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Genetics of human neural tube defects

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Neural tube defects (NTDs) are common, severe congenital malformations whose causation involves multiple genes and environmental factors. Although more than 200 genes are known to cause NTDs in mice, there has been rather limited progress in delineating the molecular basis underlying most human NTDs. Numerous genetic studies have been carried out to investigate candidate genes in cohorts of patients, with particular reference to those that participate in folate one-carbon metabolism. Although the homocysteine remethylation gene *MTHFR* has emerged as a risk factor in some human populations, few other consistent findings have resulted from this approach. Similarly, attention focused on the human homologues of mouse NTD genes has contributed only limited positive findings to date, although an emerging association between genes of the non-canonical Wnt (planar cell polarity) pathway and NTDs provides candidates for future studies. Priorities for the next phase of this research include: (i) larger studies that are sufficiently powered to detect significant associations with relatively minor risk factors; (ii) analysis of multiple candidate genes in groups of well-genotyped individuals to detect possible gene–gene interactions; (iii) use of high throughput genomic technology to evaluate the role of copy number variants and to detect 'private' and regulatory mutations, neither of which have been studied to date; (iv) detailed analysis of patient samples stratified by phenotype to enable, for example, hypothesis-driven testing of candidates genes in groups of NTDs with specific defects of folate metabolism, or in groups of fetuses with well-defined phenotypes such as craniorachischisis.

INTRODUCTION

Congenital malformations are the leading cause of infant mortality in developed countries and a major cause of health problems in surviving children. Neural tube defects (NTDs) are a common group of central nervous system anomalies affecting 0.5–2 per 1000 pregnancies worldwide. NTDs arise when the neural tube, the embryonic precursor of the brain and spinal cord, fails to close during neurulation. The cranial region (anencephaly) or the low spine (open spina bifida; myelomeningocele) are most commonly affected although, in the severe NTD craniorachischisis, almost the entire neural tube remains open, from midbrain to low spine.

Most individuals who survive with NTDs (particularly myelomeningocele) have a multiple system handicap and a limited life expectancy. However, despite the high prevalence and traumatic consequences for affected individuals and their families, the causes of NTD are poorly understood. Identification of causative factors is confounded by the fact that the majority of these malformations appear to result from a combination of genetic and environmental factors. A strong genetic component is indicated by the high recurrence risk for siblings of affected individuals (1,2). Syndromic cases of NTD also exist, often associated with chromosomal anomalies, but these represent $<10\%$ of all defects (1,3–5). The majority of NTDs are sporadic, with recurrence fitting a multifactorial polygenic or oligogenic pattern, rather than models on the basis of single gene dominant or recessives, with reduced penetrance (2).

GENETIC ANALYSIS OF HUMAN NTDS

Positional cloning strategies have been hampered by the paucity of large families with multiple affected members. Nevertheless, genome-wide studies using collections of smaller multiplex families have implicated chromosomes 2, 7 and 10 as harbouring candidate risk loci for spina bifida

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(6–8). Although the causative genes are yet to be identified, these studies may result in identification of candidate sequences that can be evaluated in larger populations. An alternative approach exploits the association of NTDs with chromosomal anomalies such as trisomies 13 and 18 (9), suggesting that gene-dosage can affect neural tube closure. Rearrangements involving deletions, duplications or balanced translocations are likely to be most informative, with fine mapping of chromosomal breakpoints enabling identification of specific loci (10).

In some studies, direct mutation screening of candidate genes has been carried out in cohorts of patients (11), but the vast majority involve statistical association analysis of sequence variants in or near candidate genes. Most work has involved case–control analysis, comparing the frequency of 'risk' alleles in affected individuals and/or mothers with a matched unaffected cohort. More sophisticated studies have used the transmission disequilibrium test (TDT) in family trios (mother, father and affected child), which is less dependent on population structure. In the remainder of this article, we review the main candidate gene studies which have arisen primarily from analysis of folate metabolic pathways and mouse models of NTDs. Boyles *et al*. (11) published a comprehensive review of this field up to 2004, and an updated candidate gene list is presented in Table 1.

CANDIDATE GENES FROM FOLATE METABOLISM

Epidemiological studies provide an opportunity to identify risk factors for NTDs, such as dietary or teratogenic agents, to which susceptibility may be modified by genetic predisposition (12–14). Among environmental factors, folate status plays a key role in determining NTD risk (15,16). Maternal supplementation with folic acid during pregnancy reduces NTD frequency (17,18) whereas reduced serum folate and/or elevated homocysteine (an inverse indicator of folate status) are observed in some mothers of NTD fetuses, and are considered risk factors for NTDs (19–21). However, NTDs are not simply a condition of folate deficiency: maternal folate levels in most human NTD-affected pregnancies are in the 'normal' range (22), suggesting that low folate status may increase susceptibility but is not directly causative. Similarly, in mice dietary folate deficiency can cause significant embryonic growth retardation but does not cause NTDs (16,23,24). Hence, sub-optimal folate status may pre-dispose to NTDs in combination with additional factors, either environmental or genetic.

The intricate interplay and cross-regulation between elements of one-carbon (folate) metabolism (Fig. 1) complicates the teasing out of events that impinge on neural tube closure. In mice, key cellular functions in the developing embryo include methylation reactions and biosynthesis of nucleotides that support rapid cellular proliferation (2,25). Cranial NTDs arise when the methylation cycle is inhibited (26,27), and in null embryos for DNA methyltransferase 3B (28). In contrast, exogenous homocysteine does not cause NTDs (29–31), even in genetically predisposed *splotch* embryos (24) and may be an indicator of impaired folate or methylation cycle activity.

If folate status interacts with genetic factors in the causation of NTDs, this could involve either folate-related or folate-independent genes. To date, most emphasis has been placed on the evaluation of folate-related genes as NTD candidates (32,33) (Table 1). Further support comes from analysis of primary cell lines obtained from NTD fetuses, which indicates that a genetically-determined abnormality of folate metabolism is present, in at least a proportion of cases (34). However, identifying specific NTD-predisposing genetic lesions has proven far from straight forward. Although a number of variants have been widely studied, inconsistent results between different cohorts and populations (Table 1) indicate that very few, if any, have a major causative effect. Below, we sub-divide the candidate folate-related genes into three functional categories.

Methylation related genes

Among folate-related genes, 5,10-methylene tetrahydrofolate reductase (MTHFR) has been the principal focus of attention, following reports that the 677C>T (A222V; rs1801133) polymorphism is associated with increased risk of NTDs in Dutch and Irish populations (35–37). Other populations show no association (38,39) or even a protective effect (40,41) (Table 1). A meta-analysis, including genotype data from 27 studies up to 2004, suggests that the 677TT genotype confers an overall 1.9 times increase in NTD risk (Odds ratio: 1.9; 95% confidence interval: 1.6–2.2) (15). A more recent meta analysis (42) found a positive association only in non-latin groups, principally the Irish population.

The action of MTHFR generates 5-methylTHF for remethylation of homocysteine, at the expense of other folates required for purine and pyrimidine biosynthesis (Fig. 1). The A222V variant protein has reduced function and is associated with elevated plasma homocysteine (36). Nullizygosity for MTHFR in mice also results in elevated homocysteine and diminished DNA methylation (43), although NTDs are not observed under either normal or folate-deficient conditions. Moreover, MTHFR nullizygosity does not exacerbate the folate-responsive *splotch* mutation (43–45). These data suggest that in populations where MTHFR is a risk factor, additional interacting factors are likely to be present.

The link between reduced methylation/elevated homocysteine and NTDs has prompted analysis of variants in other genes that could influence the methylation cycle through remethylation (*MTR*, *MTRR*, *BHMT* and *BHMT2*) or transsulfuration (*CBS*) of homocysteine (11) (Table 1). In general, mildly elevated risks have been identified in some studies but rarely replicated. MTRR (methionine synthase reductase) functions to maintain activity of MTR (methionine synthase), and a variant form (I22M, encoded by 66A>G) was reported as an NTD case and maternal risk factor in some studies, but not others (Table 1). Mouse studies do not support a role for these genes in NTDs: targeted deletion of *Mtr* is embryonic lethal prior to neurulation stages and heterozygotes do not show NTDs (46). Similarly, reduced activity of *Mtrr* and loss of *cbs* function do not cause NTDs, although elevated plasma homocysteine is observed (47,48).

Table 1. Candidate gene analysis in human NTDs

Human gene	Type of candidate	Reference	Population studied	Sample size	Type of study	Summary of results/conclusion
AHCY (S-adenosylhomocysteine hydrolase)	One carbon metabolism	(56)	Dutch	180 SB patients, 190 controls	Case–control study	No association
ALDH1L1 (Aldehyde dehydrogenase 1, member L1)	One carbon metabolism	(56)	Dutch	180 SB patients, 190 controls	Case–control study	Nominally significant association with Asp793Glu variant[a]
ALDH1A2 (Retinaldehyde dehydrogenase Type 2, RALDH2)	Retinol metabolism	(104)	USA	318 SB families	Family based association study	One polymorphism associated with increased SB risk (tentative association for two others)
AMD1 (*Adenosyl methionine decarboxylase 1*)	One carbon metabolism	(56)	Dutch	180 SB patients, 190 controls	Case–control study	No association
APE1 (apurinic endonuclease1)	DNA repair	(112)	Mixed USA	380 SB cases, 350 controls	Case–control study	Suggestion of reduced risk for Asp148Glu variant
BHMT (betaine-homocysteine methyltransferase)	One carbon metabolism	(113)	Mixed USA	252 SB cases, 337 controls	Case–control study	No association for Arg239Gln polymorphism
		(56)	Dutch	180 SB patients, 190 controls	Case–control study	No association
		(103)	Mixed USA	259 SB cases, 359 controls	Case–control study	Modest increase in SB risk associated with 1 SNP (of eight tested)
BHMT2 (betaine-homocysteine methyltransferase 2)	One carbon metabolism	(56)	Dutch	180 SB patients, 190 controls	Association study	No association detected
		(103)	Mixed USA	259 SB cases, 359 controls	Case–control study	No association for 7 SNPs tested
BRCA1 (breast cancer 1)	NTDs in mouse mutant	(114)	USA	268 SB patients and parents	Family based association study (TDT)	Association with SB for two microsatellite markers and A4956G SNP. Proposed polymorphisms affect level of lesion, not causative
CAT (catalase)	Oxidative stress	(115)	Mixed USA	507 SB cases, 185 controls	Case–control study	No association
CBS (cystathionine beta-synthase)	Folate metabolism	(116)	UK	207 NTD cases (200 mothers, 93 fathers). 601 controls, 542 control mothers	Case–control study	No association for 844ins68
		(56)	Dutch	180 SB patients, 190 controls	Case–control study	No association
		(103)	Mixed USA	259 SB cases, 359 controls	Case–control (9 SNPs)	Modest increase in SB risk associated with 2 SNPs
CFL1 (*n*-cofilin)	NTDs in mouse mutant	(117)	Mixed USA	246 SB cases, 336 controls	Case–control SNPs	Mildly elevated risk of NTDs in non-Hispanic whites
CHKA (choline kinase A)	One carbon metabolism	(118)	Mixed USA	103 SB cases, 338 controls	Case–control study	Possible association with reduced SB risk for 1 of 2 SNPs studied
CITED2	NTDs in mouse mutant	(119)	Mixed USA	64 SB cases, 72 controls	Mutation screen and case–control	No mutations. No association of three 5′-UTR SNPs with risk
COQ3 (Coenzyme Q3 homolog, methyltransferase)	Methylation	(56)	Dutch	180 SB patients, 190 controls	Case–control study	No association
CRABPI (cellular retinoic acid binding protein I)	Retinol metabolism	(104)	USA	230 SB cases, 318 SB families	Mutation screen and family based association study	No mutations. No association (3 SNPs tested)
CRABPII (cellular retinoic acid binding protein II)	Retinol metabolism	(104)	USA	230 SB cases	Mutation screen	No mutations
CTH (Cystathionase)	One carbon metabolism	(56)	Dutch	180 SB patients, 190 controls	Case–control study	No association
CUBN (cubulin)	Endocytosis (folate transport)	(56)	Dutch	179 SB patients, 190 controls	Case–control study	GG genotype for rs1907362 significantly associated with reduced SB risk
CYP26A1 (cytochrome P450)	Retinol metabolism	(104)	USA	230 SB cases	Mutation screen	No mutations
CYP26B1 (cytochrome P450)	Retinol metabolism	(104)	USA	230 SB cases, 318 SB families	Mutation screen and Family based association study	No mutations. No association (5 SNPs tested)

Continued

Table 1. *Continued*

Human gene	Type of candidate	Reference	Population studied	Sample size	Type of study	Summary of results/conclusion
DHFR (Dihydrofolate reductase)	Folate metabolism	(120)	Mixed USA	61 SB cases and parents (multi-affected families) and 219 controls	Case–control study of 19-bp intron-1 deletion	The del/del genotype was more frequent in mothers of SB cases, compared with controls. No association in fathers or patients
		(121)	Irish	283 cases (and 280 mothers, 279 fathers) and 256 controls. SB (95%) or encephalocele (5%)	Case–control study. 19-bp deletion and two 3′-UTR variants.	19-bp Intron deletion shows protective effect. May increase mRNA levels
		(122)	Dutch	109 patients, 121 mothers (SB). 234 paediatric controls, 292 control women	Case–control study. 19-bp deletion and 9-bp repeat in 5′-UTR	19-bp Intron deletion not associated with NTDs. No effect on expression
		(56)	Dutch	180 SB patients, 190 controls	Case–control study	No association
		(103)	Mixed USA	259 SB cases, 359 controls	Case–control study	No association for 9 SNPs tested. Intron deletion not tested
		(41)	Mixed UK	126 SB (open); 103 SB (closed); 49 anencephalic; 192 controls	Case–control study	No association
FOLR1 (Folate receptor 1)	Folate transport	(56)	Dutch	180 SB patients, 190 controls	Case–control study	No association
		(103)	Mixed USA	259 SB cases, 359 controls	Case–control study	No association for 3 SNPs tested
FOLR2 (Folate receptor 2)	Folate transport	(56)	Dutch	180 SB patients, 190 controls	Association study	No association
		(103)	Mixed USA	259 SB cases, 359 controls	Case–control study	No association for 3 SNPs tested
FOLR3 (Folate receptor 3)	Folate transport	(56)	Dutch	180 SB patients, 190 controls	Case–control study	No association
FPGS (Folylpolyglutamate synthase)	Cellular folate retention	(56)	Dutch	180 SB patients, 190 controls	Case–control study	No association
FTCD (Formininotransferase cyclodeaminase	One carbon metabolism	(56)	Dutch	180 SB patients, 190 controls	Case–control study	No association
GAMT (Guanidinoacetate N-methyl transferase)	One carbon metabolism	(56)	Dutch	180 SB patients, 190 controls	Case–control study	No association
GAPD (glyceraldehyde 3 phosphate dehydrogenase)	Glucose metabolism	(115)	Mixed USA	507 SB cases, 185 controls	Case–control study	No association
GART (Phosphoribosylglycinamide formyltransferase, phosphoribosyl glycinamide synthetase, phosphoribosyl aminoimidazole synthetase)	Purine biosynthesis (one carbon metabolism)	(56)	Dutch	180 SB patients, 190 controls	Association study	No association
GCPII (glutamate carboxypeptidase), *FOLH1* (Folate hydrolase)	Folate metabolism	(116)	UK	208 NTD cases (200 mothers, 92 fathers). 600 child, 531 mother controls	Case–control study	No association for 1561C>T
		(56)	Dutch	180 SB patients, 190 controls	Case–control study	No association
GGH (Gamma-glutamyl hydrolase)	Folate metabolism	(56)	Dutch	180 SB patients, 190 controls	Case–control study	No association
GLUT1 (glucose transporter 1)	Glucose metabolism	(115)	Mixed USA	507 SB cases, 185 controls	Case–control study	Pro196 silent SNP associated with risk in TDT test
GLUT4 (glucose transporter 4)	Glucose metabolism	(115)	Mixed USA	507 SB cases, 185 controls	Case–control study	No association
HK1 (hexokinase 1)	Glucose metabolism	(115)	Mixed USA	507 SB cases, 185 controls	Case–control study	Lys481 SNP variant associated with risk in TDT test
HK2 (hexokinase 2)	Glucose metabolism	(115)	Mixed USA	507 SB cases, 185 controls	Case–control study	No association
ICMT (Isoprenylcysteine carboxyl methyltransferase)	Protein methylation	(56)	Dutch	180 SB patients, 190 controls	Association study	No association
INS (insulin)	Glucose metabolism	(115)	Mixed USA	507 SB cases, 185 controls	Case–control study	No association
INSR (insulin receptor)	Glucose metabolism	(115)	Mixed USA	507 SB cases, 185 controls	Case–control study	No association
LEP (leptin)	Glucose metabolism	(115)	Mixed USA	507 SB cases, 185 controls	Case–control study	Arg109Lys variant associated with risk in TDT test
LEPR (leptin receptor)	Glucose metabolism	(115)	Mixed USA	507 SB cases, 185 controls	Case–control study	Arg109Lys variant associated with risk in TDT test
MAT1A (Methionine adenosyltransferase I, alpha)	One carbon metabolism	(56)	Dutch	180 SB patients, 190 controls	Case–control study	No association

MAT2A (Methionine adenosyltransferase II, alpha)	One carbon metabolism	(56)	Dutch	180 SB patients, 190 controls	Case–control study	No association
MGMT (O-6-Methylguanine DNA methyltransferase)	One carbon metabolism DNA methylation	(56)	Dutch	180 SB patients, 190 controls	Case–control study	No association
MTHFD1 (methylenetetrahydrofolate dehydrogenase/ methylenetetrahydrofolate-cyclohydrolase/ formyltetrahydrofolate synthetase)	Folate metabolism	(50)	Irish	176 NTD cases (Mostly SB, few encephalocele). 245 mothers, 127 fathers (also includes parents of anencephalic cases). 770 controls	Case–control to evaluate Arg653Gln (1958G>A; dbSNP rs 1801133) polymorphism	Maternal AA genotype confers increased risk to offspring
		(51)	Italian	142 NTD cases (open and closed SB) (125 mothers, 108 fathers). 523 controls.	Case–control study and family based association study (TDT) for 1958G>A	Mildly increased risk for AA and GA genotypes in cases (and mothers). TDT shows excess transmission of A allele to cases
		(52)	Dutch	103 SB cases, 113 mothers, 98 fathers. 460 controls.	Case–control and TDT for 1958G>A	No association
		(54)	Irish	509 cases (with 485 mothers and 439 fathers)—mostly open SB. 966 controls.	Case–control study for SNP rs1076991C>T	Promoter variant not independent risk factor. Case and maternal risk factor when combined with Arg653Gln variant
		(56)	Dutch	180 SB patients, 190 controls	Case–control study	No association
		(103)	Mixed USA	259 SB cases, 359 controls	Case–control (10 SNPs)	Increased SB risk for one SNP, decreased risk for three others
		(41)	Mixed UK	126 SB (open); 103 SB (closed); 49 anencephalic; 192 controls	Case–control study	No association
MTHFD2	Folate metabolism	(56)	Dutch	180 SB patients, 190 controls	Case–control study	No association
		(103)	Mixed USA	259 SB cases, 359 controls	Case–control study	No association for 8 SNPs tested
MTHFR (5,10-methylenetetrahydrofolate reductase)	Folate metabolism	(116)	UK	200 NTD cases (186 mothers, 92 fathers). 578 child, 512 mother controls	Case–control study	Mildly elevated risk for 677C>T. No association for 1298A>C
		(123)	Polish	20 NTD cases, 262 controls.	Case–control for C677T and A1298C	No association
		(124)	Irish	471 cases (451 SB, 20 encephalocele). Triads comprise >1300 samples. 922 controls.	Mutation screen. Case–control and TDT for 116C>T (P39P) and 1793G>A (R594Q)	Possible association with SB for variants tested. Unlikely to be independent risk factors (association likely due to linkage disequilibrium with 677C>T)
		(125)	Mixed USA	350 cases, 328 mothers, 245 fathers, 167 siblings	Family-based association study	No association for 677C>T
		(126)	Italian	15 cases (open SB), 18 fathers, 60 mothers. 43 control children and 100 control adults	Case–control screen for C677T and A1298C	T allele more frequent in cases than controls. A1298C not different between groups
		(127)	Mexican	118 NTD case mothers (all anencephalic), 112 control mothers	Case–control study for C677T and A1298C	Maternal TT confers higher risk than CC for anencephaly. A1298C not associated with NTDs
		(128)	Indian	83 mothers of NTD cases. 60 controls	Case–control for C677T and A1298C	677T more frequent in mothers of cases than in controls (only for lower defects). No difference in A1298C frequency

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Table 1. *Continued*

Human gene	Type of candidate	Reference	Population studied	Sample size	Type of study	Summary of results/conclusion
		(129)	Mexican (Yucatan)	108 cases (97 SB, 4 anencephalic, 7 encephalocele), with 147 parents. 120 controls	Case–control, screen for A1298C	No association
		(130)	French	77 NTD mothers. 61 controls	Case–control study	No association for 677C>T. Reduced risk for 1298A>C allele
		(131)	Chinese	38 mothers of NTD cases. 80 controls	Case–control to evaluate 677C>T	TT genotype less frequent in case mothers (but small numbers)
		(56)	Dutch	180 SB patients, 190 controls	Case–control study	No association for 677C>T or 1298A>C
		(103)	Mixed USA	259 SB cases, 359 controls	Case–control (13 SNPs)	Increased risk, OR2.0, of SB associated with 677C>T
		(41)	Mixed UK	126 SB (open); 103 SB (closed); 49 anencephalic; 192 controls	Case–control for C677T and A1298C	Slight protective effect for open SB of 677TT genotype
MTHFS (5,10-methylenetetrahydrofolate synthetase)	Folate metabolism	(56)	Dutch	180 SB patients, 190 controls	Case–control study	No association
MTR (methionine synthase)	One carbon metabolism (homocysteine remethylation)	(105)	Hispanic USA	43 NTD cases, 122 mothers. 124 control infants and 127 mothers	Case–control study for 2756A>G	Not independent risk factor. May be associated with NTDs in combination with MTRR 66A>G
		(130)	French	77 NTD mothers. 61 controls	Case–control study	No association for 66A>G
		(56)	Dutch	180 SB patients, 190 controls	Case–control study	No association
		(103)	Mixed USA	259 SB cases, 359 controls	Case–control study	No association with SB for 21 SNPs tested (including 2756A>G)
		(41)	Mixed UK	126 SB (open); 103 SB (closed); 49 anencephalic; 192 controls	Case–control study	No association
MTRR (methionine synthase reductase)	One carbon metabolism (homocysteine remethylation)	(105)	Hispanic USA	43 NTD cases, 122 mothers. 124 control infants and 127 mothers	Case–control study for 66A>G (I22M)	G allele associated with increased risk. Additional risk in combination with MTR 2756G allele
		(116)	UK	201 NTD cases (203 mothers, 88 fathers). 601 child, 532 mother controls	Case–control study	Mildly reduced risk associated with 66A>G
		(132)	Irish	575 NTD families 95% SB). 487 controls.	Case–control and family-based analysis for three variants	No association for 66A>G (I22M) with SB risk (except possible paternal effect). No association for S175L or K350R
		(133)	Dutch	109 cases (open SB) and parents. 234 control children, 292 control women.	Case–control screen for 66A>G	In this study and meta-analysis (including previous studies) maternal GG is associated with increased risk in offspring, but GG in child not associated with NTDs
		(130)	French	77 NTD mothers. 61 controls	Case–control study	Marginally increased risk associated with 66G allele
		(56)	Dutch	180 SB patients, 190 controls	Case–control study	No association
		(103)	Mixed USA	259 SB cases, 359 controls	Case–control study	Modest increase in SB risk for three linked SNPs, but not 66A>G

		(41)	Mixed UK	126 SB (open); 103 SB (closed); 49 anencephalic; 192 controls	Case–control study	Marginal increased risk for combined NTDs
MUT (methylmalonyl-CoA mutase)	One carbon metabolism	(134)	Irish	279 NTD triads (mostly SB), 256 controls	Case–control and family-based study	No association with NTDs for three variants tested
NAT1 (N-acetyltransferase 1)	Folate metabolism and acetylation reactions	(135)	Mixed USA	354 NTD families	Family based association study	1095C>A not associated with SB risk (except in combination with maternal smoking)
		(136)	Mixed USA	374 NTD families	Family based association study	Composite genotype (6 SNPs) related to reduced function associated with lower SB risk for cases and mothers
NAT2 (N-acetyltransferase 2)		(56)	Dutch	180 SB patients, 190 controls	Case–control study	No association
NNMT (Nicotinamide N-methyltransferase)	Methylation reactions	(137)	Mixed USA	252 SB cases, 335 controls	Case–control study	No association between NNMT variants and SB risk
		(56)	Dutch	180 SB patients, 190 controls	Association study	No association
NCAM1 (neural cell adhesión molecule1)	Embryonic cell adhesion	(138)	USA	204 SB families	Family based association study	Risk of SB associated with intronic SNP (of 11 tested) in first 132 families, not in further 72 families
NOS1 (nitric oxide synthase 1)	Possible effect on one carbon metabolism	(56)	Dutch	180 SB patients, 190 controls	Case–control study	No association for repeat polymorphisms
NOS2 (nitric oxide synthase 2A)		(56)	Dutch	180 SB patients, 190 controls	Case–control study	No association for SNPs and repeat polymorphism
NOS3 (nitric oxide synthase 3 endothelial)		(139)	Mixed US	301 families with SB	Family based association study	No association by TDT analysis. Maternal 894G>T associated with SB risk by log-linear modelling
		(140)	Dutch	109 SB cases, 121 mothers, 103 fathers. 500 controls	Case–control and TDT	894G>T not independent risk factor. Possible risk interaction with *MTHFR* 677TT
		(56)	Dutch	180 SB patients, 190 controls	Case–control study	No association detected for SNPs and repeat polymorphism
		(103)	Mixed USA	259 SB cases, 359 controls	Case–control study	No association
hOGG1 (*8-hydroxyguanine DNA-glycosylase1*)	DNA repair	(112)	Mixed USA	380 SB cases, 350 controls	Case–control study	No association
PAX3	NTDs in mouse mutant	(70)	Mixed USA	74 SB cases, 87 controls	Mutation screen and case–control study	rs16863657 and related haplotype associated with increased SB risk
PCMT1 (l-isoaspartate O-methyltransferase)	Methylation reactions	(141)	Mixed USA	152 SB cases, 423 controls	Case–control study of Ile120Val	Val/Val genotype associated with possible reduction in SB risk
		(56)	Dutch	180 SB patients, 190 controls	Case–control study	No association for Ile120Val
PDGFRA (Platelet derived growth factor receptor alpha)	NTDs in *Patch* mouse mutant	(142)	US Hispanic	43 NTD cases, 122 NTD mothers. 124 control infants, 127 control mothers	Case–control study for promoter haplotypes	P1 promoter haplotypes with lower activity may be associated with maternal risk. Case numbers too small for conclusion
		(143)	Mixed USA	407 parent-child triads (SB). 164 controls	Case–control and family based association (TDT)	No association for P1 promoter haplotypes
		(108)	Dutch	88 SB cases, 56 SB mothers. 74 controls, 72 control mothers	Evaluated H1 and H2 promoter haplotypes	H1 promoter may be more frequent in cases than controls. Suggestion that BMI, glucose and inositol differentially interact with H1/H2
PEMT (Phosphatidylethanolamine N-methyltransferase)	One carbon and choline metabolism	(144)	Mixed USA	360 SB cases, 595 controls	Case–control study	No association with SB for two non-synonymous SNPs

Continued

Table 1. *Continued*

Human gene	Type of candidate	Reference	Population studied	Sample size	Type of study	Summary of results/conclusion
PRKACA, *PRKACB* (cAMP-dependent protein kinase A catalytic subunits)	NTDs in mouse mutants	(145)	Mixed USA	207 SB cases, 209 controls	Mutation screen and case–control study	No mutation. No association
PRMT1 (Protein arginine methyltransferase 1)	Methylation reactions	(56)	Dutch	180 SB patients, 190 controls	Case–control study	No association
PRMT2 (Protein arginine methyltransferase 2)	Methylation reactions	(56)	Dutch	180 SB patients, 190 controls	Case–control study	No association
PYCT1A (CTP:phosphocholine cytidyltransferase)	One carbon metabolism	(118)	Mixed USA	103 SB cases, 338 controls	Case–control study	Weak association with SB risk for 1 of 2 SNPs studied
RNMT (RNA (guanine-7-) methyltransferase)	Methylation reactions	(56)	Dutch	180 SB patients, 190 controls	Case–control study	No association
SARDH (Sarcosine dehydrogenase)	One carbon metabolism	(56)	Dutch	180 SB patients, 190 controls	Case–control study	Nominally significant association with two synonymous SNPs[a]
SHMT (Serine hydroxyl methyltransferase I)	One carbon metabolism	(146)	UK	97 NTD mothers, 190 controls	Case–control study	1420C>T associated with protective maternal effect
		(56)	Dutch	180 SB patients, 190 controls	Case–control study	No association for Leu474Phe
SLCA19A1, RFC-1 (reduced folate carrier),	Folate transport	(116)	UK	206 NTD cases (186 mothers, 92 fathers). 578 child, 512 mother controls	Case–control study	No association for 80G>A (H27R)
		(125)	Mixed USA	350 cases, 328 mothers, 245 fathers, 167 siblings.	Family-based association study	No association for 80G>A
		(147)	Irish	437 NTD families, 852 controls	Case–control and family based association	No association for 80G>A. 61-bp polymorphism under-represented in cases
		(131)	Chinese	38 mothers of cases. 80 controls	Case–control for 80G>A	Association of GG genotype with risk of NTD offspring
		(148)	Chinese	104 NTD families, 100 control families	Case–control for 80G>A	Elevated risk for GG genotype of cases or mother (if folic acid not used)
		(56)	Dutch	180 SB patients, 190 controls	Case–control study	Nominally significant association for 80AA genotype and reduced risk[a]
		(103)	Mixed USA	259 SB cases, 359 controls	Case–control study	No association with risk of SB for 6 SNPs tested. 80G>A not tested
		(41)	Mixed UK	126 SB (open); 103 SB (closed); 49 anencephalic; 192 controls	Case–control study	No association
SOD2 (superoxide dismutase 2)	Oxidative stress	(115)	Mixed USA	507 SB cases, 185 controls	Case–control study	No association
T (Brachyury)	Axial development in mouse	(149)	Mixed USA	316 SB families	Family-based association study	*TIVS7* T/C allele more frequent in cases than expected
TCNII (transcobalamin II)	One carbon metabolism	(150)	Irish	∼350 NTD families, ∼700 controls.	Case–control and family based association study	No association with SB risk for 6 SNPs
		(130)	French	77 NTD mothers. 61 controls	Case–control study	No association for 776C>G
		(56)	Dutch	180 SB patients, 190 controls	Case–control study	No association for Arg259Pro
TXN2 (thioredoxin2)	NTDs (SB) in mouse knockout	(151)	Mixed USA	48 SB cases, 48 controls	Case–control study	A2 promoter allele associated with risk of NTDs but small sample size
TP53 (p53)	NTDs in mouse mutant	(152)	Irish	549 NTD cases, 532 mothers, 481 fathers. 999 controls	Case–control and family based association studies	Two non-coding variants associated with case NTD risk and two variants with maternal risk (but no multiple testing correction)
		(115)	Mixed USA	507 SB cases, 185 controls	Case–control study	No association
TRDMT1 (tRNA aspartic acid methyltransferase 1)	One carbon metabolism	(56)	Dutch	180 SB patients, 190 controls	Case–control study	Nominally significant association for one SNP[a]

TYMS (thymidylate synthase)	Folate metabolism and pyrimidine biosynthesis	(110)	Mixed USA	264 SB cases, 259 controls.	Mutation screen and case–control for 28-bp repeat in 5′-UTR and 6-bp deletion in 3′-UTR	3′-UTR polymorphism associated with increased SB risk in non-Hispanic white population. Further increased risk with 5′-UTR polymorphism
		(153)	UK	197 NTD cases, 194 mothers, 93 fathers. 179 control infants, 177 control mothers	Case–control study of 28-bp repeat	No association for TYMS
		(56)	Dutch	180 SB patients, 190 controls	Case–control study	No association
		(103)	Mixed USA	259 SB cases, 359 controls	Case–control study	Modest increase in SB risk for three linked SNPs (of 5 tested)
UCP2 (uncoupling protein 2)	Energy metabolism Previous study indicates association with NTDs	(154)	Irish	169 cases, 163 mothers, 167 fathers	Evaluated 866G/A, A55V, 3′-UTR 45-bp ins/del	UCP2 not associated with NTD risk. Different frequency of risk allele in control population compared with previous study
VANGL1 (van gogh-like 1)	PCP gene homologue; Paralogue of *VANGL2*	(95)	Mixed UK and USA	66 (21 craniorachischisis, 24 SB, 21 anencephalic) and 200 controls	Mutation screen	No causative mutations. One missense variant, present in controls
		(96)	Italian and French	144 (80 SB, 7 craniorachischisis, 22 closed spinal dysraphism, 35 caudal regression). 172 Italian and CEPH controls	Mutation screen	Three missense mutations (two in myelomeningocele, R274Q and M328T; 1 in caudal regression, V239I). V239I has functional effect on interaction with Dvl proteins
		(97)	Italian and USA	673 cases (15 open cranial dysraphisms, 456 SB, 202 closed spinal dysraphisms)	Mutation screen	Ten missense variants in 13 individuals (absent in 1187–1462 controls). Five in highly conserved residues (two in myelomeningocele, three in closed spinal dysraphism/caudal regression syndrome)
VANGL2 (van gogh-like 2)	Mouse model: Craniorachischisis in *loop-tail* mice	(95)	Mixed UK and USA	66 (21 craniorachischisis, 24 SB, 21 anencephalic) and 200 controls	Mutation screen	No causative mutations identified. 7 bp duplication in intron six in one craniorachischisis
		(96)	Italian and French	144 (80 SB, 7 craniorachischisis, 22 closed spinal dysraphism, 35 caudal regression) and 172 Italian and CEPH controls	Mutation screen	No coding mutations
XPD (DNA excision repair protein ERCC-2)	DNA repair	(112)	Mixed USA	380 SB cases, 350 controls	Case–control study	Mildly elevated risk associated with 751Gln
XRCC1 (X-ray repair cross-complementing)	DNA repair	(112)	Mixed USA	380 SB cases, 350 controls	Case–control study	No association
XRCC3 (X-ray repair cross-complementing)	DNA repair	(112)	Mixed USA	380 SB cases, 350 controls	Case–control study	No association
ZIC1	Brain defects in mouse mutant	(155)	Dutch	117 NTDs (SB, Anencephaly, Encephalocele), 364 controls	Mutation screen	No mutations
ZIC2	NTDs in mouse mutant	(155)	Dutch	117 NTDs (SB, Anencephaly, Encephalocele), 364 controls	Mutation screen; case–control study	Alanine deletion in one patient. Frequent polymorphism (1059C>T) has no association
ZIC3	NTDs in mouse mutant	(155)	Dutch	117 NTDs (SB, Anencephaly, Encephalocele), 364 controls	Mutation screen	One silent variant (858G>A) in one patient

[a]Nominally significant association which does not stand after correction for multiple testing. SB, defined as spina bifida (myelomeningocele) in study criteria. For studies labelled NTDs, populations were mixed (multiple types of NTDs) or undefined.

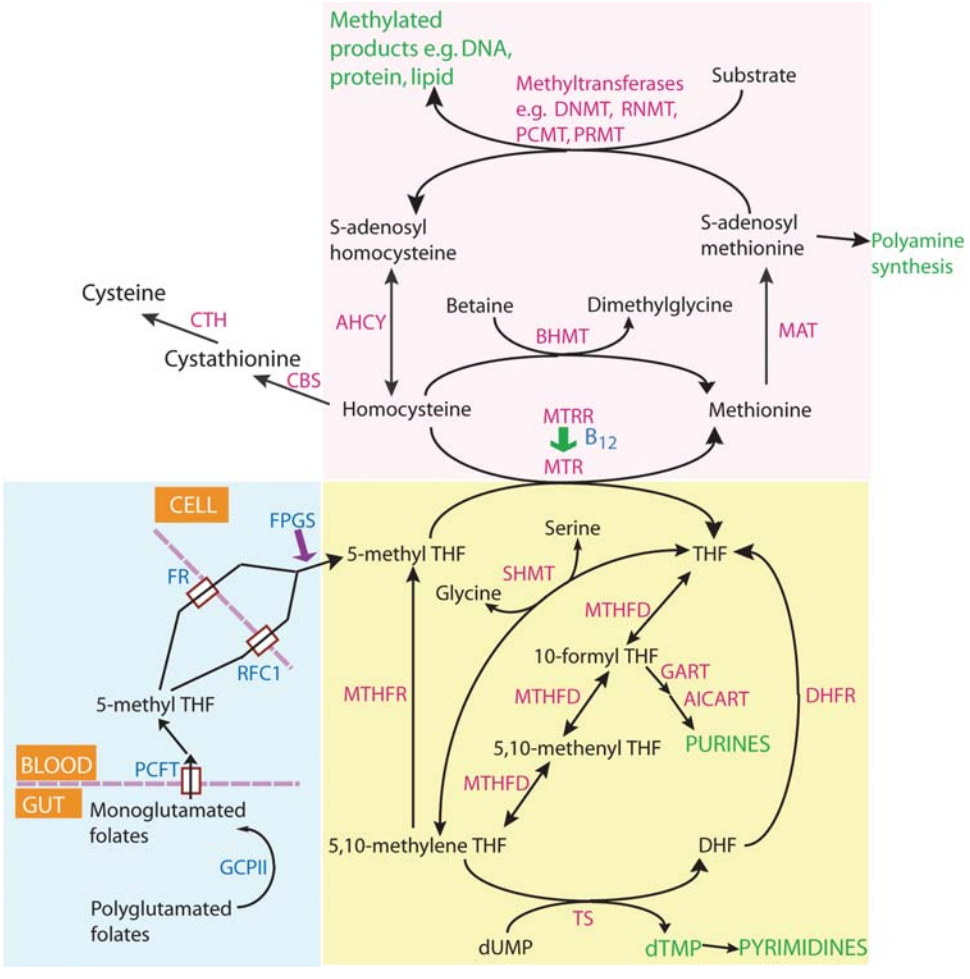

Figure 1. Summary of folate one-carbon metabolism showing the main pathways and reactions that have been subject to analysis in the context of NTDs. Blue shading: proteins involved in processing of folates in the digestive tract, transport and cellular retention (by addition of glutamates). Yellow shading: the major part of the cycle involving transfer of 1C groups between folate molecules, as required for purine and pyrimidine biosynthesis. Pink shading: reactions of the methylation cycle. For clarity, mitochondrial reactions that include generation of formate and cleavage of glycine have been omitted. For explanation of abbreviations, see Table 1.

Folate cycle enzymes required for nucleotide biosynthesis

MTHFD1 encodes the cytoplasmic trifunctional C1THF synthase enzyme. A polymorphism (1958G>A; rs2236225) which results in an R653Q substitution in the 10-formylTHF synthetase domain was found to be both a maternal and NTD case risk factor, in the Irish and Italian populations (49–51), although not in the Dutch (51,52) or British (41). The R653Q polymorphism causes reduced C1THF synthase activity in cell lines, resulting in diminished purine biosynthesis (53). A promoter polymorphism (rs1076991C>T) in *MTHFD1*, that reduces transcriptional activity *in vitro*, was also associated with NTD case and maternal risk, in combination with R653Q (54).

Folate transport

Another attractive group of candidate genes are those encoding proteins required for transport, uptake and cellular retention of folates. This includes folate receptors *FRα* (*Folr1* in mice), *FRβ* and *FRγ*, *RFC1* (reduced folate carrier), *GCPII* (folyl-γ-glutamate carboxypeptidase) and *FPGS* (folylpolyglutamate synthetase) (32,33). Increased risks associated with variants in *RFC1* and *GCPII* are not reproduced in all studies (Table 1), although the recently identified proton-coupled folate transporter *PCFT* (SLC46a1) is not required for embryonic survival or neural tube closure in mice (55), but has not yet been investigated in humans. A recent case–control study revealed a possible association with reduced risk of spina bifida for a polymorphism in *CUBN* (Cubulin), which encodes a membrane-associated multi-ligand endocytic receptor expressed in the neural folds and yolk sac (56). Together, cubulin and its partner protein megalin are involved in binding and endocytic uptake of a large number of different proteins, several of which could be important for neural tube closure, including the intrinsic factor-cobalamin complex (IF-B_{12}) and folate binding protein (folate receptor) (57,58). Intriguingly, *Cubn* was one of the most up-regulated genes in a microarray analysis of *Rfc1* null mouse embryos (59), which may

reflect a compensatory mechanism to enhance endocytic folate uptake via Folr1. Hence, *CUBN* merits further attention as a potential risk factor, especially in conjunction with *RFC1*.

In view of the apparent resilience of mouse neurulation to specific genetic disturbance of the methylation cycle, analysis of compound mutants with other folate-related or NTD susceptibility alleles would be of considerable interest. In our analysis of NTD cell lines, impaired folate cycle activity did not correlate with known variants in *MTHFR*, *MTHFD1*, *DHFR*, *GCPII*, *MTR*, *MTRR* or *RFC1* (34), encouraging the view that currently unknown genetic influences on folate metabolism remain to be identified in many NTD cases.

CANDIDATE GENES FROM THE MOUSE

The potential complexity of NTD genetics is illustrated by the fact that 200 or more different mouse genes result in NTD phenotypes either through naturally occurring, induced or targeted mutations (2,25). Many of the NTD-causing mouse mutations implicate specific signalling pathways such as non-canonical Wnt signalling (see below), maintenance of the cell cycle, regulation of the actin cytoskeleton, chromatin organization or epigenetic modifications including methylation and acetylation. Recently, NTDs were observed in mice null for *Mib2* (60), *Smurf1/2* (61) and *Hectd1* (62), which all encode E3 ubiquitin ligases, suggesting a possible role in neurulation for protein ubiquitination and targeted degradation. The human homologues of some of these mouse NTD genes have been examined in case–control association studies or directly sequenced in mutation screens, although with very few significant findings to date (Table 1).

It is important to ask how appropriate are the mouse models as paradigms for human NTDs? At the embryonic level, the events of neurulation appear extremely similar between mice and humans. For example, the initial fusion event, Closure 1, occurs at a closely similar stage and body axial level in both species, as does initiation of closure in the forebrain (Closure 3) and completion of spinal closure at the posterior neuropore. One point of variation concerns *de novo* initiation of closure at the forebrain/midbrain boundary (Closure 2 in mice) which may be absent from human neurulation (63). Hence, brain closure could be a rather simpler process in humans than mice.

Another potential difference between mouse models and human NTDs is that many gene-specific homozygous null mouse embryos exhibit phenotypes additional to NTDs, such as prenatally lethal heart defects. Such syndromic examples do not appear particularly close models for human NTDs which are primarily non-syndromic (64). On the other hand, detailed analysis of a few of the mouse mutants suggests that isolated NTDs can also result from the effect of hypomorphic alleles, combinations of heterozygous mutations, genetic background effects and/or gene-environmental interactions. This partial loss of function or multifactorial aetiologies may more closely resemble human NTDs. For example, NTDs in *splotch* mice result from homozygosity for mutations in *Pax3* (23,65) but can also occur, or be exacerbated, as a result of interaction with mutations in other genes including

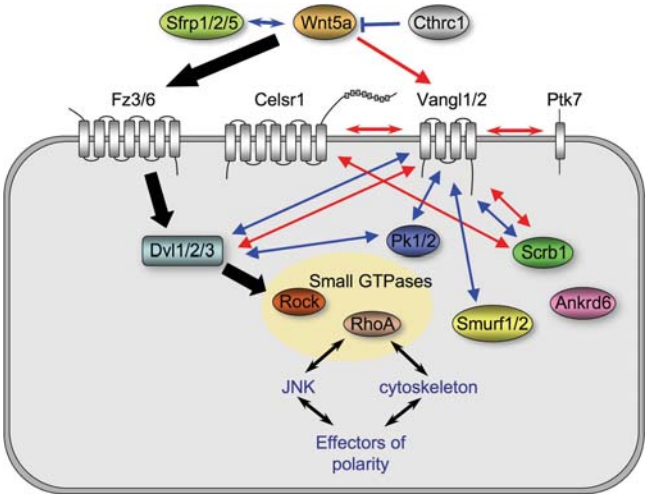

Figure 2. Diagrammatic representation of non-canonical Wnt signalling in a mammalian cell. Black arrows indicate the signalling pathway necessary for establishment of planar cell polarity (PCP). Known biochemical interactions are indicated by blue arrows and genetic interactions are shown by red arrows. Ankdr6 is the mammalian homologue of Diego which in *Drosophila* interacts with Fz, Vang and Pk, but has not been studied in vertebrates.

neurofibromin1 (66) and *grainyhead-like-3* (67). Environmental factors including folate deficiency and arsenic can exacerbate NTDs in homozygous *splotch* mutants, or induce NTDs in the usually unaffected heterozygotes (24,68). Although association studies in humans have provided little evidence to implicate *PAX3* mutations in human NTDs (69,70), the possible contribution of gene–gene and gene–environment interactions indicates that larger scale studies may be needed before a role for *PAX3* in human NTDs can be completely ruled out.

The *curly tail* mouse also exhibits features typical of the multifactorial aetiology of human NTDs (71). Spinal NTDs are partially penetrant in homozygous *ct/ct* mutant embryos, with the frequency of defects strongly affected by genetic modifiers (72). The major *ct* gene is a hypomorphic allele of *Grhl3*, whose knockouts display completely penetrant spina bifida (73–75). The *ct* mutation appears to affect a regulatory region, emphasising the need for consideration of possible non-coding mutations in human NTDs. Moreover, there is a strong effect of environmental factors, including a protective effect of supplemental inositol (76). A key role for inositol in neural tube closure is supported by the finding that inositol deficiency *in vitro* causes NTDs (77), inositol may prevent diabetes-associated NTDs (78) and the recent finding of NTDs in embryos carrying a hypomorphic allele of inositol 1,3,4-trisphosphate 5/6-kinase (*Itpk1*), a key enzyme in inositol phosphate metabolism (79).

Planar cell polarity signalling and NTDs

A major advance in understanding the genetic basis of neurulation has been the finding that initiation of closure at the hindbrain-cervical boundary (Closure 1) requires non-canonical Wnt signalling: the so-called planar cell polarity (PCP) pathway (Fig. 2). PCP signalling was defined originally

Table 2. Genes of the planar cell polarity pathway—involvement in mouse NTDs

Gene	Mutant/genotype	Protein	NTD phenotype	References
Single gene effects				
Vangl2	*Loop-tail* (*Lp, Lp*^{m1Jus}); *Lp/Lp*	Transmembrane protein	Craniorachischisis. Occasional spina bifida in *Lp/+*	(91,92,156,157)
Scrb1	*Circle-tail; Crc/Crc*	Cytoplasmic polarity protein	Craniorachischisis	(93)
Celsr1	*Crash; Crsh/Crsh*	Seven-pass transmembrane protein	Craniorachischisis	(158)
	Spin-cycle; Scy/Scy		Craniorachischisis	
Ptk7 (*CCK-4*)	*Ptk7*$^{mu/mu}$ (truncating gene-trap allele)	Transmembrane receptor tyrosine kinase-like protein	Craniorachischisis	(94)
Paralogous gene interactions				
Dvl1/2	*Dvl1*$^{-/-}$; *Dvl2*$^{-/-}$	Cytoplasmic signalling proteins	Craniorachischisis	(159)
Dvl2/3	*Dvl2*$^{-/-}$; *Dvl3*$^{+/-}$ (*Dvl2*$^{-/-}$; *3*$^{-/-}$ die before E8.5)		Craniorachischisis	(101)
Fz3/6	*Fz3*$^{-/-}$; *Fz6*$^{-/-}$	Transmembrane receptor protein	Craniorachischisis	(160)
Smurf1/2	*Smurf1*$^{-/-}$; *Smurf2*$^{-/-}$	Ubiquitin ligase	No closure at E8.5	(61)
	Smurf1$^{-/-}$; *2*$^{+/-}$ or *1*$^{+/-}$;*2*$^{-/-}$		Spina bifida and/or exencephaly	
Interactions between different genes				
Vangl2/Scrb1	*Lp/+; Crc/+*	See above	Craniorachischisis	(102)
Vangl2/Dvl3	*Lp/+;Dvl3*$^{-/-}$	See above	Craniorachischisis	(101)
	Lp/+;Dvl3$^{+/-}$		Craniorachishisis or exencephaly	
Vangl2/Vangl1	*Lp/+; Vang1*$^{gt/+}$	Transmembrane protein	Craniorachischisis	(98)
Vangl2/Ptk7	*Lp/+; Ptk7*$^{+/mu}$	See above	Spina bifida	(94)
Vangl2/Wnt5a	*Lp/+; Wnt5a*$^{-/-}$	Secreted signalling protein	Craniorachischisis	(161)
Vangl2/Sfrp1,2,5	*Lp/+; Sfrp1*$^{-/-}$; *Sfrp2*$^{-/-}$; *Sfrp5*$^{+/-}$	Secreted Wnt antagonist	Craniorachischsis	(162)
	Lp/+; Sfrp1$^{-/-}$; *Sfrp2*$^{+/-}$; *Sfrp5*$^{-/-}$		Spina bifida	
Vangl2/Cthrc1	*Lp/+; Cthrc1*$^{LacZ/LacZ}$	Secreted glycoprotein, Wnt co-factor	Exencephaly	(100)
Vangl2/Grhl3	*Lp/+; Grhl3*$^{ct/ct}$	Transcription factor	Severe spina bifida	(163)
Vangl2/cobl	*Lp/+; cobl*$^{C101/C101}$	Actin nucleator	Exencephaly	(99)

in *Drosophila*, as a genetic cascade involving the transmembrane receptor frizzled and the cytoplasmic protein dishevelled, but without a requirement for β-catenin (80–83). This pathway is required to specify planar polarity in epithelia including the wing and compound eye. In vertebrates, non-canonical Wnt signalling is highly conserved, underpinning tissue and cellular polarity during morphogenesis in systems as diverse as gastrulation and the coordinated orientation of stereociliary bundles in inner ear hair cells (84–90).

A potential role for PCP in NTDs first came to light following positional cloning of *Vangl2* (the homologue of *Drosophila strabismus/Van gogh*) in the *loop-tail* mouse mutant which exhibits the severe NTD, craniorachischisis (91,92). Subsequently, the same NTD phenotype was found in other mouse mutants and targeted gene knockouts (Table 2) almost all of which have been implicated biochemically in the PCP pathway (e.g. *Celsr1*, *Dvl*) or which interact genetically with recognized PCP genes (e.g. *Scrb*, *Ptk7*) (93,94). Interestingly, the double knockout for *Smurf1* and *Smurf2* was recently found to display craniorachischisis and other characteristic PCP defects. These genes encode ubiquitin ligases whose targets include Prickle1 (Fig. 2), supporting the crucial nature of PCP signalling for initiation of neural tube closure (61).

In view of these findings in mice, PCP genes emerge as excellent candidates for causation of craniorachischisis in humans. Nevertheless, sequence analysis has so far failed to identify mutations in human *VANGL2* or its paralogue *VANGL1* in a group of patients with craniorachischisis (95,96). Reports of other PCP gene analysis in similar patients are awaited. Although craniorachischisis is the obvious NTD phenotype for study, the *VANGL* genes have also been analysed in patients with anencephaly and open and closed spina bifida. No mutations were reported in *VANGL2* (95,96) but several highly conserved and unique, heterozygous missense variants were identified in *VANGL1* in patients with either myelomeningocele or closed spina bifida, as well as caudal regression syndrome (96,97). To date a functional effect has been demonstrated for one of these putative mutations, where V239I (identified in caudal regression syndrome) results in loss of interaction between VANGL1 and DVL proteins (96,).

Interestingly, loss of *Vangl1* function is insufficient to cause NTDs in mice, although compound heterozygotes with *Vangl2* (*loop-tail*) develop craniorachischisis (98). Nevertheless, there is increasing evidence that PCP genes can contribute to NTDs other than craniorachischisis (Table 2). For example, double heterozygotes carrying both *Vangl2* and *Ptk7* develop spina bifida (94) although *Vangl2* double mutants with *cordon-bleu*^{C101} or *Cthrc1* develop exencephaly (99,100). In contrast, *Vangl2*:*Scrb* and *Vangl2*:*Dvl3* double heterozygotes develop craniorachischisis (101,102). It remains to be determined why *Vangl2* displays this variable phenotypic behaviour when combined with different PCP and other mutants. Hence, although non-canonical Wnt signalling has been firmly linked with Closure 1 in mice, it is possible that genes in this pathway play more diverse roles in human neural tube closure.

CONCLUSIONS

The identification of genetic risk factors for human NTDs is complicated by the multiplicity of genes participating

in neurulation, and the importance of gene–environment interactions. Sequence analysis of candidate genes implicated from their role in mouse models has revealed putative mutations in a few genes, but each in only a small number of patients. Association studies of common polymorphic variants, particularly related to folate one-carbon metabolism, indicate risk factors such as *MTHFR*. However, no specific folate-related gene has yet been implicated as a major determinant of risk for NTDs. Large-scale studies will be required to provide sufficient statistical power to convincingly test whether such variants are truly NTD susceptibility factors (56,103). It will also be essential, to evaluate multiple genes (folate-related and others) in the same individuals in order to detect possible compounding effects of combinations of risk alleles that, individually, might not be statistically significant (11,39). To date, very few studies have been sufficiently large to overcome issues of multiple testing bias in screening for gene–gene interactions (39,56,104). Examination of specific hypotheses may be fruitful where fewer NTD cases are available, particularly if combined with stratified sample sets in which cases are sub-divided on the basis of phenotype. For example, NTDs with abnormal folate metabolism have enabled a combined analysis of MTR and MTRR (105), and fetuses with craniorachischisis provide a focus for determining the role of PCP genes. Gene–environment interactions appear likely to contribute to NTD predisposition, with examples including interactions of MTHFR with multivitamin use (106), MTRR with vitamin B_{12} (107) and PDGFRA with inositol and zinc (108).

One limitation of the association studies of multiple folate-related candidate genes in NTDs is the predominant focus on known polymorphisms. In future, it will be necessary also to consider the possible existence of 'private' disease-causing mutations. Moreover, the potential for deleterious gene expression changes resulting from promoter mutations or copy number variation has been addressed in relatively few studies (10,108–110). Emerging technologies for high throughput sequencing and analysis of genomic deletions and copy number variations (111) offer the prospect, in the coming years, of progress in identification of candidate genes and screening for novel mutations in human NTDs.

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Conflict of Interest statement. None declared.

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