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Genetic, Epigenetic, and Environmental Contributions to Neural Tube Closure

Jonathan J. Wilde^{1,2,*}, Juliette R. Petersen^{1,3,*}, and Lee Niswander^{1,*}

Jonathan J. Wilde: jonathan.wilde@ucdenver.edu; Juliette R. Petersen: juliette.petersen@ucdenver.edu; Lee Niswander: lee.niswander@ucdenver.edu

¹Howard Hughes Medical Institute, Department of Pediatrics, University of Colorado Anschutz Medical Campus, Children's Hospital Colorado, Aurora, Colorado 80045

²Cell Biology, Stem Cells and Development Graduate Program, University of Colorado Anschutz Medical Campus, Aurora, Colorado 80045

³Molecular Biology Graduate Program, University of Colorado Anschutz Medical Campus, Aurora, Colorado 8004

Abstract

The formation of the embryonic brain and spinal cord begins as the neural plate bends to form the neural folds, which meet and fuse to close the neural tube. The neural ectoderm and surrounding tissues also coordinate proliferation, differentiation, and patterning. This highly orchestrated process is susceptible to disruption, leading to neural tube defects (NTDs), a common birth defect. Here, we highlight genetic and epigenetic contributions to neural tube closure. We describe an online database we created as a resource for researchers, geneticists, and clinicians. Neural tube closure is sensitive to environmental influences, and we discuss disruptive causes, preventative measures, and possible mechanisms. New technologies will move beyond candidate genes in small cohort studies toward unbiased discoveries in sporadic NTD cases. This will uncover the genetic complexity of NTDs and critical gene-gene interactions. Animal models can reveal the causative nature of genetic variants, the genetic interrelationships, and the mechanisms underlying environmental influences.

Keywords

embryonic brain; embryonic spinal cord; gene-environment interactions; neural tube defects

OVERVIEW OF EMBRYONIC NEURAL TUBE CLOSURE

Neural Tube Closure: The Embryonic Beginning of the Brain and Spinal Cord

The embryonic brain and spinal cord start with the formation of a simple tube. The neural tube (NT) forms when the flat sheet of neuroepithelial cells, bordered by cells that become

*Co-first authors and equal contribution

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the epidermis, rolls up and seals together to form the closed neural tube covered by a layer of surface ectoderm (Figure 1*a–d*). Closure occurs in a progressive manner, starting at the caudal hindbrain/rostral spinal cord and then proceeding through the head and down along the spinal cord between week 3 and 4 of human gestation (Figure 1*e–g*) (embryonic day E8.75–10 in mouse).

The deceptively simple tube formation requires highly orchestrated activities, including dramatic tissue movements, especially in the brain region with its large neural folds, tight coordination between numerous cellular and molecular processes, and extensive interactions between the neural ectoderm, mesenchyme, and surface ectoderm across time and space. A few examples of individual activities are presented here. Cell proliferation must be tightly regulated and coordinated with cell differentiation: Failure of NT closure is associated with gene mutations that cause either too little or too much cell division or with alterations in the timing of differentiation [for example, *Neurofibromin*, *Pax3*, *Phactr4*, *Jumonji*, *Notch* pathway (36, 69, 70, 171)]. Cell movements and changes in cell shape critically drive the process of NT closure [see planar cell polarity (PCP) pathway below and cytoskeletal proteins such as SHROOM3, VINCULIN, COFILIN, and MENA (36, 69)]. Patterning of the neural tissue occurs during NT closure, and disruption of key patterning pathways are associated with failure of NT closure [e.g., Sonic Hedgehog signaling, bone morphogenetic protein (BMP) signaling, and retinoid signaling (36, 69)]. Movement of the neural folds toward one another requires physical forces generated by the neural, mesenchymal, and surface ectoderm tissues (3, 111, 121). The complexity of these tissue interactions is perhaps best highlighted by the final step in NT closure. As the neural folds rise up and approach each other across the physical gap, the neural ectoderm and surface ectoderm must release their contact with one another and then rapidly reestablish contact with their cognate tissue layer on the other neural fold to seal the neural tube and cover the NT with a single sheet of ectoderm (133). Concomitantly, the neural crest cells, which arise at the border between the neural and surface ectoderm, undergo an epithelial to mesenchymal transition and migrate away, whereas the neighboring neural ectoderm and surface ectoderm must maintain their epithelial character. Overall, the actions of many genes that regulate multiple cell biological and molecular events need to be tightly coordinated in time and space for proper NT closure.

Neural Tube Defects

As might be expected for such an exquisitely coordinated and dynamic morphogenetic process, NT closure is highly sensitive to perturbations, and these can result in neural tube defects (NTDs), a common and devastating birth defect (Figure 2). Failure to close the NT in the cranial region (exencephaly or called anencephaly after degradation of the exposed neural tissue) leads to death before or at birth. Infants born with caudal NTDs (e.g., myelomeningocele or spina bifida) have increased risk of mortality, and those that survive often face life-long disabilities and neurologic, cognitive, urologic, and gastrointestinal complications. NTDs occur in ~1 in 1,000 live births in the United States and resulted in 71,000 deaths globally in 2010 (28, 105). Therefore, understanding neural tube development and the causes of NTDs are among the most important health-related studies today.

The embryo grows rapidly during the period of NT closure. If the neural folds fail to rise up and come together within the correct time frame, it is likely that continued growth of the embryo will result in neural folds that are too far apart to close. It is possible that even minor changes in the timing of gene function could affect the movement and meeting of the neural folds and result in NTDs. Moreover, the highly orchestrated activities during NT closure indicate the involvement of numerous genes in this critical embryonic process. Indeed, the great majority of NTDs in humans are thought to have a multifactorial and complex etiology in which disturbances in more than one gene affect closure. Moreover, environmental factors can also alter the risk of NTDs, and this is discussed later in the review. Next, we discuss the current state of understanding of the genetic causes of NTDs, which has largely been driven by studies in animal models.

GENETIC REGULATION OF NEURAL TUBE CLOSURE

A few genetic syndromes are associated with NTDs in humans, including trisomy for chromosome 3, 18, and the X chromosome, although the responsible gene or genes that are dosage sensitive on these chromosomes are unknown (57). In the general population, the genetic risk for having a second child with an NTD is only 2–5% (40, 81). This suggests that differences in genetic makeup can affect the penetrance of a phenotype. Indeed, in mouse models, genetic background can strongly affect NTD risk, and the search for modifier loci has been an ongoing effort (69, 70, 86, 93, 95).

This genetic complexity is acknowledged, but as a starting point to understand the genetic contributions to NT closure, the efforts to date have largely focused on single gene mutations. NT closure in the mouse is similar to human neurulation at the cellular, tissue, and genetic levels, and is the favored animal model for extrapolation to humans. Research in the mouse, from classical mouse NTD mutants to targeted mutations and random mutagenesis screens, has provided considerable insights into the genes that are critically required for NT closure. Indeed, several hundred genes have been identified whose functions are required for NT closure in the mouse embryo (61, 69, 70), and this number is expected to increase as research in this field expands. These data have informed human studies, starting from the search for candidate genes in small cohorts of NTD patients to recent whole-genome or exome sequencing of NTD-affected individuals. Some prominent examples are the genes involved in the formation and signaling of cilia and the PCP pathway (see below). We also discuss the folic acid (FA) metabolism pathway, which, despite years of study and because of the action of FA in preventing NTDs, has provided very few genes that have been conclusively associated with human or mouse NTD etiology. As outlined below, it is critical to move between human and animal cell studies to functionally interpret the human data and to move toward an understanding of complex gene interactions.

Cilia and the Hedgehog Signal Transduction Pathway

Cilia are hair-like extensions from the cell surface that regulate signal transduction by mechanical and molecular cues. The evidence for a role of genes involved in cilia formation and function in NT closure converged from both human and mouse studies. In humans, NTDs have been associated with several syndromes, including Meckel-Gruber Syndrome and Joubert Syndrome (94, 103). Mapping and cloning of the genes causative for these

syndromes, and the creation of mouse genetic models that recapitulate many of the clinical features, has highlighted the importance of cilia in key embryonic and adult processes, including NT closure. Functional studies in human cells and animal systems also showed the requirement for cilia in key developmental signaling pathways, most prominently the Sonic Hedgehog pathway during NT formation (2, 91, 103, 177). In the mouse, approximately 5% of the known NTD genes are related to cilia formation and function, providing a rich source of gene candidates for analysis in human NTD samples that is only beginning to be explored (34).

Planar Cell Polarity Pathway

Over the past few years, a candidate gene approach has driven the identification of human NTD genes. A prominent focus has been on the PCP or noncanonical WNT pathway. The PCP pathway provides a prime example to illustrate insights gained, as well as the challenges and open questions discovered, from studies of human NTDs and animal models. PCP core proteins, such as VANG1/2, DISHEVELLED, CELSR1, and SCRIBBLE, and PCP effector proteins, such as FUZZY and INTURNED, have been widely implicated in driving critical cell movements during NT closure. Research in frog, mouse, and chick embryos has shown that the PCP pathway controls convergent extension cell movements, which help to lengthen the embryo along the rostral-caudal axis and narrow the neural plate (170, 185). Disruptions in the PCP pathway result in a wide neural plate, which prevents the neural folds from contacting each other. This results in the most severe NTD, which is called craniorachischisis, wherein the neural tube remains open from the brain to the spinal cord. On the basis of this severe phenotype in animal models, studies have sought to determine whether craniorachischisis in humans is also associated with mutations in the PCP pathway. Interestingly, mutations in PCP genes have been identified in human fetuses with craniorachischisis (82, 137), highlighting the importance of animal models in informing human NTD studies.

Craniorachischisis may be the rare case in which a specific pathway can be implicated in the etiology of an NTD. However, it is becoming apparent that mutations in PCP genes are also associated with a broader range of NTDs, including sporadic cases of cranial, spinal, and open and closed NTDs. Sequencing of PCP genes in a larger cohort of patients with NTDs has identified rare variants in PCP genes (82, 84). This highlights the challenges in predicting genotype/phenotype relationships. In general, if failed NT closure is the ultimate observational consequence and the initiating event is unknown, it is unlikely that strong predictions can be made as to the causative genetic defect. Similarly, NTDs that occur at a specific rostral-caudal level may not mean that the gene has a particular function in that region. There is evidence to argue for this, as well as evidence that argues for a more complex situation, including cases in which the same genetic mutation in the same genetic background may result in cranial NTD in one animal and caudal NTD in another (69, 70). Even the evidence in favor of region-specific function may be overturned as additional NTD samples are analyzed, new alleles are found, and gene-gene interactions are discovered. In reality, it may remain difficult to predict precise genotype/phenotype relationships. Nonetheless, the gene candidates revealed by animal studies provide a solid foundation for

human genetic studies, although the penetrance, expressivity, and regional specificity may depend on other genetic and environmental factors.

Unbiased Determination of the Genetics Underlying Neural Tube Defects in Humans

Considering the large spectrum of genes identified in animal models and the multigenic nature of human NTDs, significant advances in understanding the genetics of NTDs will require moving beyond single candidate gene studies to discovery-based approaches, including high-throughput sequencing of large cohorts of patients. With the decreasing costs of exome and whole-genome sequencing, investigators are beginning to explore the genomes of NTD-affected individuals and families. It is not yet known how copy number variants (CNVs) may contribute to NTDs, beyond a few genetic syndromes associated with NTDs (trisomy for chromosome 3, 18, or the X chromosome) and the recent association between CNVs in cilia genes in NTD samples (34). Protein coding mutations and potential regulatory mutations will likely form the first wave of functional analyses. However, as can be expected from studies of other human diseases, numerous gene variants will be identified and the challenge will be to evaluate the data and define possible causative mutations. Notably, the set of genes identified in animal NTD models will provide an invaluable framework to begin to decipher these large genomic data sets. Moreover, it will be important to return to animal models or simpler systems, such as cultured cells, to functionally validate gene variants.

The greater promise of the comprehensive view of the genome is the ability to reveal potential gene-gene interactions and to open avenues to interrogate the combinatorial effects of polymorphisms in NTD pathway genes across the genome. However, the challenges increase almost exponentially because of the potential for combinatorial interactions. Importantly, new technologies such as CRISPR will allow rapid genome editing, such that multiple genetic variants can be created in cells or animals to begin to decipher genetic relationships. Overall, in sporadic cases of NTDs, it is unlikely that the NTD results from a single gene mutation but rather may be attributable to the combinatorial effect of multiple genes, each with small contributions, including changes in gene and/or protein levels in time and space, in conjunction with epigenetic and environmental effects, which are discussed below.

Creation of a Publicly Available Repository of Genes Whose Function Is Required for Neural Tube Closure

One goal of this review is to establish an online, publicly available repository to provide a current and continually updated list of genes implicated in NT closure based on mutations that cause NTDs. The ultimate goal is for the NTD scientific community to contribute their information and insights to the online database to enhance the flow of knowledge between researchers, geneticists, clinicians, and epidemiologists, and to inform ongoing studies of human NTD patients. We gratefully acknowledge Dr. Muriel Harris and Dr. Diana Juriloff for their exceptional reviews (69, 70), which have provided the initial foundation of the online database, and Dr. Claudia Kappen and Dr. Michael Salbaum for providing an extraction of the MGI database related to NTDs (141). The link for the NTD Wiki database is <http://ntdwiki.wikispaces.com>.

With more than 300 genes already identified as critically required for NT closure, it may seem like a daunting prospect to attempt to understand this complex embryonic process. However, through the use of well-defined molecular markers, histology, and even newly developed live-imaging systems to visualize the dynamic cell and tissue movements of NT closure (111), it is possible to define the function of even novel genes in the process of NT closure. Thus, a framework is established to categorize the functions of new genes, and we predict that the major pathways and processes involved in NT closure have been identified. The field is coming to the point at which it should be possible to take a systems-level approach to help decipher the genomic information that is forthcoming from genome sequencing efforts.

EPIGENETIC REGULATION OF NEURAL TUBE CLOSURE

Genome-Wide Contributions to Human Neural Tube Defects

A prominent mechanism in which gene expression can be modified globally is through the action of epigenetic regulators. The mammalian epigenome consists of all modifications to the genome that confer heritable changes in gene function independently of nucleotide sequence. This includes, but is not limited to, DNA methylation, chromatin modification, and nucleosome repositioning. Mutations in a growing number of epigenetic regulators in mice have been shown to result in NTDs (Table 1), suggesting that coordinate regulation of transcriptional networks is required for proper NT closure. These observations highlight the potential of animal models to uncover pathways that will inform a global view of human NTD etiology. This, in combination with advancing technologies in whole-genome sequencing, gene expression analysis, and epigenetic profiling, provides a means for better understanding of the genetic causes of NTDs in humans. Moreover, epigenetic-related hypotheses extend to the mechanisms of FA-induced NTD prevention (discussed below) and the NTD risk conferred by disease treatments with the histone deacetylase (HDAC)-inhibitor valproic acid (136). Thus, the role of the epigenome in NT closure is of particular interest. Here, we focus on the mechanisms of epigenetic modification as contributing factors to proper closure of the mammalian neural tube.

Ground Level Changes: DNA Methylation

DNA methylation is a direct chemical modification of DNA that alters transcription of target genes via proposed mechanisms involving changes in stabilization of transcription factors and alteration of chromatin structure (115). Methylation occurs at CpG dinucleotides, is mediated by a class of enzymes known as DNA methyltransferases, and is dynamically regulated during mammalian development. Prior to implantation of the embryo, the majority of the embryonic genome, with the exception of imprinted regions, undergoes complete demethylation and is subsequently remethylated by the de novo DNA methylases DNMT3A and DNMT3B, and is maintained by DNMT1 (19). These events result in stable methylation of the majority (>90%) of CpG dinucleotides across the genome in all cell types (161). However, regions with high concentrations of CpG dinucleotides, called CpG islands, remain relatively unmethylated and are the main targets of DNA methylation-mediated transcriptional regulation (115).

Although there is a wealth of data supporting the role of CpG island methylation in transcriptional repression, recent data have painted a more complex picture. In addition to acting as a strong epigenetic suppressor of transcription through destabilization of transcription factors with their target promoters, CpG methylation of distal regulatory elements and distant promoters has been linked to binding of sequence-specific transcription factors (17). These data together support an integral role of CpG methylation in genome-wide transcriptional regulation. Moreover, due to its reversible nature, DNA methylation allows for dynamic tuning of transcriptional programs. Indeed, numerous human diseases have been linked to defects in DNA methylation and its dynamics, including Prader-Willi syndrome [loss of genomic imprinting, which confers monoallelic, parent-of-origin specific expression of genes (26)] and Rett syndrome [loss of *Mecp2* which reads the methylation state of DNA (4)]. To date, DNA methylation defects have been identified in developmental disorders, cancer, diabetes, and heart failure, suggesting that they may underlie many common human disorders (16, 63).

With respect to NTDs, mouse models lacking the de novo DNA methyltransferases DNMT3A and DNMT3B exhibit cranial NTDs, demonstrating that proper remethylation of the genome after implantation is essential for NT closure (123). The closely related DNMT3L is also required for NT closure; however, its function in DNA methylation remains poorly understood beyond its involvement in maternal imprinting (71). Other null mouse models for methyltransferases such as DNMT1, however, have been less informative about the role of DNA methylation in NT closure, largely because of early embryonic death (98). Several genes involved in one-carbon metabolism (OCM), which provides methyl groups for DNA methylation, have been studied in human NTDs; however, we only touch on these below, as they have been extensively reviewed by Greene et al. (62). Together, these data, in conjunction with the knowledge that the majority of CpG dinucleotides in the genome outside of CpG islands are constitutively methylated, suggest that methylation-related NTDs may be due to disruption of methylation at specific CpG islands. In future studies of mouse models and human NTD patients, it will be important to utilize next-generation sequencing technologies in conjunction with methylation-specific analysis to uncover the epigenetic code that helps to drive NT closure.

Altering Genomic Topology: Histone Modifications

Within the nucleus, DNA is packaged via its association with histone octamers called nucleosomes. Each nucleosome is wrapped by 147 base pairs of DNA and consists of four homodimers of the core histone proteins H2A, H2B, H3, and H4 that extend N-terminal tails from the core of the nucleosome into the nucleus (9). Histone tails can be modified with eight different covalent post-translational modifications and more than 60 different modification sites on histone tails have been identified, but this underestimates the repertoire of histone modifications, as many residues undergo di- and trivalent modification, adding to the total possible number of alterations (88). The most commonly studied histone modifications, acetylation and methylation, are generally involved in transcriptional activation and repression, respectively. However, the actual effect of any given modification depends upon the modified residue, as well as the state of neighboring residues. The predominant theory as to how these modifications affect downstream transcription is that

they alter the way that nucleosomes interact with each other (88). More specifically, they either promote or antagonize interactions between nucleosomes, therefore altering the packaging of DNA and the accessibility of transcriptional machinery to its target genomic regions (9). It is therefore reasonable to surmise that defects in the enzymatic pathways responsible for adding and removing these chemical modifications can lead to severe cellular and developmental phenotypes.

Mouse NTD models have implicated several chromatin-modifying enzymes in NT closure. Most notably, the histone acetyltransferases (HATs) GCN5 and CBP/p300, which play critical roles in transcriptional activation, are each required for NT closure (22, 128). Conversely, inhibition of HDACs with the drugs valproic acid and trichostatin A (TSA) disrupts NT closure in animal models and humans (118, 136). The H3K27me3 histone demethylase UTX is required for NT closure (179), as is the histone demethylase FBXL10 (52), but there is no direct evidence that histone methyltransferases are needed for NT closure (70). However, the polycomb repressive complex 2 (PRC2) component Jumonji, which plays a role in loading PRC2 onto target loci, is required for NT closure (162), and PRC2 has known roles in transcriptional repression through histone methylation (148). Nonetheless, the bias toward mutation of genes involved in histone acetylation and demethylation in NTDs is interesting, as histone acetylation is thought to be far more dynamic than histone methylation. The requirement for histone acetylation and histone demethylation, which often precede transcriptional activation, suggests that regulation of dynamic changes in chromatin structure and transcriptional activation is more important for proper NT closure than stable repression of gene expression through histone methylation. This is consistent with the highly dynamic yet coordinated cellular and molecular programs that drive changes in morphology, patterning, proliferation, and differentiation during NT closure.

It has been difficult to identify the underlying molecular mechanisms by which mutations in the aforementioned enzymes lead to NTDs. In the case of CBP/p300, it is possible that its role in neurulation is, in part, related to regulation of metabolic genes, as the interacting protein CITED2 is also required for NT closure and is thought to act through mechanisms that regulate cellular responses to hypoxia and energy requirements (187). Interestingly, CITED2 can regulate glucose homeostasis through PGC-1 α (140), a direct target of acetylation by GCN5 (96). Additionally, p300 has been linked to regulation of hepatic glycogen storage (72). Although the underlying cause of NTDs in mice that lack GCN5 acetyltransferase activity (*Gcn5^{hat}*) has yet to be uncovered, GCN5 plays important roles in glucose homeostasis and stress response (27, 96, 119) and the convergence of these data with those related to CBP/p300 and CITED2 and NTDs suggests that these complexes may play essential roles in regulating specific metabolic pathways that are required for NT closure. This is especially intriguing considering the NTD risk associated with maternal diabetes, which is discussed below.

Histone acetylation also appears to control the expression of patterning molecules that are essential for NT closure. By treating mouse embryos with the HDAC inhibitor TSA, it was discovered that class I and II HDAC activity is required to inhibit BMP2/4 signaling in the developing forebrain (149). Embryos treated with TSA display a distinct shift in the fate of

neural progenitor cells, and defects in neurogenesis can cause NTDs (33). Our unpublished microarray data from *Gcn5^{hat/hat}* mice also suggest defects in programs of neural differentiation, indicating that HATs and HDACs may play multiple roles within the developing neural tube and that combinatorial fine-tuning of these transcriptional programs is required to coordinate the complex morphological events that are the basis of primary neurulation. The developing NT seems particularly susceptible to changes in chromatin architecture and its dynamic remodeling, as HAT mutants are exencephalic but otherwise largely anatomically normal at the time of NT closure (22, 128).

Moving Roadblocks: Nucleosome Positioning

In addition to regulation of nucleosome aggregation via histone modification, transcriptional output is also modified via deposition and repositioning of nucleosomes along chromosomes. Nucleosome repositioning is carried out by ATP-dependent chromatin-remodeling complexes. These can shift nucleosomes to different chromosomal positions to regulate transcription by protecting the nucleosome-associated DNA from binding with transcriptional machinery (157). Genome-wide studies of nucleosome positioning have revealed that positioning can vary greatly, even within a single cell type (157). Nonetheless, it is clear that nucleosome positioning plays an important role in transcriptional regulation in conjunction with DNA methylation and histone modifications. It is therefore of no surprise that enzymes responsible for nucleosome localization are required for embryonic development and, more specifically, NT closure.

The SWI/SNF-related nucleosome remodeling BAF complex appears to play a specific role in NT closure, as mutations in multiple subunits result in NTDs in mouse. Both the core catalytic component BRG1 and the complex subunit BAF155 are required for NT closure, suggesting that proper nucleosome positioning by this complex is required for neurulation (23, 67). Complete loss of *Brg1* causes early embryonic death, highlighting the importance of nucleosome positioning in general development, but loss of a single allele results in, among other defects, exencephaly (23). An allele of BAF155 that forms an assembled but malfunctioning BAF chromatin-remodeling complex results in exencephaly and decreased neural cell survival and proliferation, yet a surprisingly small number of genes were dysregulated (67). The function of BAF155 is still not well understood. Although it is thought to be a core component of the BAF complex in all cells, it is unclear whether this BAF155 allele causes defects in nucleosome repositioning. Other studies of BAF155 function in ES cells, however, have demonstrated a direct role in regulating repression of self-renewal genes, which may help explain its roles in regulating neural development, proliferation, and cell survival (146).

Additional components of nucleosome remodeling complexes, as well as miRNAs that regulate such complexes, have also been implicated in NTDs. CECR2, which forms a specific chromatin-remodeling complex with SNF2L, causes exencephaly when mutated (10). Additionally, NAP1L2, a neuronal-specific protein closely related to nucleosome assembly factors, promotes proliferation of neuroepithelial cells and is required for cranial NT closure (138). As is the case with DNA methylation and histone modification, dynamic changes in nucleosome positioning are also important for cellular function and these

changes can be mediated by switching of subunits in chromatin-remodeling complexes (66). As such, changes in the activity of miR-9* and miR-124a cause defects in subunit switching of ATP-dependent chromatin-remodeling complexes, resulting in spina bifida in rats (178).

As may be expected, epigenetic regulators do not function independently of one another. NAP1L2, for example, can promote acetylation of H3K9 and H3K14 and loss of NAP1L2 causes overproliferation of neural stem cells, resulting in inadequate neural differentiation that has been attributed to NAP1L2 promotion of histone acetylation at the *Cdkn1c* promoter (8). Although the exact mechanistic attributes of NAP1L2 have not been discovered, it does associate with nucleosome assembly proteins such as NAP1L1 and NAP1L4 (7), suggesting that nucleosome assembly and positioning are tightly correlated with histone modification at specific loci. As another example, local demethylation of DNA is directed by the acetylation state of specific residues on locally positioned nucleosomes (31). Altogether, these concepts strongly support highly regulated mechanisms for altering genomic structure and function through multiple interconnections between epigenetic regulators.

Distal Regulatory Elements and Their Functions

In addition to gene regulatory elements located in close proximity to gene bodies, transcription is modified by distal regulatory elements. These elements include enhancers, locus control regions, insulators, and silencing elements (73). Recent data from the ENCODE project suggest that these elements are highly promiscuous, with some distal elements interacting with up to 10 different transcriptional start sites (144). Promoters also interact with numerous distal regulatory elements, even within a single cell type (144). It is therefore reasonable to hypothesize that coordinate regulation of genomic architecture is required within each cell type to bring the proper regulatory elements in close proximity to their target promoters. A theme that emerges when examining chromatin-remodeling complexes is the diversity of subunits seen within a given complex. Recent data suggest that these variable subunits may play important roles in recognizing these regulatory elements. For example, CHIP-seq experiments demonstrate that SAGA and ATAC, the two complexes in which the HAT GCN5 is the major enzymatic subunit, have significant overlap in their preferences for promoters, but significant divergence with respect to their regulatory element localization (89). Thus, SAGA and ATAC may function similarly to promote transcription via acetylation of histone residues near active promoters, but the two complexes may have tissue-specific gene regulatory functions mediated by specific preferences for distal regulatory elements. As another example, the HAT PCAF can compensate for GCN5, although these two HATs have differing affinities for their cognate regulatory complexes, and this may partially explain why *Gcn5^{hat/hat}* mutants show such specific defects in NT closure (22).

The importance of distal regulatory elements in development may also be an underlying reason why it has been difficult to identify causes for human NTDs. The discovery of genomic elements such as super enhancers that control complex traits (78) suggests that sequence variability within distal regulatory elements that control multiple aspects of neurulation could contribute to NTD risk. Unfortunately, the promiscuity of these elements, as well as data showing that distal elements interact with the nearest promoter only 7% of

the time (144), makes it extremely difficult to infer the downstream effects of sequence variability within a human regulatory element. Moreover, distal regulatory elements coincide with CpG dinucleotides whose methylation states are crucial for regulation of broad gene regulatory networks and maintenance of cellular health (5). This could help explain how distal element-specific localization of histone modification complexes interfaces with DNA methylation to regulate large transcriptional networks that are required for NT closure.

Transcriptional Variability in Development and Disease

Although the above-mentioned mechanisms are robust and redundant, transcriptional output is still subject to significant noise. This transcriptional noise might be expected to have profound negative effects upon development, but it has been postulated that transcriptional stochasticity actually plays an important role in proper development (130). During the progression from embryonic stem cells to differentiated cells, the mammalian genome undergoes massive rearrangements that require active repression of stemness genes, temporary maintenance of poised epigenetic states for differentiative genes, and activation of genes that give differentiated cells their individual traits. This process has been well-characterized in the developing nervous system, where neural stem cells undergo multiple differentiative events to give rise to more committed progenitors that eventually contribute to the diverse neuronal and glial cell populations (76). It therefore seems necessary for mechanisms that regulate stochastic gene expression within stem and progenitor cells to both allow for differentiation into multiple cell types and to repress unwanted cellular characteristics. It is hypothesized that chromatin modifiers are the main regulatory elements used to maintain order within the required transcriptional noise of development (130).

Variability of gene expression during development poses a significant problem, however, if elements that keep stochasticity in check are disrupted, which can lead to phenotypic changes. This concept was beautifully demonstrated in a paper by Raj et al. (131) that identified increased variability in gene expression as the underlying cause of incomplete phenotypic penetrance. Interestingly, gene expression data sets from the *Baf155* (67) and *Gcn5^{hat}* (J. Wilde & L. Niswander, unpublished results) mouse models of NTDs indicate that individual mutant embryos display significantly different gene expression patterns from one another, suggesting that GCN5 and the BAF complexes play important roles in controlling variability of gene expression during NT closure. As reviewed by Pujadas & Feinberg (130), the epigenome is particularly susceptible to variability, leading to the hypothesis that tight regulation of chromatin structure and dynamics is required during neurulation. This may be especially true in the developing cranial neural tube, as functional loss of almost all epigenetic modifiers identified in animal models of NTDs causes exencephaly (Table 1). This could be due to the fact that the closing cranial NT simultaneously undergoes some of the most complex and dynamic morphogenetic, patterning, and differentiative events during embryogenesis. These processes may necessitate that specific regions of the genome remain extremely plastic, through modulation of chromatin structure and inherent transcriptional variability. Future studies, therefore, may warrant a deeper look into the relationships between transcriptional variability and the regulation of chromatin structure in mouse models of NTDs.

ENVIRONMENTAL CONTRIBUTIONS TO NEURAL TUBE CLOSURE

Environmental Alteration of the Genomic Landscape

Growing evidence now suggests that environmental factors have the ability to alter the epigenetic landscape and, therefore, transcriptional activity. Several lines of study using the agouti viable yellow (A^{vy}) mouse have shown that exposure to endocrine disruptors, dietary changes, and toxic compounds can cause phenotypic changes in offspring owing to disrupted DNA methylation (47). Interestingly, gestation appears to be particularly sensitive to environmental changes, as maternal folate intake during gestation induces differential DNA methylation and phenotypic changes in A^{vy} offspring but not in their mothers (176). Even in normal animals, long-term methyl-enriched diets can lead to profound changes within the epigenome, specifically, increased transcriptional variability (97). These studies have added to a growing amount of literature suggesting that maternal diet is tightly linked to the epigenome of the offspring. This link is not just important for development, however, as rats born from mothers with suboptimal nutrition have a significantly increased risk for developing type II diabetes (143), and mice born from mothers on a low-protein diet show disrupted *Ppara* expression (102); both phenotypes were shown to be associated with alterations in the epigenome. Together, these data strongly support a significant epigenetic role for maternal nutrition in the proper development of offspring and suggest that epigenetic fidelity is imperative for proper NT closure.

Folic Acid Fortification: Reducing the Incidence of Neural Tube Defects

Although there is clearly a genetic component to NTDs, numerous environmental factors have also been strongly implicated in NTD etiology. One of the earliest records correlating environmental risks with NTDs comes from an eighteenth-century midwife, Catherina Schrader. Her exceptional records showed two clusters of increased NTD incidence: the first following unusually poor crop yields and the second primarily in lower-class, urban families, implicating socioeconomic status, nutrition, and possibly folate deficiency in the incidence of NTDs in eighteenth-century Holland (113). Since then, gene-environment interactions have been implicated in numerous diseases, including diabetes, metabolic syndrome, neurological diseases, and numerous cancers (156). As mentioned above, maternal diet can affect the developing embryo, and one of the best-studied dietary factors relative to NT closure is maternal folate.

Folate deficiency was suggested as a risk factor for early fetal death in the 1950s (163, 164), when the folate antagonist, aminopterin, was used to induce therapeutic abortions. Women who failed to abort with aminopterin treatment gave birth to babies with numerous defects, including NTDs (175). Work by Hibbard & Smithells (75) implicated maternal folate deficiency as a risk for NTD-affected pregnancies in the 1960s, and several landmark human trials in the late 1980s and early 1990s supported these findings (38, 117). The Medical Research Council Vitamin Study, published in 1991, was the first large, randomized trial to show a strong protective effect of FA supplementation. For women who had already had one NTD-affected pregnancy, supplementation with 4 mg/day of FA decreased the risk of a second NTD-affected pregnancy by 72% (6 of 593 NTDs in FA-supplemented group versus 21 of 602 in the unsupplemented group) (117). A second large-scale randomized trial in

Hungary provided evidence that FA supplementation could reduce the risk of a first NTD-affected pregnancy. Supplementation with 0.8 mg/day of FA for one month prior to conception and for the first 8 weeks of pregnancy significantly decreased the risk of having a first NTD-affected pregnancy (0 of 2,104 NTD cases in the vitamin-supplement group, compared to 6 of 2,052 NTD cases in the control group) (38).

As a result of these studies, the US Centers for Disease Control and Prevention (CDC) made the official recommendation that “[a]ll women of childbearing age in the United States who are capable of becoming pregnant should consume 0.4 mg of folic acid per day for the purpose of reducing their risk of having a pregnancy affected with spina bifida or other NTDs” (29). This supplementation campaign proved unsuccessful, however, possibly in part because physicians and patients were not sufficiently educated on the importance of supplementation (65), and less than a third of women were reported to be taking a FA supplement in 1997, despite increased public awareness (30). Given the limited effectiveness of the supplementation campaign and the fact that an estimated 50% of pregnancies in the United States are unplanned (87), the United States implemented a fortification campaign in 1998 to ensure that women of childbearing age received the daily recommended dose of 400 µg/day (46). Since fortification, the NTD incidence in the US has decreased by almost 20% overall (31% reduction in spina bifida and 19% reduction in anencephaly) (122). Similar fortification campaigns in other countries have yielded comparable results (41, 42, 104). Overall, more than 75 countries have implemented mandatory FA fortification campaigns, primarily in wheat flour, and it is estimated that FA fortification has prevented 15–25% of FA-responsive NTDs worldwide (188). Interestingly, the incidence of spina bifida did not continue to decline after the initial decrease but rates of anencephaly did (21).

Folate is a water-soluble vitamin that is found naturally in many foods, especially green, leafy vegetables (18). Dietary folates, in the form of tetrahydrofolates (THF), play an important role in OCM (Figure 3). FA is a synthetic form of folate that is more stable than the naturally occurring form. Both FA and folate must be reduced by dihydrofolate reductase (DHFR) and then converted to a biologically active form, 5-methyl-tetrahydrofolate (5-meTHF), by serinehydroxymethyl-transferase (SHMT) and 5,10-methylenetetrahydrofolate reductase (MTHFR) (60). 5-meTHF is then used in the biosynthesis of purines and thymidylate, the synthesis of methionine from homocysteine, and the biosynthesis of S-adenosylmethionine (SAM), the universal methyl donor for cellular methylation reactions, including DNA and protein methylation (14).

Drugs that act as folate antagonists have continued to be implicated as risk factors for NTDs. Women who took folate antagonists in their first trimester were more than sixfold more likely to have an NTD-affected pregnancy. These included DHFR-inhibitors, such as methotrexate, which inhibit the conversion of folate to its active form, and the antiepileptic drug valproic acid (an HDAC inhibitor) (6, 112).

After the Brownsville Cluster, in which the incidence of NTDs was four- to fivefold higher along the Texas-Mexico border than the rest of the United States, attention turned to other potential environmental risk factors. The Mexican-American population in the region was

known to consume high levels of corn, and cornmeal samples harvested around the time of the Cluster contained high levels of fumonisin contamination. Fumonisin is a mycotoxin that inhibits the biosynthesis of sphingolipids, ultimately interfering with the cellular uptake of 5-meTHF (159). In mouse studies, FA supplementation partially rescued fumonisin-induced NTDs (55, 139). Furthermore, much of the population affected in the Brownsville cluster was also folate deficient, suggesting that fumonisin is more likely to compound folate deficiency in a human population and less likely to be a significant risk factor in a folate-sufficient population.

Given the strong epidemiological evidence for the requirement of adequate FA during development, and especially at the time of NT closure, attention has focused on genes in the folate OCM pathway. More than 40 genes involved in FA metabolism have been studied in NTD patient samples (reviewed in 61). Numerous studies have focused on a common polymorphism in the *MTHFR* gene, 677C→T. *MTHFR* is required to reduce 5,10-methylenetetrahydrofolate (5,10-methylene-THF) to 5-methyl THF, which is the main circulating form of folate and is required to generate SAM (18). The 677C→T mutation generates a more thermolabile enzyme with significantly reduced activity that results in low plasma folate levels and higher levels of homocysteine (51, 165). Several meta-analyses of the epidemiological data indicate that the 677T→C mutation is an overall risk factor, with either the CT or TT allelic combinations conferring a 1.2–2-fold increased risk of an NTD-affected pregnancy (181, 191). Furthermore, the risk associated with the *MTHFR* 677C→T mutation can be alleviated by increased maternal folate (92), again highlighting the importance of gene-environment interactions. Despite the strong association with the *MTHFR* polymorphism and the intensive focus on OCM gene variants in cohorts of NTD patients, there is little additional evidence for a major causative association between genetic variants in this pathway and NTDs (61).

Consistent with the human NTD data, very few genes involved in OCM have been implicated in mouse NTDs (68), and folate deficiency in the absence of a genetic insult fails to cause spontaneous murine NTDs. A handful of mouse NTD models have been used for FA supplementation studies, with very mixed results (Table 2). For some mouse models, FA has a preventative effect (12, 13, 68, 116), whereas others are nonresponsive (68, 120), much like in the human population. Surprisingly, several models have shown a detrimental response to FA, including increased NTDs and increased fetal loss (59, 110). These studies raise an important question: In some cases, is the decreased incidence of NTDs due to an increase in early fetal loss rather than a rescue of NTDs, a possibility first raised nearly two decades ago (79)? It will be important to understand which genetic variants are favorably influenced by FA fortification, and it is expected that animal models can provide genetic, experimental, and mechanistic insights into FA-responsive and nonresponsive NTDs. Some experts have raised the idea that the dose of FA should be increased to try to further decrease the incidence of NTDs in the population (168). Indeed, FA fortification in conjunction with an increase in vitamin usage has resulted in a trend toward higher FA levels throughout the US population (80, 129). However, it is important to note that there will likely be certain genotypes that are resistant to FA, and the potential for long-term epigenetic changes remains an important consideration.

The mechanism by which FA affects NT closure is unknown, but as discussed above, changes in DNA methylation likely play a role, as do alterations in synthesis of nucleotide precursors. Methylation of the insulin-like growth factor gene differentially methylated region (*IGF2* DMR) was significantly higher in children whose mothers took FA supplements (400 µg/day) than in children of unsupplemented mothers (154). In mice, genome-wide CpG methylation patterns were found to be significantly different in the cerebral hemispheres in offspring of wild-type dams exposed to a FA-fortified diet compared with nonfortified controls (11). Furthermore, differential methylation of important developmental genes was correlated with altered levels of gene expression. Future studies are needed to determine what the link may be between FA status, changes in DNA methylation, and NTD incidence. Moreover, the OCM cycle also produces nucleotides, which are needed for proliferation, and the rate of proliferation is extremely important in NT closure. Hence, adequate folate levels likely play an important role in maintaining sufficient proliferation levels during NT closure.

Maternal Diabetes and Obesity

Maternal diabetes has long been identified as a risk factor for congenital birth defects, including NTDs (190). SNPs in maternal genes related to glucose metabolism, such as *FTO*, *LEP*, *TCF7L2*, *LEPR*, *GLUT1* and *HK1*, have been linked to an increased risk for NTDs (39, 107), and potentially informative maternal-fetal SNP interactions have also been correlated to altered NTD risk. For example, SNPs in maternal *ENPP1* and fetal *SLC2A2* correlate with increased risk of NTD (2–3.5-fold), whereas the same fetal *SLC2A2* SNPs interact with maternal SNPs in *LEP* to provide a protective effect (0.5-fold risk) (108). Fetal SNPs in *SLC2A2* alone do not appear to confer altered risks of NTDs (107). It is thought that altered maternal glucose metabolism leads to an altered intrauterine environment, which is not tolerated by the developing embryo, as pancreatic function in human embryos begins at approximately week 7 of pregnancy. Diabetic embryopathies are known to particularly affect the neural tube and heart, both of which form prior to week 7 (107).

Animal models have proven invaluable in elucidating the molecular pathophysiology of diabetic embryopathies. In mice, high glucose levels cause decreased proliferation and increased apoptosis in neural progenitor cells in the developing spinal cord (53), suggesting that a reduction in cell number contributes to diabetic embryopathies. Hyperglycemia can activate several related pathways, including altered lipid metabolism, increased generation of reactive oxygen species (ROS), and activation of apoptotic pathways (reviewed in 135). Briefly, hyperglycemia alters arachidonic acid metabolism, leading to the generation of altered levels of prostaglandins, which have been linked to adverse pregnancy outcomes. Hyperglycemia also leads to increased glucose transport into the cell, which activates the polyol pathway, causing increased accumulation of sorbitol and ultimately increased ROS levels. Increased sorbitol and the hyperglycemia-induced stress response in the endoplasmic reticulum (ER) have both been shown to activate the JNK1/2 pathway, which has been implicated in diabetes-induced NTDs (101, 183). Oxidative and ER stress have been shown to activate the PKC pathway (77), which can also activate the JNK1/2 pathway (24, 25) and induce apoptosis via Caspase-8 (192). Alleviating oxidative stress by overexpression of the antioxidant enzyme superoxide dismutase 1 (SOD1) can rescue diabetes-induced NTDs

coincident with decreased activation of the PKC and JNK1/2 pathways (99, 100, 172). Moreover, ROS in mice activates apoptosis signal-regulating kinase 1 (ASK1), which activates the transcription factor FoxO3a and Caspase-8, as well as increased levels of the apoptosis-promoting adaptor protein, TRADD, which is a direct target of FoxO3a. Interestingly, increased ASK1 phosphorylation, decreased phosphorylation of FoxO3a, increased TRADD expression, and increased cleaved Caspase-8 were also observed in neural tissue from human NTD-affected fetuses (182).

Intriguingly, a model of transcriptional variability as the underlying cause of partial penetrance has been proposed, supported by recent data from diabetic mouse models. In both a chemically induced diabetes model and a nonobese diabetic (NOD) strain, NTD-affected embryos had significantly higher variability in gene expression compared with their non-NTD littermates. Furthermore, the pathways enriched in the class of highly variable genes were different than those enriched in the differentially expressed but less-variable gene sets, suggesting a role for environmentally induced variable gene expression as a mechanism of increased NTD risk (83). Mechanistically, this may fit with recent data showing that hyperglycemia alters the epigenetic landscape. Shyamasundar et al. (153) found that neural stem cells isolated from chemically induced diabetic pregnancies had increased global levels of H3K9 trimethylation, increased DNA methylation, and decreased H3K9 acetylation compared with neural stem cells derived from normal pregnancies. As discussed above, disruption of the epigenetic landscape can increase transcriptional variability and may be an underlying factor of NTDs.

Given what is known about the molecular mechanisms involved in diabetes-induced NTDs, several measures have been proposed to protect developing embryos. There is evidence that nutritional supplements can alleviate the risks. Diabetic mice supplemented with trehalose had a much lower incidence of NTD-affected embryos than unsupplemented mice (10% versus 28%) (180). Diabetic rats supplemented with FA showed a decreased incidence of NTDs (supplemented, 6%; unsupplemented, 28%) (54). However, diabetic mice also showed premature neuronal and astrocyte differentiation when given high doses of FA compared with a moderate dose, suggesting that too much FA may cause unintended consequences (189). Human epidemiological data also suggest that FA supplementation may reduce the risk of NTDs in a diabetic pregnancy (37, 127), with a larger protective effect observed against anencephaly than spina bifida (37).

Maternal obesity, defined as a body mass index (BMI) ≥ 30 , has also been linked to an increased risk for NTDs. Meta-analyses showed an overall 1.7–1.8-fold increase in NTD-affected pregnancies for women who are obese and 3.1-fold increase for women who are severely obese (132, 150, 155). Interestingly, a prefortification study in the United States showed that the risk associated with increased BMI was independent of maternal folate status (74, 150), a finding confirmed in a Canadian study spanning pre- and postfortification, which showed that FA fortification did not alleviate the obesity-related risk of NTDs (134, 169).

Maternal Hyperthermia

Maternal hyperthermia can arise from either febrile illnesses or external exposure to heat, such as prolonged periods in a hot tub or sauna. Epidemiological studies have strongly implicated maternal fever in early pregnancy as a risk factor for NTD-affected pregnancies. A recent meta-analysis evaluated 46 studies ranging from 1990–2013 and found that the NTD risk was increased almost threefold in cases of maternal fever during the first trimester. The risk for oral clefts and congenital heart defects also increased (43). Interestingly, studies of Mexican-American women along the Texas-Mexico border (160), a more general US population (48), and Hungarian women (1) indicate that use of antifever medication can significantly alleviate this risk. Furthermore, the use of either a multivitamin or FA supplementation also appears to abrogate the risk associated with maternal febrile illness (1, 20). Surprisingly, animal models of maternal hyperthermia show a mixed response to FA supplementation. In the golden hamster, FA supplementation does not appear to rescue heat-induced NTDs (58), whereas studies indicate that FA can rescue heat-induced NTDs in mice (151).

Early studies linking maternal hyperthermia as a result of prolonged external exposure, such as in a hot tub or sauna, to increased risk for NTDs are mixed. An increased risk was found in several studies (114, 142), but another study found no correlation with sauna use and NTD risk in a Finnish cohort (145). Data collected from the large, multisite National Birth Defects Prevention Study from 1997 to 2005 show the risk for anencephaly increased by 1.7-fold for women who reported using hot tubs during early pregnancy. However, only hot tub sessions lasting more than 30 minutes had a significant effect (44). Overall, in animal and human models, the key factor appears to be raising the core temperature more than 2°C above baseline (32).

The mechanism by which heat may affect the developing embryo is unknown, but there appears to be a genetic component. In one study, five different mouse strains were exposed to hyperthermic treatment on embryonic day 8.5, just as the neural tube is closing. One strain had a 44% incidence of heat-induced NTDs in their offspring, whereas the other four strains showed no more than a 14% penetrance of NTDs (49). A follow-up study confirmed these findings and suggested the importance of maternal genotype in susceptibility to heat-induced NTDs (106).

Although it has been proposed that hyperthermia-induced NTDs may arise from changes in metabolism, research in animal models indicates that it is the heat that adversely affects development, likely as a result of increased cell death, decreased proliferation, disruption of gene expression, and damage to the embryonic vasculature (reviewed in 45). Recent work on mouse neural stem cells showed a narrow temperature range of responsiveness to heat shock (124), leading to induction of apoptosis, inhibition of proliferation, and delayed differentiation. These data highlight the importance of maintaining thermal homeostasis during the critical period of NT closure.

Maternal and Embryonic Exposure to Alcohol and Drugs

Although there is no question that exposure to alcohol in utero is detrimental to the developing fetus, the epidemiology linking maternal alcohol consumption to NTDs is less clear. A small Italian study found that mothers of children with spina bifida were 3 times more likely to have consumed 0.5 liters/day or more of alcohol in the 3 months leading up to and immediately following conception (41). Two American studies, however, showed no correlation between periconceptional maternal alcohol consumption and NTDs (109, 158). However, both American studies were retrospective, and the authors suggest that the social stigma against alcohol during pregnancy may have led to under-reporting. Animal models have shown adverse response to ethanol exposure, evidenced by increased NTDs in mice injected with ethanol around the time of NT closure (64). The authors proposed that ethanol interferes with polyamine synthesis in the developing embryo. As polyamines are known to regulate cell growth and proliferation, this may be one mechanism by which alcohol disrupts embryonic development. Alcohol can also interfere with folate in OCM, although the mechanisms are not well understood (reviewed in 90). Ethanol treatment of pigs results in decreased expression of methionine synthase (MS), a key enzyme in OCM (167), and it has been suggested that ethanol-induced inactivation of MS could lead to a pseudofolate deficiency.

Maternal smoking and maternal exposure to environmental tobacco smoke (ETS) have also been studied epidemiologically with respect to risk for an NTD-affected pregnancy, with conflicting conclusions. Some find that smoking shows no correlation and some show an increased risk of NTD-affected pregnancies. Recent meta-analyses indicate that maternal smoking confers a small increased risk for spina bifida, and a moderately increased NTD risk in cases of maternal ETS exposure (173, 174). Interestingly, the data suggest regional differences in susceptibility, with maternal smoking being a higher risk factor in Europe than in the United States (173). Both smoking and ETS are associated with decreased serum folate levels (125), so it is possible that the increased risk in Europe compared with the United States may be related to differences in FA fortification policies. It has been proposed that the risk associated with maternal smoking may be underestimated, as there is evidence that both maternal smoking and ETS are associated with a significantly increased risk of spontaneous abortion (56), and early fetal loss is rarely included in retrospective studies.

Given the prevalence of caffeine use, several studies have looked at whether periconceptional caffeine use is a risk factor for NTDs. One US study found no correlation between maternal caffeine intake and NTD-affected pregnancy (15), whereas another small study suggested that there might be a slightly elevated risk (147). Moreover, the susceptibility to caffeine as a risk factor varied by maternal race/ethnicity, with non-Hispanic whites showing no correlation and Hispanic and all other ethnicities had a 1.5–2-fold increased risk (147). An Italian study, however, showed a strong association between maternal coffee intake (3 cups/day) and spina bifida-associated pregnancy (41), but it was a relatively small study, and the overall conclusions from the study focused on folate deficiency as the most important risk factor. Tea has also been implicated in NTDs, as tea contains catechins that can inhibit DHFR activity. In northern China, daily tea consumption was shown to increase the risk of NTD-affected pregnancy by more than threefold (186),

although most of these women were also FA deficient, confounding the interpretation. However, in the United States, no correlation was seen between tea consumption and NTDs (184).

CONCLUSIONS AND FUTURE OUTLOOK

As highlighted in this review, NTDs are a complex disease impacted by genetic susceptibility, epigenetic influences, and environmental insults (Figure 4). The tools are now available to identify the genetic contributions in humans using unbiased methods to evaluate the genome and the epigenome. This, combined with expanded NTD cohorts that include familial and sporadic cases should lead to a much broader understanding of this complex genetic trait. It may be discovered that most sporadic cases of NTD in humans are not due to strong single gene contributions but instead may result from a combination of small genetic perturbations in a number of genes. Animal models will be necessary to provide experimental validation of coding and regulatory polymorphisms, the functional intersections between multiple genetic components, and mechanistic insights into the normal action of these genes and why NT closure fails upon genetic disruption. Finally, there is little insight into the mechanisms that underlie environmental contributions, whether disruptive or preventative. Research has lagged behind in using animal models to decipher how environmental factors impact NT closure and in determining genetic susceptibility to environment influences. Moreover, despite the clear beneficial impact of FA fortification on NTD risk in the general population, there are still a large number of NTD-affected pregnancies. By understanding the cellular and molecular mechanisms of neural tube closure and metabolic factors, it may be possible to identify other measures to help prevent this devastating birth defect.

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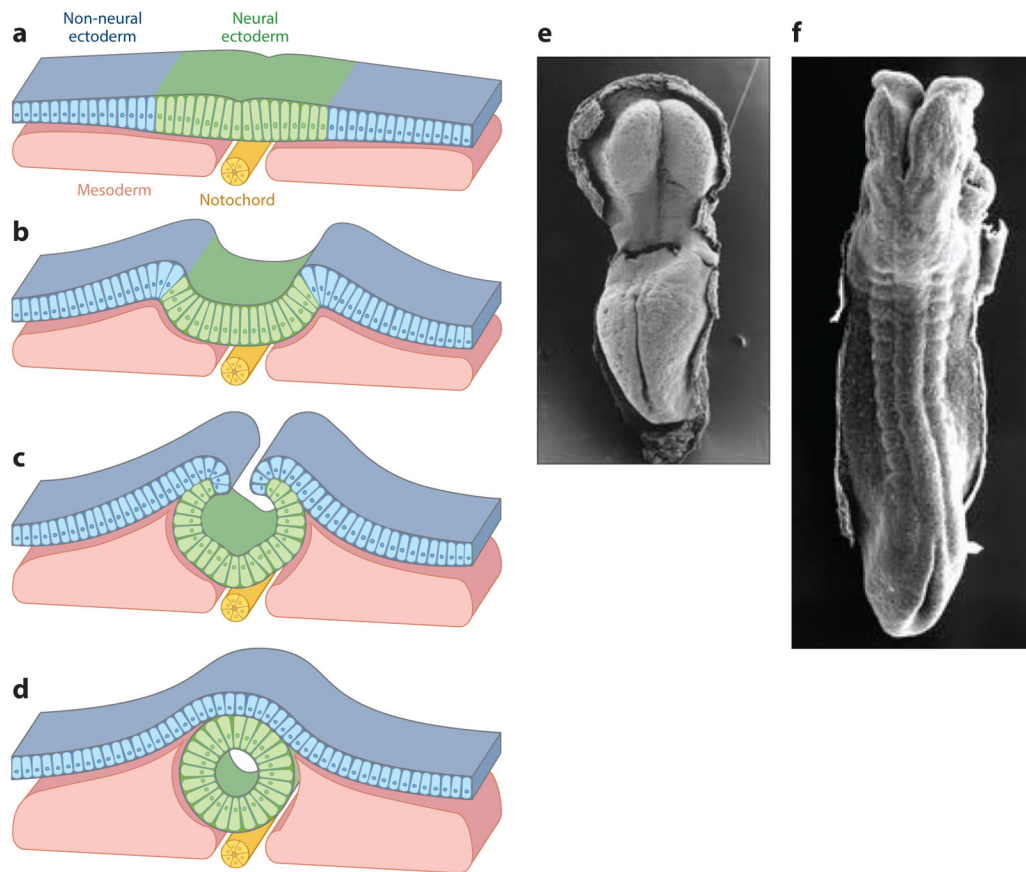


Figure 1.

Neural tube closure. (*a–d*) Schematic transverse sections that illustrate the (*a*) flat neural plate stage, (*b*) hinge-point formation and neural-fold elevation, (*c*) apposition of the neural folds with the neural ectoderm covered by the non-neural ectoderm (NNE), and the (*d*) meeting and remodeling of the neural ectoderm and NNE to form a closed neural tube covered by a single layer of NNE.

(*e–f*) Carnegie stages 9–11 human embryos (~20–24 days of gestation) just prior to the (*e*) initiation of neural tube closure and to (*f*) closure of most of the spinal region. Panels *a–d* adapted from Reference 133; panels *e* and *f* taken from scanning electron micrographs of early human embryos by Dr. K. Sulik, from embryos collected by Dr. Vekemans and T. Attie-Bitacha, and presented on http://embryology.med.unsw.edu.au/embryology/index.php?title=Embryonic_Development.

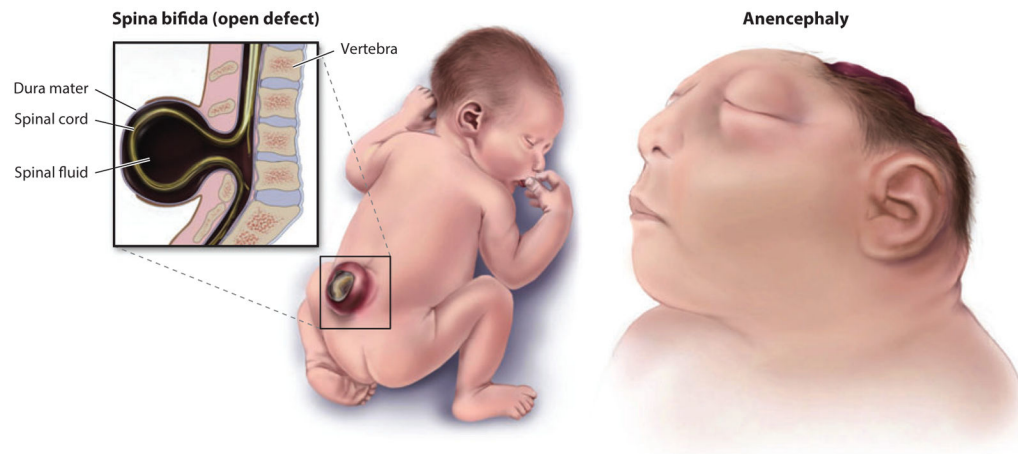


Figure 2.

Two types of open neural tube defects (NTDs). Spina bifida occurs in the spinal region, whereas failure of cranial neural tube closure is initially called exencephaly but after exposure and degradation of the brain tissue is called anencephaly. Both caudal and cranial NTDs can vary in extent of the opening and the rostral-caudal level. Adapted from Centers for Disease Control and Prevention, National Birth Defects and Developmental Disabilities.

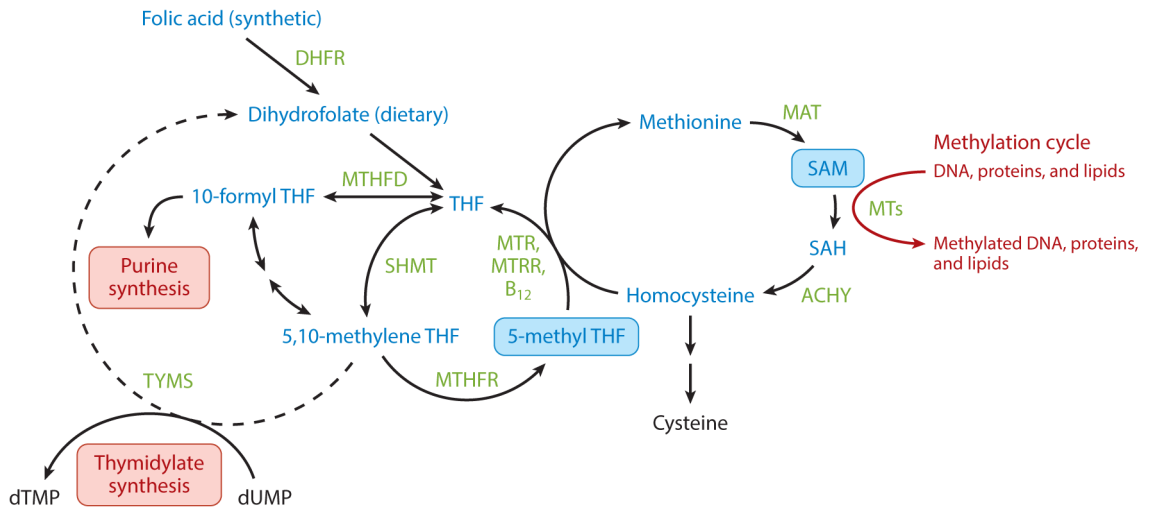


Figure 3.

Schematic of folate in one-carbon metabolism. Synthetic and dietary folates are reduced to tetrahydrofolates (THFs), which are converted to the biologically active 5-methyltetrahydrofolate (5-methyl-THF) by SHMT and MTHFR. 5-methyl-THF acts a methyl donor for purine and thymidylate synthesis and generates the major cellular methylation donor S-adenosylmethionine (SAM). Abbreviations: AHCY, S-adenosylhomocysteine hydrolase; DHFR, dihydrofolate reductase; MAT, methionine adenosyltransferase; MTHFD, methylenetetrahydrofolate dehydrogenase; MTHFR, methylenetetrahydrofolate reductase; MTR, methionine synthase; MTRR, methionine synthase reductase; MTs, methyltransferases; SAH, S-adenosylhomocysteine; SAM, S-adenosylmethionine; SHMT, serine-hydroxymethyltransferase.

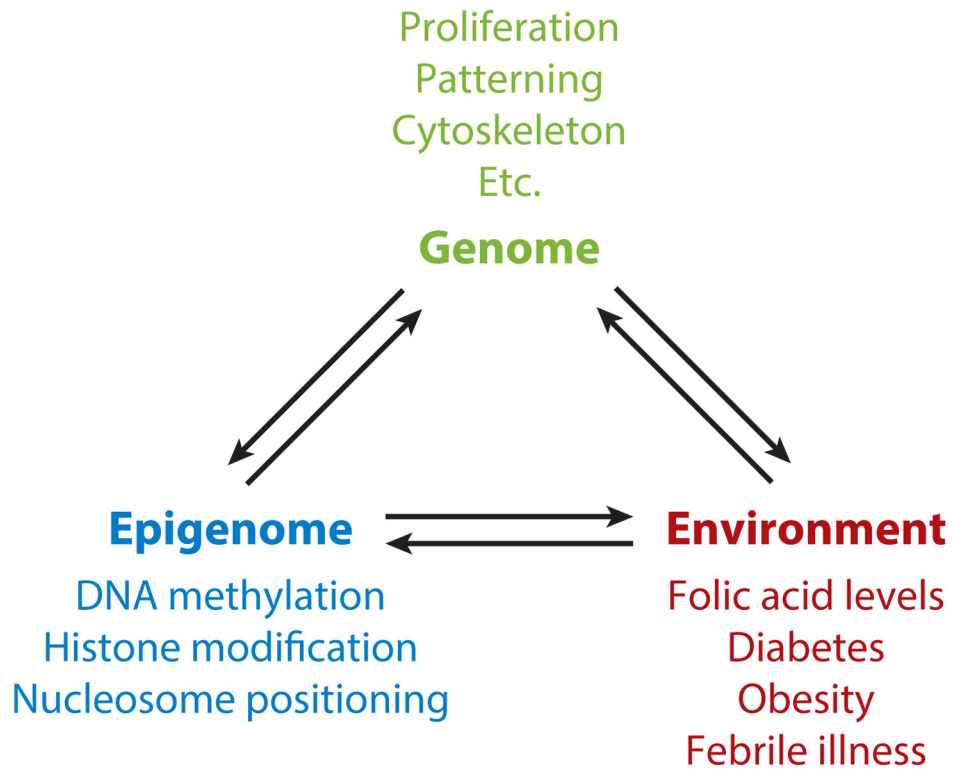


Figure 4. Interrelationship between the genome, epigenome, and environment, with respect to neural tube closure.

Table 1

Epigenetic regulators required for neural tube closure

Gene	Protein	Function	Observed Neural Tube Defect	Reference
<i>Ppm1g</i>	PPM1G	Chromatin remodeling	Exencephaly	(50)
<i>Dnmt3a</i>	DNMT3A	DNA methylation	Exencephaly	(123)
<i>Dnmt3b</i>	DNMT3B	DNA methylation	Exencephaly	(123)
<i>Dnmt3L</i>	DNMT3L	DNA methylation	Exencephaly	(71)
<i>CBP</i>	CBP	Histone acetylation	Exencephaly	(128)
<i>Kat2a</i>	GCN5	Histone acetylation	Exencephaly	(22)
<i>Ep300</i>	p300	Histone acetylation	Exencephaly	(128)
<i>Hdac4</i>	HDAC4	Histone deacetylation	Exencephaly	(166)
<i>Sirt1</i>	SIRT1	Histone deacetylation	Exencephaly	(35)
<i>Kdm2b</i>	FBXL10	Histone demethylation	Exencephaly	(52)
<i>Kdm6a</i>	UTX	Histone demethylation	Exencephaly	(152, 179)
<i>Uty</i>	UTY	Histone demethylation	Exencephaly	(152)
<i>Alkbh1</i>	ALKBH1	Histone methylation	Exencephaly	(126)
<i>Jmj</i>	JARID2/Jumonji	Histone methylation	Exencephaly	(162)
<i>Cited2</i>	CITED2	Co-regulator of CBP/p300	Exencephaly	(187)
<i>Smarcc1</i>	BAF155	Nucleosome remodeling	Exencephaly	(67, 85)
<i>Smarca4</i>	BRG1	Nucleosome remodeling	Exencephaly	(23)
<i>Cecr2</i>	CECR2	Nucleosome remodeling	Exencephaly	(10)
<i>miR-124a</i>	N/A	Nucleosome remodeling	Spina bifida	(178)
<i>miR-9*</i>	N/A	Nucleosome remodeling	Spina bifida	(178)
<i>Nap1L2</i>	NAP1L2	Nucleosome assembly	Exencephaly	(138)

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Table 2

Mouse NTD models studied for responsiveness to folic acid

Gene Name	Folic acid response			Process	Reference
	Beneficial	Detrimental	None		
<i>Amt</i> null			x	Involved in glycine cleavage system, part of the mitochondrial folate metabolism; implicated in human and mouse neural tube defects; responsive to methionine	(120)
<i>Axd</i> mutant			x	Unknown function; axial defects; responsive to methionine	(68)
<i>Cart1</i> null	x			<i>Patred</i> -class homeobox domain-containing gene, aka <i>Akt1</i>	(68)
<i>Cited2</i> null	x			Required for heart morphogenesis and left-right axis determination in mouse development	(68)
<i>Curly tail (Grhl3 mutant)</i>			x	Transcription factor; <i>Ct</i> mutation is responsive to inositol and retinoic acid	(68)
<i>Fibp8</i> null			x	Member of immunophilin protein family; involved in dorsal-ventral patterning of neural tube; inositol-resistant	(68)
<i>Folr1</i> null	x			Folate receptor; involved in transport of 5-MTF into the cell	(68)
<i>Frem2^{mi}/N^{sw}</i> allele			x	Extracellular matrix protein	(110)
<i>Grhl2^{mi}/N^{sw}</i> allele		x (het)	x (null)	Transcription factor	(110)
<i>Grhl3</i> null			x	Transcription factor	(68)
<i>Kat2a</i> hypomorph (GCN5)	x			Histone acetyltransferase	(68)
<i>L3P</i>		x		Ciliogenesis	(110)
<i>Lrp6</i> null		x		Receptor or coreceptor (with <i>Frizzled</i>) of Wnt; canonical <i>Wnt/β-catenin</i> signaling	(59)
<i>Lrp6^{Cd}</i> allele	x				(68)
<i>Map3k4</i> null			x	MAPK pathway	(68)
<i>Mthfd1^c</i> allele				Untested with folic acid; partial rescue observed upon sodium formate supplementation	(116)
<i>Nog</i> null			x	Bone morphogenetic protein antagonist; resistant to inositol and pifithrin-α (cell-death inhibitor)	(68)
<i>SELH/Bc</i> strain			x	Inbred mouse strain with a high frequency of nonsyndromic neural tube defects	(68)
<i>Shmt</i> null	x			Thymidylate biosynthesis pathway; choline-resistant	(12, 13)
<i>Shroom3^{mi}/N^{sw}</i> allele	x (short-term)		x (long-term)	Involved in actin remodeling, especially apical constriction and apicobasal elongation	(110)
<i>Slc19a1</i> null (RFC1)	x			Reduced folate carrier	(68)
<i>Sp</i> and <i>Sp^{2H}</i> mutant at <i>Pax3</i> gene	x			Transcription factor involved in ear, eye, and facial development as well as dorsal patterning of the neural tube	(68, 110)
<i>Zic2^{mi}/N^{sw}</i> allele	x			Transcriptional activator and repressor involved in organogenesis of the central nervous system	(110)