Neural tube defects and folate: case far from closed

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Abstract | Neural tube closure takes place during early embryogenesis and requires interactions between genetic and environmental factors. Failure of neural tube closure is a common congenital malformation that results in morbidity and mortality. A major clinical achievement has been the use of periconceptional folic acid supplements, which prevents ~50–75% of cases of neural tube defects. However, the mechanism underlying the beneficial effects of folic acid is far from clear. Biochemical, genetic and epidemiological observations have led to the development of the methylation hypothesis, which suggests that folic acid prevents neural tube defects by stimulating cellular methylation reactions. Exploring the methylation hypothesis could direct us towards additional strategies to prevent neural tube defects.

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Neural tube defects (NTDs) are complex congenital malformations of the CNS. Anencephaly and spina bifida are the most common and severe forms of NTD, with a prevalence of about 1 in 1,000 births, although this varies throughout the world[1]. Infants with anencephaly are stillborn or die shortly after birth, whereas infants with spina bifida can survive but are likely to have severe lifelong disabilities and are at risk of psychosocial maladjustment. The estimated lifetime medical costs are high; for example, in California, USA, the costs for children born with spina bifida exceed US$81 million per year[2].

It has been known for more than 20 years that women can reduce their risk of having an NTD-affected pregnancy by taking folic acid supplements. However, the underlying mechanisms by which folic acid contributes to a reduction in the risk of an infant being born with an NTD or other types of malformation are unclear. It is also not known why a substantial proportion of women who take folic acid supplements in the periconceptional period give birth to NTD-affected infants. These questions have directed research towards other nutritional hypotheses, and towards genetic variation in folate metabolism and transport as potential underlying mechanisms of NTDs. Epidemiological studies have indeed suggested that genetic factors can contribute to NTDs. Evidence for genetic aetiologies has emerged from studies of monozygotic twins[1,3] and rare single-gene disorders[3] that are not related to folate metabolism. Here, we discuss what is known about folate metabolism, and how knowledge of these pathways has led to the identification of candidate genes potentially involved in NTDs. For one particular candidate gene, methylenetetrahydrofolate reductase (*MTHFR*), we have performed a meta-analysis based on published studies to determine the links between particular genotypes and NTD risk. Finally, we discuss recent work suggesting that folate acts to protect against defects in methylation (transfer of a methyl (CH_3) group) that might cause NTDs.

Neurulation and neural tube defects

Neurulation has long fascinated scientists, as evidenced by a devoted literature that extends back hundreds of years. The process of neurulation occurs during early embryogenesis, starting with the formation of the neural plate from specialized ectodermal cells and culminating in the closure of the neural tube (FIG. 1), the precursor of both the CNS and most of the PNS. Considered most simply, the neural plate develops bilateral neural folds, which elevate and fuse at the midline to create the neural tube. Subsequent development, together with vesiculation within the tube, gives rise to the brain and spinal cord. Further details of this complex process are beyond the scope of this review (for reviews, see REFS 4,5).

Neurulation seems to be highly complex and tightly regulated. The development and closure of the neural tube is usually completed by 28 days post-conception. This means that by the time a woman finds out that she is pregnant, the neural tube is almost completely closed. For the neural tube to close properly, genes that regulate various morphogenetic activities must function cooperatively. These activities include programmed cell death, neural crest cell migration, neuroepithelial proliferation,

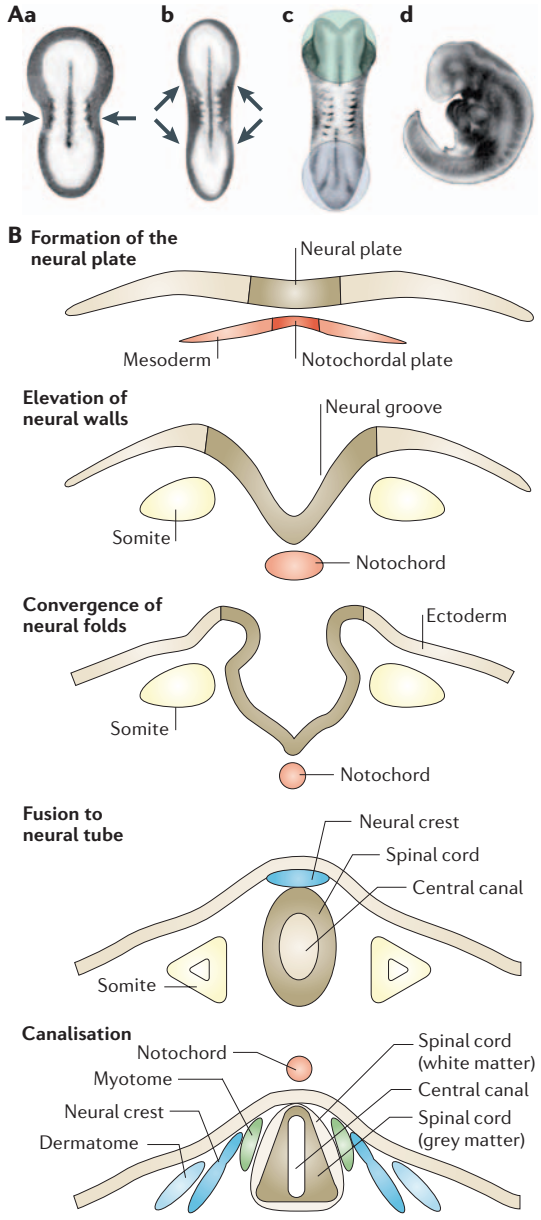


Figure 1 | **Neural tube closure in the developing mouse. A** | Four phases of primary neurulation in neural tube closure. Panels **a**–**c** are dorsal views, whereas panel **d** is a lateral view. **a**–**c** | Bidirectional fusion beginning from the original initiation site in the mid-cervical region (**a**), followed by continued bidirectional fusion (**b** and **c**) rostrally towards the anterior neuropore (blue shading) and caudally towards the posterior neuropore (green shading), which progresses as a result of convergent extension and apical constriction. Panel **d** shows a closed neural tube, with complete fusion over the rhomobencephalon, which has yet to be covered externally. Failure of the posterior neural tube to close can result in spina bifida. Defects in the closure of the anterior neural tube could result in anencephaly. **B** | Stages of neural tube closure in a transverse section. The neural plate elevates to form neural folds, which progressively appose each other, ultimately fusing to create a closed neural tube. This occurs just before the end of the first month of pregnancy. Panel **B** modified, with permission, from REF. 43 © (2001) Society for Experimental Biology and Medicine.

Anencephaly
A neural tube defect that occurs when there is a failure of neurulation, in which the neural folds do not fuse at the cranial end of the developing embryo. In these infants, most or all of the brain tissue is missing.

Spina bifida
A posterior neural tube defect that is caused by the failure of the neural tube to fuse at the caudal end. Infants with spina bifida can have an open lesion on their spine, where significant damage to the nerves and spinal cord has occurred, or the lesion can be closed.

Neural plate
Neural epithelial cells that form in the early embryo after neuronal induction and give rise to the nervous system.

Neural crest
Groups of cells that migrate from the neural tube to the periphery, where they give rise to a wide variety of cell types.

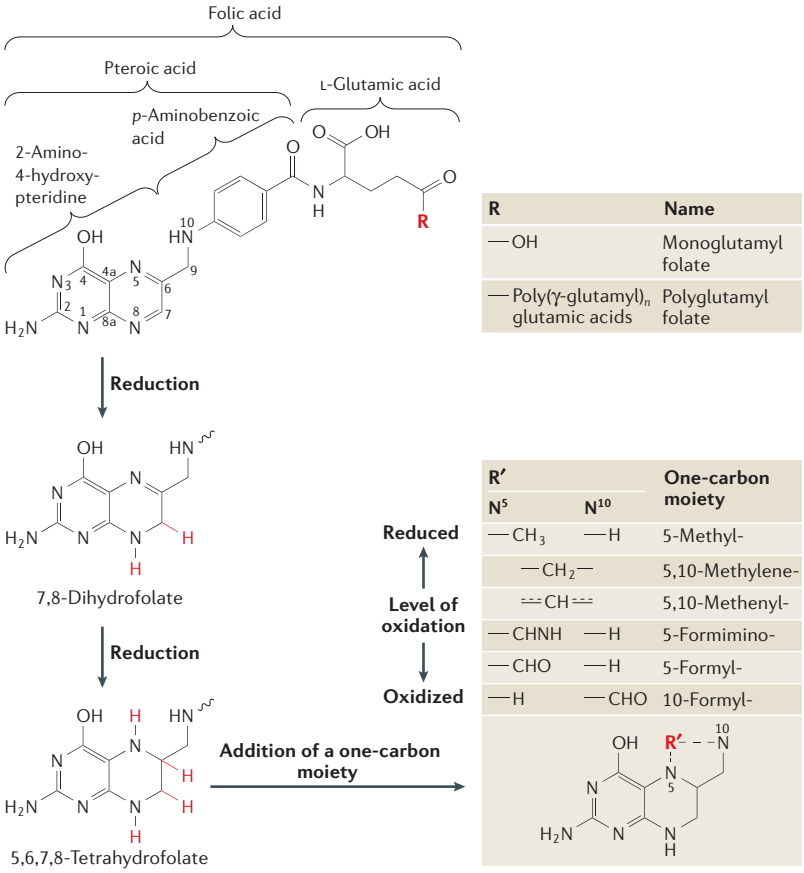
contraction of apical cytoskeletal microfilaments, and flexing at dorsolateral bending points in the developing neural tube. If these developmental mechanisms fail to take place within a critical window during embryogenesis, neural tube closure fails and the embryo develops an NTD.

Folic acid prevents neural tube defects

Maternal nutritional factors seem to contribute substantially to the complex aetiologies of NTDs. Foremost among these factors is the periconceptional use of supplements containing folic acid, which is associated with a reduction in the risk of women having NTD-affected pregnancies. More than 30 years ago, seminal work by Smithells *et al.*[6] showed that the diets and postpartum blood of women who had given birth to a foetus with an NTD were deficient in several micronutrients, particularly folate. Subsequent small non-randomized trials in women who had previously had NTD-affected pregnancies showed that taking folic acid or multivitamins containing folate during the periconceptional period resulted in a ~fourfold reduction in the risk of recurrence[7–12]. A rigorous double-blind, placebo-controlled, randomized trial, conducted by the UK Medical Research Council[13], showed that supplementation with 4 mg folic acid per day resulted in a threefold reduction in NTD recurrence risk. Taken together, these trials indicate that supplementation of folic acid in the dose range of ~0.4–5 mg per day prevents NTD births among most women who have previously had NTD-affected pregnancies.

There is also substantial evidence that folate supplementation reduces not only the recurrence but also the occurrence of NTDs. For example, several epidemiological studies have reported reductions in NTD risks associated with maternal intake of folic acid supplements or folate-rich diets[14–20]. Additional support for the importance of folate in NTD risk reduction was provided by Hernandez-Diaz *et al.*[21] Their study showed that periconceptional intake of medications that act as folic acid antagonists, such as carbamazepine or trimethoprim, could double the risk of NTD-affected pregnancies. Moreover, these investigators observed that the increased NTD risks associated with folate antagonists were attenuated by folic acid supplementation.

Evidence has emerged to suggest that maternal immunological responses that influence folate transport could affect embryonic development. Recently, Rothenberg *et al.*[22] reported the presence of auto-antibodies to the folate receptor in 75% of mothers who had given birth to NTD-affected infants, but in only 10% of mothers of non-malformed infants. This discrepancy in frequency correlated with a greater than 25-fold increase in the risk of NTDs in the infants of mothers that carried these antibodies. This small study suggests that maternal auto-antibodies that bind to the folate receptor, and so block the intracellular uptake of folate by target epithelial cells, might cause NTDs, which could explain the beneficial effect of periconceptional folate supplementation. In support of this, the lack of folate available to the developing embryo secondary to a defective or blocked



R	Name
—OH	Monoglutamyl folate
—Poly(γ-glutamyl)$_n$ glutamic acids	Polyglutamyl folate

R′		One-carbon moiety
N^5	N^{10}	
—CH_3	—H	5-Methyl-
—CH_2—		5,10-Methylene-
---CH---		5,10-Methenyl-
—CHNH	—H	5-Formimino-
—CHO	—H	5-Formyl-
—H	—CHO	10-Formyl-

Figure 2 | **Structures of folates.** The bioactive form of folate is 5,6,7,8-tetrahydrofolate. Although folic acid is not present in nature, it is more stable than other forms of folate and so is the form typically used in tablets and fortified food. Folic acid is reduced to 5,6,7,8-tetrahydrofolate by dihydrofolate reductase via 7,8-dihydrofolate. The function of folate is to provide one-carbon moieties for biosynthetic purposes. The most reduced form of folate is 5-methyltetrahydrofolate (5-MeTHF) and the most oxidized forms are 5- and 10-formyltetrahydrofolate. Polyglutamation prevents transport of folate out of the cell. Modified, with permission, from REF. 43 © (2001) Society for Experimental Biology and Medicine.

folate receptor has been shown to increase the risk of folate-responsive congenital anomalies in experimental animal model systems[23–27].

Another body of evidence that suggests that altered folate metabolism contributes to abnormal neural tube development derives from analyses of postpartum serum. Several clinical studies indicate that folate concentrations in postpartum serum and red blood cells are lower among women who have previously had a child with an NTD[28–30]; however, there have been conflicting results, with some studies failing to find such an association[31–35]. Whether it is a low maternal intake or altered absorption/metabolism of folate that is responsible for the observed association with NTD risk has yet to be established[36].

Finally, a natural population experiment provides further support for the involvement of folate in preventing NTDs. In the United States, fortification of flour and other enriched grain products with folic acid has been compulsory since 1998 (REF. 37). This has been effective in increasing folate concentration in both serum and red blood cells among women of childbearing age[38]: these increased concentrations have been attributed mainly to fortification of food, rather than to increased use of vitamin supplements among these women[38]. This supplementation has coincided with a 19% decrease in the prevalence of NTDs[39], which incorporates decreases of 31% and 16% for spina bifida[40,41] and anencephaly[40], respectively.

The preventive effects of folic acid on human NTD risk have therefore been well established. As noted above, the epidemiological evidence is robust, including consistent results from various study designs conducted in different populations: investigations showing elevated NTD risks associated with exposure to folate antagonist medications; the observation of higher auto-antibody titre to folate receptors in women who had previously had NTD-affected pregnancies; clinical studies that have reported lower postpartum folate concentration in red blood cells and serum among women who had previously had a child with an NTD; and a decline in the prevalence of NTDs since 1998, when the US food supply was fortified with folic acid.

Folate metabolism

Folate is a water-soluble vitamin that is present in a wide variety of foods, particularly in green leafy vegetables — the term folate is derived from the Latin word *folium* meaning leaf. Although according to chemical nomenclature, the difference between folate and folic acid is just one proton, in general the term folic acid is applied to the synthetic form of this B vitamin. Folic acid is the more stable of the two forms and so is more suitable for use in tablets and fortified foods (FIG. 2).

For folate metabolism that involves folic acid, this synthetic form of the vitamin must first be converted to the naturally bioactive form tetrahydrofolate (THF), which occurs through two reduction reactions catalysed by dihydrofolate reductase. Addition of a one-carbon unit and subsequent reduction steps produce 5-methyltetrahydrofolate (5-MeTHF) monoglutamate, which is taken up into the bloodstream and is the main form of folate in the circulation. From the blood, specific carriers or receptors transport 5-MeTHF into cells[42,43] (FIG. 3). Here, multiple glutamate residues are added, being linked via the γ-carboxyl peptide bond (R in FIG. 2), in a step known as polyglutamation. The incorporation of long polyglutamate chains alters the properties of the molecules such that they cannot be transported through the membrane and so they accumulate in the cells. In addition, enzymes involved in folate metabolism have higher affinities for folate polyglutamates.

Inside the cell, folate functions as both an acceptor and a donor of one-carbon units. These one-carbon units are covalently bound to folate (R′ in FIG. 2) and are present over the whole range of oxidized and reduced forms of the one-carbon moiety, including the formyl and methyl derivatives (for reviews, see REFS 42–44). These one-carbon units are donated to THF by the amino acids histidine and serine, and glycine and its derivatives dimethylglycine and methylglycine (sarcosine), or by formate, and are used in the synthesis of DNA building blocks — that is, purines (adenine and guanine) and

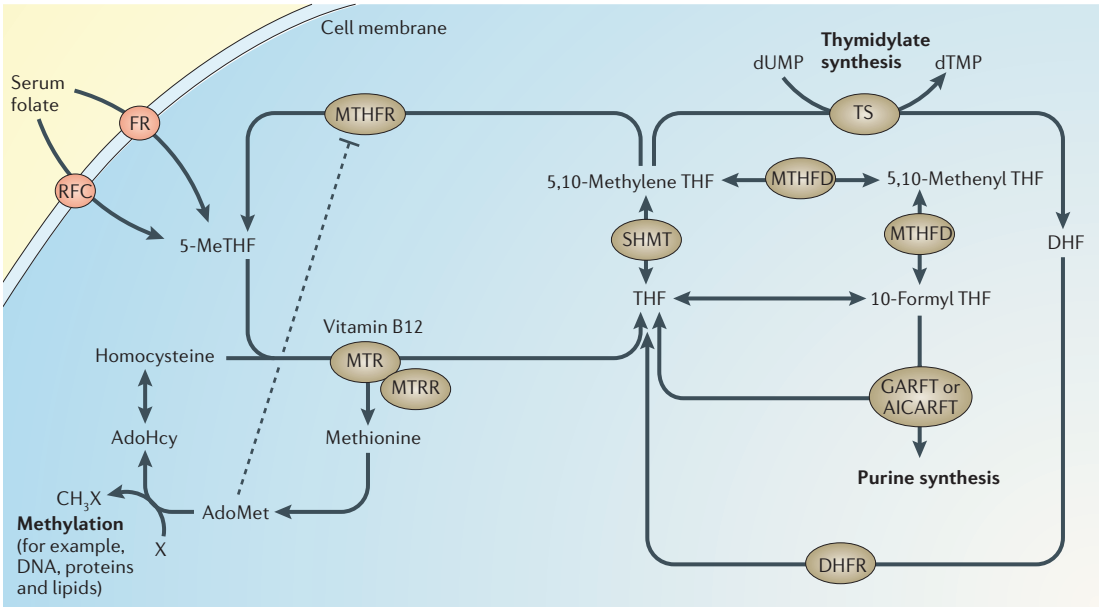


Figure 3 | **Simplified folate metabolism.** In the circulation, folate is mainly present as 5-methyltetrahydrofolate (5-MeTHF), which is taken up by cells via specific carriers or receptors. In the cell, folate donates one-carbon units for use in methylation reactions and in purine and thymidine synthesis. Alternatively, folate can also accept one-carbon units from several amino acids, including serine. Methylenetetrahydrofolate reductase (MTHFR) competes with thymidylate synthase (TS) and methylenetetrahydrofolate dehydrogenase (MTHFD) for the one-carbon units of 5-MeTHF. MTHFR activity is regulated by *S*-adenosylmethionine (AdoMet), which inhibits enzyme activity. Vitamin B12 is a cofactor of methionine synthase (MTR) and is involved in the transfer of the methyl group from 5-MeTHF to homocysteine. AdoHcy, *S*-adenosylhomocysteine; AIRCARFT, aminoimidazolecarboxamide ribonucleotide transformylase; DHF, dihydrofolate; DHFR, DHF reductase; dTMP, deoxythymidine monophosphate; dUMP, deoxyuridine monophosphate; FR, folate receptor; GARFT, glycinamide ribonucleotide transformylase; MTRR, MTR reductase; RFC, reduced folate carrier; SHMT, serine hydroxymethyltransferase; THF, tetrahydrofolate. Modified, with permission, from REF. 87 © (2003) Oxford University Press.

thymine (the thymidine precursor dTMP is shown in FIG. 3). In the methylation cycle, *S*-adenosylmethionine is the key methyl group donor. This cycle, which occurs in all cells except red blood cells, ensures adequate capacity for the methylation of numerous compounds. This includes the methylation of DNA (regulates gene expression), proteins (important post-translational modification) and lipids (important in their synthesis, for example, phosphatidylcholine). Methylation is also an important step in the metabolism of neurotransmitters and detoxification of xenobiotics. Therefore, it follows that folate metabolism is essential for cellular function, especially during periods of rapid growth.

In the methylation cycle, the methyl group of 5-MeTHF is transferred to homocysteine, via vitamin B12, to produce methionine. The reaction of ATP with methionine affords *S*-adenosylmethionine, which is the principal donor of methyl groups in cells. Loss of a methyl group from *S*-adenosylmethionine generates *S*-adenosylhomocysteine, which is a strong inhibitor of most methyltransferases and so must be removed — typically by conversion to homocysteine and adenosine — for the cycle to continue. This step produces homocysteine for methylation by 5-MeTHF, and so the methylation cycle can begin again. If inadequate amounts of 5-MeTHF or vitamin B12 are available, homocysteine accumulates in the cell. Consequently, because the equilibrium of the *S*-adenosylhomocysteine–homocysteine reaction favours *S*-adenosylhomocysteine formation, accumulation of homocysteine leads to the build up of *S*-adenosylhomocysteine, which might lead to dysregulation of gene expression, protein function, and lipid and neurotransmitter metabolism through inhibition of putative methyltransferases.

Genetic variation in folate metabolism

It is generally accepted that, in addition to environmental and dietary factors, folate metabolism and the consequent risk of NTDs is genetically determined. Elevated homocysteine concentrations have been observed in mothers who have delivered children with an NTD but who have normal folate levels, which was taken as an indicator of dysfunction of folate metabolism[30,43,45]. The strong homocysteine-lowering effect of folate supplementation[46] indicates that this form of dysfunctional folate metabolism can be overcome by additional folate intake. Because homocysteine concentrations are to a large extent under genetic control[47,48], homocysteine can be used as a biomarker to detect human NTD candidate genes that are involved in storage, transport and metabolism of folate. The identification of such genetic variants will also contribute to our understanding of the mechanisms underlying the role of folate in the prevention of NTDs.

Biomarker
A characteristic that is objectively measured and evaluated as an indicator of normal biological or pathogenic processes, or pharmacological responses to a therapeutic intervention.

Methylenetetrahydrofolate reductase. In the 1980s it was reported that most cases of relatively mild elevated homocysteine concentrations were due to heterozygosity for mutations involved in cystathionine β-synthase (CBS) deficiency. However, subsequent enzymatic and, in particular, molecular genetic studies made it clear that heterozygosity for mutations that lead to CBS deficiency is not a cause (or is at most only a minor cause) of elevated homocysteine concentrations[49,50]. Kang *et al.*[51] and, later, our group[52] described thermolabile MTHFR (a loss of enzyme activity at temperatures higher than 37°C) as a cause of decreased MTHFR activity and elevated homocysteine concentrations. Rozen and colleagues[53] cloned the *MTHFR* gene and discovered the 677C>T variant, and, in collaboration with our group, it was shown that this variant leads to the production of thermolabile MTHFR. In the same year, we showed that the presence of the *MTHFR* 677C>T variant in children with spina bifida and their mothers increases the risk of them having an NTD or having a child with an NTD, respectively[54]. Many studies in mothers and patients with NTDs followed, but these often failed to detect an association between the *MTHFR* 677C>T variant and NTD risk (Supplementary information S1 (box)).

In an attempt to clarify the relationship between MTHFR and NTD risk, we summarized the available studies pertaining to this question using a meta-analysis (Supplementary information S1 (box)). We calculated that mothers who are homozygous for the *MTHFR* 677C>T variant (677 TT) have a 60% increased risk (odds ratio of 1.6) of giving birth to an infant with an NTD, whereas homozygous offspring themselves have a 90% increased risk (odds ratio of 1.9) of being born with an NTD. Furthermore, we also found a mild increase in NTD risk in mothers and offspring who are heterozygous for the *MTHFR* 677C>T variant (677 CT), with a 10% (odds ratio of 1.1) and 30% (odds ratio of 1.3) increased risk, respectively. At the population level, this is an important observation, because the prevalence of heterozygous individuals is much higher (~40%) compared with that of homozygous individuals (~10%). Our meta-analysis not only confirms the findings of a previous study[55], but also includes data from ~50% more studies and therefore provides up-to-date estimates of the relationship between the *MTHFR* 677C>T variant and the risk of NTDs.

The *MTHFR* 677C>T variant is a good example of a susceptibility gene: the 677 TT genotype results in elevated homocysteine concentrations only if folate concentrations are in the low-to-normal range[56,57]. A study by Guenther *et al.*[58] established at the protein level that the 677C>T polymorphism (which leads to substitution of Ala by Val at amino acid number 222) results in the loss of flavin adenine dinucleotide (FAD) from MTHFR. Folate binding to MTHFR prevents this loss. As recently shown[59], riboflavin (also known as vitamin B2), which is a precursor of FAD, lowers homocysteine concentrations in individuals with the 677 TT genotype, which indicates that, in addition to folate, it could be important to investigate riboflavin intake in relation to the prevention of NTDs.

Other candidate genes. Inspired by the identification of the *MTHFR* 677C>T variant, we and others have systematically explored many genes involved in folate metabolism, in individuals with NTDs or their mothers (for reviews, see REFS 44,60–64). The methionine synthase reductase (*MTRR*) 66A>G variant has emerged as a possible genetic risk factor for NTDs. Other potential candidates are the *MTHFR* 1298A>C, methylenetetrahydrofolate dehydrogenase (*MTHFD*) 1958G>A and transcobalamin 776C>G variants[64]. Unfortunately, most single nucleotide polymorphisms (SNPs) tested have not been shown to be associated with NTD risks of substantial magnitude[64]. This indicates that many of the candidate genes studied so far might not be important in the mechanisms underlying the link between folate and NTD risk. Alternatively, NTD risk could depend on a combination of many SNPs that have little effect individually. Identifying such SNPs from the many non-functional SNPs would require the study of large populations. It is also possible that the approaches used or the candidate SNPs selected for study are responsible for the absence of identified candidate genes. For example, most studies have not explored genes by sequencing or a comparable technique, but instead have tested only published variants[60]. Therefore, the folate genes that have been tested might still harbour undiscovered potential variation. Moreover, those studies that have used sequencing examined mainly the coding region and not the untranslated and promoter regions of the gene. Most importantly, not all potential folate genes have been studied: in particular, the many methyltransferases that catalyse the transfer of methyl groups to, for example, DNA, RNA, proteins and lipids, have not, so far, been investigated. The use of dedicated SNP arrays that allow high-throughput genotyping of cases and controls, in combination with haplotype analyses, could narrow this gap in our knowledge.

Animal models. Exploring mouse models with an NTD phenotype could theoretically lead to the discovery of genes that cause human NTDs. There are more than 80 genetic mouse models of NTDs, with new models emerging on a regular basis from gene targeting studies and mutagenesis screens: these areas have been covered in detail in recent reviews[4,65–68]. Most mutated NTD-causing genes are involved in transcription or translation[68], which underlines the complex and tight regulation of neural tube closure. Mouse models in which folate transport is disrupted (including folic acid-binding protein 1 (Folbp1) and reduced folate carrier 1 (RFC1) knockouts) have NTDs. However, models in which enzymes involved in folate or homocysteine metabolism, including MTHFR, CBS and methionine synthase (MTR), are disrupted show no NTD phenotype[69–71]. This is in line with observations made in humans who have inborn errors of folate or homocysteine metabolism that are related to MTHFR and CBS or dysfunction of methionine synthase, but in whom NTDs are not necessarily over-represented[73].

In humans, most NTD cases are non-syndromic, occur with incomplete penetrance, and have an unclear transmission pattern and a high level of genetic

Single nucleotide polymorphism
(SNP). A specific location in a DNA sequence at which different people can have a different DNA base. Differences in a single base could change the protein sequence, leading to disease (for example, sickle-cell disease), or have no known consequences.

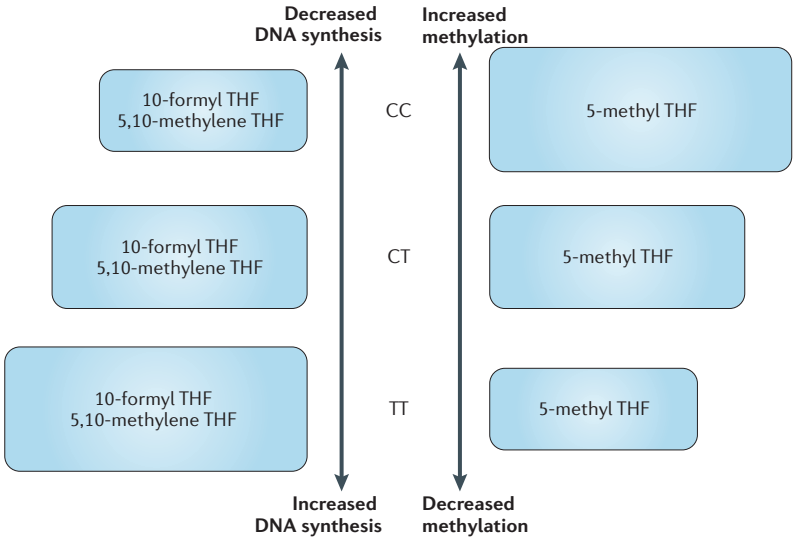


Figure 4 | **The three *MTHFR* genotypes affect methylation and DNA synthesis to different extents.** Variations in methylenetetrahydrofolate reductase (MTHFR) activity influence the flux through the different folate pathways. The *MTHFR* 677 CC genotype produces the variant with the highest enzymatic activity. This results in lower concentrations of 5,10-methylenetetrahydrofolate (5,10-methylene THF) and 10-formyl THF available for DNA synthesis and higher concentrations of 5-methyl THF available for adequate levels of methylation. The CT genotype produces an intermediate effect. Finally, the TT genotype produces the variant with the lowest MTHFR activity and leads to higher concentrations of 5,10-methylene THF and 10-formyl THF for DNA synthesis, and lower concentrations of 5-methyl THF. Consequently, there is a reduction in the extent of methylation, which could result in an increased risk of neural tube defects.

complexity. Although variable low penetrance is found in some mutant models, only a few strains, including *SELH/Bc* and *curly tail* strains, show the complex inheritance patterns and responsiveness to nutritional supplementation that are indicative of human non-syndromic NTDs[74,75]. To our knowledge, the discovery of genes that cause NTDs when disrupted in mouse models has not as yet led to the identification of a genetic basis for human non-syndromic NTDs.

In some mouse models, the NTD rate can be reduced by maternal nutrient supplements, such as folic acid (in the *Folbp1*, *RFC1*, *Cart1* and *Cd* knockouts), inositol (in $ct^{-/-}$) and methionine (in the *Axd* knockout). Some of these models have gene defects that are not related to folate metabolism (for example, $Cart1^{-/-}$, $Cd^{-/-}$ and $Axd^{-/-}$), and so it is possible that some genes involved in human NTDs that can be prevented with folate supplementation are, according to our current understanding, completely unrelated to folate metabolism. Whole-genome analyses in humans using, for example, high-density SNP arrays could help to reveal such genes. Taken together, these findings in mice and humans fit a model in which the causes of NTDs are multifactorial, and in which folate metabolism is important, but is not the only player.

Methylation hypothesis

The identification of the *MTHFR* 677C>T variant as an important genetic risk factor sheds light on the underlying mechanism by which folate prevents NTDs. MTHFR has a unique position in folate metabolism as it makes one-carbon units available for methylation reactions at the expense of purine and thymidine synthesis. As proposed in our original study[54], the reduced MTHFR enzyme activity observed in individuals with the 677 TT genotype causes a redistribution of one-carbon units. This manifests as an increase in 10-formyltetrahydrofolate and a decrease in 5-MeTHF concentrations in red blood cells[76,77] (FIG. 4). Interestingly, the 677 TT genotype results in a global reduction in the methylation of DNA[78,79]. Moreover, reduced methylation due to the *MTHFR* 677 TT genotype might be more pronounced under low-to-normal folate conditions, which would be indicated by elevated homocysteine concentrations[56,57]. Disruption of the DNA methyltransferase DNMT3B during embryonic development results in the development of a distorted neural tube, which highlights the importance of DNA methylation during periods of rapid growth[80]. This underscores the hypothesis that the *MTHFR* 677C>T variant results in increased NTD risk through decreased methylation. In addition to the reduced methylation of DNA observed in individuals with the 677 TT genotype, other methyl acceptors, such as RNA, proteins and lipids, might also be affected and therefore warrant further investigation (FIG. 5).

In our own recent *in vitro* analysis of cultured chicken embryos, we tested the hypothesis that disturbed methylation might reduce proper closure of the neural tube[81,82]. Exposure of chicken embryos to homocysteine or inhibitors of methylation, such as cycloleucine (an inhibitor of methionine adenosyl methyltransferases) or oxidized adenosine (Adox), which is an inhibitor of

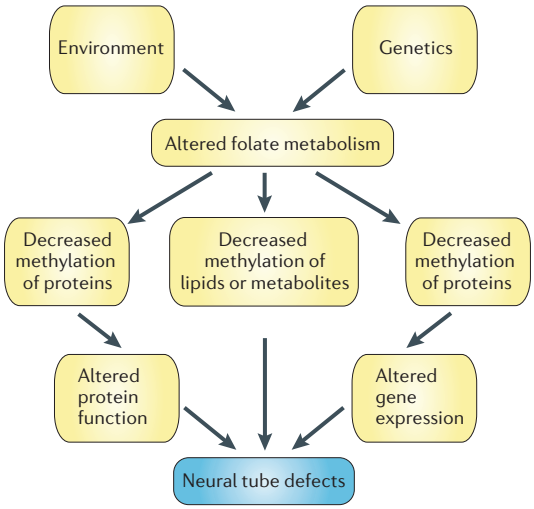


Figure 5 | **Folate metabolism is influenced by environmental and genetic factors.** Intake of B vitamins other than folate, such as vitamin B12, B6 and B2, also affects folate metabolism. This process can also be disrupted by genetic variants, with an elegant example being the *MTHFR* 677C>T polymorphism. Disruption of the methylation of lipids, DNA and proteins during early embryogenesis could lead to neural tube defects (NTDs). It is proposed that folate prevents NTDs by increasing methylation of various molecules that are essential to cellular processes.

Somites
Paired blocks of mesoderm cells along the vertebrate body axis that form during early vertebrate development and differentiate into dermal skin, bone and muscle.

S-adenosylhomocysteine hydrolase, resulted in increased concentrations of *S*-adenosylhomocysteine and a decreased *S*-adenosylmethionine/*S*-adenosylhomocysteine ratio. In these experiments, a dose-dependent delay in neurulation was observed when the embryos were treated during a relatively small window of embryonic development, at the fourth and fifth somite stages[81,82] (Supplementary information S2,S3 (movies)). Previous studies[83,84] showing that culturing rat embryos at relatively low methionine concentrations causes NTDs also support the hypothesis that disruptions in methylation underlie defects in neural tube closure. Under these culture conditions, Coelho and Klein[83] observed reduced methylation of amino acids in proteins, and proposed that this would affect actin function and so reduce microfilament contraction, which is essential for the appropriate inward folding of the neural folds. It is remarkable that administration of homocysteine to rat embryos cultured with low methionine prevented NTDs[84]. This suggests that elevated plasma homocysteine itself is not likely to cause NTDs but is instead a biomarker of a disturbed methylation cycle. These findings could also explain why administration of homocysteine to pregnant mice or cultured mouse embryos failed to induce NTDs[85,86].

If hampered methylation is the underlying mechanism in NTDs that can be prevented with folate administration, this opens up an area of research that might offer new insights into the mechanisms involved in neurulation. For example, researchers might look for differences in gene expression due to unbalanced DNA methylation, or for changes in metabolite, protein or phospholipid function (FIG. 5).

Conclusions and future directions

Here, we have summarized what is currently understood about the association between folate and NTD risks in humans from the vantage points of epidemiology, mouse genetics, human genetics and cell biology. Although there have been excellent studies in this field, the mechanism by which folic acid prevents NTDs remains largely unknown. Future research, aimed at determining the changes of methylation that contribute to epigenetics and reduce the functions of proteins, phospholipids or small molecules will hasten progress towards an understanding of this important association. Moreover, characterization of the causal mechanisms of the adverse effects of folate deficiency on human development might shed light on the pathogenic mechanisms that underlie neural tube maldevelopment or other birth defects. Therefore, further investigation of folate metabolism and its role in methylation will greatly inform future NTD prevention strategies.

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Competing interests statement
The authors declare no competing financial interests.

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