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Genetic Basis of Neural Tube Defects

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Neural tube defects (NTDs) represent a common group of severe congenital malformations of the central nervous system. They result from failure of neural tube closure during early embryonic life. Their etiology is complex, involving environmental and genetic factors that interact to modulate the incidence and severity of the developing phenotype. Despite a long history of etiologic studies, the molecular and cellular pathogenic mechanisms underlining NTDs remain poorly understood. The major epidemiologic finding in NTDs is the protective effect of perinatal folic acid supplementation that reduces their risk by 60%-70%. Genetic studies in NTDs have focused mainly on folate-related genes and identified a few significant associations between variants in these genes and an increased risk for NTDs. The candidate gene approach investigating genes involved in neurulation and inferred from animal models has faced limited success in identifying major causative genes predisposing to NTDs. However, we are witnessing a rapid and impressive progress in understanding the genetic basis of NTDs, based mainly on the development of whole genome innovative technologies and the powerful tool of animal models.
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Neural tube defects (NTDs) are the most common structural malformations of the central nervous system in human beings, affecting 1-2 infants per 1000 births. Their incidence varies among different populations.[1] They occur very early during pregnancy between gestational weeks 2 and 6 and are caused by a partial or complete failure of neural tube closure during embryogenesis at any level of the rostrocaudal axis.[1] NTDs are categorized clinically into "open," in which the affected nervous tissues are exposed to the environment or "closed," in which the defect is covered by skin. Open NTDs represent the most common forms of NTDs and include anencephaly and myelomeningocele (spina bifida) that result from the failure of fusion in the cranial and spinal region of the neural tube, respectively. Anencephaly is characterized by a partial or total absence of the cranial vault and cerebral hemispheres and is invariably lethal. Spina bifida can lead to mild or severe lifelong physical and developmental disabilities, depending on the size and location of the spinal defect.[2] Other open dysraphisms include myeloschisis, hemimyelomeningocele, and hemimyelocele, and are sometimes associated with a Chiari II malformation.[3] Another rare form of open NTD is craniorachischisis that results from failure of neural tube closure over the entire body axis.[2] Closed (skin-covered) NTDs are further categorized clinically, depending on the presence or absence of a lower back subcutaneous mass. Closed NTDs with a mass are represented by lipo-myeloschisis, lipo-myelomeningocele, meningocele, and myelocystocele. Closed NTDs without a mass include simple dysraphic states (intradural lipomas, diastematomyelia, teratoma, dermoid, epidermoid, tight filum terminale, persistent terminal ventricle, and dermal sinus) and complex dysraphic states (dorsal enteric fistula, neurenteric cysts, split cord malformations, caudal regression syndrome, and spinal segmental dysgenesis).[3] For a detailed clinical review of spinal NTDs, please see the review by Rossi et al.[3]

Population and family studies indicate a complex etiology to NTDs, involving both environmental and genetic factors. Environmental factors implicated in increasing the risk for NTDs include geography, epidemic trends, socio-economic class, maternal age, maternal diet, maternal diabetes and obesity, and drug exposure mainly to antiepileptic drugs.[4,5] This review focuses on the genetic factors predisposing to NTDs in human beings. We first present a brief review of the normal process of neurulation, including the underlying molecular and cellular mechanisms, which will help the reader to better understand how lesions in this process could lead to NTDs. Next, our current knowledge of the genetic basis of NTDs is summarized, with particular emphasis on studies of candi-

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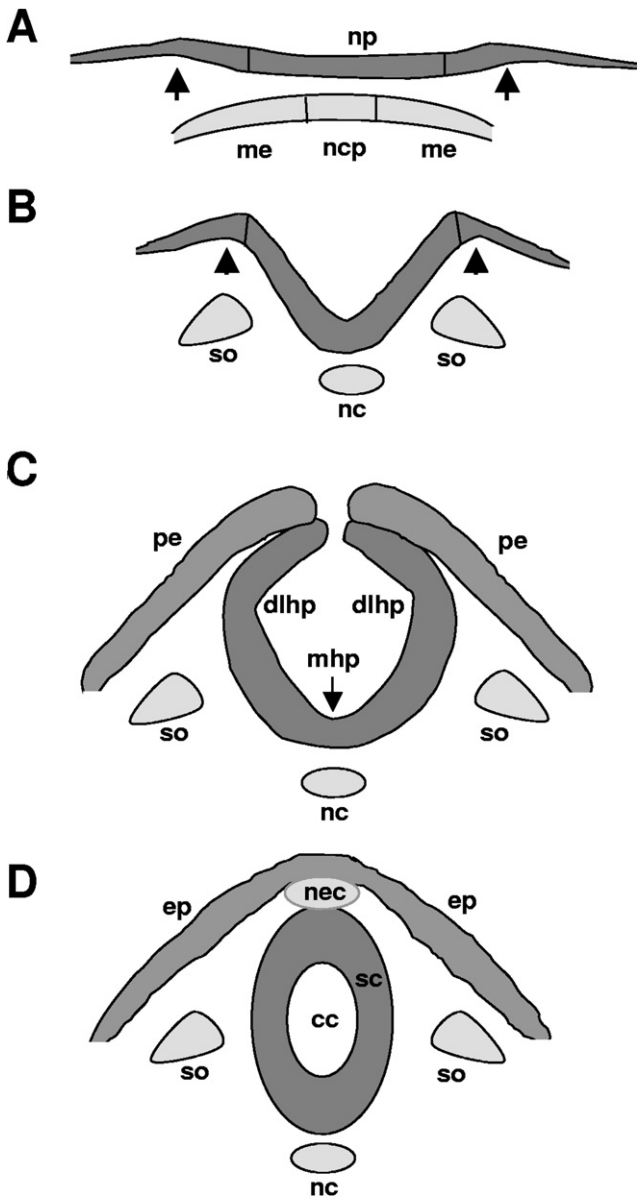

Figure 1 A simplified diagram of neural tube closure. (A) Neural folds form at the lateral extremes of the neural plate (A, arrows), elevate (B, arrows) and converge towards the dorsal midline (C), and fuse at their dorsal tips to form the closed neural tube (D). Bending or hinge points form at two sites: the median hinge point (mhp) overlying the notochord and the paired dorsolateral hinge points (dlhp) at the lateral sides of the folds (C). cc, central canal; ep, epidermis; me, mesoderm; nc, notochord; ncp, notochordal plate; nec, neural crest; np, neural plate; pe, presumptive epidermis; sc, spinal cord; so, somites.

date genes that are either folate-related or derived from animal models. Finally, a projection on future directions for gene identification studies in NTDs is presented.

Overview of Vertebrate Neurulation

Neurulation is a fundamental embryonic process that leads to the development of the neural tube, which is the precursor of the brain and spinal cord. Building a neural tube is an extremely complex phenomenon in which cells need to change in shape, migrate, and differentiate to transform a flat sheet of thickened epithelial cells (the neural plate) into a hollow tube (neural tube). In human beings, neurulation occurs through 2 distinct phases that occur at distinct sites along the rostrocaudal axis of the embryo: primary neurulation (weeks 3-4) that leads to the formation of the brain and most of the spinal cord till the upper sacral level, followed by secondary neurulation (weeks 5-6) that is limited to the tail bud and creates the lowest portion of the spinal cord, including most of the sacral and all the coccygeal regions.[3,6]

Primary Neurulation

This process is dynamic and involves complex morphogenetic events controlled by distinct molecular pathways (Fig. 1). The initial step is formation of the neural plate whereby the dorsal midline ectoderm is differentiated into the neuroepithelium to form the neural plate. Normally, bone morphogenetic proteins prevent the ectoderm to follow its default pathway to form neuroectoderm and instead instruct it to form epidermis. Neural induction occurs by suppression of this epidermal fate where bone morphogenetic proteins antagonists, including chordin, noggin, and follistatin, emanating from the primitive node, allow the ectoderm to form neuroectoderm.[7-9] Other signaling pathways are also implicated in driving neural induction; these are known to involve fibroblast growth factors, canonical Wnt signaling, and insulin-like growth factor.[7-9]

The neural plate is next converted into an elongated structure that is broad at the cranial and narrow at the spinal regions. During this process of shaping, the major driving force is a morphogenetic event called convergent extension (CE). The CE describes the narrowing and lengthening of a group of cells that could be the embryonic axis during gastrulation or the neural plate during neural tube closure. In this complex process, cells elongate mediolaterally and produce polarized cellular protrusions that enable them to move directionally and to intercalate with other neighboring cells.[10,11] This change in shape and movement results in convergence toward the midline and extension of the tissue along the anteroposterior axis (Fig. 2). CE is controlled by the noncanonical Wnt/frizzled pathway, in contrast to the canonical Wnt pathway that acts through β-catenin stabilization controlling cell fate specification, and is equivalent to the so-called planar cell polarity (PCP) pathway in the fly.[12] The PCP, also called tissue polarity, is the process by which cells become polarized within the plane of an epithelium. This form of polarization has been well studied in the adult epithelial tissues of *Drosophila* where it can be observed in the distal orientation of wing hairs, the posterior orientation of the abdominal bristles, and the more complex organization of the ommatidia (eye units) in the adult eye. Genetic studies in the fly have identified a group of so-called "core" PCP genes required for PCP signaling in all tissues and which include *Frizzled* (*Fz*), *Disheveled* (*Dsh*), *Strabismus/Van Gogh (Stbm/Vang), Flamingo* (*Fmi*), *Prickle (Pk),* and *Diego* (*Dgo*).[12] These PCP proteins are highly conserved in vertebrates

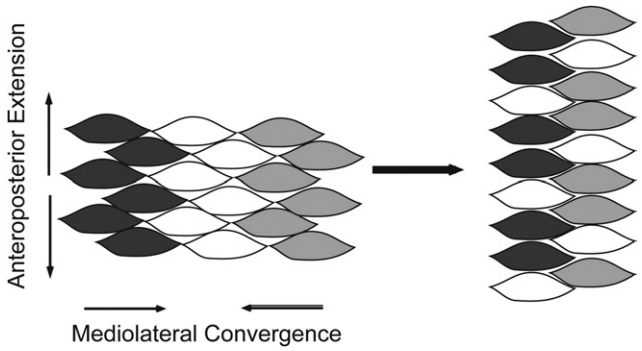

Figure 2 A simplified diagram of the morphogenetic process of convergent extension during gastrulation and neural tube closure. Cells become polarized, intercalate and converge together on the mediolateral axis leading to extension of the developing tissue on the anteroposterior axis.

where they have been implicated in controlling the process of CE during gastrulation and neural tube formation.[12] The exact mechanisms by which the PCP pathway regulates CE remain poorly understood.

Shaping of the neural plate is accompanied by its bending that involves the formation of hinge points at 2 sites: the median hinge point (MHP) overlying the notochord and extending along the rostrocaudal axis, and the paired dorsolateral hinge points (DLHP) at the lateral sides of the folds and predominantly at the future brain levels (Fig. 1).[6] The MHP is the only bending point at the upper spinal level, whereas the DLHP forms in the lower spine and cranial region. This differential bending at different axial levels is controlled by signals emanating from the notochord, including the signal transduction protein sonic hedgehog.[13-16] Expansion of the underlying mesenchyme seems to be essential for the complex bending process at the cranial region, whereas cytoskeletal integrity may be necessary for neurulation at cranial and spinal regions.[6] After formation of the hinge points, the neural plate rotates by elevation around the MHP and by convergence around the DLHP (Fig. 1). The major morphogenetic event that takes place in bending of the neural plate is a process termed apical constriction where columnar cells in the neural tube are converted into wedge-shaped cells.[17] Two actin-related genes have been implicated in this process: *p190RhoGap*, a negative regulator of Rho GTPase involved in regulating actin dynamics,[18] and *Shroom* that codes for an actin-binding protein.[19,20]

Neural tube formation is completed by fusion of the neural plate when dorsal tips of the neural folds come into contact at the dorsal midline and adhere to each other (Fig. 1). The cellular and molecular mechanisms underlying fusion of the folds remain poorly understood. As the neural folds approach, cellular protrusions expand from apical cells and interdigitate, followed by formation of permanent cell contacts. Mouse studies have implicated Ephrin-A5 and EphA7 receptors in this fusion process in the cranial epithelium.[21,22] Eph tyrosine kinase receptors and their membrane-bound ephrin ligands mediate cell interactions and participate in several developmental processes.[23]

Theories of Neural Tube Closure

Neural tube closure is a discontinuous process occurring at multiple initiation sites of neural tube closure in the developing embryo. In mouse, 3 sites of closure have been described (Fig. 3).[6] Closure 1 is initiated at the hind-brain and/or cervical boundary and then proceeds rostrally into the future brain and caudally into the spinal region. Closure 2 is initiated at the forebrain and/or midbrain boundary and closure 3 occurs at the rostral extremity of the forebrain.[6] The open regions of neural folds between these initial sites are called neuropores. As closure proceeds from these sites, neuropores shorten and eventually close, leading to an intact closed neural tube. The positions of closures 1 and 3 are invariant among mouse strains, whereas position of closure 2 is polymorphic where it could be relatively caudal or rostral in the midbrain.[24] However, mouse strains with a rostral position of closure 2 have higher predisposition to cranial NTDs (exencephaly) than those with more caudal positions.[25] In human beings, multiple sites of neural tube fusion were hypothesized on the basis of observational studies of fetuses, but the exact number of these sites remains controversial, varying between 2, 3, and 5 sites (Fig. 3).[26-28] Only closures 1 and 3 were proven to definitely occur.

Secondary Neurulation

Secondary neurulation begins after closure of the posterior neuropore and completion of primary neurulation. The secondary neural tube arises from the tail bud that corresponds to a mass of pluripotent cells derived from the remnant of the primitive streak and located at the caudal end of the embryo. These cells undergo proliferation and condensation, followed by cavitation and fusion with the central canal of the neural

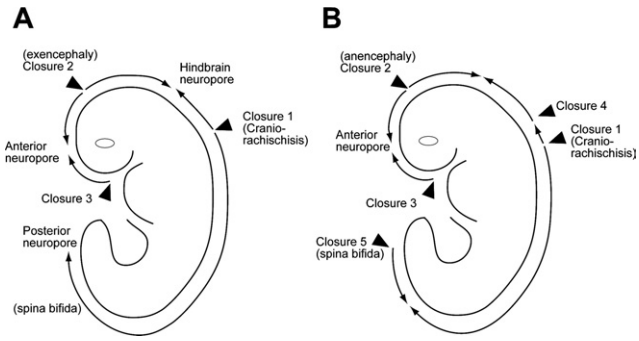

Figure 3 Multiple and sequential initiation sites of neural tube closure in mouse (A) and human (B) embryos. Panel A shows a diagrammatic sideview of a mouse embryo with the initiation sites of neural tube closure (arrowheads): closure 1 at the hindbrain/cervical boundary; closure 2 at the forebrain/midbrain boundary and closure 3 at the rostral extremity of the forebrain. Panel B shows a diagrammatic sideview of a human embryo with the initiation sites of neural tube closure (arrowheads) based on Van Allen's model.[26] In addition to closures 1, 2 and 3 equivalents to those described in mice, closure 4 in humans occur at the caudal end of the hindbrain and closure 5 in the lunbar region. Fusion proceeds bidirectionally from closure 1 and 2 and unidirectionally from closures 3, 4 and 5. NTDs resulting from failure of closure at various sites are shown in parentheses.

tube formed by primary neurulation.[29] Recent fate mapping studies in the avian embryo suggest that both primary and secondary neural tubes originate from the neural plate through similar molecular and cellular mechanisms.[29]

Failure of Neural Tube Closure Leads to NTDs

NTDs occur at various levels of the embryonic axis, reflecting the occurrence of multiple closure sites. Failure of neural tube closure during primary neurulation at any level of the body axis from the brain down to the sacral spine leads to "open" NTDs[1,6] (Fig. 3). Failure of closure 1 leads to craniorachischisis where the neural tube remains open throughout the brain and spinal cord. Failure of the caudal spread of fusion from closure 1 results in open spina bifida or myelomeningocele. Failure of closure 2 leads to exencephaly that by late gestation converts into anencephaly, where the skull vault is missing and the brain tissue is degenerated. Failure of closure 3 leads to anencephaly confined to the forebrain region and that is often associated with split-face malformation. Failure of secondary neurulation leads to some forms of "closed" NTDs where the developing neural tube fails to separate from other tissues of the tail bud, for example, dysfunctional secondary neurulation can lead to an inappropriately low-lying spinal cord. Patients with such distal spinal cord anomalies may manifest symptoms, including lower limb abnormalities and bowel and bladder dysfunction, which result from inappropriate innervation from the malformed spinal cord. Such distal spinal cord anomalies coupled with lower limb, bladder, and bowel anomalies are commonly classified as tethered cord syndrome.[30] It is important to note that other forms of "closed" NTDs can be caused by defects in the developing axial mesoderm at any level of the neuraxis, despite the completion of primary and secondary neurulation. Encephalocele and meningocele, for example, may result from bony malformations where the closed neural tube herniates through the affected regions.[6] Alternatively, heterotopic fat can lead to tethering of the spinal cord at any level.

Evidence for a Genetic Basis to NTDs

Several lines of evidence support a genetic component to NTDs. First, NTDs are associated with chromosomal abnormalities, most frequently trisomy 13, trisomy 18, and triploidy. NTDs are also associated with known genetic syndromes, including Meckel's syndrome, anterior sacral meningocele, Currarino syndrome, and anal stenosis.[31] Second, NTDs show ethnic and racial differences in incidence rates. In the United States, the incidence of spina bifida, for example, is very low in African Americans as compared with the white Hispanic and non-Hispanic populations.[32] Third, NTDs show increased risk for a second affected child for couples with 1 affected infant (3-5 fold) and for siblings of affected individuals as compared with the general population (10 fold).[31,33] Finally, the occurrence of familial settings or distributions further strengthens the evidence for a genetic basis to NTDs.[33,34] The inheritance patterns and recurrence risks for such congenital anomalies in these multiplex families do not follow a Mendelian pattern; instead they have been suggested to follow a multifactorial threshold model where a continuous variable "liability" exists in a population with a "developmental" threshold value beyond which the individual becomes affected. Few studies have shown that maternal genetic factors and sex-influenced factors contribute to the risk for NTDs.[35,36] Heritability in NTDs is estimated to be 60% with multiple susceptibility genes involved; however, the number, identity, and relative contribution of such genes to NTDs remain unknown.[33]

Identification of genes predisposing to NTDs in human beings using positional cloning is difficult because of the underlying complex etiology and the scarcity of large families with multiple affected members. A genome wide linkage screen in NTDs has identified few chromosomal regions of interest; however, results from such studies were not further pursued and should be interpreted cautiously as they are complicated by probable genetic heterogeneity and variable penetrance.[37,38] Research studies of gene identification in NTDs have mainly adopted a candidate gene approach and focused on genes of folic acid pathway and on candidate genes from animal studies.

Studies of Folate-Related Genes in NTDs

The most significant epidemiologic finding relevant to NTDs is the protective effect of periconceptional administration of folic acid, which reduces their incidence as much as 60%-70%.[39] This finding was initiated by a series of clinical studies that demonstrated an association between reduced folate status and elevated maternal homocysteine and an increased risk for NTDs, followed by randomized control trials and population-based folate fortification programs that confirmed the efficacy of folic acid supplementation in reducing NTD occurrence.[40] Despite an impressive number of folate-related investigative studies, the mechanism(s) by which folate deficiency predisposes to NTDs remains unclear. Human studies focused on analyzing the proteins that bind, transport, or metabolize folate for the presence of genetic variants that would act as susceptibility NTD factors and on gene-nutrient interactions that would result in NTD pathogenesis.

Folic acid is an inactive water-soluble B vitamin that is absorbed in the proximal small intestine through a proton-coupled, high-affinity folate transporter. After it is in the bloodstream, folate is transported into cells mainly through the folate receptor (*FR-α*, *-β*, and *-γ*) and through the reduced folate carrier.[41] The intracellular intake of folate through *FR-α* is critically important for embryogenesis as demonstrated in mice, where functional knock out of folate binding protein-1 (Folp1, ortholog of *FR-α*) leads to exencephaly and is embryonically lethal.[42] It has been shown that mothers with an NTD pregnancy produce auto-antibodies that bind to FRs on the placental membrane and, therefore, block the binding of folic acid. Folate supplementation in

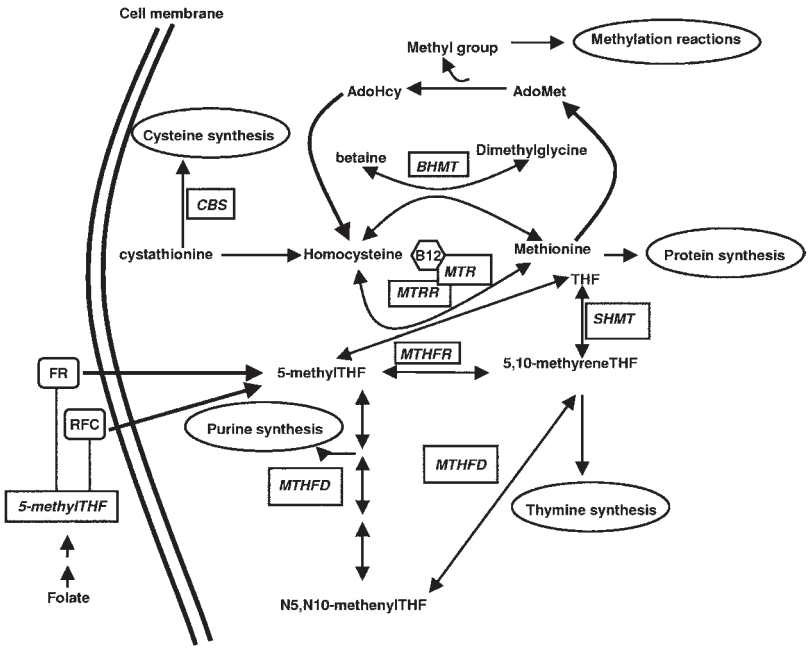

Figure 4 Simplified diagram of folate and homocysteine metabolic cycles. AdoHcy, S-adenosylhomocysteine; AdoMet, S-adenosylmethionine; *BHMT*, betaine-homocysteine methyltransferase; *CBS*, cystathionine β-synthase; FR, folate receptor; *MTHFD*, methylenetetrahydrofolate dehydrogenase; *MTHFR*, methylenetetrahydrofolate reductase; *MTR*, methionine synthase; *MTRR*, methionine synthase reductase; RFC, reduced folate carrier; *SHMT*, serine hydroxymethyltransferase; THF, tetrahydrofolate.

such cases would present an alternative cellular entry of folate or would increase competition with the auto-antibodies binding to FRs to restore folate homeostasis.[43] No coding variants and very few noncoding variants were identified in *FR-α* and *FR-β*; the latter were not associated with an increased NTD risk.[41,44,45] By contrast, a common polymorphism, c.80A>G in *RFC-1* has been demonstrated as a moderate genetic risk factor for NTDs, especially under conditions of maternal folate deficiency.[46,47]

After the folate is in the cell, it acts as a methyl donor for methionine synthesis through homocysteine (Hcy) remethylation. Methionine is the single most important methyl donor for the methylation of DNA and tRNA. Folate also acts a donor of 1-carbon groups for synthesis of thymidine and purines, the building blocks of DNA (Fig. 4). Folate and Hcy metabolic cycles are closely related and involve over 25 proteins, many of which have been investigated for association with an increased NTD risk.[41] This review will present the major genetic findings related to the role of folate and Hcy metabolism in NTDs. For more detailed reviews, readers are invited to read references 40, 41 and 48.[40,41,48]

The 5,10-methylene-tetrahydrofolate reductase gene (*MTHFR*) is the first and most extensively studied gene as a potential risk factor for NTDs. This gene is of particular importance as it regulates the levels of 5-methyl-THF available for Hcy remethylation (Fig. 4). A common single nucleotide polymorphism (SNP) in this gene, c.677C>T, is associated with reduced levels of enzyme activity, elevated levels of plasma Hcy, and an increased NTD risk in some populations. Meta-analysis studies strongly implicate the *MTHFR* 677TT genotype as a risk factor for NTDs in mothers (50%-70% increase) and fetuses (80%-90% increase).[49,50] Another SNP within the *MTHFR* gene, c.1298A>C, was found to be associated with a reduced level of enzyme activity (not as severe as with c.677C>T) and an increased risk for NTDs, but with no effect on Hcy plasma levels.[49,51] However, knockout mice for the *MTHFR* gene failed to show an NTD phenotype despite a high level of homocysteine and even under folate deficient conditions.[52,53] Consequently, the mechanism by which the SNP c.677C>T confers a high risk for NTDs remains elusive.

Two other genes involved in the methionine and/or homocysteine remethylation cycle, methionine synthase (*MTR*), and methionine synthase reductase (*MTRR*), were also extensively studied in NTDs. *MTR* catalyzes the transfer of the methyl group from 5-methyl-THF to Hcy, using vitamin B12 (cobalamin, cbl) as a cofactor[41] (Fig. 4). One coding SNP, c.2756A>G, identified in *MTR* in the helix involved in cofactor binding, generated inconsistent results in association with an increased NTD risk.[41,45,48] The active complex MTR bound to Cbl, called cbl(I)*MTR*, can bind to the methyl group of 5-methylTHF to form cbl(III)*MTR* that transfers the methyl group to Hcy. Cbl(I)*MTR* can be oxidized into the inactive cbl(II)*MTR* that can be reactivated by the enzyme *MTRR* (Fig. 4). One variant identified in the *MTRR* gene, c.66A>G, can be considered as a maternal risk factor for NTD.[41,48] A meta-analysis study implicates the maternal *MTRR* 66GG genotype as a risk factor for developing NTDs.[41] Transgenic mice with partial or complete knock out for the *MTR* or *MTRR* genes display no developmental NTD phenotype, adding inconclusive evidence for the role of these genes in the pathogenesis of NTDs.[54,55]

The tri-functional enzyme methylene-THF dehydrogenase/

formyl-THF synthase/methenyl-THF cyclohydrolase (MTHFD) plays a central role in folate metabolism as it is involved in many conversion reactions of substrates used in purine and thymidine synthesis (Fig. 4). One *MTHFD* SNP, c.1958G>A, was found to be strongly associated with an increased maternal risk for NTDs.[56-58] This is particularly interesting as a defective de novo nucleotide synthesis represents one of the earliest proposed mechanisms for the association between folate and NTDs, whereby a decrease in mitotic and/or increase in mutation rates during critical morphogenetic events could result in failure of neural tube closure.[59]

Association studies conducted in NTDs suggest that variations in folate-related genes might increase the risk for NTDs through gene-gene and gene-environment interaction and through either the maternal or embryonic genotype. For example, the *MTHFR* c.677C>T is related to decreased *MTHFR* activity, low plasma folate, and high plasma homocysteine and has a variable penetrance affected by dietary and supplemental folate.[48,60] The *RFC-1* c.80A>G variant may interact with low red blood cell folate status and *MTHFR* mutations to increase NTDs risk.[61] A recent study showed a significant association between the *CUBN* gene that encodes the intrinsic factor-cobalamin/vitamin B12, and a decreased spina bifida risk, and this protective effect was related to increased vitamin B12 and red blood cell folate.[45] Additional studies are needed to better define the nature and extent of the interaction between folate-related genetic variants and nutrient factors in modulating the incidence and penetrance of the NTD phenotype.

Candidate NTD Genes From Animal Studies

Although genetic variants have been identified in folate-related genes that increase the risk of developing an NTD, the total genetic variation identified to date in these genes does not account for the overall genetic contribution to NTD incidence observed in human populations. It is clear that other genes that are not related to folate play an important role in the pathogenesis of NTDs. As briefly described in this review, neurulation is a complex developmental process that involves distinct morphogenetic events that, in turn, involve a wide range of molecular and cellular mechanisms and signaling pathways. Further complications are that aberrations in cellular mechanisms or genes that are extrinsic to the neurulation "machinery" can have deleterious effects on neural tube formation. These include, among others, apoptotic cellular events, premature neurogenesis in the neural fold, and failure of emigration of neural crest cells.[6]

Faced with this level of complexity, researchers have turned to animal models to identify and characterize genes involved in normal and abnormal neurulation. Extensive studies in the frog, fish, and mice were instrumental in dissecting the molecular pathways underlying the 2 major cellular events of CE and apical construction during neurulation. The mouse model is particularly powerful where identification of defective genes in mouse mutants provides an entry point for identifying homologs and/or orthologs involved in NTDs in human beings.[62] To date, over 190 naturally occurring or experimentally induced mouse mutants with NTDs have been described, providing researchers with important tools for gene identification studies in NTDs.[63] A long list of candidate genes derived from animal studies has been investigated in human NTDs and only a few yielded positive results with no major causative gene identified.[1,44] Table 1 lists candidate genes that were derived from animal studies and that were found to be associated with human NTDs.[64-81] These genes are involved in a wide range of functions at all steps of neural tube formation and patterning.

A recent study of the PCP gene *VANGL1* in human NTDs proves the efficacy of the mouse model for gene identification in NTDs.[79] The candidacy of *VANGL1* for involvement in human NTDs was deduced from previous work on the looptail (*Lp*) mouse mutant. *Lp* is a well-established model for the study of NTDs in human beings where heterozygotes develop a looped tail appearance and homozygous embryos develop the severe NTD form of craniorachischisis and die in utero. *Lp* was actually the first mutant to implicate a role of PCP and CE in NTDs. The gene defective in *Lp* was identified as *Vangl2* and is a mammalian homologue of the *Drosophila* gene *Stbm/Vang* that forms part of the PCP pathway.[82] *Vangl2* has another homolog in vertebrates, *Vangl1*, that shares ~68% identity, and overall similar structure. *Vangl1* shows a dynamic expression in the developing neural tube and genetically interacts with *Vangl2*.[83] Resequencing efforts of *VANGL1* in a cohort of 144 NTD cases identified 3 rare missense mutations in familial and sporadic cases of NTDs, including a *de novo* mutation appearing in a familial setting. These mutations were hypothesized to be pathogenic on the basis of genetic and functional data.[79] A follow-up study on *VANGL1* by the same group in a larger cohort identified 5 novel missense variants in *VANGL1,* affecting evolutionary conserved residues and absent from all controls analyzed.[80] These studies provide evidence supporting the role of *VANGL1* as a risk factor in the development of spinal NTDs.

Novel candidate genes from animal models are continuously being discovered. Particularly, genes of the PCP pathway implicated in CE and neural tube formation present strong candidates for human NTDs. In mouse, mutations at several PCP loci, including *Vangl2*[82], *Scribble* (*Scrb1*),[84] *protein kinase 7* (*PTK7*),[85] and *Celsr1* (a mouse *Fmi* ortholog),[86] and combined mutations at *Dvl1/Dvl2*[87], *Dvl3/Vangl2,*[88] and at *Fz3/Fz6*[89] cause various forms of NTDs, further confirming the involvement of PCP in the pathogenesis of these diseases. The recent findings of novel mutations in *VANGL1* associated with human NTDs[79,80] should set the pace for similar studies of other PCP genes in large human NTD cohorts.

Gene Identification in NTDs: Challenges and Future Directions

Candidate gene studies in human NTDs have faced limited success primarily because of the multifactorial causation of NTDs where the genotype at 1 locus cannot explain the re-

Table 1 Candidate Genes Derived From Animal Studies That Were Associated With NTDs

Gene	Summarized Results	References
ALDH1A2: aldehyde dehydrogenase required for synthesis of retinoic acid during patterning of the neural tube	Significant association with three SNPs	64
CYP26A1: cytochrome P450 enzyme involved in metabolism of retinoic acid during patterning of the neural tube	One frameshift mutation, disease-specific	64,65
MSX2: transcription factor induced by retinoic acid during neurulation	One exonic deletion, disease-specific	66
NCAM1: neural cell adhesion molecule implicated in fusion of the neural tube	Significant association with an intronic SNP	67
PAX: transcription factors important for specification of neural crest-derived structures during patterning of the neural tube	*PAX-1*: one missense mutation, disease-specific; significant association with a flanking marker	68,69
	PAX-3: 5-bp deletion leading to frameshift mutation, disease-specific; one haplotype associated with an increased risk for NTD	70,71
	PAX7: significant association with an intragenic marker	69
	PAX8: significant association with an intragenic marker	69
PDGFRA: receptor alpha for platelet-derived growth factor, a potent mitogen involved in cell morphology and migration	Specific combinations of promoter haplotypes with inconsistent evidence for association	72-74
PRKACA: catalytic subunits of protein kinase A, a downregulator of SHH signaling	One missense mutation, disease-specific	75
SLUG: zinc finger transcription factor of the snail family, implicated in dorsalization of the neural tube	One missense mutation in one affected case and unaffected father	76
T box (Brachyury): transcription factor essential for mesoderm formation and axial development	One variant in intron 7: inconsistent evidence for association	77,78
VANGL1: homolog of *VANGL2*, a core gene of the PCP mediating CE	Three rare novel missense mutations, including a de-novo mutation that abrogates interaction with another PCP member (DVL); 5 rare novel missense mutations	79,80
ZIC2: zinc-finger transcriptional regulator of SHH activity	One alanine deletion from the amino terminal alanine stretch, disease-specific	81

SHH, sonic hedgehog.

sulting phenotype. Other limiting factors reside in the nature of such studies, where, to date, 2 major kinds have been adopted: association studies of candidate SNPs and direct resequencing. Association studies are often underpowered by small sample size that often leads to negative or inconsistent results. Concerted efforts with larger cohorts or a meta-analysis approach are needed for detecting significant associations. Resequencing studies in NTD cohorts have not typically included promoter or intronic regions that might contain regulatory elements important for transcription or splicing, respectively. A thorough analysis of these regions might help discover additional regulatory variants that predispose to NTDs.

In terms of future directions, powerful advances in genomics technologies have the potential of revolutionizing the exploration of NTDs by allowing genetic analysis of lesions beyond point mutations in known coding exons. Exciting data have recently emerged on the role of submicroscopic genomic imbalance or DNA copy number variants (CNVs) as an important cause of neurodevelopmental conditions and birth defects.[90-92] The novel technology of Array Comparative Genomic Hybridization (aCGH) can survey the whole genome for the detection of large segments of genomic imbalance that are usually detectable by karyotyping as well as smaller CNVs.[91] Using whole genome aCGH, several groups have shown that pathogenic CNVs can be identified in 15%-25% of patients who are mentally retarded. However, pathogenic CNVs appear to be more frequent in MR patients with structural birth defects, raising the possibility that they are also a frequent cause of structural malformations in fetuses and newborns.[93,94]

DNA-methylation epigenetic changes could play an important role in the development of NTDs. This is particularly interesting with the development of the methylation hypothesis that suggests that folic acid prevents NTDs by protecting against defects in methylation reactions that would otherwise lead to alterations in expression levels of genes essential for proper neurulation.[48] Assays that interrogate the whole ge-

nome for epigenetic regulatory modifications, for example chromatin immunoprecipitation combined with DNA microarrays (CHIP-chip), will enable us to explore the epigenomic influences on NTDs.[95] Another key regulator of gene expression is the recently discovered class of small RNA molecules, known as microRNAs (miRNAs), that play important regulatory roles in developmental timing and patterning, cellular differentiation, organogenesis, and apoptosis.[96] A recent study investigating the molecular mechanisms defective in *p53*-deficient mouse embryos displaying an increased frequency of exencephaly has identified genes and miRNAs that may be involved in the mechanisms causing NTDs.[97] Microarray approaches have been recently developed to study the expression levels of miRNAs in developing mouse embryos. A practical problem with DNA methylation and miRNA expression studies is that it is necessary to test the affected tissue(s), limiting the use of such studies in human patients. However, one could foresee the power of such studies in animal models for elucidating the potential role of these pathogenic mechanisms in NTDs.

Finally, we are heading to a "whole genome" sequencing era where new sequence technologies will allow whole genome mutation analysis with no a priori assumption regarding gene function. A similar approach focuses on sequencing only protein-coding exons that comprises about 1% of the human genome sequence. In the latter, exon-containing genomic fragments are isolated using oligonucleotide capture libraries, followed by next generation sequencing instrumentation.[98] A major challenge involved in such innovative technologies is the management and analysis of the massive datasets that will be generated.

Conclusions

We are still in the early stages of identifying the genetic factors predisposing to NTDs, due mainly to the complexity of this trait and the kinds of etiologic studies conducted so far. However, we are witnessing an explosion of high-resolution technologies that, coupled with animal models, will help define these genetic factors and better understand the pathophysiology of NTDs. The ultimate goal and hope is to develop better counseling and management strategies for the thousands of families that are still affected with these devastating conditions.

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