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Cross-talk between miRNAs from the *Dlk1-Dio3* locus and histone methylation to protect male cerebellum from methyl donor deficiency.

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Abbreviations

1C: one-carbon ; DEA: differential expression analysis; DemiRs: differentially expressed microRNAs ; Er α : Estrogen receptor alpha; FDR: false discovery rate; GO ID: gene ontology identification ; KMT: lysine methyltransferase; KDM: lysine demethylase; MDD: methyl donor deficient or methyl donor deficiency; miRNA: microRNA; MTHFR: methylene tetrahydrofolate reductase; QC: quality control; SAM: S-adenosylmethionine.

Keywords: epigenetics, folate, histone, microRNA, rat cerebellum

Abstract

Scope: Disruption of the one carbon metabolism during development, *i.e.* following a gestational vitamin B9 and B12 deficiency, is involved in birth defects and brain development delay. Using a rat nutritional model, consisting of pups born to dams fed a vitamin B9 and B12 deficient diet (MDD), we previously reported molecular and cellular alterations in the brain, in a sex dependent manner, with females being more affected than males. We hypothesized that epigenetic modifications could participate in the sex differences we observed.

Methods and Results: We investigated lysine methylation of histones and expression of microRNAs in the cerebellum of MDD male and female pups.

We report a differential regulation of H3K36Me2 and H4K20Me3 between males and females, in response to MDD. Moreover, distinct regulation of *Kmt5b* and *Kdm2a* expression by miR-134-5p and miR-369-5p from the *Dlk1-Dio3* locus, contributes to the maintenance of expression of genes involved in synaptic plasticity.

Conclusion: These results could explain the neuroprotection to MDD that male pups display. Our work will contribute to the understanding of the consequences of vitamin starvation on brain development, as well as how the epigenome is affected by one carbon metabolism disruption.

Introduction

In addition to DNA methylation, much of the epigenetic information is stored in the covalent modifications along the chromatin. For example, methylation of lysine residues of H3 (K4, K36, and K79) is associated with active transcription, whereas methylation of K9, K27 on H3, or K20 on H4, indicates repressed transcription with inactive chromatin ^[1]. Moreover, these epigenetic modifications can reflect variations in the extracellular environment through changes in gene expression. Modifications in the epigenome are physiologically crucial for brain development and can fluctuate depending on the developmental stage, the considered brain structure, or the sex. Sexual differentiation of the brain is mostly mediated by gonadal steroid hormones, with indirect effects involving epigenetic modifications. Transient production of steroid hormones like estradiol induces chromatin remodeling in the developing brain, resulting in lasting changes in gene expression and establishment of the neuronal circuits^[2]. Analysis of gene expression in whole-brain from adult mice showed that almost 15 % (655 out of 4508 genes, fold change greater than 1) of the measured genes displayed a male- or a female-biased expression ^[3]. In addition, the active mark H3K4me3 is enriched in female mice in specific parts of the brain, affecting gene expression associated with the synaptic function ^[4]. Therefore, epigenetic modifications contribute to sex-biased gene expression in the brain. Similar findings were described in humans. During infancy, the development of the prefrontal cortex is accompanied by modifications of expression of 8061 transcripts, with gender accounting for ~8% of these changes ^[5]. These differences, furthermore, have been observed at the molecular scale. They could partially explain why males and females show different prevalence and symptomatology to several major neuropsychiatric and neurological pathologies ^[6,7]. For instance, women are more susceptible to stress-related disorders, whereas men are more prone to early-life social/cognitive behavior-related disorders ^[8]. Similarly, the risk of Alzheimer's disease is higher in women than in men ^[9], and in contrast, males are more likely to develop Parkinson's disease^[10].

The metabolic state of cells is also integrated into the regulation of epigenetic mechanisms and can impact gene expression. The connection between one-carbon (1C) metabolism and neurodegenerative diseases has been well established ^[11]. While studies on transmethylation and 1C metabolism have been conducted so far mostly in the context of DNA methylation, other epigenetic modifications, such as miRNAs or protein histone modifications, are also affected ^[12]. Nevertheless, the 1C metabolism relies on a sufficient supply of vitamin B9

(folates) and B12 (cobalamin) to effectively remethylate homocysteine into methionine, which is the precursor of S-adenosylmethionine (SAM), the universal methyl donor involved in almost all of the methylation reactions in the cell. In the case of either folate or cobalamin deficiencies, neurotoxicity is caused by the tissular accumulation of homocysteine in the brain and/or dysregulations of epigenetic and epigenomic mechanisms, leading to cerebral dysfunctions, as well as development abnormalities^[13]. Furthermore, the female brain seems more sensitive to the methyl donor deficiency (MDD) caused by folate or cobalamin deficiencies. For example, homocysteine levels are higher in the hippocampus of older women^[14]. In rodent models, a gestational folic acid supplementation alters the expression of imprinted genes and transcription factors involved in brain development in a sexually dimorphic manner^[15]. We previously reported molecular, cellular, and structural alterations in the cerebellum in a rat nutritional model, consisting of pups born to dams fed either control or B9 and B12 deficient diet (the MDD diet). Female pups showed a higher sensitivity to the MDD diet than males, especially regarding neurobehavioral disturbances, as well as abnormal gene expression and activation signaling pathways^[16–18]. For instance, neurosteroidogenesis, Wnt signaling pathways, and *Egr1/Erα* pathway were dysregulated in female but not in male rats^[16–18]. However, these sexually dimorphic effects of MDD on brain function remain partly uncharacterized at the molecular level. MDD leads to the dysregulation of SAM synthesis, which is essential for all methylation reactions, particularly those involved in epigenetic modifications. Indeed, a transcriptomic microarray experiment performed on the cerebellum of 21 days old female rats (P21) born to dams fed either a control or an MDD diet^[19] revealed dysregulations of genes involved in histone methylation (Histone Demethylase Activity, GO ID 32452, FDR 7.00E-04), and especially lysine methylation (Histone Lysine Demethylation, GO ID 70076, FDR 0.0019) in MDD animals compared to controls, suggesting that histone modifications could be affected by MDD in female pups. Therefore, we hypothesize that epigenetic modifications could participate in the sex differences that we observed. In this study, we investigated lysine methylation of histone proteins in the cerebellum of male and female pups born to dams fed either an MDD or a control diet.

We report here a different regulation of histone methylation in the male and female cerebellum in response to MDD. Notably, differential regulation of lysine methyltransferase 5b (*Kmt5b*) and lysine demethylase 2a (*Kdm2a*) expression by miRNAs from the locus delineated by the delta like homolog 1 (*Dlk1*) and the type III iodothyronine deionidase (*Dio3*) genes contributes to the maintenance of expression of genes involved in synaptic

plasticity such as *Grid2* (glutamate ionotropic receptor delta type subunit 2) or *Gria3* (glutamate ionotropic receptor AMPA type subunit 3), as well as the protective mechanism observed in males.

Experimental section

Animals

A validated rat model of early methyl donor deficiency was used, as previously described^[19,20]. Experiments were performed on Wistar rats (Janvier labs, Le Genest-Saint-Isle, France) following the National Institute of Health Guide for the Care and Use of Laboratory Animals in an accredited establishment (Institut National de la Santé et de la Recherche Médicale, INSERM U1256). Project approval was obtained from the French Ministry of Research (authorization APAFIS#2436-2015102612288709 v5). Animals (8 weeks old) were maintained on a 12h light-dark cycle and were housed at 22-23°C, with *ad libitum* access to food and water. One month before mating, females were fed either a standard diet (A04 Standard Diet, Safe, Augy, France) or a diet deficient in folate and vitamin B12 and lowered in choline (Special Diet Service, Saint-Gratien, France). These diets were maintained until the weaning of the offspring at day 21 (P21). On postnatal day 2, litters were adjusted to a normal litter size of 8 pups per dam.

Rat pups were euthanized at P21 with an overdose of isoflurane. Cerebellums were rapidly harvested and frozen in liquid nitrogen. Biochemical validation of the vitamin B9 and B12 deficiency was performed as previously described^[19](Table S1). Pups from different litters (N = 5) were analyzed.

RNA extraction

Total RNA was extracted from 20 mg of frozen cerebellum with 1 mL of TRIzolTM Reagent (ThermoFisher Scientific, Waltham, Massachusetts, USA), according to the manufacturer's recommendations. An additional DNase treatment was performed using TURBOTM DNase (ThermoFisher Scientific, Waltham, Massachusetts, USA), followed by an acid-phenol extraction and precipitation with ethanol. The quality of total RNAs was attested by O. D. 260 nm/O. D. 280 nm and O. D. 260 nm/ O. D. 230 nm determination by spectrophotometry using a MultiskanTM GO microplate spectrophotometer (Waltham, Massachusetts, USA) and a Bioanalyzer (Agilent Technologies, Massy, France). The samples with an RNA integrity number > 8 were selected. RNA samples were aliquoted and stored at -80°C for subsequent analyses.

Microarray expression profiling

Microarray experiments were performed at the Proteome and Genome Research Unit at the Luxembourg Institute of Health (Strassen, Luxembourg). The miRNA expression profile between male and female control cerebellum was analyzed using the Affymetrix Gene-Chip miRNA 4.0 Arrays and the FlashTagTM Biotin HSR RNA Labeling kit (Genisphere, USA). Pre-processing of microarray data was performed using Partek® Genomics suite version 6.5 (Partek® GS) using the robust multichip analysis algorithm, which performs background adjustment, quantile normalization, and probe summarization [21,22]. Normalized miR expression was then imported into R environment for filtering, quality control (QC) and differential expression analysis (DEA). Standard methods such as analysis of distribution, boxplot of relative log expression, principal component analysis were applied for QC. Filtering were applied to keep only mature *Rattus norvegicus* miRNAs with an expression level above 4 (log₂ scale) in at least one sample.

Quantitative RT-PCR analysis

One microgram of total RNA was subjected to 2-step RT-qPCR using the PrimeScriptTM RT Master Mix and the TB GreenTM Premix Ex TaqTM following the manufacturer's recommendations (Takara Bio Inc, Kusatsu Shiga, Japan). Primer sequences are listed in Table S2.

For miRNA analysis, 500 ng of total RNA were first incubated with 5 U of poly(A) polymerase (New England BioLabs, Evry, France) for 30 min at 37°C, in the presence of 1 mM ATP. After denaturation at 95°C for 2 min, samples were reverse-transcribed with 200 U of M-MLV (ThermoFisher Scientific, Waltham, Massachusetts, USA) using OTG3 primer (Table S2). Two µL of the reaction were used for qPCR analysis using the TB GreenTM Premix Ex TaqTM. A forward primer corresponding to the sequence of the mature miRNA was used in combination with OTG4 (Table S2).

The expression of the genes or the miRNAs of interest was determined using the $2^{-\Delta\Delta Ct}$ method by using the geometric mean of two reference genes. *Gapdh* and *Map1b* were used for mRNA quantification, whereas *rno-miR-652-3p* and *rno-miR-195-5p* were used for miRNA quantification. Following the miRnome results, we selected these two miRNAs as internal controls since their expression was not altered by MDD or sex. For mRNA quantification, reference genes were tested and selected based on their stable expression between sex and whatever the diet.

Cell culture

C6 rat glioma cells were maintained in DMEM (Invitrogen, Waltham, Massachusetts, USA) supplemented with 10 % fetal bovine serum FBS (Invitrogen, Waltham, Massachusetts, USA) and 0.1 % penicillin/streptomycin. Cells were grown in a humid atmosphere under 5 % CO₂.

Cells were seeded in 6-well plates to reach 40-50 % confluency on the day of transfection. Cells were then transfected with 10 nM of miR-134-5p and miR-369-5p mimics (miScript miRNA mimics, Qiagen, Hilden, Germany) using Lipofectamine RNAiMax (Invitrogen, Waltham, Massachusetts, USA). After 72 hours, cells were harvested in PBS 1X. The AllStars Negative Control siRNA (Qiagen, Hilden, Germany) was used to transfect cells as a negative control.

Histone methylation analysis

Histone proteins were extracted from 20 mg of frozen cerebellum or transfected cells. Samples were resuspended in PBS 1X Triton X-100 0.5 % supplemented with a cocktail of protease inhibitors (Invitrogen, Waltham, Massachusetts, USA). After 20 strokes in a Dounce homogenizer followed by centrifugation at 1,000 *xg* at 4°C for 5 min, pellets were resuspended in 3 volumes of extraction buffer (0.5 N HCl, 10 % glycerol) and incubated for 30 min on ice. After centrifugation of 5 min at 12,000 *xg*, 8 volumes of acetone were added to the supernatant, followed by overnight incubation at -20°C. After final centrifugation of 5 min at 12,000 *xg*, histone proteins were resuspended in 20 µL water.

Five µg of proteins were separated on a 15 % SDS-PAGE and transferred onto a 0.2 µm nitrocellulose membrane using the semi-dry transfer (Biorad, Hercules, CA, USA). Membranes were blocked in TBST-milk 5% and incubated overnight with the primary antibody: anti-H3 (ab10799, ChIP grade, Abcam, Cambridge, United Kingdom), anti-H3K4me2 (Ab7766, ChIP grade, Abcam), anti-H3K36me2 (ab9049, ChIP grade, Abcam), anti-H3K36me3 (ab9050, ChIP grade, Abcam), anti-H3K9me3 (ab176916, Abcam), anti-H4 (ab10158, ChIP grade, Abcam), anti-H4K20me3 (ab177190, ChIP grade, Abcam). The corresponding secondary antibody coupled to HRP (Jackson ImmunoResearch, West Grove, PA, USA) was added for 1 hour at room temperature. Chemiluminescent signals were

acquired using the FusionFx7 (Vilber Lourmat, Marne-la-Vallée, France) and quantified using ImageJ v1.53 (National Institute of Health).

ChIP analysis

Fifteen mg of frozen cerebellum were cut into pieces and resuspended in 1 mL cold PBS 1x with 1.5 % formaldehyde. Samples were cross-linked 2 min at room temperature before stopping the reaction by adding glycine to a final concentration of 0.125 M. After centrifugation for 5 min at 500 *xg* 4°C; tissues were washed in cold PBS 1X and resuspended in 1 mL of FA buffer lysis buffer (50 mM HEPES-KOH pH 7.5, 140 mM NaCl, 1 mM EDTA pH8, 1 % Triton X-100, 0.1 % sodium deoxycholate, 0.1 % SDS, protease inhibitors). After 10 strokes in a Dounce homogenizer, lysates were sonicated using an M220 Focused-ultrasonicator (Covaris LLC, Woburn, MA, USA). One μ l of antibody (H3K36me2 or H4K20me3, ChIP grade, Abcam, Cambridge, United Kingdom) was added and the samples were incubated for 1 hour at 4°C. After adding 25 μ L DynabeadsTM protein G (Invitrogen, Waltham, Massachusetts, USA), samples were further incubated overnight at 4°C. Beads were washed in low salt buffer (0.1 % SDS, 1 % Triton X-100, 2 mM EDTA, 20 mM Tris-HCl pH 8, 150 mM NaCl), high salt buffer (0.1 % SDS, 1 % Triton X-100, 2 mM EDTA, 20 mM Tris-HCl pH 8, 500 mM NaCl) and LiCl buffer (0.25 M LiCl, 1 % NP-40, 1 % sodium deoxycholate, 1 mM EDTA, 10 mM Tris-HCl pH 8). After elution (1 % SDS, 100 mM NaHCO₃) and reversal of DNA cross-links, DNA was purified using the PCR purification kit (Qiagen, Hilden, Germany). The enrichment of the DNA template was analyzed by qPCR. Primer sequences are given in Table S2. The relative enrichment was calculated with the fold enrichment method using the no-antibody signal.

Statistical analysis

Statistical analyses were performed with GraphPad Prism 9.3 software (GraphPad Software, Inc, La Jolla, CA, USA). All data are expressed as means \pm SEM. Two-group comparisons (controls vs. MDD or males vs. females from the same diet group) were performed using the Mann-Whitney test for non-parametric data or an unpaired *t*-test for parametric data. *p*-values \leq 0.05 were considered significant. In miRNA experiments, to identify DEA, two-ways ANOVA followed by Tukey Honest Significant Difference test were performed. miRs with Tukey's adjusted *p*-value $<$ 0.05 were considered as differentially expressed.

Results

Histone methylation in the cerebellum is affected by MDD.

To investigate whether histone methylation could be involved in the alterations of gene expression previously observed in the cerebellum, we tested the expression level of several methylation marks of histone proteins in control and MDD pup cerebellum by immunoblot. We observed a decreased level of H3K4me2 in MDD pups compared to controls, being more pronounced in the males than the females (50% reduction vs. 25%; Figure 1). The methylation of H3 on K36 (H3K36me2 and H3K36me3) was increased in MDD males but H3K36me3 levels were decreased in MDD females (Figure 1). In contrast, methylation of the repressive marks H4K20 or H3K9 remained unaffected in female pups born to dams fed the MDD diet (Figure 2, right panel). In males, methylation of H3K9 (H3K9me3) was increased following MDD, whereas we observed a decreased level of H4K20me3 (Figure 2 left panel).

These dysregulations of histone marks, which were much more notable in males, could be due to an altered expression of the corresponding writers and erasers of histone methylation. Therefore, we next measured by RT-qPCR the gene expression levels of several methylases and demethylases acting on the marks previously investigated (Figure 3A, supporting information Figure S1). Expression was relatively stable in females following MDD, except for an increase of *Kmt3b* (Figure 3B) and *Kdm2b* (Figure 3C), both enzymes regulating mono- and dimethylation of H3K36 (Figure 3A). However, the level of H3K36me2 remains unaffected by MDD in females (Figure 1). In contrast, in males, the expression of most of the enzymes we tested was dysregulated following MDD, with a decrease in expression. Only *Kmt1d* and *Kmt3c* (also called *Smyd2*) expression was increased in males born to dams fed the MDD diet (Figure 3B).

There was no clear correlation between the decrease in the activating mark H3K4me2 in MDD males and the expression levels of the corresponding writers and erasers, as *Kmt2a* and *Kmt2c* or *Kdm5a* and *Kdm2b* were all less expressed (Figure 3B-C). However, the increase in H3K9 trimethylation could be explained by the decrease in the demethylase *Kdm4c* in MDD males compared to controls (Figure 3C). Similarly, the decrease of *Kmt5b* expression could relate to the decrease in the repressive mark H4K20me3 (Figure 3B). Despite the reduced level of *Kmt2c*, the decrease in dimethylation of H3K36 could be explained by the diminished

expression of both *Kdm2a* and *Kdm4c* (Figure 3C), whereas the decrease in *Kmt3c* correlated with the decrease in trimethylation of the same residue (Figure 3B).

In conclusion, histone marks are more impacted by MDD in males' cerebellum than in females. This could be partly due to the changed expression of some methylases and/or demethylases. Whereas two activating marks were decreased in females, we observed both an increase and a decrease in activating and repressive marks in the male cerebellum following MDD. Notably, the increase of H3K36me2 and the decrease of H4K20me3 could be explained by the altered expression of *Kdm2a* and *Kmt5b*, respectively.

Micro-RNAs of the Dlk1-Dio3 locus are up-regulated in the male cerebellum following MDD

It was previously shown that MDD could affect miRNA expression during brain development [23,24]. However, to the best of our knowledge, a potential sex-biased response to MDD was not investigated. We therefore determined whether miRNAs could contribute to the sex-biased response to MDD in the cerebellum. We first performed miRNA microarray on the males' and females' cerebellum of pups born from dams fed a control diet. We found that 21 miRNAs were differentially expressed (DemiRs) in the cerebellum depending on the sex (Table 1). Among these 21 DemiRs, we selected 8 candidates displaying high expression levels in the cerebellum, and performed RT-qPCR on independent samples in order to validate microarray results, and see how they respond to MDD (Figure 4, Figure S2B). However, we were not able to validate most of the miRNAs we tested by RT-qPCR for differences of expression between control males and females (Figure 4, Figure S2B). Nonetheless, we observed a differential expression for some of them in males depending on the mother's diet. Two of them, miR-134-5p and miR-369-5p, were significantly overexpressed in the cerebellum of males born to dams fed the MDD diet but not in females (Figure 4). These two miRNAs belong to the *Dlk1-Dio3* locus. We tested two other miRNAs from this cluster, miR-410-3p and miR-380-3p, and they also displayed the same response to the MDD diet (Figure 4, Figure S2A). Strikingly, no difference in the expression of these miRNAs was observed in females in response to MDD (Figure 4).

We then used TargetScan to determine the list of putative or known targets of these miRNAs with functions in chromatin remodeling (Table 2; [25]). Several predicted targets were down-regulated in the male cerebellum following MDD (Table 2). Notably, miR-134-5p displays a higher level of expression in males, whereas two of its predicted targets (*Kdm2a* and *Kmt5b*)

are significantly less expressed, with levels of their substrate histone marks also affected, an increase for H3K36me2 and a decrease for H4K20me3 respectively. This negative correlation was also found for miR-369-5p, *Kdm2a*, and H3K36me2.

In order to validate whether the increased expression of miR-134-5p and miR-369-5p could indeed affect the expression of *Kdm2a* and *Kmt5b* and result in changes in the corresponding histone marks in male cerebellum, we overexpressed both miRNAs in a cellular system. C6 rat glioma cells were transfected for 72 hours with miRNA mimics and expression levels of both writer/eraser and histone marks were assessed by RT-qPCR and western blot, respectively. C6 glioma cells do not express miR-134-5p nor miR-369-5p endogenously (data not shown). Overexpressing miR-134-5p resulted in decreased mRNA levels of both *Kdm2a* and *Kmt5b*, whereas only *Kdm2a* expression was affected when overexpressing miR-369-5p (Figure 5A). These results are in agreement with TargetScan prediction.

Using immunoblot, we also evaluated the histone marks H3K36me2 and H4K20me3 following miRNA overexpression. H3K36me2 and H4K20me3 levels were decreased in cells transfected with the miR-134-5p mimics (Figure 5B). Unexpectedly, overexpressing either miR-134-5p or miR-369-5p leads to decreased H4K20 trimethylation compared to cells transfected with negative control (Figure 5B).

In conclusion, miR-369-5p seems to target *Kdm2a* and miR-134-5p *Kmt5b*, resulting in an increase of the histone mark H3K36me2 or a decrease in H4K20me3, respectively.

Genes involved in synaptic plasticity are less associated with the repressive mark H4K20me3 in males' cerebellum.

We previously reported that the expression of genes involved in synaptic plasticity was more dysregulated in female cerebellum than in males following MDD^[19]. We hypothesized that the alteration of histone methylation might contribute to these observations. *Grid2* codes for a subunit of ionotropic glutamate receptor and is selectively expressed in Purkinje cells. *Gria3* codes for a subunit of ionotropic glutamate receptor of the AMPA type. Their expression was reduced in females but not in males' MDD cerebellum (Figures 6A and 7A). To test whether differences were observed in H3K36 or H4K20 methylation in chromatin associated with the *Grid2* or *Gria3* genes, we performed a ChIP experiment using antibodies specifically directed against H3K36me2 and H4K20me3. We observed no significant difference in the level of

H3K36me2 either in the promotor or the coding region of *Grid2* or *Gria3* following MDD (Figures 6 and 7). However, H4K20me3 levels were reduced in both the promotor and the coding region of *Grid2* in the male MDD cerebellum compared to male controls, with no difference in females (Figure 6). Similar results were obtained on *Gria3* (Figure 7). This decrease of the repressive mark H4K20me3 in the male cerebellum may contribute to the maintained expression of *Grid2* and *Gria3*.

Discussion

Methyl donor deficiency caused by vitamin B9 and B12 deficiency is associated with an increased risk for several neurodegenerative diseases and neurological pathologies [26]. We developed a rat nutritional model to investigate the underlying molecular mechanisms. The cerebellum is particularly affected by MDD in rat pups at P21, with decreased levels of $Era\alpha$, accompanied by an impairment of neurosteroidogenesis [17] and alterations in the $Egr1/Era\alpha$ and Wnt signaling pathways [18,19]. Interestingly, we repeatedly observed a sex-biased sensitivity to MDD, with females being more affected than males. We hypothesized that epigenetic modifications could contribute to this sex-biased response to MDD. A previous transcriptomic analysis performed in the lab on the cerebellum of P21 female pups born to dams fed the MDD diet indicated that clusters of genes involved in histone methylation were displaying a decreased expression [19]. To further validate this result, we measured the levels of several histone methylation marks and found that, contrary to what was expected, males displayed more changes than females following MDD. Notably, the greatest differences between the two sexes were observed for the activating mark H3K36me2, as well as the repressive mark H4K20me3. Although less studied than DNA methylation, methylation of histones is known to be affected by 1C metabolism disturbances, in particular, H3K9, H3K27, and H3K4 sites, which were the most investigated [27]. As the impairment of 1C metabolism leads to dysregulation of SAM synthesis, the universal methyl group donor used for transmethylation reactions, a decrease in histone methylation is generally observed under MDD conditions. For instance, we have already reported a decrease in H4K20me3 in the liver of E20 MDD pups [28], which was also observed following the inactivation of the MTHFR enzyme, a major component of the 1C metabolism responsible for the conversion of 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate [29]. However, in some cases, histone methylation could also be increased following MDD [30–32]. We report here for the first time an effect of MDD on histone methylation levels in the cerebellum.

Variations in methylation levels at specific histones could be the consequence of a reduced availability of SAM, but also due to altered expression levels of the modifiers, the lysine methylases and demethylases enzymes. The expression of these histone writers and erasers was also globally more affected by MDD in males than in females. After investigating several histone marks, we found a decreased expression of the methylases and demethylases responsible for their modifications, suggesting potential regulation mechanisms in males'

cerebellum but not in females in response to MDD. In the case of H3K36me2 and H4K20me3, their levels were negatively correlated with those of the respective modifier enzymes, *Kdm2a* and *Kmt5b*.

In addition to histone methylation, we also investigated the effects of MDD on another epigenetic mechanism, the miRNAs. These may serve a still underappreciated role in organizing the sexually dimorphic brain. Indeed, the miRNA environment is dynamically responsive to testosterone^[33] and estrogen^[34]. In the human brain, male-biased miRNAs tend to be enriched for functions like histone modifications and circadian rhythm and are often clustered in the human genome^[35]. We identified several miRNAs belonging to the *Dlk1-Dio3* locus that were specifically showing an increased expression in MDD males but not in females. This locus contains three protein-coding genes (*Dlk1*, Retrotransposon-Like 1 *Rtl1*, and *Dio3*) as well as the largest known miRNA cluster in vertebrates, which spreads over 30-kb and is sub-divided into two clusters: the miR-127/135 and the miR-379/410 clusters^[36,37]. The *Dlk1-Dio3* locus is imprinted, and the miRNAs it contains are maternally expressed in a range of embryonic and extraembryonic cell types, with postnatal expression being found predominantly in the brain^[37-39]. Evidence suggests that miRNAs from the miR-379/410 cluster are processed from a long non-coding polycistronic RNA, called *Mirg* in mice^[37,40]. It was reported that the expression of miR-379/410 was induced by neuronal activity in primary rat neurons^[40]. Additionally, the same study underlined that the transcription of the miR-379/410 cluster, mediated by the transcription factor Mef2, led to the dendritic outgrowth of hippocampal neurons. In our nutritional rat model, we measured an increased expression of 4 miRNAs of the cluster (miR-134-5p, miR-410-3p, miR-369-5p, and miR-380-3p). However, the expression of miR-485, located between miR-134 and miR-369, was not affected by MDD (Figure S2A), suggesting a post-transcriptional effect rather than a transcriptional effect on the putative long primary transcript. In the developing or the adult brain, miRNAs from this cluster display expression differences^[41]. Indeed, clustered organization of miRNA genes does not always imply their co-expression. For example, differential regulation of several miRNAs from this cluster was observed in a mouse model of ischemia in the hindlimb, with expression of some miRNAs remaining unaffected, and others with an early or a late-upregulation following ischemia^[42]. In this case, expression seemed to be regulated by binding of different RNA-binding proteins to aid in the processing of the miRNA precursors. Therefore, upregulation of some miRNAs from this cluster following MDD might be explained by a differential regulation in the maturation process of the miRNA precursors.

Maternal deletion of the miR-379/410 imprinted cluster in induced neurons resulted in an up-regulation of the transcriptional program regulating synaptic transmission and neuronal function through the increase of paternally expressed transcripts *Plagl1* and *Peg3*, as well as additional effects on *NeuroD1* ^[39]. Consistent with the persistent brain expression of miRNAs belonging to this cluster in adults, several studies have pointed out regulatory roles in neuronal functions, especially for miR-134 ^[41]. *In silico* analysis predicted that miRNAs of the miR-379/410 cluster could target several mRNA coding for chromatin modifiers (Table 2). Our results in P21 MDD male cerebellum and rat glioma cells overexpressing either miR-134-5p or miR-369-5p suggest that they target *Kdm2a* and *Kmt5b* transcripts, consistent with the increase in H3K36me2 and the decrease in H4K20me3 respectively. ChIP experiments further support the regulation of *Kmt5b* by miR-134-5p. Upon MDD, miR-134-5p expression is increased in the male cerebellum but not in the female. It, therefore, down-regulates *Kmt5b*, leading to a decrease in the repressive mark H4K20me3, enabling a maintained expression of several genes targeted by the Wnt signaling pathways and involved in synaptic plasticity, such as *Grid2* or *Gria3*. This mechanism could contribute to the protective effect we repeatedly observed in males in this nutritional rat model^[17,18] but also in other rodent genetic models ^[43] or humans ^[14]. Future work will explore the molecular mechanisms leading to the deregulation of miRNAs belonging to the miR-379/410 imprinted cluster upon MDD. Preliminary investigations have revealed that expression of miR-134-5p, miR-369-5p, and miR-485-3p in neuroblastoma rat cells is not affected by dihydroxytestosterone treatment (data not shown). However, estradiol remains an interesting candidate involved in these regulations, and future work will also explore this possibility. Finally, one could imagine that MDD leads to differential methylation of genomic DNA in the region of the miR-379/410 cluster, and for this reason, we will consider this hypothesis as well.

To conclude, we report for the first time a sex differential expression of miRNAs from the *Dkl1-Dio3* locus in the rat cerebellum in response to gestational MDD, which in turn could modulate chromatin modifications, and contribute to a maintained expression of genes involved in the synaptic plasticity. This could partly explain why males pups are less sensitive to MDD than female pups.

Data availability

Data are to be shared upon request to ND.

Supporting information

This article contains supporting information.

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Conflict of interest

The authors declare no conflict of interest.

Author contributions

ND designed the research. JW, PM, NBO, EL, PD, FB, ND conducted the experimental research. TK, LV curated data. JW, PM, PD, NBO, MR, LL, BL, JLG, BLM, ND. contributed to the experimental design and interpretation of data. JW, ND drafted the manuscript. All authors read and approved the final version of the manuscript.

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Tables

Table 1: List of differentially sex-expressed miRNAs in the cerebellum (miRnome)

ID	FeatureSymbol	Sex_FC	Sex_Tukey.pval
MIMAT0000840	rno-miR-134-5p	-1.63	0.02
MIMAT0003174	rno-miR-540-3p	-1.50	0.03
MIMAT0025048	rno-miR-3099	-1.45	0.03
MIMAT0001628	rno-miR-433-3p	-1.43	0.04
MIMAT0000568	rno-miR-330-3p	-1.41	0.05
MIMAT0012839	rno-miR-668	-1.36	0.05
MIMAT0005337	rno-miR-760-3p	-1.33	0.05
MIMAT0000903	rno-miR-320-3p	-1.33	0.01
MIMAT0005329	rno-miR-674-5p	-1.31	0.03
MIMAT0000847	rno-miR-142-5p	-1.22	0.00
MIMAT0000796	rno-miR-26a-5p	1.15	0.03
MIMAT0000570	rno-miR-331-3p	1.46	0.05
MIMAT0025073	rno-miR-6332	1.49	0.04
MIMAT0003206	rno-miR-369-5p	1.58	0.04
MIMAT0024851	rno-miR-1306-5p	1.77	0.00
MIMAT0017882	rno-miR-219b	1.82	0.02
MIMAT0017222	rno-miR-485-3p	1.89	0.03
MIMAT0017095	rno-miR-18a-3p	2.31	0.02
MIMAT0017885	rno-miR-702-3p	2.57	0.00
MIMAT0024844	rno-miR-1839-3p	4.16	0.01
MIMAT0003208	rno-miR-374-5p	4.86	0.02

Bold: miRNA belonging to the *Dlk1-Dio3* locus

Females were compared to males.

Table 2: List of miRNA targets.

miR-134-5p	miR-380-3p	miR-369-3p	miR-410-3p	mir-369-5p
KDM2A	SALL1 [#]	KMT3A	<i>KMT2D</i>	<i>KMT7</i>
KMT3C	ATXN7	<i>NCOA3</i>	KDM2A	<i>KDM1B</i>
<i>KMT2D</i>	<i>SMYD1</i>	<i>ARID4A^{##}</i>	RCOR1	<i>SETD3</i>
KMT5B	<i>ARID1A</i>	KMT2A	ARID4B	KDM2A
<i>HDAC5</i>	MECP2	<i>CDC73</i>	<i>NR3CA</i>	<i>BRD3</i>
	KMD5A	<i>ARID2</i>	<i>KDM7A</i>	
	<i>KDM2B</i>		<i>SETD3</i>	
	KDM3C			

A list of potential mRNA targets of some miRNAs of the *Dlk1-Dio3* cluster was determined using TargetScan^[25]. Genes with a function in chromatin modifications are indicated. The genes that were less expressed in MDD males than controls, and not affected in females, whatever the diet, are indicated in bold (determined by RT-qPCR, N = 5 animals per group, *p*-value < 0.05). Genes with no expression variation, whatever the sex and the diet, are in italics.

reduced expression in MDD females only (N= 5 animals per group, *p-value* < 0.05), ## reduced expression in both MDD males and females, compared to controls(N= 5 animals per group, *p-value* < 0.05). Genes in gray were not evaluated.

Figure legends

Figure 1: Levels of activating histone marks in male and female cerebellum.

(A) H3K4me2, H3K36me2, and H3K36me3 levels were analyzed by western blot and (B) quantified by normalization to total histone H3 using ImageJ (A.U. arbitrary units). Dark blue: control (CTRL) males, light blue: methyl donor deficient (MDD) males, purple: control (CTRL) females, pink: methyl donor deficient (MDD) females.

N= 5 per group, * $p < 0.05$, ** $p < 0.01$.

Figure 2: Levels of inhibiting histone marks in male and female cerebellum.

(A) H3K9me3 and H4K20me3 levels were analyzed by western blot and (B) quantified by normalization to total histone H3 or H4 using ImageJ (A.U. arbitrary units). Dark blue: control (CTRL) males, light blue: methyl donor deficient (MDD) males, purple: control (CTRL) females, pink: methyl donor deficient (MDD) females.

N= 5 per group, * $p < 0.05$.

Figure 3: Expression levels of histone methylases and demethylases in P21 rat cerebellum.

(A) The different amino acids methylated on H3 or H4 tails are indicated, with their respective methylases or demethylases^[44]. The number of dots refers to the lysine's mono-, di- or trimethylation.

(B) mRNA expression levels of histone lysine methylases (*Kmt*) were quantified by RT-qPCR on total RNA extracted from control (dark blue) or methyl donor deficient (MDD, light blue) males or control (purple) or methyl donor deficient (MDD, pink) females.

(C) mRNA expression levels of histone lysine demethylases (*Kdm*) were quantified by RT-qPCR on total RNA extracted from control (dark blue) or methyl donor deficient (MDD, light blue) males or control (purple) or methyl donor deficient (MDD, pink) females.

N = 5 per group, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Figure 4: Several miRNAs of the miR379/410 locus are overexpressed in the male cerebellum following methyl donor deficiency.

The miRNA expression level was quantified by RT-qPCR on total RNA extracted from P21 rat pups fed the control or the deficient (MDD) diet. Dark blue: control males, light blue: MDD males, purple: control females, and pink: MDD females.

N = 5 per group, * $p < 0.05$, ** $p < 0.01$, A.U. arbitrary units.

Figure 5: Overexpressing miR-134-5p or miR-369-5p alters the expression of Kdm2a and Kmt5b.

(A) Expression levels of *Kdm2a* (green) and *Kmt5b* (red) were quantified by RT-qPCR on total RNA extracted from cells overexpressing either miR-134-5p or miR-369-5p and compared to cells overexpressing a scramble sequence.

N = 3, ** $p < 0.01$, *** $p < 0.001$, A.U. arbitrary units.

(B) H3K36me2 (green) and H4K20me3 (red) levels were analyzed by western blot, and signals normalized to total H3 or H4. Quantification results are depicted on the right.

N > 4, * $p < 0.01$, A.U. arbitrary units.

Figure 6: Association with H3K36me2 and H4K20me3 with Grid2 DNA in male and female cerebellum.

(A) *Grid2* expression level was quantified by RT-qPCR in male and female cerebellum.

(B, C) The association between H3K36me2 or H4K20me3 with regions from the promotor or the coding sequence (ORF) of *Grid2* was analyzed by ChIP followed by qPCR. ChIP signals are represented as the fold increase relative to the background (no antibody) signal.

N = 5 per group, ** $p < 0.01$. A.U.: arbitrary units.

(B) dark blue: control (CTRL) males, light blue: methyl donor deficient (MDD) males, (C) purple: control females, pink: MDD females.

Figure 7: Association with H3K36me2 and H4K20me3 with Gria3 DNA in male and female cerebellum.

(B) *Gria3* expression level was quantified by RT-qPCR in male and female cerebellum.

(B, C) The association between H3K36me2 or H4K20me3 with regions from the promotor or the coding sequence (ORF) of *Gria3* was analyzed by ChIP followed by qPCR. ChIP signals are represented as the fold increase relative to the background (no antibody) signal.

N = 5 per group, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. A.U.: arbitrary units.

(B) dark blue: control (CTRL) males, light blue: methyl donor deficient (MDD) males, (C) purple: control females, pink: MDD females.