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Maternal Vitamin B Deficiency and Epigenetic Changes of Genes Involved in the Alzheimer's Disease Pathogenesis

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Abstract

Alzheimer's disease (AD) is characterized by progressive neurodegenerative impairment of the central nervous system and is the most prevalent form of dementia. Considering the influence of maternal nutrition on fetal programming, which consequences usually come later in life, we investigated whether maternal vitamin B deficiency during early development alters the offspring expression of genes related to AD etiopathogenesis. Mice dams were submitted to experimental diet one month before and during pregnancy or pregnancy/lactation and, after birth, their offspring were distributed into three groups: control "CT", deficient pregnancy "DP" and deficient pregnancy and lactation "DPL". At postnatal day (PND) 0, a significant decrease of *App* in females (p=0.007) and *App* and *Bace1* in males (p=0.030 and p=0.040, respectively) was observed when compared to CT group. At PND 28, DPL female presented an increase of *App, Bace1* and *Ps1* gene expression when compared to CT (p=0.003, p=0.003 and p=0.002, respectively) and DP groups (p=0.017, p=0.005 and p=0.002, respectively). In males at PND 28, a decrease of *App* and *Ps1* was observed in both DP (p=0.012; p=0.001) and DPL (p=0.001; p=0.04) when compared to CT group. No differences were observed in females and males at PND 210. Regarding APP, BACE1 and PS1 protein expression and global DNA methylation pattern, no difference was observed throughout development in female or male offspring. Regarding behavioral evaluations, no changes were observed in the object recognition task, but the DPL males presented lower locomotor activity when compared to DP (p=0.028) and CT (p=0.003) groups. In conclusion, the early exposition to vitamin B deficiency alters the expression of genes related to AD.

Keywords: Maternal programming; Homocysteine; Vitamin B12; Folate; Alzheimer's disease

Introduction

Epidemiological and experimental studies have demonstrated that fetal and neonatal environment have a great influence on physiological functions and may increase the risk of developing chronic diseases in adult life [1,2]. The fetal origin of adult diseases hypothesis, also known as fetal programming, suggests a correlation between an adverse uterine environment (nutritional deficit or environmental stimuli) and adaptive responses in the fetus [3].

Studies performed on experimental animals have shown that parental diet may harm fetal health by affecting DNA methylation pattern, which is one of the epigenetic mechanisms of gene expression regulation [4]. The prevalence of folate and cobalamin deficiency during pregnancy is relatively high in some countries of Sub-Saharan Africa, northern Europe and in Brazil [5-8]. Disorders in the maternal-fetal homocysteine (Hcy) metabolism due to folate and/or cobalamin deficiencies are related to a wide array of pathological conditions, such as recurrent miscarriages, placental abruption, preeclampsia, neural tube closure defects and intrauterine growth retardation [9-13]. Additionally, chronic maternal hyperhomocysteinemia may impair brain development and consequently the cognitive functions of the fetuses by the increase of neuronal vulnerability to excitotoxicity, oxidative stress and apoptosis [14,15]. High Hcy levels also constitute a risk factor for the development of cardiovascular diseases, stroke and neurodegenerative disorders, such as Alzheimer's disease (AD) [16-18].

Alzheimer's disease is characterized by β-amyloid (Aβ) peptide deposition in senile plaques and cerebral vessels, intracellular lesions associated with τ (TAU) protein on neurofibrillary tangles, synaptic loss, neuronal death and the consequent brain atrophy [19-22]. β-amyloid peptides originate from proteolysis of amyloid precursor protein (APP), a trans-membrane glycoprotein widely expressed in the brain which products have important physiological roles in the nervous system, such as formation and consolidation of synaptic connections, neuronal plasticity and cellular response to stress [23]. In the nonamyloidogenic pathway, APP is cleaved by γ-secretase (PS1 and PS2) and α-secretase (ADAM10 and TACE); however APP processing by γ-secretase and β-secretase (BACE1 and BACE2) shows features associated with AD pathogenesis [24]. The PS1 and BACE have their genes regulated by methylation and a reduction of folate and cobalamin in the culture medium can cause reduction of methyl donors and the subsequent increase concentrations of the Aβ peptide [25].

Based on these information and considering that maternal hyperhomocysteinemia modifies expression of neural adhesion molecules, delays brain maturation and leads to an impairment of learning and memory performance in the offspring [26], the present study aims to explore the outcome of maternal hyperhomocysteinemia, due to vitamin B complex deficiency, in the programming of genes regarded as risk factors for AD development in offspring.

Materials and Methods

Animal treatment protocol

Animal experiments were conducted according to the Guide for the Care and Use of Laboratory Animals (8th edition, National Academy Press, Washington D. C., 2011), and were approved by the Institutional Animal Care and Use Committee of the Universidade Federal de São Paulo (#1169/08). Animal room was maintained on a 12-hour light/dark cycle, with food and water available ad libitum. Adult female mice at 3 months of age (n=90) were distributed in two groups and received different diets one month prior to mating. A control group (n=46) was fed with a standard diet (AIN-93M, LabDiet®, St. Louis, MO) containing the following levels of vitamins: B2 (6.2 mg/kg), B9 (2.2 mg/kg), B12 (25 μg/kg), and choline (1.250 mg/kg); while the experimental group (n=44) received a deficient diet (LabDiet®, St. Louis, MO) containing: B2 (0.938 mg/kg), B9 (0.290 mg/kg), B12 (2.37 μg/kg), and choline (0.1736 mg/kg) [27]. The respective diets contain 3.75 and 3.78 kcal/g and they were provided during pregnancy and lactation. The deficient diet was previously associated to increase plasma Hcy levels in dams and to a significant methyl-group deficit in the brain fetus, caused by vitamin B and choline deficiency. Additionally, offspring fed by mothers on a deficient diet showed high levels of homocysteine at PND 28 [27].

A male mouse was introduced in female home cages with two or three females for mating, and the onset of pregnancy was established after vaginal plug observation [28]. After birth, the offspring were distributed into the following groups: control "CT" (offspring of control, breastfed by control mothers), deficient pregnancy and lactation "DPL" (offspring of deficient mothers, breastfed by deficient mothers) and deficient pregnancy "DP" (offspring of deficient mothers, breastfed by control mothers). Dams of DP offspring were provided with deficient diet during pregnancy, and after birth (PND 0) their offspring were cross-fostered by control dams during lactation, fed with standard diet. To minimize the fostering effect, offspring of CT and DPL groups were also cross-fostered by CT and DPL dams, respectively. The litter size was adjusted to n=8 animals per dam (n=4, females; n=4, males) in all groups. All offspring were breastfed until PND 28, and after weaning they began to receive standard diet.

Behavior and cognition analysis

The object recognition task was adapted from Dewachter and co-workers [29]. Animals were habituated for one hour to a white empty box (20 × 20 × 40 cm) with a beige floor. Twenty-four hours later, during the first trial, mice were placed individually in the box with the presence of the object A (cube) for 10 min, and the time spent exploring this object (when the animal's snout was directed toward the object at a distance <1 cm) was measured. Three hours later, the second trial was carried out with a novel object (object B: pyramid) placed together with the familiar object (object A) and observed during 10 min. The time (tA and tB) the animal spent exploring the two objects was recorded. The recognition index (RI), defined as the ratio of the time spent exploring the novel object over the time spent exploring both objects [(tB/(tA + tB)) × 100] was used to measure non spatial memory.

Locomotor activity box

The locomotor analysis was performed on Opto-Varimex locomotor activity cages (20 × 30 × 40 cm), surrounded with photoelectrical horizontal detection sensors (Columbus Instruments, Columbus, OH, USA). Mice locomotor activity was assessed by the instrument every 5 min for the entire 30 min duration of the test.

Sample collection

Male and female mice were euthanized by decapitation at different developmental stages (PND 0, 28, 90 or 210), and the whole brain was rapidly collected and stored at -80°C. For the following analysis, total brain or total cerebral cortex were dissected and homogenized in PBS using a tissue homogenator (T10 basic IKA, Staufen, Germany).

DNA, RNA and methylation evaluations

DNA and RNA were extracted from the aliquots of total brain or dissected cerebral cortex by Ilustra triplePrep kit (GE HealthCare, Chicago, IL, USA). Regarding global DNA methylation assay, Imprint™ Methylated DNA Quantification kit (Merck, Kenilworth, NJ, USA) was used.

App, *Ps1* and *Bace1* gene expression

The extracted RNA was measured afterwards in NanoDrop 8000 spectrophotometer equipment (Thermo Scientific, Waltham, MA, USA). For the cDNA synthesis, one μg of total RNA was used in a mix containing ImProm II enzyme, Random Primers, deoxynucleotides (dNTP) and RNAse inhibitor (RNAsin) (Promega, Fitchburg, WI, USA). Real time quantitative polymerase chain reaction (RT-PCR) was carried out by means of Maxima SYBR Green PCR Master Mix (Fermentas, Waltham, MA USA) and primers (*App, Ps1* and *Bace1*) in the Step One Plus equipment (Thermo Scientific, Waltham, MA, USA).

Western blotting

Aliquots of homogenized total brain or total cerebral cortex was used for western blotting analysis of APP, BACE1, PS1 and Aβ (primary antibodies from Cell Signaling Technology, Danvers, MA, USA). About 180 μg of protein sample was loaded for detection of APP and BACE1 in 10% polyacrylamide gels, while PS1 and Aβ levels were evaluated in 15% polyacrylamide gels. Glyceraldehyde 3-phosphate dehydrogenase protein (GAPDH) was used to normalize all data (primary antibody from Sigma-Aldrich, Saint Louis, MO, USA). All information regarding reagents, buffers and electrophoresis conditions were previously described by Viana and co-workers [30].

Statistical analysis

Analysis of offspring at PND 0 was performed using t test for independent groups. For the analysis of offspring at PND 28, PND 90 and PND 210, we performed analysis of variance (ANOVA) followed by post hoc Fisher's test when necessary. Data is presented as mean ± standard error. The level of significance considered was $p \leq 0.05$. The program STATISTICA 10.0 was used to perform the analysis.

Results

Global DNA methylation

The B-complex vitamins (B2, B9, B12 and choline) contribute directly to s-adenosylmethionine (SAM) synthesis and the last one is the primary methyl donor for methylation of DNA. Considering the maternal deficiency of components involved in SAM synthesis, we

evaluated the global DNA methylation pattern in brain (PND 0) and total cerebral cortex (PND 90 and 210) of female and male offspring (Figure 1). Regarding brain global DNA methylation analysis at PND 0, no statistically significant differences in females (t test, p=0.160) and males (t test, p=0.820) were observed. Moreover, at PND 90 and 210 no statistically significant differences were observed in females (ANOVA, p=0.194; p=0.961) and males (ANOVA, p=0.705; p=0.551), respectively.

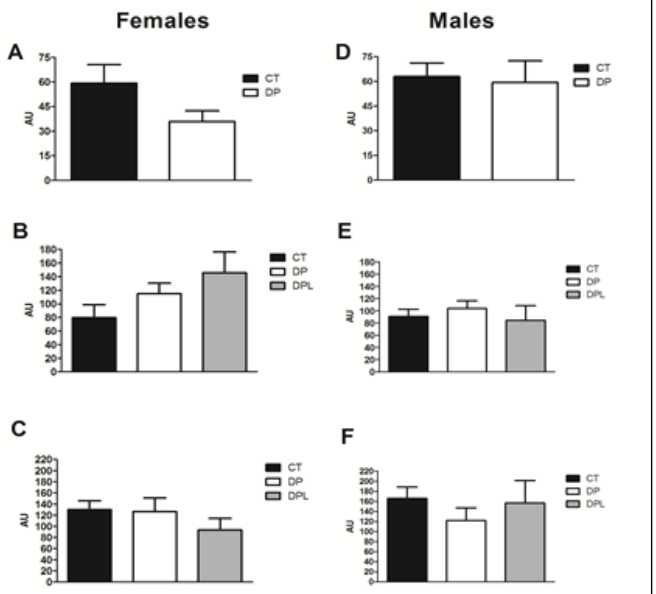

Figure 1: Quantification of global DNA methylation in brain and total cerebral cortex of offspring from dams on vitamin B deficient diet during pregnancy and pregnancy/lactation. n=6-8; CT=control; DP=deficient diet during pregnancy; DPL=deficient during pregnancy and lactation; PND=postnatal day; AU=arbitrary unit. Female, A: PND 0; B: PND 90; C: PND 210. Male, D: PND 0; E: PND 90; F: PND 210. ANOVA. Data are presented as the mean ± standard error.

Gene expression

Figure 2 summarize the results of *App, Bace1* and *Ps1* gene expression during development. In PND 0 females, there was a statistically significant decrease of App gene expression in DP when compared to CT group (t test, p=0.007); no differences were observed in *Bace1* and *Ps1* genes expression (t test, p=0.474) and p=0.640, respectively). In PND 0 males, there was a statistically significant decrease of *App* and *Ps1* genes expression when comparing DP and CT groups (t test, p=0.030 and p=0.040, respectively). There was no statistically significant difference between CT and DP offspring in relation to Bace1 gene expression (t test, p=0.090).

In contrast to PND 0, PND 28 females presented a statistically significant increase of *App, Bace1* and *Ps1* genes expression when comparing the DPL to CT (Fisher, p=0.003, p=0.002 and p=0.002, respectively) and DP groups (Fisher p=0.017, p=0.002 and p=0.002, respectively). In males, there was a statistically significant decrease of *App* and *Ps1* genes expression in both DP (Fisher, p=0.012; p=0.001) and DPL (Fisher, p=0.001; p=0.04) when compared to CT group. Regarding *Bace1* gene expression, there was no significant difference (ANOVA, p=0.319).

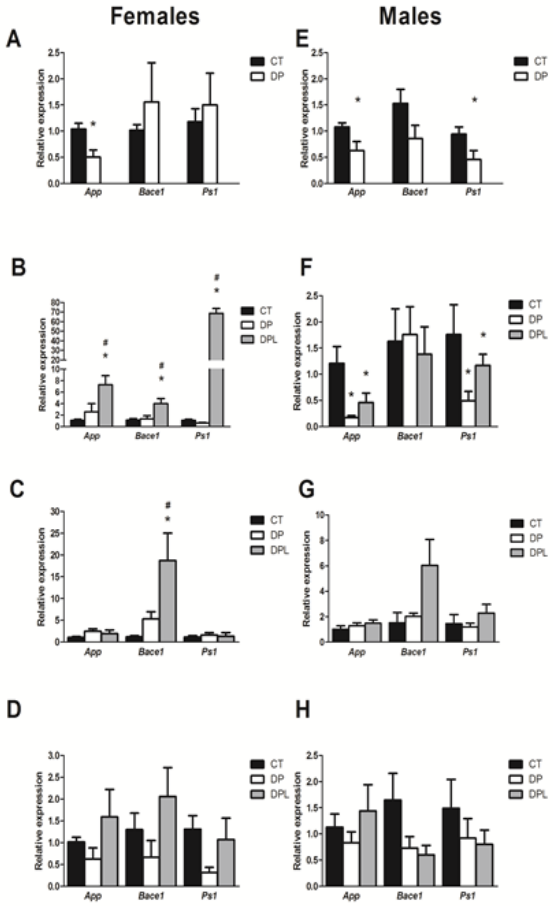

Figure 2: App, Bace1 and Ps1 gene expression in brain and total cerebral cortex of offspring from dams on vitamin B deficient diet during pregnancy and pregnancy/lactation. n=5-8, except to PS1 at PND 28 and 90, n=4-6; CT=control; DP=deficient diet during pregnancy; DPL=deficient during pregnancy and lactation; PND=postnatal day. Female, A: PND 0; B: PND 28; C: PND 90; D: PND 210. Male, E: PND 0; F: PND 28; G: PND 90; H: PND 210. ANOVA. Data are presented as the mean ± standard error. *Different from the CT group (P ≤ 0.05). #Difference between DP and DPL groups (P ≤ 0.05).

In PND 90 females there was a statistically significant increase of *Bace1* gene expression in DPL when compared to CT (Fisher p=0.005) and DP groups (Fisher p=0.019). There were no statistically significant differences in *App* and *Ps1* genes expression (ANOVA, p=0.158 and p=0.783, respectively). Regarding males, there were no statistically significant differences in *App*, *Bace1* and *Ps1* genes expression (ANOVA, p=0.483, p=0.057 and p=0.398, respectively).

At PND 210, there were no significant differences when comparing CT, DP and DPL offspring in relation to *App*, *Bace1* and *Ps1* genes expression in females (ANOVA, p=0.226 and p=0.166 and p=0.138, respectively) or males (ANOVA, p=0.511, p=0.132 and p=0.521, respectively).

Western blotting

Considering the differences in *App*, *Bace1* and *Ps1* genes expression throughout development, we evaluated the corresponding protein in females and males at PND 0, 28, 90 and 210 period (Figures 3 and 4, respectively). There were no differences in females for APP, BACE and PS1 at PND 0 (t test, p=0.474; p=0.431; p=0.639, respectively), PND 28 (ANOVA, p=0.349; p=0.394; p=0,371, respectively), PND 90 (ANOVA, p=0.734; p=0.753; p=0,671, respectively) and PND 210 (ANOVA, p=0.898; p=0.773; p=0,441, respectively) or in males PND 0 (t test, p=0.955; p =0.216; p=0.109, respectively), PND 28 (ANOVA, p=0.643; p=0.453; p=0,587, respectively), PND 90 (ANOVA, p=0.605; p=0.520; p=0,954, respectively) and PND 210 (ANOVA, p=0.829; p=0.245; p=0,388, respectively). We also labeled Aβ peptide in all periods with the corresponding positive control, but no peptide was observed in any group (data not shown).

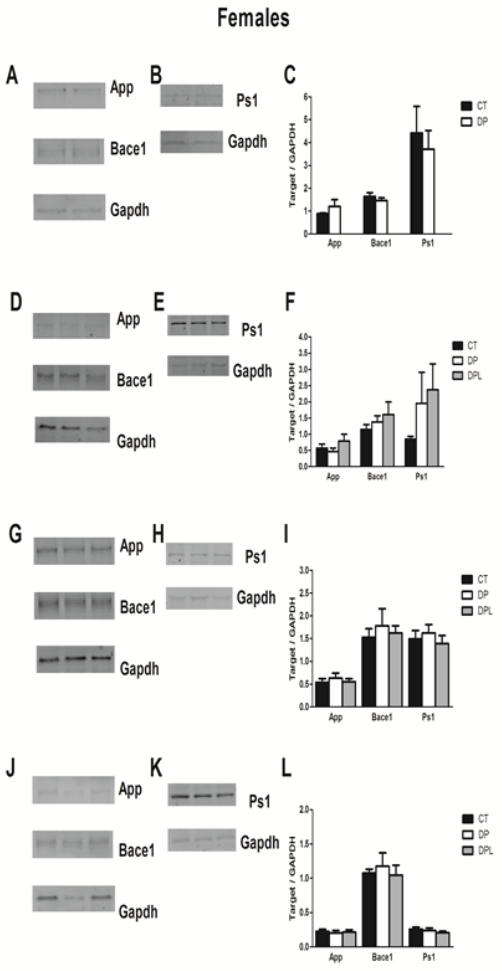

Figure 3: Western blotting analysis in brain and total cerebral cortex of offspring from dams on vitamin B deficient diet during pregnancy and pregnancy/lactation. n=2-3 (A-C), 5 (D-L); CT=control; DP=deficient diet during pregnancy; DPL=deficient during pregnancy and lactation; PND=postnatal day. A-C: PND 0; D-F: PND 28; G-I: PND 90; J-L: PND 210. ANOVA. Data are presented as the mean ± standard error.

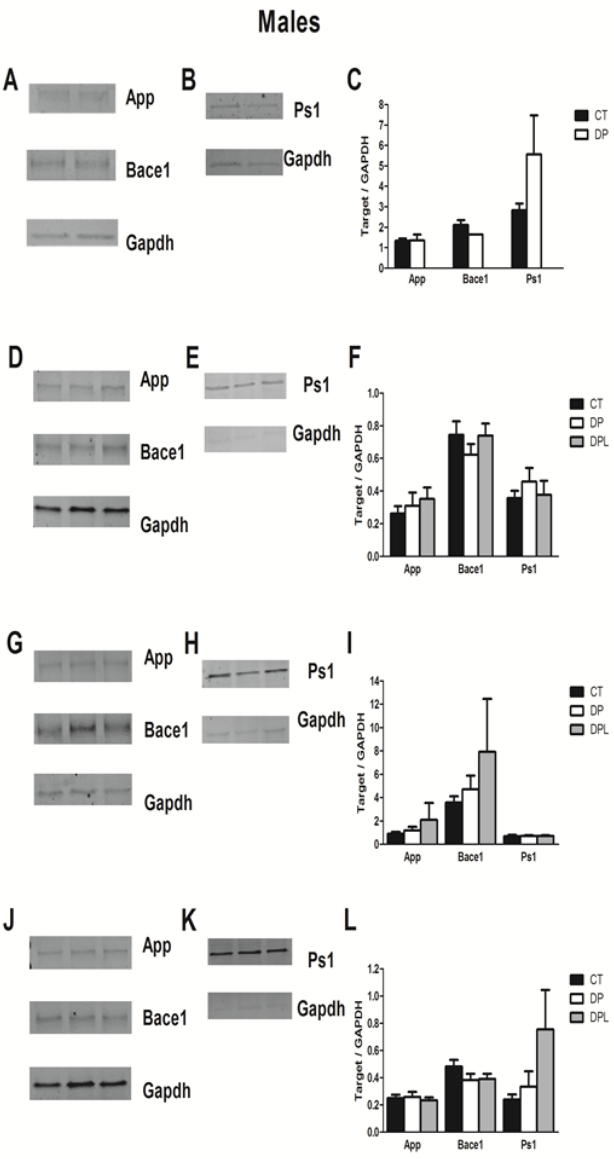

Figure 4: Western blotting analysis in brain and total cerebral cortex of offspring from dams on vitamin B deficient diet during pregnancy and pregnancy/lactation. n=3-5 (A-C), 5 (D-L); CT=control; DP=deficient diet during pregnancy; DPL=deficient during pregnancy and lactation; PND=postnatal day. A-C: PND 0; D-F: PND 28; G-I: PND 90; J-L: PND 210. ANOVA. Data are presented as the mean ± standard error.

Behavior and cognition analysis

To assess whether maternal B-complex vitamins deficiency during pregnancy or pregnancy/lactation affected offspring cognitive abilities, we subjected offspring at PND 210 to a novel object recognition task, dependent of hippocampal activity (Figure 5). The results showed that DP and DPL offspring were not significantly different from CT group

in both females (ANOVA, p=0.893) and males (ANOVA, p=0.112) evaluations.

Locomotor activity box

As demonstrated in Figure 6, males from DPL group had lower locomotor activity when compared to DP (Fisher, p=0.028) and CT groups (Fisher, p=0.003). No difference was observed in locomotor activity of females (ANOVA, p=0.161).

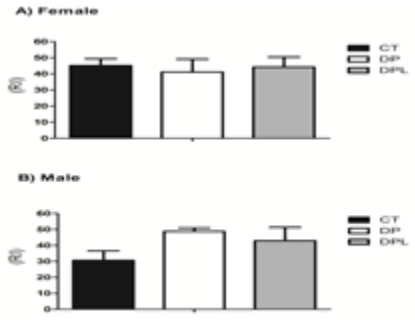

Figure 5: Object recognition task in PND 210 offspring from dams on vitamin B deficient diet during pregnancy and pregnancy/lactation. n=5-8; CT=control; DP=deficient diet during pregnancy; DPL=deficient during pregnancy and lactation; PND=postnatal day; RI=recognition index. ANOVA. Data are presented as the mean ± standard error.

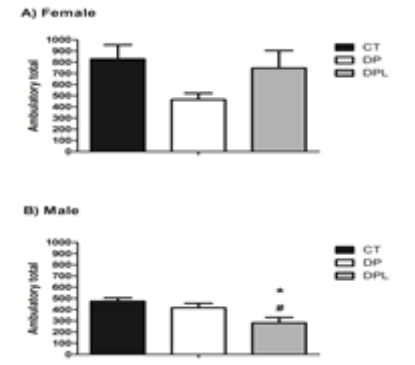

Figure 6: Locomotor activity in PND 210 offspring from dams on vitamin B deficient diet during pregnancy and pregnancy/lactation. n=6-8; CT=control; DP=deficient diet during pregnancy; DPL=deficient during pregnancy and lactation; PND=postnatal day. ANOVA. Data are presented as the mean ± standard error.

Discussion and Conclusion

DNA methylation and histone acetylation are among the epigenetic mechanisms that regulate gene expression and genomic imprinting during embryogenesis, which are essential for the maintenance of body gene expression profile [31]. The pattern determined by these mechanisms is transmitted through mitosis, thus specific to each cell type and tissue; however, it is susceptible to the interference of maternal environment during development such as uteroplacental insufficiency, malnutrition, diabetes and toxic components of cigarette smoke [32].

It is believed that the role of nutritional profile in gene expression programming by one-carbon metabolism is governed by methionine-homocysteine flow. In this route, methyl groups available from s-adenosylmethionine (SAM), among other purposes, to DNA and protein methylation are sensitive to amino acids, folic acid, vitamin B12 and vitamin B6 supply. After transmethylation reactions, SAM is converted into S-adenosylhomocysteine (SAH) and then hydrolyzed to adenosine and Hcy. In fact, Fuso and co-workers demonstrated that β-secretase (*Bace*) and presenilin 1 *(Ps1*) are regulated by methylation and also that low levels of folate and vitamin B12 in culture medium can cause a reduction of SAM levels, and a consequent increase in PS1 and BACE levels with increased Aβ peptide production. In an experimental model, folate-deficient diet supplemented by Hcy decreased SAM concentration and SAM/SAH ratio in both placenta and liver of pregnant rats, and these changes were linked to a decrease in DNA methylation [33]. Based on these studies, we developed an experimental model to evaluate the effects of maternal vitamin B deficiency on offspring programming of genes involved in AD predisposition. In this study, we had observed a decreased gene expression in female (*App*) and male (*App* and *Ps1*) mice offspring. However, it is interesting to point out that we observed previously that vitamin B deficiency during pregnancy and lactation period was associated to a decreased brain SAM/SAH ratio in offspring at PND 0. Considering the published work by Fuso and co-workers, the exposition to vitamin B deficient diet generated a decrease in SAM/SAH ratio and an increase in *Bace1* and *Ps1* gene expression in the mice submitted to diet [34]. It is important to emphasize that in our experiment, these evaluations were performed on offspring (PND 0) of dams in deficient diet during pregnancy and, although we have observed similarities between the two results, such as decreased SAM/SAH ratio, other mechanisms related to maternal-fetal metabolism may have contributed to the differences in gene expression. We did not evaluate the methylation pattern in *App*, *Bace1* and *Ps1* promoters, but no statistically significant difference was observed in relation to global DNA methylation pattern. Moreover, these changes in mRNA expression did not affect protein expression, since no significant difference was observed on Western blottings in female and male mice. On the other hand, when we evaluated the offspring at the end of the lactation period (PND 28), we have observed different responses between males and females, with increased expression of *App*, *Bace1* and *Ps1* in DPL females and a decrease in *App* and *Ps1* in DP and DPL males. However, these changes do not affect the expression of APP, BACE and PS1 proteins in both genders. Fetal programming appears to be sexually dysmorphic and in our previous study we have also observed a gender difference in response to maternal manipulation [35]. In this study, although both newborn females and males have shown a decrease in SAM/SAH ratio, this change was determined by the increase of SAH levels in females and a decrease of SAM levels in males. Besides that, we observed decreased plasma levels of glutathione and folate only in adult male [27], corroborating that female and male offspring responded differently to methyl donor deficiency in utero. Indeed, studies on epigenetic mechanisms have shown that males may be more susceptible in adulthood due to epigenetic marks generated during embryonic period [36,37]. The offspring's care, usually associated to females, is suggested as a selective pressure agent [38].

In evaluations performed on PND 90, an increase in *Bace1* gene expression was observed only in females, and similarly to previous results, we did not observe changes in global DNA methylation pattern or in protein expression for APP, BACE and PS1.

At PND 210, we did not observe changes in the global DNA methylation pattern neither in gene nor in protein expression. Early deficiency in methyl donors has specifically affected the gene expression from PND 0 until PND 90. However, it is likely that epigenetic markers in these evaluated genes have been lost along development given that only PND 90 females presented changes in *Bace1* gene expression and no further changes are observed at PND 210 in both females and males. Indeed, epigenetic marks present a certain level of remodeling and are inherently reversible [39]. Concerning no correlation between gene expression and protein expression, several factors may have contributed, such as mRNA and protein production/degradation. In mammalian cells, mRNAs are produced on average of two copies per hour, while a copy of mRNA produces dozens of copies of the respective protein in the same period [40]. Moreover, mRNAs showed an average half-life of 2.6–7 hours versus 46 hours to protein [40].

Regarding the behavioral pattern, Blaise and co-workers [41] observed a loss in exploratory behavior, learning and memory abilities of adult young rats whose mothers were exposed to a vitamin B deficient diet during pregnancy and lactation. In our PND 210 litters no differences were observed in spatial visual recognition memory, known to be dependent of the hippocampal activity, regardless of gender. Moreover, we evaluated mice locomotor activity and observed a difference in DPL males; these animals showed lower locomotor activity when compared to CT and DP groups. Previously, we had found that these animals showed low plasma folate levels and lower perigonadal white adipose tissue, associated with decreased body weight [42]. Considering that folate plays important roles in different metabolic pathways, such as methionine, histidine and glycine-serine cycles, it is possible that the lowest locomotor activity would be associated with neurological or bioenergetic problems, since serine deficiency leads to malfunctioning of central nervous system [43]. Folic acid deficiency is also associated with impairment in RNA and DNA synthesis, fat and fatty acid metabolism, and muscle formation [44-46].

In conclusion, vitamin B deficiency during pregnancy and lactation altered *App*, *Bace1* and *Ps1* genes expression in brain cortex of female and male offspring mice during development and even after the inclusion of the standard diet. However, we did not notice more changes in gene expression in analyzes performed at PND 210 as seen before. In addition, protein expression data revealed no changes in any of the periods analyzed. However, we cannot rule out other epigenetic marks that could have occurred in this model, since these animals had a lower locomotor activity and that biochemical and biometric changes were previously reported [27,42].

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Conflict of Interest

On behalf of all authors, the corresponding author states that there is no conflict of interest.

Authors Contributions

Vanessa Cavalcante-Silva: Conceived, designed and performed the experiments. Also analyzed the data and wrote the paper; Leandro Fernandes: Together with Cavalcante-Silva V performed the experiments and analyzed the data; Eduardo Jun Haseyama: Together with Cavalcante-Silva V performed the experiments and wrote the paper; Ana Luiza Dias Abdo Agamme: Together with Cavalcante-Silva V performed the experiments and analyzed the data; Maria Tereza Cartaxo Muniz: Together with Cavalcante-Silva V conceived and designed the experiments; Vânia D'Almeida: Together with Cavalcante-Silva V conceived and designed the experiments and contributed with reagents, materials and analysis tools. All authors contributed by reading critically the manuscript.

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