The Emergent Role of Epigenetic and Environmental Factors in Promoting Neural Tube Defects

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The molecular mechanisms driving neural tube closure are highly complex. This is demonstrated by the implication of many genes, environmental factors, and epigenetic mechanisms in the development of neural tube defects (NTDs). NTDs are birth defects that appear in many forms, with some variations being universally fatal, and, collectively, they affect up to 2 in every 1000 pregnancies. The combination of high prevalence and high mortality rate highlights the importance of ongoing research with the objective of elucidating the extensive etiology of NTDs. This review will outline the current understanding of epigenetic and environmental factors in NTD pathogenesis, as well as suggest some potential therapeutic targets for NTD prevention.

Contents

1 Introduction

Neural tube defects (NTDs) are critical birth conditions associated with the development of the brain and spinal cord (Avagliano et al., 2019). Collectively, they exist as one of the most common congenital malformations in humans affecting central nervous system (CNS) development, appearing in up to 2/1000 established pregnancies worldwide (Mitchell, 2005). Both genetic, epigenetic and environmental causes of NTDs have been extensively researched, and evidence suggests that NTD pathogenesis follows a complex, multifactorial, oligogenic etiology (Copp and Greene, 2013) for which each individual risk factor is insufficient in driving independently (Harris and Juriloff, 2007). This review will investigate the extent to which the dysregulation of epigenetic factors and the presence of environmental factors contributes to the development of neural tube defects, and will also consider the potential of these factors as therapeutic targets for NTD prevention and treatment.

1.1 Neural Tube Defects

NTDs arise upon a failure of the morphogenetic mechanisms involved in neural tube closure, at any level of the rostrocaudal axis, during embryogenesis (Copp and Greene, 2013). As a result, the brain and spinal cord may not develop as intended, which leads to a range of physical and neurological disorders. NTDs may be characterized as open or closed; the difference exists in the fact that open NTDs display an open lesion after failed closure, leaving segments of the spinal cord or brain exposed, but for closed NTDs, there is no visible opening in the neural tube or its derivatives post-closure. For example, tethered spinal cord syndrome, a closed NTD, is a condition for which the closed neural tube does not exhibit any gaps, but the resultant spinal cord is abnormally attached to surrounding tissue (Yamada et al., 2007). Mutations in more than 200 different genes are known to contribute to NTDs in mice (Copp and Greene, 2010), such as those associated with the Sonic hedgehog (Shh) and Bone morphogenetic protein (BMP) pathways (De Marco et al., 2006; Murdoch and Copp, 2010). In particular, environmentally induced folic acid deficiency, or mutations in folate-related genes, are understood to promote critical neurodevelopmental instability on the basis that perinatal folic acid supplementation reduces the incidence of NTDs by 60–70% (Kibar et al., 2007). There is a plethora of additional

evidence supporting the genetic component of NTD causation; for example, NTDs are linked to the occurrence of trisomies 13 and 18, as well as the rearrangements of some chromosomes (Lynch, 2005). Beyond this, epidemiological research indicates that genetic factors associated with NTDs are transferred preferentially from the maternal line (Byrne and Carolan, 2006), and the estimated recurrence risk of NTDs in siblings, 2-5%, is up to 50 times higher than that of the general population (Risch, 1990).

During early embryogenesis, the neural tube exists in the form of the neuroepithelium, which is a unilayered structure of proliferating epithelial cells that are characterized as multipotent, primary neural stem cells (NSCs). These NSCs can be identified by the expression of markers such as the transcription factors Sox2 and Pax6, as well as nestin, an intermediate filament (Solozobova et al., 2012). The NSCs derived from the neuroepithelium drive neurulation by first giving rise to the neural plate, which then forms the neural tube through a series of complex folding and fusion events. Naturally, if the population of NSCs is dysregulated at a genomic, epigenomic, or post-transcriptional level, this may lead to abnormal differentiation, excessive proliferation, or excessive apoptosis, from which the resultant overgrowth, or lack of, neural tissue interferes with the process of neural tube closure.

Apoptosis is a crucial component of neural development that, as with the other listed mechanisms, must be regulated effectively to prevent NTDs. Studies suggest that embryonic progenitor cells undergo apoptosis upon caspase activation (D'Sa-Eipper and Roth, 2000), and in response to a trigger of the Fas death receptor (Cheema et al., 1999). Overexpression of the Bcl-2 regulatory protein family in a neural stem cell line is just one of the mechanisms by which the rate of apoptosis may be decreased (Esdar et al., 2001). With regards to abnormal differentiation, the Notch, Shh, and Wnt signalling pathways are all understood to greatly influence cell fate specification during the development of the nervous system (Sivakumar et al., 2011). Dysregulated expression of a combination of the genes involved in the aforementioned pathways has the potential to disrupt the evolution of the neural tube, leading to NTDs.

Prenatal identification and evaluation of neural tube defects is carried out using both bio-

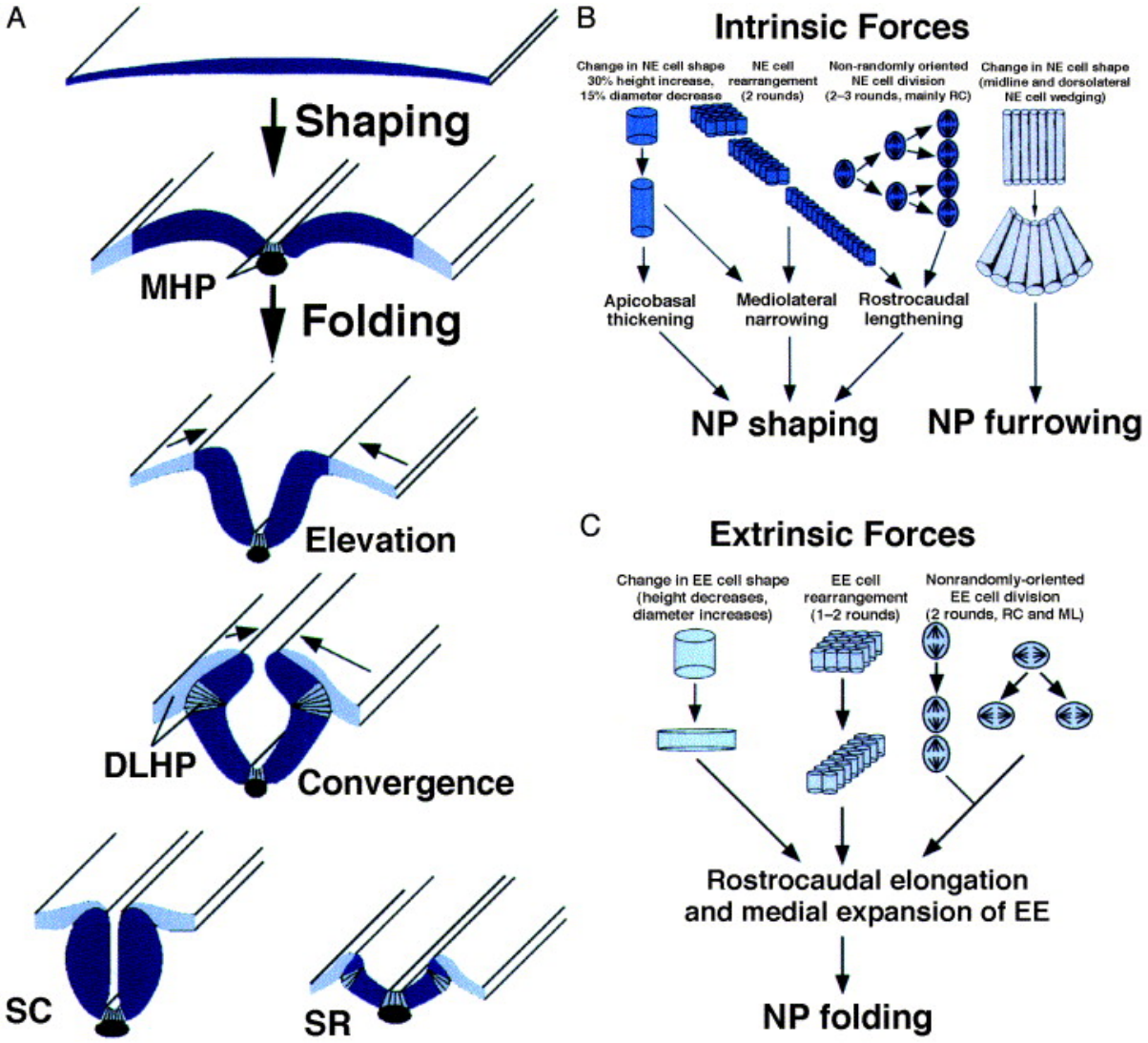

Figure 1: (A) The sequence of morphogenetic events during neurulation. (B) Intrinsic forces driving neural plate shaping and furrowing. (C) Extrinsic forces causing folding of neural plate and closure of neural groove. Abbreviations: EE, epidermal ectoderm; NE, neuroepithelial; NP, neural plate; ML, mediolateral; RC, rostrocaudal. Figure from Smith and Schoenwolf (1997).

chemical and imaging methods. In recent times, the enhancement of MRI techniques has allowed for the capture of high quality images of fetuses, devoid of artifacts from fetal motion (Egloff and Bulas, 2015). As well as MRI, ultrasound imaging has long since been used to identify the presence of NTDs in fetuses by evaluating surface-level structural characteristics of the fetus such as the shape and size of the head and spine (Harmon et al., 1995). Biochemical markers offer a potential target for the identification of NTDs as characteristic substances are known to appear in maternal blood or amniotic fluid during the gestation period. Alpha-fetoprotein (AFP) is an alpha-1 protein specific to fetal life with a genetic origin on chromosome 4 (Muller, 2003). There is an established relationship between abnormally high levels of AFP in the amniotic fluid

or maternal serum, and open neural tube defects (Brock and Sutcliffe, 1972; Brock et al., 1974). This relationship is reliable enough that 75–90% of open NTDs can be detected by elevated maternal serum alpha-fetoprotein (MSAFP) levels (Wald et al., 1977). In addition, raised levels of acetylcholinesterase (AChE) in amniotic fluid is closely associated with open NTDs, especially when appearing in confluence with abnormal levels of AFP (Smith et al., 1979). The most common NTDs are anencephaly (open), spina bifida aperta (open), and spina bifida occulta (closed) (Botto et al., 1999), and so the majority of the relevant literature presents findings regarding NTD etiology based on studies of fetuses and patients affected by these particular disorders.

Anencephaly

Anencephaly is a pathology of development that is estimated to appear in 1 out of every 4600 births worldwide (Mai et al., 2019). However, there is a lack of consistency in this statistic across various sources, and prevalence does vary geographically. It must also be noted that there is a high rate of prenatal fetal demise from anencephaly that is not accounted for here. Anencephaly is considered a severe malformation as the mortality rate without extreme medical intervention is 100% from initial incidence to a matter of days after birth (Munteanu et al., 2020). It is identified, using ultrasound or MRI, by the partial or total absence of cerebral structures and of the cranial vault, and by abnormal development of the skull base (Naidich et al., 1992). The effect of this is that the fetus lacks most, or all, of it's brain tissue. Anencephaly occurs when the cranial end of the neural tube fails to close completely during embryonic development, which results in the exposure of the brain tissue to amniotic fluid (Avagliano et al., 2019). It is the contact with the intra-amniotic environment that reclassifies the defect from exencephaly to anencephaly, as exencephaly is simply the condition describing failed neural tube closure at the cranial end in isolation. Although the exact etiology of neural tube defects, including anencephaly, is not fully understood due to their sheer complexity and multifactorial nature, there is still a substantial amount of research investigating particular genes that provide susceptibility to NTDs if they are subjected to a mutation or change in expression.

Spina Bifida

Spina bifida, which directly translates to 'split spine', is not only the most common NTD (as a family), but also one of the most common malformations of any kind pertaining to human structural development (Mitchell et al., 2004). In the United States (varies worldwide), approximately 1 in every 3000 births were affected by spina bifida between 2010 and 2014 (Mai et al., 2019), with the mortality rate reported as up to 60% by adulthood (Oakeshott et al., 2010). Spina bifida is a general term that encompasses a set of open and closed NTDs, classified as spina bifida aperta and spina bifida occulta, respectively. The most notable forms of spina bifida are myelomeningocele (open) and meningocele (may be open or closed). Extensive screening tests have unveiled 48 genes as potential risk factors for human spina bifida (Greene et al., 2009), which presents a strong basis for further discussion and research into the underlying genetic, epigenetic, and environmental mechanisms involved.

Myelomeningocele (Figure 2b) is a form of spina bifida aperta (formerly known as spina bifida cystica) that is identified by the formation of a cyst filled with cerebrospinal fluid (CSF) that breaches through the fetal spine after failed neural tube closure. In addition, there is abnormal extension of the meninges through the spinal opening, and the spinal cord protrudes from the spinal canal into the fluid-filled sac (Mohd-Zin et al., 2017). Myelomeningocele is often linked to derivative disorders such as hydrocephalus, ventriculomegaly, and type II Chiari hindbrain malformation (Stevenson, 2004; Williams, 2008). These associated disorders offer reasoning as to why myelomeningocele is considered the most severe form of spina bifida.

Meningocele (Figure 2c) is frequently introduced as a less severe form of myelomeningocele; the CSF-filled sac still forms, and the meninges protrude through the spinal opening, however, the spinal cord tissue and nerve roots are unaffected with respect to normal development (Northrup and Volcik, 2000). Given that the outer layer of skin may or may not be present during meningocele formation, it is possible that an open lesion is displayed in some cases and not others, therefore giving rise to the ongoing debate as to whether meningocele represents spina bifida aperta or occulta (Mohd-Zin et al., 2017).

With the ability to identify NTDs during prenatal development, and an understanding of the genetic origin of NTDs, a foundation is set for treating these disorders.

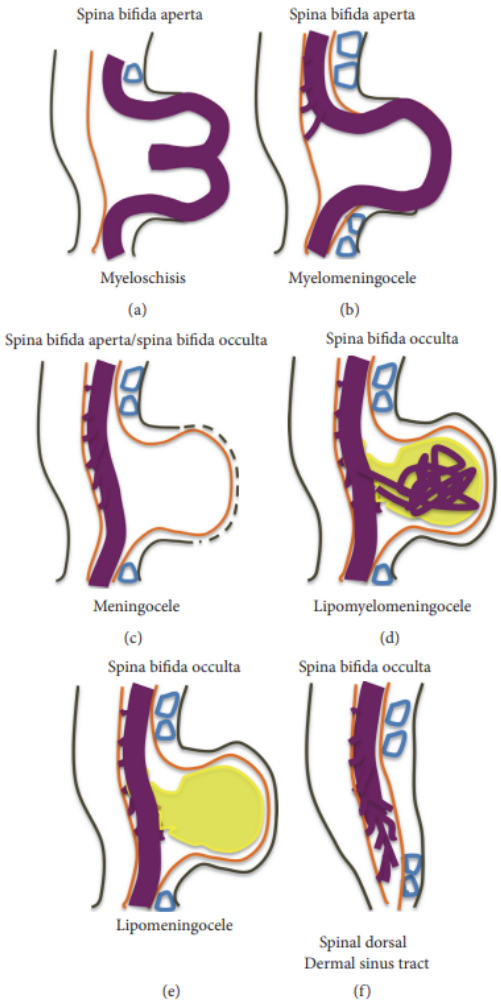

Figure 2: Schematic diagrams of different forms of spina bifida. Figure from Mohd-Zin et al. (2017).

1.2 Epigenetic Mechanisms

The term 'epigenetics' was first proposed in 1939 to describe the molecular influence on gene expression in early embryonic development (Waddington et al., 1939). Epigenetics is now universally accepted as the study of changes in gene expression that do not arise from alterations in DNA sequence, but instead from chemical modifications that allow the same genome to show alternative phenotypes (Felsenfeld, 2014). These epigenetic regulatory mechanisms, collectively referred to as epigenetic marks, include DNA methylation, histone modifications, chromatin remodelling, and non-coding RNA molecules. Together, they define each cell's dynamic, and unique, environmentally-affected epigenome. Dysregulation of epigenetic markers may arise as a result of environmental factors, such as dietary changes, aging, or stress, or from a failure of the mechanisms associated with epigenetic memory in cell division (Saze, 2008). It is the role of

epigenetic writers to add genetic markers, while erasers remove them from one cell generation to the next. Another set of enzymes known as readers then bind to and recognize the regulated epigenetic modifications (Nicholson et al., 2015). The evolving state of dynamic epigenomes through cellular generations seen during early development is essential in determining the structure of the neural tube (Wilde et al., 2014).

Although methyl groups can be added to other nucleotides, cytosine is the most common target for DNA methylation. Cytosine methylation frequently occurs at CpG dinucleotide sites (cytosine followed by guanine nucleotide), accounting for approximately three quarters of all DNA methylation in human and mouse cells (Ziller et al., 2011). DNA methyltransferase 1 (DNMT1), DNMT3A and DNMT3B, are three conserved enzymes that function to maintain and regulate cytosine methylation, and are crucial in ensuring normal development (Li et al., 1992; Okano et al., 1999). DNMT1, an epigenetic writer enzyme, is known to reliably propagate symmetrically methylated CpGs through mitosis by recognising the nascent strand opposing a previously methylated site (Smith and Meissner, 2013), which is crucial for neural development. It must be noted that there are instances in which methylation must be inhibited or removed, rather than conserved, in order to ensure error-free development.

Histones are highly conserved proteins that define the structure of chromatin and package DNA into nucleosome particles. They follow the form of an octamer core of H2A, H2B, H3, and H4 histone proteins that, based on amino acid sequencing results, have been conserved from archaea to humans (Zhou et al., 2019). These proteins are epigenetically and post-translationally modified through acetylation, methylation, phosphorylation, ubiquitination, glycosylation, and so on (Henikoff and Smith, 2015). The addition of these chemical groups affects how tightly the chromatin is packed within a cell's nucleus, which controls the accessibility of transcription factors to DNA and regulates gene expression. Cells have various enzymatic mechanisms in place to catalyze the addition or removal of histone modifications, and the enzymes involved in histone modification exhibit a high degree of substrate specificity that differentiates between both the histone subtypes and the individual side chains of each histone (Costa, 2008). Histone demethylases (HDMs) are a class of enzymes that remove methyl groups from histone tails and have been observed in regulating neurodevelopment (Park et al., 2022). In particular, the Ju-

monji demethylases (JMDs), which belong to the HDM2/7 subfamililes, target lysine residues on histones H3 and H4. This enzymatic action holds a great influence on determining NSC cell fate (Fueyo et al., 2015), which, as has been established, must be regulated in order to prevent imperfect neural tube closure and NTDs.

There is increasing evidence to show that non-coding RNA (ncRNA) is involved in many epigenetic mechanisms of gene expression (Costa, 2008), which is clear in the fact that ncRNAs are capable of directing DNA methylation and histone modifications in complex organisms (Bernstein and Allis, 2005). Micro RNAs (miRNA) and circular RNAs (circRNA) are key to neurodevelopment but they are not considered true epigenetic factors (Lee, 2012), though they may regulate other epigenetic layers. For example, miR-483-5p and miR-132 regulate methyl CpG-binding protein 2 (MeCP2) levels, which itself is an epigenetic regulator (Han et al., 2013). On the other hand, long non-coding RNAs (lncRNA), which are RNA transcripts longer than 200 nucleotides in length, have been described as direct, critical regulators of epigenetic mechanisms, particularly in neurodevelopment (LaSalle et al., 2013).

2 Epigenetic & Environmental Factors Contribute to NTDs

Both open and closed NTDs arise during neurulation, between three and four weeks after conception (Frey and Hauser, 2003). Comprehensively elucidating the genetic etiology of NTDs is challenging due to the low recurrence patterns and lack of simple Mendelian inheritance. However, it appears increasing likely that NTD cases arise from a combination of genetic, epigenetic, and environmental factors, so, given the evidence supporting the involvement of ncRNA, DNA methylation, histone modifications and folic acid in forming epigenomes and influencing neurodevelopment, there is a clear motivation for research into the role of epigenetic dysregulation and environmental factors in promoting NTDs. This section will seek to elucidate the particular epigenetic mechanisms and environmental associations that have emerged in recent years as candidates for promoting frequently occurring NTDs, such as anencephaly and spina bifida, as well as other less common forms of NTDs.

2.1 Folate Deficiency

Folate deficiency is one of the most crucial nutritional factors involved in the pathogenesis of NTDs (Kibar et al., 2007). The methylenetetrahydrofolate reductase (MTHFR) gene encodes the suggested enzyme, that functions to convert 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate, the circulating form of folate, in folic acid metabolism (Yaliwal and Desai, 2012). This reaction, in which vitamin B12 operates as a cofactor, is catalyzed by methionine synthase (Lacasana et al., 2012), which itself is key to a plethora of biochemical pathways associated with DNA synthesis, cell division, tissue growth, and DNA methylation. MTHFR activity can be downregulated by two genetic polymorphisms in *MTHFR*: *677C-T* and *1298A-C*. The former causes an alanine to valine substitution that increases the risk of anencephaly by a factor of 2 to 4 in homozygous patients, and the latter leads to a glutamate-alanine substitution that also increases the risk of NTD development, though the relative risk is lower than that of the *677C-T* polymorphism. The network of biochemical pathways leading to NTDs as a result of the *677C-T* polymorphism is clear as the associated decrease in enzymatic activity leads to low levels of readily available active folate, which compromises neural tube closure as active folate is essential for normal neurodevelopment (Gunnarsdottir et al., 2019). It has been found that the *MTHFR* promoter is particularly susceptible to hypermethylation (Khazamipour et al., 2009), and this specific epigenetic aberration may strongly promote NTDs. One study group of children with NTDs were epigenetically profiled and the results showed a statistically significant (P-value = 0.001) association between epimutated *MTHFR* and NTDs (Stolk et al., 2013).

2.2 Defects in Planar Cell Polarity Pathway

Another pathway implicated in NTDs that is a compelling candidate for investigation is the planar cell polarity (PCP) pathway, which is diagrammatically represented in Figure 4. PCP is a noncanonical signalling pathway for the Wnt morphogen in vertebrates (Gao et al., 2011) involved in the development of cell polarity, which is a crucial stage of neurulation, and therefore affects neural tube closure (Gao et al., 2012). The PCP mechanisms function to provide a coordinated polarized orientation for the cells involved in neurulation, which is necessary in forming the distinct apical-basal axis in the transition from neural plate to neural tube (Wang

et al., 2012). This axis is set such that, in normal neural development, apical cell surfaces are directed at the lumen of the neural tube, and basal surfaces outline the neural tube. There exists an extensive set of proteins that enable this process by operating within the PCP pathway as mediators of cell-cell adhesion (Gao et al., 2012). The key genes in the PCP pathway have been identified by observing the single nucleotide polymorphisms (SNPs) capable of dirsupting the cell polarity mechanisms, which occur most notably in Vang-like 1 (*VANGL1*) and Vang-like 2 (*VANGL2*). It was the discovery of the *VANGL2* mutant, 'Looptail', in mice, that first established a link between PCP and NTDs (Kibar et al., 2001; Murdoch et al., 2001). From the same study that identified a strong association between methylation of *MTHFR* and NTDs, particularly anencephaly, the data shows that NTD-affected children had a higher methylation of *VANGL1* than the control group. However, the P-value returned 0.063, suggesting that, despite the likely association, further research must be done to establish a statistically significant relationship between epimutated *VANGL1* and NTDs. However, the environmentally-induced C1543T and T1796C variants of *VANGL2* show a promising association with the development of NTDs (Lei et al., 2010).

Although they are involved in the development of all NTDs, the PCP genes and mechanisms discussed are heavily implicated in spina bifida in particular (Kibar et al., 2009). Beyond those seen in *VANGL1* and *VANGL2*, there are several other PCP-associated polymorphic variants that are likely to be associated with spina bifida, which highlights the importance of normal regulation of PCP genes in preserving healthy neurodevelopment and avoiding NTD occurrence.

Table 1: PCP-associated polymorphic variants returning $p < 0.01$ for association with spina bifida (Wen et al., 2010)

Gene	Ethnic Group	Variant
CELSR1	Hispanic	rs3788723, rs4823831, rs3788706, rs5768773
FZD6	White	rs2131860
	Hispanic	rs3808558
PRICKLE1	Hispanic	rs3895894
PRICKLE2	Asian	rs153717, rs149105, rs153726, rs695936, rs888405
	Hispanic	rs2306380

Of these genes, *FZD6* is of greatest interest as the DNA methylation patterns of its promoter region are found to be significantly altered in fetuses affected by spina bifida (Juriloff and Harris, 2012). Despite some evidence suggesting that *PRICKLE1* and *PRICKLE2* gene expression is dysregulated in NTD-affected human fetuses, the precise epigenetic mechanisms driving this process are still unknown and require further research.

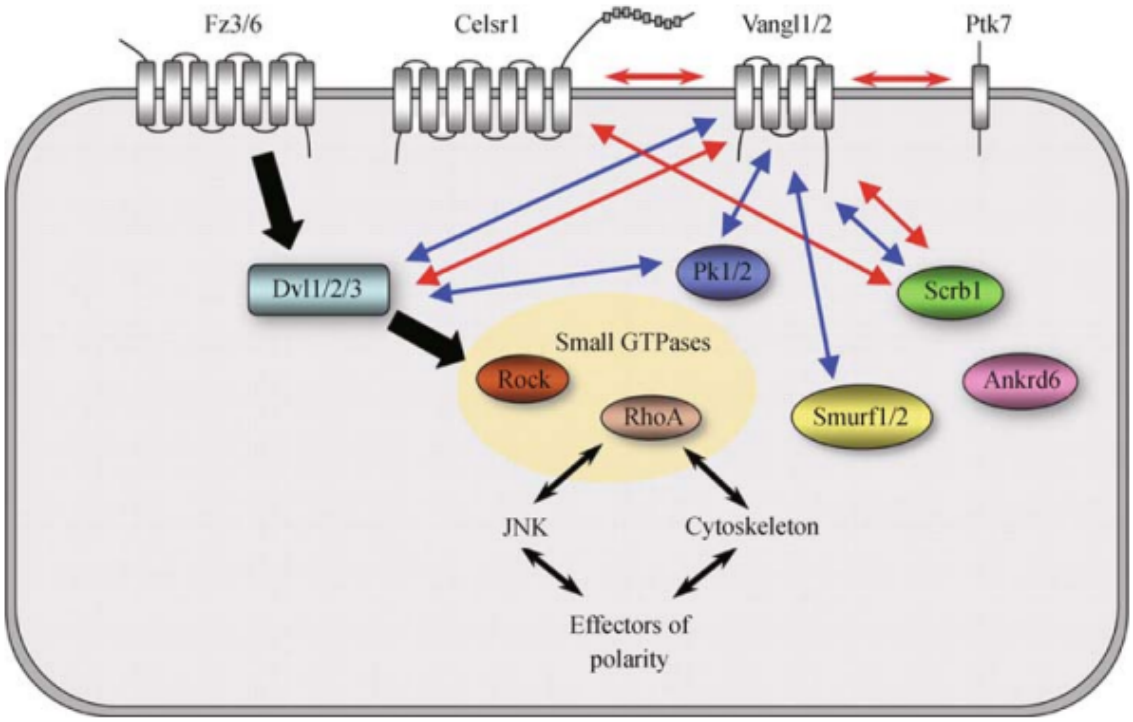

Figure 3: PCP signaling pathway in a mammalian cell. Black arrows represent the integral signaling pathways in PCP establishment. Blue arrows show known biochemical interactions, and red arrows connect genetic interactions. Figure from Cai and Shi (2014).

2.3 Environmental Disruption of *VANGL2* Causes Severe NTDs

VANGL2 is a core PCP gene, as was established in Section 2.2, that encodes the Van Gogh-like 2 (*Vangl2*) protein. *Vangl2* was originally identified in *Drosophila*, and, like its mammalian homologues, is a transmembrane protein responsible for transmitting signals from the cell surface to the cell interior (Hatakeyama et al., 2014) in the interest of supporting the greater PCP pathway objective of forming the apical-basal axis during neurulation. Of all the PCP-associated genes, *VANGL2* is discussed in particular due to the recent research showing that interactions

between *VANGL2* and specific environmental factors cause severe NTDs. Nychyk et al. (2022) showed that the interaction between hyposulfated glycosaminoglycans (GAGs) and the protein product of the Looptail (*Lp*) allele of *VANGL2* causes craniorachischisis in cultured mouse embryos, and normal development may be restored with supplementation of exogenous sulphate. Craniorachischisis is the most severe form of NTD and is characterised by an open neural tube from the midbrain to low spine (Copp et al., 1994). Murdoch et al. (2014) also found that double heterozygosity of *Lp* in combination with PCP gene mutations leads to craniorachischisis. In addition, when this double heterozygosity of *Lp* appears in combination with genes outside the PCP pathway, other NTD variations may form. For example, spina bifida is known to develop in cases displaying the double heterozygous mutant combination of *Lp* and grainyhead-like transcription factor 3 (*GRHL3*) (De Castro et al., 2018), and exencephaly arises in *Lp*/*Cobl* mutants (Carroll et al., 2003), where *Cobl* represents cordon-bleu WH2 repeat, a protein involved in actin dynamics and cytoskeletal organization. These observations verify the involvement of *VANGL2* dysregulation in promoting NTDs. By investigating the mechanisms of GAG hyposulfation, a link may be established between specific epimutations and the environmental factors associated with *Lp*. Human sulfatase 1 (SULF1) is an enzyme that catalyzes the removal of sulfate groups from various substrates, and has recently been shown to desulfate GAGs (Lai et al., 2008). Furthermore, *SULF1* has been repeatedly observed to be a target of epigenetic upregulation (Junnila et al., 2010). This offers a pathway between epimutated SULF1 and the occurrence of NTDs via the hyposulfation of GAGs. However, as the research is still active, there is insufficient data to definitively claim that this mechanism leads to NTDs.

2.4 Abnormal Upregulation of *Wnt2b* & *Wnt7b* Induces Spina Bifida

Abnormal alterations of *Wnt* genes are already understood to be linked with NTDs (Mulligan and Cheyette, 2012), which is supported by the demonstration that a null mutation of *Wnt3a* gave rise to spina bifida aperta in mice (Greco et al., 1996). Furthermore, studies investigating the effects of knockout *Wnt* genes such as *Wnt5a* and *Wnt7a* observed aberrant development, particularly in the apical-basal axis established during neurulation (van Amerongen and Berns, 2006). This suggests that other members of the *Wnt* family are also implicated in the pathogenesis of

NTDs. By employing chromatin immunoprecipitation assays to assess histone modifications, and the MassARRAY platform to identify abnormal levels of methylation in Wnt genes, Bai et al. (2016) obtained a set of novel results that greatly improve the understanding of the role of epigenetically dysregulated *Wnt* genes in NTDs. Initially, a check was done to ensure that, in mice affected by spina bifida, the transcription levels of *Wnt2b* and *Wnt7b* were actually altered in order to justify research into epigenetic dysregulation. This required measurement of mRNA expression levels, which showed that the transcriptional levels of these genes were upregulated by a factor of 3.1 and 2.8, respectively (data shown in Figure 4). It was then shown that histone 3 lysine 4 (H3K4) acetylation was enriched in GC-rich promoters of the *Wnt2b* and *Wnt7b* genes, whereas H3K27 trimethylation was reduced. In addition, notable hypomethylation was observed in multiple CpG sites belonging to the altered histone modification of target regions. In summary, the results suggest that the overexpression of *Wnt2b* and *Wnt7b* promotes NTDs, and that this dysregulation arises from clear epigenetic mechanisms such as DNA methylation, histone acetylation, and histone trimethylation. Therefore, a rare, direct link between dysregulated epigenetics and the occurrence of NTDs has been established.

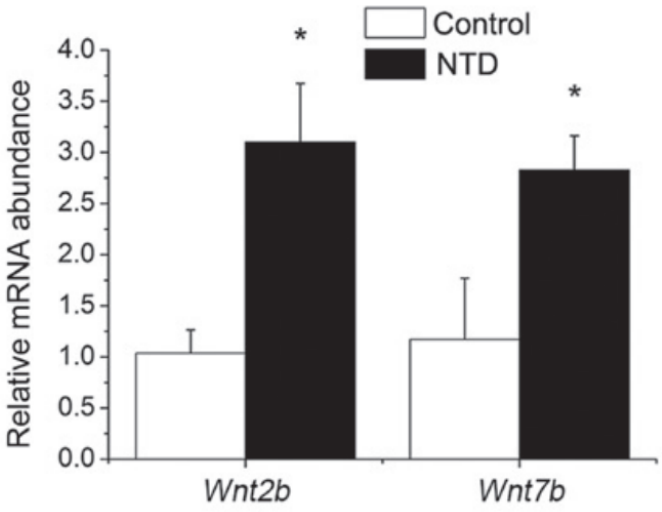

Figure 4: Relative levels of mRNA expression for Wnt2b and Wnt7b in mouse fetuses with NTDs against control groups. Figure from Bai et al. (2016).

3 Targets for NTD Treatment & Prevention

As the role of genetic and epigenetic factors in NTDs is yet to be fully understood, the current set of clinically approved NTD treatment and prevention mechanisms is limited to the supplementation of environmental factors. Folic acid supplementation is the most widely adopted and clinically approved strategy for NTD prevention, and is so effective that, as a result, many countries have implemented mandatory folic acid fortification of staple foods (Cornel and Erickson, 1997). In fact, the US public health service recommends that anyone that may become pregnant consumes 400 μg/day of folic acid. Furthermore, periconceptional administration of multivitamin supplements has been shown to reduce the incidence of NTDs from 5% to 0.6% in fetuses of women who had formerly given birth to an infant with a NTD (Smithells et al., 1980).

3.1 Potential Epigenetic Targets

There are currently no actively administered, epigenetically-based NTD prevention methods due to the recency of all research detailing specific epigenetic mechanisms that promote NTDs. However, these known mechanisms and associated pathways that lead to NTDs can be broken down and inspected for interactions that could hypothetically be interfered with in order to restore normal development and prevent NTDs.

HAT Inhibition

Section 2.4 explains that the dysregulation of *Wnt2b* and *Wnt7b* expression arises from H3K4 acetylation. From this process, it can be inferred that the inhibition of H3K4 acetylation in affected fetuses should reduce the risk of NTDs as this would occur in association with the nullified overexpression of *Wnt2b* and *Wnt7b*. Histone acetyltransferases (HATs) are a family of enzymes that add an acetyl group to the lysine residues of histones in histone acetylation. Arif et al. (2010) suggests a novel water-soluble HAT inhibitor (HATi), known as CTK7A, that has been shown to inhibit oral tumour cell growth, a condition that also arises as a result of histone H3 hyperacetylation. CTK7A itself is a salt form of HBC, a complex benzoic acid-based compound, that inhibits HAT p300/CBP (promotes oral cancer when hyperactive) without

affecting the behaviour of other histone modifying enzymes, making it a strong candidate for clinical trials and perhaps testing within the context of NTD prevention.

Upregulation of H3K27 Trimethylation

As discussed above, H3K27 trimethylation (H3K27me3) is reduced in cases of spina bifida where *Wnt2b* and *Wnt7b* expression is upregulated. This is expected as H3K27 trimethylation is associated with gene silencing. Therefore, if action can be taken to increase H3K27 trimethylation, then *Wnt2b* and *Wnt7b* expression may be partially silenced and returned to normal levels, subsequently preventing the pathogenesis of spina bifida. Enhancer of zeste homolog 2 (EZH2) is a subunit of the polycomb repressive complex 2 (PRC2) and is the primary enzyme responsible for trimethylation of histone H3 at lysine 27 (Fujii et al., 2011), so artificially increased H3K27 trimethylation may theoretically be achieved by enhancing the activity of EZH2. Yin Yang 1 (YY1) is a zinc-finger transcription factor capable of upregulating EZH2 expression. EZH2 overexpression is associated with various cancers (Kim and Roberts, 2016), and the network of regulatory mechanisms that controls EZH2 activity is complex, however, YY1 does still present a possibility for a target within embryos that may be artificially upregulated in order to downregulate *Wnt2b* and *Wnt7b* expression and lower the risk of NTDs.

Ethical Considerations

It is noted that interfering with embryonic development in humans to treat or prevent NTDs based on the risk of development of cognitive and behavioural difficulties raises some ethical concerns. It can be argued that taking medical action suggests that the value of a life is lower in those affected by such conditions, an idea that many people may object to.

4 Conclusion & Future Perspectives

Due to the complex multifactorial etiology of NTDs, research into the genetic and epigenetic mechanisms, as well as the relevant environmental factors underpinning their development, is still ongoing and incomplete. Despite the necessity for more research to provide a complete net-

work of known genetic and biomolecular pathways, there is still sufficient emerging evidence to show that exposure to particular environmental factors and dysregulation of epigenetic modifications promotes NTDs. For example, folate deficiency, which may be caused by dietary changes or an *MTHFR* polymorphism; defects in the PCP pathway caused by SNPs of key genes; interactions between hyposulfated GAGs and *Lp*; and epigenetically-induced upregulation of *Wnt2b* and *Wnt7b*, are all implicated in NTD pathogenesis.

Further elucidation of the genetic and epigenetic pathogenesis of NTDs could be achieved by the advancement of exome and whole-genome sequencing technologies. This could enable large-scale studies that identify genetic variants associated with NTDs, which could each be studied and tested for associations with epigenetic mechanisms such as ncRNA, histone modifications or DNA methylation. In the context of NTD prevention, folic acid, as well as multivitamin supplementation provide some methods by which the incidence of NTDs may be reduced by utilising knowledge of the role of environmental factors in causing NTDs. Although there is no clinically approved basis for epigenetic mechanisms as targets, inhibitors of HATs, such as CTK7A, as well as factors that cause upregulation of H3K27me3, offer an interesting direction for further testing and research.

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