Gene expression levels of DNA methyltransferase enzymes in *Shank3*-deficient mouse model of autism during early development

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Objectives. The balance between DNA methylation and demethylation is crucial for the brain development. Therefore, alterations in the expression of enzymes controlling DNA methylation patterns may contribute to the etiology of neurodevelopmental disorders, including autism. SH3 and multiple ankyrin repeat domains 3 (*Shank3*)-deficient mice are commonly used as a well-characterized transgenic model to investigate the molecular mechanisms of autistic symptoms. DNA methyltransferases (DNMTs), which modulate several cellular processes in neurodevelopment, are implicated in the pathophysiology of autism. In this study, we aimed to describe the gene expression changes of major *Dnmts* in the brain of *Shank3*-deficient mice during early development.

Methods and Results. The *Dnmts* gene expression was analyzed by qPCR in 5-day-old homozygous *Shank3*-deficient mice. We found significantly lower *Dnmt1* and *Dnmt3b* gene expression levels in the frontal cortex. However, no such changes were observed in the hippocampus. However, significant increase was observed in the expression of *Dnmt3a* and *Dnmt3b* genes in the hypothalamus of *Shank3*-deficient mice.

Conclusions. The present data indicate that abnormalities in the *Shank3* gene are accompanied by an altered expression of DNA methylation enzymes in the early brain development stages, therefore, specific epigenetic control mechanisms in autism-relevant models should be more extensively investigated.

Key words: epigenetic mechanisms, methylation, autism, *Shank3*

The balance between the DNA methylation and demethylation in the brain is dynamically regulated by specific epigenetic enzymes. Epigenetic processes are involved in the brain development. Therefore, alterations in the expression of enzymes controlling DNA methylation patterns may contribute to the etiology of the neurodevelopmental disorders.

Most significant enzymes catalyzing the transfer of a methyl group to DNA belong to DNA methyltransferases (DNMTs), which modulate several cellular processes in the neurodevelopment and synaptic plasticity (Srancikova et al. 2021). The DNA methylation is intensively studied process and it appears

that belongs to those ones, which could be implicated in the pathophysiology of autism. A recent study has indicated that genetic variations in the enzymes catalyzing the transfer of a methyl group to DNA are associated with phenotype variations in the autism spectrum disorder, characteristic with a developmental disability that can cause significant social, communication, and behavioral challenges (Alex et al. 2019). Moreover, increased methylation at the gene promoter of the methyl CpG binding protein 2 has been observed in the brains of the autistic patients (Nagarajan et al. 2008). A genome-wide analysis has revealed differential DNA methylation regions in the

sperm of the autistic children fathers, which might be another component leading to the understanding of the origin of the pathophysiology of autism spectrum disorder (Garrido et al. 2021).

Autism is associated with *de novo* or inherited mutations of genes involved in the control of synaptic function. In this context, genetic disorders that are associated with an increased risk of autism are considered as “synaptopathies”. Despite a wide phenotypic heterogeneity of the autism spectrum disorder, the knowledge on the consequences of alterations in synaptic genes in relation to abnormal social behavior and autism pathophysiology is rapidly improving. For instance, it is known that mutations in SH3 and multiple ankyrin repeat domains 3 (*SHANK3*) gene, which encodes a scaffolding protein of the postsynaptic density of excitatory synapses, are associated with autism etiology (Zhu et al. 2018). Deletions or mutations in the *SHANK3* gene cause Phelan-McDermid syndrome, a neurodevelopmental disorder accompanied by an autistic behavior. In a recent study, specific DNA methylation signature associated with large 22q13.3 deletions have been demonstrated in samples from patients suffering from Phelan-McDermid syndrome (Schenkel et al. 2021). In the experimental conditions, the *Shank3*-deficient mice are commonly used as a well-characterized transgenic model to investigate the molecular mechanisms of autistic symptoms (Peca et al. 2011; Reichova et al. 2020). In this study, we aimed to reveal, whether the gene expression of the major *Dnmts* in the brain of *Shank3*-deficient mice is changed during the early development. For this study, the 5th postnatal day of mice, as the most relevant developmental time-point (because of an intensive neuronal cell proliferation and synaptogenesis during the first postnatal week), was selected.

Materials and methods

Animals. Animal experiments were approved by the State Veterinary and Food Administration of the Slovak Republic and performed in accordance with the European Communities Council Directive of 24 November 1986 (86/609/EEC) and relevant legislation. *Shank3*-deficient (B6.129-Shank3tm2Gfng/J) mice, with deletion of the PDZ domain (exons 13 to 16), were purchased from the Jackson Laboratory. Wild-type (WT) and *Shank3*-deficient (hereafter called *Shank3*^{-/-}) mice were housed under a 12h light-dark cycle at constant room temperature (22°C) with free access to water and food. The male pups (WT, n=6; *Shank3*^{-/-}, n=3) were sacrificed on the 5th postnatal day by decapitation. Thereafter, the brains were quickly removed and three brain structures including the prefrontal cortex, the hippocampus, and the hypothalamus were separated, frozen in liquid nitrogen, and stored at -80°C until used.

RNA isolation and quantitative Real Time PCR (qRT-PCR). Total RNA was isolated by using a phenol-chloroform method (TRI-reagent; MRC, Germany) according to the manufacturer's instructions. Total RNA was reverse transcribed with High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific, Slovakia) based on the manufacturer's instructions. qRT-PCR in QuantStudio5 Real-Time PCR System (Life Technologies, USA) was carried out with Power SYBR Green PCR Master Mix (Thermo Fisher Scientific, Slovakia) and gene-specific primers (Table 1). Relative gene expression levels were calculated by using the Livak method, the 2^{-ΔΔCt} value of each sample was calculated with reference control gene, glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*).

Table 1
List of primers used in this study

Name	Primers	Gen Bank Accession
Gapdh	FW: CGGTGCTGAGTATGTCGTGGAGTC	NM_001289726.1
	RV: CTTTGGCTCCACCCTTCAAGTG	
Dnmt1	FW: CCTAGTTCGGTGGCTACGAGGAGAA	NM_001314011.1
	RV: GCCGAATTGTGTCTTGGTGGATGACA	
Dnmt3a	FW: GCCGAATTGTGTCTTGGTGGATGACA	NM_007872.4
	RV: CCTGGTGAATGCACTGCAGAAGGA	
Dnmt3b	FW: GGATGTTTCGAGAATGTTGTGG	NM_001271744.1
	RV: GTGAGCAGCAGACACCTTGA	

Abbreviations: *Gapdh* – glyceraldehyde-3-phosphate dehydrogenase; *Dnmt1*, *Dnmt3a*, and *Dnmt3b* – DNA methyltransferase enzymes.

Statistical analysis. Statistical comparisons were performed by SigmaPlot 10 using the Student's t-test. Results are expressed as the mean $\pm$ SEM. The value of $p < 0.05$ was considered as statistically significant.

Results and discussion

The present study demonstrates differential gene expression levels of *Dnmts* (*Dnmt1*, *Dnmt3a*, and *Dnmt3b*) enzymes in three brain regions (frontal cortex, hypothalamus, and hippocampus) in *Shank3*^{-/-} mice compared to WT mice on the 5th post-

natal day. We found significantly lower *Dnmt1* and *Dnmt3b* mRNA levels in the frontal cortex (Figure 1) in *Shank3*-deficient mice. However, no such changes were observed in the hippocampus (Figure 1).

It is known that DNA methylation determines several steps of neurogenesis, neuronal stem cell proliferation, and differentiation (Jobe and Zhao 2017). Therefore, the role of changes in both gene and protein DNMTs levels during the cell proliferation and their potential interference with postnatal brain development could be suggested in *Shank3*^{-/-} mice. Moreover, it is known that both DNMT1 and DNMT3 enzymes are highly expressed in the mouse frontal cortex (Feng et al. 2010). Conditional *Dnmt1* and *Dnmt3a* gene deficiency within mouse fore-brain resulted in demethylation of neuronal genes with corresponding alterations in synaptic function and behavior (Feng et al. 2010).

The interplay between the consequences of *Shank3* gene deficiency and further epigenetic modifications of synaptic genes comes into the play when explaining autism behavior. For instance, combined pharmacological inhibition of epigenetic enzymes has been shown to reverse social deficits in a different model of adult *Shank3*-deficient mice (Zhang et al. 2021). Although these authors consider compensation of SHANK3 protein expression at synapses unlikely by epigenetic mechanisms, there could be a biological relationship between DNA methylation and SHANK3 gene abnormalities, as it has been revealed by a study using human samples from patients with Phelan-McDermid syndrome (Schenkel et al. 2021). These authors have found significant DNA methylation difference in regions located across multiple chromosomes in patients with large 22q13.3 deletions. Even though, the involvement of at least a part of the SHANK3 gene could be suggested, Schenkel et al. (2021) did not find such extensive DNA methylation differences in the individuals with small deletions or SHANK3 intragenic variants.

It is interesting that tissue-specific SHANK3 protein expression can also be regulated by epigenetic mechanisms. It has been found that SHANK3 DNA regions are highly methylated in tissues (peripheral blood lymphocytes), where SHANK3 protein expression is low or even absent (Beri et al. 2007). The *Shank3* gene uses multiple promoters and differential splicing to generate alternative mRNA isoforms, therefore, the *Shank3*^{-/-} mice used in the present study (deletion of the exons 13 to 16) could also be accompanied by altered methylation patterns. Nevertheless, alterations in gene expression of DNA methylation

Figure 1. Gene expression levels of three DNA methyltransferase enzymes (*Dnmt1*, *Dnmt3a*, and *Dnmt3b*) in the three brain areas (frontal cortex, hippocampus and hypothalamus) of *Shank3*^{-/-} mice evaluated at the 5th postnatal day. Bars indicate mean $\pm$ SEM, $n=3-6$ per group. Significant differences are expressed vs. control animals (WT) marked as * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$.

enzymes seem to be brain region-specific as far as we found significantly higher expression of *Dnmt3a* and *Dnmt3b* in the hypothalamus in the *Shank3*^{-/-} mice (Figure 1).

These results raise further questions on the potential hypermethylation of synaptic genes in some brain regions under conditions related to autism. Moreover, we need to keep in the mind the isoform-specific expression of *SHANK3* gene in the autistic subject brains (Zhu *et al.* 2014).

The results of the present study indicate that abnormalities in the *Shank3* gene are accompanied by an altered expression of DNA methylation enzymes during the early brain development stages.

These data, however, are still preliminary due to the low number of samples obtained from homozygous *Shank3*^{-/-} mice. Actually, specific epigenetic control mechanisms in the *Shank3*-deficient mice and other autism-relevant models should prospectively undergo more extensive studies.

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References

- Alex AM, Saradalekshmi KR, Shilen N, Suresh PA, Banerjee M. Genetic association of DNMT variants can play a critical role in defining the methylation patterns in autism. *IUBMB Life* 71, 901–907, 2019.
- Beri S, Tonna N, Menozzi G, Bonaglia MC, Sala C, Giorda R. DNA methylation regulates tissue-specific expression of *Shank3*. *J Neurochem* 101, 1380–1391, 2007.
- Feng J, Zhou Y, Campbell SL, Le T, Li E, Sweatt JD, Silva AJ, Fan G. *Dnmt1* and *Dnmt3a* maintain DNA methylation and regulate synaptic function in adult forebrain neurons. *Nat Neurosci* 13, 423–430, 2010.
- Garrido N, Cruz F, Egea RR, Simon C, Sadler-Riggleman I, Beck D, Nilsson E, Ben Maamar M, Skinner MK. Sperm DNA methylation epimutation biomarker for paternal offspring autism susceptibility. *Clin Epigenetics* 13, 6, 2021.
- Jobe EM, Zhao X. DNA methylation and adult neurogenesis. *Brain Plast* 3, 5–26, 2017.
- Nagarajan RP, Patzel KA, Martin M, Yasui DH, Swanberg SE, Hertz-Picciotto I, Hansen RL, Van de Water J, Pessah IN, Jiang R, Robinson WP, LaSalle JM. MECP2 promoter methylation and X chromosome inactivation in autism. *Autism Res* 1, 169–178, 2008.
- Peca J, Feliciano C, Ting JT, Wang W, Wells MF, Venkatraman TN, Lascola CD, Fu Z, Feng G. *Shank3* mutant mice display autistic-like behaviours and striatal dysfunction. *Nature* 472, 437–442, 2011.
- Reichova A, Bacova Z, Bukatova S, Kokavcova M, Meliskova V, Frimmel K, Ostatnikova D, Bakos J. Abnormal neuronal morphology and altered synaptic proteins are restored by oxytocin in autism-related *SHANK3* deficient model. *Mol Cell Endocrinol* 518, 110924, 2020.
- Schenkel LC, Aref-Eshghi E, Rooney K, Kerkhof J, Levy MA, McConkey H, Rogers RC, Phelan K, Sarasua SM, Jain L, Pauly R, Boccuto L, DuPont B, Cappuccio G, Brunetti-Pierri N, Schwartz CE, Sadikovic B. DNA methylation epi-signature is associated with two molecularly and phenotypically distinct clinical subtypes of Phelan-McDermid syndrome. *Clin Epigenetics* 13, 2, 2021.
- Srancikova A, Bacova Z, Bakos J. The epigenetic regulation of synaptic genes contributes to the etiology of autism. *Rev Neurosci* 32, 791–802, 2021.
- Zhang F, Rein B, Zhong P, Shwani T, Conrow-Graham M, Wang ZJ, Yan Z. Synergistic inhibition of histone modifiers produces therapeutic effects in adult *Shank3*-deficient mice. *Transl Psychiatry* 11, 99, 2021.
- Zhu L, Wang X, Li XL, Towers A, Cao X, Wang P, Bowman R, Yang H, Goldstein J, Li YJ, Jiang YH. Epigenetic dysregulation of *SHANK3* in brain tissues from individuals with autism spectrum disorders. *Hum Mol Genet* 23, 1563–1578, 2014.
- Zhu W, Li J, Chen S, Zhang J, Vetrini F, Braxton A, Eng CM, Yang Y, Xia F, Keller KL, Okinaka-Hu L, Lee C, Holder JL Jr, Bi W. Two de novo novel mutations in one *SHANK3* allele in a patient with autism and moderate intellectual disability. *Am J Med Genet A* 176, 973–979, 2018.