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REVIEW ARTICLE

Etiology, pathogenesis and prevention of neural tube defects

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ABSTRACT **Spina bifida, anencephaly, and encephalocele are commonly grouped together and termed neural tube defects (NTD). Failure of closure of the neural tube during development results in anencephaly or spina bifida aperta but encephaloceles are possibly post-closure defects. NTD are associated with a number of other central nervous system (CNS) and non-neural malformations. Racial, geographic and seasonal variations seem to affect their incidence. Etiology of NTD is unknown. Most of the non-syndromic NTD are of multifactorial origin. Recent *in vitro* and *in vivo* studies have highlighted the molecular mechanisms of neurulation in vertebrates but the morphologic development of human neural tube is poorly understood. A multisite closure theory, extrapolated directly from mouse experiments highlighted the clinical relevance of closure mechanisms to human NTD. Animal models, such as *circle tail, curly tail, loop tail, shrm* and numerous knockouts provide some insight into the mechanisms of NTD. Also available in the literature are a plethora of chemically induced preclosure and a few post-closure models of NTD, which highlight the fact that CNS malformations are of hetergeneitic nature. No Mendelian pattern of inheritance has been reported. Association with single gene defects, enhanced recurrence risk among siblings, and a higher frequency in twins than in singletons indicate the presence of a strong genetic contribution to the etiology of NTD. Non-availability of families with a significant number of NTD cases makes research into genetic causation of NTD difficult. Case reports and epidemiologic studies have implicated a number of chemicals, widely differing therapeutic drugs, environmental contaminants, pollutants, infectious agents, and solvents. Maternal hyperthermia, use of valproate by epileptic women during pregnancy, deficiency and excess of certain nutrients and chronic maternal diseases (e.g. diabetes mellitus) are reported to cause a manifold increase in the incidence of NTD. A host of suspected teratogens are also available in the literature. The UK and Hungarian studies showed that periconceptional supplementation of women with folate (FA) reduces significantly both the first occurrence and recurrence of NTD in the offspring. This led to mandatory periconceptional FA supplementation in a number of countries. Encouraged by the results of clinical studies, numerous laboratory investigations focused on the genes involved in the FA, vitamin B12 and homocysteine metabolism during neural tube development. As of today no clinical or experimental study has provided unequivocal evidence for a definitive role for any of these genes in the causation of NTD suggesting that a multitude of genes, growth factors and receptors interact in controlling neural tube development by yet unknown mechanisms. Future studies must address issues of gene-gene, gene-nutrient and gene–environment interactions in the pathogenesis of NTD.**

Key Words: animal models, clinical reports, folate supplementation, mechanisms of NTD, neural tube closure, neural tube defects

INTRODUCTION

Neural tube defects (NTD) are a group of heterogenous and complex congenital anomalies of the CNS. Commonly included in this group are anencephaly, spina bifida and encephaloceles. CNS anomalies are by far the most common birth defects and they are surpassed in frequency only by congenital cardiovascular abnormalities (Manning & Archer 2001). Several neural and non-neural malformations are commonly associated with NTD (Gardner 1980; Kallen *et al*. 1998; Davies & Duran 2003). The most frequent open NTD are anencephaly with cranioschisis and spina bifida aperta with myeloschisis, which arise during the process of neurulation, between the 17th and 30th postfertilization days. NTD arising as a result of a primary failure of the neural tube closure are clinically apparent by being open, i.e. leaving tissues of the unclosed neural tube exposed, in contrast to postneurulation NTD, which are skin covered. Encephaloceles and other skin covered lesions are examples of the post-closure NTD (Campbell *et al*. 1986; Dias & Partington 2004). The neural tissue in encephaloceles connects to the brain through a narrow stalk. About 70–80% of encephaloceles occur in the occipital region and the nasal and parietal ones are less common (Bozinov *et al*. 2005).

Anencephaly is a lethal malformation. Individuals with spina bifida have substantially enhanced survival rate thanks to recent improvements in medical and surgical management. However, these patients continue to be at increased risk for morbidity and mortality throughout their life. Treatment does not completely restore normal life. Individuals with lumbosacral spina bifida continue to experience varying degrees of motor and sensory dysfunction of lower limbs and failure of anal and urethral sphincters. Essentially all individuals with thoracic spina bifida and most of the patients with lumbosacral spina bifida are at increased risk for hydrocephalus and Chiari type II malformations (Rintoul *et al*. 2002; McLone & Dias 2003). Congenital malformations in general and NTD in particular contribute significantly to neonatal mortality during the first year of life (Pinar 2004). Medical care and management of NTD patients require a multidisciplinary team with special skills and cost substantial amounts of money. Periconceptional counseling can result in significant savings in life time costs for NTD affected individuals (de Weerd *et al*. 2004). A thorough understanding of the normal development of the neural tube, and pathophysiology, prognosis and susceptible periods of NTD are particularly important for planning strategies for effective prevention of NTD. This review endeavors to outline our current

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understanding of normal neural tube development and alludes to possible etiology, pathogenesis and prevention of NTD.

Embryonic development of the neural tube

Our current knowledge of the morphological and molecular mechanisms of neural tube development stems largely from experiments in chick and mouse embryos (Juriloff *et al*. 1991; Fleming *et al*. 1997; Schoenwolf & Smith 2000; Colas & Schoenwolf 2001, 2003; Lawson *et al*. 2001; Schoenwolf 2001; Ybot-Gonzalez *et al*. 2002; Copp *et al*. 2003). In recent years, there has been some important advancement in our understanding of such complex issues as how different signaling pathways converge in the induction of neural crest cells, their sequential migration-activation process, actual migration and transformation into mesenchyme and their diverse morphologic derivatives in *Xenopus*, chick and mouse embryos. A host of genes have been implicated in the networking of crest cell induction, migration, specification, and differentiation (Gammill & Bronner-Fraser 2002, 2003; Knecht & Bronner-Fraser 2002; Basch *et al*. 2004; Farlie *et al*. 2004). However, as far as human neural tube is concerned, only a cursory idea of the sequence of its normal morphologic development is available. Fundamental questions of molecular and cellular mechanisms of neural tube closure in human embryos remain largely unanswered (O'Rahilly & Muller 1989, 1994, 2002).

Neurulation

The initial step in neural tube development is a characteristic thickening of the ectoderm from the level of the primitive node of Hensen caudally to the prochordal plate rostrally at the beginning of the 3rd week of embryonic life. This slipper-shaped structure is called the neural plate. Studies in *xenopus* embryos led to the belief that the ectoderm is preprogrammed towards a neural fate and endogenous BMPs (bone morphogenetic proteins) inhibit this default system. However, neural induction in higher vertebrates appears to be a more complex process, in which inhibition of BMP-4 involves an intricate interplay of FGF (fibroblast growth factors), Noggin, Chordin, Wnt-3, β-catenin and possibly calcium transients (Jessell & Sanes 2000; Linker & Stern 2004; Webb *et al*. 2005). These genes appear to be conserved across species ('Mice are people'). Differential rates of cell proliferation, cell movement and changes in cell shape in the neural plate result in the formation of neural groove in the median plane and neural folds on either side. By complex processes of cell-shaping (apicobasal elongation, apical narrowing and basal expansion), cell movement and cell adhesion, aided by the forces provided by the underlying mesenchyme and overlying surface ectoderm, the neural folds elevate themselves, converge along the dorsal midline and fuse with each other to form the neural tube (Colas & Schoenwolf 2001). Fusion also involves the surface ectoderm at the edges of the neural folds dorsal to the neural tube. Neural crest-derived ectomesenchyme in the cranial region and somitic mesenchyme in the trunk region spread around the neural tube under the surface ectoderm and form the primordia of the meninges, axial skeleton and muscles attached to the axial skeleton.

Median and dorsolateral hinge-points

Preceding closure, the initially elliptical neural plate becomes elongated which results in an elongated keyhole-shaped structure, with a broad rostral and a narrow caudal region. This dramatic change in shape of the neural plate results from convergent extension (CE) and planar cell polarity (PCP) and involves a number of genes (Torban *et al*. 2004). The PCP and CE pathways function to establish polarized tissue patterns as well as to coordinate complex cell migrations and asymmetric cell division during gastrulation and neurulation. CE in neurulation is a medially directed movement of cells with intercalation in the midline, which results in both lengthening of the neural plate craniocaudally and relative narrowing transversely. Change in shape is accompanied by a median bending in the neural plate first in the area of future cervical region, called the median hinge-point (MHP) and one on each side, known as the dorsolateral hinge-point (DLHP) along the junction between neuroectoderm and surface ectoderm. Cells in the MHP of chick embryos are predominantly wedge-shaped, occupy a median furrow and remain attached to the notochord/notochordal plate (Smith *et al*. 1994). Coordinated changes in cell-shape instruct hinge-point formation (Wallingford *et al*. 2002). Changes in cell-shape associated with DLHP also include an apical narrowing and basal widening of neuroepithelial cells with cytoskeletal protein concentration under the apical membrane (Hildebrand & Soriano 1999). Cytoskeletal integrity of the neural plate cells plays a major role in the process of hinge point formation and convergence of the neural folds in the dorsal midline.

In *loop-tail* (*Lp*) mouse embryos, disruption of the process of CE results in a neural plate, which has an abnormally broad floor plate with no MHP. The elevated neural folds remain too far apart to contact with each other and the entire neural tube remains unclosed (craniorachischisis). They carry a point mutation in *Vangl2*, a gene known to control PCP in *Drosophila*. Both *crash* and *circletail* mutants resemble *Lp* phenotypically. They carry a mutation in *Celsr1* and *Scrb1* genes, respectively. Doudney and Stanier (2005) recently demonstrated a genetic interaction between all these three genes, where double heterozygotes exhibit the same homozygous phenotypes. *Circletail* and *Loop-tail* mutants were previously shown to interact and result in a phenotype similar to that of individual homozygotes (Murdoch *et al*. 2003). Viewed together, these studies show that interactions between independent recessive alleles may explain some of the complex inheritance in human NTD. They also indicate that CE and PCP are crucial developmental mechanisms that control neural tube closure in mammalian embryos. In wild type embryos, *Shh* expression remains high, while in the *Ltap/Lpp1* the expression is reduced in the floor plate region at the time of neural tube closure. However, in the $Shh^{-/-}$ embryos, *Ltap/Lpp1* expression remains high, the floor plate is indistinct and midline structures are defective, whereas a low expression of *Ltap/Lpp1*correlates with broad floor plate. These embryos subsequently develop defects of ventral midline cells of the neural tube and cyclopia indicating that *Shh* plays a strong role as an extracellular signaling molecule during development (Chiang *et al*. 1996). In *shroom (shrm)* mutation, cytoskeletal polarity within the neuroepithelium is perturbed and both DHLP formation and convergence of the neural folds are severely affected resulting in exencephaly, acrania, facial clefting and spina bifida (Hildebrand & Soriano 1999). Dorsal elevation of the edges of the neural plate into neural folds is largely aided by significant proliferation and expansion of the underlying mesenchyme. Brook *et al*. (1991) hypothesized that an enhanced axial curvature of the posterior neuropore (PNP) retards the elevation and convergence of the neural folds and thereby delays PNP closure in curly tail mouse embryos. In an elegant study, Peeters *et al*. (1998) compared caudal axial curvature and closure rates of PNP in chick, rabbit, mouse and human embryos at developmentally comparable stages and adduced evidence that a decrease in axial curvature due to unbending is accompanied by an increased PNP closure rate in these species. PNP closure process is fastest in chick embryos because they do not have an axial curvature. Decrease in axial curvature and embryonic body elongation in the caudal region also possibly involve changes in cell shape, cell proliferation and cell rearrangement.

Neural tube closure

Morphologically closure of neural tube involves apposition of the dorsal edges of the neural folds along the median plane, epithelial breakdown at contact sites accompanied by apoptosis and merger of the neuroepithelium. Studies in animal embryos indicate that *Shh* emanating from the notochord and region-specific expression of Gli1, Gli2 and Gli3 play major roles in the spatially and temporally controlled processes of neural fold fusion. Gli1 is expressed in the midline neural plate cells and in immediately adjacent cells. Gli2 and Gli3 are expressed in all but midline neural plate cells. Gli1 mediates *Shh* signaling. *Shh* represses Gli2 transcription. Gli3 and *Shh* repress each other. Gli1 induces ventral forebrain neuronal differentiation. In contrast, ventral spinal motor neurons are induced by Gli1 and Gli2. Thus Gli proteins also integrate positional information and neuronal type-specific differentiation in the CNS (Lee *et al*. 1997; Ruiz i Altaba 1998; Stamataki *et al*. 2005).

Theories of neural tube closure

Five sites of initiation of neural fold-fusion

The 'zipper theory' according to which the first site of contact between the dorsal edges of the neural folds occurs in the cervical region and fusion progresses cranially and caudally until the entire tube is formed, is no longer tenable. Multiple sites of initiation of neural folds fusion was reported in the mouse by Sakai (1989) and later by others (Juriloff *et al*. 1991; Golden & Chernoff 1993). Van Allen *et al*. (1993; Van Allen 1996) proposed a similar multisite closure model for the human embryos (Fig. 1). Their theory was based on the observations on therapeutic abortuses and still born fetuses who had fusion defects at different sites along the neural axis. According to their postulate (Van Allen *et al*. 1993; Van Allen 1996), a variety of NTD could be classified by closure sites where closure-failure occurs. Consistent with this model, the first site of contact between the cranial neural folds, known as *closure 1*, occurs at the presumptive rhombencephalon-spinal cord junction and progresses bidirectionally. *Closure 2* starts at prosencephalon-mesencephalon junction and also extends rostrally and caudally. *Closure 3* begins at the rostral tip of the neural plate adjacent to the stomodeum and progresses caudally to meet *closure 2*. *Closure 4* starts between *closure 1* and *closure 2* over the rhombencephalon and completes the closure of the cranial portion of the neural tube from which the brain develops. *Closure 5* starts at the caudal end of the neural groove and proceeds cranially to meet *closure 1* thus completing closure of the spinal portion of the neural tube.

Three sites of initiation of neural fold-fusion

Van Allen and colleagues (Van Allen *et al*. 1993; Van Allen 1996) and Seller (1995) were possibly the first to give a clinical flavor to the existence of more than one initial site of neural fold fusion in human embryos. However, their five-site theory was not supported by other contemporary studies in mouse embryos (Juriloff *et al*. 1991; Copp & Bernfield 1994) and by observations on human embryos (Sulik & Sadler 1993). For instance, mouse embryos are found to vary widely in the timing and location of sites of initiation of neural fold fusion (Copp & Bernfield 1994; Fleming & Copp 2000). The cranial neural tube only presents three closure sites: Site 1 and Site 3 are similar to those of Van Allen *et al*. (1993). Site 2 appears to vary in position along the rostrocaudal axis in inbred and outbred strains and backcrosses of *splotch* (Sp^{2H}) to DBA2 and NZW (Fig. 2). Generally strains with a morphological shift of Site 2 to more rostral positions have a greater predisposition to NTD than those with more caudal positions (Fleming & Copp 2000). Closure 2 is absent in exencephaly prone SELH/Bc strain.

Nakatsu *et al*. (2000) studied recently 68 normal embryos of Carnegie stages 10–12 and 98 embryos of Carnegie stages 11–23 with NTD stored at the Congenital Anomalies Research Center in Kyoto. They observed three sites: *Site A, Site B and Site C* (Fig. 3). The situations of Site A and Site C closely resemble *Closure 1* and

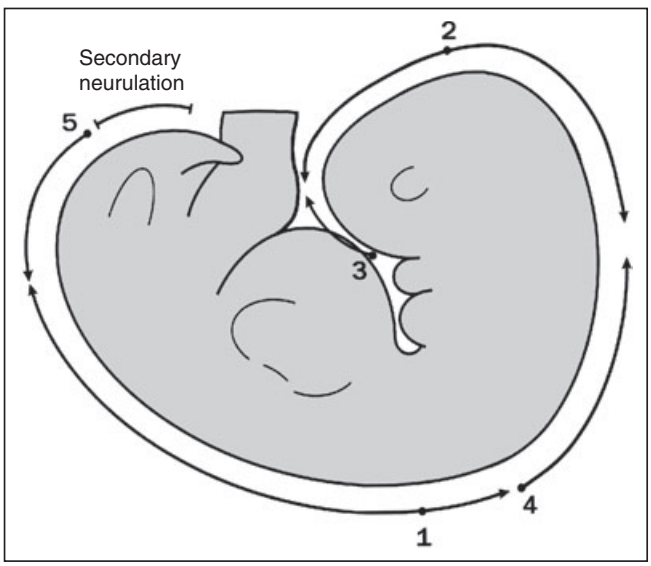

Fig. 1 (**A**) Extrapolated from mouse to human embryos, Van Allen's (1996) model has 5 sites of initial fusion of neural folds ('closure' 1–5): (1) Rhombencephalon-spinal cord junction; (2) Prosencephalon-mesencephalon junction; (3) Rostral extremity of neural folds; (4) Caudal end of rhombencephalon; (5) Between L2 and S2. Fusion spreads bidirectionally from Closure 1 and 2 and unidirectionally from Closure 3, 4 and 5. (Van Allen [1996]. Kind permission of John Wiley & Sons.)

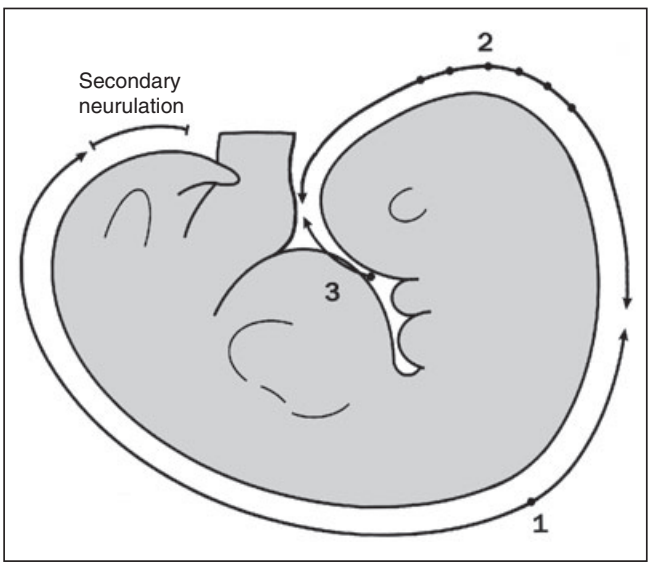

Fig. 2 Mouse models (Juriloff *et al*. 1991; Copp & Bernfield 1994) have 3 sites of initial fusion of neural folds: Site 1 and Site 3 are similar to those of Van Allen (1996). Site 2 appears to vary in position (block dots) along the rostrocaudal axis in inbred and outbred strains and backcrosses of *splotch* (Sp^{2H}) to DBA2 and NZW, generally more rostral positions predisposing to NTD and caudal positions being resistant to NTD (Fleming & Copp 2000). (Copp & Bernfield [1994]. Kind permission of Lippincott Williams & Wilkins.)

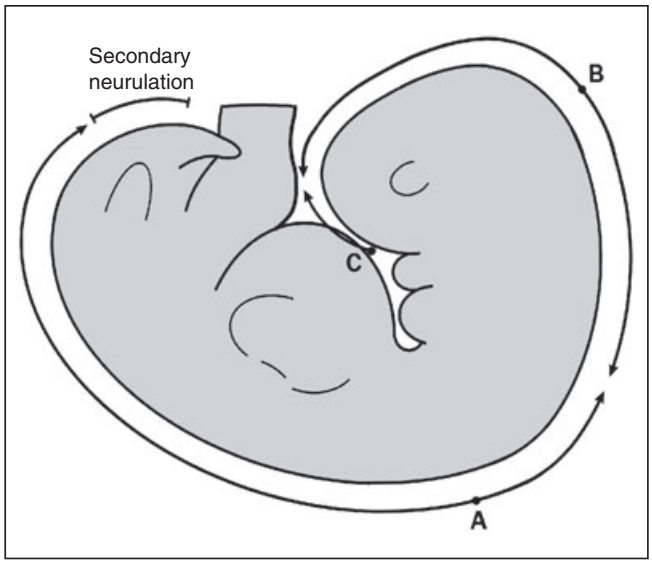

Fig. 3 The Kyoto Collection of Human Embryos of Carnegie Stages 10–12 present 3 sites of initial fusion of neural folds (Nakatsu *et al*. 2000). Site A initiates at upper cervical region, Site B at rhombencephalon-mesencephalon junction and Site C at the rostral end of the neural groove. Fusion spreads bidirectionally from Site A and B and caudally from Site C. The caudal fusion from Site A spreads towards the caudal neuropore and involves the entire spinal portion of primary neural tube. Secondary neurulation from the neural cord forms the distal neural tube which connects with primary neural tube at S2. (Nakatsu *et al*. [2000]. Kind permission of Springer Science and Business Media.)

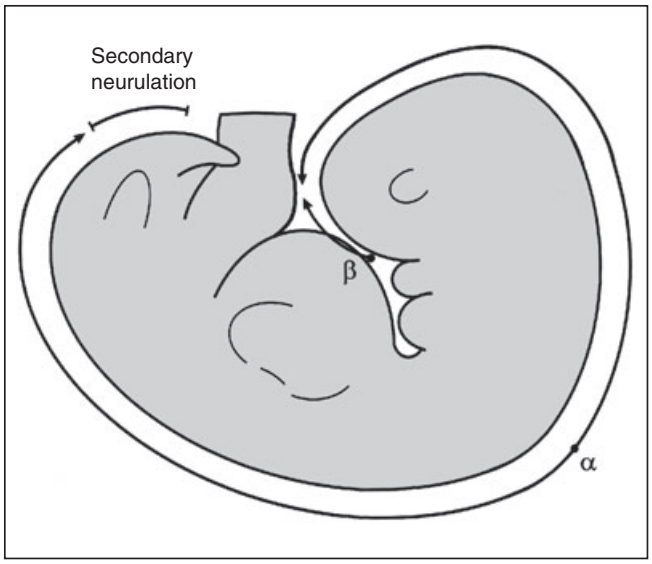

Fig. 4 Graphic reconstruction of the Carnegie Collection of human embryos of Stage 8–13 reveal mainly 2 fusion sites: Site α and Site β. Site α initiates at the caudal rhombencephalic level and Site β at the rostral extremity of the neural plate. Fusion of neural folds spreads from Site α caudally to the caudal neuropore and rostrally to the caudal limit of fusion from Site β to close the rostral neuropore (O'Rahilly & Muller 2002). Accessory sites of neural fold fusion are sometimes seen in the cervical, rhombencephalic and thoracic regions (not illustrated). (O'Rahilly & Muller [2002]. Kind permission of John Wiley & Sons.)

Closure 3, respectively, of Van Allen *et al*. (1993). Fusion of neural folds at *Site A* begins in the upper cervical region at late Carnegie stage 10 and progresses rostrally and caudally. *Site B* of neural fold-fusion is recognized in a few of the Carnegie stage 10 embryos at the rhombencephalon-mesencephalon junction and extends bidirectionally and rather rapidly. Therefore, the opening between *site A* and *site B* is so small that it possibly does not last longer than a few hours. Van Allen *et al*. (1993) on the other hand assumed *Closure 2* to initiate at prosencephalon-mesencephalon junction, particularly because their postulate was based on mouse studies. *Site C* of Nakatsu *et al*. (2000) initiates at the rostral tip of the neural groove in Carnegie stage 11 embryos and spreads caudally over the prosencephalon. This one meets with the rostral extension of neural fold-fusion from *site B* and completes the closure of the rostral neuropore, the opening between the rostral portions of neural folds of the prosencephalon (Fig. 3). In the meantime, fusion between neural folds from *site A* progresses caudally all the way to the caudal neuropore in the lumbosacral region. The neural groove becomes progressively shallow toward the caudal neuropore, which is completely closed at Carnegie stage 12. However, *Closure 4* and *Closure 5* of Van Allen *et al*. (1993) were not observed. Also fusion starting at *Site A* was found to be bidirectional; its caudal extension continued to the entire length of the spinal portion of the primary neural tube.

Two sites of initiation of neural fold-fusion

On the other hand, O'Rahilly and Muller (2002) observed only two sites of initiation of neural folds-fusion by graphic reconstruction of paraffin sections of Carnegie collection of human embryos of Stages 8–13: *Site* α in the region of the rhombencephalon and *Site* β in the region of the rostral tip of the prosencephalon (Fig. 4). The earliest of their embryos with fusion of neural folds had 4 pairs of somites. Therefore, the initial fusion must have occurred in their embryos in the occipital rather than in cervical region of the neurotaxis. Fusion of neural folds starting at *Site* α was seen to progress bidirectionally and that at *Site* β caudally. The fusion of the surface ectoderm was seen to occur first followed by fusion of the neural folds thus implying a significant role for the surface ectoderm. Accessory sites of neural fold apposition were observed along the neural axis independently and simultaneously but they were highly inconsistent in incidence, limited to Stage 10 and did not follow a specific pattern. Therefore, they interpreted Nakatsu *et al*.'s (Nakatsu *et al*. 2000) Site B as an example of 'accessory loci' which had not 'been overlooked by previous investigators'. That the fusion of neural folds in the region between Site A and Site B (of Nakatsu *et al*. 2000) is too rapid might be the reason why O'Rahilly and Muller (2002) did not observe site B in their embryos. The inference from these studies is that the phenomenon of multisite closure of developing neural tube, extrapolated directly from mouse embryos is of limited clinical significance because the number of initial fusion sites is possibly limited to 2 or 3 in human embryos (Nakatsu *et al*. 2000; O'Rahilly & Muller 2002). Different combinations of closure failure defects reported in the literature possibly suggest a causal heterogeneity of NTD origin (Martinez-Frias *et al*. 1996). The 2 or 3 fusion sites (and the occasional accessory sites) observed in human embryos are adequate to interpret logically most of the NTD resulting from a primary closure failure of the neural tube. The reported differences in the number of initial sites of contact between neural folds in human embryos is possibly a reflection of the fact that: (a) neural tube closure is a rapid process; (b) neural tube closure is a more complex process than what we currently understand; (c) the number of human embryos of neurulation stage available for study is inadequate; (d) we need to observe more embryos to fill the gaps in our knowledge;

and (e) since severely dysraphic embryos die early *in utero*, one doesn't get to see all theoretically possible combinations of non-contiguous malformations of the CNS at or after term.

Species variation in neural folds fusion

Neural tube closure has been investigated extensively in chick (Van Straaten *et al*. 1996), mouse (Sakai 1989; Juriloff *et al*. 1991; Golden & Chernoff 1993; Juriloff & Harris 2000), rat (Edwards 1968), hamster (Marin-Padilla 1970), rabbit (Peeters *et al*. 1998) and pig (van Straaten *et al*. 2000) embryos. Not only are there a certain degree of homologies between species but also there are noticeably significant species and strain differences in the number of sites of initiation of closure, direction of fusion progression, and sequence of onset of fusion points. Accessory sites of neural fold fusion reported occasionally (O'Rahilly & Muller 2002) need to be investigated further because they might help us explain why sometimes both rostral and caudal dysraphic spinal NTD develop in the same embryo. Neural crest-derived ectomesenchyme in the cranial region and somite derived paraxial mesoderm in the spinal portion have both mechanical and inductive influence on the neural plate differentiation, neural fold elevation and subsequent fusion of the neural folds and surface ectoderm of either side. This influence is apparent from the fact that parts of the axial skeleton are grossly malformed when neural tube fails to close in human and animal embryos (Padmanabhan & Hameed 1985; Padmanabhan 1989; Marin-Padilla 1991).

Secondary neurulation

The spinal portion of the primary neural tube from which most of the spinal cord develops ends approximately at the level of somite 31, the future sacral segment 2 (S2) (Muller & O'Rahilly 1987). The caudal neuropore closes during Carnegie Stage 12 when there are approximately 25 pairs of somites. The most distal segment of the spinal cord develops by a process, called *secondary neurulation* from the *caudal eminence* (not to be confused with the 'tail bud' of chick embryos). Caudal eminence comprises pluripotent cells derived from the regressing primitive streak. These cells give rise to the *neural cord*, a portion of the hindgut, caudal somites, caudal notochord, etc. (O'Rahilly & Muller 1989). The mesenchymal neural cord then becomes an epithelial cord, acquires a lumen and connects itself with the primary neural tube and forms the remaining sacral and coccygeal segments of the spinal cord. The uncertainty of our knowledge of caudal neural tube development is largely compounded by a paucity of properly preserved human embryos of the stages during which this part of the tube develops. In a recent study on the Kyoto collection of human embryos, Saitsu *et al*. (2004) observed no direct extension of the lumen of the primary neural tube into that of the neural cord. Multiple cavities were observed as was the case in RA-treated mouse embryos (Padmanabhan 1998; Shum *et al*. 1999). These cavities possibly coalesce to form the definitive lumen of the caudal neural tube. The entire neural tube formation is completed during Carnegie stage 11 and 12, within a span of about 10 days starting from postfertilization day 18. This critical window of neural tube closure is the period during which any primary failure of neural tube closure must occur. Additional studies are required to clarify mechanisms of caudal neural tube formation in order to understand why this part of the tube is frequently involved in NTD, the consequences of which seem to vary in severity and clinical manifestation. One recent fate mapping study in chick embryos suggests that the neural plate gives rise to both the primary and the secondary neural tubes and that the distal part of the axis which forms last suffers most as a result of perturbation of axis elongation and mesodermal tissue deficiency possibly caused by altered expression of T-box, Wnt and HLXB9 (Catala 2002).

Neural tube defects

Pathogenetic mechanisms of NTD

According to Von Recklinghausen (1886), arrest of closure of the embryonic neural tube is the mechanism of open NTD - hence the term 'dysraphia'. Morgagni (1769), on the other hand, believed that increased intraventricular pressure due to excessive production of cerebrospinal fluid (*hydrops cerebri et medullaris*) might lead to reopening of an already closed neural tube resulting in NTD (reviewed Padmanabhan 1984). Although most of the NTD are often reported to result from a primary failure of the embryonic neural tube to close, there is some compelling clinical and experimental evidence in support of the possibility of a closed neural tube secondarily reopening, resulting in NTD (Gardner 1980; Padmanabhan 1984, 1988, 1989; Padmanabhan & Hameed 1985; O'Rahilly & Muller 1988, 2002; Padmanabhan 1990a,b; Sulik & Sadler 1993; Van Allen *et al*. 1993; Van Allen 1996; Ikenouchi *et al*. 2002). Both experimental and human embryo studies indicate that post-closure defects are of relatively late onset (Padmanabhan 1990a,b; Ikenouchi *et al*. 2002) and might occur over an extended period of time during development. Most textbooks of embryology and NTD review papers (Van Allen *et al*. 1993; Van Allen 1996; Copp *et al*. 2003; Cabrera *et al*. 2004) appear to describe NTD as single developmental abnormalities and seek pathogenetic mechanisms as a direct result of closure failure of the neural tube rather than seeing them as a part of a spectrum maldevelopment affecting the neural tube and associated meninges and axial skeletal structures. Depending on the timing of onset during development, NTD affect not only different regions of the neural tube but several non-neural organs (Gardner 1980; Seller & Kalousek 1986). Myelomeningoceles are almost always associated with Chiari II malformations (McLone & Dias 2003). In an elegant study, Seller and Kalousek (1986) compared the frequency and pattern of isolated NTD and those of the NTD associated with other abnormalities. They note that significant clustering of developmental defects are associated with total craniorachischisis and upper thoracic spina bifida, less frequently with anencephaly and lumbosacral spina bifida and never with sacral spina bifida. This definitive pattern possibly implies a connection between the mechanisms by which NTD and associated anomalies develop. They postulate that additional abnormalities arise as a result of mechanical induction by the specific disturbance of the neural tube and its surrounding tissues. The developmental disruption at neurulation is more far reaching in NTD with associated anomalies than in isolated NTD.

Incidence and prevalence of NTD

The incidence of open NTD is reported to vary in different parts of the world depending on the geographic region, seasons at conception, gender of the affected infants, ethnicity, and socioeconomic status of the parents, maternal age and parity (Laurence *et al*. 1968; Xiao *et al*. 1990). The occurrence of NTD in abortuses and stillbirths is manifold higher than that in otherwise normal population (Nishimura *et al*. 1987; Shiota *et al*. 1987). A declining trend in NTD frequency has been reported recently in some areas, while the incidence has remained stable in other parts of the world (McDonnell *et al*. 1999; Chan *et al*. 2001; Busby *et al*. 2005)**.** While the reason for this decrease is unclear, there seems to be some effect from prenatal diagnosis, selective termination of NTD pregnancies, genetic counseling, and possibly nutritional supplementation during pregnancy (Lemire 1988a,b; EUROCAT Working Group 1991;

Stevenson *et al*. 2000; Chan *et al*. 2001; Williams *et al.* 2005). There is some robust data which indicates that the European countries vary widely in the availability and use of prenatal screening and its quality, as well as the 'culture' in terms of the decision to continue or terminate an NTD pregnancy. This necessarily contributes to notable variations between countries in childhood prevalence and cost to health care services of congenital anomalies (Garne *et al*. 2005).

Etiology of neural tube defects

As outlined above, development of the neural tube is a multi-step process strictly controlled by genes and modulated by a host of environmental factors. It involves gene–gene, gene–environment and gene–nutrient interactions. Despite years of intensive epidemiological, clinical and experimental research, the exact etiology of NTD remains rather complex and poorly understood. Genetic and environmental factors contribute to NTD. However, it is generally agreed that most NTD cases are of multifactorial origin, having a significant genetic component to their etiology that interacts with a number of environmental risk factors (Volcik *et al*. 2002a; Frey & Hauser 2003).

Genetic causes of neural tube defects

There are quite a few lines of evidence in favor of a strong genetic contribution to the causation of NTD in a proportion of all NTD prevalence. A compelling piece of evidence is the observation that NTD show familial aggregation, although they do not follow a strict Mendelian pattern of inheritance. The recurrence risk for NTD in siblings of patients with myelomeningocele is reported to range from 2 to 5% (Sebold *et al*. 2005). The incidence of NTD among first and second degree relatives of affected infants appears to be significantly higher than that reported to involve general population. Females and monozygotic twins appear to be particularly prone to NTD (Windham & Sever 1982). The prevalence of both encephalocele and anencephaly are increased, whereas spina bifida is decreased in twins as compared to singletons suggesting that twins and singletons vary in their response to etiologic factors and that there may be different factors influencing development of specific types of NTD. The etiology could be different in areas of high and low prevalence.

Several chromosomal and single-gene disorders have been reported to be associated with NTD (Online Mendelian Inheritance in Man 2000). Spina bifida occurs more frequently in autosomal trisomies. Animal studies have shown that there are as many as 100 mutant genes affecting neurulation and almost all of them have their homologs in humans (Juriloff & Harris 2000; Klootwijk *et al*. 2004). NTD are associated with several single gene disorders (e.g. cerebrocostomandibular syndrome. Fraser syndrome, Meckel–Gruber syndrome, Waardenburg syndrome). However, so far we know of no single gene, which is solely responsible for NTD in humans. It must be admitted that the genetics of human NTD is complex and poorly understood. One of the reasons for this is the paucity of families with several NTD-affected members. Perinatal mortality and morbidity of individuals with NTD are significant and the reproductive fitness of the survivors is poor. In addition to chromosomal anomalies, congenital heart diseases, certain skeletal dysplasias, NTD contribute significantly to neonatal deaths (Petrini *et al*. 2002; Pinar 2004). Anencephalic offspring surviving to term die at birth or soon after (Pinar 2004) thus a pattern of inheritance cannot be established.

Anencephaly is reported to be particularly more prevalent in certain communities with a high rate of consanguinity (Zlotogora 1997a,b; Al-Gazali *et al*. 1999). Spontaneous abortuses with NTD have a significant association with chromosomal aberrations suggesting a genetic component to their etiology (Coerdt *et al*. 1997; McFadden & Friedman 1997). Spontaneous abortions are often followed by term infants with NTD in the subsequent pregnancies (Carmi *et al*. 1994). Despite the declining prevalence rates of NTD in many parts of the world, there seems to be no decline in NTD recurrence within affected families (Czeizel & Metneki 1984). A Hungarian study (Papp *et al*. 1997) shows a recurrence risk of 3.47% and 2.95% for NTD and hydrocephalus, respectively, after excluding the obviously monogenically inherited cases from a total of 1285 isolated and 177 multiple forms of craniospinal anomalies. The risk was seen to be influenced mainly by anatomical severity of the malformation, degree of relationship and the number of affected relatives in the family (Papp *et al*. 1997).

Understandably anencephalic fetuses are so severely malformed that they do not survive to establish a parent-child transmission. The genes regulating normal development of the neural tube have not been delineated definitively and therefore one does not know what genes to look for in index children. The recurrent risk for anencephaly in siblings ranges from 2 to 5%, much lower than the 25 and 50% expected under strict Mendelian recessive and dominant inheritance, respectively. However, there are some studies that report anencephaly involving autosomal recessive genes, with some environmental influence (Demenais *et al*. 1982; Shaffer *et al*. 1990; Zlotogora 1995). The timing and nature of such influence might explain why a significant number of recurrences involve an NTD phenotype that is different from the case phenotype. An autosomal dominant gene has been implicated in a familial aggregation of spina bifida occulta (Fineman *et al*. 1982). Based on the knowledge that BMP4 and its specific inhibitor NOG are involved in normal neural tube development in mice, Felder *et al*. (2002) performed a single-strand conformation analysis mutation screen for both genes in 179 German spina bifida patients. There were four cases of missense mutations in BMP4 and one in NOG. It was suggested that these mutations acted in concert with other gene variants and contributed to susceptibility for NTD in these patients (Felder *et al*. 2002). Further studies involving larger affected populations are required before some meaningful conclusions can be drawn on the heritability of open NTD. Meckel–Gruber syndrome, which is a rare autosomal recessive disorder, has an encephalocele component in addition to several severe CNS malformations (Ahdab-Barmada & Claassen 1990). Patients with interstitial deletion of chromosome 2 (2q35–36.2), including the PAX3 gene (Waardenburg syndrome-type 3), are reported to have myelomeningocele (Nye *et al*. 1998). Waardenburg syndrome (WS) is a pleiotropic, autosomal dominant condition with variable penetrance and expressivity. Therefore it is not surprising that PAX3 mutations have been associated with only a subset of WS patients. It is also worth noting here that not all PAX3 mutations are associated with a Waardenburg phenotype and that additional regional loci may modify or regulate the PAX3 locus and/or the development of a Waardenburg phenotype (Pasteris *et al*. 1993).

Twinning appears to be associated with a significant increase in NTD over the rest of the population (Windham & Sever 1982). There is an increased frequency of twinning in the near relatives of probands with upper NTD and upper NTD families with twins have a higher rate of NTD siblings than do families without twins (Garabedian & Fraser 1994). Monozygotic twinning is more frequently concordant for congenital anomalies than dizygotic twinning (Windham & Sever 1982). Some genetic or environmental factors were thought to make NTD families susceptible to twinning (Garabedian & Fraser 1994). Since supplemental folate (FA) protects some fetuses against NTD, it was suggested that FA might play

some role in twinning. However, there is no robust evidence in support of the presence of an increased frequency of twins following FA supplementation (Li *et al*. 2003).

Consanguinity appears to allow clustering of liability genes each of which possibly contributes to NTD in some way (Carter *et al*. 1968). A recent study on a highly consanguineous community (Al-Gazali *et al*. 1999) indicates that whereas some rare syndromes occur relatively more frequently, NTD *per se* do not occur at a high frequency in this population.

Environmental causes of neural tube defects

Physical and chemical environment

Differential incidence of NTD depending on the geographic areas, socioeconomic status of the parents, seasonal variations, discordance in monozygotic twinning, etc. point to the possibility of an environmental component to the etiology of NTD. Alternatively, these variations in frequency might point to possible gene–environment interactions at critical stages of neural tube development. Epidemiological and experimental studies on NTD provide some evidence that a host of physical agents (e.g. X-irradiation, hyperthermia, stress), drugs (e.g. thalidomide, folate antagonists, androgenic hormones, antiepileptics such as valproate and carbamazepine, and hypervitaminosis A), substance abuse (e.g. alcohol), chemical agents (e.g. organic mercury, lead), maternal infections (e.g. rubella, cytomegalovirus, *Toxoplasmo gondii*, syphilis), maternal metabolic conditions (e.g. phenylketonuria, diabetes mellitus, endemic cretinism), etc. are capable of causing congenital malformations of the central nervous system structures (Shenefelt 1972; Wilson 1973; Committee on Developmental Toxicology, National Research Council 2000; Shepard *et al*. 2002; Ray *et al*. 2004). Parental socioeconomic class, occupation (e.g. nursing, processing food and beverages, farming, textile dye and leather industries, spraying pesticides) and possible occupational exposure to noxious agents such as organic solvents, anesthetic agents, sterilants, viruses, pesticides, paints, X-radiation, have been reported to be associated with a high risk for NTD (Brender & Suarez 1990; Matte *et al*. 1993; Sever 1995; Shaw *et al*. 2002; Blanco Munoz *et al*. 2005). Maternal health status (e.g. obesity, diabetes) has been attributed to enhance the risk for NTD in the offspring (Ray *et al*. 2004; Dietl 2005). Such studies need to be extended so as to include a large and statistically acceptable number of cases for further confirmation of the conclusions made thus far. The antenatal screening study (Ray *et al*. 2004) should include women with spontaneous pregnancy loss which is known to be associated with a high incidence of NTD. It is important to point out here that contrary to frequent claims, it is CVS anomalies and not NTD which are more common in infants of diabetic mothers (Schaefer-Graf *et al*. 2000)). This observation is in agreement with our diabetic rat study (Padmanabhan & Al-Zuhair 1988)

Ambient exposures

Ambient exposures include exposure to airborne chemicals living in close proximity to their source (e.g. polyvinyl chloride) (Theriault *et al*. 1983; Uzych 1988) and toxic wastes from landfill sites located within 3 km of residence (Croen *et al*. 1997; Marshall *et al*. 1997; Dolk *et al*. 1998). Some studies have reported negative results for maternal exposures from landfill sites located at distance of over 3 km (Vrijheid *et al*. 2002; Morris *et al*. 2003). Landfill sites contain a range of chemicals which might further contaminate surface and ground water, plants and cattle grown in the vicinity and the air. Therefore, it was not possible for these studies to determine the type and amount of exposure. Additional confounders include the socioeconomic status of mothers living in this area and potential exposure to other drugs and chemicals outside the sites in the background. Maternal exposure to contaminated drinking water with carbon tetrachloride, trichloroethylene, and benzene has been reported to confer an increased risk of NTD and major cardiac defects (Bove *et al*. 1995). Methods of assessment and misclassification of pregnancy outcomes are possibly the reason for certain of the discrepancies observed across studies.

Hyperthermia

Hyperthermia has been suspected to be a neuroteratogen for human embryos by an analogy from laboratory investigations. Results of *in vivo* and *in vitro* experimental studies on guinea pig, rat, mouse and chick embryos indicate that the neural tube is particularly sensitive to heat stress. Enhanced core body temperature appears to interfere with several critical developmental events such as cell proliferation, migration, differentiation and apoptosis. The response to heat appears to depend on the species, strain, embryonic developmental stage at which heat exposure occurs, dose and duration of exposure (Edwards *et al*. 1995, 2003). A similar dose–response relationship has not been established for human embryos. Direct extrapolation of animal data to human embryos is not possible. However, a recent meta-analysis indicates that maternal hyperthermia during gestation is associated with an enhanced incidence of neural tube defects (odds ratio 1.95) showing that the neural tube is heat-sensitive in human embryos too (Moretti *et al*. 2005) Maternal exposure to non-physiological levels of high temperature (e.g. in sauna, hot water tub) and treatment with antipyretic agents during critical period of neurulation have been found to increase the risk of NTD in the offspring (Suarez *et al*. 2004). In addition to parental and embryonic genetic susceptibilities, the actual level of exposure, gestational stage at which the exposure occurred and the genotypes of the parents and offspring might have contributed substantially to the variabilities between studies. It is important to point out here that NTD are of heterogenous nature with regards to their etiology. The pathogenetic mechanisms of all CNS anomalies are not the same and different anomalies might be related to not only the agent to which exposure occurs but also to the pathogenetic differences between anomalies (e.g. anencephaly, spina bifida and encephaloceles). Future studies must address these variables in the context of evaluating NTD incidence and risk factors.

Environmental and genetic interactions

A host of suspected teratogens is also available. These agents might be teratogenic given the susceptible genetic background. Since NTD are of multifactorial origin, an understanding of the possible interaction-effects of teratogens with susceptible genes is of particular interest. One of the practical ways to look at this issue is to use animal models in which the confounding variables inherent to human studies could be maximally avoided while studying the pathogenetic mechanisms. Currently there are over 100 mouse models of NTD. Most of these mouse NTD phenotypically resemble human NTD to a large extent (Harris 2001). Among mouse and human homolog genes may be genes with alleles of partial function, which might combine to induce the risk of some of the common non-syndromic NTD. In this context, it needs to be understood that in addition to gene–gene interactions, there might also be instances where susceptible genes interact with teratogens thus enhancing the NTD risk (Finnell *et al*. 2004). These interactions might contribute to the seasonal or geographic variations often attributed to NTD incidence. Of the numerous opportunities that exist for interactions, at least some are important from a teratologic

point of view. For instance, food and beverages contain ingredients which might interact, particularly with orally administered-drugs via drug-metabolizing enzymes. Foods containing phytochemicals and herbal supplements have the greatest potential to induce enzymes, which could affect the bioavailability of the active molecules in the drug necessitating dosage adjustments (Harris *et al*. 2003). Dietary modifications are also known to alter the plasma concentrations of drugs. Dietary habits and nature of the diets have ethnic and geographic variations. Thus exposures to a drug considered safe in a given dosage for use in pregnancy in a given situation might reach teratogenic concentrations in some pregnant women.

Nutritional factors

The role of maternal nutrition on normal fetal development and growth is well known. Nutrional status of the parents can affect the quality of the gametes and fertilization capacity. There are some reports that suggest that polymorphisms in folate metabolizing enzymes might be associated with enhanced likelihood of meiotic non-disjunctions (O'Leary *et al*. 2002; Wong *et al*. 2002). Genotype of the embryo and nutrient environment of the mother play decisive roles in growth and development of the embryo. With the discovery that periconceptional FA supplementation has beneficial effects in terms of reduction of 50–70% in the occurrence of NTD (MRC Vitamin Study Research Group 1991; Czeizel & Dudas 1992) and substantial reduction in the incidence of several other non-neural anomalies, enthusiasm for search for the mechanisms of FA-responsiveness to NTD increased. As a result, a number of FA-related genes have been studied in some detail. The following genes in the folate metabolic pathway are noteworthy because they have been studied more extensively than others.

- 5,10-methylene tetrahydrofolate reductase (MTHFR);
- Methylene tetrahydrofolate dehydrogenase (MTHFD);
- Cystathionine β-synthase (CBS);
- Methionine synthase (MTR);
- Methionine synthase reductase (MTRR);
- Folate Receptor α and β.

MTHFR (5, 10-methylene tetrahydrofolate reductase)

C677T and A1298C are two well known mutations of MTHFR gene implicated in human NTD. These mutations have been observed in variable per centage of individuals in different populations both with and without NTD. Whitehead *et al*. (1995) observed the C677T thermolabile variant gene in 18% of spina bifida patients and 6% of control population in Ireland and indicated the possible contribution of this mutation to NTD risk. van der Put *et al*. (1995) showed the presence of C677T mutation in 15% of spina bifida patients and 16% of mothers and 10% of fathers of patients as well as 5% of controls in the Netherlands. These results would suggest that a 'suboptimal maternal folate status imposes a biochemical stress on the developing embryo, a stress it is ill-equipped to tolerate if it has a TT genotype' (Estkes 1998; Shields *et al*. 1999). Data from several areas of the world have shown that the thermolabile variant C677T is indeed associated with an increased risk of NTD but comparing with the prevalence of this mutation, it can only account for a small proportion (about 12%) of observed NTD (Morrison *et al*. 1998; Richter *et al*. 2001; De Marco *et al*. 2002). Data from most studies do not support a role for A1298C polymorphism in NTD, nor does it have a combined effect with C677T polymorphism (Parle-McDermott *et al*. 2003a). Search for defects in other genes controlling FA metabolism became necessary when MTHFR variants could not account for all or most of the 50–70% rescue effects attributed to periconceptional intake of FA on NTD.

Methylenetetrahydrofolate-dehydrogenase (MTHFD)

It is a trifunctional (methylenetetrahydrofolate-dehydrogenase, methenyltetrahydrofolate cyclohydrolase, formyltetrahydrofolate synthetase) nicofinamide adenine dinucleotide phosphate (NADP)-dependent cytoplasmic enzyme (also referred to as C1-THF synthase), which catalyzes the conversion of tetrahydrofolate to the corresponding 10-formyl, 5, 10-methynyl, and 5, 10-methylene derivatives important for the *de novo* biosynthesis of purines and pyrimidines and thus DNA biosynthesis. A Dutch study on spina bifida patients and their parents could not obtain strong evidence in favor of a risk for NTD in patients homozygous for R293H and R653Q polymorphisms (Hol *et al*. 1998), whereas Brody *et al*. (2002) showed evidence for a role for the maternal genotypic MTHFD1 R653Q variant in abnormal neural tube development. In this instance, the case mother carries the risk genotype. This is an important observation that highlights not only the significance of FA-related genes, but also the influence of the maternal genotype on neural tube development. The differences in the data produced in geographically different parts of the world may be explained by not only the genetic characteristics of the populations studied but also the differences in their nutritional environment. Further work is needed to elucidate the complexity of this issue, because it still doesn't account for the entire rescue property attributed to folate with regards to NTD.

Vitamin B12 and homocysteine

Even those NTD patients and their parents who are not homozygous or heterozygous for MTHFR C677T mutation have low plasma FA and elevated homocysteine levels (van der Put *et al*. 1997a). In fact mothers of NTD infants are only low in folate levels but not deficient. Therefore, in addition to low FA levels or polymorphisms in FA metabolizing enzymes, lower vitamin B12 (or cobalamin) concentrations during pregnancy may also be independently contributing to an increased risk for NTD (Kirke *et al*. 1993). Homocysteine (Hcy) is formed from demethylation of methionine. It can be irreversibly degraded into cystathionine and cysteine catalyzed by cystathionine β-synthase or remethylated into methionine by methionine synthase (MTR) or betaine-homocysteine methyl transferase (BHMT). Women carrying an NTD affected fetus have been observed to have mildly elevated Hcy concentrations in plasma and amniotic fluid, in response to methionine loading (Mills *et al*. 1995; Steegers-Theunissen *et al*. 1995). Plasma Hcy levels and NTD incidence are known to be determined by both genetic and nutritional factors. However, common variant alleles of the cystathionine β-synthase gene do not seem to constitute a definitive risk for NTD (Ramsbottom *et al*. 1997). Methionine synthase reductase (MTRR) is required to maintain the methionine synthase cofactor, methylcobalamin derived from vitamin B12 in an active state (Brody *et al*. 1999). Thus vitamin B12-dependent remethylation of homocysteine involves transcobalamin (TC), methionine synthase (MTR) and MTR reductase (MTRR). Mutations in these genes might be involved in elevation of total plasma homocysteine (tHcy) concentrations and in the causation of NTD. In a study of South Italian NTD patients, Gueant-Rodriguez *et al*. (2003) observed a strong association between MTR 2756 AG/GG, TC 777 CG/GG/MTHFR 677 CC and MTRR 66 GG/MTHFR 677 CC genotypes and increased risk for NTD. This study also indicates that genetic factors that can limit cellular availability or MTRR-dependent reduction of B12 may augment the risk of spina. bifida. Zhu *et al*. (2003) reported a similar association of polymorphisms of MTR and MTRR in a population along the US-Mexico border.

A recent study from northern UK, where a high frequency of NTD is known to occur indicates that the *MTHFR* 677C→T polymorphism constitutes a risk factor and that the *MTRR* 66→A G polymorphism exerts a protective effect in NTD cases. When statistical tests for interaction were performed, three genotype combinations in cases (*MTRR*/*GCPII*; *MTHFR* 677/*CβS*; *MTHFR* 677/*MTRR*) and one combination in case mothers (*CβS*/*RFC-1*) were found to elevate NTD risk. Maternal–fetal interaction was also detected when offspring carried the *MTHFR* 677C→T variant and mothers carried the *MTRR* 66A→G variant, resulting in a significant elevation of NTD risk. These data suggest that both independent genetic effects and gene–gene interaction play important roles in relation to NTD risk. It appears that multilocus rather than single locus analysis might provide an accurate measurement of genetic susceptibility to NTD (Relton *et al*. 2004). Studies on the Irish and Dutch NTD patients suggest that inherited variations in MTR and MTRR either alone or in combination with other folate enzyme polymorphisms are not associated with elevated NTD risk and that methionine synthase gene is not involved in the etiology of NTD (van der Put *et al*. 1997a,b; Brody *et al*. 1999; O'Leary *et al*. 2002; O'Leary *et al*. 2005). Conclusions have to wait further elucidation of the complex gene functions in FA metabolism. Methylmalonyl-CoA mutase is another B12-dependent enzyme which utilizes adenosylcobalamin to catalyze the isomerization of L-methylmalonyl-CoA to succinyl-CoA. Moderately elevated methylmalonic acid (MMA) in women carrying an NTD affected fetus has been reported (Adams *et al*. 1995). However common variations in the methylmalonyl-coA mutase are not found to be risk factors for NTD in an Irish study (Parle-McDermott *et al*. 2003b).

Folate carrier and folate receptor genes

Now that variants of the genes that code for the enzymes of the folate metabolic pathway could not account for all the 50–70% reduction in NTD incidence attributed to FA-supplementation, attention is turned to the hypothesis that individuals with defective FA absorption, transport and cellular internalization might be at increased risk for NTD. Folates are poorly transported across plasma membrane by diffusion. Reduced folate carrier (RFC) protein is expressed in the brush border of small intestinal epithelium and in hepatocytes. It preferentially mediates transport of reduced FA such as 5 methyltetrahydrofolate (MTF). RFC transcripts and proteins are expressed in the trophoblast, yolk sac, neuroepithelium, limb buds and cardiac primordium in mouse embryos (Maddox *et al*. 2003). Targeted mutation of RFC in the mouse results in fetal death at mid-gestation indicating its developmental importance (Zhao *et al*. 2001). FA receptor gene variants might impose a reduction in FA uptake by embryonic cell populations involved in neural tube development and thus confer an increased liability to NTD. Folate receptor genes occur as a family of paralogous glycoproteins: FR-α, FR-β, FR-χ and FR-δ. FR-α is described to possess the greatest affinity for 5-methyltetrahydrofolate, the physiological form of folic acid. The binding of 5-methyl tetrahydrofolate to a folate receptor is an important step in folate uptake and delivery into the cells by endocytosis. Expression of Folbp1 (folate binding protein-1, the mouse equivalent of human FR-α) is reported to follow a pattern by first being expressed at the initiation sites of neural fold fusion, particularly along the dorsal edges of neural folds as well as in the yolk sac thus suggesting a possible role in neural tube closure and maternofetal transport of FA at the yolk sac (Piedrahita *et al*. 1999; Saitsu *et al*. 2003). Functional inactivation of Fbp1 and Fbp2 in mice shows that Fbp1 is important for embryo survival, neuroepithelial differentiation and fusion and neural crest migration and differentiation (Piedrahita *et al*. 1999). Mouse embryos, in which FBP1 has been knocked out, develop NTD and die at mid-gestation (Spiegelstein *et al*. 2004). Folb$^{2-/-}$embryos develop normally. However, it should be pointed out here that the role of RFC and FBP in human neural tube closure has not been clarified as yet. Studies in European and US NTD populations have not established FR-α, or FR–β gene association with NTD (Barber *et al*. 1998; Heil *et al*. 1999; Trembath *et al*. 1999; O'Leary *et al*. 2003).

Other nutrients

Another nutrient important for proper functioning of many proteins, enzymes and transcription factors is Zinc (Zn). A relationship between Zn and NTD was demonstrated experimentally several years ago by Warkany and Petering (1972). Later several Zn-dependent transcription factors were shown to be risk factors for NTD in mouse models (Purandare *et al*. 2002). Maternal and offspring Zn and myo-inositol concentrations have been reported to be lower in human spina bifida (Groenen *et al*. 2003). However, a recent human study shows that genetic variants in ZIC1, ZIC2, and ZIC3 are not major risk factors for NTD (Klootwijk *et al*. 2004). It appears that Zn deficiency might act on vulnerable embryonic primordia via as yet unknown mechanisms and contribute to NTD in susceptible embryos. Realizing the importance of cholesterol biosynthesis in normal NTD development, some studies focussed on the genetic variations in the apoE and apoB genes, known to regulate cholesterol metabolism. The results did not show that these genes contribute substantially to the risk of spina bifida in infants (Volcik *et al*. 2002b).

SUMMARY AND CONCLUSION

The last three decades have seen a remarkable accumulation of clinical and experimental data in search of the causes, mechanisms and manifestations of neural tube malformations. However, due to the paucity of human embryos of early stages of neural tube development, our knowledge of the morphologic and molecular bases of normal development of human neural tube has remained limited. Neural tube defects have a narrow definition in the literature and do not include post closure defects with the exception of encephaloceles. Search for polymorphic genes that might contribute to NTD is largely limited to those known to be involved in FA pathway and none has been shown to play decisive roles in causing arrest of neural tube closure. It appears that gene-gene, gene-nutrient and gene-(non-nutrient) environment interactions might contribute to NTD. Future studies must be directed at unraveling the possible interaction or combinations of such interactions, prenatal medical and surgical correction of NTD and effective preventive strategies.

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