Neural tube defects: recent advances, unsolved questions, and controversies

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Neural tube defects are severe congenital malformations affecting around one in every 1000 pregnancies. An innovation in clinical management has come from the finding that closure of open spina bifida lesions in utero can diminish neurological dysfunction in children. Primary prevention with folic acid has been enhanced through introduction of mandatory food fortification in some countries, although not yet in the UK. Genetic predisposition accounts for most of the risk of neural tube defects, and genes that regulate folate one-carbon metabolism and planar cell polarity have been strongly implicated. The sequence of human neural tube closure events remains controversial, but studies of mouse models of neural tube defects show that anencephaly, open spina bifida, and craniorachischisis result from failure of primary neurulation, whereas skin-covered spinal dysraphism results from defective secondary neurulation. Other malformations, such as encephalocele, are likely to be postneurulation disorders.

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Introduction

Neural tube defects (NTDs) affect an average of one in every 1000 established pregnancies worldwide,[1] although variations in prevalence have been reported, ranging from 0·2 to 10 per 1000 in specific geographical locations. Higher frequencies occur in miscarriage material.[2] NTDs rank among the commonest categories of birth defects, alongside congenital heart anomalies and genitourinary defects,[3] and reports of fetuses and infants with anencephaly, myelomeningocele, and craniorachischisis extend back to ancient Egyptian times.[4] Moreover, a striking progression of diverse biomedical advances has maintained interest in the topic over the the past 60 years.

Record and McKeown's report[5] in 1949 first raised awareness of the complex epidemiological features of NTDs. These features include marked variations in NTD prevalence between geographical locations, ethnic groups, socioeconomic status, and pregnancy order in multiparous women, and the preponderance of anencephaly in girls.[6] Carter's analysis in 1974 of the epidemiological data[6] led to the present view of a multifactorial cause of NTDs with modern findings suggesting that multigenetic predisposition and environmental factors, such as diabetic milieu or folate status, are important.

In this Review we consider the clinical management and primary prevention of NTDs, advances in the understanding of NTD causation, and NTD pathogenesis. We note a number of unsolved questions and controversial topics.

Clinical features and management

Clinical severity of NTDs varies greatly (table 1). Open lesions affecting the brain (anencephaly, craniorachischisis) are invariably lethal before or at birth. Encephalocele can also be lethal depending on the extent of brain damage during herniation. Open spina bifida is generally compatible with postnatal survival, although the resulting neurological impairment below the level of the lesion can lead to absence of sensation, inability to walk, and incontinence. Associated disorders include hydrocephalus, which often needs CSF shunting, vertebral deformities, and genitourinary and gastrointestinal disorders. Closed spinal lesions are generally less severe and can be asymptomatic, as with spina bifida occulta, which is regarded as a normal variant. However, lumbosacral spinal cord tethering can be present in spinal dysraphism, and can lead to lower-limb motor and sensory deficits, and a neuropathic bladder.

Before the 1970s, management of open spina bifida consisted solely of palliative surgical and medical support. Although children generally survived if their lesion was closed surgically, thereby avoiding ascending infection, neurological outcome varied greatly with the vertebral level of lesion (ie, higher defects meant greater neurological deficit than lower defects), leading to suggestions that surgery should be offered only to patients with a good prognosis.[11] An ethical debate ensued about whether surgical treatment should be withheld, but this was superseded in the 1970s by the development of methods for prenatal diagnosis of open NTDs. Initially, diagnosis was based on measurement of α-fetoprotein concentration in the amniotic fluid and maternal blood,[12,13] but later technological improvements enabled ultrasonography to replace α-fetoprotein measurement as the mainstay of prenatal diagnosis.[14] Nowadays, most fetuses with NTDs are diagnosed prenatally in developed countries, and many are aborted therapeutically. By contrast, many babies with NTDs continue to be born in developing countries where prenatal diagnosis is not routine, and in countries where therapeutic abortion is either illegal or not practised because of religious or cultural views.

In human and mouse embryos, the persistently open spinal cord undergoes relatively normal neuronal differentiation during the embryonic period, including development of spinal motor and sensory function below the lesion,[15] which shows that neural tube closure is not needed for subsequent events of neuronal differentiation. As gestation progresses, however, neurons die within the exposed spinal cord, suggesting that the amniotic fluid environment is toxic for cells

	Anencephaly	Myelomeningocele	Craniorachischisis	Spinal dysraphism	Encephalocele
Relative frequency (%)*	40%	50%	3%	Unknown	7%
Epidemiological features	Mostly sporadic	Mostly sporadic	Unknown; high prevalence noted in north China[7]	Unknown	Usually sporadic, but can be syndromic (eg, in Meckel syndrome)
Sex ratio	Marked female excess (3:1)	Variable in different populations; approximately equal overall	Female excess	Equal	Female excess among occipital lesions
Clinical presentation	Lack of brain and cranial vault; fetal loss or stillbirth	Open spinal cord covered by meningeal sac (spina bifida cystica) or exposed (spina bifida aperta); most commonly thoracolumbar, lumbar, or lumbosacral; usually livebirth; frequently associated with hydrocephalus postnatally	Anencephaly continuous with complete open spina bifida; fetal loss or stillbirth	Skin-covered lesion involving two or more vertebrae, apparent only on radiography; hair tuft, lipoma, or other cutaneous features often coexist	Meningeal sac, often containing brain tissue, protrudes from skull; commonly in occipital, parietal, or frontoethmoidal locations
Prenatal diagnosis	Ultrasonography from first trimester; increased serum AFP	Ultrasonography from first trimester; increased serum AFP	Ultrasonography from first trimester; increased serum AFP	No	Ultrasonography, dependent on size of lesion
Surgical treatment	None: lethal beyond birth	Surgical closure postnatally or in utero in some centres; insertion of CSF shunt for hydrocephalus	None: lethal beyond birth	Untethering of spinal cord, usually in childhood	Repair by removal of sac and closure
Non-surgical treatment	None	Long-term treatment of hydrocephalus and skeletal, renal, gut, and other secondary disorders	None	Treatment of genitourinary disorders as common sequelae	Treatment of epilepsy and learning disorders as common sequelae
Genetic causation	Genes as for NTDs as a whole	Genes as for NTDs as a whole	PCP genes are only positive findings	Unknown	*MKS1*, *TMEM67*, *TMEM216*, *RPGRIP1L*, and *CEP290* identified as causal for Meckel syndrome
Non-genetic causation	Increased risk in diabetic pregnancy; no specific associations	Valproic acid exposure increases risk ten times; increased risk in diabetic pregnancy	None known	None known	None known
Primary prevention	Folic acid, as for NTDs as a whole	Folic acid, as for NTDs as a whole; inositol prevention of open spina bifida in *Grhl3* mouse model	Folic acid may prevent; frequency in north China decreased after folic acid introduced	Folic acid resistant? Lipomyelomeningocele frequency shows no reduction after food fortification with folic acid[8]	Folic acid may prevent; some evidence of reduction after food fortification with folic acid
Embryonic origin	Failure of cranial neural tube closure	Failure of caudal neuropore closure	Failure of closure 1	Defective secondary neurulation	Postneurulation disorder?
Pathogenesis	Originates after failed closure as exencephaly; converted to anencephaly by degeneration of neural tissue and absence of cranial vault formation	Degeneration of exposed neural tissue after failed closure; origin of meningeal sac in spina bifida cystica is not clear	Combined anencephaly and myelomeningocele	Unknown	Herniation of meninges with or without brain tissue through defect in skull

NTD=neural tube defect. AFP=α-fetoprotein. PCP=planar cell polarity. *Relative frequencies relate to NTDs in established pregnancies.[9,10] Spinal dysraphism is not usually included within epidemiological studies of NTDs and, because its prevalence is poorly documented, a relative frequency is not given.

***Table 1*: Characteristics of the main types of NTDs**

that would normally be contained within the closed neural tube; axonal connections are interrupted and function is lost.[15,16] Hence, neurological disability in open spina bifida is a two stage process of failed neural tube closure followed by neurodegeneration in utero. This finding has encouraged attempts to arrest or prevent further neurodegeneration by covering the persistently open neural tube as early as possible during fetal development.

Surgical repair in utero for early open spina bifida has been practised in several centres in the USA for the past 15 years.[17] An important development was the report of a controlled clinical trial to assess the success of this procedure. Investigators of the Management of Myelomeningocele Study (MOMS)[18] randomly assigned fetuses with prenatally diagnosed myelomeningocele to either in-utero surgery or standard postnatal repair. The trial showed that fetal surgery brings significant short-term benefits for the newborn baby, including a 50% reduction in shunting for hydrocephalus and a large improvement in spinal neurological function. However, investigators recorded a significantly higher rate of premature birth and maternal complications, such as uterine dehiscence at the operation site, in the in-utero surgery group compared with the non-surgical group. Although long-term outcomes for children after this surgical intervention remain unknown, these pioneering studies will no doubt encourage other centres to think about implementation of in-utero surgery.

Primary prevention of NTDs

In the 1970s, Smithells and colleagues[19] noted that mothers who were pregnant with fetuses with NTDs had reduced serum concentrations of several vitamins (folic acid, riboflavin, and vitamin C). The researchers did an intervention trial of periconceptional Pregnavite Forte F, a multivitamin supplement containing 0·36 mg folic acid, to assess its possible effect in prevention of NTD recurrence in high-risk women with a previously affected pregnancy.[20] The ethics committees for the study did not think a randomised controlled trial was justified in view of the evidence that vitamins might help to prevent NTDs, so the findings of significant prevention by Pregnavite Forte F were not accepted as definitive.[21] In due course, a randomised, double-blind trial (the Medical Research Council [MRC] Vitamin Study[22]) was done to specifically assess 4 mg folic acid separately from multivitamins, and showed that folic acid is the essential factor for prevention of NTD recurrence. Subsequently, a randomised controlled trial in Hungary of a multivitamin supplement containing 0·8 mg folic acid significantly prevented the first occurrence of NTDs,[23] an important finding considering that 95% of all NTD cases are first occurrences in a family. A further clinical trial, based in China, showed a fall in NTD prevalence subsequent to the introduction of folic acid supplementation.[24]

The MRC trial led to the recommendation that all women planning a pregnancy should consume 0·4 mg folic acid per day (the dose used in the Smithells trials), and that women at high risk of having a baby with an NTD should receive 4–5 mg per day. However, despite widespread public health education efforts in the UK and other countries, the incidence of NTDs did not decrease during the decade after publication of the MRC trial.[25] In the USA, a campaign of government lobbying[26] eventually achieved its aims, and mandatory fortification of bread flour with folic acid was introduced in 1998. Fortification was introduced soon after in Canada, and then throughout South America, South Africa, Australia, and other countries. Although NTD rates were falling in several countries before this period,[27] most authorities agree that the decision to fortify with folic acid, ensuring a more favourable folate status in women who become pregnant, has contributed substantially to a reduction in the number of pregnancies affected by NTDs.[28] No European countries, including the UK, have implemented food fortification. Other strategies to improve supplementation include incorporation of folic acid within oral contraceptives[29] and the use of supplements that contain a more bioavailable form of folic acid. Compounds such as 5-methyltetrahydrofolate (6S-5-MTHF) could offer a means to enhance folate status more effectively than folic acid in some individuals.[30] However, an appropriate formulation that retains its stability in food is needed,[31] and a formal trial for prevention of NTDs has not yet been undertaken.

Surveys of NTD prevalence show that the decrease after food fortification has been smaller than expected from the MRC trial.[28] Although some experts have advocated increasing doses of folic acid to prevent a larger proportion of total NTDs,[32] most authorities accept that a proportion of cases, perhaps 0·7–0·8 per 1000 pregnancies, are likely to persist irrespective of folic acid use, and that little additional benefit will accrue from a further increase in dose.[33,34] In other words, some NTDs are probably resistant to folic acid, and perhaps of different cause from the subgroup sensitive to folic acid. This notion is well established in mouse models of NTDs in which some genetic types are prevented by folic acid, whereas others are folic acid resistant.[35] One potential adjunct therapy that has been identified from mouse studies is inositol, which is effective in prevention of a large proportion of spinal NTDs in the *Grhl3* (curly tail mutant) mouse, for which folic acid is ineffective.[36] Uniquely among vitamins, inositol deficiency leads to NTDs in rodent embryos.[37] A randomised clinical trial to investigate inositol for prevention of human NTD recurrence is currently underway in the UK.[38]

Controversies and unsolved questions in NTD prevention

Are NTDs a vitamin deficiency disorder?

The finding that exogenous folic acid can prevent many NTD cases is often interpreted as showing NTDs to be a vitamin deficiency disorder.[39] Indeed, both folate and vitamin B12 deficiency are statistical risk factors for NTDs.[40] However, maternal folate concentrations in most affected pregnancies are within the normal range,[40,41] arguing against a simple folate deficiency model. In mice that are severely folic acid deficient, wild-type mothers do not have embryos affected by NTDs, although intrauterine growth retardation is routinely reported.[42,43] The frequency of cranial NTDs is exacerbated by maternal folate deficiency in mutant splotch (*Pax3*) embryos, whereas wild-type littermates are never affected by NTDs.[44] Similarly, in the *Shmt1* knockout mouse, the development of NTDs is noted solely in mothers that are folate deficient.[45] Clearly, folate deficiency is a risk factor for NTDs, but only in the presence of a predisposing genotype.

Could folic acid prevent NTDs by killing affected embryos?

The notion that folic acid could prevent NTDs by killing affected embryos, termed terathanasia, is theoretically possible because the disappearance of a proportion of NTD cases, due to early pregnancy loss, might be interpreted as primary prevention.[46] Investigators noted a small excess of miscarriages in the multivitamin-treated group of the Hungarian randomised trial,[23] but this excess has been interpreted as a rise in the number of pregnancies reaching a stage when their loss can be recognised as miscarriage because of folic acid supplementation. In several mouse models, folic acid prevents the development of NTDs in embryos with genotypes that would have

otherwise destined them to develop the disorder.[47,48] Moreover, in the splotch (*Pax3*) mouse model, folate deficiency exacerbates cranial NTDs, which is consistent with true primary prevention by folic acid.[44] However, exposure of several mouse NTD strains to dietary folic acid supplementation has produced a diverse range of responses, with apparent exacerbation of NTDs in some cases.[49] This variation in response could result partly from the widely varying doses of folic acid used in different mouse strains, often amounting to a 100 times excess compared with human supplementation.[50] However, serum folate concentrations are generally similar in supplemented mice and people.[49] A further consideration is that various pathogenic mechanisms are known to underlie NTDs in mice, and probably in humans; hence, the interaction of folic acid supplementation with these mechanisms is probably heterogeneous.

How does folic acid promote normal neural tube closure?

The fundamental question of how folic acid promotes closure of the neural tube has received little attention compared with the hundreds of publications on the clinical and epidemiological aspects of folic acid supplementation. Folic acid is known to have a direct effect on the neurulation-stage embryo, because treatment of genetically predisposed mouse embryos in vitro can normalise neural tube closure.[47] Folic acid enters one-carbon metabolism, which has two main outputs: production of pyrimidines and purines for DNA replication during cell proliferation; and donation of methyl groups to macromolecules, including DNA, proteins, and lipids (figure 1). Cell multiplication has an important role in neural tube closure,[52,53] encouraging the hypothesis that enhanced cell proliferation could be a key effect of folic acid. However, methylation of genomic DNA and histones is increasingly being implicated in the epigenetic regulation of gene expression,[54] and could underlie the action of folic acid in prevention of NTDs. Detailed analysis of folic acid prevention of NTDs in animal models could resolve this issue in the coming years.

Causes of NTDs

Both genetic and non-genetic factors are implicated in the causes of NTDs, with up to 70% of the variance in NTD prevalence attributable to genetic factors.[55] Evidence for genetic causation includes an increased recurrence risk for siblings of index cases of 2–5% compared with the 0·1% risk in the general population, together with a gradually decreasing frequency in more distant relatives. Women with two or more affected pregnancies have a higher risk (around 10%) of further recurrence.[56] NTD prevalence is greater in same-sex twins (assumed to include all monozygotic cases) compared with different-sex pairs, which is consistent with a pronounced genetic component. Nevertheless, NTDs rarely present as multiple cases in families; instead a sporadic pattern is usually recorded. Taken together with the high prevalence of NTDs worldwide, this finding is consistent with a multifactorial polygenic or oligogenic pattern of inheritance and an important role for non-genetic factors.

The genomics revolution of the past 15 years has affected NTD research in two major ways: first, in providing the methods necessary to investigate candidate genes for NTD causation; and second, in providing a wealth of mouse genetic NTD models that have stimulated many studies of causation in human disease. In the near future, a full genome-wide assessment of interacting variants, including coding, regulatory, and epigenetic marks, will be possible to establish an embryo's risk of developing an NTD. Such a strategy might need to be used to fully understand the complex multifactorial causation of these disorders. However, in view of the probable causal heterogeneity within sporadic NTDs, the implementation of this approach is highly demanding, and requires large numbers of samples for analysis. Genes in two main pathways have yielded positive findings with regard to the causation of NTDs: folate one-carbon metabolism, and non-canonical Wnt signalling (the planar cell polarity pathway).

Folate-related genes

Given the historical links between folic acid and NTDs, folate pathway genes have been most intensively studied (figure 1). Positive associations have been reported between specific folate-related gene variants and NTDs in several case-control studies.[51,57,58] For example, *MTHFR* encodes a key cytoplasmic enzyme of folate metabolism (methylenetetrahydrofolate reductase) that generates 5-methyltetrahydrofolate for homocysteine remethylation. The *MTHFR* polymorphism 677C→T (rs1801133) is associated with a roughly 1·8 times higher risk of NTDs, although the predisposition is detectable only in non-Hispanic populations.[59] A further risk factor is the Arg653Gln variant (rs2236225) of *MTHFD1*, which encodes a trifunctional enzyme that catalyses the conversion of tetrahydrofolate to 5,10-methylene-tetrahydrofolate.[58,60]

Genes that encode enzymes that catalyse mitochondrial one-carbon metabolism have also been implicated in the causes of NTDs. An intronic polymorphism in *MTHFD1L*, the gene for mitochondrial 10-formyl-THF synthetase, is associated with increased risk of NTDs,[61] whereas two genes, *AMT* and *GLDC*, encoding enzymes (aminomethyltransferase and glycine dehydroxylase) of the glycine cleavage system harbour various mis-sense genomic changes in NTD cases but not in unaffected controls.[62] In the case of *GLDC*, these variants diminish enzyme activity, indicating a functional effect on folate metabolism. Each of these enzymes greatly affects flux of formate from the mitochondrion into the cytoplasm, which accounts for roughly 75% of one-carbon units entering folate metabolism.[63]

Hence, genetic variants that reduce the efficiency of folate one-carbon metabolism increase the risk of NTDs.

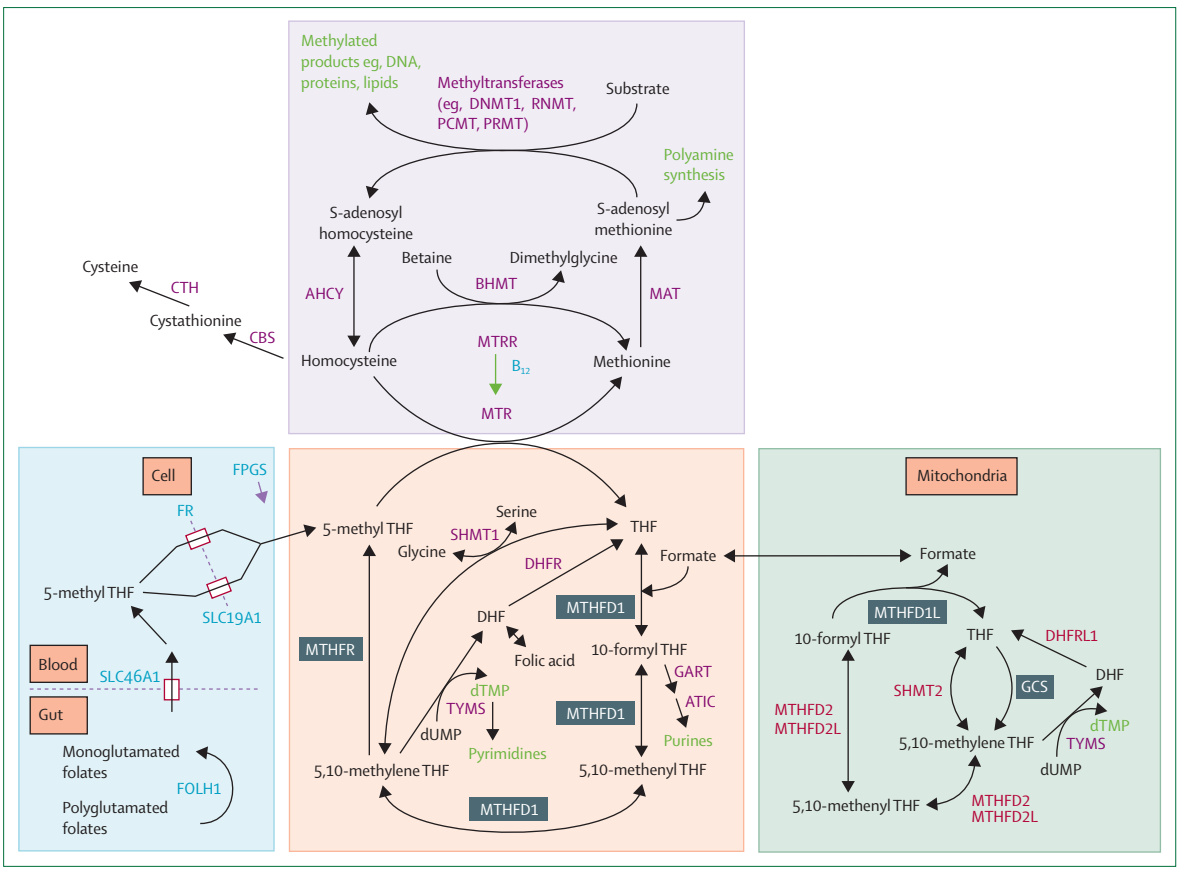

***Figure 1*: Summary of folate one-carbon metabolism showing the main pathways and reactions**
Blue shading: processing of folates in the digestive tract, transport, and cellular retention (by addition of glutamates). Orange shading: transfer of one-carbon groups between folate molecules for purine and pyrimidine biosynthesis. Purple shading: reactions of the methylation cycle that generate S-adenosyl methionine, the universal methyl group donor. Green shading: mitochondrial reactions that generate formate via cleavage of glycine. Green text: main outputs of folate one-carbon metabolism. Enzymes whose genetic variations have been implicated in human NTDs are indicated in black boxes. MAT=methionine adenosyltransferases. FR=folate receptors. THF=tetrahydrofolate. DHF=dihydrofolate. MTHFD=methylenetetrahydrofolate dehydrogenase. MTHFR=methylenetetrahydrofolate reductase. TMP=thymidine monophosphate. dUMP=deoxyuridine monophosphate. GCS=glycine cleavage system. NTD=neural tube defect. Figure modified from Greene et al, 2009,[51] by permission of Oxford University Press.

These findings are consistent with a study of cell lines derived from fetuses with NTDs, in which a subset exhibited apparent inborn errors of folate metabolism, as shown by diminished thymidylate biosynthesis.[64] Strikingly, under folate-replete dietary conditions only the mitochondrial enzymes encoded by the genes *Mthfd1l* and *Amt* caused NTDs in knockout or gene trap mice.[62,65] Disruption of the cytoplasmic enzymes encoded by *Mthfr* and *Mthfd1* do not cause mouse NTDs,[66,67] whereas exencephaly occurs in *Shmt1* null embryos under folate-deficient conditions.[45] These findings could indicate that mammalian neural tube closure is particularly sensitive to changes in the mitochondrial contribution to folate metabolism.

Planar cell polarity genes

At the onset of neurulation, the embryo undergoes lengthening and narrowing of the disc-shaped neural plate to ensure that the neural folds are sufficiently close together for closure to begin.[68,69] This elongation of the neural plate and underlying mesoderm needs a lateral to medial displacement and intercalation of cells, termed convergent extension.[70] At the molecular level, the cell movements of convergent extension are dependent on non-canonical Wnt signalling through the planar cell polarity (PCP) pathway (figure 2), which signals via frizzled membrane receptors and cytoplasmic dishevelled protein, but does not include downstream stabilisation of β catenin.[71]

Indications of a possible role of the PCP pathway in human NTDs came from the discovery that genes in the pathway underlie severe NTDs in several mouse mutants. Mutations in the transmembrane proteins encoded by *Vangl2*, *Celsr1*, *Ptk7*, and *Fzd3/6* (double mutant), and the cytoplasmic proteins encoded by *Dvl1/2/3* and *Scrib*, all result in craniorachischisis, a severe NTD (table 1) in which closure fails along most of the body axis, yielding an open neural tube from midbrain to low

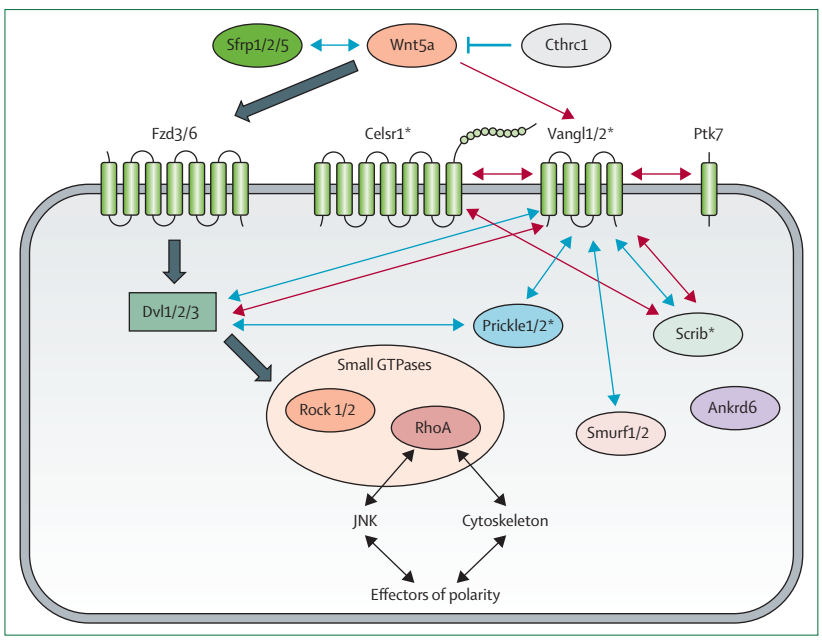

***Figure 2*: Summary of non-canonical Wnt signalling in a mammalian cell**
Black arrows indicate the signalling pathway necessary for establishment of PCP. Known biochemical interactions are indicated by blue arrows and genetic interactions are shown by red arrows. JNK=c-Jun NH(2)-terminal kinase signalling pathway. PCP=planar cell polarity. NTD=neural tube defect. *Proteins whose genes that have been implicated in human NTDs. Figure modified from Greene et al, 2009,[51] by permission of Oxford University Press.

	Phenotype in mouse mutant homozygotes	Phenotype associated with mutations in human gene
Celsr1[73,74]	CRN	CRN
Dvl1/2 or *Dvl2/3*[75,76]	CRN	*DVL2*: varying types of NTDs, not CRN
Fuz[77]	Exencephaly	Varying types of NTDs, not exencephaly
Fzd3/6[78,79]	CRN	*FZD6*: varying types of NTDs, not CRN
Prickle1[80]	None	Varying types of NTDs, not CRN
Ptk7[81]	CRN	No reports
Scrib[73,82]	CRN	CRN
Sec24b[83]	CRN	No reports
Vangl1[84-86]	None	Varying types of NTDs, not CRN
Vangl2[87-90]	CRN	Varying types of NTDs, not CRN

PCP=planar cell polarity. CRN=craniorachischisis. NTD=neural tube defect.

***Table 2*: PCP genes and their mutant phenotypes in mice and humans**

spine.[72] Many studies have subsequently reported unique and predominantly mis-sense variants in PCP genes of patients with NTDs as probable causal alleles (table 2). However, the finding that aminoacid changes are present in affected individuals but absent from controls, although suggestive, does not prove their causal role in NTDs. Functional evidence is needed to show whether the specific human mutations actually cause protein dysfunction, or the NTD phenotype needs to be reproduced in an animal model that incorporates the same genetic alteration.

Assays of PCP protein function, including interaction with dishevelled[84,87] and translocation to the plasma membrane,[73] have identified functional defects caused by NTD-associated variants of *VANGL1*, *VANGL2*, *CELSR1*, and *SCRIB*. Several human *VANGL1* mis-sense variants block the rescuing effect of vangl1 mRNA on the *vangl2* mutant phenotype in zebrafish.[91] Whether any of the putative NTD-causing variants in human PCP genes also cause an NTD phenotype in laboratory mammals, such as knock-in mouse strains, is unknown.

Genetic causes of encephalocele

By contrast with open NTDs, occipital encephalocele is often syndromic, most commonly as part of Meckel syndrome. Several genes have been identified to cause this disorder: *MKS1*, *TMEM216*, *TMEM67*, *RPGRIP1L*, and *CEP290*.[92] MKS proteins play a key part in the structure and function of primary cilia, protrusions of the cell surface that are rooted in the centrosome and undergo a disassembly and reassembly cycle as the cell proliferates. Primary cilia are essential for signalling pathways, such as downstream of hedgehog proteins. Many rare disorders are now known to be causally associated with genes needed for ciliary structure and function, which has led to the notion of ciliopathies. How encephalocele results from disordered ciliary function is unknown.

Environmental factors

Few non-genetic factors have been definitively associated with human NTDs, in contrast with the wide range of teratogenic agents known to cause the defects in rodents.[93] Of particular clinical significance is valproic acid, an anticonvulsant that increases the risk of spinal NTDs by roughly ten times when taken early in pregnancy.[94] Although the teratogenic mechanisms are hypothesised to involve anti-folate effects, particularly for carbamazepine,[95] studies of valproic acid suggest potent histone deacetylase inhibitory activity. This activity could disturb the balance of protein acetylation versus deacetylation, similar to the action of the histone deacetylase inhibitor trichostatin-A, which causes NTDs in mice.[96] Another environmental teratogen with proven effect in humans is the fungal product fumonisin, which caused a doubling of NTD incidence along the Texas–Mexico border in the early 1990s.[97] Fumonisin is a potent NTD-causing teratogen in mice, with marked effects on sphingolipid metabolism and downstream embryonic gene expression.[98] Other environmental factors implicated in the cause of NTDs include maternal diabetes,[99] maternal obesity,[100] and exposure to high temperatures during early pregnancy.[101]

Although environmental causes of birth defects are perhaps the most preventable of predisposing factors, only a very small proportion of all congenital disorders

have a known environmental cause—estimated at 0·12 cases per 1000 births (0·5% of all defects) in a survey of European pregnancies.[3] Moreover, genetic variation probably plays an important part in determining the susceptibility of a particular pregnancy to non-genetic factors. For example, substantial differences are routinely noted between different inbred mouse strains for many teratogenic factors including valproic acid and fumonisin.[98,102] In humans, single nucleotide polymorphisms in genes associated with type 1 and type 2 diabetes mellitus have been associated with NTDs.[103,104]

Controversies and unsolved questions in NTD causation

Can folic acid be targeted to so-called sensitive pregnancies?

Inheritance of a predisposing folate enzyme variant, perhaps affecting *MTHFR* or *MTHFD1*, might be predicted to affect an individual's response to exogenous folic acid supplementation. Female carriers could need a larger dose of folic acid than wild-type individuals to protect against NTDs in preganancy. Hence, genotyping for known or novel risk variants might provide a means of folic acid targeting. A multivariate analysis of NTD cases (or mother–fetus pairs) is needed to examine this possibility, with assessment of all known folate-related genetic variants, indices of folate supplementation, and serum or red cell folate, homocysteine, and other metabolites.

Do human and mouse phenotypes correspond when the same gene is mutated?

Despite the finding of PCP gene mutations in both mice and human NTDs, the phenotypes do not always correspond closely. For example, human NTDs associated with heterozygosity for *VANGL2* variants have included anencephaly, holoprosencephaly (not a defect of neural tube closure), and closed spina bifida.[87,88] By contrast, mouse *Vangl2* mutants exhibit craniorachischisis in homozygotes and low, open spina bifida in compound heterozygotes with other gene mutations.[51] Furthermore, human mutations have been discovered in *VANGL1* and *PRICKLE1*,[80,84] despite there being no NTD phenotype in homozygous mouse mutants for these genes (table 2). Only two PCP genes, *CELSR1* and *SCRIB*, have been associated with the same NTD phenotype (craniorachischisis) in both mice and people,[73] whereas *VANGL2* mutations were not identified in individuals with craniorachischisis.[105] On the basis of a polygenic model of NTD causation, we predict that present genetic findings are incomplete and that additional interacting genetic variants remain to be discovered. The precise combination of predisposing variants could determine whether an individual develops anencephaly, spina bifida, or craniorachischisis.

NTD pathogenesis

NTDs comprise a diverse set of birth defects that are usually thought to arise during the third and fourth weeks after fertilisation. However, many questions remain about the precise timing of origin of specific anomalies that are included in these disorders. Moreover, understanding of the cellular and molecular mechanisms by which human NTDs arise during embryonic development is poor. In fact, investigators of the clinical, epidemiological, and folic acid prevention aspects of NTDs tend to ignore embryonic development. However, as genetic risk factors start to emerge from modern genomics research, it is essential to understand when and how such gene variants might exert their effects. Similarly, in attempting to optimise prevention mediated by folic acid, and to introduce new preventive therapies, it is important to appreciate the precise embryonic mechanisms that might be the targets for therapeutic intervention.

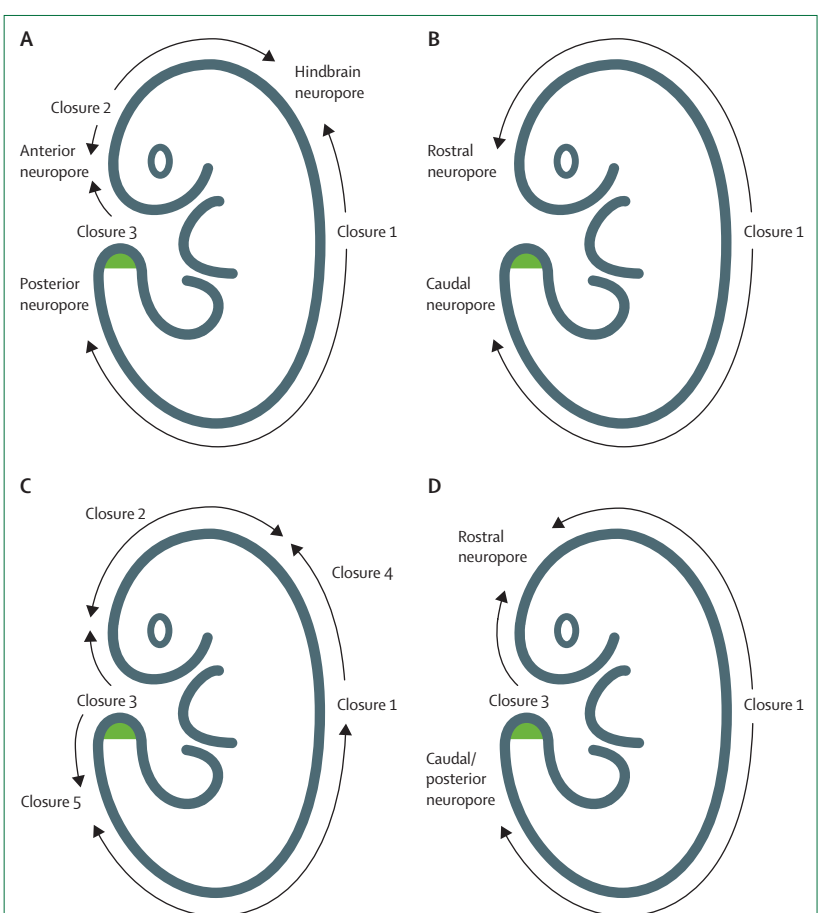

***Figure 3*: Primary neurulation in mouse and human embryos**
(A) Pattern of mouse neural tube closure, as experimentally verified in many mouse strains.[35] (B) The original concept of human closure in which bidirectional zippering occurs from an initiation site (closure 1) towards the rostral and caudal extremities. (C) Modified human concept based on mouse multisite closure, as used for retrospective interpretation of human neural tube defects.[112] (D) Pattern of human neural tube closure based on embryo observation.[113] The site of secondary neurulation in the tailbud is indicated by green shading.

Advances in developmental studies of mouse neurulation

The availability of more than 250 different models of open NTDs in mice[106] enables increasingly advanced analysis of the neurulation events at tissue, cellular, and molecular levels. Because this large range of mouse models covers most types of human NTDs, it has been possible to build a picture of events that is specific to every stage of neural tube formation, and therefore to the NTDs that arise from defects at particular stages. One potential limitation to mammalian research is the relative inaccessibility of the developing embryo within the uterus. However, access to neurulation stages is greatly helped by use of whole-mouse embryo culture, in which intact midgestation embryos encased within their extraembryonic membranes can be grown in vitro during the entire period of neurulation.[107] As live imaging techniques based on confocal microscopy become increasingly advanced and adaptable, a new era is beginning of real-time information about the dynamic events of neurulation in mammals through the imaging of cultured mouse embryos.[108] Non-mammalian models, especially chick, frog, and zebrafish, continue to provide insight into some of the key pathways and cellular mechanisms of neural tube formation.[109–111]

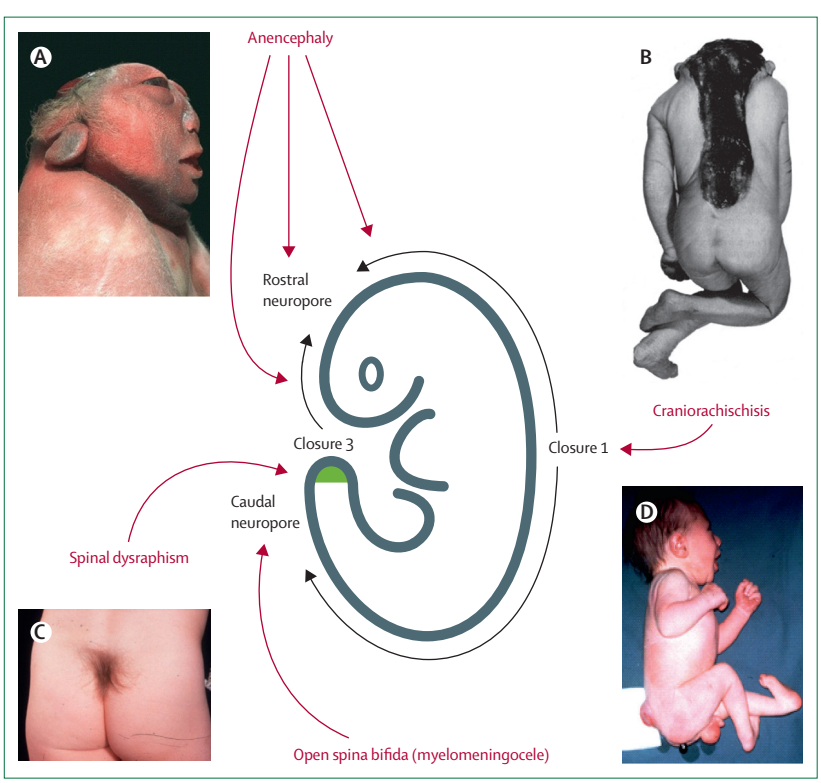

***Figure 4*: Sites of origin of neural tube defects in the human embryo resulting from disturbance of primary or secondary neurulation**

(A) Anencephaly is the consequence of faulty cranial closure events. (B) Craniorachischisis arises when closure 1 fails. (C) Skin-covered spinal dysraphism arises through disturbance of the secondary neurulation process. (D) Open spina bifida results from failure of caudal neuropore closure. Figure modified from: Copp, 2005 (B,C),[125] and Copp, 2008 (D),[126] by permission of John Wiley and Sons.

Events of mouse neural tube formation

Neural tube formation in mice is divided into primary (closure) and secondary (canalisation) phases. Primary closure is initiated at several discrete points along the body axis (figure 3A): first, at the boundary between future hindbrain and cervical spine (closure 1); then, around 12 h later, at the boundary between future forebrain and midbrain (closure 2); and soon afterward at the rostral extremity of the future forebrain (closure 3).[35,114] The open regions of neural folds between the sites of initial closure are termed neuropores, and close progressively as the neural tube zips up bidirectionally from the sites of closures 1 and 2, and in a caudal direction from the site of closure 3. The anterior and hindbrain neuropores complete closure within a few hours of closures 2 and 3, whereas spinal neurulation continues zipping caudally along the growing spinal region until the posterior neuropore finally closes, marking the end of primary neurulation. In mice, the process takes around 48 h to be completed and ends during embryonic day 10.

Secondary neurulation follows on seamlessly from primary neurulation, and is the process by which the neural tube forms in the lower sacral and coccygeal regions.[115,116] The caudal end of the embryo comprises the tail bud, which contains self-renewing stem cells whose derivatives condense into longitudinal cell masses. The most dorsal of these undergoes canalisation, converting the solid neural precursor into a hollow secondary neural tube. The stem cell population within the tail bud is multipotent,[117] giving rise to all non-epidermal tissues of the postlumbar body including neural tube and vertebrae. Probably for this reason, malformations and tumours (eg, teratomas) of the sacral and coccygeal regions often comprise several tissue types.

Origin of NTDs during neural tube formation

Analysis of the mouse mutant loop-tail (*Vangl2*) has shown that craniorachischisis, the most severe NTD, results from failure of closure 1.[118] However most embryos that develop NTDs complete closure 1 but fail later during neurulation, presenting NTDs as separate open lesions of the cranial neural tube (exencephaly, progressing to anencephaly), spinal neural tube (open spina bifida), or both. The wave of zippering closure down the body axis can arrest at any stage, yielding an open spina bifida of varying length. Hence, *Zic2* mutant mice fail early in spinal neurulation, owing to absence of dorsolateral neural plate bending.[119] These mice exhibit large spina bifidas from the thoracic level downwards. By contrast, spinal closure in the curly tail (*Grhl3*) mutant fails later because of enhanced axial curvature of the body axis.[120] This failure produces a spina bifida confined to the lumbosacral region. When secondary neurulation is disturbed, closed defects occur at sacrococcygeal levels causing spinal dysraphism, in which the spinal cord is characteristically

tethered to adjacent tissues, showing faulty tissue separation during tailbud development.

Controversies and unsolved questions on neurulation

Do similar neurulation events occur in human and mouse embryos?

The human neural tube begins to close at 17–18 days after fertilisation and, as in mice, closure is discontinuous (figure 3). The site of closure initiation in humans occurs at the same level as closure 1 in mice, and the onset of closure from the extreme rostral end of the neural plate appears similar to mouse closure 3.[113] Whether an event similar to closure 2 exists in human embryos is disputed, but there is probably no independent closure initiation event in the midbrain or forebrain.[113,121,122] Human brain formation is therefore achieved by neurulation progressing directly between closures 1 and 3, with completion of a single rostral neuropore (figure 3). Closure 2 is also absent in the SELH/Bc mouse strain[123] but more than 80% of embryos successfully complete brain formation, suggesting that closure 2 is not obligatory even in mice.

Do all NTDs result from defective neural tube closure?

Various malformations are included under the overall description of NTDs (table 1) but whether they all arise directly from abnormalities of primary or secondary neurulation is unclear. Historically, neurulation was thought to start halfway along the embryonic body and progressed by zippering towards the cranial and caudal ends, with closure of anterior and posterior neuropores, respectively (figure 3). A major change in this view happened after the introduction of the multisite closure concept, on the basis of studies of neurulation-stage mouse embryos.[124] The origin of human NTDs[112] was re-interpreted based on appearance of late fetuses and the closure events that were thought to have been defective (figure 3). However, extrapolation from a late-stage fetus to its embryonic origins is largely guesswork, and subsequent studies showed that closures 4 and 5 exist in neither mouse nor human neurulation.[35,113] Closure 2 probably does not occur in human embryos either. What remains is a fairly simple pattern of human neurulation, most akin to the original model (figure 3). Although craniorachischisis, anencephaly, open spina bifida, and closed secondary neurulation lesions can be explained on the basis of key embryonic events (figure 4), other NTDs including encephalocele, meningocele, and iniencephaly are unlikely to arise directly from failure of neural tube formation, but more likely as postneurulation disorders. This explanation is consistent with the finding of a distinct cause for encephalocele as part of Meckel syndrome.

Conclusions

NTDs continue to provide a multifaceted challenge to epidemiologists, clinicians, and developmental biologists alike. Although their imminent eradication was predicted when prenatal diagnosis was introduced, and again after the discovery of the preventive effects of folic acid, in fact NTDs remain one of the commonest categories of birth defects worldwide. Their clinical severity and uncertain cause make them priorities for further research, whether to better target primary preventive measures, to improve in-utero surgery for prenatal repair, or to identify the causative genes to provide an objective basis for individual genetic counselling. In this Review, we provide evidence that NTDs are not vitamin-deficiency disorders in the way that rickets results from early vitamin D deficiency. Rather, folate one-carbon metabolism is a key mechanism in the development of NTDs that is affected by, and interacts with, both genetic and environmental factors. The application of new genomic technologies to NTDs should herald the identification of many further risk factors, enabling understanding of the entire range of causative factors that affect the mother and her neurulation stage embryo. Being accurate about exactly how the neural tube is formed during embryogenesis is important, and we have shown how extrapolation backwards from late fetal appearance to presumed early embryonic events is hazardous and, in the case of NTDs, has led to misconceptions about the developmental origin of these disorders.

Search strategy and selection criteria

We searched PubMed from January, 1940, to April, 2013, with the search terms: "neural" AND "tube" AND "defect"; "NTD"; "congenital" AND "malformations"; "folic" AND "acid"; "prevention"; "anencephaly"; "spina bifida"; "neurulation"; "convergent" AND "extension"; "planar" AND "cell" AND "polarity". Studies were also identified through searches of the reference lists of the articles found with these search terms and of our own files. Only papers published in English were reviewed. The final reference list was generated on the basis of originality and relevance to the scope of this Review.

Contributors

All authors did the literature search and provided one or more of the figures. AJC wrote the Review. PS and NDEG edited the Review.

Conflicts of interest

We declare that we have no conflicts of interest.

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