Epigenetic consequences of *in utero* exposure to rosuvastatin include altered histone methylation patterns in newborn rat brains

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II) Legradi A, **Dulka K**, Jancsó G, Gulya K (2020) Orofacial skin inflammation increases the number of macrophages in the maxillary subregion of the rat trigeminal ganglion in a corticosteroid-reversible manner. **Cell Tissue Res**. 382(3):551-561. doi: 10.1007/s00441-020-03244-3. (**IF: 5.249**) (Q1)

III) Lajkó N, Kata D, Szabó M, Mátyás A, **Dulka K**, Földesi I, Fülöp F, Gulya K, Vécsei L, Mihály A (2020) Sensitivity of rodent microglia to kynurenines in models of epilepsy and inflammation *in vivo* and *in vitro*: Microglia activation is inhibited by kynurenic acid and the synthetic analogue SZR104. **Int J Mol Sci**. 21(23):9333. doi: 10.3390/ijms21239333. (**IF: 5.923**) (Q1)

IV) Szabo M, **Dulka K**, Gulya K (2016) Calmodulin inhibition regulates morphological and functional changes related to the actin cytoskeleton in pure microglial cells. **Brain Res Bull**. 120:41-57. doi: 10.1016/j.brainresbull.2015.11.003. (**IF: 3.37**) (Q2)

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III) **Dulka K**, Nacsa K, Lajkó N, Gulya K (2018) Nagy tisztaságú mikroglia kultúra készítése primer és szekunder tenyészetekből. **Magyar Élettani Társaság Vándorgyűlése**, Szeged, Hungary, 2018.06.27-30. *(P1.56)*

IV) **Dulka K**, Szabo M, Gulya K (2015) Calmodulin inhibition affects proliferation and cell viability in unchallenged and LPS-challenged pure microglial cultures. **XII European Meeting on Glial Cell Function in Health and Disease**, Bilbao, Spain, 2015.07.15-18. *(TO2-04B)* **GLIA**: 63 (1) pp. E95-E95

OTHER PUBLICATION NOT RELATED TO THE THESIS

I) Kata D, Nacsa K, Légrádi Á, **Dulka K**, Gulya K (2015) Állati sejtek és szövetek tenyésztése. Egyetemi jegyzet. SZTE, Szeged, pp. 1-233. https://elearning.szte.hu/mod/szte/course.php?id=84 (in Hungarian)

1. INTRODUCTION

In the nucleus, DNA is packed in a chromatin structure which determines its accessibility for functions such as transcription, replication, and DNA repair. Chromatin composed of a specialized set of proteins, the so-called histones (H), that organize DNA into the nucleosome. The nucleosome is the fundamental unit of chromatin and it is composed of four core histones (H2A, H2B, H3, and H4), around which 146 base pairs of DNA is coiled. Chromatin architecture, nucleosomal positioning, and ultimately access to DNA for gene expression, is strongly controlled by histone proteins.

In addition to playing a vital role in chromatin structure and dynamics, histones undergo posttranslational modifications (PTMs), which provide mechanisms for mediating diverse cellular processes. The histones contain many amino acids that can be modified posttranslationally. Histone modification is one of the main mechanisms of epigenetic modifications that regulate gene expression. Epigenetics, in a broad sense, is a hereditary phenomenon that changes gene expression and thereby alters the cellular phenotype without changing the DNA sequence. Besides the above-mentioned enzymatic modifications of histones, epigenetic alterations that affect gene expression include DNA methylation and the synthesis of 19-30 nucleotide-long non-coding RNAs. The N-terminal tails of histones are subject to a number of highly site- and residue-specific posttranslational modifications, including methylation, acetylation, phosphorylation, ubiquitylation, and SUMOylation, which are implicated in influencing gene expression and genome function. This epigenetic modification requires several different histone modifying enzymes, including “writers” that attach modifications to histone tails, “erasers” that remove modifications and “readers” that recognize these modifications.

The location and the degree of methylation of the Lys residue on a histone tail are associated with differential gene expression status. Lys methylation marks, such as H3K4 and H3K36, are implicated in the activation of transcription and linked to open chromatin, whereas H3K9, H3K27, and H4K20 Lys methylation sites are associated with transcriptional repression and they are characteristic of condensed chromatin. The complexity of the methylation patterns of histone proteins and the methylation state at any given Lys residue (unmethylated, mono- (me1), di- (me2), or trimethylated (me3)) also influence gene expression.

Statins (3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors (HMGCRIs)) are a class of lipid-lowering agents used in the treatment of high blood

cholesterol levels. Of all the commercially available statins, RST is one of the highest-selling prescription drugs on the market and it has the greatest inhibitory effect on cholesterol biosynthesis. Statins act by blocking the cholesterol synthesis through inhibition of the enzyme necessary for the production of L-mevalonate. The inhibition of the first and rate-limiting step of cholesterol synthesis also reduces the pools of intermediate metabolites in the pathway that regulate a wide range of cellular functions including hormonal communication, protein synthesis, cell membrane maintenance and lipid modifications. The main effects of statins are related to lipid metabolism, however, apart from the inhibition of cholesterol synthesis, reduction of circulating levels of low-density lipoproteins and triglycerides, and stimulation of the expression of high-density lipoproteins, they also strongly modulate inflammatory cells surrounding atherosclerotic plaques. Statins improve vascular endothelial function, they have antitumor properties and induce apoptosis in various cancer cell lines.

Although it has additional beneficial effects, it also has some unwanted side effects. For instance, it has potentially harmful but not well-documented effects on embryos, and RST is contraindicated during pregnancy. During pregnancy, the use of statins requires caution because of their interference with cholesterol biosynthesis. Proper HMG-CoA reductase activity and cholesterol levels are essential for cell proliferation and early embryonal development. The inhibition of this enzyme by statins may disrupt membrane synthesis, cellular proliferation and growth, metabolism and protein glycosylation, which are crucial for the normal development of the placenta and the embryo. As a result of their potentially harmful, but not well documented effects on the embryo, statin treatments for women should be discontinued 3 months before attempting to become pregnant, and they should not be used during pregnancy or breastfeeding. Since about half of all pregnancies are unplanned, there is a possibility that a pregnant woman may be taking RST at least for a while.

Mammalian embryonic/fetal development involves precise molecular interactions between intrinsic factors such as genetics and epigenetics, and extrinsic maternal factors such as environmental perturbations. As recent studies have emphasized the importance of maternal effects on chromatin structure and the interrelationship between the genome, epigenome, and environment on one hand, and because we have very limited data on any epigenetic study involving RST on the other, the aim of this study was to investigate

whether RST elicited molecular epigenetic events such as histone methylation in the brains of the newborn rats whose mothers had been treated over time with the drug.

2. SPECIFIC AIMS

Epigenetic regulation allows the alteration of the phenotype or transcriptional state of a cell or an organism without affecting its genome, and this leads to adaptation to various developmental and/or environmental cues. Histone modification is one of the main mechanisms for epigenetic modifications. Examining histone modifications at a particular genomic region, or across the genome, can reveal the structure of chromatin, thereby permitting DNA accessibility and gene activation states, or even locations of gene regulatory elements. During the embryonal development histone methylation landscapes of brain cells are sensitive to a wide range of environmental perturbations.

Currently, the use of statins is contraindicated during pregnancy. The main clinical concern is the teratogenic effect of statins on the fetus, though there is no evidence to suggest that the use of statins in pregnancy increases the risk of fetal abnormalities. As regards epigenetics, we have limited data on epigenetic studies involving RST. In an attempt to shed more light on the epigenetic effects of RST, we set out to characterize, partly by applying quantitative techniques, and determining the distributions and levels of selected histone PTMs in newborn rat brains. Our specific aims were:

1) To determine whether RST induces molecular epigenetic events in the brains of newborn rats whose mothers were pretreated with the drug during their pregnancy;
2) To determine whether *in utero* RST treatment could be related to cytoarchitectonic alteration and/or influence cell proliferation in newborn brains;
3) To quantitatively analyze the changes in methylation patterns as a consequence of *in utero* RST exposure, and identify the cell types that display these epigenetic changes in newborn brains.

3. MATERIALS AND METHODS

Pregnant Sprague–Dawley rats were divided into three groups. Besides the absolute controls (no supplements at all), the vehicle-treated control animals received a small amount (650 mg) of liver pâté in pellet form once a day, whereas treated rats were given daily oral doses of RST (0.25 mg/kg body weight) mixed into pellets of liver pâté. Both

groups received this liver pâté supplement (with or without RST) from the 11th day of pregnancy for 10 days (or until delivery). On postnatal day 1, the cerebral hemispheres of absolute control, vehicle-treated control, and RST-treated rats were removed and either homogenized for western blot analysis or embedded in paraffin for hematoxylin and eosin (H&E) staining and fluorescent immunohistochemistry/confocal microscopy.

Eleven antibodies specific for Lys methylation sites and states of H2A, H2B, H3, and H4 histone proteins were selected for western blot analyses and fluorescent immunohistochemistry. Antibodies for cell-specific markers were used to detect neurons, astrocytes, oligodendrocytes and microglial cells, as well as to check for possible changes in their ratios. The anti-Ki67 antibody was used to visualize proliferating cells.

Tissue sections (6 μm thick) were deparaffinized, rehydrated and used for hematoxylin/eosin staining and fluorescent immunohistochemistry/confocal microscopy.

Brains from newborn rats were dissected, homogenized and western blot analysis was performed. In these immunoblotting experiments the grayscale digital images were processed at identical settings to allow comparisons of western blots obtained from different samples. The bands were analyzed by densitometry via the computer program ImageJ. All statistical comparisons were made using SigmaPlot software.

4. RESULTS

4.1. Histone methylation patterns of absolute and vehicle-treated controls do not differ significantly in the newborn brain

When investigating epigenetic events in the newborn brain after the *in utero* administration of RST, we assumed that liver pâté, the vehicle used to deliver RST, did not elicit changes in histone methylation patterns. To ascertain such possible effects of the liver pâté, we assayed histone methylation levels using western blots from absolute control and vehicle-treated control newborn rat brain samples. In this study, we focused on methylations of the Lys (K) residues of the four core histones (H2AK118me1, H2BK5me1, H3, H3K4me1, H3K4me3, H3K9me3, H3K27me3, H3K36me2, H4, H4K20me2, and H4K20me3). Our data ruled out potential effects of this diet supplement on the methylation patterns of these sites, as they did not significantly change their levels between the absolute and vehicle-treated control newborn rat brains. As all vehicle-based controls had values between 93.2% ± 7.7% and 106.4% ± 10.7% of the absolute controls,

with no significant differences among them from at least five separate experiments, we refer to vehicle-treated controls henceforth as merely "controls", and further data presentation uses vehicle-based controls as a reference point.

4.2. *In utero* RST exposure does not affect cell composition in the newborn brain

RST exposure *in utero* did not bring about structural abnormalities in the newborn brain at the level of light microscopy, as evidenced by H&E staining. Double immunofluorescent staining for microglial and neuronal cell markers revealed that the ratios of these cells did not change between the control and RST-treated group. A quantitative western blot analysis of cell-specific markers showed that prenatal exposure to RST did not cause abnormalities in cell composition in the newborn brains, as the ratios of these cells did not change significantly between control and treated groups. These observations were supported by Ki67 fluorescent immunohistochemistry. Analyses of immunohistochemical data found roughly the same incidence of Ki67-immunopositive cells among the DAPI-labeled cell nuclei for both control and prenatally RST-treated rats.

4.3. *In utero* RST exposure alters histone methylation patterns in the newborn brain

We found that prenatal RST treatment induced a general, nonsignificant increase in H2AK118me1, H2BK5me1, H3, H3K9me3, H3K27me3, H3K36me2, H4, H4K20me2, and H4K20me3 levels, to 101.0%–111.7% of the control levels. However, the levels of histone H3 mono- and tri-methylation at Lys 4 (H3K4me1 and H3K4me3) were significantly elevated (134.3% ± 1.6% and 127.8% ± 8.5%, respectively) when compared to the control values. These modifications are known to play roles in transcription activation.

4.4. The increase in H3K4me1 and H3K4me3 is localized mainly to neuronal cell nuclei

Cell-specific markers were used to localize the increased H3K4me1- and -me3-immunopositivities within the newborn brain. Most of the fluorescent immunoreactivity for these methylation marks was detected in NeuN-immunopositive neuronal cell nuclei, which constitute the vast majority of the tissue parenchyma at this time of postembryonic development. Interestingly, besides neurons, few Iba1-positive microglia and GFAP-

positive astrocytes exhibited increased H3K4me1and H3K4me3 immunopositive signals, although the numbers of these cells in the neonatal brain were negligible.

5. DISCUSSION

Based on the sporadic human data available, and because of the potential teratogenic *in utero* effects of statins observed in initial animal experiments the use of statins in human pregnancy is currently not recommended primarily as a precaution. Animal models have provided some evidence for the teratogenic effects of statins on pregnancy outcomes, including decreased fetal body weight, survival rate, and unusual neonatal development. Exposure to lipophilic statins (such as simvastatin, lovastatin, atorvastatin, cerivastatin, and fluvastatin) is hypothesized to be of greater risk to the fetus than hydrophilic statins, because of their greater ability to reach the fetus in larger concentrations as a result of placental transport. For example, *in utero* exposure to simvastatin reduced offspring viability and permanently disrupted reproductive tract development in males. As RST is relatively hydrophilic, its transplacental passage is probably reduced.

About half of all pregnancies are unplanned, with concomitant chances of exposure to statins. The risk to the fetus needs to be weighed against the risk to the mother. The effect of prolonged exposure of the mother to a hypercholesteremic environment throughout pregnancy and lactation on her cardiovascular status is a subject of concern. Occasionally, studies have reported beneficial effects from statin use. Statins proved beneficial in preventing preeclampsia, thus ameliorating the risks of structural abnormalities to the fetus. A reconsideration of the use of statins in high-risk mothers, therefore, seems inevitable.

Mammalian embryonic development involves precise molecular interactions between intrinsic factors such as genetics and epigenetics, and extrinsic maternal factors, such as environmental perturbations, drugs, or even maternal nutrition, as nutritional components could influence the epigenetic landscape in the fetus and hence developmental processes. Numerous environmental factors could elicit long-term consequences for the adult phenotype and they might influence and promote adult-onset diseases. Diet is now recognized as a major environmental factor that may contribute to regulate physiological and pathophysiological aspects of homeostasis, metabolism, and gene expression. It was therefore necessary to exclude any interference of liver pâté, the vehicle for RST, on epigenetic mechanisms.

The nervous system comprises several different cell types that are defined by morphology, function, anatomical location, and specific patterns of gene expression. In the developing mammalian brain, neurons are generated first, followed by the supporting glia. On postnatal day 1, the newborn rat brain contains over 90% of neurons and about 6% non-neuronal cells, among which approximately 15-30 % are microglia. We did not find any signs of RST histotoxicity or changes in the cellular compositions of newborn brains through light microscopy. This supports the opinion that the use of statins in pregnancy poses no risks of developing fetal CNS abnormalities.

It should be added that most of the histone methylation marks we detected in this study were localized to neurons, and to a much smaller extent, to microglia. During embryonal development, the histone methylation landscape of the brain cells is sensitive to a wide range of environmental cues. Alterations in histone methylation have recently been linked not only to a number of neurological and psychiatric disorders but also to proper brain development and function.

H3K4me1 and H3K4me3 marks, commonly located in euchromatin, are broadly associated with transcriptional regulation and the epigenetic tagging of promoters and enhancer sequences, and they are known to allow the DNA to adopt a more “open” conformation and recruit chromatin-modifying factors. The proper regulation of H3K4 methylation is pivotal for healthy brain development, as mutations associated with the loss and gain of H3K4 methylation could potentially result in intellectual disability, autism, microcephaly, seizure disorders, and other neurological diseases in early childhood. Perhaps this epigenetic mark is involved more broadly in the pathophysiology of some neurodevelopmental disorders.

The present study showed that prenatal RST administration from embryonic day 11 led to regulation of the methylation processes at K4 of histone H3 during CNS development. RST selectively affected methylated forms of H3K4, namely it increased the levels of both the mono- and trimethylated forms of H3K4 in the newborn brain. The epigenetic changes elicited by RST are not good or bad unless definitely proven either way. Although there are no such data available yet on humans, these data could contain a message. Prenatal statin therapies, should be used with caution and warrant further investigation until a clearest picture of their precise effects on the epigenetic spectrum emerges.

6. CONCLUSION

In this study, we provided evidence that in newborn rat brains prenatal exposure to RST alters the methylation landscape in general. We found that RST significantly elevated the levels of H3K4me1 and H3K4me3 marks compared to the controls. The elevation of these methylation marks was most dominant in neurons after RST treatment. We did not find any histological structural alteration in newborn brains that could be related to RST treatment. We speculate that changes in histone marks, more precisely the elevated levels of H3K4me1/me3 may be responsible for weakening heterochromatin structure and inducing a "relaxed" state of chromatin in the developing brain cells. This might allow heterochromatinization of new genomic loci, and perhaps lead to or reflect a new program of gene expression. The precise mechanisms underlying this process requires further study. It remains to be determined in future studies whether the changes observed in the H3K4 methylation patterns: (1) are linked to specific loci or are genome-wide, (2) reflect an adaptive or maladaptive response to RST, or (3) represent the outcomes of secondary or tertiary processes in response to RST treatment. The identification of the genomic sequences involved in the control of the embryonic development of offspring whose mothers had been treated with RST remains a formidable challenge. The epigenetic changes elicited by RST are possibilities that should be taken on board. Although there are no such data available yet on humans, our data could provide an adverse message.

7. THE MAIN FINDINGS OF THE STUDY

1) We demonstrated that RST induced molecular epigenetic events in the brains of newborn rats when pregnant mothers were treated daily with oral RST from the 11th day of pregnancy;
2) By analyzing cell-type-specific markers in the newborn brains we demonstrated that prenatal RST administration did not affect the light microscopic cytoarchitecture and cell type ratios of the nervous tissue as compared to the controls;
3) We found that prenatal RST administration induced a general, nonsignificant increase in H2AK118me1, H2BK5me1, H3, H3K9me3, H3K27me3, H3K36me2, H4, H4K20me2, and H4K20me3 levels, as compared to the controls;

4) We found that significant changes were detected in the number of H3K4me1 and H3K4me3 sites (134.3% ± 19.2% and 127.8% ± 8.5% of the controls, respectively), which are generally recognized as transcriptional activators;
5) Using fluorescent/confocal immunohistochemistry for cell-type-specific markers and histone methylation marks on tissue sections, we demonstrated that most of the increase at these sites belonged to neuronal cell nuclei;
6) We concluded that prenatal RST treatment induced epigenetic changes that might affect neuronal differentiation and development, and such possibilities should be taken into account when human RST therapy is recommended.

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