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Studijní program: Speciální chemicko-biologické obory

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Genetika a molekulární podstata vzniku spina bifida

Genetics and molecular basis of spina bifida

Bakalářská práce

Vedoucí závěrečné práce:

doc. RNDr. Ing. Vladimír Krylov, Ph.D.

Praha, 2019

Prohlášení:

Prohlašuji, že jsem závěrečnou práci zpracovala samostatně a že jsem uvedla všechny použité informační zdroje a literaturu. Tato práce ani její podstatná část nebyla předložena k získání jiného nebo stejného akademického titulu.

V Praze, ...

Alice Kim

Poděkování

Chtěla bych v první řadě poděkovat svému školiteli doc. RNDr. Ing. Vladimíru Krylovovi, Ph.D., který mi po celou dobu psaní pomáhal, radil a vždy se mi snažil vyjít vstříc. Samozřejmě bych chtěla poděkovat i své rodině, která mě podporovala během celého studia, byla mi morální oporou a během psaní závěrečné práce mi poskytovala potřebné zázemí.

Abstrakt

Spina Bifida patří mezi vady vznikající během neurulace, konkrétně při uzávěru neurální trubice. Neurální valy nejčastěji v bederní oblasti nefúzují a zůstává zde otvor. Recentní studie naznačují, že naše chápání tohoto procesu, tj., že uzavírání neurální trubice probíhá nezávisle na několika místech, by mohlo být chybné a akcentují iniciaci z jednoho bodu. K vytvoření Spina bifida vedou různé faktory, jako je výživa, genetické predispozice a prostředí. Kyselina listová a vitamin B12 se ukázali jako účinné doplňky stravy, pokud jde o snížení rizika vzniku této vady. Z genetického pohledu, mutace v genech MTHFR, CUBN, CHKA, SARDH, MTRR, Grhl-3, které jsou zapojeny v procesu methylace, jsou považovány za důležité rizikové faktory pro vznik Spina bifida u člověka. V rámci myšího modelu byla prokázána role nesprávné methylace nebo hypomethylace Hox a Vangl genů. V neposlední řadě tuto vadu způsobují taktéž mutace v genech Par1 / Par2.. Neurální valy v daném místě nefúzují a zůstává zde otvor. Recentní studie naznačují, že naše chápání tohoto procesu, tj., že uzavírání neurální trubice probíhá nezávisle na několika místech, by mohlo být chybné a akcentují iniciaci z jednoho bodu. K vytvoření Spina bifida vedou různé faktory, jako je výživa, genetické predispozice a prostředí. Kyselina listová a vitamin B12 se ukázali jako účinné doplňky stravy, pokud jde o snížení rizika vzniku této vady. Z genetického pohledu, mutace v genech MTHFR, CUBN, CHKA, SARDH, MTRR, Grhl-3, které jsou zapojeny v procesu methylace, jsou považovány za důležité rizikové faktory pro vznik Spina bifida u člověka. V rámci myšího modelu byla prokázána role nesprávné methylace nebo hypomethylace Hox a Vangl genů. V neposlední řadě tuto vadu způsobují taktéž mutace v genech Par1 / Par2.

Klíčová slova: Spina Bifida, neurální trubice, vady neurální trubice, Myelomeningokéla, neurulace

Abstract

Spina bifida is a neural tube defect that evolves during neurulation when the neural folds fail to fuse and result in an open neural tube. New studies have shown that our understanding of neural tube closure might be wrong and suggested a single site neural tube closure in humans. Various factors like nutrition, genetics, and environment lead to the formation of a neural tube defect. Folic acid and Vitamin B12 have been shown as effective supplements when it comes to lowering the risk of developing NTD and. Genetic mutations of MTHFR, CUBN, CHKA, SARDH, MTRR, Grhl-3, which are all involved in methylations are considered important risk factors for NTD's. Wrong methylation or hypomethylation of Hox and Vangl genes have shown to be also playing a role in NTD's. Par1/Par2 mutations in mice have shown to cause Spina Bifida.

Keywords: Spina Bifida, NTD, Neural tube defects, Myelomeningocele, Neurulation

List of shortcuts

SB	**Spina Bifida**
SBO	**Spina Bifida Occulta**
SBM	**Spina Bifida Meningocele**
MMC	**Spina Bifida Myelomeningocele**
NTD	**Neural tube defect**
AFP	**Alpha-fetoprotein**
THF	**Tetrahydrofolate**
MTHFR	**Methylenetetrahydrofolate**
5-MeTHF	**5-Methyltetrahydrofolate**
Adox	**oxidized adenosine**
CHKA	**Choline kinase alpha**
CDP-choline	**Cytidine diphosphocholine**
PC	**Phosphatidylcholine**
DMAE	**2-dimethylaminoethanol**
ET-18-OCH_3	**1-O-octadecyl-2-O-methyl-rac-glycerol-3-phosphocholine**
SARDH	**Sarcosine dehydrogenase**
Ghrl-3	**Grainyhead-like-3**
GPCR	**G-protein coupled receptors**
PCP	**Planar cell polarity**
VANGL	**Van Gogh like**
CE	**Convergent extension**

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1. Introduction to Spina Bifida

Spina bifida is a defect which a child born with. The primary defect is, that the tissues and bones around the spinal cord are not fully closed, which causes in the most severe case, that the neural cord is leaning out of the body. Spina bifida usually occurs on the lower part of the back, but on rare occasions, it can develop in the middle too. The three major types are Spina bifida occulta, spina bifida meningocele, and spina bifida myelomeningocele.

It is believed, that the occurrence of spina bifida leads back to the years 2000 bc., that is the approximate period from which this defect is known to the humankind. Around the year 1760, the Italian anatomist Giovanni Battista Morgagni said, that the spina bifida could occur with the hydrocephalus simultaneously, but that both could develop without the other. In the year 1841, the famous surgeon Nicholaas Tulp mentioned in his book "Observationes Medicae," the way he treats spina bifida. He tried to punctuate the sac, which unavoidably has lead to death. He also noticed that the sac contained neural elements, from which he stated that the sac must be treated with more caution. Fast forward to 1875 when Rudolf Ludwig Karl Virchow started using the term spina bifida occulta and shortly after, in 1892, the first surgery was performed on spina bifida myelomeningocele, the most common form of spina bifida. C. Bayer placed the neural tissue inside the spinal canal, and by using multiple rotating techniques and overlapping tissues, he covered the area until it fully closed, which was by that time a substantial surgical advance. Before that, most cases were treated with extraction of the fluid, by using a small needle, which needed to be inserted from the side of the sac to not damage the neural elements. Further, in the 20th century, the techniques of treating spina bifida were slowly evolving, but all surgical treatments were based on C. Bayers first operation. In our current century, in some cases, we are capable of treating even spina bifida myelomeningocele without further complications, by performing multiple surgeries.

This bachelor thesis will focus on the genetics and causes of this defect and try to conclude, what reactions, genes, and factors could lead to this defect.

2. What is Spina Bifida?

Spina bifida (further SB) is considered a neural tube defect (NTD), which means that the cause of this malformation is the improper or incomplete closure of the neural tube or damage in the tissues surrounding the neural elements within the first four weeks of development. The presence of this defect is detectable from AFP (alpha-fetoprotein) and afterward from an ultrasound (Dash et al., 2006). In case of an NTD, the AFP levels increase in the mother's serum, because of the leakage of AFP from the child's open neural tube defect, so naturally, Spina Bifida occulta (further SBO, Fig. 1, 1st image) is not visible from this screening. SBO is the mildest form of spina bifida and at the same time most common. Humans born with SBO are often not aware of their condition until further in life.

Spina bifida meningocele (further SBM, Fig. 1, 3rd image) is a more severe and least common form of SB. The meninges are creating a sac and are leaning out of the vertebrae, but the nervous system stays in its usual position, so people with SBM have a lesser risk of suffering from long term health problems.

Spina Bifida myelomeningocele (further MMC, Fig. 1, 4th image) is the most severe form of SB and is often accompanied by hydrocephalus. The meninges are creating a sac outside of the body and contain nervous system tissues, which increases the risk of having long term incapabilities and problems throughout the life of the disabled individual. Issues that might occur are bladder and bowel control issues, urinary infections, skin irritations, orthopedic abnormalities, and in some cases, even paralysis.

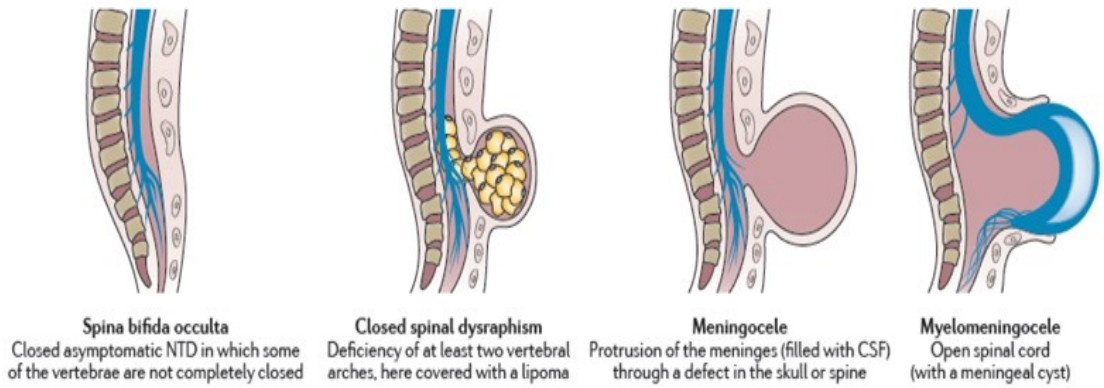

Fig. 1 (Copp et al., 2015)

2.1. Neural tube closure

Neurulation is the process of neural tube formation, which will eventually become the brain and spinal cord. In humans, it starts forming within the 3rd week after fertilization and requires that the neural plate starts elevating and creating folds, which fuse in the midline and form the neural tube. The exact molecular mechanisms are yet not known, but various studies show what pathways might influence the neural tube closure.

After the gastrulation, there are three main layers, the ectoderm, mesoderm, and entoderm. The neural tube starts forming from the ectoderm. The first event of neurulation is the formation of a thickened area of cells, called the neural plate. It forms at the cranial end of the embryo and grows towards the caudal end. In the future, the brain will form at the cranial end and the spinal cord at the caudal end. By the end of the 3rd week after fertilization, the lateral edges of the neural plate start elevating and form the neural folds, in between those folds, the neural groove is formed. Eventually, the neural folds start fusing from the middle outwards and form the neural tube (Visualization in Fig. 2).

Often, the malfunction of the fusion causes NTD's for instance, also Spina Bifida, which is in the caudal end.

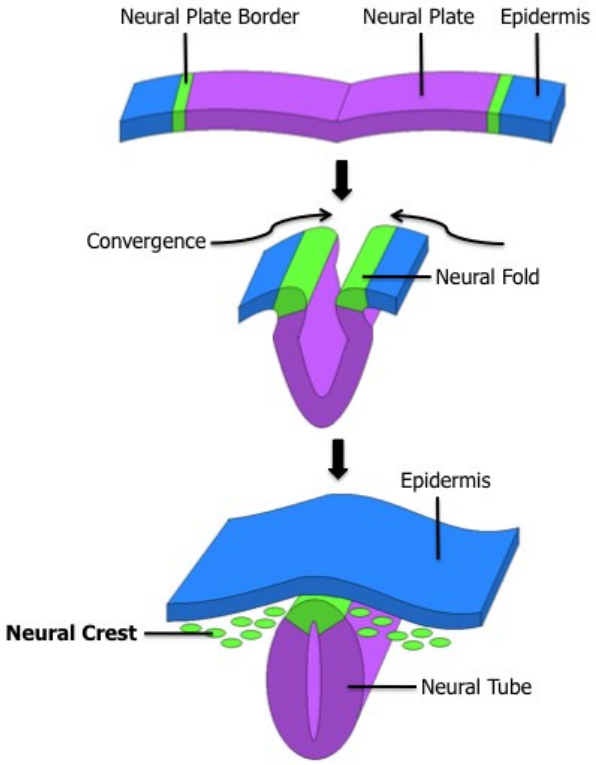

Fig.2 (source: English Wikipedia "Neural tube")

2.2. Multiple site closure theory

Nowadays, the widely supported idea of the neural tube closure is the one, where there are multiple closing sites, precisely three in humans (a theory supported in the study (Nakatsu et al., 2000), which means, the closure itself is initiated at various positions along the neural folds. The first initiation site is at point A, the future cervical region, in Fig. 3 and continues to close in the caudal and rostral direction. The second initiation is around the mesencephalic-rhombencephalic boundary, which is site B in Fig. 3. The last site, site C, in the illustration, begins at the rostral end of the neural groove and reaches the closure from site B shortly after initiation (Nakatsu et al., 2000). This theory supports the idea that neural tube defects are caused by the improper closure in between the initiation sites (between C and B, B and A and at the posterior neuropore). An older study by van Allen (1993) suggests that there are five initiation points in human neural tube closure. Each closure failure is shown in Fig. 4 (dots show where the failure occurred) is responsible for a different location of SB.

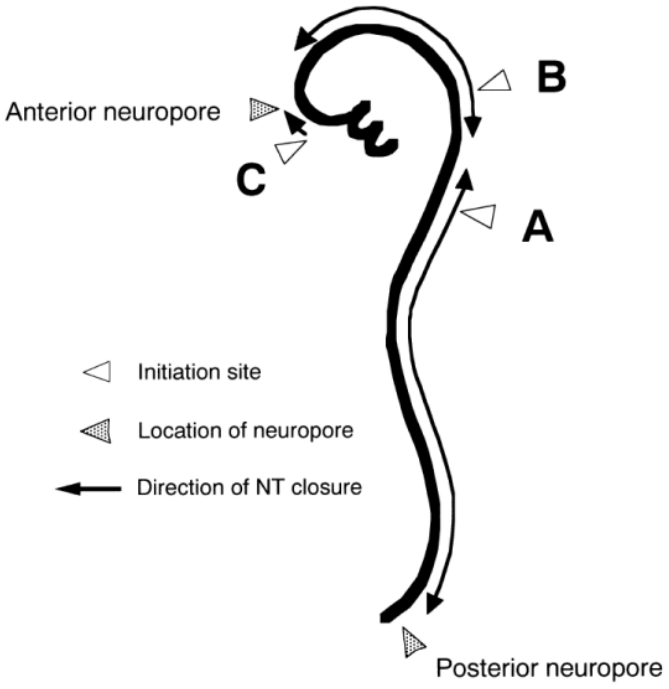

Fig.3 (Nakatsu et al., 2000)

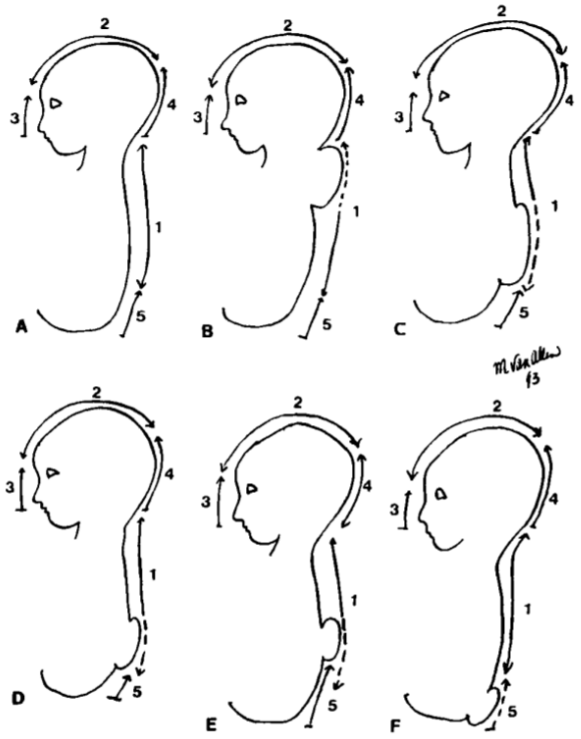

Fig. 4 (Van Allen et al., 1993)

2.3. Single site closure theory

Another study suggests though that there is only one initiation site and that the closure itself progresses similar to a zipper. Some cases of human NTD could not even be explained with the 5-site model of Van Allen and suggested even a sixth closing site (Srinivas et al., 2008). The researchers studied multiple human and mouse embryos during neurulation and could not find any signs of multiple closing sites. The theory is that neural tube defects might not be caused solely by the incomplete closure of the neural tube, but also by detaching after fusion. A study has hypothesized that the inability of apoptotic neural cells to migrate in *Nell-1* transgenic mice, might have lead to acrania in this model (X. Zhang et al., 2006). Acrania is a postneurulaion NTD, which is very similar to exencephaly except that it evolves after the neurulation process. There are two hypotheses of how an NTD might occur with a single site closure in various

locations, so not only at the neuropores. The first theory is that the fusion starts at one point and continues towards the neuropores but that the closure of the next spot in line is not dependent on the previous one. Instead, it is dependant on the signal, which means that it is very similar to a zipper, where a tooth can be skipped, but the rest is still able to close.

The second theory suggests that even after fusion, the neural folds detach and cause the NTD due to apoptosis in that specific area (Huang et al., 2002; X. Zhang et al., 2006). These ideas support the assumption that an NTD can occur at any part of the neural tube.

3. Factors that influence the occurrence of Spina Bifida

To fully understand Spina Bifida, we need to take a closer look at what it is and what factors may or may not play a role in the development of the neural tube. Three known factors influence the occurrence of Spina Bifida: Nutrition, genetics, and environmental factors. Moreover, the correlation of these factors is relevant and might increase the risk of Spina Bifida occurrence. The correlation impacts will be described further in this chapter.

3.1. Nutrition

Nutrition plays a significant role in the development of an NTD. The deficit of specific nutrients might cause NTD's, but studies have yet to confirm which mechanisms lead to which defects and the molecular mechanisms behind it.

3.1.1. Folate/Folic acid

Folate is a water-soluble B vitamin, which our body does not produce, which means, we have to obtain it through food or supplements. Folic acid is a synthetic version of folate and is more stable and sometimes even better absorbed. Thus we

are using it as a supplement instead of folate. After consumption, folic acid is transformed into an active form of folate (majorly 5-Methyltetrahydrofolate) and enters the folate pathways. Neural crest cells show high expression of folate receptors from which we can deduce that it is highly demanded in these types of cells (Običan et al., 2010). Folic acid plays a role in the one-carbon metabolism that produces pyrimidines and purines for DNA synthesis. It is also needed for the generation of S-AdenosylL-methionine, which is a methyl donor for DNA, RNA, and protein methylation (Rochtus et al., 2015). There is a methylation theory that suggests; folic acid prevents NTD's by enhancing cellular methylation reactions. Therefore it is believed that a disbalance of methylation might disturb neural tube folding (Greene et al. 2011).

In 1998 it became mandatory in the US to enrich grain cereals with folic acid with the concentration 1,4mg/kg ((FDA), 1996). Based on the studies shown in table No.1, except for one, the overall occurrence of NTD's has decreased after the fortification, which shows a link between folic acid intake and NTD's. It must be remarked though that results differ among different ethnicities. The highest prevalence is amongst Hispanic, less in non-Hispanic white people, and the least in non-Hispanic black people (Williams et al., 2005).

Reduction in NTD rates with folic acid fortification.

Reference	Area/State	Period studied		Total NTD prevalence rate (/1,000 births)		% Reduction
		Before fortification	After fortification	Before fortification	After fortification	
Ray *et al*. [72]	Ontario	1994–1997	1998–2000	1.13	0.58	49
Honein *et al*. [73]	USA	1995–1996	1998–1999	0.38	0.31	19
Persad *et al*. [74]	Nova Scotia	1991–1997	1998–2000	2.58	1.17	55
Williams *et al*. [75]	USA	1995–1996	1998–1999	0.76	0.56	26
De Wals *et al*. [76]	Quebec	1992–1997	1998–2000	1.89	1.28	32
Palomaki *et al*. [77]	Maine	1993–1996	1998–2000	1.23	1.07	13
Lambert-Messerlian *et al*. [78]	Rhode Island	1991–1996	1998–2000	3.8	3.3	13
Simmons *et al*. [79]	Arkansas	1993–1995	1999–2000	1.09	0.82	25
Liu *et al*. [64]	Newfoudland	1991–1997	1998–2001	4.36	0.96	78
Chen *et al*. [80]	Costa Rica	1996–1998	1999–2000	9.7	6.3	35
Hertrampf *et al*. [81]	Chile	1999–2000	2001–2002	1.70	1.01	41
Lopez-Camelo *et al*. [82]	Chile	1982–1991	2001–2002	1.57	0.80	49
Canfield *et al*. [83]	USA	1995–1996	1999–2000	0.71	0.5	30
De Wals *et al*. [69]	Canada	1993–1997	2000–2002	1.58	0.86	46
Chen *et al*. [84]	California	1989–1996	1998–2003	0.59	0.70	No decline
Sayed *et al*. [85]	South Africa	2003–2004	2004–2005	1.41	0.98	31
Amarin *et al*. [86]	Jordan	2000–2001	2005–2006	1.85	1.07	49

Table 1. summarized in Review (Imbard et al., 2013)

3.1.2. Vitamin B12

It is not entirely clear what role Vitamin B12 might play in the process of neurulation, but it is a cofactor of the enzyme methionine synthase. It influences the incorporation of folate into the cellular pool, and the transition of folate derived 1-carbon units, which are further used in DNA synthesis or methylation. It has been now shown multiple times that Vitamin B12 deficiency plays its role in the development of an NTD and is independent of the folic acid intake (Ray et al., 2007; Molloy et al., 2009). Women with concentrations of Vitamin B12 <200 ng/L are at three times higher risk of having a child with an NTD than those with concentrations >400 ng/L (Molloy et al., 2009) but the specifics are yet unknown.

3.2. Genetic factors

It is yet not known which specific genetic mutations for sure cause NTD's, but it is known that having a child with an NTD increases the risk by 2-5% for the next offspring, the (Copp & Greene, 2008). SB is most probably not caused by only one or two genes but by multiple which are also influenced by the environmental factors and nutrition, such as the intake of folic acid (Volcik et al., 2002). There are though four pathways in the methylation cycle; folate pathway, B12 pathway, choline pathway and remethylation pathway that have been studied, in which genetic mutations might increase/decrease the probability of an NTD (Imbard et al., 2013).

3.2.1. Vangl1/Vangl2 (PCP)

PCP is a molecular mechanism which plays a role in the directional movement of cells during gastrulation and neurulation, by giving them coordinated polarized orientation and its disruption causes NTD's (Bayly & Axelrod, 2011). PCP supports the movement of mesoderm, and neural plate cells from the sides of the embryo towards the midline. These cells push in between the former cells of the midline and cause, therefore an elongation and narrowing of the embryo in the rostrocaudal axis. This process of elongation and narrowing is called "convergent extension," and the importance of PCP in this process was first shown in Xenopus (Wallingford & Harland, 2002) and afterward in mice (Wang et al., 2006). Six proteins are included in the "core" signaling module: the multi-domain protein Dishevelled (Dsh) (Theisen et al., 1994), the serpentine receptor Frizzled (Fz) (Vinson & Adler, 1987), the Lim domain protein Prickle (Pk) (Gubb et al., 1999), the 4-pass transmembrane protein Van Gogh (Vang) (Taylor et al., 1998; Wolff & Rubin, 1998), the Ankyrin repeat protein Diego (Dgo) (Feiguin et al., 2001), and the seven-transmembrane atypical cadherin Flamingo (Fmi) (Usui et al., 1999). The drosophila has been the leading model organism on which the PCP mechanisms have been studied. Mutations in these core proteins lead to disruptions of cell polarity and result for example on the Drosophila in an incorrect position of pre hair growth

(closer to the center or in the center, rather than on the side) (Usui et al., 1999; Axelrod, 2001; Feiguin et al., 2001; Strutt, 2001; Tree et al., 2002; Bastockn et al., 2003). The Vangl2 gene is the mammalian homolog to the *Vang* gene in Drosophila, which causes craniorachischisis in homozygous Loop-tail (Lp) mouse mutants (Kibar et al., 2001; Murdoch et al., 2001). Vangl1 is a vertebrate homolog of Vangl 2, which it also interacts with genetically, and has a dynamic pattern of expression in the forming neural tube (Torban et al., 2008a). The injection of Vangl1 mRNA in Zebrafish suppresses a CE defect in *tri*/*Vangl2* mutant embryos, which suggests that Vangl1 and Vangl2 have very similar biochemical activities (Jessen & Solnica-Krezel, 2004).

In a study where 673 patients with Spina Bifida were examined for Vangl1 mutations, seven were heterozygous five novel missense mutations, which affect amino acid residues which are distributed on the entire length of the Vangl1 protein. Since there were no signs of null mutations, which would silence the whole protein, further investigations are needed to examine the effect of those mutations on the protein function (Zoha Kibar et al., 2009). Since all mutations detected in Vangl1 so far are heterozygous, it is assumed that these variants may cause only a partial loss of function and genetic and environmental factors might influence the expression of an NTD phenotype, which is also supported by a study where Vangl1 interacts with Vangl2 to cause craniorachischisis in mice (Torban et al., 2008b; Kibar et al., 2009).

The same research was applied to the study of the Vangl2 gene. Eight patients had seven rare missense mutations (all heterozygous), which have not been described previously. An interesting finding was the fact that the p.Arg177His, and p.Arg270His mutations caused an exchange of two basic amino acids which is not considered a significant chemical change, but these two mutations lie within the two conserved regions of Vangl2, which suggests intolerance of amino acid substitution across evolution (Z Kibar et al., 2011). Interestingly, five patients had a closed NTD, and two had an open NTD, which implicates that Vangl2 could have a

significant influence on closed NTD's rather than open, but this theory contradicts the fact that PCP knockout mice have craniorachischisis and no occurrence of closed NTD's (Torban et al., 2008a; Z Kibar et al., 2011).

Genetic mutations of these genes have been found only in a small number of patients with NTD's. Zoha Kibar et al. (2009) suggested that one of the reasons might be DNA methylation changes at these genes, which could support the methylation theory mentioned in chapter 3.1.1.

3.2.2. MTHFR (folate pathway)

Methylenetetrahydrofolate reductase (MTHFR) is an enzyme in the folate metabolism that is involved in the synthesis, methylation, and repair of DNA (Hosseini et al., 2011; Zeisel, 2009). The most studied and probably most significant polymorphism of this enzyme is the C677T polymorphism (Sameer et al., 2011). In this point mutation, at the nucleotide 677 in exon 4, cytosine is converted into thymine, which results in the amino acid exchange from alanine to valine (Sharp & Little, 2004). Due to the 677 TT (homozygote) mutation, the concentration of 5-Methyltetrahydrofolate(5-MeTHF), which plays a vital role in the homocysteine metabolism, in the plasma and red blood cells decreases, and the total concentration of 10-formyltetrahydro-folate increases (Put & Blom, 2000; Bagley & Selhub, 2002) which is shown in the illustration No.4. With the 677 TT mutation also decreases the overall methylation of DNA and is lower in comparison to the wild type 677 CC genotype (Friso et al., 2002;Castro et al., 2004) and since the disruption of DNA methyltransferase DNMT3B during embryo development causes a disrupted neural tube it is clear that methylation is needed during periods of rapid growth (Okano et al., 1999). This hypothesis was tested with an *in vitro* analysis of cultured chicken embryos which were given homocysteine and other inhibitors of methylation like cycloleucine (an inhibitor of methionine adenosyl methyltransferases) and oxidized adenosine (Adox), which is an inhibitor of *S*-adenosylhomocysteine hydrolase. The results were an increase of *S*-

adenosylhomocysteine, and a decrease of *S*-adenosylmethionine/*S*-adenosylhomocysteine and a dose-dependant delay in neurulation was visible (Afman et al., 2003). S-adenosylmethionine is involved in the methylation of proteins, lipids and DNA and the decrease of it might lead to a lower methylation capacity of the cell, which could explain the hypomethylation of genomic DNA in the brain of NTD affected fetuses (Chang et al., 2011). Other studies also have shown (Coelho & Klein, 1990; Vanaerts et al., 1994) that culturing mice with low methionine levels not only causes them to have NTD's, which supports the methylation theory, mentioned in the chapter 3.1.1., but also reduced amino acid methylation in proteins. Based on that, it is proposed that it affects actin function and can reduce microfilament contraction, which is needed for the proper inward folding of neural folds (Coelho & Klein, 1990).

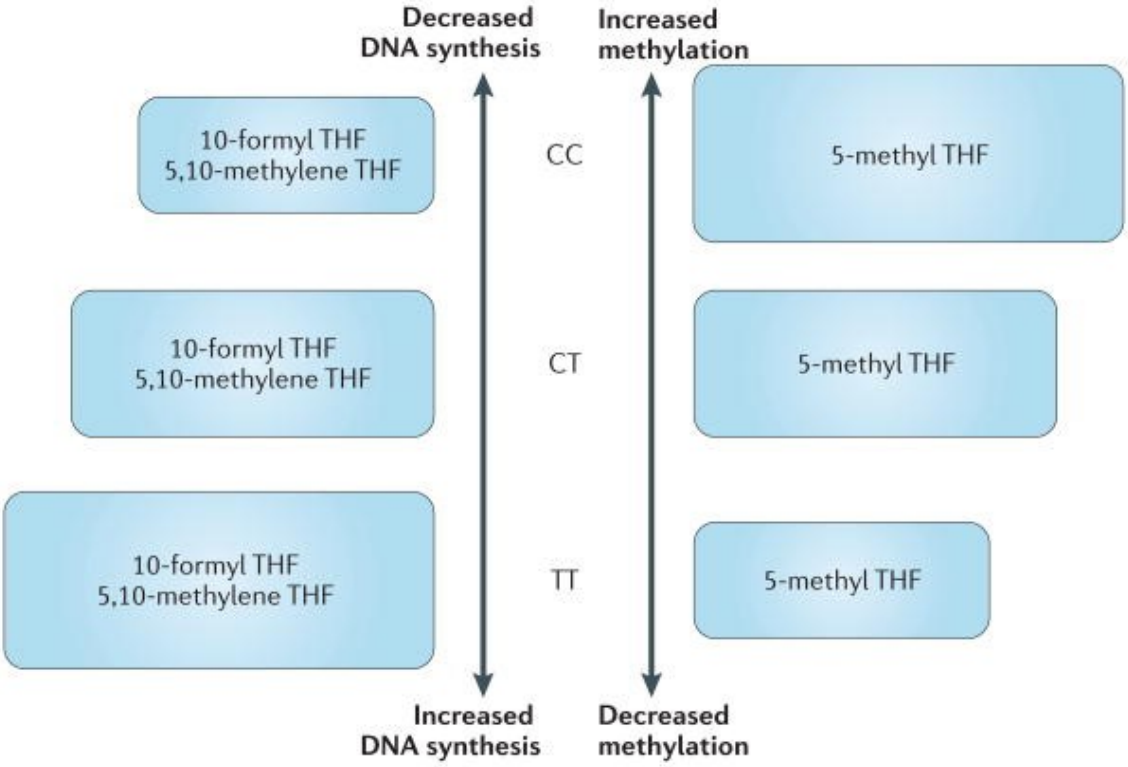

Fig.5 (Blom et al., 2006)

A study, where all of the data on the correlation of MTHFR and NTD's was gathered and analyzed, has shown, that there indeed is an increased risk of an NTD in Asian, Caucasian and mixed ethnicities though the correlation was not found in African groups, which might have been caused by small sample size and limited studies. Although the correlation was found, only 13% of NTD's attribute to the C677T mutation, which is not enough for the polymorphism to be alone at fault (Boduroğlu et al., 1999).

Factors that might influence the impact of the C677T polymorphism are, for instance, gene to gene interactions, where the complex folate metabolism might be altered by other genes, thus resulting in a balance shift in metabolites (Robien & Ulrich, 2003). Another factor is the genetic-nutritional interaction since most homozygotes carrying the C677T mutation do not have NTD's, which suggests a genetic-nutritional interaction (Gibson & Bottuglieri, 2000).

Although C677T polymorphism is the most studied mutation, several other types might enhance the risk of an NTD: A1289C (De Marco et al., 2002; van der Put et al., 2002), C116T and G1793A (O'Leary et al., 2005).

3.2.3. CUBN (Vitamin B12 pathway)

The CUBN gene encodes Cubilin; a peripheral membrane glycoprotein which is an intrinsic factor–cobalamin receptor. It acts as a receptor for intrinsic factor–vitamin B12 complexes (Christensen & Birn, 2002) and is also involved in the reabsorption of Vitamin B12 (Pangilinan et al., 2012). During embryonic development, this gene is expressed in developing neuroepithelial cells and the neural tube and also mediates ligand endocytosis by absorptive epithelia at the maternal-fetal interfaces, trophectoderm, and visceral endoderm (Franke et al., 2009). So far only the rs1907362 polymorphism in CUBN is associated with Spina Bifida and Vitamin B12 levels in Dutch patients, so the influence of the mutation in mothers is yet not known (Franke et al., 2009). The CUBN gene is not well studied yet, and further

research is needed to understand whether it is indeed a risk factor for Spina Bifida.

3.2.4. CHKA (choline pathway)

Choline is vital in the methyl metabolism and acts as a methyl donor in the methylation of homocysteine to methionine, just like folate (Zeisel et al., 1994), and the deprivation of this nutrient even leads to apoptosis (Albright et al., 2018). Humans are, in fact, capable of synthesizing choline de novo especially in the liver through sequential methylation of phosphatidylethanolamine using S-adenosylmethionine as the methyl donor (Bremer & Greenberg, 1961), though it is not enough to balance out a choline-deficient diet (Zeisel, 2000).

In the cytidine diphosphocholine (CDP-choline) pathway, choline is used for the de novo synthesis of phosphatidylcholine (PC) and sphingomyelin (Ishidate, 1997). In neurulating mice, choline is primarily used for PC synthesis through the CDP-choline pathway. A demonstration by Fischer showed that the inhibition by 2-dimethylaminoethanol (DMAE) and 1-O-octadecyl-2-O-methyl-rac-glycerol-3-phosphocholine (ET-18-OCH_3), an inhibitor of PC synthesis, causes cell death and NTD's in neurulatig mice (Fisher et al., 2001). Although precautionary choline intake was associated with a decrease of NTD risk (Shaw et al., 2004), this study suggests that with the CHKA single nucleotide polymorphism rs939883 mutation, which causes an NTD risk, it might be ineffective since no gene-nutrient interactions were found (Enaw et al., 2006). This result indicates that choline metabolism and dietary choline intake might be two separate risk factors, though this theory does not need to be true since it has low statistical power due to small sample size.

3.2.5. SARDH (choline and folate pathway)

The SARDH gene encodes the enzyme sarcosine dehydrogenase, which catalyzes the oxidative demethylation of sarcosine to glycine and is also involved in the breakdown of choline via betaine (Franke et al., 2009). It is also an intermediate in

the folate-dependent one-carbon metabolism and promotes the folate-mediated transfer of one-carbon units, which is necessary for DNA synthesis and repair (Green et al., 2013). Besides, it was found that SARDH is implicated in plasma homocysteine and Alu methylation phenotypes and since elevated homocysteine levels are associated with NTD risks, SARDH might be a valuable risk factor (Wernimont et al., 2011; Tang et al., 2015). In a case-control study of the Chinese Han population, two potential polymorphisms loci were found; rs2797840 and rs2073817, in which rs2797840 is associated with increased fetal folate content (Piao et al., 2016). A Dutch study had different results, though, they have associated rs573904 in the gene as a risk factor in the Dutch population (Franke et al., 2009). These differences might occur due to different studied ethnicities, which has also been observed by Liebert et al. (Feuchtbaum et al., 1999), and different studied NTD's; all Spina Bifida in Franke's and mixed NTD's in Piao's. It is hypothesized that the protective allele G of rs2797840 associates with an increase of folate content in the brain from which Piao et al. deducted that it alters the transcription factor accessibility of the gene, resulting in an increase of transcription of SARDH, which further increases the level of glycine. Importantly, cleavage of glycine produces besides others a carbon unit for the methylation of THF, which could potentially charge the folate cycle and contribute to an increased folate level in the brain, which could lead to a decrease of NTD occurrence (Piao et al., 2016). The G/A nucleotide polymorphism of the other found locus rs2073817 leads to an Arg/His amino acid polymorphism, which affects the function of the protein and increases the risk of an NTD (Piao et al., 2016). Further studies of the exact mechanisms are needed though since they are yet not fully understood.

3.2.6. MTRR (remethylation pathway)

MTRR is a gene, that codes methionine synthase reductase, which is involved in homocysteine metabolism and methionine production by keeping the methionine synthase, which is coded by the MTR gene, in an active state (Linden & Heijer,

2006; Zhang et al. 2017). The methionine synthase catalyzes homocysteine to methionine by taking 5-methyltetrahydrofolate as a methyl donor and remethylating homocysteine with cob(I)alamin (Vitamin B12) as a cofactor (Li et al., 1996). Since by occasional oxidation of its cofactor cob(I)alamin to cob(II)alamin the enzyme gets deactivated, methionine synthase reductase reactivates the methionine synthase by reductively methylating cob(II)alamin to methylcob(I)alamin with S-adenosylmethionine (AdoMet) as a methyl donor (Li et al., 1996; Proc, 2001; Zhang et al., 2017). The best-studied mutation of MTRR which could be a risk factor for spina bifida is the A66G polymorphism, an N-terminal I22M substitution, which causes an amino acid exchange from isoleucine to methionine. At first, it was believed that this mutation is a risk factor only when the concentration of vitamin B12 is low (Wilson et al., 1999) but later a risk increase in mothers and daughters with the MTRR 66AG/GG genotype alone was described (Zhu et al., 2003). Another meta-analysis has shown that mothers with MTRR 66GG genotype have a two times higher risk of having a Spina Bifida affected a child but that this genotype is not associated with Spina Bifida risk in children (van der Linden et al., 2006). Mothers with MTRR 66GG genotype who have at the same time elevated levels of methylmalonic acid(MMA) in their plasma, which indicates a Vitamin B12 deficiency (Savag et al., 1994) have a 5,5 times higher risk of having a child with Spina Bifida (van der Linden et al., 2006).

3.2.7. Grhl-3

Curly tail mice mutants (ct/ct) have been studied for NTD's for over 50 years, and recently a new gene candidate grainyhead-like-3 (Ghrl-3) has been detected as an influence of the curly tail phenotype (Ting et al., 2003). Studies have shown that the intake of inositol in curly tail mice mutants reduces the severity and incidence of NTD's by 53% (Ting et al., 2003) but that this syndrome is not necessarily caused by inositol deficiency. On the other hand, surprisingly, folate intake did not have any influence on the ct/ct mouse mutants, which indicated a folate resistance in this

mutation (Greene et al., 1997). All *Grhl3*$^{-/-}$ mutants display NTD's including Spina Bifida, but *Grhl3*$^{+/-}$ are indistinguishable from the wild type phenotype, and it was shown that Spina Bifida was caused by the primary failure of neural tube closure where the neural fold elevation did not occur (Ting et al., 2003). It has also been found that there is a lower level of Grhl3 mRNA in ct/ct mutants, approximately 30% of the expression of the Grhl3 heterozygotes or wild types (Ting et al., 2003). Interestingly, the severity or incidence in *Grhl3*$^{-/-}$ mutants could not be influenced by folate nor inositol intake, which suggests that Grhl3 could influence inositol actions in neurulation and that ct/ct mutants could be rescued by overexpression of Grhl3 (Ting et al., 2003).

A case study suggests that the rs2486668 (C27G) polymorphism could increase the risk of Spina Bifida in a Chinese population. The GG genotype is associated with a 2.59-fold risk than the CC genotype (Yang et al., 2019). An interesting fact is that a strong association of rs2486668 and NTD's was found only among those who never took folic acid supplements (Yang et al., 2019).

3.2.8. Hox genes

Homeobox genes, also known as Hox genes are transcription factors which play a significant role in the development of the anterior-posterior body axis during embryogenesis and are expressed in the brain and spinal cord (Volcik et al., 2002). It is hypothesized that in cases where folic acid played no role in the evolvement of an NTD, the mother might have a folic acid resistance, which leads to a disturbed methylation cycle and might lead to abnormal embryonic development. Based on the study (Volcik et al., 2002), it was found that patients with MMC and their siblings have HOXB7 hypomethylation. Though, there was no correlation between the MTHFR activity and HOXB7 methylation found, which suggests that the folic acid pathway related to MTHFR was not involved. Furthermore, failure in proper methylation of HOX genes HOXA and HOXB clusters may result in wrong cell identity and lead to neural malformations (Rochtus et al., 2015).

3.2.9. Par1/Par2

Protease-activated receptors are G-protein coupled receptors (GPCR), which allow cells to recognize proteases in their environment. Par1 and Par2 are both, in humans and mice, and can be found on mouse chromosome 13 (Coughlin, 2000). Par1 and Par2 knockout mice were studied, and these are the results: Embryos with *Par1*$^{-/-}$: *Par2*$^{-/-}$ that occasionally survived sometimes showed signs of spina bifida. Though the same results were not seen in single knockout mice, only occasionally was late gestational lethality present in *Par2*$^{-/-}$ knockouts, from which was concluded that these two phenotypes interact in the development of an embryo (Camerer et al., 2010). The expression of Par2 has been observed in the surface ectoderm at the time of fusion though Par1 expression has not been detected in the surface ectoderm, only in the endocardium, endothelium, and a subset of hematopoietic cells. Only the mRNA of Par1 could be detected in the surface ectoderm, which leads to believe that it is expressed below detection levels (Camerer et al., 2010). These findings lead to further investigation of which protease(s) might lead to the activation of Par2. Extracellular serine proteases have shown to cleave PARs thus Camerer et al. focused on integral membrane proteins, which are secreted and have a GPI linkage with an extracellular serine protease domain. The results have shown that matriptase, which is a type-2 transmembrane protein, is a strong candidate for Par2, but not Par1, activation during neural tube closure (Camerer et al., 2010) although no signs of NTD's were found in matriptase knockout mice (List et al., 2002). It has been suggested that Par2 is activated by prostasin through a matriptase-dependent mechanism in the surface ectoderm, where Par2 and matriptase are both expressed (Camerer et al., 2010). The physiological roles of these mechanisms are yet not fully understood and require further investigation.

4. Conclusion

The first step to finding what exactly causes Spina Bifida probably has to be the determination of whether humans have multiple closing sites or just one, because of the proposed theory that NTD's can occur not only from improper closure but also deadhesion after the fusion, mentioned in chapter 2.3. This could lead to new findings since all mutations and theories support the idea of improper closure and not deadhesion. All of the mentioned genetic and nutritional factors, either directly or indirectly interact with each other. The first remarkable candidate in the evolvement of Spina Bifida could be methylation, so the methylation theory in general, since many pathways lead to a dysfunctional methylation cycle. Low folate intake leads to low concentrations of 5-Methyltetrahydrofolate and thus lower the number of methylations, and so does the MTHFR gene mutation. Vitamin B12 intake also influences methylation indirectly since it influences the integration of folate into the cellular pool. Even though folate and Vitamin B12 are two separate risk factors, they might contribute to this same error, because Vitamin B12 is involved in the folate metabolism and thus also the CUBN mutation, which is involved in the reabsorption of Vitamin B12. The CUBN rs1907362 polymorphism might lead to lower levels of Vitamin B12, and that again could lead to a decrease of 5-Methyltetrahydrofolate levels. The SARGH gene is indirectly involved in the methylation of THF where the protective allele G of rs2797840 might lead to an increased folate content in the brain, and the overexpression could lead to a prevention of NTD, but rs2073817, on the other hand, causes a disfunction of sarcosine dehydrogenase and thus to an NTD risk. Choline is also responsible for methylation of homocysteine to methionine just like folate, which means that a defect of it could lead to lower methionine levels. The intake of Choline supplements was associated with a decrease of NTD risk, but the CHKA single nucleotide polymorphism rs939883 is seen as a separate risk factor since Choline intake does not influence NTD's caused by this mutation.

Hox genes though have no linkage to the folate metabolism, and yet the abnormal methylation of Hox A and Hox B clusters may result in wrong cell identity and increase the risk of an NTD, just as in Vangl genes. Grhl-3 mutations showed in mice mutants that it might

influence inositol actions, which might rescue folate resistant mice. This leads to believe that rs2486668 (C27G) polymorphism, which showed an increased risk in Chinese populations might be independent of folate intake, although the most significant association of this polymorphism was in women who never took folate supplementations.

Many case studies had different outcomes, though, from which can be deduced that different genetic mutations have a different impact on various ethnicities.

Overall little is understood when it comes to molecular mechanisms of Spina Bifida or neural tube closure, though the methylation theory might be an excellent start to the discovery of specific mechanisms of Spina Bifida.

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