# Mouse Models of Neural Tube Defects

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## 2.1 Overview

During embryonic development, the central nervous system forms as the neural plate and then rolls into a tube in a complex morphogenetic process known as neurulation. Neural tube defects (NTDs) occur when neurulation fails and are among the most common structural birth defects in humans. The frequency of NTDs varies greatly anywhere from 0.5 to 10 in 1000 live births, depending on the genetic background of the population, as well as a variety of environmental factors [1–3]. The prognosis varies depending on the size and placement of the lesion and ranges from death to severe or moderate disability, and some NTDs are asymptomatic. This chapter reviews how mouse models have contributed to the elucidation of the genetic, molecular, and cellular basis of neural tube closure, as well as to our understanding of the causes and prevention of this devastating birth defect.

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## 2.2 Types of NTDs

The neural tube initially forms as a flat epithelial plate that must roll into a tube to form the brain and spinal cord. Defects in this process result in NTDs, a constellation of malformations of the central nervous system (Fig. 2.1). The most common NTD in humans is spina bifida, which results from failure of closure in the spinal region. The consequence of spina bifida varies greatly, depending on the size and placement of the lesion, the involvement of the spinal nerves and meninges, as well as the presence of associated conditions such as hydrocephalus, Chiari malformation, genitourinary, and gastrointestinal disorders. Spina bifida can manifest as myelomeningocele, meningocele, or spina bifida occulta. Myelomeningocele is the most common and severe form of spina bifida and involves protrusion of the meninges and spinal cord through an opening in the vertebrae. Meningocele occurs when the meninges but not the spinal cord protrude. Spina bifida occulta can be asymptomatic and occurs when the dorsal part of vertebrae does not properly form. More severe open NTDs include craniorachischisis and anencephaly. Craniorachischisis is the most serious NTD, resulting from failure of neural tube closure along the entire neural plate. Exencephaly (the embryonic precursor to anencephaly) occurs when closure fails in the anterior neural plate or future brain. Anencephaly and craniorachischisis



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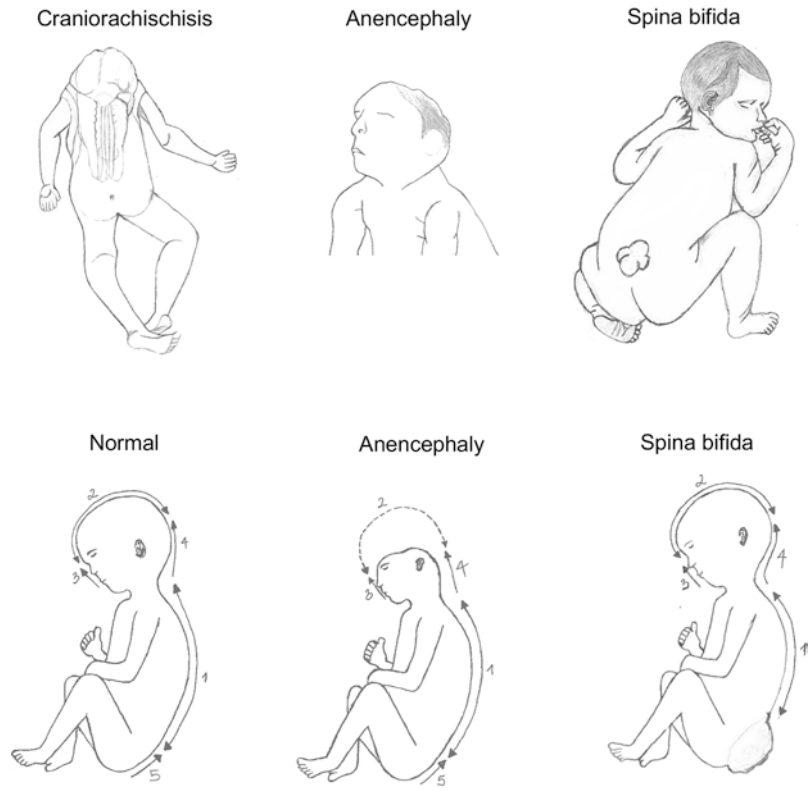


**Fig. 2.1** Top panels. Types of neural tube defects that originate from failure of neural tube closure. Craniorachischisis occurs when the neural tube fails to close along the entire length of the neural plate. Anencephaly occurs when closure fails in the cranium and spina bifida at the posterior end of the neural tube. Bottom Panels. Regions of neural tube closure postulated by analysis of defects in human embryos superimposed on newborn body. During normal neural tube formation, multiple zones of neural tube closure extend in anterior and posterior directions from distinct closure points. Zone 1 is in the spinal cord; zones 2, 3, and 4 in the cranium; and zone 5 in the most posterior of the neural tube. Anencephaly is caused by the failure of neural plate fusion in regions 2–4 and spina bifida by the failure of regions 1 and 5. Illustrations are after reference [328] and courtesy of Claris Nde

are fatal, resulting in the prenatal death or demise of the newborn shortly after birth. Spina bifida occulta and multiple abnormalities are classified clinically as NTDs; however, the developmental origins of these malformations are not due to failure of neural tube closure. Closed NTDs include encephalocele, iniencephaly, hydrocephalus, microcephaly, and holoprosencephaly. Encephalocele occurs when the cranial vault fails to form properly around a closed neural tube, leading to protrusion of the brain and meninges through an opening in the skull, whereas other NTDs such as iniencephaly, hydrocephalus, microcephaly, and holoprosencephaly result from improper growth of the closed neural tube.

## 2.3 Diagnosis and Treatment of NTDs

Most NTDs are diagnosed before birth by standard prenatal screening tests. High levels of alpha fetal protein (AFP) in maternal serum or in amniotic fluid are correlated with NTDs and signal the need for further testing. Most NTDs can be identified by ultrasound during the routine anatomy scan between 18 and 22 weeks. Babies with spina bifida are typically delivered by cesarean section, and the lesion is surgically corrected either in utero or shortly after birth [4, 5]. However, secondary defects frequently occur with spina bifida, including Arnold-Chiari malformations with hindbrain herniation, hydrocephalus requiring

placement of a shunt, and tethering of the spinal cord leading to progressive pain, incontinence, and weakness of the lower extremities, as well as spinal deformities [6–11]. Nerve damage can result in neurogenic bladder and bowel or paralysis of lower extremities requiring the need to use a wheelchair, braces, or crutches [5]. Because of reduced sensation to lower extremities, patients are susceptible to unrealized infections, which may necessitate amputation of damaged limbs. Other complications include learning disabilities, social issues, and latex allergies [5]. In spite of these complications, with improvements in care, the majority of patients survive well into adulthood [5, 8].

## 2.4 The Etiology of NTDs

While the cause of individual cases of NTDs are rarely known, the vast majority of NTDs are due to complex interactions of multiple genetic and environmental factors with an estimated 60–70% of NTDs having a genetic contribution [12–14]. Evidence for the genetic causes of NTDs comes from the finding that chromosomal abnormalities are often present in NTD-affected fetuses, and NTDs are noted in spontaneous abortions with abnormal karyotypes [15–18]. NTDs also occur at higher rates in certain genetic syndromes, including Meckel-Gruber, Waardenburg, and 22q11.2 deletion syndromes [19–33]. Finally, twin studies indicate a 5% concordance rate, and NTD risk is significantly increased in NTD patients or individuals with a previously affected pregnancy [18, 34–37]. In spite of a clear genetic component, few causative genes have been identified. This is in part due to complex etiology of the malformation, the number of genes that could cause the defect, as well as the existence of few multiplex families for genetic studies. While thus far a handful of genes associated with NTDs were identified in small cohorts of patients, few definitive causative genes are known [38]. Interestingly, the majority of variants identified to date are linked to the noncanonical Wnt pathway that controls planar cell polarity or to folic acid metabolism, implicating these as key pathways driving NTDs in humans [39, 40]. This chapter will focus on Wnt signaling and folic acid metabolism to illustrate how the study of mouse models has been essential in elucidating the central role of these pathways in neurulation.

In addition to the large number of genes that could cause NTDs, another complicating factor in finding the genetic causes of NTDs in humans is the complex etiology of these defects. The majority of genetic mutations involved in NTDs do not likely cause a defect unless combined with other genetic or environmental factors. The multifactorial threshold model (Fig. 2.2) is proposed to account for the pattern of NTD inheritance observed in humans where multiple factors of small effect interact to cause a disease [41, 42]. This model postulates that neural tube closure is a threshold event that occurs either successfully or not, resulting in either normal neural tube closure or defects. A single genetic insult or environmental exposure might not cross the threshold to cause NTDs, but one or more factors in combination result in failure of neural tube closure. The mouse model is a tractable experimental system in which to test the multifactorial threshold model and test gene–gene, gene–environment, and environment–environment interactions [41, 43]. Digenic inheritance can be modeled in mouse in compound mutants, or modifier variants do not cause NTDs themselves but increase the penetrance and/or severity of defects in combination [42, 44–50]. Gene–environment interactions are also tractable in the mouse model. For example, the impact of alterations of either macro- or micronutrients on the incidence and severity of NTDs can be studied in models [51]. Varying macronutrients such as dietary protein, fat, and carbohydrate composition of the mouse chow can influence NTD risk [51–54]. Micronutrient supplementation with folic acid, inositol, retinoic acid, iron, as well as nutrients that feed into the folate pathway, including vitamin B12, choline, methionine, formate, and glycine, can also impact NTDs in a variety of mutant mouse models [51, 55–59]. Studies of mouse models of diabetes provide novel insight as to the genes and pathways that interact with hyperglycemia to cause NTDs [51, 60, 61]. Exposure to teratogens, including

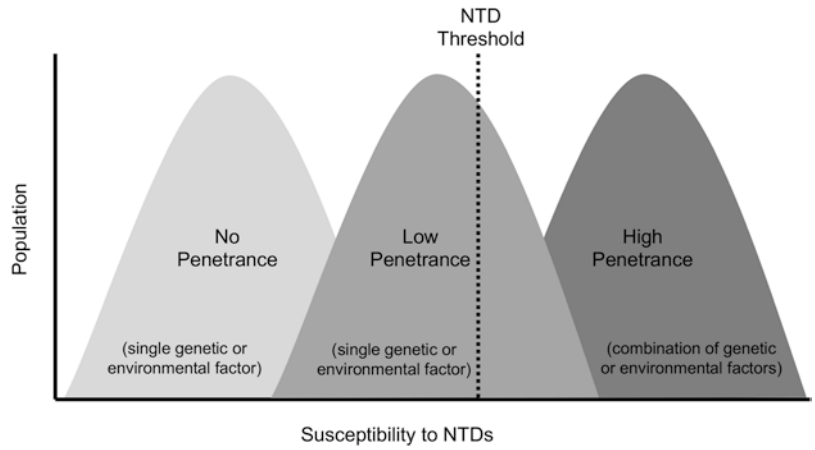


**Fig. 2.2** Multifactorial threshold model illustrating the complex inheritance of NTDs. Multiple genetic and environmental factors contribute to the susceptibility for NTDs. Defects result when neurulation is significantly disrupted so that a threshold event, represented by the dotted line (NTD threshold), is surpassed. Susceptibility to NTDs follows a normal distribution, and in isolation, factors may not be sufficient to cause NTDs (no penetrance) or only a few individuals with a particular contributing factor show NTDs (low penetrance). However, factors in combination can interact to surpass the NTD threshold, resulting in a high percentage of individuals showing NTDs

medications (e.g., valproic acid), arsenic, the mycotoxin fumonisin, or hyperthermia, as a result of hot tub usage or maternal fever can induce NTDs in mouse models [62–65].

## 2.5 NTDs Result from Failure of Neural Tube Closure

Primary neurulation is a complex morphogenetic process that results in the transformation of the flat neural plate into the neural tube (Fig. 2.3). Neural tube formation involves the coordinated growth and morphogenesis of multiple tissues. Forces that drive neural tube closure arise from the neural tissue itself (intrinsic forces), as well as from the adjacent surface ectoderm and underlying mesoderm (extrinsic forces; [66]). Primary neurulation begins after gastrulation as the neuroepithelium is induced from the embryonic ectoderm. Following induction, the neural plate forms as individual neuroepithelial cells elongate, resulting in a thickening of the ectoderm on the dorsal side of the embryo. Two coordinated morphogenetic movements intrinsic to the neural plate drive elevation of the neural folds by facilitating the rolling of the plate into a tube. Convergent extension (CE) movements drive lengthening and narrowing of the neural plate and direct formation of hinge points around which the neural plate bends. A single hinge point forms in the midline of the neural plate (medial hinge point (MHP)), followed by the formation of paired dorsal lateral hinge points (DLHPs) in lateral regions. Extrinsic forces from the surface epithelium and surrounding mesenchyme also promote elevation of the neural folds. As the paired neural folds meet in the dorsal midline, they fuse and the neural and surface epithelium remodels to form two separate epithelial sheets.

Broadly speaking, two mechanisms of neurulation are employed to form a neural tube, primary and secondary neurulation. Primary neurulation is when a flat neural plate rolls into a tube, whereas secondary neurulation occurs when mesenchymal cells coalesce into a tube. In amniotes, the majority of the central nervous system is formed by primary neurulation, whereas the most posterior portion of the spine caudal to the sacral vertebrae forms by secondary neurulation [67–69]. In primary neurulation, the neural plate does not roll into a tube all at once; rather, closure is initiated at discrete points, followed by "zipping" to fuse the neural folds together (Fig. 2.1; [70]). Closure 1 initiates at the hind-

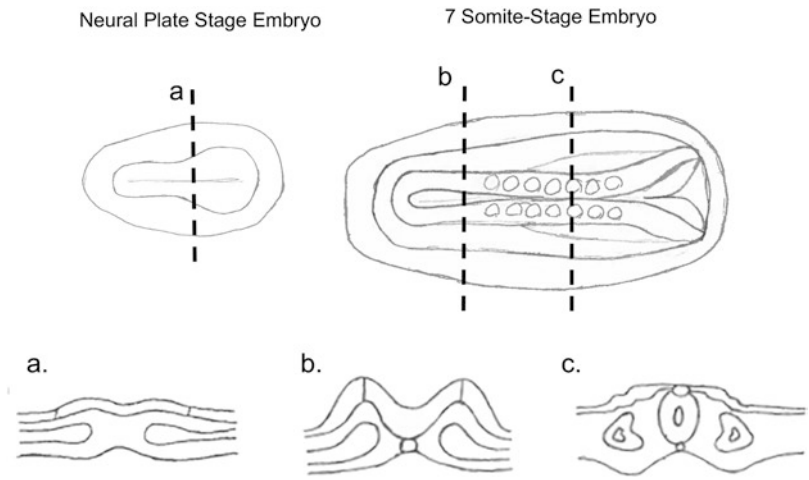


**Fig. 2.3** Neural tube closure in the human embryo. The top-left panel shows an illustration of a neural plate stage embryo where the neural plate and neural grove has formed but the neural folds have not yet begun to elevate. The top-right panel shows a seven-somite stage embryo with a neural tube that has begun to form in the spinal region but the posterior neural pore is not yet closed and neural fold elevation is just beginning in the cranium. Bottom panels show cross-sectional views of the neural plate in different stages of closure from positions delineated by the dotted lines in the top panels. (**a**) Cross-section of a neural plate stage embryo where the neural groove is formed but the neural folds have not elevated. (**b**) Cross-section of neural plate where neural folds are in the process of elevating. (**c**) The neural tube has closed, and the neural ectoderm and nonneural ectoderm are in the process of separating. Illustrations courtesy of Claris Nde

brain/spinal cord boundary and extends in both anterior and posterior directions. This is followed by the formation of closure points in the cranial region: Closure 2 at the midbrain/forebrain boundary and Closure 3 at the anterior aspect of the forebrain. The position of Closure 2 is variable between mouse strains, and its position is correlated with strain-specific susceptibility to exencephaly [71, 72]. Closure 2 may also be variable during human neurulation, as it has been identified in some but not other human embryo samples [73]. Another closure point then forms at the caudal end of the spine as closure of the posterior neuropore becomes imminent [74]. As primary neurulation ceases, there is a transition zone where the dorsal portion of the neural tube undergoes elevation and folding, whereas cells of the ventral neural tube delaminate and then integrate into the neural tube [75]. As neurulation proceeds further, this transition zone gives way to purely secondary neurulation where neuromesodermal progenitors undergo mesenchymal to epithelial transitions to incorporate into the forming neural tube [76]. Disruptions in any of these processes can result in NTDs. The remainder of this chapter will review the molecular and cellular basis of these processes, illustrating how studies in animal models reveal their integration to provide a basis for the interaction of genetic lesion impacting these processes in human NTDs.

## 2.6 Mouse Models Have Been Instrumental in Elucidating the Mechanics of Neural Tube Closure

While multiple animal models are used to study neurulation, the mouse has several advantages. First of all, as opposed to that in frogs (African clawed frog, *Xenopus laevis*) and fish (zebrafish, *Danio rerio*), neural tube closure in chickens and mice is most similar to that in humans, where primary neurulation occurs in the majority of the neural tube. In contrast, zebrafish employs a modified secondary neurulation process along the entire neural axis in which deep and superfi-

cial mesenchymal cells converge toward the midline and coalesce into a neural keel intermediate. Deep and superficial cells then undergo radial intercalation to form an epithelial tube [77]. The *Xenopus* neural plate is also stratified into deep and superficial layers [78], and apical constriction occurs in the superficial layers to drive neural fold elevation [79]. Once the folds fuse in the dorsal midline, deep and superficial cells undergo radial intercalation to form a pseudostratified epithelium. While the pathways that control cell shape changes, such as convergent extension and apical constriction are conserved between these animal models, overall difference in morphogenesis between these models makes the mouse and chicken most broadly relevant for understanding human neural tube closure.

The mouse also has the advantage of being amenable to genetic approaches to study the genes required for neural tube closure. The availability of numerous mouse mutants with NTDs provides a rich source of diverse models for study to elucidate the genes and pathways required for neural tube closure [42, 44, 45, 59]. However, because the mouse embryo develops in utero, examination of the cell behaviors that underlie neurulation presents significant challenges compared to models that develop exteriorly. Thus, historically most of what is known about the dynamic cell movements and behaviors that drive neurulation comes from studies in the frog, fish, and chicken. Yet recent advances in live-imaging approaches combined with improved *ex utero* culture conditions are beginning to overcome these hurdles, providing new insight as to the cell and tissue movements that underlie neural tube formation in the mouse and how genetic mutations disrupt this process [74, 80–90].

## 2.7 Convergent Extension Movements and the Planar Cell Polarity Pathway

Following a thickening of the neuroepithelium, the neural plate undergoes convergent extension movements, resulting in lengthening and narrowing along the anterior-posterior and medial-lateral axes. Polarized cell behaviors that mediate convergent extension movements are controlled by the planar cell polarity (PCP) pathway [91–93]. PCP was first described in *Drosophila*, where it controls the polarity of cells within an epithelium and the positioning of asymmetrically localized structures such as wing hairs [94]. The PCP pathway is conserved in vertebrates and is controlled by the non-canonical Wnt pathway leading to asymmetrical distribution of protein complexes within an epithelium (Fig. 2.4). During neurulation, PCP regulates the polarization of mediolateral protrusions that drive convergent extension movements [95]. Best studied in the *Looptail* (*Lp*) mouse line with mutation of *Van Gogh like-2* (*Vangl2*), defective convergent extension leads to craniorachischisis, where the neural tube fails to close along the entire anterior-posterior axis accompanied by shortening of the embryo and a wider midline and floorplate [96–104]. Interestingly, human embryos with craniorachischisis are short with a broad floorplate [105], suggesting that similar mechanisms may underlie craniorachischisis in humans. *Vangl2* is necessary for convergent extension movements in the notochord and neural plate [98], and mutations in other PCP pathway genes also result in NTDs in the mouse. For example, compound mutants for the vertebrate homologues of *Disheveled* or *Frizzled* receptors show craniorachischisis [106–108], as do targeted knockouts of other PCP pathway components such as *Celsr1*, *Wnt5a*, *and Ptk7* [109–112]. Mutations of PCP genes can also result in spina bifida and exencephaly [106, 113–117].

Consistent with the multifactorial threshold model for NTDs, a number of genes can interact with $Vangl2^{Lp}$ heterozygotes, resulting in NTDs in compound mutants. For example, $Vangl2^{Lp}$ can genetically interact with other PCP genes, including *Wnt5a*, *Vangl1*, *Dvl2*, *Dvl3*, *Celsr1*, *Fz1, Fz2*, *Daam1*, and *Protein tyrosine kinase-7* (*Ptk7*) in compound mutants to cause NTDs [76, 97, 107–112, 118–121]. Additionally, $Vangl2^{Lp}$ can genetically interact with mutations in genes not previously identified as regulating PCP pathways to give NTDs. These include *Grhl3*, *Bardet-Biedl syndrome-1 (BBS1)*, *BBS4*, *BBS6*, *cordon bleu* (*cobl*) and *Scribble* (*Scrbl*), Syndecan 4

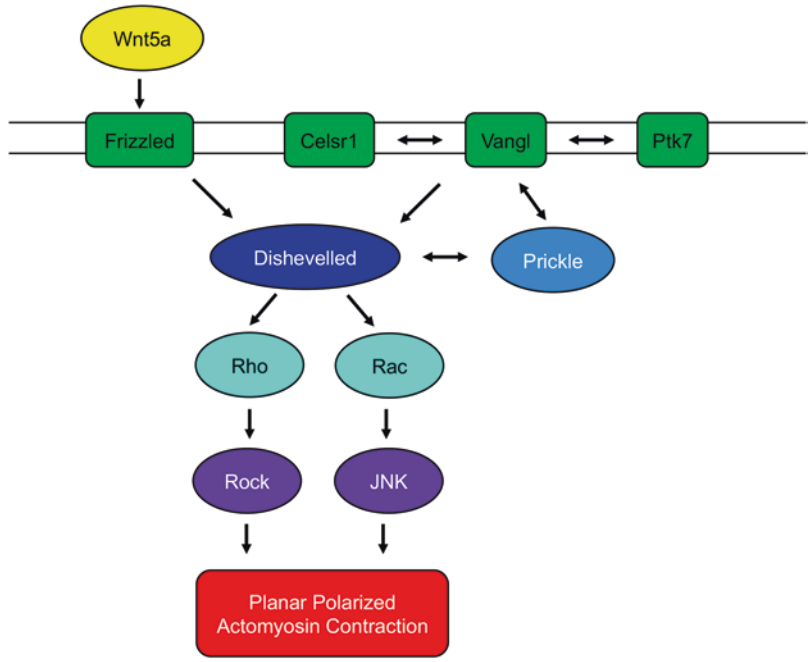


**Fig. 2.4** Key elements of the noncanonical Wnt/planar cell polarity pathway signaling pathway involved in neural tube closure in humans and mice. Wnt5a stimulates the PCP pathway by binding to Frizzled that interacts with Celsr1, Vangl, Prickle, and the coreceptor Ptk7 to recruit disheveled (Dvl). Dvl activates the small GTPases Rho and Rac, leading to planar polarized actomyosin contraction

(Sdc4), and Sec24b [121–127]. Interestingly, heterozygous *Vangl2*$^{Lp/+}$ embryos show a slightly wider and shorter midline and delayed neural tube closure [98], providing the basis for the development of NTDs in heterozygous embryos and in genetic interaction experiments.

PCP genes are also associated with NTDs in humans. Thus far, multiple mutations in a variety of PCP-related genes are associated with NTDs in humans, including predicted and/or proven deleterious mutations in *CELSR1*, *CELSR3*, *FZD6*, *PRICKLE1*, *VANGL1*, *VANGL2*, *FUZ*, *SCRIB*, *PTK7*, and *DACT1* [40, 128–148]. The deleterious nature of a handful of these sequence variants has been verified in a variety of assays to test the ability to rescue PCP phenotypes in zebrafish, binding to known interacting proteins or altered localization in polarized epithelium [129, 134, 136, 137, 139, 140]. Remarkably, digenic inheritance has also been found involving PCP genes in human patients [40, 141, 142, 145, 148].

## 2.8 Hinge Point Formation

The medial point in the spinal cord is formed as cells of the neural epithelium become wedge shaped eliciting bending of the neural plate around these hinge points (Fig. 2.5). The pseudostratified neuroepithelium is comprised of bipolar neural progenitors with a nucleus that moves between apical and basal positions dependent upon the phase of the cell cycle. During mitosis, the nucleus is localized at the apical surface, and during other phases of the cell cycle, it is positioned more basally. As hinge points form, the cell cycle is prolonged, resulting in greater numbers of cells in the hinge point in nonmitotic phases and nuclei localized in basal positions [149–152]. The majority of cells in the MHP have basally positioned nuclei, resulting in multiple wedge-shaped cells that contribute to the bending of the epithelium. This in combination with local destabilization of adherens and tight junctions at the hinge points allows bending of the rigid neural plate at the hinge points [149–151]. The rigidity of the neural plate is maintained by apical constriction involving nonmuscle myosin that contracts the

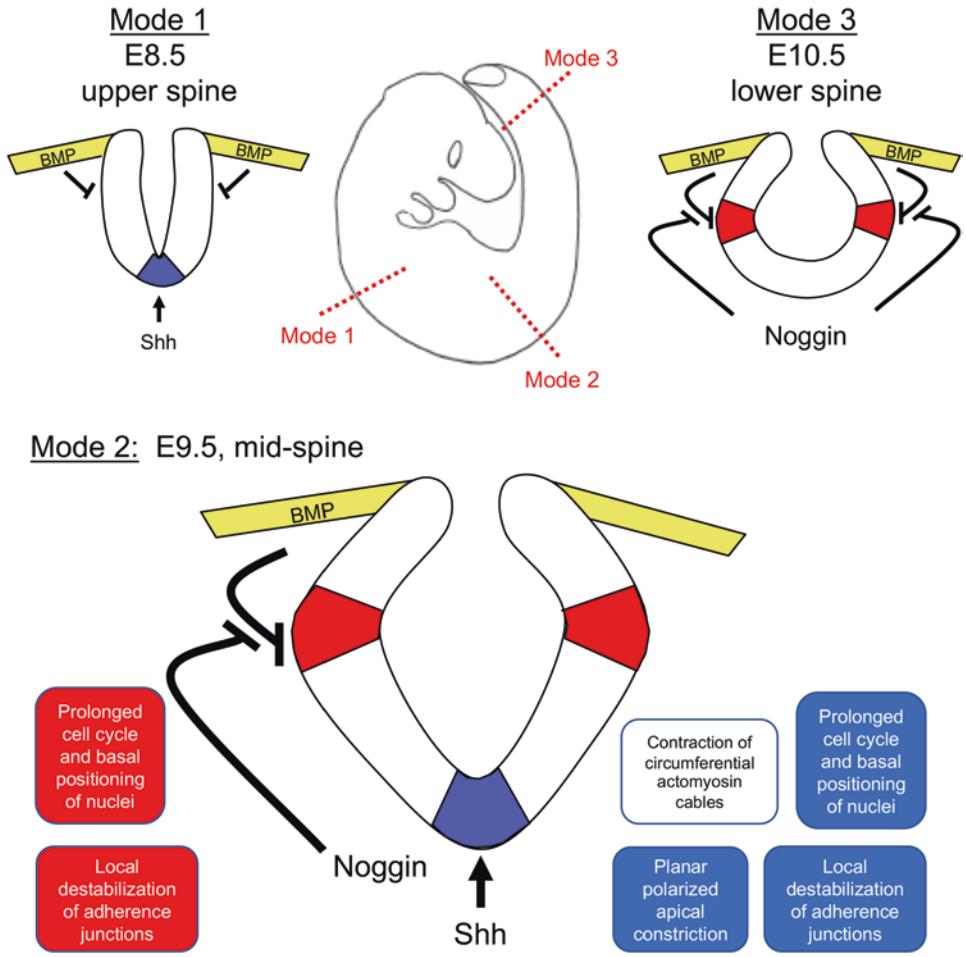


**Fig. 2.5** Interaction of BMP and Shh signaling results in different modes of neurulation along the anterior-posterior axis. In the anterior spinal cord (Mode 1), only the medial hinge point (MHP) (blue) forms. In the mid-spinal cord (Mode 2), both MHP and paired dorsal lateral hinge points (DLHP) (red) form. In the posterior spinal region (Mode 3), only exaggerated DLHPs are found. The formation of the MHP is promoted by Shh from the notochord and that of DLHPs is inhibited by BMP from the nonneural ectoderm. BMP expression is consistent along the anterior/posterior axis, but Shh is not expressed in the lower spinal cord and the BMP antagonist Noggin is not expressed in the anterior spinal cord. In Mode 1 neurulation, BMP and Shh are expressed and inhibit DLHP but promote MHP formation. In the mid-spinal cord region, Mode 2 neurulation involves both MHP and DLHPs. Here Noggin blocks the DLHP inhibiting activity of BMP. In the posterior spinal region, Shh expression is weak or nonexistent and no MHP forms. The absence of Shh and the presence of Noggin promote the formation of prominent DLHPs. BMP and Shh influence hinge point formation by regulating cellular behaviors. In the DLHP (red) and MHP (blue), inhibition of BMP prolongs the cell cycle, resulting in increased number of cells with basal positioned nuclei, as well as local destabilization of adherence junctions, which leads to buckling of the neural plate around regions (white) where circumferential contraction of actomyosin cables promote a rigid neural plate. Planar polarized apical constriction also contributes to formation of the medial hinge point

circumferential actomyosin cables anchored at the adherens junction [153, 154].

In the cranial region, coordinated apical constriction of the neural epithelium also contributes to hinge point formation and bending of the neural plate [149, 153]. The contractile force needed for apical constriction is also generated by myosin contracting the actin filaments, a process involving the small GTPase RhoA, ROCK, and myosin light chain kinase [155–158].

Inhibition of this kinase cascade or mutation of actin-binding proteins disrupts neural tube closure [159–164]. One of the best studied regulators of apical constriction in the cranial region is Shroom3 [155, 156, 158, 165, 166]. Importantly, putative loss of function sequence variants in *SHROOM3* are associated with NTDs in humans [167–169].

## 2.9 Apical Constriction Is Coordinated with PCP Activation in the Neural Plate

Dynamic integration of PCP and apical constriction pathways drives simultaneous convergent extension and bending of the neural plate [158, 170–173]. Asymmetrical enrichment of PCP components with apical constriction pathways at the mediolateral facing edge of neuroepithelial cells results in the tightening of actomyosin cables preferentially along the mediolateral axis to allow for the rolling of the neural plate [170]. Narrowing and lengthening of the neural plate also involves the coordination of PCP and apical constriction as epithelial rosettes resolve in a preferred direction [174, 175]. This complex and intimate link between the dynamic localization of core PCP proteins, actomyosin assembly, and polarized junction shrinking during cell intercalation is key for neural tube closure [176]. This interaction also provides a basis for genetic interaction of the basal-lateral Scribble and the core PCP protein Vangl2, which results in craniorachischisis in $Vangl2^{Lp/+}$;$Scrbl^{Ccr/+}$ compound mutants [122].

## 2.10 Formation of Hinge Points Is Regulated by Shh and BMPs

The relative contribution of the MHP and DLHPs to neurulation differs along the anterior-posterior axis of the spinal cord (Fig. 2.5; [177]). In the anterior spinal cord, MHPs are most prominent and DLHPs fail to form, resulting in the neural plate folding over the MHP and the neural folds meeting in the dorsal midline. This pattern of neurulation is referred to as "Mode 1." In more caudal regions, both the MHP and paired DLHPs are prominent and the neural plate rolls around these hinge points. This is referred to as "Mode 2" neurulation. In the posterior spinal cord, "Mode 3" neurulation predominates where a prominent MHP does not form and the neural folds roll around the DLHP. In the cranial region, both MHP and DLHPs form and DLHP formation is a dynamic process, as evident in live-imaging experiments where DLHPs form, disappear, and then reform as the neural folds elevate [81].

The dynamic activity of Shh and BMPs along the anterior-posterior axis of the spinal cord influences the mode of neurulation (Fig. 2.5; [178]). *Shh* is expressed at highest levels in the anterior regions of the spinal cord and is almost nonexistent in the most caudal regions [178]. Moreover, Shh and BMPs inhibit formation of the DLHPs [178, 179]. BMPs are secreted from the surface ectoderm, and their expression remains essentially constant along the spinal neural plate. However, the BMP antagonist Noggin is expressed in middle and posterior regions, where it promotes DLHP formation by inhibiting BMPs and destabilizing adherens and tight junctions [149–151]. While disruption of BMP signaling results in NTDs [180–186], loss of Shh signaling results in exaggerated hinge points, and the neural tube still closes. On the other hand, activation of Shh signaling by loss of negative regulators results in failure of DLHP formation and neural tube closure in regions of the neural tube where DLHPs are critical [187]. Importantly, sequence variants in negative regulators of Shh signaling, including *SUFU*, *PTCH1*, *PKA*, and *GPR161*, are associated with spina bifida in humans [188–191].

## 2.11 PCP, Ciliogenesis, and Shh Signaling

PCP signaling also influences the positioning of cilia on the cell [192]. Many of the genes that interact with $Vangl2^{Lp}$ to cause NTDs in mouse

models are involved in cilia, including BBS (Bardet–Biedl syndrome) proteins. While NTDs are not commonly described as features of BBS, mouse mutants in some of the genes that cause BBS show a low penetrance of NTDs or interact with other genes to cause NTDs [126, 193]. Similarly other ciliopathies, such as Meckel-Gruber (MKS) and Joubert syndromes are also associated with NTDs in mouse models but not the human syndrome [194–196]. Mutations of the PCP effector proteins *Fuzzy* and *Inturned* result in defects in cilia and Shh signaling and neural tube closure [115–117]. Because cilia play an essential role in the transduction of Shh signaling [197, 198], the PCP pathway can potentially interact with Shh signaling to cause NTDs.

## 2.12 Role of the Nonneural Ectoderm in Neural Fold Elevation and Fusion

The nonneural ectoderm is required for neural tube closure by providing an inductive signal for DLHP formation, a driving force for the elevation of the neural folds and participating in the fusion of the neural folds [199–203]. In chicken embryos, removal of the surface epithelium results in failure of DLHP formation and neural fold elevation [202]. This could reflect either an inductive or a mechanical role in DLHP formation and elevation of the neural folds. In support of an inductive role, removal of all but a small strip of surface epithelium is sufficient to induce DLHPs [202]. BMP and Noggin are expressed in the surface ectoderm, and culture with a Noggin-coated bead will induce DLHPs [179]. On the other hand, oriented cell divisions in the epidermis of the chicken embryo drive medial-lateral expansion of the tissue [204], and the surface epithelium in *Xenopus* migrates medially during neural tube closure [203], potentially providing a mechanical force for neural fold elevation. The surface ectoderm differentially contacts the neural tube along the anterior posterior neural axis and it is likely that the role of the surface ectoderm changes as well [173].

*Grhl2* and *Grhl3* are expressed almost exclusively in the surface ectoderm during neurulation and are required for the proper development of the epidermis and neural tube closure [205–212]. *Grhl3* is also expressed in the hindgut epithelium, and mutation of *Grhl3*$^{ct}$ in a hypomorphic mouse line creates an imbalance in proliferation between the posterior neural tube and the underlying hindgut epithelium resulting in spina bifida [213]. *Grhl3* and *Grhl2* null mouse mutants show defects in more anterior regions of the spinal cord and failure of DLHP formation in spite of normal expression of epidermally derived factors involved in DLHP formation, such as *BMP2* and *Noggin* [205–212]. Importantly, *GRHL* genes are implicated in human NTDs [167, 168, 214].

During fusion of the neural folds, cells extend finger-like projections that contact protrusions on the opposing neural folds, intercalate, draw the folds closer, and fasten them together [81, 82]. The neural folds are comprised of neural and nonneural ectoderm, which extend different projections in regionally distinct areas of the neural tube [85, 90, 153]. Live-imaging experiments in the mouse suggest that closure in the hindbrain/midbrain region does not occur by "zipping" but rather formation of multiple intermediate closure points that "button up" the folds together [82, 89]. The tissue layer that makes initial contact differs based on the anterior-posterior level. Between closure points 1 and 2, fusion is initiated by cells of the nonneural ectoderm, followed by cells of the neural ectoderm [82, 215]. Between closure points 2 and 3, both layers contact at the same time while initiation at closure 3 is mediated by the neural ectoderm [215]. Scanning electron microscopy revealed that protrusions are predominantly filopodia during early stages of spinal neurulation, then replaced by membrane ruffles and filopodia [90, 153]. The PCP pathway is also required for directional protrusive activity of the neural epithelium during fusion [76]. *Grhl2* is also required for neural fold fusion evident in live-imaging experiments where elevation and apposition of the neural folds can occur but fusion fails [208]. As the neural folds meet in the midline, extensive tissue remodeling separates the neural and nonneural

ectoderm joining the opposing folds. Molecularly, GRHL transcription factors influence expression of multiple proteins that can influence neural fold fusion, including adherens junctions, as well as proteins that suppress EMT to reinforce the epithelial properties of the nonneural ectoderm during tissue remodeling [86].

## 2.13 Prevention of NTDs by Micronutrient Supplementation

Maternal diet is a key environmental factor influencing the incidence of NTDs, and by the 1960s, folic acid emerged as a key micronutrient with reports that women with NTD-affected pregnancies had reduced intake of folate, as well as lower folate levels in blood, than in normal pregnancies [216, 217]. This led to a series of clinical trials to test if folic acid supplementation could prevent NTDs [218–223]. In 1991, results of a double-blind randomized trial demonstrated a 72% reduction of NTDs in a large trial involving women with previous NTD-affected pregnancies [224]. Further trials to determine if folic acid supplementation could prevent NTDs in women of average risk demonstrated that improvement is greater depending on the initial NTD rate of the population [225]. For example, in Northern China, where the NTD rate is very high (48 in 10,000 live births), the incidence was reduced to 7 in 10,000 with supplementation. But in Southern China, the NTD rate was rather low (10 in 10,000) and was only reduced to 6 in 10,000 [226]. Many countries now fortify grains and cereals with folic acid, and in the United States, studies show that fortification results in increased folate status of the population, and an estimated 30% reduction in the incidence of NTDs [227–229]. The *MTHFR* gene encodes methylenetetrahydrofolate reductase, which is essential for the conversion of homocysteine to methionine, a key reaction in the folate pathway. Common polymorphisms in the *MTHFR* gene that reduce enzyme function are associated with increased risk of NTDs [230]. For example, 40% of the general population is heterozygous and 10% homozygous for the hypomorphic MTHFR 667C>T allele. Another common mechanism impacting folate metabolism is the production of function-blocking autoantibodies against the folate receptor, which are found at higher levels in maternal serum from NTD-affected pregnancies [231–234]. Folic acid supplementation can overcome the increased risk associated with MTHFR 667C>T polymorphism or the presence of folic acid receptor autoantibodies [235, 236]. However, folic acid supplementation does not prevent all NTDs in humans, and supplementation typically only reduces the incidence to 5–7 per 10,000 live births [225].

Folic acid supplementation can also prevent NTDs in mouse models, including lines with deletion of *Folbp1*, *Rfc1*, *Cart1*, and *Gcn5* or mutation in $Lrp6^{Cd}$ and $Pax3^{2H}$ [237–246]. The maternal genotype also impacts the risk of NTDs and response to supplementation. For example, NTDs in the *Lrp2* mouse model are prevented by the injection of folic acid but not dietary folic acid [247]. Since Lrp2 plays an important role in folate uptake with folate deficiency [248], this result highlights the impact of the maternal genotype on folate status. This is echoed in human data where mothers who are heterozygous for the MTHFR 667C>T allele have a slightly increased risk of having an NTD-affected pregnancy, whereas the risk increases to 60% for homozygous mothers and to 90% for homozygous females from homozygous mothers [230].

Similar to NTDs in humans, many mutant mouse lines are not rescued by folic acid supplementation [181, 210, 249–252]. Interestingly, this may be influenced by the impact of the particular mutant allele rather than the gene involved. For example, NTDs in the $Lrp6^{cd}$ mouse line are prevented by supplementation with folic acid, whereas supplementation in the $Lrp6^{null}$ mouse line results in more severe NTDs and embryo loss [253]. In fact, folic acid supplementation results in the early loss of mutant embryos in some mouse lines [253, 254]. Furthermore, high levels of dietary folic acid intake results in activation of negative feedback loops, leading to overrepression of folic acid metabolism [255, 256]. The adverse effects of folic acid supplementation are

cumulative, with long-term but not short-term supplementation being detrimental [254].

Importantly, folic acid deficiency is not sufficient to induce NTDs in humans or mouse models [257–263]. Rather, gene–environment interactions (e.g., suboptimal folate status plus a genetic predisposition) likely combine to result in NTDs. For example, folate deficiency increased the frequency of NTDs in *Pax3*$^{Sp}$ mutants and other susceptible mouse background strains [259, 260]. Similarly, mutation of a gene required for folate metabolism (*Shmt1*) does not result in NTDs, but with folate deficiency, NTDs occur [257, 264]. Altered folate metabolism has been documented in cell lines derived from NTD-affected human fetuses, as well the *Pax3*$^{Sp}$ and *Lrp6*$^{Cd}$ mouse models of NTDs [243, 265, 266]. Finally, *Pax3*$^{Sp/+}$;*Shmt1*$^{-/+}$ compound mutants show increased penetrance and severity of NTDs, indicating an interaction of *Pax3* mutation with the folate pathway [264]. This may be relevant to human NTDs as spina bifida and anencephaly are associated with *PAX3* mutations in the autosomal dominant Waardenburg syndrome, as well as in nonsyndromic NTDs [22, 32, 167, 267–277].

## 2.14 Mechanisms by Which Folic Acid Prevents NTDs

In spite of the clear benefit for folic acid supplementation, it is not clear how folic acid prevents NTDs [230]. Folates are not synthesized by the body and need to be included in the diet. Folic acid feeds into the folate one carbon metabolism pathway (Fig. 2.6), a network of interlinked reactions that generates key metabolites required for several cellular processes, including the synthesis of nucleic and amino acids; the production of methyl donor S-adenosyl methionine (SAM) used for methylation of histones, proteins, lipids, and DNA; as well as influencing homocysteine production [278–280]. These outputs can directly impact apical constriction and the cytoskeletal dynamics necessary for neural fold elevation, as well as cilia formation [281–283]. The emerging picture is that a variety of functional outputs of folate metabolism are required for normal development. Impaired flux of metabolites through these reactions may be the key factor responsible for NTDs with deficiency and prevention with supplementation. The specific metabolites required are likely due to individual metabolic need based on how flux through the pathway is perturbed by genetic mutations and environmental factors.

## 2.15 Folate is a Cofactor Required for Synthesis of DNA, Amino Acid and Methyl Donors

The *Pax3*$^{Sp2H}$ mutant mouse strain, which has a metabolic deficiency in the supply of folate for the biosynthesis of pyrimidine, is susceptible to NTDs with folate deficiency, and NTDs in this strain are prevented by folate supplementation [243, 264]. Either Folic acid supplementation or deficiency have measurable effects on DNA methylation impacting gene expression [284, 285]. Importantly, both global DNA hypomethylation and hypomethylation at specific genes are associated with an increased risk for NTDs [286]. One of these genes is *Pax3*, which exhibits reduced expression and altered methylation with exposure to polycyclic aromatic hydrocarbon, as well as oxidative stress in diabetic pregnancy that induces NTDs [287, 288]. Similar to the *Pax3* mutant models, supplementation of diabetic mice with folic acid can prevent NTDs [289]. The greater susceptibility of females to NTDs and prevention by folic acid supplementation suggests an epigenetic requirement for folate metabolism to provide methyl donor groups. Data from both humans and mouse demonstrate that anencephaly affects more females than males, and NTDs in females are reduced to a greater extent with folic acid supplementation [290]. Epigenetic inactivation of the X chromosome is proposed to act as a sink for methyl donors, resulting in less methyl donor groups available for other functions. Folic acid supplementation potentially increases available methyl groups and preferentially rescue NTDs in females [290–292].

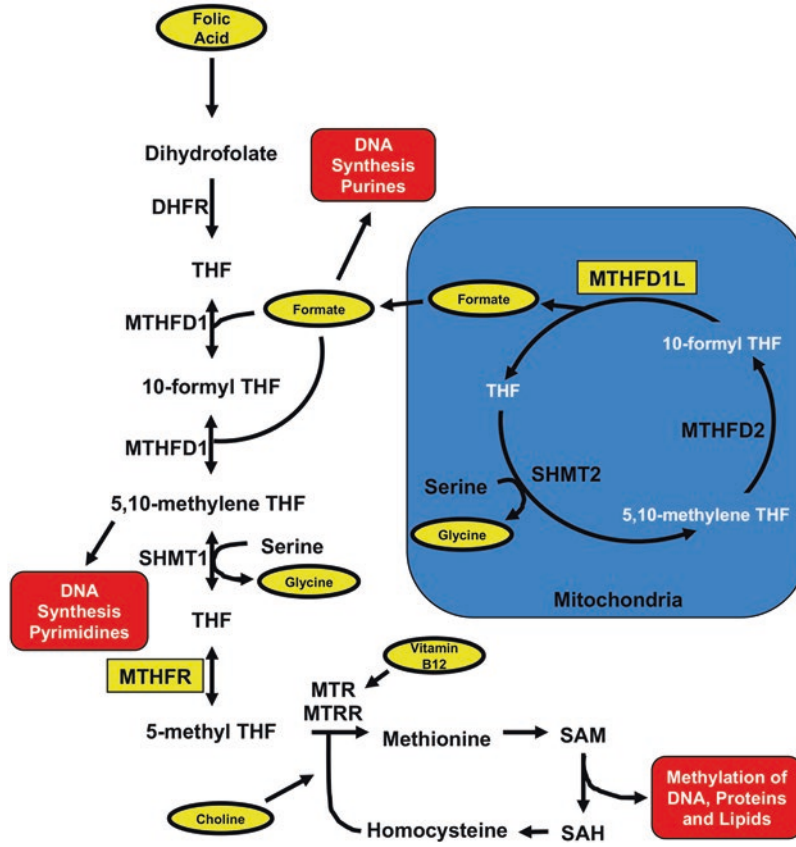


**Fig. 2.6** The folate pathway and neural tube defects. Schematic of the folate metabolic pathway showing key enzymes involved in the cytoplasm and mitochondria (blue). Key outputs of the folate cycle hypothesized to modulate neural tube closure are shown in red boxes and include regulation of DNA synthesis by providing the building blocks for pyrimidines and purines, as well as production of methyl donors required for methylation of DNA, proteins, and lipids. Metabolites that can prevent NTDs when supplemented in mouse models are highlighted by yellow ovals and key enzymes implicated in NTDs in humans, such as MTHFR and MTHFD1L, and are also highlighted by yellow boxes

## 2.16 Folate and Homocysteine

Another possible mechanism by which folic acid supplementation might prevent NTDs is by reducing homocysteine levels [293]. Elevated maternal homocysteine during pregnancy is associated with an increased risk for NTDs [294]. Homocysteine accumulation leads to homocysteinylation of a slew of proteins increasing their antigenicity. Folate deficiency in a mouse model increases homocysteine levels and expression of autoantibodies against homocysteinylated proteins that was reversible with folate supplementation [295]. In humans, genotypes associated with reduced folate uptake or metabolism result in elevated antifolate receptor autoantibodies further impacting folate status of the mother [296]. Furthermore, homocysteinylated H3K79 was increased in brain tissue from NTD cases along with alterations in gene expression [297].

## 2.17 Studies in Mice Suggest Supplementation with Inositol or Formate May Prevent Folate-Resistant NTDs

Aside from *MTHFR*, other enzymes in the folate one carbon metabolism pathway have not consistently been associated with NTDs in human populations or in mouse models [39]. On the other hand, the glycine cleavage branch of the pathway that links folate one carbon metabolism in the mitochondria with reactions in the cytoplasm through the transfer of formate is emerging as key for NTD susceptibility in both mouse models and humans [148, 298–300]. In human populations, sequence variants in either the mitochondrial methylenetetrahydrofolate reductase (*MTHFD1L*) or the mitochondrial inner membrane folate transporter (*SLC25A32*) are associated with increased risk for NTDs [298, 299, 301, 302]. Mutation of

mouse homologues of these genes also results in NTDs [299, 300, 302–306]. Importantly, NTDs in many of these models are prevented by supplementation with formate but not folate. These findings provide important preclinical data suggesting that formate supplementation in conjunction with folate should be considered in the prevention of folate-insensitive NTDs in humans.

Another supplement with a promise to prevent folate-resistant NTDs is inositol, a simple carbohydrate naturally found in many foods [307]. Inositol acts as an insulin-sensitizing agent, and supplementation improves glucose and lipid profiles with positive effects on fertility in assisted reproduction and in women with polycystic ovary syndrome [308]. Hyperglycemia results in inositol depletion, and inositol supplementation suppresses diabetes-induced NTDs in mouse models [309, 310]. Mouse embryos grown in culture and *Grhl3*$^{ct}$ mutants, in particular, develop NTDs with reduced inositol in the growth media, and the incidence of NTDs in *Grhl3*$^{ct}$ mutants is reduced by inositol but not folic acid supplementation [311–315]. Additionally, mutation of genes involved in inositol metabolism *results in NTDs* [316, 317]. Studies in humans also provide support for inositol in the prevention of NTDs. Low serum concentrations of inositol are associated with increased NTD risk and are also found in children with spina bifida. Preliminary trials where dual supplementation of inositol and folate is given to women with previous NTD-affected pregnancies suggest that this treatment is highly effective as no NTDs have occurred in the dual supplementation group, whereas some NTDs did occur with folate supplementation alone. However, the sample size of these studies is still too low to draw definitive conclusions [318–323].

## 2.18 Future Directions

In recent years, next-generation sequencing approaches such as whole-genome and whole-exome sequencing, as well as targeted sequencing of extensive panels of candidate genes in large NTD patient cohorts, have been employed to identify the genes responsible for NTDs in humans [38, 141, 142, 145, 324–326]. These approaches have the potential to identify new candidate genes, as well as multiple sequence variants, in a single individual that might contribute to NTD in a multifactorial fashion. In fact, a recent whole-genome sequencing study concluded that the genetic basis for NTD is omnigenic involving genes spread across almost the entire genome [326]. Furthermore, this study concluded that predicted loss of function variants in almost all genes had some minor impact on NTD risk, and NTD risk was associated with increased numbers of rare loss of function variants. Surprisingly, there was no significant enrichment of damaging variants in human orthologs of the 249 mouse NTD-associated genes previously implicated in NTDs [42, 44–46, 59, 327]. These findings indicate that previous efforts using targeted genomic screens that rely heavily on the candidate genes identified in animal models represent only the tip of the iceberg in terms of the genes that contribute to NTDs. As new candidate genes are identified in these human screens, the mouse model will be essential for modeling the complex interaction of variants leading to NTDs.

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