CHAPTER

27

Neural-Tube Defects

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OUTLINE

27.1 NEURAL-TUBE DEFECTS

After heart defects, neural-tube defects (NTDs) are the second most common birth defect, occurring in approximately 300 000 newborns worldwide (~1:1000 live births) (Botto et al., 1999). The prevalence of NTDs varies considerably between different racial and ethnic groups (Carmichael et al., 2004; Feldman et al., 1982; Feuchtbaum et al., 1999). In the United States, the two most common types of NTDs, spina bifida and anencephaly, are estimated to affect ~3000 pregnancies each year, with caudal NTDs occurring at a higher rate than cranial NTDs (Canfield et al., 2009; CDC, 2004). Both cranial and caudal NTDs can be associated with craniofacial defects (Rittler et al., 2008), but the majority of NTDs are nonsyndromic (Hall et al., 1988). NTDs, especially those of the cranial region, seem to have a higher prevalence among females (Rogers and Morris, 1973; Seller, 1987), although the reason for this sex difference is not well understood.

27.1.1 Types of NTDs

NTDs are divided into two major groups, cranial NTDs and spinal NTDs, and the nomenclature and classification of NTDs in humans are based on position and severity of the NTDs. Cranial NTDs result from failure of the neural tube to close in the cranial region and are classified as follows:

1. Encephalocele, defined as herniation of the cranial vault. Encephalocele can be either a meningocele,

Neural Circuit Development and Function in the Brain: Comprehensive Developmental Neuroscience, Volume 3 http://dx.doi.org/10.1016/B978-0-12-397267-5.00041-8

if the herniation contains only cerebral spinal fluid (CSF) and meninges, or a meningoencephalocele, if it also contains neural tissue (McComb, 1997).
2. Anencephaly, where the cranial vault is absent and the neural tissue degenerates by week 8 of gestation (Calzolari et al., 2004).

Spinal NTDs also are referred to as spinal dysraphisms. This term was coined by Lichtenstein in 1940 to describe incomplete fusion or malformations of structures in the dorsal midline of the back, particularly congenital abnormalities of the vertebral column and spinal cord (Tavafoghi et al., 1978). These can be further divided into three groups:

1. Spina bifida occulta, which in its strict definition refers only to bone-fusion defects in the spine.
2. Spina bifida cystica, which refers to meningocele and myelomeningocele, where the herniation contains not only CSF, but also neuronal tissue.
3. Spina bifida aperta (SBA), in which the neural tissue is exposed to the environment (Kaufman, 2004).

A relatively rare form of dysraphism that is open or exposed results from the failure of closure of the neural tube throughout its entire length and is called craniorachischisis (Coskun et al., 2009). The term *spina bifida* has become commonly associated with the open spinal dysraphism of a myelomeningocele.

27.1.2 Clinical Aspects and Prognosis of NTDs

NTDs can be fatal; all exencephalic embryos are stillborn or die shortly after birth, whereas the mortality rate of babies with spina bifida is especially high over the first year of life. Individuals with less severe myelomeningocele suffer from lifelong disabilities, including reduced mobility, little or no bowel and bladder control, and urological infections, and they often require surgical interventions to control the effect of hydrocephalus, the build-up of fluid inside the skull caused by obstruction of CSF circulation (Simeonsson et al., 2002). NTDs pose a considerable monetary burden on the health care system (Kinsman and Doehring, 1996), as well as a significant emotional burden on the affected individual and his/her family. Therefore, understanding neural-tube development and the causes of NTDs are among the most important health-related studies today.

27.1.3 Etiology of NTDs

The etiology of NTDs is complex and involves both genetic and environmental factors, which makes NTDs a classic example of a multifactorial disorder.

27.1.3.1 Genetics of NTDs

The genetic risk of a recurrent NTD in a family with one child with an NTD is 2–5%, a 50-fold increase compared with the rest of the population (Forrester and Merz, 2005). Despite the evidence that genetics plays a pivotal role in the occurrence of NTDs, there are few data on single-gene defects directly associated with NTDs in humans. A number of chromosome rearrangements, such as trisomies 13 and 18, and known genetic syndromes, for example, Meckel syndrome, are associated with NTDs (Lynch, 2005). Chromosomal abnormalities, such as aneuploidy, are present in 5–17% of cases with NTDs (Kennedy et al., 1998), and a 13q deletion, with a critical region at 13q33-34, is strongly correlated with the occurrence of NTDs (Luo et al., 2000). These data can be used to extract clues in an effort to determine the identity of genes involved in neural-tube closure. Clues as to candidate genes for association studies of human NTDs also have been derived from research in animal models of NTD, as described below and as outlined in comprehensive reviews by Harris and Juriloff in 2007 and 2010.

27.1.3.1.1 ENVIRONMENT AND NTDS

Maternal diabetes during pregnancy is a key environmental factor, as there is a greater than tenfold increase in NTD frequency in the offspring of diabetic mothers as compared to the general population (Milunsky et al., 1982). The mechanism by which diabetes contributes to failure of neural-tube closure in humans remains unclear. However, a mouse model of diabetes showed a correlation between elevated blood glucose in the mother, increased apoptosis of neural cells, and reduced levels of Pax3, a gene required for neural-tube (NT) formation (Fine et al., 1999; Phelan et al., 1997). Genomics studies show that maternal diabetes can alter transcriptional programs in the developing mouse embryo, potentially affecting genes that directly and indirectly control NT formation (Pavlinkova et al., 2009). Maternal hyperthermia during pregnancy can increase the risk of NTDs up to twofold, although the mechanism responsible for this increase is unknown (Lynberg et al., 1994). Pharmacological agents, among them valproic acid (Nau et al., 1991) and antiepileptic drugs, are associated with an increased incidence of NTDs. For example, antiepileptic drugs can increase NTD risk by 10- to 20-fold (Lindhout et al., 1992). Lifestyle choices such as drinking, smoking, and recreational drugs also increase the risk of NTDs through unknown mechanisms (Suarez et al., 2008). Finally, studies have provided a link between dietary choices and risk for NTDs. In general, vitamin intake is not correlated with reduced risk for NTDs (Carmichael et al., 2003); however, there is one dietary supplement, folic acid, which can reduce the risk

for NTDs when added to the maternal diet periconceptionally. Folic acid is the environmental factor that has attracted the most attention from developmental biologists, epidemiologists, and the general public due to the view that it has a protective effect against NTDs (Honein et al., 2001). Epidemiological studies started in the early 1980s showed that maternal folic-acid supplementation led to a significant reduction in NTD incidence (Laurence, 1985). This evidence dramatically influenced public-health policies and led to food-fortification programs in the United States and a number of other countries. The mechanism by which folic acid affects the incidence of NTDs remains largely unknown, despite the investigative efforts of a number of laboratories worldwide concerning its effect on the developing embryo.

As noted, there is a gap in knowledge of the mechanisms that lead to NTDs with respect to both genetic and environmental causes. This lack of knowledge significantly limits efforts of the scientific and medical communities toward the prevention of NTDs, both through genetic counseling and through control of the embryonic environment before and during NT formation.

27.2 VERTEBRATE NEURULATION

27.2.1 Animal Models for Vertebrate Neurulation Studies

Neural-tube development requires the coordination of multiple tissues (neural ectoderm, the neighboring mesenchyme, and the overlying ectoderm) in both space and time. For over 50 years, a number of animal systems have been used to study NT development. Model systems such as birds (Averbuch-Heller et al., 1994; Bel-Vialar et al., 2002; Schoenwolf et al., 1989) and amphibians (Clarke et al., 1991; Davidson and Keller, 1999; Roffers-Agarwal et al., 2008) and zebrafish (Nyholm et al., 2009; Puschel et al., 1992; Sumanas et al., 2005) have contributed considerable insight into neurulation. The mouse is the most extensively studied mammalian experimental model for neurulation and NTD, and it most closely recapitulates human neurulation. However, even mice have limitations as a model of human NT development. For example, there are thought to be differences between mice and humans in the number of closure initiation sites, the sites where the neural folds first meet. Moreover, very few mouse models of NTD are representative of nonsyndromic human NTDs (Juriloff and Harris, 2000). Despite these differences, the mouse undergoes neurulation in a manner that is closer to human than to other animal models used to study neurulation to date. Moreover, similar genes are likely responsible for NT closure in humans as in mice (Harris and Juriloff, 2007, 2010). Finally, the great number of mouse NTD mutants provides an invaluable tool for understanding the underlying genetic, molecular, and cellular basis of NTD.

27.2.2 Primary and Secondary Neurulation

The process that forms the primordium of the central nervous system, the neural tube, is called neurulation. Modes of neurulation vary across vertebrate species; however, cellular comparisons reveal conserved mechanisms. This reinforces the idea that study of neurulation in different animal systems can contribute to our understanding of the molecular basis of NT formation and NTDs in humans.

There are two different modes of neurulation: primary and secondary neurulation (Figure 27.1). During primary neurulation, the flat and thickened epithelial layer on the dorsal surface of the embryo called the neural plate bends, and the neural folds seal together to form a hollow neural tube. Secondary neurulation occurs in the most caudal region of the embryo. During secondary neurulation, a cluster of mesenchymal cells condenses below the surface ectoderm to form a cord-like structure. A central lumen forms in this compact structure through

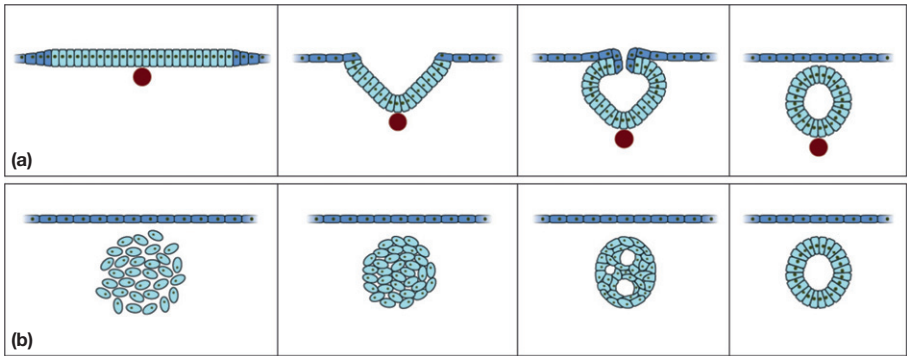

FIGURE 27.1 Primary and secondary neurulation. (a) Primary neurulation and (b) secondary neurulation in amniotes. Organization of cells from onset (*left*) to completion (*right*) of neurulation.

a process known as cavitation, giving rise to the NT (Griffith et al., 1992; Schoenwolf, 1979; Schoenwolf and Delongo, 1980). All amniotes studied to date, including humans (Saitsu et al., 2004), undergo both primary and secondary neurulation.

Although anamniotes have provided considerable information on how neurulation occurs and have furthered our understanding of neurulation in mammals, for the purposes of this chapter, we will focus on amniotes and more specifically mice due to their similarity in development and genetics to human neurulation.

27.2.3 Neurulation in Amniote Model Systems

Amniote neurulation occurs in at least four stages: (i) the acquisition of a neural fate, (ii) the elevation of the neural folds, (iii) the bending of the neural plate, and (iv) the meeting of the neural folds at the midline and fusion that forms the closed neural tube (Figure 27.2).

These four stages occur concurrently along the rostral–caudal axis and in coordination with movements of the primitive streak. Following neural induction, the neuroectoderm becomes morphologically distinct from the non-neural ectoderm through apicobasal thickening of the neuroectoderm to form a tall and thick epithelial sheet that is relatively wide along the medial–lateral axis and short along the anterior–posterior (AP) axis (Figure 27.2(a)). Subsequently, the neural plate undergoes convergent extension (see below) to narrow along the medial–lateral axis and elongate along the AP axis.

Bending of the neural plate at the lateral edges forms the neural folds, which continue to elevate and converge to meet in the midline and form a closed tube. Forces from the neural ectoderm, the mesenchyme and the adjacent non-neural ectoderm promote elevation of the neural folds (Moury and Schoenwolf, 1995). Further bending of the neural folds is driven by formation of "hinge" points involving apical constriction of a limited number of cells in three positions along the neural plate. The first hinge point, called the floor plate or medial hinge point (MHP), forms in the middle of the neural plate above the notochord (posterior) or prechordal mesoderm (anterior). The next two hinge points form in pairs on the dorsolateral sides of the elevating neural folds and are called dorsolateral hinge points (DLHPs; Figure 27.2(b) and 27.2(c)) (Colas and Schoenwolf, 2001; Shum and Copp, 1996). The inductive signal for MHP formation comes from the notochord/prechordal mesoderm and is the secreted protein Sonic Hedgehog (Shh) (Smith and Schoenwolf, 1989; van Straaten et al., 1988). Shh, in a dose-dependent manner, also participates in positioning of the DLHPs (Ybot-Gonzalez et al., 2002). Bending the neural folds is critically important in bringing the neural folds in close proximity to the midline and thus facilitates closure.

The final step of neurulation is when the neural folds meet in the midline, the non-neural ectoderm separates from the neural ectoderm, and each tissue layer seals together to form a continuous sheet of ectoderm overlying the closed neural tube (Figure 27.2(d)). This closure is traditionally called fusion, although the cells do not actually fuse. Very little is known about this last step of neurulation in any organism. For instance, in the mammalian embryo, it has been debated whether the neural or non-neural ectoderm initiates closure. Recent live imaging of the mouse embryo during neurulation showed that the non-neural ectoderm cells are highly dynamic and extend thin, long, and fast-moving cellular extensions that connect the two folds as they approach each other, indicating that the non-neural ectoderm initiates and possibly drives neural-fold fusion (Pyrgaki, 2010b). A role for adhesion molecules is hypothesized in fusion. For example, mutations in adhesion molecules, such as ephrinA5 and ephrinA7, can cause NTDs in mice (Holmberg et al., 2000). However, a direct involvement of these molecules in neural-fold fusion has not been demonstrated.

The fusion of the neural folds starts from one or more initial closure points, depending on the species examined. The classical view has been of a zipper-like closure that proceeds rostrally and caudally from these initial closure points. Recent live imaging of the mouse embryo cranial

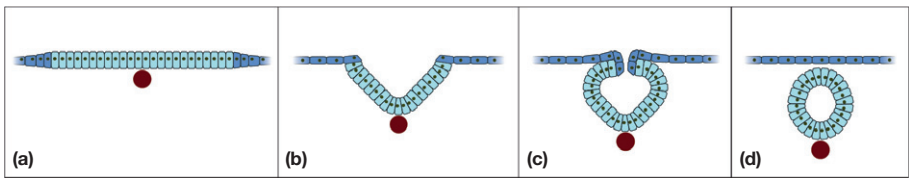

FIGURE 27.2 Four distinct steps of neural-tube formation in amniotes. (a) Acquisition of a neural fate and shaping of the neural plate. (b) Elevation of the neural folds. The non-neural ectoderm exerts force on the neural folds to contribute to neural-fold elevation. (c) Bending of the neural plate and formation of hinge points. In three positions, the neural ectoderm cells change their shape from cuboidal to wedge-shaped to form the medial hinge point and dorsolateral hinge points. These cellular changes facilitate further bending of neuroectoderm to bring the neural folds close together along the midline. (d) Meeting of the neural folds at the midline and fusion to form the closed neural tube and the overlying ectodermal sheet.

region supports a zipper-like closure in the hindbrain. However, closure in the mouse midbrain suggests a different mechanism in which intermediate closure points facilitate closure of the folds in a "buttoning-up" manner (Pyrgaki et al., 2010b).

Migration of the neural crest cells, which form at the boundary between the neural and non-neural ectoderm and then migrate laterally to different parts of the body, occurs concurrently with neural-tube closure in some rostral–caudal regions or after neural-tube closure in other regions.

27.2.4 Neurulation in Humans

Practical and ethical considerations limit our ability to study neurulation in humans, and animal models are therefore needed to provide insight into the cellular and molecular bases of human neurulation. However, there is sufficient information regarding neurulation in humans to suggest that some of the principles of neurulation in amniotes also apply to the human embryo.

Neural-tube formation and closure in humans occurs within the first month of pregnancy, between weeks 3–4 of gestation. In humans, primary neurulation is responsible for generating most of the neural tube. The caudal eminence of the human embryo is formed via secondary neurulation, which will be the point of origin of the terminal parts of the spinal cord, notochord, somites, vertebrae, hindgut, nerves, and blood vessels. This ectoderm-covered mass of pluripotent tissue is a continuation of the primitive streak (at stages 9–12), and it gradually replaces the streak (at stages 12 and 13 of the development of the human embryo, at about 4 weeks post fertilization) and forms a solid cellular mass called the neural cord. The neural cord forms the lower sacral and coccygeal parts of the spinal cord (Muller and O'Rahilly, 1987). The rostral and caudal neuropores close when approximately 20 and 25 pairs of somites are visible, respectively.

One potential difference between human and mouse neural-tube formation may be the number of initial closure points. The mouse has three initial fusion points. In humans, there is an ongoing debate about the number of fusion points. Clinical studies of human embryos with NTDs have argued for 3–5 closure points (Martinez-Frias et al., 1996; Nakatsu et al., 2000; Srinivas et al., 2008). These data are disputed in a study published in 2002 that supports the idea that the human embryo has only two initial closure sites and two neuropores (O'Rahilly and Muller, 2002). It is unclear whether the question of the number of closure points in the human embryo can be answered by case reports. Therefore, one cannot be sure that animal models fully represent the human condition, and it is very likely they do not. We need to be aware that, despite their usefulness, animal models do have limitations, and one needs to be careful when using them to infer analogies to human neurulation.

27.3 GENETIC APPROACHES USED TO UNCOVER REGULATORS OF NEURAL-TUBE CLOSURE IN MICE

Genetic approaches in model organisms are powerful tools for elucidating the biological bases of NTDs and NT development. The mouse provides an excellent model for studies of mammalian neurulation, as the technology of genetic manipulation is very advanced, allowing for a specific gene to be altered and studied with respect to its effect on the organism (Sedivy and Joyner, 1992).

Targeted mutagenesis approaches in the mouse have dramatically assisted efforts to understand better the processes underlying human disease. A great number of mouse models of human conditions are used in research to provide insight into the mechanisms of disease and to explore novel treatments for these conditions.

Phenotype-driven approaches (also referred to as forward genetic screens) start from animals that have been selected for a phenotype of interest and then move to identifying the gene that has been mutated. Many of the forward genetic screens utilize mutagenic agents, ENU being the agent most widely used, that create point mutations and hence can result not only in the inactivation of genes, but also in the generation of novel alleles (i.e., neomorphic, hypomorphic, and alteration of a conserved protein domain). These novel alleles may better represent a disease phenotype than a complete loss-of-function allele.

Over the past decade, forward genetic screens in mice from a number of laboratories worldwide have provided a new resource for the study of multiple biological systems, including neural-tube closure (Nishimura et al., 2003; Zohn et al., 2005). Additional studies to determine the molecular and cellular processes regulated by these key developmental genes have promoted new levels of understanding of the mechanisms that underlie embryonic development. In terms of neural-tube development, forward genetic approaches in the mouse have revealed a number of novel genes that oftentimes had not been functionally analyzed in any system, thus highlighting the effectiveness of phenotype-based screens in elucidating novel aspects of NT development.

27.4 MOLECULAR BASIS OF NTDs

The following sections will describe the molecular mechanisms that control normal neurulation and how these can be disturbed and subsequently lead to NTDs, with the focus on mice and humans.

Following neural induction, the early neural plate is initially specified to give rise to rostral brain tissues (i.e., the forebrain). Posterior character is then imparted to the neural tissue such that it acquires more caudal fates (midbrain, hindbrain, and spinal cord). Further rostrocaudal patterning of the neural plate then begins to take place, as well as patterning in the dorsoventral axis. As the process of neural induction, patterning, and formation of the neural tube is covered in detail in Rubenstein and Rakic, 2013. We will focus here on how disruptions of genes that regulate these processes lead to NTDs both in mice and humans.

27.4.1 Genes Controlling Convergent Extension/PCP Pathway and NTDs

After neural induction, the neural plate narrows and elongates along the embryo's medial–lateral and AP axes, respectively. This shaping of the neural plate is achieved mainly via the morphogenetic process called convergent extension: a medially directed movement and intercalation of the neural-plate cells toward the midline resulting in the lengthening and narrowing of the neural plate (Figure 27.3(a) and 27.3(b)).

It is well established that the planar cell polarity (PCP) pathway controls convergent extension of both neural and mesodermal tissues in vertebrates (reviewed in Wallingford et al., 2002). Vertebrate cognates of the PCP cascade are required for neural-tube closure in frog, zebrafish, and mouse embryos. In the mouse embryo, mutations in five different genes of the PCP pathway lead to the most severe form of NTD, in which the NT remains open throughout its length and mimics the human condition known as craniorachischisis. These embryos have an abnormally wide neural plate, and the two folds are physically too far apart to be joined to each other, due to defects in convergent extension.

The *loop tail* (*Lp*) mouse is the oldest mouse model of craniorachischisis, first described in 1949 by Strong and Hollander. Heterozygous *Lp* mice are viable with a characteristic looped tail, whereas homozygotes exhibit craniorachischisis (Strong and Hollander, 1949). The neural folds of *Lp* homozygous embryos at 8.5 days after fertilization (E8.5) are elevated, but the MHP is absent and the midline region assumes a U shape, in contrast to the normal "V" shape. The gene mutated in *Lp* is homologous to *Drosophila strabismus* and is called *Vangl2* (also *Lpp1* or *Lptap*) (Murdoch

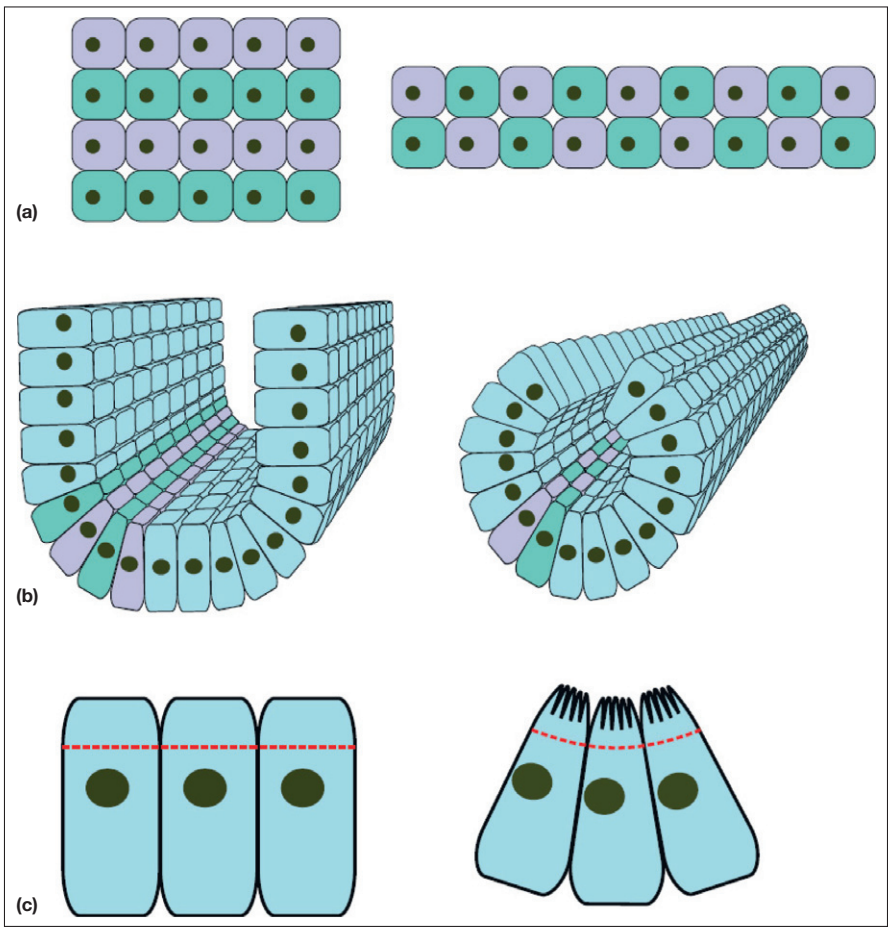

FIGURE 27.3 Convergent extension and apical constriction. (a) Convergent extension cell movements result in tissue elongation via intercalation of adjacent cells (depicted by *dark* and *light squares*) in an epithelial sheet to form a narrower, longer strip of tissue. (b) Convergent extension facilitates the narrowing of the neural plate and brings the neural folds in closer proximity. (c) Apical constriction. Contraction on the apical side of neural cells leads to formation of hinge points and bending of the neural tube.

et al., 2001a). *Vangl2* encodes a transmembrane protein with a C-terminal PDZ-binding motif and is expressed broadly in the early neuroectoderm (Jessen and Solnica-Krezel, 2004). Mice null for *Vangl1*, another Vangl family member in mammals, show subtle alterations in the polarity of inner-hair cells of the cochlea (another manifestation of a PCP defect), but trans-heterozygotes for *Vangl1* and *Vangl2* exhibit craniorachischisis, similar to *Vangl2* null mice (Torban et al., 2008). Genetic interactions have been also observed between *Lp* and *circletail* (*Crc*) mice (Murdoch et al., 2001b). Mice heterozygous for both *Lp* and *Crc*, though not allelic, display the same phenotype of craniorachichis that each of these mice displays when homozygous for each individual gene. The *Crc* mouse harbors a stop mutation in the cell polarity gene *Scribble*, which is the mouse orthologue of *Scribble* in flies (Murdoch et al., 2003). *Lp* also genetically interacts with a regulator of the PCP pathway, PTK7. PTK7 is highly conserved among species and encodes a transmembrane tyrosine kinase protein that, when mutated, leads to NTD and defects in stereociliary bundle orientation in mice (Lu et al., 2004). Two other mouse mutant lines, *Crash* and *Spin cycle*, display NTD and inner-ear defects that result from disruption of the PCP gene *Celsr1*, a large protocadherin seven-pass transmembrane molecule, orthologous to *Flamingo*, a *Drosophila* gene also known as *Starry night* (Curtin et al., 2003). Finally, double mutants of mouse *Disheveled1* and *Disheveled2* (*Dsh1* and *Dsh2*) exhibit NTDs very similar to the craniorachischisis observed in other PCP mutants. *Dsh1* and *Dsh2* share 65% sequence identity, and the fact that *Dsh1* null mice have a far less severe phenotype (Hamblet et al., 2002) than the double mutant indicates functional redundancy between the two proteins.

Recent studies indicate another role for the PCP proteins in cilia biogenesis. In *Xenopus*, loss of function of *Fuzzy* or *Inturned*, two orthologues of fly PCP effector proteins, results in NTD and failure of ciliogenesis due to incorrect microtubule orientation (Park et al., 2006). Disruption of *Fuz* in mice also causes NTDs, apparently due to disrupted ciliogenesis that in turn leads to defective Shh signaling (Gray et al., 2009). Furthermore, *inturned* in mammals is an important regulator of cilia formation, NT closure, and embryonic development, and *inturned* mutations affect Shh signaling (Zeng et al., 2010). A connection between ciliary function and NT closure has also been identified in humans. Meckel syndrome, the most frequently identified syndromic NTD, is associated with disruption of *MKS1* and *MKS3* (Kyttala et al., 2006; Smith et al., 2006). Mks1 is required for ciliogenesis and Hedgehog signaling in mice (Weatherbee et al., 2009). Genes involved in Bardet–Biedl syndrome (BBS), a human ciliary defect, have been disrupted in mice, leading to phenotypes shared with PCP mutants, including NTDs (exencephaly), open eyelids, and disrupted cochlear stereociliary bundles. BBS genes interact with *Vangl2* in mice and zebrafish and Vangl2, like BBS proteins, is localized to the basal body and axoneme of the cilia (Ross et al., 2005). Within the PCP pathway genes, the coding region and splice sites of *Vangl1* and 2 have been analyzed in a population of 66 human patients with NTDs, and only one variant in *Vangl1* was identified, which is predicted to result in a nonsynonymous change that is unlikely to have functional significance (Doudney et al., 2005). More recently, however, three patients with NTD were found to be heterozygotes for mutated VANGL1 alleles and five novel mutations in VANGL1 have also been found in patients with NTDs (Kibar et al., 2007, 2009). Moreover in a 2010 study, three novel missense mutations in VANGL2 were found in three different human fetuses with severe NTDs (Lei et al., 2010). Recent studies took advantage of this PCP pathway-specific knowledge generated from animal models to interrogate a larger set of PCP genes for mutations in samples from patients with craniorachischisis. This identified eight potentially causative mutations in CELSR1 and SCRIB. Overall, these studies make a strong case for a connection between PCP genes and NTDs.

27.4.2 Neural-Tube Patterning

Patterning of the neural tube along the AP and dorsal–ventral (DV) axes relies on tightly regulated molecular cascades. The patterning centers of the early embryo as well as their mechanisms of actions are described in Rubenstein and Rakic, 2013. Here, we will describe how disruption of patterning-related genes alters NT development and leads to NTDs in humans and mice.

27.4.2.1 Anterior–posterior Patterning Genes and NTDs

The major players of AP patterning include graded signals from fibroblast growth factors (FGFs), Wnt, and retinoic acid (RA). FGFs are key players in caudal transformation of anterior neural tissue to a more posterior fate (Kudoh et al., 2004; Rentzsch et al., 2004). FGFs are expressed in the regressing primitive streak, which continues to promote the progressive caudal patterning of the neural tissue (Dasen et al., 2003; Liu et al., 2001).

Along with FGFs, a number of other molecules, such as Wnts, Nodal, and RA, are present in the posterior of the embryo, and they refine the AP fate of the neural tissue (reviewed in Gamse and Sive, 2000). RA signaling acts to promote the neural character of the hindbrain and the anterior spinal cord (Bel-Vialar et al., 2002; Liu et al., 2001). RA and FGF act in opposition to each other to impart AP fates along the spinal cord. Studies in *Xenopus* embryos indicate a requirement for RA

signaling for expression of posterior markers (Hoxb9, N-tubulin, and Xlim1) and for establishment of posterior fate (Blumberg et al., 1996), and overexpression of Cyp26, an enzyme that mediates the degradation of RA, causes anterior structures to develop where normally there should be posterior structures (de Roos et al., 1999; Hollemann et al., 1998). The mammalian homologue, Cyp26A1, when ablated in mice, causes embryonic lethality, spina bifida, and truncation of the tail and lumbrosacral region. A higher risk for NTDs in humans has been associated with polymorphisms in RALDH2, an enzyme in the biosynthetic pathway of RA. Other enzymes which function in RA synthesis or metabolism, such as ALDH1, CYP26A1, CYP26B1, and CYP26C1, as well as cellular RA-binding proteins, such as CRABP1 and CRABP2, have been examined as potential NTD risk factors, but none of these genes has so far shown significant correlation with NTD risk in humans (Deak et al., 2005b).

Graded activity of Wnt signaling is also required for proper positioning of the midbrain–hindbrain border. The mechanism by which Wnt leads to posteriorization of the neural tissue is indirect and requires FGF signaling (Domingos et al., 2001). Mutations in components of the Wnt pathway can lead to NTDs. For example, a missense mutation of a highly conserved amino acid in the low-density, lipoprotein-receptor-related protein 6 (Lrp6), a co-receptor required for Wnt canonical signaling, causes exencephaly in homozygous mutant mice. Mice heterozygous for this mutation display the characteristic phenotype of a crooked tail (Carter et al., 2005).

27.4.2.2 Dorsal–ventral Patterning Genes and NTDs

Dorsoventral patterning largely utilizes a common mechanism throughout the length of the embryonic body. Four different classes of secreted factors influence DV patterning: Shh, bone morphogenetic proteins (BMPs), FGFs, and RA.

Correct patterning of the ventral half of the NT largely relies on the activity of Shh, whereas the dorsal half requires the inductive activities of the transforming growth factor-beta superfamily, which includes the BMPs, expressed in the overlying ectoderm and the roof plate. The roof plate is the dorsal equivalent of the floor plate, and it expresses BMP4, 5, and 7, which in turn induce dorsal genes, such as the transcription factors Pax3 and Msx (Liem et al., 1995, 1997). BMPs also act to set the borders of expression of bHLH (*Math*, *Mash*, and *Ngn*) and LIM (*Lbx* and *Lmx*) transcription factors (Helms and Johnson, 2003).

Tissues from humans with SBA have been evaluated for mutations in Bmp4 or the BMP inhibitor Noggin. Four heterozygous missense mutations in Bmp4 inherited from unaffected heterozygous parents in four different patients have been identified (Felder et al., 2002). Three out of the four missense mutations in Bmp4 (S91C, T225A, and R226W) are in the protein region responsible for dimerization, and the sequence alterations in this region have been proposed to influence the stability of the mature protein. The fourth missense mutation (S367T) was found within the C-terminal region that is physiologically important and hence might disrupt the function of the mature BMP4 protein. Moreover, one missense mutation (G92E) was found in Noggin, which replaces a highly conserved glycine in a region essential for proper structure and function of the Noggin protein. The sequence variants in these SBA patients may cause changes in protein stability and/or activity and therefore contribute to the failure of NT development (Felder et al., 2002).

Shh signaling, from the notochord and floor plate, acts on the surrounding neural cells to regulate the expression of transcription factors that control the formation of ventral cell types, such as motor neurons and ventral interneurons. As described above, genes involved in ciliogenesis are also needed for Hedgehog signaling and disruptions in this process are associated with NTD in mice and humans.

The Pax family of patterning molecules has been extensively studied for association with NTD. Many Pax genes are tightly regulated in both space and time during NT development (Stoykova and Gruss, 1994), and BMPs act in conjunction with Shh to set the correct expression boundaries of Pax genes. Mutations of *Pax3* in mice cause the *splotch* (*sp*) phenotype, which includes NTD (exencephaly and spina bifida) and dysgenesis of spinal ganglia, limb, and heart structures (reviewed in Gruss and Walther, 1992). *Pax3* mutations are associated with the Waardenburg syndrome in humans (pigmentation, limb, and enteric nervous-system defects and deafness) (Baldwin et al., 1994; Tassabehji et al., 1993). Attempts have been made to associate NTD in humans with mutations in *PAX 1*, *3*, *7*, or *9*. In two studies of 79 and 38 familial cases of NTDs, one mutation in the paired domain of *Pax1* was found in a patient with spina bifida (Hol et al., 1996); however, no further Pax mutations have been detected. Thus, the data to date do not show a strong association between Pax gene mutations and human NTDs.

27.4.2.3 Bending of the Neural Plate and NTDs

Another crucial step during NT formation is the bending of the neural plate in order to elevate and bring the two folds into close proximity along the midline. The MHP and two DLHPs are differentially used at different axial levels. Moreover, their formation is differentially controlled at a molecular level. For example, lack of *Zic2* function in mice causes loss of DLHP, although the MHP forms properly (Ybot-Gonzalez et al., 2007), and this results in extensive spina bifida (Elms et al.,

2003; Nagai et al., 2000). In mouse, the posterior neuropore completes NT formation, although it lacks a MHP; thus, the presence of the DLHP is sufficient for closure. The MHP may be dispensable for spinal neural-tube closure, as mice that lack Shh, HNF3β, Gli2, or Gli1 and Gli2 do not develop spinal NTD, despite failure to form the MHP, although less-acute bending still occurs by an unknown mechanism (Ang and Rossant, 1994; Chiang et al., 1996; Ding et al., 1998; Matise et al., 1998; Park et al., 2000; Weinstein et al., 1994).

The Shh pathway is critical in the bending of the neural plate. High Shh levels suppress DLHP formation (Ybot-Gonzalez et al., 2002), and low Shh levels induce their formation (Echelard et al., 1993), thus helping to set the position of DLHP formation. Loss of Patched, the Shh receptor that keeps the Shh pathway in an "off" state when a ligand is absent, causes both cranial and spinal NTDs in mice (Goodrich et al., 1997). One aspect of the phenotype could be that loss of Ptch constitutively actives the pathway and, like Shh overexpression, prevents DLHP formation. Loss of PKA, a protein kinase that downregulates Shh signaling, leads to spinal NTD (Huang et al., 2002). Mutation of Rab23, another negative regulator of the Shh pathway, disrupts DLHP formation and neural patterning leading to cranial NTD (Gunther et al., 1994).

The cellular basis for NT bending is apical constriction. The neuroepithelium, a pseudostratified epithelium of a single-layer thickness, begins to change its shape from columnar to wedge, first in the medial region and then at dorsolateral positions, where the hinge points will form (Figures 27.2(b), 27.2(c), and 27.3(c)). Our understanding of the cellular basis of this highly regulated constriction is limited. Two molecules, Shroom3 and p190RhoGAP, regulate apical constriction in the NT. Shroom3 belongs in the Shroom family but to date only Shroom3 has been associated with NTDs in mice. Shroom3 controls apical constriction via binding of filamentous actin. Mice null for *Shroom3* display exencephaly and loss of actin that would normally be localized on the apical-cell surface (Hildebrand and Soriano, 1999). P190RhoGAP is a negative regulator of Rho GTPase, which regulates actin microfilaments. Mice lacking p190RhoGAP have a number of defects, including NTD, and an excessive accumulation of polymerized actin in cells of the neural plate (Brouns et al., 2000). In addition to apical constriction, basal expansion, a process that is potentially also relying on cytoskeletal rearrangements, has been thought to assist NT bending (Smith and Schoenwolf, 1987); however, no mutants that disrupt this process have been identified.

Like *Shroom3*, other genes that regulate actin rearrangement at the cell surface are associated with NTDs. For example, cranial NTDs result from the disruption of protein kinase C, which targets a number of cytoskeletal regulators including the actin-binding protein vinculin, the cytoskeleton-related genes *mena* (*Enah*)/*profilin1* (*pfn1*), and *Macs* and *Mlp* (Koleske et al., 1998; Lanier et al., 1999; Stumpo et al., 1995; Xu et al., 1998). These data support the involvement of actin in NT formation. However, both older (Schoenwolf et al., 1988) and more recent (Ybot-Gonzalez and Copp, 1999) studies indicate that the MHP and DLHP are not affected by cytochalasin treatment, which blocks actin polymerization and elongation. This suggests that actin microfilaments may act to stabilize the hinge points after their formation, rather than to drive their formation.

Some of the genes involved in apical constriction and bending of the neural plate have been examined in human NTDs. In the Shh pathway, GLI3 mutations cause two different syndromes, the Greig cephalopolydactyly syndrome and the Pallister–Hall syndrome (Johnston et al., 2005, Radhakrishna et al., 1997), but neither syndrome presents with NTD. The lack of NTD is perhaps not surprising, given that the *Gli3* mutant mouse (*extratoes*) shows cranial NTDs with low penetrance and only in homozygous mutant embryos, and this also results in perinatal lethality (Winter and Huson, 1988). Mutations in *Shh* (Nanni et al., 1999) and in its receptor, *patched-1*, are associated with holoprosencephaly (Ming et al., 2002), a structural forebrain anomaly found with high frequency in humans. In studies conducted on human embryos with craniorachischisis, abnormal *Shh* expression was found (Kirillova et al., 2000), similar to altered expression of *Shh* in *Lp* mice (Murdoch et al., 2001a). However, the primary event leading to abnormal *Shh* expression was not identified. Variations in the *Shh* gene have not been correlated with other human NTDs, although these studies have largely been limited to the N-terminus part of the Shh protein, but not the C-terminus, which contains the autocatalytic cleavage site (Zhu et al., 2003). Variations in genes such as *PKA*, *Zic1*, *2*, *3* also failed to show correlation with human NTDs. In terms of apical constriction, only *MLP* and *MACS* have been examined as potential candidates for NTDs, but no correlation was found at least in 43 Caucasian simplex families in which the affected child had a lumbrosacral myelomeningocele (Stumpo et al., 1998).

27.4.2.4 NTDs due to Disruption of Neural-fold Fusion

The last step of NT closure, the joining of the two folds along the midline, also called fusion, is the least studied and least understood. It has been hypothesized that adhesion molecules facilitate neural-fold joining. However, whether such molecules play a direct role in fusion is unknown, although as outlined below, a few adhesion molecules are required for NT closure. In addition, it has been suggested that components of the extracellular matrix (ECM) might play a role in fusion (Moran and

Rice, 1975; Sadler, 1978), although it is unclear which ECM components may be involved.

Defects in a few adhesion molecules have been linked to NTDs. For example, in mice, inactivation of the ephrin receptor subfamily members *Eph-A5* or *Eph-A7* causes NTDs (Holmberg et al., 2000). Both ephrin receptors are expressed in the cranial, but not spinal, neuroepithelium. Ephrin signaling is thought to act through components of the PCP pathway. According to Lee et al. (2006), ephrinB1, a cell-surface-ephrin ligand, controls migration of cells during frog development by acting through PCP-pathway components and clustering of Dishevelled proteins. Two members of the cadherin family of adhesion molecules, N-cadherin and E-cadherin, have complementary expression patterns during neurulation, with N-cadherin expressed in the neural plate and E-cadherin expressed in the non-neural ectoderm (Detrick et al., 1990). Mice null for *N-cadherin* (*Cdh2*) undergo normal neurulation, showing that N-cadherin is not essential for NT formation (Radice et al., 1997). A role for E-cadherin has been more difficult to discern, as loss of E-cadherin results in embryonic lethality prior to neurulation. However, two pieces of evidence support a role for E-cadherin in NT closure. First, the culture of rat embryos with an antisense oligonucleotide against E-cadherin leads to cranial NT malformations (Chen and Hales, 1995). Secondly, when E-cadherin is downregulated in the non-neural ectoderm as occurs in Grhl2 mutants, the neural folds, face, and body wall fail to fuse (Pyrgaki et al., 2010a; Werth et al., 2010). NCAM1 is another adhesion molecule expressed in the neuroectoderm. Although *NCAM1* null mice do not display NTD (Cremer et al., 1994), NCAM1 has been studied in 132 families with spina bifida, and variations in this molecule may contribute to NTD risk (Deak et al., 2005a). Finally, NTD is observed in mouse embryos lacking protease activator receptor 1 and 2 (PAR1 and PAR2), which are expressed in the non-neural ectoderm during NT formation (Camerer et al., 2010). Thus, both cellular and molecular evidence support the idea that the non-neural ectoderm is involved in initiating and perhaps driving neural-fold fusion.

27.4.2.5 NTD due to Disruption of Apoptosis

During NT development, as during embryonic development in general, apoptosis and cell proliferation must be tightly regulated for development to proceed normally. Apoptosis or programmed cell death occurs in the neuroepithelium during neurulation. This was first documented by ultrastructural studies (Geelen and Langman, 1979; Schluter, 1973) and subsequently shown to be apoptotic cell death (Lawson et al., 1999). Either an increased or decreased rate of apoptosis has been associated with NTDs in mice. For example, NTDs in embryos lacking *ApoB* (Homanics et al., 1995), *Bcl10* (Ruland et al., 2001) or *Mdm4* are associated with increased apoptosis. In these cases, the phenotype is attributed to insufficient numbers of cells participating in neurulation.

Conversely, mutations in genes such as *Trp-53* (Sah et al., 1995), *Casp3* (Kuida et al., 1998), and *Apaf1* (Cecconi et al., 1998) lead to NTDs associated with reduced levels of apoptosis. In these cases, excessive numbers of cells that would otherwise be reduced by apoptosis are thought to prevent the normal morphogenesis of the neural plate. Despite the dramatic phenotypes caused by misregulation of apoptosis, experimental studies to block apoptosis with pharmacological agents during mouse neurulation indicates that apoptosis facilitates NT closure but is not necessary for its completion (Massa et al., 2009; Yamaguchi et al., 2011). To date, there have been no studies that attempt to correlate the risk for human NTDs with molecules of the apoptotic pathway.

27.4.2.6 NTD due to Disruption of Proliferation

Proliferation must be tightly controlled, as either increased or decreased rates of proliferation can lead to NTDs. During cranial neural closure, proliferation is differentially regulated along the dorsoventral axis of the NT, with the dorsal half displaying more proliferation than the ventral half (Copp et al., 2003). As proliferation and differentiation are inversely correlated, this difference is reflected by a greater number of differentiated cells in the ventral NT at this early stage of neurulation.

Changes in expression of genes that control proliferation often lead to abnormal NT development. For example, overexpression of *Notch3* results in exencephaly, and these mice have increased numbers of neuronal progenitors (Lardelli et al., 1996). Tumor-suppressor genes, when mutated in mice, can cause abnormal neural-cell proliferation and lead to exencephaly. Examples include loss of function of *p53* (Sah et al., 1995) and a hypomorphic mutation in *Brca1* (Gowen et al., 1996). A study of an Irish population showed that two noncoding variants of *p53* are associated with NTD risk and two different variants with maternal risk (Pangilinan et al., 2008). In addition, variations in *Brca1* in a family-based association study correlate with spina bifida, although this may not be causative (King et al., 2007). Mutations in *Tsc2*, another tumor-suppressor gene, cause exencephaly and embryonic lethality in mice (Kobayashi et al., 1999). Mice with heterozygous mutations for genes of the chromatin-remodeling complex, such as *Brg1* and *Srg1*, are predisposed to exencephaly, and they often get tumors of the nervous system (Kim et al., 2001). Hyperproliferation also causes exencephaly in the mouse *Phactr4*^{humdy} mutant, which indirectly regulates the

tumor-suppressor protein Rb (Kim et al., 2007). Hyperproliferation of the neuroepithelium before and during closure creates excess tissue, which inhibits bending of the NT and/or prevents the two folds from reaching each other. Reduced proliferation, on the other hand, leads to a number of cells inadequate to complete NT closure. Loss of mLin41 causes cranial NTD due to reduced proliferation and premature differentiation of the neuroectoderm, thus acting as a temporal regulator of neural progenitor maintenance (Chen et al., 2012).

Changes in cell number in tissues that surround the neuroepithelium can indirectly have a deleterious effect on NT formation. One example is reduced proliferation in the hindgut of *curly tail* mice, which likely carry a mutation in *Grhl3* (reviewed in Brouns et al., 2005). This hypoproliferation causes growth imbalance between the ventral and dorsal tissue, which in turn leads to excessive curvature within the caudal region and the failure of the posterior neuropore to close (Gustavsson et al., 2007; van Straaten and Copp, 2001). To date, the connection between the disruption of *Grhl3* and the proliferation defect remains elusive. Another example is the *open mind* mutation, which disrupts Hectd1 ubiquitin ligase, and results in inappropriate numbers of head-mesoderm cells and exencephaly (Zohn et al., 2007). Finally, *Twist1* mutant mice display exencephaly and severe craniofacial defects (Soo et al., 2002). *Twist1* is expressed in the head mesenchyme, where it regulates both proliferation (Ota et al., 2004) and apoptosis (Hjiantoniou et al., 2003), as well as neural-crest delamination and migration (Vincentz et al., 2008). The Saethre–Chotzen syndrome (SCS) in humans is due to TWIST1 mutations (Elanko et al., 2001; Kress et al., 2006). The SCS phenotype includes craniofacial defects but not NTD, although these phenotypes are thought to be due to haploinsufficiency rather than complete loss of TWIST1 function (el Ghouzzi et al., 1997).

27.5 FUTURE DIRECTIONS

In mice, there are currently >200 genes that are known to be required for neural-tube closure. However, surprisingly few of these models have been used to go beyond the question of the function of an individual gene. An interesting next level of exploration will be to determine the interplay between these genes. NTD in humans is largely thought to be a multigenic disease in which small changes in multiple genes may combine to lead to NTDs. Although the analysis of model organisms with homozygous mutations or severe loss-of-function phenotypes has been extremely useful, these models do not adequately reflect the complex genetics of human NTD. Therefore, it will be of interest to probe the phenotypic interplay between different gene mutations further, including that between genes that do not obviously fit within a particular pathway or cellular process.

The genetic complexity of human NTD leads to a cautionary note with respect to genetic counseling. Based on the already large number of gene candidates identified from animal NTD models and the fact that a significant portion of the genome is still not functionally analyzed, there will likely be on the order of 500–1000 genes required for NT closure and which, when mutated, can lead to NTD. This highlights the difficulty in trying to identify causative single-gene mutations in human NTD. Moreover, it becomes a daunting task to conceive of a comprehensive genetic screen. Ultimately, advances in personalized medicine and whole-genome analysis will open this field of research to a more comprehensive overview. Studies are underway to determine whether genome copy-number variations are associated with NTD, but these data are currently lacking. The potential to identify a large number of polymorphisms leads to the next question, namely, how to determine whether a polymorphism identified from association studies in humans may create a meaningful change in gene expression or protein function. This question can now be quite readily addressed by techniques that allow sophisticated manipulation of the mouse genome such that specific polymorphisms can be created and tested *in vivo* for changes in phenotype and/or protein function.

Another area ripe for exploration lies in determining the interplay between mouse NTD models and known environmental factors. For example, would NTD risk increase in mice heterozygous for a mutation in a gene required for NT closure when gestation occurs in a mother that is a model for diabetes or obesity? If so, this would greatly aid research in determining the molecular basis of NTD risk defined by epidemiological studies.

Epidemiological research lies at the heart of one of the most profoundly influential studies that has changed our approach to NTD risk. Epidemiological studies have clearly shown a decreased incidence of NTD as diets across the world have improved, and this positive response has been strongly correlated with a higher level of folic acid in the diet. Although the preventative effect of folic acid on NTD incidence has been recognized since the 1980s, surprisingly little basic research has been done to understand the mechanistic basis for this effect. To date, only 18 mouse NTD models have been tested for their responsiveness to folic acid. Of these, FA supplementation reduced the incidence of NTDs in six cases, was not beneficial in nine cases, and, contrary to expectations, exacerbated NTDs in three cases (Gray et al., 2010; Harris 2009; Marean et al., 2011). Testing of many more of these mouse models will provide critical information for understanding the molecular logic that may underlie responsiveness to folic-acid

supplementation. For example, are there particular pathways or cellular processes that are associated with responsiveness to folic-acid supplementation? Is the response allele-specific, or can a null allele be rescued, the latter perhaps suggesting that folic acid may bypass the defect? Folic acid provides the building blocks for many cellular reactions, including cell proliferation, gene expression, and genome stability, through its effects on nucleotide biosynthesis and as a methyl donor. Mouse NTD models can be used to tease out the complex function of folic acid, for instance, by targeting one arm of the biosynthetic pathway (i.e., supplementation with purines or biasing the diet toward methyl donors) in order to determine the mechanisms of folic-acid responsiveness better. Furthermore, there is a need to reconsider the treatment traditionally done in the mouse studies, which consists of short-term (days) exposure to folic-acid supplementation. This is not reflective of the trend in long-term folic-acid exposure in the U.S. population, as mandatory FA fortification began in 1998 and periconceptual use has been strongly recommended since the early 1990s. Indeed, recent long-term and multigenerational studies in mice indicate that the incidence of NTD for a given genetic allele can differ between short- and long-term folic acid diets and that the epigenetic profile can vary widely in wild-type mice fed a methyl diet for six generations (Li et al., 2011; Marean et al., 2011). These unexpected findings highlight the need to understand how FA influences NT closure and the mechanisms and genetics underlying the response to FA. Finally, in both mice and humans, there are a relatively large proportion of folate-resistant NTDs. One goal will be to utilize the knowledge gained from understanding the normal function of the mutated gene and its downstream pathways to determine whether other periconceptual therapies can be designed based on this insight. The types of studies outlined above will be critical to a better understanding of the complex genetic interplay that likely represents non-syndromic NTD in humans, as well as the intriguing but poorly understood intersection between genes and environmental influences in the highly complicated process of neural-tube closure.

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