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Advances in Genetics
of Non Syndromic Neural Tube Defects

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1. Introduction

This chapter revisits established and emerging information on the causes of the human Neural Tube Defects (NTD). These congenital malformations, affecting 1 per 1000 births, have adverse consequences that afflict society as well as the affected individuals. The mechanisms by which they arise are still unknown.

Investigators have focused their efforts in many research fields (developmental and metabolic pathways and animal models) to understand the mechanisms underlying the etiology of these devasting defects. Nevertheless, few candidate genes have proved to have significant impact on the development of NTDs. Moreover, the existing body of literature is still fragmented and constrained by limited size, lack of power, and lack of replicated and independent studies.

The review attempts to describe the results obtained by the analysis of candidate genes from three types of evidences: developmental pathway and biochemical pathways (folate metabolism). The update of the literature includes the most recent findings.

This chapter aims to improve the knowledge of the readers on a such complex multifactorial disease outlining that, even though the remarkable recent progresses, further studies are needed to clarify the molecular basis of NTDs.

The knowledge of the etiology con help us in developing strategies for prevention of the NTDs, by the identification of parents who have a higher chance of having an affected child. In these families it may be possible to clarify the mode of inheritance and estimate the recurrence risk of the disorder. Actually, we are far to have immediate clinical benefits for NTDs, but providing clinicians with a better knowledge of the development of the disease, we could speed up the search for a focused prevention and a more complete familial counselling.

Neural tube defects (NTDs) are congenital malformations that involve failure of the neural tube closure during the early phases of development at any level of the rostro-caudal axis. The incidence of NTDs in European countries excluding UK and Ireland was 0.1-0.6 per 1000 births in the period 1989-2002. [1] Each year, more than 4500 pregnancies in the European Union are affected by NTDs. [2]

In order to gain insights into the embryonic basis of NTDs, it is important to understand the morphogenetic processes and the underlying molecular mechanisms involving the neural tube closure. Neurulation begins with formation of the neural plate, a thickening of the ectoderm on the dorsal surface of the post-gastrulation embryo. Neurulation occurs in a two-part process: i) primary neurulation (weeks 3-4) resulting in formation of the neural tube that will develop into brain and most of spinal cord; ii) secondary neurulation (weeks 5-6) that creates the neural tube caudal to the mid-sacral region. [3] During the primary neurulation, the neural plate is subject to shaping and folding, with fusion along the midline to form the tube. The secondary neural tube is derived from a population of mesenchymal cells, the tail bud, which undergo proliferation and condensation followed by cavitation and fusion with the primary neural tube. [4] Neurulation is driven by redundant mechanisms both at the tissue and cellular level. [5]. Active processes required for neural tube closure include convergent extension cell movements, expansion of the cranial mesenchyme, contraction of actin filaments, bending of the neural plate, and adhesion of the neural folds. Insight into these mechanisms are being provided by the many mouse models in which NTDs occur as a result of genetic mutations. [6] Neural tube closure is a discontinuous process and proceed bi-directionally in a zipper-like fashion. Five closure sites exist in human embryos and on the basis of the study of clinical cases, van Allen et al. [7] proposed a multisite model in which the initial site of closure starts at the rhomboencephalic segment and the caudal end is the last to close.

There are several different types of NTDs. The distribution of different types may vary in different populations and can also vary by the birth prevalence of the defects. [8] The most common classification includes anencephaly, myelomeningocele (also called spina bifida) and encephalocele. Craniorachischisis and iniencephaly are considered to be rarer in most populations. NTDs group includes other conditions, broadly described as occult spinal dysraphisms (lipoma, tight filum terminale) and some complex dysraphic states as diastematomelia, spinal segmental dysgenesis, and caudal regression syndrome. [9] Failure of the closure at any level of the body axis leads to open NTDs where the affected nervous tissues are exposed to the surface. Failure of the secondary neurulation leads to closed forms of NTDs, where the defect is covered by the skin and it is not exposed to the surface of the body.

The majority of NTD cases are non syndromic or isolated NTDs, meaning that the NTD is the only defect. A number of anomalies including clubfoot, deformation of the lower limbs, hydrocephalus and Arnold Chiari II malformation have been widely accepted as secondary abnormalities that may accompany NTDs. Aetiology of non syndromic NTDs is multifactorial with both environmental and genetic factors implicated. Non syndromic NTDs have an important genetic component and it is important that research is focused at determining the basis of this genetic aetiology. The aim of this chapter is provide evidences for genetic contribution to non syndromc NTDs along with the discussion of different approaches for identifying such genes. The identification of genetic factors for NTDs is a relatively a new area of research. In fact, thare are many different mutations in mice resulting in NTDs, but only few well characterized genetic factors in humans. Here we provide an overview of the major causative genes and the more recent genetic advances emerging thanks to the powerful tool of mouse models.

2. Evidence for genetic contribution of non syndromic NTDs

There are strong evidence supporting a genetic component to NTDs. NTDs can be associated with various chromosome rearrangements, the most common include trisomy 13, trisomy 18, tetraploidy, and deletions involving chromosomes 2, 3, 11, 13, 22. Genetic syndromes with NTDs include Meckel-Gruber syndrome and Currarino syndrome. [10] Overall hereditabilty in NTDs is estimated to be 60% with multiple genes involved. Recurrence risk for sibling is 2-5%, which represents up to 50-fold increased risk respect to that observed in the general population [11]. Positive family history families with many affected children have implied an autosomal recessive model of inheritance. [12] Inspection of multiplex families demonstrated that affected relative pairs are related at either the second- or third-degree, suggesting oligogenic inheritance. [13]. The recurrence risk and the inheritance pattern in families have been demonstrated to follow a multifactorial threshold model in which the liability to these malformations follows a normal distribution pattern: additive effects of several genetic and environmental factors cumulate to increase the liability until a threshold value beyond which the phenotype become expressed. Twin studies demonstrated that concordance rate is relatively low among cotwins of affected cases. [11]. Recurrences in families in which the case is affected with spina bifida tend to be spina bifida and families; recurrence in families in which the case is anencephaly tend to be anencephaly. [14-15] However, one third of recurrent cases involve a NTDs phenotype that is different from the cases phenotype. This intra-familial heterogeneity may be the consequence of the pleiotropic effect of a underlying causative gene. NTD occurrence vary among different ethnic groups and it is unknown if this population-specific incidence is due to differences in the frequency of risk-associated alleles or to specific dietary habits or exposures. The highest incidence is in Northern China (7/1000) and the lowest in Japan (0.1/1000). In Europe, the highest rate is reported in Ireland (3/1000). Furthemore, an Irish study demonstrated that predisposing genetic factors may be transmitted from the mother's side of the family. [16] Another important line of evidence for a genetic basis for NTDs comes from the numerous mouse models, both spontaneous and induced via a gene-driven process. [17].

3. Identification of susceptibility genes involved in NTDs

The number and identity of genes predisposing to NTDs remain unknown. Once genes implicated in the development of NTDs are identified, it will be essential to understand potential additive or multiplicative effects of genetic and environmental factors. Candidate genes for NTDs can be classified into two categories according to how they have been identified: biological and positional. Candidate genes based on biological plausibility have resulted from research done in animal models and from biological pathways important for NTDs. Candidate genes can also be identified through positional techniques via chromosomal rearrangements in NTD patients or genomic screen in linkage analysis.

A number of genes have been identified from research done in experimental animals, most notably from mouse models. The mouse model remains one of the most well studied animal models for NTDs. More than 190 genes in mouse produce varying NTDs phenotypes. [17]. There are both naturally occurring and induced strains of mice. The two most common mouse phenotypes are exencephaly, that is comparable to human anencephaly, and craniorachoischisis, in which the neural tube remains open along the entire body axis. The mouse system is useful for the study of NTDs because the genetic map of mouse is noted

and many of the human homologues are known. Moreover, mouse has a short gestational period and does not require the same ethical considerations as the studies in humans. Another advantage is that both genetic and environmental factors may be studied. For example three mouse mutants have been demonstrated to have a reduced risk for NTDs after maternal folate supplementation: *crooked* (*Cd*), *splotch* (*Sp*) and *cartilage homeoprotein1 (Cart1)*. [17] Nevertheless, there are obvious differences between mouse and human, so that it is possible that genes contributing to NTDs in mouse not always do the same in humans. The majority of human NTDs are isolated a show a multifactorial inheritance. In contrast, NTDs in mouse are a lethal condition in the homozygous knock-out state and NTDs is a part of a larger syndrome. Mice have a four-sites model of neural tube closure. In humans controversies have been arisen about the number of closure sites , although there is a general agreement on the presence of rostral and caudal sites. The majority of candidate genes from mouse are involved in a wide range of functions, in crucial steps of neurulation and only few are implicated in cellular functions such as genome stability and DNA repair. Despite a such long list of candidate genes, studies failed to show positive association in humans. Another system that can be used is the zebrafish (*Brachydanio rerio*), that is a cost-effective model system for studying NTDs since fertilized eggs can develop rapidly into transparent embryos, allowing all stages of vertebrate development to be visualized easily. [18] Zebrafish has proven to be very useful for studying embryological processes, including morphogenesis, lineage specification, and mechanisms of acquired cell fate. Furthemore, assays have been developed to perform both gain and loss of function experiments. Partial loss of function phenotypes can be generated by microinjection of antisense morphplino. [19] A forward genetic approach (random mutagenesis) will led to identification of potential NTDs genes. Such a screen would be useful in expanding the collection of potential NTDs risk genes.

The approach for identifying positional candidate genes is through a genomic screen in which genetic markers at known locations are tested in families with two or more affected individuals. Thanks to advances in the density and polymorphisms of genetic markers, highthroughput genotyping methods, and powered statistical genetic linkage analysis, it is possible to perform large-scale investigation of human complex traits such as NTDs. Regions with excess sharing of alleles among affected individuals are considered region of interest and may harbour genes that predispose to NTDs. Few studies have performed genome-wide linkage studies in families with multi le affected individuals, given the scarcity of these large families. In fact, high morbidity and mortality rates associate with the disorder, and pregnancy termination after case identification are factors that limit the ascertainment of multiplex families. [20] A recent genomic screen was performed on 44 multiplex American NTDs families identifying linkage to chromosome 7 and 10. Two candidate genes (Meox2 and Twist1) were identified on chromosome 7 and three candidates (FGFR2, GFRA1, Pax2) were found on critical region of the chromosome 10. [21]

Statistical test are employed to assess evidence of allelic association for NTDs. Infact allelic association is population-specific. There are two methods of testing allelic association in NTDs candidate genes: case-control and family-based studies. Traditionally, NTDs studies have employed case-control studies, where the allelic frequency in an unrelated group of patients are compared to a group of controls individuals. This study can be flawed, if the controls are not properly matched with patients for age, gender, and geographic origin. This population admixture may result in

biased results and spurious associations. In family based studies, the frequencies of alleles transmitted by a parent to the affected child are compared with that of the allele not transmitted. The family studies can also detect a paternal or maternal effect. The most used family-based test is the Transmission disequilibrium test (TDT). [22] When one parent is not available for the analysis unaffected sibling can be used as controls. Association studies in patients with NTDs were often underpowered by a small sample size, leading to inconsistent results. Larger cohorts of patients and controls are needed to reach definitive conclusion regarding the role of the most of studied genes. Meta-analysis for variants of genes are also warrented.

4. Genetic variations in folate metabolism

A large number of experimental and observational clinical research studies over the past half century demonstrated that folic acid supplementation decreases both the occurrence and recurrence risk of NTDs by approximately 70%. [23-24] The recommended intakes are 4 mg/day for high risk women and 0.4 mg/day for all others. Maternal periconceptional folate supplementation may act to overcome insufficient maternal folate levels. However, in most cases, mothers of affected foetuses have either normal folate status or are, at most, mildly folate-deficient, arguing against maternal folate deficiency as a major causative factor. [25-26] Alternatively, it is postulated that supplemental folic acid may act to overcome an underlying defect in folate metabolism that results from a genetic mutation in the mother or in the foetus. Although a protective effect of folate is recognized, the mechanism by which some women develop low folate levels and predispose their offspring to NTDs and other congenital malformation is still debated.
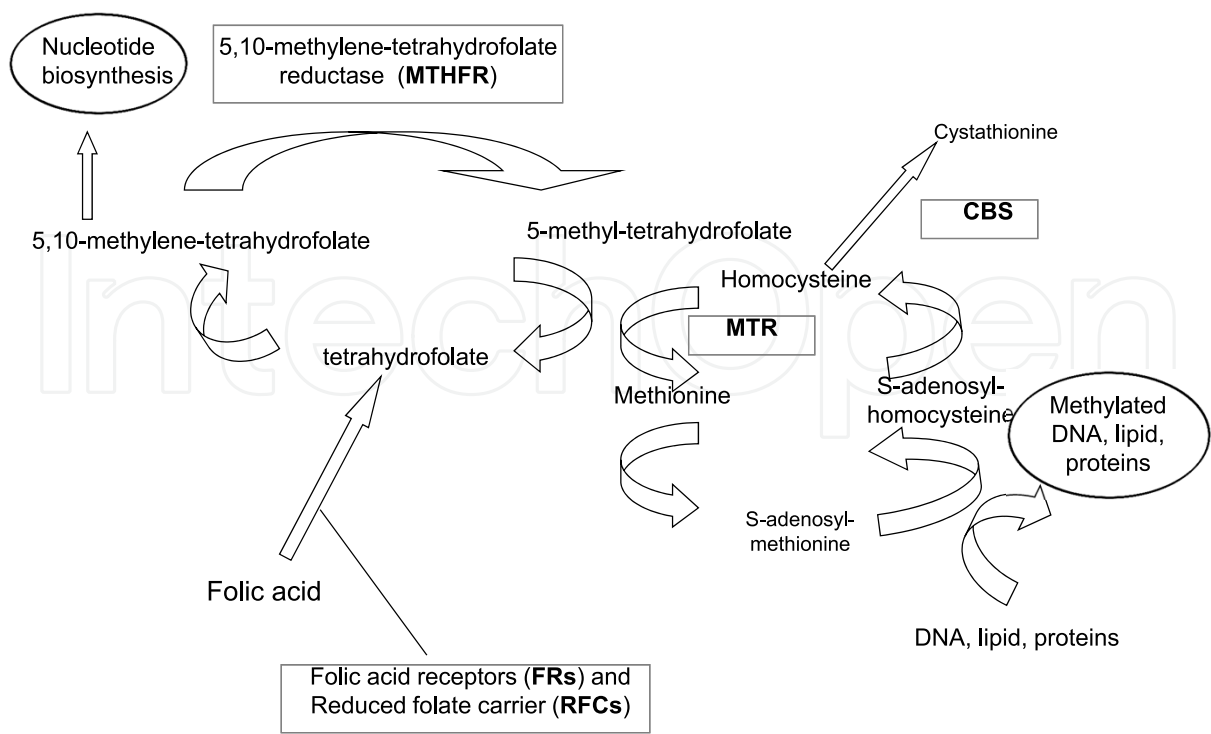

Fig. 1. Folate and homocysteine metabolism

Folic acid is a water soluble B vitamin that partecipates in transfer of single-carbon units in several critical pathways (Figure 1). These pathways include synthesis or catabolism of amino acids (serine, glycine, histidine, and methionine) and synthesis of nucleotides. Through their role in methionine synthesis, folates are also critical for the methylation cycle, since methionine is the methyl donor for many important methylation reactions, including DNA methylation. Folate-dependent conversion of homocysteine to methionine begins early in development in all tissues and provides a link between folate and homocysteine metabolism. An elevation of plasma homocysteine, mild hyperhomocysteinemia, has become recognized as a risk factor for NTDs [25, 27].

Proteins that mediate, or are functionally associated with, folate metabolism have provided candidates for genetic analysis in human NTDs. There are over 25 proteins involved in the folate and closed-related homocysteine pathways. Several of the corresponding genes have been examined as risk factors for NTDs, but few have showed a positive association (Table 1).

Variant	Association with NTDs risk
MTHFR C677T	+
MTHFR A298C	+/-
MTHFR C116T	+ (only in one study)
MTHFR G1793A	+ (only in one study)
MTHFD1 G19658A	+
MTHHD1L 781-6823ATT(7-9)	+
MTR A2756G	-
MTRR A66G	+ (in mothers with low levels of vitamin B12)
CBS 844ins68	+
BHMT G742A	+
RFC-1 A80G	+
FR alpha/Betas	+
TCII A67G; G280A; A701G; C776G; C1043T; G1196A	+
GCPII C1561T	-

Table 1. Variants of genes involved in folate and homocysteine metabolism studied for association with NTDs risk

Methylenetetrahydrofolate reductase (MTHFR) converts 5,10methyleneTHF to 5-mthylTHF, the methyl donor for homocysteine conversion to methionine. The $677C \rightarrow T$ variant converts an alanine residue to a valine residue. Biochemical studied confirmed that this variant is characterized by residual activity after heating. [28] The variant is extremely common, with homozygosity frequencies ranging between 5% and 15% in many populations. [29] The association of the mutant 677T/T genotype with hyperhomocysteinemia occurs only in individuals with low folate status. Folate has been shown to stabilize the mutant human enzyme and should prevent hyperhomocysteinemia in mutant individuals [30]. The MTHFR 677T/T Homozygous mutant genotype first emerged as a possible risk factor for NTDs in some populations [31-33], even if other studies failed to demonstrate an association. [34-35] Two meta-analysis were performed in 1997 [36] and 2000 [29]. The latter study found a pooled odds ratio for cases 677T/T homozygous cases of 1.7 (95%CI 1.4-2.2), with a pooled attributable fraction of NTDs cases of 6%. A second mutation in the *MTHFR* gene

(1298A→C) has also been positively associated with NTDs [37-39]. The 1298 variant is in strong linkage disequilibrium with the 677 variant, such that 1298C and 677T changes are rarely seen on the same allele. Therefore, the two mutations can occur on separate alleles in the same individual who may be predisposed to hyperhomocysteinemia, if folate status is low. Compound heterozygosity for the 677T and 1298C alleles (677C/T/1298A/C) has been suggested to increase the risk, although the results have been conflicting. [37, 40-41] Moreover, a very recent report identified other two polymorphisms of *MTHFR* gene a 116C→T (P39P) and 1793G→A (R594Q). A possible association between NTDs cases and these two new polymorphisms was found; further analysis demonstrated that this association is driven by the linkage disequilibrium with the 677C→T NTD risk factor [42]. The MTHFR 677 variant has been suggested to account for approximately 10% of the NTDs risk. [29]. Since up to 70% of NTD cases may be prevented by the folate, other variants in folate-related genes have to be involved.

Given its important role in folate metabolism, the trifunctional enzyme methylenetetrahydrofolate dehydrogenase/methenyltetrahydrofolate cyclohydrolase/ formyltetrahydrofolate synthetase (*MTHFD1*) may play a role in NTD pathogenesis. Brody et al. demonstrated that the polymorphism 1958G→A (R653Q) within the *MTHFD1* gene resulting in the substitution of a conserved arginine by glutamine is a maternal risk factor for NTDs. [43] Mothers who possess two copies of the Q allele have an increased risk of an NTD-affected pregnancy. There was also a suggestion that the Q allele decreases fetal viability. Very recently, the impact of the *MTHFD1* 1958G>A polymorphism on NTD risk was elevaluated in the Italian population both by case-control and family-based studies. An increased risk was found for the heterozygous 1958G/A (OR=1.69; P=0.04) and homozygous 1958A/A (OR=1.91; P=0.02) genotypes in the children. The risk of an NTD-affected pregnancy of the mothers was increased 1.67-fold (P=0.04) only when a dominant effect (1958G/A or 1958A/A vs 1958G/G) of the 1958A allele was analysed. Family-based tests also confirmed a significant excess of transmission of the 1958A allele to affected individuals, demonstrating that this variant is a genetic risk factor for Italian NTD cases. [44] A potential role of the mitochondrial paralogue MTHFD1L as candidate gene for NTDs association has been also investigated. In particular, a common triallelic deletion/insertion variant 781-6823ATT(7-9) which influences splicing efficiency was tested and a significant increasead risk for allele 1 [ATT(7)] was identified. [45]

The methionin synthase (MTR), a vitamin B$_{12}$-dependent enzyme, utilizes 5-methyltetrahydrofolate, generated by MTHFR, for homocysteine remethylation to methionine. A polymorphism of the MTR gene, the 2756A→G (D919G) that lead to the substitution of an aspartic acid with a glycine residue, has been studied in several studies, but the majority have not reported a significant association [36, 40, 46-48].

A common variant in the gene methionine synthase reductases (MTRR), the 66A→G (I22M), that converts a isoleucine into a methionine residue, has been shown to confer a four-fold increased risk for NTDs in mothers having low vitamin B$_{12}$ levels [49]. Other studies have also suggested an effect on NTDs risk, both for mothers and children with 66GG mutant genotype, but interactions with nutrients has not been examined. [50-51]. The MTRR mutant genotype combined with MTHFR mutant genotype conferred a significant four-fold increased risk in children [49]. An interaction of MTR and MTRR genotypes has also recently reported. [50]

Cystathionine beta synthase (CBS) catalyzes the first step in the transsulfuration pathway of homocysteine. The 68-bp insertion in the exon 8 of the gene appeared to be associated with an increased risk in several studies. [46, 52-53]. Multiplicative interaction of the CBS 68-bp insertion with the homozygosity for the MTHFR 677C→T variant was showed to confer a five-fold increased risk for NTDs children, The MTHFR mutation alone had a two-fold increased risk, whereas there was no risk for CBS mutant genotype alone. [54].

Betaine homocysteine methyltransferase (BHMT) catalyzes homocysteine remethylation to methione, using betaine as the methyl donor. Homozygosity for the BHMT R239Q variant is associated with a decreased risk in mother and children [55]. The 80GG genotype for the 80A→G variant of the reduced folate carrier (RFC1), which transports 5-methyltetrahydrofolate across the plasma membrane, confers an increased risk for mothers with low red blood cell folate [55] and for children whose mothers do not use vitamins [56]. A recent study by Rothenberg et al. showed that some mothers with a NTDs-pregnancy produce autoantibodies that bind to folate receptors (FRs) on the placental membrane [57] The authors suggest that folate supplementation would bypass autoantibody formation that mediates the placental FRs blockage.

Several variants have been identified in trancobalamin II (*TCII*) gene (67A→G, 280G→A, 701A→G, 776C→G, 1043C→T, 1196G→A), but none of the SNPs were associated with a significant increased risk for NTD. Among them, three *TCII* SNPs (67A→G, 776C→G, 1196G→A) affected the transcobalamin concentration, even if these variants only partially accounted for the reduced proportion of vitamin B_{12}. [58].

The glutamate carboxypeptidase II (GCPII) is localized in the jejunum and catalyzes the conversion of folate polyglutamates to monoglutamates prior to absorption. The GCPII C1561T (H475Y) variant was identified and studied as risk factor for NTDs, but no study demonstrated an association. [59-60]

In general, there are many different polymorphism in genes invoved in folate transport or metabolism that have been identified and studied for association with NTDs risk, but data are not yet convincing for most of them. The only one well characterized genetic risk factor is the MTHFR C677T. Many studies are biased by small sample size and insufficient statistical power. The interaction between genes and non genetic factors is an intriguing area of investigation, but it requires larger cohort of patients and controls (Table 2).

MS A2756G/MTHFR C677T	(Morrison et al., 1998)
MS A2756G/MTRR A66G	(Zhu et al., 2003)
MTRR A66G/MTHFR C677T	(Relton et al., 2004)
MTRR A66G/FGCP C1561T	(Relton et al., 2004)
MTRR A66G/MTHFD1 G1958A	(van der Put et al., 1997; Hol et al., 1998)
MTHFR A1298C/RFC-1 A80G	(DeMarco et al., 2003)
RFC-1 A80G/CBS 844ins68	(Relton et al., 2004)
MTFR C677T/CBS 31 bp VNTR	(Afman et al., 2003)
MTHFR C677T/CBS 844ins68	(Botto and Mastroiacovo 1998; de Franchis et al. 2002;Relton et al., 2004)

Table 2. Gene-gene interactions between variants of genes involved in folate/homocysteine metabolism

5. From animal models to humans

Mouse mutant strains that exhibit NTDs have arisen by spontaneous mutations, or are the result of mutagen- and gene-trapped induced mutations. Other mutants have arisen through manipulation of an already identified and characterized gene that is manipulated to alter its function. The majority of the known mouse models are those generated by gene knock-out technology, that mainly develop cranial defects. [17, 61-62] The penetrance of NTDs in mouse models is frequently affected by both genetic background and environmental influences. Mouse mutant genes produce NTDs, for the most part, when they are homozygous. Mouse with double heterozygosity for two mutant genes also develop NTDs, demonstrating that gene-gene interaction may promote the development of NTDs. [63] A variety of exogenous agents when administrated to embryos exert teratogenic effects with production of abnormalities that include NTDs. Drugs, chemicals, or other substances extraneous to pregnancy cause NTDs. In addition, molecules derived from maternal metabolism, such those produced in maternal diseases condition, may induce NTDs. For example, elevated glucose and ketone body concentrations, as found in diabetes mellitus, increased the NTD risk. [64] Teratogens interact directly with genetic mutant effects in the causation of NTDs. The frequency of exencephaly in the *curly tail* strain is elevated by administration of all-trans retinoic acid. [65] This demonstrates that exogenous agents can exacerbating the severity of the phenotype interacting with a genetic mutation. An exogenous agent that ameliorates the genetic predisposition to NTDs in mouse models is a candidate therapeutic agent. Several NTDs mouse mutant, *Sp*, *Cart1*, *Cd*, *Splotch*, have shown to interact with folic acid, exhibiting a reduction of NTDs incidence. [66-68] But not all mouse models are folate responsive, demonstrating that there is a proportion of NTDs defects that require another preventive therapy. Inositol was shown preventive in folate-resistante mouse models, such as *curly tail* strain, an action that operates via stimulation of protein kinase C activity. [69]

Many of the genes that are mutated or disrupted in NTD mouse models are now being identified. Few genes have been identified as contributing to human NTD aetiology. Recently, animal models demonstrated an essential role of for the planar cell polarity (PCP) signaling pathway in the process of the convergent extension. Sistematic analysis of human homologues PCP genes is giving promising results for our understanding of molecular basis of human NTDs.

6. Genes of PCP pathway and NTDs

Several studies showed that early, during embryogenesis of CNS, the major driving force essential to the shaping of the neural plate is a process which is referred to as convergent extension (CE). This is a morphogenetic process by which cells elongate medio-laterally and intercalate with other neighboring cells forming a longer and narrower array. This changes lead to the conversion of an initially wide and short neural plate into a narrow and elongated one. [70] A crucial role in CE has been assigned to the Planar Cell Polarity (PCP) pathway, a highly conserved, non-canonical Wnt-frizzled-dishevelled signaling cascade (Figure 2). PCP signaling plays a key role in establishing and maintaining polarity in the plane of epithelia in Drosophila and in epithelial and non-epithelial tissues in vertebrates.

[71-72] Genetic studies of mutants affecting complex structures in the fly have identified a group of proteins referred to as "core PCP" components, that include transmembrane proteins such as Frizzled (Fz), Strabismus/Van Gogh (Stbm/Vang) and Flamingo (Fmi), as well as cytoplasmic proteins, including Dishevelled (Dsh/Dvl), Prickle (Pk), and Diego (Dgo). [73-74] Downstream of the core PCP members, additional factors mediate the PCP signaling in different tissues, the so-called "PCP effectors", that include the proteins Inturned, Fuzzy and Fritz. [75-77] Evidence for the involvement of the PCP pathway in CE process in vertebrate has emerged from studies of a wide range of mutants of orthologs of Drosophila PCP genes in several animal models such as zebrafish, Xenopus and mouse. [78-83] In mouse, *Loop-tail* (*Lp*) was the first mutant to implicate a role of PCP pathway and CE process in NTD pathogenesis. [84-85] *Lp* heterozygotes are characterized by a "looped" tail appearance, while homozygotes develop a severe NTD resembling human craniorachischisis. [86] NTD in *Lp* is caused by independent missense mutations S464N (*Lp*) and D255E (*Lpm1Jus*), localized in the proposed C-terminal cytoplasmic domain of a gene, now referred to as Van Gogh-like 2 (Vangl2). [84-85; 87] A novel experimentally induced allele, *Lp* (*m2Jus*), having a missense mutation, R259L, in Vangl2 has been recently reported. This mutation segregates in a recessive manner, with all heterozygotes appearing normal, and 47% of homozygotes showing a looped-tail. Homozygous *Lp* (*m2Jus*) embryos showed spina bifida in 12%. [88] Seven other mutant mice carrying mutations in some of PCP genes and fail to complete neural tube closure leading to craniorachischisis have been described; they include: *circle-tail* (*Crc*), *crash* (*Crsh*), *Ptk7*, and *dishevelled1-/-/dishevelled2-/-* (*Dvl1-/-/Dvl2-/-*), *Frizzled3-/-/Frizzled6-/-* (*Fzd3-/-/Fzd6-/-*), *dishevelled3/Lp* (Dvl3+/-/Lp/+) double-knockout mice, and *Fuzzy*. [89-94] The *Crsh* mouse harbors a mutation in Celsr1 that encodes a protein orthologous to Drosophila Flamingo. [93] Like Vangl2, this gene functions in the PCP pathway. In the *Crc* mouse, a point mutation was identified introducing a stop codon into the apical cell polarity gene scribble (Scrb1), a PDZ domain-containing gene that is the ortholog of Drosophila scribble. [95] Scribble was not known, at a first time, to be a PCP component in Drosophila. However, a polarity defect is observed in the inner ear of the Crc mice, suggesting a role for Scrb1 in establishment of polarity in vertebrates. [96] A mutation in the protein tyrosine kinase 7 (Ptk7) gene, wich encodes a conserved transmembrane protein with tyrosine kinase homology, disrupts neural tube closure and stereociliary bundle orientation and shows genetic interaction with *Lp*. [93] Double null homozygous embryos for both dishevelled1 and dishevelled2 genes *(Dvl1-/-/Dvl2-/-)* as well as Frizzled3 and Frizzled6 genes (*Fzd3-/-/Fzd6-/-*), two members of highly homologous seven-pass receptor family, also exhibit NTDs that closely resemble the craniorachischisis observed in single knock-out mice. [91, 97] Although neurulation appeared normal in both Dvl3-/- and *LtapLp*/+ mutants, combined mutants Dvl3+/-/*LtapLp*/+ displayed incomplete neural tube closure. [94] *Fuzzy* knockout mice exhibit both NTDs and defective primary cilia. [98] The exact mechanism by which the PCP pathway regulates CE cellular movements remains poorly understood. A recent study reported that PCP signalling was requested for re-establishing cell polarity that is transiently lost in dividing cells during neurulation. In fact, during mitosis, dividing cells loose their polarized features threatening the overall organization of the developing tissue; PCP signaling acts through quickly repolarization of the daughter cells and directs their integration into the neuroepithelium. [99] Evidence for an intrinsic role of ciliogenesis in PCP is derived from study of Bardet-Biedl syndrome

(BBS), a genetically heterogeneous human disorder with pleiotropic manifestations including obesity, polydactily, endocrine dysfunction, cystic renal disease, progressive photoreceptor degeneration and hearing loss. [100-101] BBS genes share the common features that the encoded proteins localized to the cilium or its cellular anchor, the basal body. [102] Targeted disruption of Bbs1, Bbs4 or Bbs6 in mouse lead to phenotype shared with PCP mutants, including NTDs (14% of Bbs4-/- mice display exencephaly) [103]. A second link between PCP and cilia has come from the identification of the mouse Inversin (Invs) gene, which encodes a large adaptor-like protein with homology to Drosophila PCP protein Diego. Recent studies in zebrafish and Xenopus embryos revealed that knock-down of Invs disrupts CE process. [104] The subcellular localization of inversin is complex and dynamic, and includes the basal bodies, primary cilia and, during metaphase and anaphase, the spindle poles. [105-107] A recent study in Xenopus demonstrated a link between ciliogenesis, Hedgehog signalling (HH) and PCP [108]. Disruption of orthologs of two Drosophila PCP effectors, Inturned (In) and Fuzzy (Fy), leads to failure of ciliogenesis, resulting from incorrect orientation of ciliary microtubules. In Xenopus, the absence of In and Fy elicited prominent rostral NTDs in addition to more caudal NTDs, predicted to result from disruption of PCP signalling. These caudal defects were shown to arise from failure of CE in the neural plate, whereas the rostral defects were shown to stem from a failure of Hedgehog signalling. [108]. Finally, it is tempting to speculate that both PCP and Hedgehog phenotypes may be linked in humans to NTDs. Mutations in two genes, MKS1 and MKS3, that were predicted to be involved in cilia formation have been recently found in patients with Meckel syndrome (MKS), characterized by bilateral renal cystic dysplasia, central nervous system malformations, bilateral upper and lower limb polydactyly and fibrocystic changes of the liver. [109-112]. The gene mutated in type 1 MKS encodes a protein associated with the base of the cilium in vertebrates and nematodes. Loss of function of mouse Mks1 results in an accurate model of human MKS, with structural abnormalities in the neural tube, biliary duct, limb patterning, bone development and the kidney that mirror the human syndrome. Analysis of patterning in the neural tube and the limb demonstrates altered Hedgehog pathway signaling underlies some MKS defects.[113]

7. PCP pathway and human NTDs

In humans mutations in VANGL1 and VANGL2 genes cause NTDs and abrogate the physical interaction between VANGL proteins and other members of PCP pathway. In a first study three Italian patients who were heterozygotes for missense mutations of VANGL1 (V239I, R274Q, and M328T) were identified. The V239I mutation was identified in a girl with a severe form of caudal regression The girl's mother also carried the mutation, but showed no clinical signs of NTDs. Her brother having a milder form of the disorder, a dermal sinus, also carried the V239I mutation. The V239I mutation was absent in the father, in the maternal aunt and in the maternal grandparents, indicating that it had arisen *de novo* in the germline of one of the maternal grandparents or somatically in the mother, with subsequent transmission through the mother's germline. Valine at position 239 is located in the fourth predicted transmembrane domain of the VANGL1 protein. It is invariant and part of a 'VLLE' motif, which is conserved across all known VANGL proteins. In vitro studies demonstrated that V239I mutation abrogated interaction between VANGL1 and their binding partners, Dvl1, Dvl2, and Dvl3. The R274Q mutation was found in a girl with

myelomeningocele. Her mother, who carried the R274Q mutation, and maternal aunt had vertebral schisis, a minimal sign of NTD. Arg274 is in the cytoplasmic domain of VANGL1 and is invariant in all known orthologs except in C. elegans, in which it is replaced by glutamate. The M328T mutation was carried by a child with myelomeningocele, hydrocephalus, and Chiari II malformation. M328T mutation occurs in the predicted cytoplasmic domain of VANGL1. We did not detect these variants in 150 ancestrally matched controls. Thus, these are the first genetic mutations clearly linked to NTDs such as spina bifida in humans. [114].
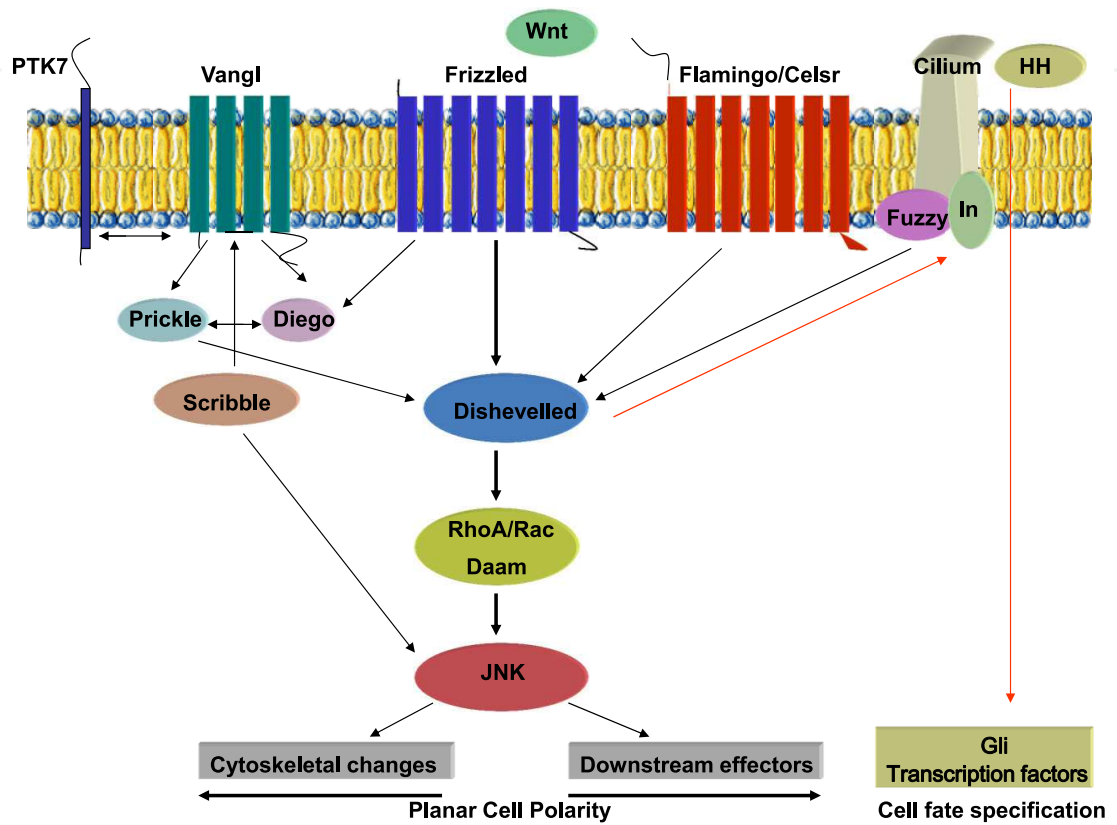
Fig. 2. Planar Cell Polarity (PCP) pathway.

Wnt proteins bind to its receptors, Frizzled (Fz), leading to recruitment and activation of Dishevelled, that is a multimodule protein PCP activation requires formation of a multimodule protein-complex including two transmembrane proteins, Vangl (1/2) and Flamingo/Celsr, and three cytoplasmic proteins, Dishevelled, Diego and Prickle. These proteins acquire an asymmetric localization at the plasma membrane that is crucial for proper PCP signaling. Scribble is a cytoplasmic protein binds to and genetically interacts with Vangl, suggesting a role in PCP signaling. Ptk7 receptor is a regulator and shows genetic interaction with Vangl. Downstream effectors include small GTPases Rho or Rac, which are active when bound to GTP. The Formin homology protein, Daam1, as an important link between Dishevelled and the Rho GTPase for cytoskeletal modulation. Alternatively, Dvl can initiate Rac signaling and its downstream effector c-Jun N-terminal kinase (JNK) that promote sites of actin polymerization modulating lamellipodia extension. Inturned (In) and Fuzzy that are down-stream regulator of PCP pathway do influence convergent extension and they also are broadly required for Hedgehog (HH) signaling

Validation of the potential pathogenic effect of VANGL1 V239I, M328T, and R274Q mutations *in vivo* was performed by investigating their effect on CE in zebrafish. Knocking down the expression of tri, the ortholog of Vangl2, using an antisense morpholino (MO), led to a defective CE manifested by a shortened body axis and widened somites. Co-injection of the human VANGL1 with the tri-MO was able to partially rescue the tri-MO induced phenotype in zebrafish. In contrast, co-injection of V239I and M328T, failed to rescue this phenotype. Overexpression studies evaluating the ability of the human VANGL1 alleles to induce a CE phenotype when injected at high doses in zebrafish embryos have been carried out. While overexpressing the wild-type allele led to a severely defective CE, overexpression of either V239I and M328T variants failed to do so. Thus, results from both tri-MO knockdown/rescue results and overexpression assays suggest that these two variants most likely represent "loss-of-function" alleles that affect protein function during embryonic development. [115].

Overall, human VANGL1 gene has been sequenced in a cohort of 810 NTD patients with various ethnic origin. Eight missense mutations both in familial (V239I, R274Q, S83L, and R181E) and sporadic (M238T, F153S, L202F, and A404S) cases have been identified. These mutations affect evolutionary conserved amino residues that are distributed along the entire length of the VANGL1 protein. Since these many of these variants do not represent obvious null mutations (like stop codon, deletions), functional experiments are needed to investigate their effect on the protein function. All mutations detected so far in VANGL1 in NTDs are heterozygous, leading to the speculation that these variants may act as partial loss of function alleles and interact with other environmental and genetic factors to cause the NTD phenotype. The finding of VANGL1 mutations in both open and closed NTDs support this hypothesis of common underlying molecular mechanisms. [116]

Sequencing of *VANGL2* in the same cohort of patients led to the identification of six novel heterozygous missense mutations in seven patients, that could be pathogenic based on genetic and initial validation data [117]. Four of these mutations, R135W, R177H, L242V, R270H, were predicted to be damaging to protein function using bioinformatics' tools, and two others, T247M and R482H, affect highly conserved residues across evolution. Five mutations were identified in patients affected with closed spinal NTDs, suggesting that VANGL2 mutations may predispose to NTDs is approximately 2.5% of closed spinal NTDs. A Chinese study recently reported the identification of three other missense mutations in VANGL2 in fetuses with a cranial NTDs: S84F, R353C , and F437S. [118]

Recently, three non-synonymous Fuzzy amino acid substitutions in some patients with NTDs have been identified, resulting in alteration of the length of primary cilia and cell movement. Since Fuzzy knockout mice exhibit both NTDs and defective primary cilia and Fuzzy is expressed in the emerging neural tube, mutations in Fuzzy may account for a subset of NTDs in humans. [98]

Thus, the evidence is accumulating for an important contribution of PCP genes to the pathogenesis of human NTDs, necessitating a detailed analysis of other not yet explored PCP genes in large cohorts of patients.

8. Conclusions

NTDs are a group of severe congenital malformations having a profound physical, emotional, and financial effects on families and communities. Despite their importance in

terms both of human suffering and cost to the health care system, the causes of most cases of NTDs are not known. There is growing evidence that many NTDs have a genetic background. Elucidation of genetic causes predisposing to NTDs are important challenge in light of preconception care program that aim to reduce reproductive risks before conception and to improve the chance for a healthy birth outcome. Further, the genetic screening done for research setting may become available for diagnostic purposes, allowing to identify the predisposition before conception and to provide reproductive options to minimize the chance of having affected children in the future.

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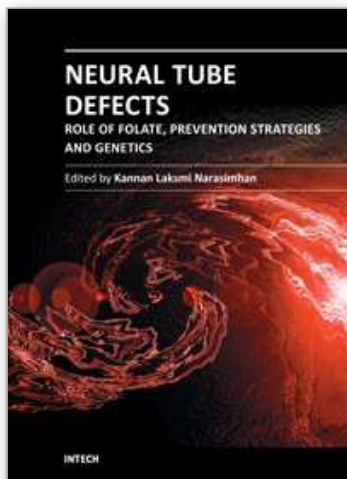
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