

Pathobiology and Genetics of Neural Tube Defects

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Summary: *Purpose:* Neural tube defects (NTDs), including spina bifida and anencephaly, are common congenital malformations that occur when the neural tube fails to achieve proper closure during early embryogenesis. Based on epidemiological and clinical data obtained over the last few decades, it is apparent that these multifactorial defects have a significant genetic component to their etiology that interacts with specific environmental risk factors. The purpose of this review article is to synthesize the existing literature on the genetic factors contributing to NTD risk.

Results: To date, there is evidence that closure of the mammalian neural tube initiates and fuses intermittently at four discrete locations. Disruption of this process at any of these four sites may lead to an NTD, possibly arising through closure site-

specific genetic mechanisms. Candidate genes involved in neural tube closure include genes of the folate metabolic pathway, as well as those involved in folate transport.

Conclusions: Although extensive efforts have focused on elucidating the genetic risk factors contributing to the etiology of NTDs, the population burden for these malformations remains unknown. One group at high risk for having children with NTDs is epileptic women receiving antiepileptic medications during pregnancy. Efforts to better understand the genetic factors that may contribute to their heightened risk, as well as the pathogenesis of neural tube closure defects, are reviewed herein. **Key Words:** Birth defects—Spina bifida—Epilepsy—Anticonvulsant drugs.

Neural tube defects (NTDs) are among the most common of human congenital malformations, affecting 0.6 per 1,000 live births in the United States (1), where there are approximately 4,000 NTD-complicated pregnancies annually. NTDs include all congenital anomalies that involve a failure of the neural tube to close during the fourth week of human embryogenesis, with defects occurring at any point along the formation of the spinal cord, rostrally from the developing brain and caudally to the sacrum. While analysis of the inheritance of NTDs has shown some familial aggregation, for the most part these defects do not follow a pattern of simple Mendelian inheritance. Although easily diagnosed, NTDs possess a complex etiology, which is probably due to their multifactorial nature, comprising both environmental and genetic components. Given that these malformations occur frequently and represent a significant public health problem, particularly among specific population subgroups, such as women with drug-treated seizure disorders, there is a great incentive to identify the genetic factors contributing to NTD susceptibility. This will serve as the basis of

better prenatal screening methods for the detection and ultimately the prevention of NTDs.

Disorders of neural tube closure (NTC) involve abnormalities in the region-specific NTC junctures within the cranial and caudal levels of the neural tube, often resulting in the frank exposure of neural tissue. These defects vary in their severity, depending on the type and level of the lesion. The most severe and common of the anterior defects is anencephaly, which leads to partial or total secondary brain degeneration from a lesion caused by incomplete fusion of the neural folds in the second NTC site (closure site II). This defect comprises ~50–65% of all human NTDs and is invariably lethal (2,3). Exposure of brain tissue without secondary degeneration is the analogous murine condition, commonly termed exencephaly. Spina bifida constitutes the general category of caudal defects (below the level of T12) involving spinal cord tissue. The clinical spectrum of NTDs also includes cranio-rachischisis, in which the neural folds never elevate to fuse along the entire length of the embryo, and iniencephaly, in which there is lack of proper formation of the occipital bones, with a short neck and a defect of the upper neural tube.

Despite exhaustive research efforts now spanning several decades, little is known about the actual genetic mechanisms governing the primary events involved in

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NTC. It is now widely appreciated that this is a complex process, occurring at four distinct initiation sites in the mouse and presumably in humans, that coordinates multiple morphological and cellular events (4,5). This multisite NTC pattern provides an additional level of complexity to neural tube formation, so that a comprehensive understanding of the pathogenesis of NTDs is currently beyond our grasp.

EMBRYOLOGY

Genetic regulation of mammalian neural tube morphogenesis is a highly complicated process that most certainly involves a multitude of genes. These genes have vital functions in a wide range of biological activities that are currently thought to involve signaling molecules, transcription proteins and factors, cytoskeletal and gap junction proteins, and tumor suppressor genes (6–9). At the earliest stages, the epithelia of the two-layered embryo must be induced by signaling molecules to become a neural plate, which is anchored over the notochord and eventually bends at the midline in what has been referred to as the medial hinge point (10). In a general sense, the lateral portions of the neural plate will elevate, and in the rostral regions, the neural folds bend inward at the dorsolateral hinge points (10). The neural tube, which forms as a result of these movements, fuses at several discrete points, with closure proceeding in a rostral-to-caudal direction from these sites of initiation (4,11). Specifically in the mouse, at gestational day (GD) 7.0, the neural folds begin to fuse at the caudal portion of the hindbrain, marking closure I. The site at the forebrain–midbrain boundary, referred to as closure II, begins to close around GD 8.0. These two closure sites spread bidirectionally once NTC has been initiated. At GD 9.0, the neural plate at the most rostral position of the developing embryo fuses to form the anterior forebrain at closure III. By GD 9.5, the neural tube in the midbrain–hindbrain junction undergoes closure IV, leaving only the posterior neuropore to close. By GD 10.0, the neural tube has closed completely, except at the caudal end, where the posterior neuropore becomes increasingly small and ultimately disappears. Closures II and IV proceed bidirectionally until they meet to complete the formation of the cranial neural tube. Thus, GDs 9.0 and 10.0 are the critical period of anterior NTC.

In humans, the process of NTC is quite similar to that described previously for the mouse. The primary neural tube is thought to close during Carnegie stage 12, whereas the secondary neural tube develops by a process of differentiation and canalization with the primary neural tube. This whole process occurs between Carnegie stages 13 and 20 (12,13). It has very recently been proposed that human embryos have two successive sites of fusion of the neural folds, one in the rhombencephalic region and

one in the prosencephalic portion of the embryo. This line of reasoning is based on a reassessment of 98 histologically sectioned (stages 8–13) human embryos from the Carnegie Collection (14).

O’Rahilly and Müller (14) argue that fusion sites of the neural folds appear in succession, with fusion site α in the rhombencephalic region and fusion site β in the prosencephalic region, adjacent to the chiasmatic plate. Fusion from site α proceeds bidirectionally (rostral and caudal), whereas that from site β is unidirectional (caudal only). The first fusion of the neural folds occurs when four to six somitic pairs are present, initiating from α at the level of somites 2 and 3. This initial fusion extends rapidly in both directions so as to involve the rhombencephalic and spinal levels. Continued extension proceeds rostrally and caudally, with extensions resulting in the inclusion of cervical, thoracic, mesencephalic, and prosencephalic levels (14). The observed α and β fusions terminate in two neuropores, one rostral and one caudal. In addition, accessory loci of fusion, without positional stability and of unknown frequency, may be encountered in older, Carnegie stage 10 embryos. The presence of these accessory loci is confined to stage 10 and seems to be quite inconstant, with no specific pattern to their appearance; therefore, their importance is rather speculative (14).

During primary neurulation, the fusion of the neural folds involves surface ectoderm, followed by the neural ectoderm, and subsequently the interposition of mesenchyme. Initially, the seam of the surface ectoderm contacts neural ectoderm without intervening mesenchyme. The subsequent interposition of mesenchyme in the neural folds derives from the primitive streak/node and is responsible for elevation of the folds before their contact and fusion. The presence of mesenchyme in the crest, at stages 9 and 10, most likely is a requirement for normal neurulation; thus, impairment of the primitive node and streak could lead to NTDs. The study by O’Rahilly and Müller (14) confirms that in the human embryo the rostral neuropore closes bidirectionally from the dorsal and terminal lips. The dorsal lip, which is the rostral limit of fusion site α , proceeds caudorostrally. The rostral neuropore becomes gradually defined between these two lips, and growth of the lips is closely related to the appearance of successive somites. Human NTC is thought to be complete when approximately 19 or 20 somite pairs have appeared (14).

PATHOGENESIS OF SPINA BIFIDA

There are several ongoing controversies concerning the pathogenesis of NTDs. The best data currently available come from animal models rather than reconstructions made from human embryos and fetuses. One particular model system that has been widely studied in efforts to better understand the pathogenesis of posterior NTC

defects is the mouse curly-tail mutant (15). This is largely due to the striking phenotypic similarities between the lesions in these mice and human spina bifida. It has long been appreciated that there is a relationship between a delay in the closure of the posterior neuropore in the curly-tail (ct/ct) homozygotes and the development of spina bifida (16,17). These studies clearly demonstrated that spina bifida was always preceded by nonclosure of the posterior neuropore. Further, van Straaten and co-workers (18) found that the posterior neuropore in the mutant embryos was up to five times longer than that observed in wild-type control embryos. This confirmed that the spina bifida defect observed in this mouse model did not occur from a reopening of the neural tube; rather, the neural tube never closed during early embryogenesis (15).

It has been proposed that the spinal defects are secondary to a dorsoventral cell proliferation imbalance, which leads to a transiently increased ventral curvature of the posterior neuropore of the curly-tail mutant embryos. This curvature is responsible for mechanical stresses opposing the dorsolateral bending movements that normally occur during neurulation; consequently, the closure of the posterior neuropore is delayed and the mice are born with both spina bifida and curly tails (15). More recently, the pathogenesis of spina bifida has been defined in molecular terms for this model system. The first signs that development has gone astray are seen at the 22-somite stage in the tail bud region of the embryo, when both cell proliferation and *Wnt5a* gene expression are visibly reduced in the homozygous curly-tail embryos. These defects precede the ventral curvature of the caudal embryonic axis, which should be significantly enlarged by the time the embryo has 27 to 29 somites (15). At this developmental stage, both *RARβ* and *RARγ* are down-regulated, which is of interest given that both retinoic acid and *myo*-inositol can rescue the normal phenotype in curly-tail mutants and do so by up-regulating *RARβ* expression. It may well be that the curly-tail mutant phenotype is the result of dysregulated *Wnt5a* and *RARβ* gene expression, or a product of more complex gene-nutrient interactions.

The cell proliferation imbalance that has been observed in the posterior neuropore and produces the exaggerated ventral curvature of the caudal body axis that serves to delay the neuropore's closure may do so by acting as a counterbalance to the normal dorsolateral bending of the neural tube. As the angle of the curvature is reduced with the growth and development of the embryo, a small proportion of mutant embryos are able to successfully complete posterior neuropore closure. Nonetheless, these embryos ultimately develop a curly tail that may be the result of inadequate *RARβ* expression in this caudal region. In other embryos, the delay of closure is so prolonged that the embryo cannot recover sufficiently and the lesion cannot be avoided.

GENETIC RISK FACTORS

Given the highly complex etiology of NTDs, the identification of the genes directly involved in determining susceptibility to these malformations remains exceedingly difficult (19). Recent evidence linking folic acid to the prevention of NTDs has stimulated a great deal of new research to determine the protective mechanism of this B vitamin (20,21). Although supplemental folic acid may reduce the incidence of NTDs by up to 70%, the molecular mechanisms by which it exerts its protective effect remain unknown and are a matter of considerable speculation. The remarkable ability of folic acid to reduce the incidence of NTDs has made those genes encoding the ~150 proteins directly involved in folic acid metabolism and transport the target of extensive investigation. These folate pathway candidate genes include folate receptor alpha (*FRα*), reduced folate carrier (*RFC*), the 5,10-methylene-tetrahydrofolate reductase (*MTHFR*), cystathionine β-synthase (*CBS*), methionine synthase (*MTR*), methionine synthase reductase (*MTRR*), methylenetetrahydrofolate dehydrogenase (*MTHFD*), and serine hydroxymethyltransferase (*SHMT*), to name just a few (22–38). A more comprehensive list of candidate genes is given in a recent review by Juriloff and Harris (39).

To date, few polymorphisms identified within candidate genes appear to be causally related to NTDs or to serve as significant risk factors (19,27). In several mothers, single nucleotide polymorphisms have been identified that, together with environmental or nutritional factors, can increase the risk for NTDs (27). The association studies that are currently being conducted at a number of institutions around the world will become more able to identify true risk factors as the number of affected families, as well as individual subjects available for study, increases.

Folate receptor alpha

Human folate receptor alpha (hFR-α) is a glycosylphosphatidylinositol (GPI)-anchored membrane-bound protein with a high affinity for 5-methyltetrahydrofolate (5-Me-THF). The receptor, located within caveolae (40), transports folates intracellularly across the cellular membrane in the only folate-specific step in folate transport. As such, it is easy to imagine how a slight conformational change in this protein that alters its ability to transport sufficient quantities of folate to the embryo at critical periods of development might make an embryo susceptible to NTDs. This is further supported by the high concentration of these receptors in the maternal placenta, as well as the syncytiotrophoblast and fetal neuroepithelium (41). Because most embryonic cells do not express hFR-α, the presence of these receptors in cells of the developing neural tube suggests a critical role for folic acid during normal NTC. Additional evidence of this gene's involvement in susceptibility to NTDs comes from mouse studies in which its homolog, the folate binding protein-1 gene (*Folbp1*),

was inactivated, and the nullizygous mice exhibited NTDs and other folate-dependent congenital defects (42,43).

Investigation of this gene in human subjects has focused on the exons responsible for protein function and the promoter region. Barber and colleagues (28,32) conducted an exhaustive search for nucleotide polymorphisms within the coding region of *hFR-α* using spina bifida cases and matched control samples collected as part of a live-born phenylketonuria screening program in California. In the initial study, exons 3, 5, and 6 were screened in over 1,000 DNA samples using single-stranded conformational polymorphism (SSCP) analysis. A follow-up investigation involved direct DNA sequence analysis of *hFR-α* exons 5 and 6 in 50 NTD-affected individuals. Finally, the entire *hFR-α* coding region was screened by dideoxy fingerprinting in a sample of 219 individuals that was stratified by folate status and pregnancy outcome (28). These studies failed to reveal any polymorphic nucleotides within either the coding region or the intronic bases surrounding the intron–exon boundaries of the *hFR-α* locus (28). An explanation put forward by the authors for the lack of detectable polymorphism within the *hFR-α* locus was that gene conversion had taken place within the FR gene family. Barber et al. (28) argued that gene flow between homologous chromosomes would have the effect of cleansing mutations at the genomic level, including selectively neutral substitutions that normally accumulate within DNA over time. A total of 163 intronic bases immediately adjacent to the intron–exon boundaries within the coding region and 122 base pairs of the 3′ untranslated region (3′UTR) immediately downstream of the stop signal were screened in this study. The complete lack of observed nucleotide variation in these regions was cited as supporting the possibility of gene conversion. Additionally, the high degree of sequence identity between *hFR-α* and the pseudogene suggested that recombination had occurred between these loci. Subsequently, work by De Marco and colleagues in Italy (29) provided further evidence of de novo pseudogene-specific mutations in *hFR-α* alleles arising by gene conversion events, documenting their occurrence in three unrelated NTD patients. In fact, the presence of a high degree of homology between tandemly repeated pseudogene and *hFR-α* sequences would facilitate unequal chromosome pairing, resulting in more frequent nonallelic sequence exchanges. *hFR-α* and pseudogene sequences are <20 kilobases apart, and their homology approaches 90% within exons 6 to 7 and the 3′UTR. The identified mutations in the three patients were the consequence of the introduction of a single 214 base pair insertion (patient 1) from one locus into another, resulting in at least a single nucleotide difference (patients 2 and 3). Patient 1 was heterozygous for chimeric exon 7 with the introduction of three missense mutations in *cis*. All of these mutations affected the carboxy-terminal amino acid membrane tail or the GPI-anchor region. Additionally, the

replacement of the entire 3′UTR with the corresponding pseudogene sequence in this patient, and the insertion of a pseudogene-specific nucleotide in 3′UTR in patient 3, might compromise *hFR-α* mRNA stability. Furthermore, the gene conversion event in patient 2, although limited to the insertion of a single pseudogene nucleotide, affected a tightly conserved lysine residue, suggesting significant consequences for the efficiency of folate binding.

In a subsequent investigation, Trembath et al. (30) screened the *hFR-α* coding region by SSCP in a sample of 154 NTD cases and their relatives obtained from Midwestern NTD clinics. Only a single case presented with a gene alteration, which consisted of a single, silent mutation (TGA → TAA) within the stop codon (30). This mutation was identified in a male, nonHispanic Caucasian with a meningomyelocele. Analysis of parental DNA verified paternity and determined that the mutation was a de novo event. A separate population of 326 control individuals composed of newborn infants from Iowa revealed a lone atypical sequence with an amino acid substitution of serine to asparagine within exon 6 of *hFR-α*; this atypical sequence occurred in a phenotypically normal individual and thus had no significant association with NTD risk (30).

In addition to studies of the protein-coding region, evaluations of the *hFR-α* promoter region have revealed that the nucleotides encompassing a segment of the 5′UTR that have been shown to be necessary for maintenance of normal transcription rates (31) contained three different polymorphisms (631T → C, 610A → G, and 762 G → A). These three polymorphic sites formed only two variant alleles, as two of the substitutions (631T → C and 610A → G) were always observed together. No statistically significant association or trend was observed for either a risk or a protective effect for children or mothers with any of the different allelic forms of this gene ($p > 0.05$). Although the frequency of the polymorphic alleles showed significant variation between sampling locations, there was no significant association of either allele with NTD risk. These polymorphic promoter alleles appear to represent neutral variation within the promoter region of *hFR-α*. Therefore, unlike the coding region of this gene, which showed very little variation, the promoter region does not appear to be under the same degree of selective pressure to remain invariant; it is possible that clinically significant single nucleotide polymorphisms exist within these regions that could account for the population burden of NTDs (32).

5,10-Methylenetetrahydrofolate reductase

An important component of the folic acid metabolic pathway is the amino acid homocysteine; even mildly elevated levels of maternal homocysteine are often associated with an increased risk for an NTD-affected pregnancy (44–46). This information is helpful as it provides a focus on one portion of the folic acid biosynthetic pathway

for which there are several different enzymes that result in mild to severe hyperhomocysteinemia. Homocysteine itself has a dual role in the folate pathway: (a) it is the precursor of methionine via the remethylation pathway, and (b) it is the precursor of cystathionine via the transsulfuration pathway. S-adenosylmethionine (SAM), acting as a regulator between remethylation and transsulfuration, is an activator of CBS-mediated homocysteine transsulfuration and an inhibitor of MTHFR, thereby inhibiting remethylation (47). Thus, MTHFR serves as one of the cell's principal means of regulating intracellular concentrations of methionine and homocysteine. MTHFR is the rate-limiting step in homocysteine remethylation, and thus mutations in this gene can lead to elevations in homocysteine concentrations. The gene that codes for the MTHFR enzyme is the one gene that has been most often described as a potential risk factor for NTDs. The MTHFR gene, comprising 11 exons with known alternative splicing, has been mapped to the short arm of chromosome 1 (1p36.3) and produces a protein product that is ~77 kDa.

One well-documented mutation in the MTHFR gene (677C → T) has been described and is associated with reduced enzymatic activity that has been linked to increased plasma homocysteine levels in homozygous (TT) individuals if their folate intake is low (33,34). This occurs in the homozygous state in 10–25% of the population and results in a thermolabile form of the enzyme associated with 50–60% reduced enzyme activity (35). Over the last several years, several different groups have reported between a threefold and a sevenfold increased NTD risk associated with this MTHFR mutation, especially if the mutation was in the homozygous state, in Holland and Ireland (33,48). However, other larger studies have found either a smaller or no association with NTDs (36). Shaw and colleagues (36) examined this common MTHFR polymorphism in a population-based, case-control set of samples that included >200 cases of spina bifida. In addition to the genotype information, data were collected on maternal vitamin consumption during the periconceptional period. When the homozygous 677T/677T variant genotype was observed in an infant whose mother took multivitamins, there was only a modest tendency toward an increased odds ratio [OR = 1.2; 95% confidence interval (CI), 0.4–4.0] relative to the reference group, who were infants with the homozygous 677C/677C genotype whose mothers took multivitamins. For cases in which the mother did not take multivitamins and the infant had the 677TT (homozygous) genotype, there was a slightly higher odds ratio (OR = 1.6; 95% CI, 0.8–3.1). These results are consistent with an interaction between genotype and maternal vitamin use, as the risk for NTDs appears to be higher among the offspring of women who did not take folic acid, but the difference between the two odds ratios is not statistically significant. Therefore, this interaction does not appear to have a major biological impact on NTD risk.

Studies focusing on maternal rather than infant MTHFR genotype have suggested that folate deficiency and the homozygous TT maternal genotype are important risk factors for NTDs (37,38). Since the genotype leads to higher homocysteine levels only if the folate status is low, it is highly unlikely that it is a significant factor contributing to the overall population burden of NTD risk.

Botto and Yang (35) performed a meta-analysis on MTHFR data from a number of different countries and ethnic groups with regard to the frequency of the C677T allele. They reported a pooled odds ratio of 1.7 for NTD risk among infants with the 677T/677T homozygous genotype, with a slightly lower odds ratio for infants who were heterozygous for the variant C677T allele, suggesting a relationship between the number of variant 677T alleles and risk for NTDs. Furthermore, they calculated a pooled attributable risk of 6% for infants who were homozygous for the variant 677T allele. This calculation assumes that there is a causal relationship between the MTHFR gene and the development of NTDs. With regard to homozygosity for the MTHFR 677T variant allele, it appears that it is only *associated* with an increased risk for spina bifida in highly selected populations, and only in certain studies within those populations.

In a study by Dean and co-workers (49), the interaction between MTHFR genotypes and in utero exposure to antiepileptic drugs (AEDs) was examined in a small cohort of 57 patients and their parents. Of these, 46 infants were exposed to valproate (VPA), 11 to carbamazepine (CBZ), and 9 to phenytoin (PHT). Fifteen of the infants were exposed to AED polytherapy. The control group used for comparison in this study included 152 samples taken from individuals either matched to the parents of the infants or randomly identified as “adult samples.” Because of the limitations of the study design, interpretation by the authors that there was a statistically significant excess of 677C → T homozygotes associated with susceptibility to fetal anticonvulsant drug syndromes involving these three antiepileptic medications remains questionable. Among the patients, there was an excess of individuals with the heterozygous 677CT genotype, although this was not statistically significant. Clearly, other MTHFR mutations or mutations in other genes in the folic acid or other enzymatic pathways may contribute to the risk for adverse pregnancy outcomes, which include NTDs.

Methionine synthase and methionine synthase reductase

MTR, also known as 5-methyltetrahydrofolate homocysteine methyltransferase, catalyzes the remethylation of homocysteine to methionine with the co-factor methylcobalamin acting as an intermediate methyl carrier. MTR plays a critical role in maintaining adequate intracellular methionine levels, as well as regulating the folate pool. Severe deficiency of MTR activity leads to megaloblastic

anemia, homocysteinuria, and hyperhomocysteinemia. The human MTR gene has been cloned and mapped to chromosome 1q43. A polymorphism of the MTR gene (A2756G), which changes the 919th amino acid from aspartic acid to glycine, has been widely found in the general population (50). However, this polymorphism does not seem to change either the plasma folate/B₁₂ level or the homocysteine level (51–53) or to increase the risk for spina bifida (54–56). A recent study showed that individuals with the GG genotype tend to have slightly lower plasma total homocysteine (tHcy) levels (57).

The cobalamin co-factor of MTR becomes oxidized over time and the enzyme is inactivated. Regeneration of the functional enzyme requires reductive methylation catalyzed by MTRR. Phenotypic expression of MTRR deficiency is considered to be similar to that of patients with *cbIE* (58). The MTRR gene has been localized to chromosome 5p15.2-15.3 (59). A common polymorphism of this gene (A66G), which changes the 22nd amino acid from isoleucine to methionine, has been found to be associated with increased NTD risk when either the cobalamin status is low or the MTHFR 677TT genotype is present in an infant (60). This is true even though this polymorphism alone does not change the patient's homocysteine levels. The G allele at this locus has also been associated with an increased risk for Down's syndrome when it occurs in combination with the MTHFR 677CT or TT genotypes (61,62). Those individuals with the AA genotype have higher levels of tHcy and a 4% increase of cardiovascular disease risk (63).

More recently, Zhu et al. (64) found that infants with the MTRR AG genotype had a threefold higher risk of NTDs when compared with those with the AA genotype (OR = 2.98, 95% CI = 1.26–7.05, $p < 0.05$). Infants with the MTR AG genotype also had a 2.7-fold higher risk of NTDs compared with those who had the AA genotype, although this difference was not statistically significant (OR = 2.65, 95% CI = 1.12–6.26, $p > 0.05$). It is noteworthy that infants who are heterozygotes for both MTRR and MTR (AG + AG) had exceptionally elevated NTD risks, with an odds ratio of 14.7 compared with wild type (AA + AA) (OR = 14.7, 95% CI = 3.2–67.5, $p < 0.05$) (63). A comparable result was observed in the mothers of NTD cases (OR = 9.1, 95% CI = 1.9–43.1, $p < 0.05$). These results suggested that the MTRR and MTR genes interact to increase the infant's NTD risk (63).

ANTI-EPILEPTIC MEDICATIONS AS RISK FACTORS FOR NTDs

Valproate-induced NTDs

The anticonvulsant drug VPA has been directly implicated as a potent neural tube teratogen, producing a 1–2% spina bifida response frequency in exposed human fetuses (65). This represents a 10- to 20-fold increase in

prevalence over the spina bifida rates observed in the general population (66). Since spina bifida develops in only a small percentage of VPA-exposed fetuses, the data suggest that the affected fetuses have a genetically determined predisposition for VPA-induced NTDs. In utero VPA exposure in humans also has been associated with craniofacial, cardiovascular, and skeletal defects (62,67,68), although the developing nervous system appears to be particularly sensitive to disruption after exposure to this drug.

Humans are not unique in their response to VPA, as this drug has been shown to induce exencephaly and spina bifida in rodents and other laboratory animals (69,70). In the various animal species exposed in utero to VPA, abnormalities of the skeletal system were the most commonly reported developmental defect. The principal malformation associated with VPA exposure in utero in experimental animals has been NTDs, including exencephaly and spina bifida. Nau (71) produced exencephaly in mouse embryos from dams exposed to sufficiently high dosages of VPA to produce maternal plasma concentrations in excess of 230 $\mu\text{g/ml}$, irrespective of the route of administration. This concentration represents a two- to fivefold increase over the recommended human therapeutic level (72). Probit analysis indicated that a single subcutaneous injection of the drug administered on gestational day 8 must produce maternal plasma concentrations of 445 $\mu\text{g/ml}$ to produce a 10% increase in the rate of exencephaly over that observed in the controls (71). A multiple-injection treatment regimen will produce the same increased response frequency for NTDs at maternal plasma concentrations of only 225 $\mu\text{g/ml}$, whereas osmotic minipumps can be used to induce this same NTD response frequency while delivering a steady state plasma VPA concentration of 248 $\mu\text{g/ml}$ (71).

With respect to posterior NTDs, Ehlers and colleagues (73,74) demonstrated that multiple doses of VPA (200 mg/kg, i.p.) administered 6 h apart beginning on gestational day 9.0 can produce a 10% response frequency of spina bifida occulta, which increases to 95% with a VPA dosage increase to 500 mg/kg body weight. A significant degree of malformation of the ribs and vertebrae was apparent when the exposed fetuses were examined after alcian blue-alizarin red skeletal staining (73,74). A low frequency (4–6%) of spina bifida aperta was also induced by the same VPA treatment regimen in the Han:NMRI mouse strain, resulting in a highly disorganized and necrotic spinal cord within the vertebral canal in the lumbosacral region of the developing fetus. The absence of neuronal tissue indicated an almost complete localized ablation of the neural tube in the VPA-exposed fetuses (74).

Murine model systems have been exploited in an effort to learn more about the genetic basis of susceptibility to VPA-induced NTDs. Such studies have revealed a strain-dependent hierarchy of NTD susceptibility

after single maternal intraperitoneal injections of 600 mg/kg VPA on gestational day 8.5 (70). In these studies, SWV/Fnn mice demonstrated high susceptibility to exencephaly, LM/Bc/Fnn embryos demonstrated a more modest NTD response, and C57BL/6J and DBA/2J mice were completely resistant (70). There are several possible theories to explain a genetically regulated mechanism for susceptibility to VPA-induced NTDs, one of which involves the documented inhibition of folate metabolism by VPA (75,76). Interference with selected steps in the folate pathway could potentially result in a decreased rate of methylation of essential, developmentally regulated genes during critical periods of embryogenesis. This would significantly enhance the sensitivity of the embryos to specific malformations. Such a difference in methylation patterns between embryos of several inbred strains might explain their different sensitivity to VPA-induced NTDs. However, definite interactions between folate metabolism, VPA therapy, and gene regulation have yet to be documented.

The pathogenesis of VPA-induced NTDs may also arise from alterations in neuroepithelial mitotic rates that drive the normal timing of neurulation. Thus, at discrete time points VPA exposure may perturb mitosis, leading to insufficient neuroepithelial cellular proliferation that culminates in a failure of neural fold elevation and fusion. VPA exposure has been shown to inhibit the proliferation of neuronal cells in culture. At concentrations that had previously been reported to be teratogenic in both humans and mice, VPA caused a 50% reduction in the proliferation rate of C6 glioma cells by impeding the cell cycle during the G₁ phase (77,78). If exposure of C6 glioma cells to VPA occurred after this specific cell cycle restriction point, the proliferation of these cells was not affected (77). Furthermore, agents that inhibited cell proliferation in the C6 glial cell line within twice their therapeutic dose were consistently associated with major NTDs (78). Collectively these data illustrate the necessity for stable cellular proliferation within the developing neuroepithelia in order for NTC to occur, and provide compelling evidence of a potential mechanism for VPA teratogenicity.

As previously outlined, the process of neurulation is complex and requires elevation, apposition, and fusion of the neural folds to form the neural tube. This process involves many genes and their corresponding proteins. To ascertain whether changes in the expression of genes might play a role in determining the susceptibility to VPA exposure, the transcriptional activity of genes known to encode proteins involved in NTC was analyzed in embryos exposed to VPA. With the advent of newer molecular biological approaches, such as *in situ* transcription and antisense RNA amplification, it was possible to examine gene expression directly in the neural tubes of developing embryos (79). The experimental protocols for

the gene expression studies have been previously reviewed in some detail (80,81). Briefly, NTC stage embryos from control- or VPA-treated dams were harvested at selected timepoints, generally gestational days 8.5, 9.0, and 9.5. The gene expression patterns in the embryos were analyzed by univariate and multivariate statistical approaches. In general, it appeared that teratogenic concentrations of VPA elicited strain-dependent effects on the expression of several genes that are important to normal embryonic development. These genes included cell cycle and apoptosis genes such as *bcl-2* and *p53*. Strain-dependent changes were also observed in a number of growth factor genes, including brain-derived neurotrophic factor (*bdnf*), nerve growth factor (*ngf*), and its receptor (*ngf-R*). Folate pathway genes, including the *folbp-1* and *folbp-2* genes, as well as the *MTHFR* gene, were examined (79). The gene expression data collected to date suggest that subtle collective changes in several molecules, each of which by itself may be developmentally harmless, together produce the adverse phenotypic changes that may result in the observed NTDs. Clearly, cell cycle and growth factor genes are involved, and these changes may well be folate responsive.

Carbamazepine-induced NTDs

The literature concerning the teratogenic potential of the front-line AED CBZ is much more limited than that which exists for either PHT or VPA (for review see ref. 82). Nonetheless, it has become increasingly clear in recent years that the risk for NTDs in infants exposed in utero to CBZ rivals that of VPA. Hernandez-Diaz and colleagues (83) observed a sevenfold increased risk for NTDs among women who used CBZ during pregnancy between 1976 and 1998. This supports the earlier work of Rosa (84), who first reported the association between CBZ and the risk for NTDs. In a meta-analysis of the literature on CBZ teratogenicity, Matalon and colleagues (85) reviewed the outcomes of 1,255 prospectively ascertained gestations compromised by the drug. They reported a significantly increased rate of congenital malformations, primarily NTDs, among the CBZ-exposed infants. Evaluating the effects of polytherapy, the same authors failed to identify any increased rate of malformations when CBZ was administered with one other AED ($p > 0.05$); however, when CBZ exposure occurred in the presence of two or more other AEDs, the teratogenic risk to the exposed infant was significantly elevated (85). In summary, the teratologic evidence collected in the past 25 years suggests that in utero exposure to CBZ, whether the drug is used as monotherapy or polytherapy by a pregnant woman with epilepsy, poses a significant risk for NTDs. The evidence will become more clear with the maturing collection and analysis of data from the AED pregnancy registries that exist in both the United States and Europe (EURAP).

CONCLUSIONS

It is known that the risk for having a child with an NTD is largely the result of genetic factors interacting with a variety of environmental agents, including AEDs. The complexity of the etiology should not deter efforts to better understand the genetic factors contributing to NTD susceptibility, which are a first step in providing better prenatal counseling to pregnant patients with epilepsy. At present, variation in the many folate pathway genes is a focus of investigation, although it is possible that these genes are primarily modifiers of risk and not truly causative. The number of known single gene alterations in mice that produce an NTD phenotype is growing rapidly, and this provides a compelling rationale for examining the human homologs that may be associated with NTD risk. Such an approach has prompted one investigator to publish a list of "best picks" among the available murine models for investigation in human data sets (19). Among the genes suggested are those involved in actin organization (Macs and p190 RhoGAP; Mlp), methylation (Dnmt3b), and cell cycling (Trp53, Gadd45a). Clearly, other gene families, including those involved in drug metabolism and detoxification, must be considered. There is every reason to believe that genetic variation within major genes contributes to the risk for NTDs, and that investigators will soon reach a new level of understanding of the interaction between genetic and environmental risk factors, and thus elucidate the etiology of NTDs in epilepsy-complicated pregnancies.

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REFERENCES

- Nakano KK. Anencephaly: a review. *Dev Med Child Neurol* 1973; 15:383-400.
- Thomas JA, Markovac J, Ganong WF. Anencephaly and other neural tube defects. *Front Neuroendocrinol* 1994;15:197-201.
- Hunter AG. Brain and spinal cord. In: Stevenson RE, Hall JG, Goodman RM, eds. *Human malformations and related anomalies*. New York: Oxford University Press, 1993:109-37.
- Golden JA, Chernoff GF. Intermittent pattern of neural tube closure in two strains of mice. *Teratology* 1993;47:73-80.
- Van Allen MI, Kalousek DK, Chernoff GF, et al. Evidence for multi-site closure of the neural tube in humans. *Am J Med Genet* 1993;47:723-43.
- Echelard Y, Epstein DJ, St-Jacques B, et al. Sonic hedgehog, a member of a family of putative signaling molecules, is implicated in the regulation of CNS polarity. *Cell* 1993;75:1417-30.
- Ewart JL, Cohen MF, Meyer RA, et al. Heart and neural tube defects in transgenic mice overexpressing the Cx43 gap junction gene. *Development* 1997;124:1281-92.
- Sah VP, Attardi LD, Mulligan GJ, Williams BO, Bronson RT, Jacks T. A subset of p53-deficient embryos exhibit exencephaly. *Nat Genet* 1995;10:175-80.
- Zhang J, Hagopian-Donaldson S, Serbedzija G, et al. Neural tube, skeletal and body wall defects in mice lacking transcription factor AP-2. *Nature* 1996;381:238-41.
- Shum AS, Copp AJ. Regional differences in morphogenesis of the neuroepithelium suggest multiple mechanisms of spinal neurulation in the mouse. *Anat Embryol (Berl)* 1996;194:65-73.
- Macdonald KB, Juriloff DM, Marris MJ. Developmental study of neural tube closure in a mouse stock with a high incidence of exencephaly. *Teratology* 1989;39:195-213.
- Lemire RJ. Neural tube defects. *JAMA* 1988;259:588-62.
- Haque M, Takami T, Soures SB Jr, Aree SN, Hakuba A, Hara M. Development of lumbosacral spina bifida: three-dimensional computer graphic study of human embryos at Carnegie stage twelve. *Pediatr Neurosurg* 2001;35:247-52.
- O'Rahilly R, Müller F. The two sites of fusion of the neural tube and the two neuropores in the human embryo. *Teratology* 2002;65:162-70.
- van Straaten HW, Copp AJ. Curly tail: a 50-year history of the mouse spina bifida model. *Anat Embryol (Berl)* 2001;203:225-37.
- Copp AJ, Seller MJ, Polani PE. Neural tube development in mutant (curly tail) and normal mouse embryos: the timing of posterior neuropore closure in vivo and in vitro. *J Embryol Exp Morphol* 1982;69:151-6.
- Copp AJ. Relationship between timing of posterior neuropore closure and development of spinal neural tube defects in mutant (curly tail) and normal mouse embryos in culture. *J Embryol Exp Morphol* 1985;88:39-54.
- van Straaten HW, Hekking JW, Copp AJ, Bernfield M. Deceleration and acceleration in the rate of posterior neuropore closure during neurulation in the curly tail (ct) mouse embryo. *Anat Embryol (Berl)* 1992;185:169-74.
- Harris MJ. Why are the genes that cause risk of human neural tube defects so hard to find? *Teratology* 2001;63:165-6.
- Czeizel AE, Dudas I. Prevention of the first occurrence of neural-tube defects by periconceptional vitamin supplementation. *N Engl J Med* 1992;327:1832-5.
- Shaw GM, Schaffer D, Velie EM, Morland K, Harris JA. Periconceptional vitamin use, dietary folate, and the occurrence of neural tube defects. *Epidemiology* 1995;6:219-26.
- Finnell RH, Greer KA, Barber RC, Piedrahita JA, Shaw GM, Lammer EJ. Neural tube and craniofacial defects with special emphasis on folate pathway genes. *Crit Rev Oral Biol Med* 1998;9:38-53.
- Ramsbottom D, Scott JM, Molloy A, et al. Are common mutations of cystathionine beta-synthase involved in the aetiology of neural tube defects? *Clin Genet* 1997;51:39-42.
- Morrison K, Papapetrou C, Hol FA, et al. Susceptibility to spina bifida: an association study of five candidate genes. *Ann Hum Genet* 1998;62(pt 5):379-96.
- Hol FA, van der Put NM, Geurds MP, et al. Molecular genetic analysis of the gene encoding the trifunctional enzyme MTHFD (methylene tetrahydrofolate-dehydrogenase, methenyltetrahydrofolate-cyclohydrolase, formyltetrahydrofolate synthetase) in patients with neural tube defects. *Clin Genet* 1998;53:119-25.
- Heil SG, Van der Put NM, Waas ET, den Heijer M, Trijbels FJ, Blom HJ. Is mutated serine hydroxymethyltransferase (SHMT) involved in the etiology of neural tube defects? *Mol Genet Metab* 2001;73:164-72.
- Shaw GM, Lammer EJ, Zhu H, Baker MW, Neri E, Finnell RH. Maternal periconceptional vitamin use, genetic variation of infant reduced folate carrier (A80G), and risk of spina bifida. *Am J Med Genet* 2002;108:1-6.
- Barber RC, Shaw GM, Lammer EJ, et al. Lack of association between mutations in the folate receptor-alpha gene and spina bifida. *Am J Med Genet* 1998;76:310-7.
- De Marco P, Moroni A, Merello E, et al. Folate pathway gene alterations in patients with neural tube defects. *Am J Med Genet* 2000;95:216-23.
- Trembath D, Sherbondy AL, Vandyke DC. Analysis of selected folate pathway genes, PAX3, and human T in a Midwestern neural tube defect population. *Teratology* 1999;59:331-41.
- Saikawa Y, Price K, Hance KW, Chen TY, Elwood PC. Structural and functional analysis of the human KB cell folate receptor

- gene P4 promoter: cooperation of three clustered Sp1-binding sites with initiator region for basal promoter activity. *Biochemistry* 1995;34:9951–61.
32. Barber R, Shalat S, Hendricks K, et al. Investigation of folate pathway gene polymorphisms and the incidence of neural tube defects in a Texas hispanic population. *Mol Genet Metab* 2000;70:45–52.
 33. Frosst P, Blom HJ, Milos R, et al. A candidate risk factor for cardiovascular disease: a common mutation in methylenetetrahydrofolate reductase. *Nature Genet* 1995;10:111–3.
 34. van der Put NMJ, van den Heuvel LP, Steegers-Theunissen RPM, et al. Decreased methylenetetrahydrofolate reductase activity due to the 677 C-T mutation in families with spina bifida offspring. *J Mol Med* 1996;74:691–4.
 35. Botto LD, Yang Q. 5-10 Methylenetetrahydrofolate reductase variants and congenital anomalies: a HuGE review. *Am J Epidemiol* 2000;151:862–77.
 36. Shaw GM, Rozen R, Finnell RH, Wasserman CR, Lammer EJ. Maternal vitamin use, genetic variation of infant methylenetetrahydrofolate reductase, and risk for spina bifida. *Am J Epidemiol* 1998;148:30–7.
 37. Martinez de Villarreal LE, Delgado-Eneiso I, Valdez-Leal R, et al. Folate levels and N(5),N(10) methylenetetrahydrofolate reductase genotype (MTHFR) in mothers of offspring with neural tube defects: a case-control study. *Arch Med Res* 2001;32:277–82.
 38. Volcik KA, Blanton SH, Tyerman GH, et al. Methylenetetrahydrofolate reductase and spina bifida: evaluation of level of defect and maternal genotype risk in Hispanics. *Am J Med Genet* 2000;95:21–7.
 39. Juriloff DM, Harris MJ. Mouse models for neural tube closure defects. *Hum Mol Genet* 2000;9:993–1000.
 40. Kamen BA, Wang MT, Streckfuss AJ, Peryea X, Anderson RG. Delivery of folates to the cytoplasm of MA104 cells is mediated by a surface membrane receptor that recycles. *J Biol Chem* 1988;263:13602–9.
 41. Weitman SD, Lark RH, Coney LR, et al. Distribution of folate receptor GP38 in normal and malignant cell lines and tissues. *Cancer Res* 1992;52:3396–401.
 42. Piedrahita JA, Oetama B, Bennett GD, et al. Mice lacking the folic acid-binding protein Folbp1 are defective in early embryonic development. *Nat Genet* 1999;23:228–32.
 43. Finnell RH, Spiegelstein O, Wlodarczyk B, et al. DNA methylation in Folbp1 knockout mice supplemented with folic acid during gestation. *J Nutr* 2002;132(suppl 8):2457S–61S.
 44. Rosenquist TH, Ratashak SA, Selhub J. Homocysteine induces congenital defects of the heart and neural tube: effect of folic acid. *Proc Natl Acad Sci USA* 1996;93:15227–32.
 45. Steegers-Theunissen R, Boers G, Tribels FJ, Eskes TK. Neural-tube defects and derangement of homocysteine metabolism. *N Engl J Med* 1991;324:199–200.
 46. Langman LJ, Cole DE. Homocysteine. *Crit Rev Clin Lab Sci* 1999;36:365–406.
 47. Selhub J. Homocysteine metabolism. *Annu Rev Nutr* 1999;19:217–46.
 48. Gelineau-van Waes J, Finnell RH. Genetics of neural tube defects. *Semin Pediatr Neurol* 2001;8:160–4.
 49. Dean JC, Moore SJ, Osborne A, Howe J, Turnpenny PD. Fetal anticonvulsant syndrome and mutation in the maternal MTHFR gene. *Clin Genet* 1999;56:216–20.
 50. Leclerc D, Campeau E, Goyette P, et al. Human methionine synthase: cDNA cloning and identification of mutations in patients of the cblG complementation group of folate/cobalamin disorders. *Hum Mol Genet* 1996;5:1867–74.
 51. Ma J, Stampfer MJ, Christiansen B, et al. A polymorphism of the methionine synthase gene: association with plasma folate, vitamin B12, homocysteine, and colorectal cancer risk. *Cancer Epidemiol Biomarkers Prev* 1999;8:825–9.
 52. Christiansen B, Arbour L, Tran P, et al. Genetic polymorphisms in methylenetetrahydrofolate reductase and methionine synthase, folate levels in red blood cells, and risk of neural tube defects. *Am J Med Genet* 1999;84:151–7.
 53. van der Put NMJ, Eskes TKAB, Blom HJ. Is the common C677C → T mutation in the methylenetetrahydrofolate reductase gene a risk factor for neural tube defects? A meta-analysis. *Q J Med* 1997;90:111–5.
 54. Brody LC, Baker PJ, Chines PS, et al. Methionine synthase: resolution mapping of the human gene and evaluation as a candidate locus for neural tube defects. *Mol Genet Metab* 1999;67:324–33.
 55. Morrison K, Edwards YH, Lynch SA, Burn J, Hol F, Mariman E. Methionine synthase and neural tube defects. *J Med Genet* 1997;34:958.
 56. Shaw GM, Todoroff K, Finnell RH, et al. Infant methionine synthase variants and risk for spina bifida. *J Med Genet* 1999;36:86–7.
 57. Chen J, Zhang I, Cheng L, Li Y. The effect of polymorphisms of MTHFR gene and vitamin B on hyperhomocysteinemia. *J Tongji Med Univ* 2001;21:17–20.
 58. Rosenblatt DS, Cooper BA, Pottier A, Lue-Shing H, Matiaszuk N, Grauer K. Altered vitamin B12 metabolism in fibroblasts from a patient with megaloblastic anemia and homocystinuria due to a new defect in methionine biosynthesis. *J Clin Invest* 1984;74:2149–56.
 59. Leclerc D, Wilson A, Dumas R, et al. Cloning and mapping of a cDNA for methionine synthase reductase, a flavoprotein defective in patients with homocystinuria. *Proc Natl Acad Sci USA* 1998;95:3059–64.
 60. Wilson A, Leclerc D, Rosenblatt DS, Gravel RA. Molecular basis for methionine synthase reductase deficiency in patients belonging to the cblE complementation group of disorders in folate/cobalamin metabolism. *Hum Mol Genet* 1999;8:2009–16.
 61. Hobbs CA, Sherman SL, Yi P, et al. Polymorphisms in genes involved in folate metabolism as maternal risk factors for Down syndrome. *Am J Hum Genet* 2000;67:623–30.
 62. O'Leary VB, Parle-McDermott A, Molloy AM, et al. MTRR and MTHFR polymorphism: link to Down syndrome? *Am J Med Genet* 2002;107:151–5.
 63. Gaughan DJ, Kluijtmans LA, Barboux S, et al. The methionine synthase reductase (MTRR) A66G polymorphism is a novel genetic determinant of plasma homocysteine concentrations. *Atherosclerosis* 2001;157:451–6.
 64. Zhu H, Wicker NJ, Shaw GM, et al. Double mutants of MTR A2756G and MTRR A66G with increased risk of neural tube defects (NTDs). *Mol Genet Metab* 2003;78:216–21.
 65. Lammer EJ, Sever LE, Oakley GP Jr. Teratogen update: valproic acid. *Teratology* 1987;35:465–73.
 66. Bjerkendal T, Czeizel A, Goujard J, et al. Valproic acid and spina bifida. *Lancet* 1982;2:1096.
 67. Jager-Roman E, Deichl A, Jakob S, et al. Fetal growth, major malformations, and minor anomalies in infants born to women receiving valproic acid. *J Pediatr* 1986;108:997–1004.
 68. Lindhout D, Schmidt D. In-utero exposure to valproate and neural tube defects. *Lancet* 1986;1:1392–3.
 69. Nau H, Hendrickx AG. Valproic acid teratogenesis. *ISI Atlas of Science: Pharmacology* 1987;1:52–6.
 70. Finnell RH, Bennett GD, Karras SB, Mohl VK. Common hierarchies of susceptibility to the induction of neural tube defects in mouse embryos by valproic acid and its 4-propyl-4-pentenoic acid metabolite. *Teratology* 1988;38:313–20.
 71. Nau H. Teratogenic valproic acid concentrations: infusion by implanted minipumps vs conventional injection regimen in the mouse. *Toxicol Appl Pharmacol* 1985;80:243–50.
 72. Niedermeyer E. *Epilepsy guide: diagnosis and treatment of epileptic seizure disorders*. Baltimore-Munich: Urban & Schwarzenberg, 1983.
 73. Ehlers K, Sturje H, Merker H, Nau H. Valproic acid-induced spina bifida: a mouse model. *Teratology* 1992;45:145–54.
 74. Ehlers K, Sturje H, Merker H, Nau H. Spina bifida aperta induced by valproic acid and by all-trans-retinoic acid in the mouse: distinct differences in morphology and periods of sensitivity. *Teratology* 1992;46:117–30.
 75. Wegner C, Nau H. Diurnal variation of folate concentrations in mouse embryo and plasma: the protective effect of folinic acid on valproic-acid-induced teratogenicity is time dependent. *Reprod Toxicol* 1991;5:465–71.
 76. Wegner C, Nau H. Alteration of embryonic folate metabolism by valproic acid during organogenesis: implications for mechanism of teratogenesis. *Neurology* 1992;42(4 suppl 5):17–24.
 77. Martin ML, Regan CM. The anticonvulsant valproate teratogen restricts the glial cell cycle at a defined point in the mid-G1 phase. *Brain Res* 1991;554:223–8.

78. Regan CM, Gorman AM, Larsson OM, et al. In vitro screening for anticonvulsant-induced teratogenesis in neural primary cultures and cell lines. *Int J Dev Neurosci* 1990;8:143–50.
79. Finnell RH, Włodarczyk BC, Craig JC, Piedrahita JA, Bennett GD. Strain dependent alterations in the expression of folate pathway genes following teratogenic exposure to valproic acid in a mouse model. *Am J Med Genet* 1997;70:303–11.
80. Finnell RH, Vacha SJ, Mackler SA. Nucleic acid amplification technologies. In: Daston G, ed. *Molecular and cellular methods in developmental toxicology*. Boca Raton, FL: CRC Press, 1997:93–125.
81. Finnell RH, Junker WM, Wadman LK, Cabrera RM. Gene expression profiling within the developing neural tube. *Neurochem Res* 2002;27:1165–80.
82. Finnell RH, Dansky LV. Parental epilepsy, anticonvulsant drugs, and reproductive outcome: epidemiologic and experimental findings spanning three decades; 1: Animal studies. *Reprod Toxicol* 1991;5:281–99.
83. Hernandez-Diaz S, Werler MM, Walker AM, Mitchell AA. Neural tube defects in relation to use of folic acid antagonists during pregnancy. *Am J Epidemiol* 2001;153:961–8.
84. Rosa FW. Spina bifida in infants of women treated with carbamazepine during pregnancy. *N Engl J Med* 1991;324:674–7.
85. Matalon S, Schechtman S, Goldzweig G, Ornoy A. The teratogenic effect of carbamazepine: a meta-analysis of 1255 exposures. *Reprod Toxicol* 2002;16:9–17.