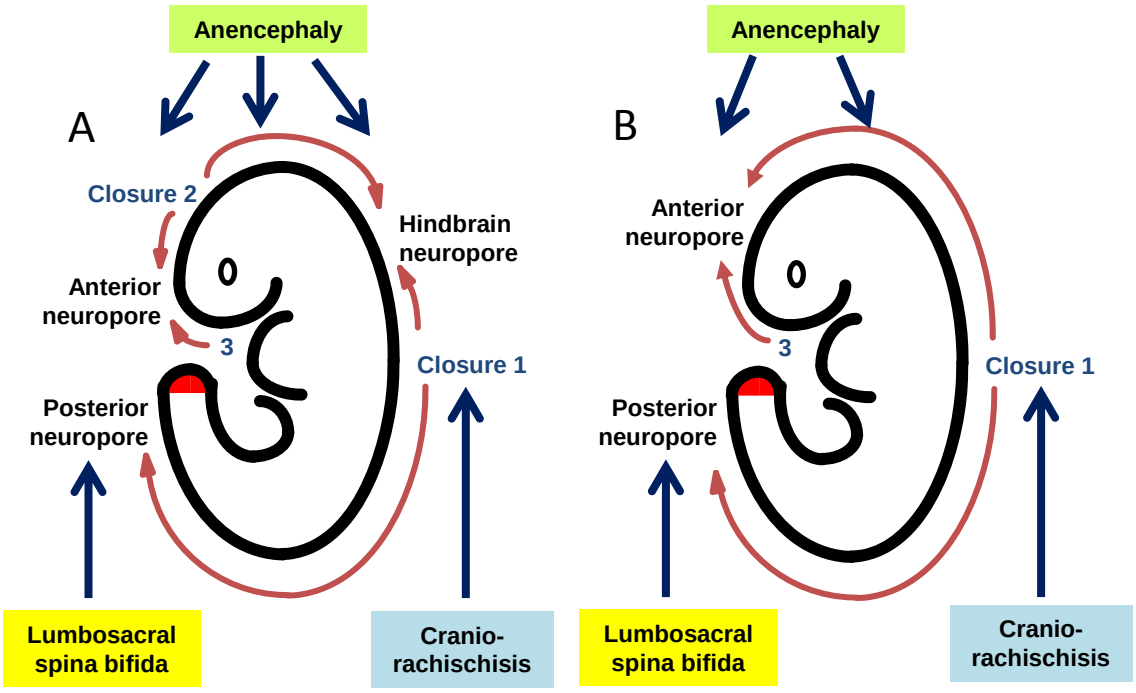


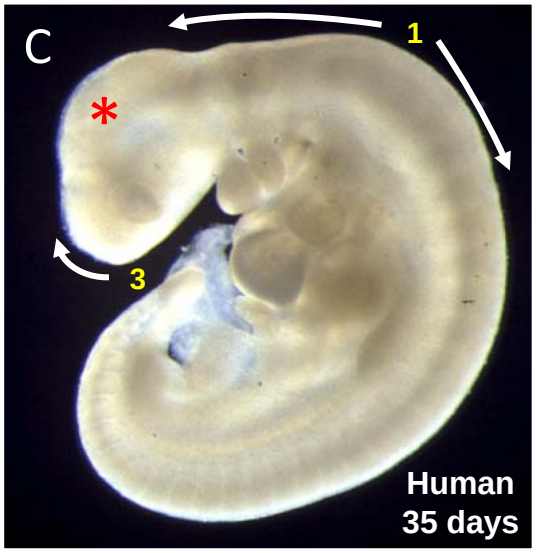

Failed spinal neurulation

Early spina bifida

Myelo(meningo)cele

Figure 2


A
Anencephaly
Closure 2
Hindbrain neuropore
Anterior neuropore
0
3
Closure 1
Posterior neuropore
Lumbosacral spina bifida
Cranio-rachischisis
B
Anencephaly
Anterior neuropore
0
3
Closure 1
Posterior neuropore
Lumbosacral spina bifida
Cranio-rachischisis


C
1
*
3
Human
35 days